MASARYKOVA UNIVERZITA
PŘÍRODOVĚDECKÁ FAKULTA
ÚSTAV BOTANIKY A ZOOLOGIE

Význam polyploidie, hybridizácie a asexuálneho rozmnožovania v evolúcii cievnatých rastlín

Habilitačná práca

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Obsah

Poďakovanie

Hoci sa rodičom a blízkym zvykne ďakovať až na konci tejto kapitoly ja začnem práve nimi, nakoľko to bol môj otec, ktorý vo mne vzbudil záujem o prírodu. Spolu s bratom nás každodenne brával na prechádzky prakovskými lesmi, dolinami a dolkami, kde sme pozorovali všetko, čo sa hýbalo aj nehýbalo, a to bez ohľadu na prítomnosť či neprítomnosť chlorofylu v bunkách. Za to, že tento záujem pretrval podnes, a ktorý sa mi stal aj životným povolaním ďakujem aj mamine, manželke Vierke a mojim deťom. Tí všetci ma podporovali v mojom bádateľskom úsilí a vytvárali nevyhnutné zázemie potrebné pre normálny život. Im patrí moja najväčšia vďaka.

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Abstract

Interspecific hybridization and polyploidization and, in a lesser extent, apomixis (asexual seed reproduction) have played a substantial role in the evolution of vascular plants. Although all three processes are often closely associated, their contribution to speciation is different. Using examples from three genera of sunflower family (*Centaurea*, *Hieracium* and *Pilosella*), we briefly summarize in this thesis the different aspects of these evolutionary mechanisms, which have been studied by the author and his collaborators over the last ten years.

Interspecific hybridization results in phenotypic and genotypic alterations, and if such an 'evolutionary novelty' is reproductively isolated from parental taxa and its reproduction is assured then a new hybridogeneous species can arise. We provide molecular evidence for hybrid origin and morphological differentiation of the allotetraploid *Centaurea stoebe* and the allopolyploid *Pilosella alpicola* s. str. (Appendices B, C, M, N). These hybridogeneous species were reproductively isolated from their ancestors either by a ploidy barrier (*C. stoebe*) and / or by apomictic reproduction (*P. alpicola* s.str.) (Appendices A, C). We hypothesize that interspecific hybridization has triggered an important shift from a monocarpic life cycle in the diploid *C. stoebe* to a polycarpic perennial life cycle in tetraploid cytotype. This in turn might explain the colonization success of a tetraploid cytotype in North America, where it has become invasive. In contrast, autopolyploidization in *Pilosella rhodopea* did not lead to morphological nor genetic differentiaton, probably due to very effective and intensive intercytotype gene flow (Appendix D).

Our results show that the triploid cytotype of *P. rhodopea* creates an important intercytotype bridge, forming tetraploids through frequent unreduced gamete formation (Appendix C). Furthemore, cytogeographic and genetic data suggest multiple origins of autopolyploidy and a primary diploid-polyploid contact zone in *P. rhodopea*. The primary contact zones where new polyploids arise from resident diploids have only rarely been reported in vascular plants (Apendices C, D). The intercytotype interactions were also studied in five mixed-ploidy populations of *Centaurea stoebe*. By combining different methodological approaches, we found strong microspatial and microhabitat segregation of cytotypes in every site. Such segregation was driven by anthropogenic disturbance and later immigrations of tetraploids into already established diploid populations. Our results thus highlight the importance of non-adaptive spatio-temporal processes in explaining microhabitat and microspatial segregation of cytotypes. (Appendix A).

The issue of natural hybridization in the genus *Hieracium*, its frequency and direction, was addressed in three studies (Appendices L, O, P). They provided the first convincing evidence on recent, though rare, homoploid hybridization in the genus. In addition to natural hybridization, we also performed a pionier experimental hybridization in the same genus. The most important result was a discovery of induced autogamy in *Hieracium* – a phenomenom which might play an important role in reproductive isolation of otherwise strictly self-incompatible taxa (Appendices Q, R, S).

Using karyological and flow-cytometric methods, we assessed new or corroborated previous chromosome counts / ploidy levels for many *Pilosella* and *Hieracium* taxa (Appendices C, E, F, G, H, I, J, K). In addition, for many hawkweed species we have determined the mode of reproduction by performing either a castration experiment or flow-cytometric seed screening analyses (Appendices C, G, I, Q). Interestingly, we have found a correlation between hybridization and apomictic reproduction in the allopolyploid *P. alpicola* s.str. but not in the autopolyploid *P. rhodopea* that exclusively reproduces sexually (Appendix C). These results thus stress the importance of polyploidy and apomictic reproduction as the stabilization mechanisms in the evolution of *Hieracium* and *Pilosella*.

1. ZOZNAM PUBLIKÁCIÍ – PRÍLOH UCHÁDZAČA ZARADENÝCH DO HABILITAČNEJ PRÁCE

A. **Mráz P**, Španiel S, Keller A, Bowmann G, Farkas A, Šingliarová B, Rohr RP, Broennimann O, Müller-Schärer H. Anthropogenic disturbance as a driver of microspatial and microhabitat segregation of cytotypes in diploid-tetraploid contact zones. (rukopis pripravený na odoslanie).

Autorský podiel uchádzača: 80%.

PM navrhol a koordionoval štúdiu, podieľal sa na zbere rastlinného materiálu, na meraniach rastlín v teréne, spolupodieľal sa na cytometrických a karyologických analýzach, izolácii DNA a analýze genetických dát, vykonal štatistické analýzy, interpretoval výsledky a napísal štúdiu.

B. **Mráz P**, Garcia-Jacas N, Gex-Fabry E, Susanna A, Barres L, Müller-Schärer H. 2012. Allopolyploid origin of highly invasive *Centaurea stoebe* sl (Asteraceae). *Molecular Phylogenetics and Evolution* 62: 612–623.

Autorský podiel uchádzača: 70%.

PM navrhol a koordionoval štúdiu, nadviazal kontakt so španielskym tímom, podieľal sa na zbere a pestovaní rastlinného materiálu a cytometrických a karyologických analýzach, izolácii DNA a analýze dát, interpretoval výsledky a napísal štúdiu.

C. Šingliarová B, Hodálová I & **Mráz P.** 2011. Biosystematic study of the diploid-polyploid *Pilosella alpicola* group with variation in breeding system: patterns and processes. *Taxon* 60: 450–470.

Autorský podiel uchádzača: 50%

PM navrhol a koordinoval štúdiu, čiastočne sa podieľal na zbere rastlinného materiálu, previedol molekulárne analýzy a významne sa podieľal na interpretácií výsledkov a na písaní textu.

D. Šingliarová B, Chrtek J, Plačková I & **Mráz P.** 2011. Allozyme variation in diploid, polyploid and mixed-ploidy populations of the *Pilosella alpicola* group (Asteraceae): relation to morphology, origin of polyploids and breeding system. *Folia Geobotanica* 46: 387–410.

Autorský podiel uchádzača: 30%.

PM navrhol a koordinoval štúdiu, čiastočne sa podieľal na zbere rastlinného materiálu, na vyhodnotení genetických dát, ich interpretácií a na písaní textu.

E. **Mráz P**, Chrtek J & Šingliarová B. 2009. Geographical parthenogenesis, genome size variation and pollen production in the arctic-alpine species *Hieracium alpinum*. *Botanica Helvetica* 119: 41–51.

Autorský podiel uchádzača: 90%.

PM navrhol a koordinoval štúdiu, podieľal sa na zbere väčšiny rastlinného materiálu, previedol cytometrické a karyologické analýzy, interpretoval výsledky a napísal prácu.

F. **Mráz P**, Šingliarová B, Urfus T & Krahulec F. 2008. Cytogeography of *Pilosella officinarum* (Compositae): Altitudinal and longitudinal differences in ploidy level distribution in the Czech Republic and Slovakia and the general pattern in Europe. *Annals of Botany* 101: 59–71.

Autorský podiel uchádzača: 60%.

PM navrhol a koordinoval štúdiu, podieľal sa na zbere rastlinného materiálu, cytometrických a karyologických analýzach a na interpretácii výsledkov, významne sa podieľal na konečnej podobe rukopisu.

G. **Mráz P** & Szeląg Z. 2004. Chromosome numbers and reproductive systems in selected species of the genera *Hieracium* L and *Pilosella* Hill (*Asteraceae*) from Romania. *Annales Botanici Fennici* 41: 405–414.

Autorský podiel uchádzača: 80%.

PM navrhol a koordinoval štúdiu, nazbieral analyzovaný materiál, previedol všetky karyologické analýzy, spolupodieľal sa na interpretácií výsledkov a napísal text práce.

H. Chrtek J, **Mráz P** & Severa M. 2004. Chromosome numbers in selected species of *Hieracium* s.str. (*Hieracium* subgen. *Hieracium*) in the Western Carpathians. *Preslia* 76: 119–139.

Autorský podiel uchádzača: 50%

PM sa podieľal na zbere rastlinného materiálu, na karyologických analýzach a interpretácií výsledkov a prispel písaním príslušných častí textu.

I. Rotreklová O, Krahulcová A, **Mráz P**, Mrázová V, Mártonfiová L, Peckert T & Šingliarová B. 2005. Chromosome numbers and breeding systems in some species of *Hieracium* subgen *Pilosella* from Europe *Preslia*. 77: 177–195.

Autorský podiel uchádzača: 20%.

PM sa podieľal na zbere materiálu a karyologických analýzach a písaní príslušných častí textu.

J. Niketić M, Vladimirov V & **Mráz P.** 2006. Chromosome numbers and taxonomic-chorological notes on selected species of *Hieracium* s str (*Asteraceae*) from Montenegro. *Phytologia Balcanica* 12: 85–97.

Autorský podiel uchádzača: 10%.

PM podieľal sa na karyologických analýzach a prispel k záverečným korektúram finálneho textu.

K. Chrtek J, **Mráz P**, Zahradníček J, Mateo G & Szeląg Z. 2007. Chromosome numbers and DNA-ploidy levels of selected species of *Hieracium* s.str. *Folia Geobotanica* 42: 411–430.

Autorský podiel uchádzača: 30%.

PM sa podieľal na zbere rastlinného materiálu, cytometrických a karyologických analýzach a prispel písaním príslušných častí textu.

L. **Mráz P**, Chrtek J & Fehrer J. 2011. Interspecific hybridization in the genus *Hieracium* s str – evidence for bidirectional gene flow and spontaneous allopolyploidization. *Plant Systematics and Evolution* 293: 237–245.

Autorský podiel uchádzača: 80%.

PM navrhol a koordinoval štúdiu, podieľal sa na zbere rastlín, previedol časť molekulárnych laboratórnych prác a analýz (ITS a AFLP), interpretoval výsledky a napísal štúdiu.

M. **Mráz P**, Bourchier RS, Treier UA, Schaffner U & Müller-Schärer H. 2011. Polyploidy in phenotypic space and invasion context: a morphometric study of *Centaurea stoebe* s.l. *International Journal of Plant Sciences* 172: 386–402.

Autorský podiel uchádzača: 80%.

PM koordinoval štúdiu, čiastočne sa podieľal pestovaní rastlín a ich meraní, previedol štatistické analýzy, interpretoval výsledky a napísal štúdiu.

N. Henery ML, Bowman G, **Mráz P**, Treier UA, Gex-Fabry E, Schaffner U, Müller-Schärer H. 2010. Evidence for a combination of pre-adapted traits and rapid adaptive change in the invasive plant *Centaurea stoebe*. *Journal of Ecology* 98: 800–813.

Autorský podiel uchádzača: 10%.

PM sa spolupodieľal sa na morfologických meraniach a na písaní diskusie.

O. **Mráz P**, Chrtek J, Fehrer J & Plačková I. 2005. Rare recent natural hybridization in the genus *Hieracium* sstr – evidence from morphology, allozymes and chloroplast DNA. *Plant Systematics and Evolution* 255: 177–192.

Autorský podiel uchádzača: 60%.

PM navrhol a koordinoval štúdiu, nazbieral analyzovaný materiál, previedol časť karyologických analýz, spolupodieľal sa na interpretácií výsledkov a napísal text práce.

P. Chrtek J, **Mráz P** & Sennikov AN. 2006. *Hieracium* ×*grofae* – a re-discovered diploid hybrid from the Ukrainian Carpathians. *Biologia, Bratislava* 61: 365–373.

Autorský podiel uchádzača: 50%.

PM navrhol a koordinoval štúdiu, podieľal sa na zbere rastlinného materiálu, previedol merania peľu, podieľal sa na interpretácii výsledkov a písaní textu.

Q. **Mráz P.** 2003. Mentor effects in the genus *Hieracium* sstr (Compositae, Lactuceae). *Folia Geobotanica* 38: 345–350.

R. **Mráz P** & Tomčíková D. 2004. Experimental hybridization in the genus *Hieracium* s. str. – crosses between diploid *H. umbellatum* and triploid *H. sabaudum. Thaiszia – Journal of Botany* 14, Supplement 1: 15–16.

Autorský podiel uchádzača: 90%.

PM navrhol a koordinoval štúdiu, nazbieral rodičovské druhy, previedol medzidruhové kríženia, interpretoval výsledky a zostavil výsledný text práce.

S. **Mráz P** & Paule J. 2006. Experimental hybridization in the genus *Hieracium* s.str. (Asteraceae): crosses between selected diploid taxa. *Preslia* 78: 1–26.

Autorský podiel uchádzača: 80%.

PM navrhol a koordinoval štúdiu, nazbieral rodičovské druhy, previedol medzidruhové kríženia, interpretoval výsledky a zostavil výsledný rukopis práce.

T. Chrtek J jr, Tomková M, **Mráz P**, Marhold K, Plačková I, Krahulcová A & Kirschner J 2007 Morphological and allozyme diversity in the *Hieracium nigrescens* group (Compositae) in the Sudety Mountains and the Western Carpathians. *Botanical Journal of the Linnean Society* 153: 287–300.

Autorský podiel uchádzača: 10%.

PM sa podieľal na zbere rastlinného materiálu, izoenzýmových analýzach a prispel k záverečným korektúram finálneho textu.

2. ÚVOD

Polyploidia, medzidruhová hybridizácia a v menšej miere aj apomiktický spôsob rozmnožovania sa považujú za jedny z hlavných mechanizmov v evolúcii vyšších rastlín (Rieseberg & Willis 2006). Hoci sú tieto tri procesy veľmi často prepojené, v mnohých aspektoch sa líšia.

Medzidruhové kríženie ako zdroj novej fenotypovej variability, ktoré môže viesť v určitých prípadoch ku vzniku nových taxónov, bolo rozpoznané prinajmenšom už Linném v 18. storočí (Coyne & Orr 2004). Na druhej strane, význam polyploidie pre okamžitú tvorbu reprodukčných bariér ako nevyhnutnej podmienky speciácie a jej význam pri stabilizovaní rozmnožovania inak sterilných hybridov bol formulovaný až oveľa neskôr Wingem v roku 1917 (Winge 1917). Objav polyploidnej evolúcie bol na tú dobu tak revolučný, že populačný genetik Haldane vyhlásil, že polyploidná evolúcia je najpodstatnejší doplnok, ktorý musí byť zaradený do Darwinovej teórie o pôvode druhov (Coyne & Orr 2004). Pochopiteľne, Darwin v období, v ktorom tvoril, nemal žiadnu potuchu o význame bunkového jadra, chromozómov či polyploidie pre evolúciu. Drvivá väčšina cievnatých rastlín sa rozmnožuje sexuálne tvoriac veľké množstvo genotypov a fenotypov, ktoré majú v heterogénnom prostredí rôzne optimum (fitness) a len tie s najlepšou lokálnou adaptáciou môžu prežiť. Predpokladá sa, že vznik sexuálneho rozmnožovania súvisí práve s lepšou schopnosťou organizmov prispôsobovať sa meniacemu prostrediu. Na druhej strane existujú skupiny rastlín, u ktorých prevláda asexuálna tvorba semien (agamospermia, apomixia) vedúca ku klonálnej štruktúre druhov. Apomixia (podobne ako polyploidia) má poistnú funkciu v rozmnožovaní – tzv. „escape from sterility“ (Darlington 1939), nakoľko jej vznik je často úzko spätý s medzidruhovým krížením, ktorého výsledkom sú zväčša sterilné hybridy.

Hoci základná funkcia všetkých troch procesov v evolúcii cievnatých rastlín je dobre známa, mnohé detaily a čiastkové pochody týchto mechanizmov zostávajú nevyriešené. V poslednom desaťročí, vďaka rozvoju molekulárnych technológií, možno badať nebývalý záujem o štúdium týchto procesov. Použitie molekulárnych, cytometrických a sofistikovaných štatistických prístupov tak umožňuje presvedčivé rozpoznanie hybridov a hybridogénnych druhov, a ich rodičovských kombinácií, časový odhad ich vzniku, geografický pôvod, genetické a epigenetické zmeny po chromozómovej duplikácii či hybridizácii. Na druhej strane objasnenie ekologického významu týchto zmien sprevádzajúcich duplikáciu genómu či

hybridizáciu výrazne zaostáva za pokrokom dosiahnutým molekulárnymi metódami (Soltis et al. 2010).

Predkladaná práca si kladie za cieľ stručne zhrnúť význam týchto procesov v evolúcii cievnatých rastlín a prezentuje konkrétne výsledky získané štúdiom modelových rodov *Centaurea*, *Hieracium* a *Pilosela*.

3. POLYPLOIDIA

Polyploidia je stav, kedy má jedinec v jadrách buniek viac ako dve úplne chromozómové sady. Vzhľadom na vysokú frekvenciu polyploidie medzi cievnatými rastlinami, niektoré odhady vravia až o 70%-ách (Masterson 1994, Otto and Whitton 2000), sa tento mechanizmus považuje za jeden z najdôležitejších v evolúcii vyšších rastlín. Polyploidia prispieva k vzniku nových taxónov troma hlavnými spôsobmi. Prvým je **reprodukčná izolácia** vznikajúca doslova okamžite medzi ancestrálnym diploidom / diploidmi a novo vzniknutým polyploidom. Druhým je **obnova normálneho párovania chromozómov,** a tým stabilizácia rozmnožovania u hybridogénnych taxónov. A napokon tretím je **fenotypická zmena** sprevádzajúca polyploidizáciou *per se*.

3.1. Význam polyploidie pre tvorbu reprodukčných bariér

Reprodukčná izolácia predstavuje súbor rôznych mechanizmov, ktoré zabraňujú kríženiu medzi spoluvyskytujúcimi sa druhmi, a tak sa podieľajú na udržiavaní integrity druhov. Všeobecne môžme izolačné mechanizmy rozdeliť na prezygotické, ktoré zabraňujú oplodneniu (napr. geografická alebo ekologická segregácia, preferencia rôznych opeľovačov, posun v kvitnutí, peľová kompetícia), alebo postzygotické, ktoré fungujú po oplodení a zvyčajne spôsobuju sterilitu hybridov.

V diploidno-polyploidných komplexoch bola pozorovaná prítomnosť silných postzygotických bariér (Coyne et Orr 2004). Krížením medzi novo vzniknutým polyploidom, najčastejšie tetraploidom, a rodičovským diploidom dochádza k tvorbe cytotypovo intermediárnych hybridov s nepárnym počtom chromozómov. Takéto intermediárne hybridy majú vážne problémy s tvorbou gamét, nakoľko je narušené párovanie a následná segregácia nepárneho počtu chromozómov počas prvej fázy meiotického delenia. Tento abnormálny proces vyúsťuje do tvorby aneuploidných, často neživotaschopných, gamét a spôsobuje tak

sterilitu triploidnej rastliny. Tento jav sme potvrdili u jedného triploidného hybrida (2n = 3x = 27) nájdeného v diploidno-tetraploidnej populácii (2n = 2x = 18, 2n = 4x = 36) druhu *Centaurea stoebe* (**Príloha A**). Triploid tvoril len veľmi málo semien, ktoré boli takmer bez výnimky abnormálneho guľovitého tvaru naznačujúceho problémy v embryogenéze alebo aj v tvorbe endospermu. Vyklíčilo len jedno semeno, ktoré malo na rozdiel od ostatných semien normálny podlhovastý tvar. Karyologické analýzy ukázali, že klíčenec bol aneuploidný (2n = 21) a vznikol pravdepodobne fúziou aneuploidného vajíčka (n = 12) triploidnej matky a haploidného peľu (n = 9) diploidného otca.

Veľmi často však kríženie diploidných a tetraploidných rastlín neprináša žiadne potomstvo. Marks (1966) prišiel s vysvetlením, že skorá aborcia vajíčok je zrejme spôsobená nevyrovnanou dávkou materského a otcovského genómu v endosperme intermediárnych hybridov – tzv. **hypotéza triploidného bloku**. Kým u normálne vzniknutého potomstva diploidných rastlín je tento pomer v endosperme 2 materské a 1 otcovský genóm, v prípade triploidných hybridov je temto pomer zmenený a závisí od ploidie materskej a otcovskej gaméty (Ehlenfeldt et Ortiz 1995). Takýto mechanizmus vedie k silnej postzygotickej izolácii diploidných a tetraploidných rastlín a k vzácnosti intermediárnych triploidov. Podobný jav sme zistili v zmiešaných populáciách *Centaurea stoebe*, kde triploidy vznikali len vo veľmi nízkej frekvencii – 0.2% (**Príloha A**). Navyše naše nepublikované dáta z kontrolovaného kríženia medzi diploidnými a tetraploidnými rastlinami svedčia takisto v prospech silnej postzygotickej bariéry spôsobenej zrejme efektom triploidného bloku (Mráz et al. nepubl.).

3.2. Význam polyploidie z hľadiska tvorby nových fenotypov

Zdvojenie, resp. zmnohonásobenie počtu chromozómových sádok v bunkovom jadre spúšťa kaskádu mechanizmov, ktoré sa prejavujú od úrovne jadra a bunky až po pletivá a orgány. Najmarkantnejším príkladom zmeny po duplikácii genómu je napr. zväčšenie objemu (veľkosti) buniek – u rastlín pozorovaných často u peľových zŕn alebo buniek prieduchového aparátu (Müntzig 1936). Polyploidia však môže výrazne ovplyvniť aj biochemické a fyziologické procesy, čo sa často využíva pri zámernej polyploidizácii mnohých poľnohospodárskych plodín. Takéto zmeny môžu ovplyvniť aj adaptačné schopnosti organizmov, a tým ich rozšírenie (Levin 1983).

3.3. Typ polyploidie a jeho rozlíšenie

Polyploidu môžme podľa pôvodu rozdeliť na **autopolyploidiu** a **alopolyploidiu**. V prvom prípade vznikajú polyploidy v rámci jedného druhu. V druhom je polyploidizácia zvyčajne dôsledkom predchádzajúcej medzidruhovej hybridizácie, kedy duplikáciou chromozómov dochádza k obnoveniu fertility inak vysoko sterilných hybridných diploidov (Clausen et Goodspeed 1925). Tradične sa oba spôsoby rozlišovali cytologicky. Autopolyploidy tvoria počas prvej meiózy multivalenty homologických chromozómov (autotetraploidy tvoria tetravalenty). U alotetraploidov, vzhľadom na ich hybridogénny pôvod, sa naopak pozorovala tvorba bivalentov, keďže obsahujú heterologické chromozómy. Takéto meiotické správanie má výrazný vplyv na segregáciu chromozómov, a tým aj genotypové zloženie gamét a vzniknutého potomstva: alotetraploidy v kontrolných kríženiach tvoria menšie množstvo odlišných genotypov ako autoteraploidy. Z tohto dôvodu sa aj segregačné pomery potomstva využívajú na stanovenie vzniku polyploidov.

V súvislosti s prudkým rozvojom molekulárnych techník sa v poslednom období čím ďalej tým viac využívajú rôzne molekulárne markery na zistenie auto-, resp. alopolyploidného pôvodu druhov. Prítomnosť dvoch (alebo viacerých) rozdielnych genómov u alopolyploida môže byť zistená jadrovými DNA markermi, ktorá sú dedené od oboch rodičov. Klonovaním jadrovej ribozomálnej DNA (ITS1-5.8S-ITS2) sme zistili, že tetraploidný cytotyp druhu *Centaurea stoebe* je hybridogénneho pôvodu, keďže takmer všetky analyzované tetraploidy mali dva odlišné ribotypy, kým diploidný cytotyp, jeden z predpokladaných rodičov tetraploida (druhý rodič je neznámy), mal len jeden hlavný ribotyp (**Príloha B**). Datovaním vzniku tetraploidného ribotypu sme odhadli aj jeho mladý – pleistocénny pôvod. Podobný pattern – prítomnosť väčšieho množstva aditívnych polymorfizmov (10-11 na sekvenciu) v priamych sekvenciách ribozomálnej DNA u alpského druhu *Pilosella alpicola* s. str. svedčí o jeho hybridogénnom pôvode (**Príloha C**). Porovnanie sekvencií s iným druhmi rodu ukázalo, že tento druh vznikol pravdepodobne krížením alpského druhu *P. glacialis* a balkánskeho druhu *P. rhodopea*. Hoci tieto rodičovské druhy v súčasnosti majú neprekrývajúci sa areál, k hybridizácii mohlo dôjsť počas posledných zaľadnení, kedy tieto druhy mohli prísť do sekundárneho kontaktu. Na rozdiel od *P. alpicola* s. str. polyploidné cytotypy druhu *P. rhodopea* sa neodlišujú v množstve a charaktere ITS nukleotidových polymorfizmov od diploidov a majú výrazne nižší počet aditívnych polymorfizmov na sekvenciu (2-4) ako *P. alpicola* s.str. Navyše 2 aditívne polymorfizmy boli fixované

u všetkých analyzovaných zástupcov tejsto skupiny. Tieto výsledky ukazujú, že polyploidné cytotypy *P. rhodopea* vznikli autopolyploidne z diploidného cytotypu. Zdieľanie rovnakých alozýmových alel diploidmi a polyploidmi *P. rhodopea* potvrdilo tento scénar (**Príloha D**).

3.4. Polytopický vznik polyploidov a geografické paterny

Zavedenie molekulárnych techník do evolučno-taxonomického výskumu prinieslo významný kvalitatívny pokrok. Jedným z najdôležitejších objavov, ku ktorým molekulárne metódy prispeli bol dôkaz o mnohonásobnom – **polytopickom pôvode polyploidov** vznikajúcich z rôznych rodičovských genotypov (napr. Brochmann et al. 1992, Segraves et al. 1999, Schmizu-Inatsugi et al. 2009). Tento proces, ktorý bol neskôr dokázaný u ďalších rastlinných druhov, je zásadný, lebo dokazuje, že polyploidizácia je všeobecný jav, ku ktorému dochádza opakovane a nezávisle, a to tak v čase ako aj v priestore. Polytopický pôvod polyploidných populácií a jedincov možno zistiť z miery ich genetickej diferenciácie. Polytopicky vzniknuté polyploidné jedince sú geneticky differencovanejšie ako jedince a populácie monotopického pôvodu (pozri napr. Schneller et al. 1998). Ako ukázali izozýmové analýzy, tetraploidné jedince druhu *Pilosella alpicola* s.str. zo švajčiarskych Walliských Álp vznikli z iných rodičovských genotypov ako pentaploidy v talianskych Dolomitoch (**Príloha D**), hoci v oboch prípadoch vznikli z rovnakej kombinácie rodičovských druhov (**Príloha C**). Podobný je prípad tetraploidného cytotypu *Centaurea stoebe,* u ktorého možno na základe značnej variability chloroplastovej DNA usudzovať, že vznikol mnohokrát a nezávisle (**Príloha B**). Nielen alopolyploidné druhy môžu vznikať mnohonásobne a polytopicky. Polytopický pôvod sme zistli aj pre autopolyploidné populácie *Pilosella rhodopea* (**Príloha D**).

Hoci najpresvedčivejšou metódou na zistenie polytopického resp. monotopického pôvodu polyploidov je nepochybne molekulárny prístup, odlišné geografické rozšírenie cytotypov môže takisto napovedať o ich nezávislom pôvode (viď príklad tetra- a pentaploidnej *P. alpicola* s.str. rozobratý vyššie). Alopatrické rozšírenie diploidného a triploidného cytotypu jastrabníka alpínskeho (*Hieracium alpinum*) naznačuje, že triploidné apomiktické populácie museli pradepodobne vzniknúť z lokálnych – dnes už neexistujúcich diploidných populácií niekde v blízkosti alpského glaciálneho refúgia, odkiaľ kolonizovali prevažnú časť areálu druhu, a takmer určite nevznikli z reliktných diploidných populácií rastúcich vo východných a južných Karpatoch (**Príloha E**, Mrát et al. nepubl.). Ako ukázalo podrobné cytogeografické štúdium druhu *Pilosella officinarum* založené na už publikovaných

a nových údajoch reprezentujúcich spolu takmer 1000 európskych lokalít, pentaploidné a hexaploidné populácie osídľujúce alpský, karpatský a zrejme aj Balkánsky priestor vznikli pravdepodobne nezávisle od škandinávskych penta- a hexaploidov, nakoľko obe arely sú oddelené rozsiahlym územím s výraznou prevahou tetraploidných populácií (**Príloha F**). Táto štúdia navyše ukázala, že územím bývalého Československa, konkrétne Moravským úvalom prebieha kontaktná zóna penta- a hexaploidných karpatských a panónskych populácií na jednej strane a tetraploidných západoeurópskych populácií na strane druhej. Takýto cytogeografický pattern vznikol pravdepodobne v dôsledku nezávislého pôvodu jednolivých cytotypov a následnej postglaciálnej kolonizácie.

3.5. Frekvencia polyploidie

Využitie prietokovej cytometrie spôsobilo doslova revolúciu v štúdiu polyploidných komplexov, keďže umožňuje spoľahlivé určenie ploidie u mnohonásobne väčšieho počtu rastlín za rovnaký čas, ako je to možné klasickým cytologickým prístupom (Kron et al. 2007). Tento posun na kvantitatívnu – populačnú úroveň umožnil odhaliť pomerne častú vnútropopulačnú cytotypovú variabilitu, a teda existenciu ploidne zmiešaných populácií. Napríklad na území bývalého Československa a severného Maďarska sme zistili, že 32 populácií *Pilosella officinarum* bolo cytotypovo zmiešaných, pričom predchádzajúce údaje uvádzali spoločný výskyt dvoch cytotypov len na troch lokalitách (**Príloha F**). Ešte prekvapujúcejšie bolo zistenie, že balkánsky druh *Pilosella rhodopea*, pre ktorý bol v dovtedajšej literatúre publikovaný len jeden triploidný a jeden diploidný údaj, pozostáva takmer výhradne z cytotypovo zmiešaných populácií, ktoré sú najčastejšie zložené až z troch cytotypov – di-, tri- a tetraploidov (**Príloha C**). Analýza veľkého počtu jedincov pomohla odhaliť aj existenciu zriedkavých cytotypov ako penta- a hexaploidov (**Príloha C**, a Šingliarová et Mráz nepubl.). Klasickým cytologickým, ale aj cytometrickým prístupom sme pre desiatky taxónov rodu *Hieracium* a *Pilosella* stanovili buď úplne nové chromozómové počty alebo ploidné úrovne alebo sme zlepšili poznanie o ich karyologickej premenlivosti (**Prílohy G-K**).

3.6. Mechanizmy vzniku polyploidov

Polyploidy môžu vznikať duplikáciou chromozómov v skorom štádiu zygoty (tzv. **somatická polyploidizácia**) alebo splynutím **redukovaných a neredukovaných gamét**, pričom druhý proces sa považuje z hľadiska frekvencie polyploidizácie za dôležitejší (Bretagnolle et Thompson 1995, Ramsey et Schemske 1998, Grant 2002). Tvorba neredukovaných gamét je pomerne častý jav a je spôsobený poruchami v prvých štádiách meiózy. Z diploidov môžu reprodukčne stabilizované tetraploidy vzniknúť buď v jednom kroku splynutím dvoch neredukovaných gamét, alebo častejšie v dvoch krokoch ako navrhli Harlan a de Wet (Harlan et de Wet 1975). Pri tomto dvojkrokovom spôsobe dochádza v prvej fáze k tvorbe intermediárneho triploidného jedinca, ktorý však musí byť aspoň sčasti fertilný. Keďže meióza je u triploida vážne narušená (viď vyššie), ten produkuje aj neredukované triploidné gaméty, ktoré po splynutí s haploidnou gamétou diploidnej rastliny dávajú vznik tetraploidnej zygote. V tejto hypotéze o dvojkrokovom pôvode tetraploidov má teda významnú funkciu intermediárny triploidný cytotyp plniaci funkciu tzv. **triploidného mosta** (triploid bridge). Dôkazov pre tento proces je však v prírode veľmi málo (Husband 2004). Analýzou plodie embrya a endospermu v semenách (tzv. flow cytometric seed screening – FCSS; cf. Matzk et al. 2000) triploidného cytotypu *Pilosella rhodopea* sme zistili, že tento cytotyp tvorí veľmi často neredukované (apomeiotické) triploidné zárodočné miešky, z ktorých po dvojitom oplodnení haploidným peľom vznikajú spravidla tetraploidné rastliny – v súlade s hypotézou Harlana a de Weta (**Príloha C**). V menšej miere však triploidy tvoria aj diploidné, triploidné aj pentaploidné potomstvo. Analýzy semien diploidných a tetraploidných rastlín na druhej strane ukázali, že okrem potomstva vlastnej plodie, sú tieto cytotypy schopné v malej miere produkovať aj triploidné rastliny, ktoré sú s najväčšou pravdepodobnosťou výsledkom kríženia diploidných a tetraploidných rastlín. Naše údaje tak jasne ukazujú, že nové cytotypy môžu vznikať krížením medzi rôznymi cytotypmi, a že pomerne významnú úlohu tu zohrávajú triploidy produkujúce okrem redukovaných gamét aj gaméty neredukované. Výsledky z cytotypovo zmiešaných prírodných populácií *Pilosella rhodopea* (**Príloha C**) tak potvrdzujú údaje z kontrolovaného kríženia iného druhu *Pilosella echioides* (Peckert et Chrtek 2006), naznačujúc tak pravdepodobne všeobecnejšiu platnosť tohto procesu u chlpánikov. Na rozdiel od triploidov sme však zatiaľ nezistili produkciu neredukovaných gamét u diploidného cytotypu, bez ktorej by primárne nevznikol ani cytotyp triploidný. V budúcnosti by sa teda ďalšie úsilie malo koncentrovať práve na kľúčový mechanizmus

vzniku neredukovaných gamét (ako často, za akých podmienok a u akých rastlín) u diploidného cytotypu *Pilosella rhodopea*.

Paun et al. (2009) ukázali, že alopolyploidia je častejšia v prípadoch, kedy sú rodičovské druhy fylogeneticky odlišnejšie. Tento jav sa dáva do spojitosti s častejšou tvorbou neredukovaných gamét u týchto hybridov v dôsledku problémov s párovaním a segregáciou heterologických chromozómov (Ramsey et Schemske 1998). V rode *Hieracium* sme spontánnu polyploidizáciu zistili raz u medzidruhového kríženca *H.* ×*krasani* vzniknutého z kríženia medzi fylogeneticky veľmi divergentnými druhmi *H. alpinum* a *H. transilvanicum* (**Príloha L**).

3.7. Mechanizmy koexistencie cytotypov

Levin (1975) vyslovil hypotézu, že tetraploidný cytotyp vznikajúci zriedkavo v čisto diploidnej populácii bude z tejto populácie rýchle vytesnený v dôsledku neefektívneho kríženia s početnešími diploidnými rastlinami ústiaceho do nízkej produkcie tetraploidného potomstva. Tento mechanizmus označovaný ako **minority cytotype exclusion** by mal okrem primárnych zón, kde polyploidy vznikajú *in situ*, prebiehať aj v sekundárnych kontaktných zónach, kedy po predchádzajúcej geografickej separácii zvyčajne jeden cytotyp imigruje do populácie druhého cytotypu. Sekundárnych kontaktných zón je známych ďaleko viac ako primárnych (Petit et al. 1999). Udomácnenie sa minoritného cytotypu v populácii dominantného cytotypu bude možné jedine vtedy, ak bude znížená možnosť medzicytotypového kríženia prostredníctvom prezygotických bariér, alebo ak minoritný cytotyp bude kompetične silnejší. Medzi najvýznamnjšie prezygotické bariéry patria: priestorová segregácia, posun v kvitnutí, posun v spektre opeľovačov, posun v spôsobe rozmnožovania (napr. apomicktická alebo autogamická tvorba semien minoritiného cytotypu), alebo iné ekologické nároky cytotypov (Petit et al. 1999). V prírodných kontaktných zónach sa však takéto bariéry skúmali len veľmi zriedka.

Koexistenciu diploidného a tetraploidného cytotypu *Centaurea stoebe* sme podrobne skúmali v niekoľkých zmiešaných populáciách na území Slovenska a Rakúska (**Príloha**). Molekulárne a cytometrické dáta ukázali, že sa jedná o sekundárnu zónu spoluvýskytu, a že diploidné rastliny sú ďaleko početnejšie ako tetraploidné. V každej populácii sme zistili výraznú mikropriestorovú segregáciu cytotypov. Táto preistorová segregácia bola korelovaná s výskytom lokálnych antropogénnych disturbancií (okraje ciest, bývalé lomy, jamy po

štrkovej ťažbe), ktoré boli osídľované prevažne tetraploidným cytotypom. Naopak diploidy sa vyskytovali v širšom spektre mikrohabitatov; od pomerne zapojených suchých trávnikov až po prirodzene otvorené či antropicky disturbované stanovištia. Napriek predpokladanej väčšej kompetičnej schopnosti tetraploidov, ktoré sú spravidla mnohobyľové, v zapojených porastoch boli menej početné ako zvyčajne jedno- alebo málobyľové diploidy. Frekvencia cytotypov, genetická a priestorová štruktúra tak naznačuje, že tetraploidy „imigrovali" do už udomácnených diploidných populácií a prednostne osídľovali miesta narušené človekom, ktoré im poskytli voľnú niku. Ich prežitie a zotrvanie na týchto miestach bolo oproti diploidnému cytotypu zvýhodnené polykarpickým – viacročným životným cyklom, zatiaľčo diploidy sú spravidla monokarpické jedno- alebo dvojročné rastliny. Naše výsledky tak vyzdvihujú neadaptívny, priestorovo-časový aspekt mikropriestorovej a mikrostanovištnej separácie cytotypov (**Príloha A**). Z ďalších dôležitých výsledkov tejto štúdie je hodné spomenúť závislosť množstva semien tvorených tetraploidnými rastlinami od ich frekvencie v diploidných populáciách potvrdzujúcich tak Levinovu hypotézu (Levin 1975) a experimentálne výsledky (Husband 2000).

Iným úplne kontrastným príkladom spolužitia cytotypov je druh *Pilosella rhodopea*, ktorý sa vyskytuje takmer výlučne v cytotypovo zmiešaných populáciách, predstavujúc tak jeden z ojedinelých do teraz známych prípadov v rastlinnej ríši (**Príloha C**). Takýto cytogeografický pattern, ktorý je podporený aj molekulárnymi dátami nasvedčuje, že sa jedná o zriedkavo dokázanú zónu primárneho kontaktu (**Prílohy C, D**). Koexistenicia cytotypov *P. rhodopea* na týchto lokalitách je možná jednak vďaka paradoxnej neprítomnosti prezygotických bariér (!), ktorá umožňuje intenzívne intercytotypové kríženie, a jednak vďaka častej tvorbe neredukovaných gamét triploidmi. Tento príklad potvrdzuje teoretický model navrhnutý Felberom (Felber 1991), ktorý ukázal, že aj častá produkcia neredukovaných gamét môže prispievať ku koexistencii cytotypov.

4. HYBRIDIZÁCIA

Kríženie medzi dvoma druhmi vyúsťuje do tvorby potomstva majúceho celú škálu intermediárnych znakov. Časť novovzniknutého potomstva však môže vykazovať aj úplne nové, tzv. transgresné znaky (Rieseberg et Ellstrand 1993, Rieseberg 1997). Medzidruhové kríženie tak prispieva k vzniku nových fenotypov, ktoré ak sú reprodukčne izolované od rodičovských druhov, môžu dať základ novým druhom (napr. Ownbey 1950, Abbott et Lowe

2004). Aj keď poznáme len jedného z rodičovských druhov tetraploidnej *Centaurea stoebe* (**Príloha B**), domnievame sa, že polykarpický viacročný cycklus pozorovaný u tetraploida a chýbajúci u diploidného cytotypu (**Prílohy M, N**), vznikol skôr následkom medzidruhovej hybridizácie ako polyploidizácie (**Príloha B**). A to buď „prenosom" z druhého neznámeho rodičovského druhu, ktorý sa mohol rozmnožovať polykarpicky, alebo polykarpia vznikla ako úplne nový znak. Rovnaký jav bol pozorovaný napr. u diploidných hybridov v rode *Tragopogon*, kde oba rodičovské druhy boli monokarpické, ale kríženec polykarpické (Krahulec et al. 2005). Takáto zmena v životnom cykle tetraploidného cytotypu *C. stoebe* mohla uľahčiť jeho šírenie sa mimo jeho prirodzených stanovíšť v Európe (**Príloha A**, pozri tiež Ochsmann 2000), či spôsobiť rozsiahle inváziе v severnej Ameriky, kde bol cytotyp zavlečený (napr. Sheley et al. 1998, Treier et al. 2009). Kvantitatívne zmeny niektorých znakov sme pozorovali aj u hybridogénneho alopolyploidného druhu *Pilosella alpicola* s.str. (**Príloha C**).

Hoci hybridizácia je jedným z najjednoduchších spôsobov tvorby evolučných „noviniek", Burke a Arnold (Burke et Arnold 2001) ukázali, že mnohé hybridy sú sterilné. Úspešný vznik nového hybridogénneho taxónu teda závisí od jeho skorej reprodukčnej stabilizácie a zároveň reprodukčnej izolácie od rodičovských taxónov. Medzi najdôležitejšie mechanizmy prispevajúce k takejto stabilizácii patrí apomiktické rozmnožovanie (viď nižšie), alopolyploidia (viď vyššie) a vznik ďalších pre- a postzygotických bariér (ako napr. chromozómová translokácia, ekologická alebo geografická segregácia a pod.) (Grant 1981). Predpokladá sa, že homoploidná hybridná speciácia (bez zmeny ploidnej úrovne – rodičovské ako aj hybridogénny taxón majú rovnakú ploidnú úroveň) je v prírode vzácnejšia ako alopolyploidná (Rieseberg 1997, Abbott et al. 2010).

Tempo hybridogénnej evolúcie závisí okrem vyššie spomenutých faktorov aj od frekvencie spontánnej medzidruhovej hybridizácie v prírode. Ako ukázali Ellstrand et al. (1996), spontánne kríženie u cievnatých rastlín je síce bežné, ale je nerovnomerne zastúpené medzi jednotlivými taxonomickými skupinami. Napríklad v rode *Hieracium* (Asteraceae) sa predpokladalo, že väčšina apomiktických polyploidných druhov vznikla dávnym medzidruhovým krížením (Zahn 1921-1923), čo potvrdila aj nedávna molekulárna štúdia (Fehrer et al. 2009). Dôkazy o recentnom medzidruhovom krížení však chýbali. Ako sme ukázali v troch našich prácach, recentná hybridizácia medzi diploidnými sexuálne sa rozmnožujúcimi druhmi je v prírode možná, aj keď je skutočne zriedkavá (**Prílohy L, O, P**). Zriedkavosť medzidruhových recentných krížencov v rode *Hieracium* je daná najmä

zriedkavosťou diploidných taxónov (polyploidné sú striktne asexuálne – viď nižšie), ich geografickou a / alebo ekologickou segregáciou, ale tiež efektom indukovaného samoopelenia – tzv. **mentor efektom**, kedy prítomnosť peľu iného druhu môže prelomiť bariéry zabraňujúce samoopeleniu (Richards 1997). Tento jav sme dokázali v experimentálnom krížení viackrát, a to jednak v kríženiach medzi diploidnými druhmi ako aj medzi diploidným a polyploidnými druhmi (**Prílohy Q, R, S**). Indukovaná autogamia tak môže prispievať k reprodukčnej izolácii sympatrických druhov.

Pri dokazovaní hybridného pôvodu morfologicky intermediárnych rastlín v rode *Hieracium* sme použili celú škálu molekulárnych markerov (AFLP – amplified fragment length polymorphisms, sekvencie jadrového ITS – internal transcribed spacer, izozýmy a chloroplastové sekvencie lokusu trnT-trnL a jeho reštrikčnú variabilitu – PCR-RFLP). Tento kombinovaný prístup dokázal nielen hybridný pôvod predpokladaných krížencov (*Hieracium alpinum* × *H. transilvanicum*), ale ukázal, že medzidruhová hybridizácia môže byť obojsmerná, a že disperzia peľu je vzdialenostne efektívnejšia ako disperzia semien, napriek ich adaptácii na šírenie sa na dlhú vzdialenosť (long-distance dispersal) (**Prílohy L, O**).

Poznatky získané zo štúdia prírodných hybridných zón v rode *Hieracium* sme overovali v pionierskej štúdii venovanej experimentálnemu kríženiu (**Príloha R, S**). Zväčša morfologicky intermediárne F1 hybridy boli vysoko sterilné, a často vykazovali nezvyčajný charakter vetvenia. Pri krížení diploidného *H. umbellatum* (matka) a triploidného *H. sabaudum* (otec) sme získali z takmer všetkých krížení len matroklinné potomstvo dokazujúce význam indukovaného samoopelenia ako významnej reprodukčnej bariéry v rode *Hieracium* (**Príloha R**). Hybridy, ktoré vznikli len z jedného kríženia, boli diploidné, čo poukazuje na produkciu a funkčnosť haploidného peľu u triploidného apomikta *H. sabaudum* (**Príloha R**). Naše výsledky sú zatiaľ jedinými úspešnými a interpretovateľnými v rode *Hieracium*, nakoľko predchádzajúce pokusy zlyhávali na použití apomiktických polyploidných rodičovských kombinácií (Ostenfeld 1921, Zlatník 1938, Mendel 1950).

5. APOMIXIA

Apomixia, resp. presnejšie agamospermia je nepohlavná tvorba semien (Asker and Jerling 1992). Hoci presný spúšťač takého rozmnožovania nie je stále známy a je predmetom diskusií (Bicknell et Koltunow 2004), predpokladá sa, že agamospermia je dôsledkom zmien v regulácií génov zapojených do tvorby zárodočných mieškov a okolitých pletív (napr. Carman

1997). Takáto deregulácia génov môže vzniknúť zo stretu dvoch odlišných genómov po hybridizácii a / alebo duplikáciou génomu (tzv. genome collison; cf. Carman 1997), nakoľko takmer všetky doteraz známe apomiktické cievnaté rastliny sú polyploidného pôvodu (Asker et Jerling 1992). Agamospermia prebieha v dvoch etapách. Prvou je tvorba neredukovaného zárodočného mieška, a tým aj neredukovanej vajcovej bunky (apomeióza). Druhou etapou je autonómny vývin embrya bez predchádzajúceho oplodnenia vajcovej bunky (partenogenéza). Podľa toho, kde apomeiotický miešok vzniká, možno agamospermiu rozdeliť na dva základné typy: diplospóriu a apospóriu (Asker and Jerling 1992). V prvom prípade dochádza k vzniku neredukovanej megaspóry a neskôr zárodočného mieška z materskej bunky megaspóry. Takýto typ apomixie je známy v rode *Hieracium* (Rosenberg 1927, Bergmann 1941) a bol mnohokrát potvrdený aj u zástupcov iných rodov z čeľade Asteraceae (*Taraxacum*, *Antennaria*, *Chondrilla*, cf. Gustaffson 1946). Pri apospórii dochádza k tvorbe redukovanej megaspóry a k vývinu redukovaného zárodočného mieška, ktorý však rýchlo abortuje v dôsledku oveľa rýchlejšieho vývinu apospórických neredukovaných mieškov vznikajúcich zo somatických pletív v okolí nucella. Apospória je v rastlinnej ríši ďaleko častejšia ako diplospória a vyskytuje sa aj v rode *Pilosella* (Rosenberg 1907, Pogan and Wcisło 1995). V prípade apospórie nie vždy dochádza k vytesneniu redukovaného mieška neredukovaným apospórickým a potomstvo tak môže vzniknúť pohlavne. V takomto prípade hovoríme o tzv. fakultatívnej apomixii, ktorá je v rode *Pilosella* veľmi bežná (Krahulcová et al. 2000). Naopak v prípade rodu *Hieracium* sa predpokladá, že apomiktické rozmnožovanie je obligátne, keďže zatiaľ neexistuje spoľahlivý dôkaz pre pohlavné rozmnožovanie u apomiktov (Gustafsson 1946). Apomiktický spôsob rozmnožovania možno dokázať viacerými spôsobmi – embryologicky, geneticky, cytometricky alebo v prípade čeľade Asteraceae pomerne jednoducho – kastráciou (Richards 1997). Posledne uvedený test je založený na odrezaní hornej časti úboru tesne pred jeho otvorením. Keďže týmto úkonom dôjde aj k odstráneniu blizny, opelenie a následne ani opelenie nie je možné. Ak aj napriek tomu úbory vytvoria semená, je to dôkaz o ich apomiktickom pôvode. Týmto spôsobom sme určili apomiktický spôsob rozmnožovania u viacerých druhov a cytotypov rodu *Pilosella* (**Prílohy C, I**) a *Hieracium* (**Príloha G**). Je pritom zaujímavé, že doteraz všetky testované polyploidy (tri- a tetra-) rodu *Hieracium* sa rozmnožujú apomikticky, kým mnohé najmä párne polyploidné druhy rodu *Pilosella* sa rozmnožujú sexuálne (**Prílohy C, I**). V rode *Hieracium* tak môžme zistením ploidnej úrovne nepriamo indikovať aj spôsob rozmnožovania.

Sofistikovanejšiu metódu do štúdia apomixie zaviedol Matzk et al. (2001). Je založená na analýze ploidie embrya a endospermu v jednotlivých semenách prietokovou cytometriou. Pri sexuálnom rozmnožovaní krytosemenných rastlín v dôsledku dvojitého opelenia je pomer ploidie endospermu a embrya 3:2. Tento pomer sa však mení u semien vzniknutých nepohlavne. V rodoch *Hieracium* a *Pilosella* je pomer ploidie endospermu a embrya v apomikticky vzniknutých semenách 2:1. Túto metódu sme využili pri skúmaní rozmnožovania, apomeiózy a vzniku cytotypov v skupine *Pilosella alpicola* (**Príloha C**).

Evolučný význam agamospermickej tvorby semien možno vidieť v stabilizácii rozmnožovania hybridogénneho potomstva („escape from sterility hypothesis“) (Darlington 1939). Ten istý autor však tvrdí, že takýto únik pred sterilitou je len únikom do slepej uličky („evolutionary blind alley“), keďže nepohlavné rozmnožovanie výrazne redukuje genetickú variabilitu, a tým znižuje adaptačné schopnosti apomiktov (Darlington 1939). Apomiktické organizmy pri absencii rekombinácie a selekcie navyše hromadia letálne mutácie (efekt, ktorý sa označuje ako „Muller ratchet“, Muller 1932). Oba procesy by teda mali viesť ku krátkej životaschopnosti apomiktov. Ako však ukázali niektoré recentné štúdie, existujú aj apomiktické línie, ktoré vznikli už pred mnohými miliónmi rokov (Schurko et al. 2008, Schwander et al. 2011).

Popri nevýhodách má nepohlavná tvorba semien nesporne aj výhody (napr. Asker et Jerling 1992, van Dijk et Vijverberg 2005, Hörandl 2006). (i) Apomikty sú vďaka unisexuálnemu rozmnožovaniu schopné založiť populáciu s jedným jedincom – majú teda lepšiu kolonizačnú schopnosť ako sexuálne sa rozmnožujúce organizmy. (ii) Apomikty nie sú odkázané na prítomnosť resp. aktivitu opeľovačov – sú teda reprodukčne lepšie zabezpečené. (iii) Keďže mnohé apomikty sú hybridogénneho pôvodu, môžu byť zvýhodnené aj fixovanou heterozygozitou. Lepšiu kolonizačnú schopnosť apomiktických triploidov *Hieracium alpinum* naznačuje ich ďaleko väčší areál – jav označovaný v literatúre ako **geografická partenogenéza** (**Príloha E**). Na druhej strane veľmi malý areál apomiktickej *Pilosella alpicola* s.str. vo švajčiarskych Alpách naznačuje buď recentný vznik a nedostatok času na šírenie sa, alebo obmedzenú schopnosť šírenia sa, alebo určitý selekčný mechanizmus zabraňujúci kolonizácii okolitých a na prvý pohľad vhodných miest na osídlenie (**Príloha C**). Tak ako vo väčšine apomiktických komplexov (Gornall 1999), aj v rodoch *Hieracium* a *Pilosella* apomikty vykazujú ďaleko menšiu mieru genetickej variability ako sexuálne sa rozmnožujúce druhy (**Prílohy D, R**). Je pritom zaujímavé, že kým v rode *Hieracium* majú morphologicky blízko príbuzné taxóny vždy odlišné multilokusové alozýmové genotypy

a možno ich tak spoľahlivo použiť na ich determináciu (**Príloha D**), v rode *Pilosella* tomu tak nie je (**Príloha T**). Je to zrejme v dôsledku ďaleko väčšej miery medzidruhovej a introgresívnej hybridizácie v rode *Pilosella* ako v rode *Hieracium*, kde sú tieto javy veľmi zriedkavé (viď vyššie).

6. ZÁVER

Predložená habilitačná práca stručne zhŕňa význam troch vzájomne súvisiacich mechanizmov a to hybridizácie, polyploidizácie a apomiktického rozmnožovania v evolúcii cievnatých rastlín, a ilustruje dôsledky týchto procesov na konkrétnych príkladoch v rodoch *Centaurea*, *Hieracium* a *Pilosella*. Mnohé tu prezentované výsledky priniesli nové poznatky týkajúce sa vzniku polyploidov a hybridov v študovaných rodoch, taktiež pre- a postzygotických reprodukčných bariér, kontaktných zón cytotypov a cytotypových interakcií, ktoré snáď pomohli posunúť naše poznanie o trochu ďalej, ak už nie vo „veľkej“ vede, tak aspoň v modelových rodoch.

7. LITERATÚRA

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Príloha A

Mráz P, Španiel S, Keller A, Bowmann G, Farkas A, Šingliarová B, Rohr RP, Broennimann O, Müller-Schärer H

Anthropogenic disturbance as a driver of microspatial and microhabitat segregation of cytotypes of *Centaurea stoebe* and cytotype interactions in secondary contact zones

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Anthropogenic disturbance as a driver of microspatial and microhabitat segregation of cytotypes of *Centaurea stoebe* and cytotype interactions in secondary contact zones

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- *Background and Aims* In a mixed ploidy population, strong frequency-dependent mating will lead to the elimination of the less common cytotype, unless prezygotic barriers enhance assortative mating. However, such barriers favouring cytotype coexistence have only rarely been explored in natural mixed ploidy populations.
- *Methods* We investigated microspatial and microhabitat distribution; competitive, life history and fitness traits; flowering phenology and genetic relatedness (cpDNA and microsatellites) of co-occurring diploid plants and their closely related allotetraploid derivates from the *Centaurea stoebe* complex (Asteraceae) to determine the mechanisms involved in coexistence of cytotypes and their interactions at small spatial scale.

- *Key Results* Diploids and tetraploids were genetically differentiated thus corroborating secondary origin of contact zones. The cytotypes were spatially segregated at all sites studied, with tetraploids colonizing microhabitats created by human-induced disturbances. Conversely, they were rare in more natural sites and sites with denser vegetation despite their superior persistence (polycarpic life cycle) and competitive ability (increased number of shoots). The seed set of tetraploid plants was strongly influenced by their frequency in mixed ploidy populations. Triploid hybrids were extremely rare and almost completely sterile, indicating a strong postzygotic barrier between cytotypes.

- *Conclusions* Our findings suggest that tetraploids are recent immigrants into diploid populations and that anthropogenic activities creating open niches and mediating propagule introductions were the major factor shaping the non-random distribution of cytotypes at fine spatial scale. Establishment and spread of tetraploids was further facilitated by their superior persistence through perennial life cycle. Our results

highlight the importance of non-adaptive spatio-temporal processes in explaining microhabitat and microspatial segregation of cytotypes.

Key words: assortative mating, *Asteraceae*, *Centaurea stobe*, cpDNA, cytotype coexistence, disturbance, flow cytometry, microsatellites, polyploidy, reproductive isolation, triploid block.

INTRODUCTION

Mixed ploidy populations consisting of different cytotypes of the same or closely related species offer unique opportunity to study the intercytotype interactions and reproductive isolation mechanisms which may affect the establishment and coexistence of a newly emerged polyploid from sympatric diploids in primary contact zones or a new cytotype immigrated to the population of other cytotype in secondary contact zones (Petit *et al.*, 1999). Regardless the type of contact zone, in both situations the minority cytotype will be subjected to strong frequency-dependent mating with the more frequent cytotype. As a consequence, the rarer cytotype may be progressively eliminated from the mixed ploidy population, since it will produce mainly aborted seeds or sterile hybrids due to strong postzygotic isolation frequently observed in diploid-polyploid crosses ('minority cytotype exclusion'; Levin, 1975).

Although frequency-dependent mating should lead to single cytotype populations, recent studies have shown that sympatric occurrence of cytotypes is more common than previously anticipated (Baack, 2004; Suda *et al.*, 2007; Halverson *et al.*, 2008; Li *et al.*, 2010; Šafářová and Duchoslav, 2010; Šingliarová *et al*., 2011). The disadvantage of the minority cytotype may be overcome by several mechanisms that increase the probability of assortative mating. Such barriers include: (i) microspatial segregation (Baack, 2004; Kolář *et al.*, 2009; Šafářová and Duchoslav, 2010; Trávniček *et al.*, 2011*a*, *b*), which may be correlated with habitat differentiation (Lumaret *et al.*, 1987; Felber-Girard *et al.*, 1996, Hardy *et al.*, 2000, Hülber *et al.*, 2009); (ii) flowering time displacement (Petit *et al.*, 1997; Husband and Sabara 2004); (iii) shift in floral phenotype and physiology, which may alter the spectrum of pollinators (Segraves and Thompson, 2005; Kennedy *et al.*, 2006; but see Jersáková *et al.*, 2010 or Castro *et al.*, 2011); (iv) pollen competition (Husband *et al.*, 2002; Peckert and Chrtek, 2006); (v) shift in breeding system (e.g. switch from allogamy to autogamy, or from

sexual reproduction to parthenogenesis; Kao, 2007) or (vi) break-down of a strict self-incompatibility system and subsequent induced autogamy due to a mixture of heteropolyploid pollen ('mentor effect'; Tas and van Dijk, 1999; Mráz, 2003; Brock, 2004; Hörandl and Temsch 2009). In addition, superior competitive ability (e.g. Buggs and Pannell, 2006; Besnard and Baali-Cherif 2009) or frequent immigration of the minority cytotype may enhance its establishment (Felber, 1991).

Given the high incidence of polyploidy in vascular plants (Levin, 2002) and despite growing number of studies on natural mixed ploidy populations in the last decade, we still know little about the mechanisms underlying the establishment and coexistence of cytotypes in diploid-polyploid contact zones (Soltis *et al.*, 2010). In our study we address these issues using the *Centaurea stoebe* complex as a model system.

The Eurasian *Centaurea stoebe* complex is represented by diploid and tetraploid cytotype occurring in predominantly single cytotype populations (Treier *et al.*, 2009; Mráz *et al.*, unpubl.). Interestingly, several mixed ploidy populations have been recorded in Central Europe and were interpreted as a putative primary contact zone with recurrent formation of tetraploids (Španiel *et al.*, 2008). However, Mráz *et al.* (2012) recently found that tetraploid cytotype originated from hybridization between the diploid *C. stoebe* and one still unknown closely related taxon. Therefore, an allopolyploid origin of tetraploid cytotype questions the hypothesis on *in situ* evolution of tetraploids and rather suggests a secondary contact zone in Central Europe. Despite the higher frequency of diploid populations in Europe and a largely sympatric distribution with tetraploid populations, only the latter have been recorded in the introduced range in North America (Treier *et al.*, 2009; Mráz *et al.*, 2011), where they have become highly invasive (Sheley *et al.*, 1998). Such a pattern may indicate either stochastic introduction or possible post-introduction selection that favoured the tetraploid cytotype (Müller-Schärer *et al.*, 2004). Importantly, both cytotypes differs in their life history. While

diploids are monocarpic, tetraploids are predominantly perennial polycarpic (Bogs and Story 1987; Ochsmann, 2000; Mráz *et al.*, 2011). Such difference might provide tetraploids with a superior colonization ability when comparing to diploids through a longer life span and repeated seed reproduction, which in turn might lead to the replacement of diploids. Indeed, this process has recently been observed at the margin of the species range in Germany where tetraploids replaced the diploids on deluvial sediments (Wells *et al.*, 2008). The natural mixed ploidy populations of *C. stoebe* thus provide a unique opportunity to study pre-zygotic barriers allowing cytotype coexistence and their potential interactions under natural conditions at small spatial scale.

The main goal of the study was to explore the microspatial distribution (from a few centimetres to several tens of meters) and frequency of cytotypes in narrow contact zones and to infer whether there is any correlation between cytotype distribution and microhabitat characteristics (soil moisture, vegetation density). We assessed also the relative fitness of both cytotypes by measuring of some vegetative (number of bolting stems, number of accessory rosettes) and reproductive traits (seed set, germination). In addition, using two molecular markers we aimed to provide molecular evidence on secondary contact zone in Central Europe and assess the intrecytotype gene flow and the origin of intermediate cytotypes.

MATERIAL AND METHODS

Study species

Centaurea stoebe is a herbaceous species distributed from westernmost Asia to Western Europe with centre of distribution confined to South-eastern and Eastern Europe (Meusel and Jäger, 1992). It is represented by two cytotypes, diploid ($2n = 2x = 18$) and the allotetraploid

(2*n* = 4*x* = 36), which are treated sometimes as separate taxa (Ochsmann 2000), but their nomenclature is still unresolved (Mráz *et al.*, 2011). The cytotypes are morphologically very similar, especially in the field (Španiel *et al.*, 2008). However, typical representatives of cytotypes can be distinguished as shown a recent morphometric study based on plants grown from seeds and cultivated under uniform conditions (Mráz *et al.*, 2011). Besides differences in the shape of capitula, the number of inner florets or branching pattern, the most striking difference between cytotype is the life history. While the diploids have a predominantly annual-biannual monocarpic life-cycle, the tetraploids are usually shortly perennial polycarpic plants forming overwintering accessory rosettes (Bogs and Story 1987; Ochsmann, 2000; Mráz *et al.*, 2011). Both cytotypes are pollinated by a wide spectrum of insects and are strictly self-incompatible (Harrod and Taylor, 1995; Beil *et al.*, 2008; Mráz *et al.*, unpubl.). The seeds of *Centaurea* species have only a very short pappus, not allowing effective wind dispersal. As a consequence, most of the seeds are dispersed within a few decimetres from their mothers by falling down from open dehydrated flower heads or by flicking the loosely held achenes due to a movement of the stem by wind or passing animals (Sheldon and Burrows, 1973; Witztum *et al.*, 1996; Colas *et al.*, 1997). At a longer, more than 1 m distance the *Centaurea* seeds may be dispersed by ants (Imbert, 2006; Mráz pers. observations), grazing animals (Wessels-de Wit and Schwabe, 2010) or anthropogenic activities (attached to undercarriages of vehicles, to shoes etc., Sheley *et al.*, 1998).

Sampling

The study was performed in August 2007-2009 in south-western Slovakia and north-eastern Austria where four mixed ploidy populations out of eleven known in total (Mráz *et al.*, unpubl.) have been reported in the literature (Španiel *et al.*, 2008; Treier *et al.*, 2009). Since

we suspected more mixed ploidy populations in this region, we performed additional field surveys of six other populations from which two were revealed to be mixed and four to be pure diploid populations. In total, ten populations occurring in various types of habitats and with different land use history were studied for cytotype structure (Table 1, Fig. 1, Fig. S1). In each site we first performed an initial cytotype screening of 30 plants along a transect across a mosaic of heterogeneous microhabitats to determine cytotype frequencies and the position of the contact zone. Subsequently, in six mixed ploidy populations we labelled at least 100 randomly selected plants at the flowering and rosette stage. The exact positions of plants were determined triangularly using a measure tape. From each labelled plant we sampled fresh leaf tissue to determine its ploidy level. From a subset of plants, leaf material was also sampled and stored in silica-gel for molecular analyses. Microspatial cytotype distribution was studied in five populations only, as one site (GLA) was partially destroyed in 2009. For that population we noted only the approximate position of 30 plants sampled during the initial transect study in 2008. In 2009, we localized 120 plants at this site, but all plants were diploid, because the small patch with tetraploid plants found in 2008 was destroyed (Table 1, Fig. S1).

Traits and environmental variables

For each labelled plant at mixed-cytotype sites, with the exception of MAR, we recorded the presence (number) / absence of accessory rosettes and the number of stems of flowering plants as a proxy for perenniality and competitiveness, respectively. To estimate the reproductive output per plant we determined the seed set of all capitula that were mature at the sampling time at four sites. As environmental variables we estimated vegetation cover around each individual (20×20 cm) using a semi-quantitative scale ranging from 1 (0-5% of

vegetation cover) to 5 (75-100%) corresponding to the Braun-Blanquet scheme for vegetation plots (Braun-Blanquet, 1928). In addition, at SAND, WEIT and KOP we took 2-3 measurements of soil moisture in the vicinity (20 × 20 cm) of randomly-selected plants with known ploidy using a Theta Probe soil moisture sensor (Delta T Devices, UK) equipped with 12 cm long rods. The soil moisture measurements were performed before noon and lasted for 1-2 hours maximum to avoid temporal variations in soil humidity at the study site. Only the mean value of soil moisture around each measured plant was used for statistical analyses. Because of strong differences in flowering phenology between cytotypes at KOP on the sampling day (14 August 2009), we recorded the number of withering plants without any open flower and flower-head bud, and the number of plants having at least one still flowering capitulum or flower head bud before anthesis on all study plants of both cytotypes.

Germination experiment

To compare the rate and speed of germination between cytotypes, we sowed 20 seeds from each of 20 randomly-selected mother plants per cytotype collected at SAND in 2008. The seeds stored at room temperature were exposed to cold treatment (4°C) before sowing for three weeks. On 7 January 2009 the seeds were planted in 2×2 cm cells at 1cm depth in seedling trays filled with a 2:1 mixture of sterilized compost and sand. The seedlings were cultivated in a heated greenhouse with 16h artificial light per day and 23°C day / 15°C night temperature, and watered every 3–4 days. Emergence time (i.e. the number of days from sowing to germination) was inspected every day between 15-18 p.m. over a period of 60 days. At the end of the experiment (8 March 2009) we calculated the proportion of germinated seeds per plant and per cytotype.

Flow cytometry and karyology

The samples were cytometrically analysed using a Partec Cyflow cytometer (Partec GmbH., Münster, Germany) equipped with a mercury lamp in the Institute of Botany in Bratislava, while the ploidy of seedlings from the germination experiment were assessed using a Partec Cyflow SL cytometer equipped with a green laser at the Department of Biology in Fribourg. In the first case, the samples consisting of fresh leaf tissue were prepared in a two-step procedure using Otto's extraction buffer and staining buffer containing 4′,6-diamidino-2-phenylindole (DAPI) (Otto, 1990; Doležel and Göhde, 1995); in the second case, we used general purpose buffer and propidium-iodide (PI) as a stain following the protocol of Loureiro *et al.* (2007). *Bellis perennis* L. was used as an internal reference standard in both procedures (Španiel *et al.*, 2008). The DNA-ploidy level was inferred as a relative position of the G1 peak of sample to the position of G1 peak of internal standard. The exact chromosome number of one germinated seedling (DK-293-P10) produced by triploid plant (DK-293) was determined by chromosome counting (for method see Mráz *et al.*, 2011).

DNA extraction and molecular analyses

DNA was extracted from 10–15 mg of silica-dried leaf tissue with the DNeasy 96 Plant Kit and DNeasy Plant Mini Kit (Qiagen) following the manufacturer protocol.

Plastid DNA. The *trnT-trnL* and *atpB-rbcL* loci were sequenced usually in six plants per population and cytotype. The loci were amplified using primers developed by Taberlet *et al.* (1991) and Chiang *et al.* (1998), respectively. Amplifications were performed in 25 µl volume containing 5 µl of genomic DNA (4 ng.µl^{-1}), 2.5 µM buffer (PCR Buffer 10×), 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, 0.25 µM BSA and 1 U of Taq

polymerase (Qiagen). The cycle profile included the initial denaturation at 94°C / 3 min followed by 36 cycles of 94°C / 30 s, 50 °C / 30 s, 72 °C / 1 min, and ended with 72 °C / 5 min and 4 °C thereafter. Sequences were edited manually using Chromas Lite 2.01 (http://www.technelysium.com.au/chromas_lite.html) and assembled using Mega 4.01 (Tamura *et al.*, 2007).

Microsatellites. Microsatellites were analysed in 248 plants collected at SAND to estimate the relatedness between ploidies as well as the origin of one triploid plant. In addition, SSRs were used to determine the origin of rare progeny found in two tetraploid plants at GLA, as we supposed that mixture of pollen from predominant diploids and rare tetraploids could induce selfing in tetraploids ('mentor effect', see above). Details of four SSRs loci developed using expressed sequence tags (Barker *et al.*, 2008) were kindly provided by R. Kesseli and D. Tsirelson (Boston) and are given in Table S1. Amplifications were performed in 25µl reaction volume containing 1×PCR buffer, 2.5 mM $MgCl_2$,1x BSA, 0.25 mM of each dNTP, 0.08 µmol of forward primer with M13(−21) tail, 0.4µMol of reverse primer and 0.32 µmol of fluorescently labelled M13(−21) universal primers, 1U Taq polymerase (Qiagen), and 2µl of diluted (20 $ng.\mu l^{-1}$) genomic DNA. The cycle profile included the initial denaturation at 94°C / 5 min followed by 36 cycles of 94°C / 30 s, 55°C / 30 s, 72 °C / 30 s and ended with 72 °C / 5 min and 4 °C thereafter. PCR products were multiplexed and analysed using an ABI 310 genetic analyser (Applied Biosystems). Only presence / absence of alleles and not their dosage were used to genotype individual plants.

Statistical and data analyses

Spatial aggregation of cytotypes within each site was assessed using Mantel tests where the correlation between pair-wise geographical distances of plants and pair-wise cytotype

'distances' (0 for the plants of the same cytotype and 1 for the different one) was computed and statistically evaluated using 9999 randomizations. Association between cytotypes and the five vegetation cover classes was tested by chi-square tests for the whole dataset and separately for each site. Chi-square test was used to assess differences in flowering between cytotypes at KOP. Differences in soil moisture between micro-sites of diploids and tetraploids, as well as the germination rate from the germination experiment, were assessed using t-tests. Difference in the probability of forming accessory rosettes for each cytotype for the whole data set was tested using a generalized linear mixed effect model, with binomial distribution and logit link function with cytotype as a main factor and population nested in cytotype as a random factor. Wilcoxon non-parametric tests were used to test differences between the cytotypes in seed production, number of shoots and accessory rosettes per plant, and germination speed from the germination experiment. To test whether the seed production of tetraploid plants depends on theirs proportions in mixed ploidy populations, we used a linear mixed effect model with log transformed values of number of seeds plant as response variable and ploidy and proportion of tetraploids per site and their interaction as explanatory variables, with population as a random factor. Genetic relatedness between cytotypes was assessed by principal component analysis based on presence / absence of the alleles at four SSRs loci. All statistical analyses and plotting were carried out using several packages implemented in R (R Development Core Team, 2009). To visualize microspatial cytotype distribution in different vegetation classes at SAND we interpolated geographically the vegetation density plots using the inverse distance weighted technique (IDW function in Spatial Analysts extension for ArcGIS$_{TM}$ (ESRI, 2011), with a variable search radius including 12 points (i.e. standard parameters)). The microspatial distribution of cytotypes was then overlaid onto this layer. A haplotype network based on substitution and insertion-

deletion polymorphisms of two assembled cpDNA loci was constructed using the median-joining algorithm in Network v. 4.6.0.0 (www.fluxus-engineering.com; Bandelt *et al.*, 1999).

RESULTS

Cytotype frequency

Diploid plants were more abundant than tetraploids in five of the six mixed-ploidy populations studied (Table 1, Fig. 1). Although at MAR we sampled more tetraploids in 2007 (Table 1), inspection of this site in 2010 revealed that diploids were more common than tetraploids when also considering the area outside of the transect studied in 2007 (Mráz pers. observation). Frequent pure diploid populations in the close vicinity of mixed-cytotype sites confirm the general predominance of the diploid cytotype in the region (Table 1, Fig. 1). Single triploid plants were found at two sites (SAND and MAR) representing 0.3 and 0.8 % of plants analysed at these sites, respectively. These data are the first record of triploidy in the *C. stoebe* group. The very low frequency of triploids was corroborated by analysing 449 seedlings from the germination experiment. Mother plants of the SAND site exclusively produced progeny of their own ploidy level, with the exception of one triploid seedling (DK-189-P4) from one tetraploid mother plant (DK-189). Similarly, only tetraploid offspring (24 in total) were produced by two tetraploid plants from the predominantly diploid GLA population. Flow cytometry analysis of one seedling (DK-293-P10) of a triploid mother plant (DK-293) from the SAND site indicated an aneuploid ploidy level (Fig. 2) and counting of the exact chromosome number showed $2n = 21$. Thus, the seedling had three extra chromosomes when compared to the diploids ($2n = 18$, Fig. 2).

Spatial and habitat segregation of cytotypes at the micro-scale

Spatial analyses revealed significant segregation of diploid and tetraploid cytotypes at all sites (Table 2, Fig. 3). Though not studied in detail due to destruction of the site, three tetraploids at GLA were also found clustered together within one patch. In general, the ratio between the proportion of diploid plants and the proportion of tetraploid plants significantly increased with increasing vegetation density (linear regression, $p = 0.0055$, $r = 0.96$) (Fig. 4). However, when analysing each site separately, this pattern was significant only at SAND, where the 4x plants had a clear tendency to occupy more open sites (Table 2, Fig. S2). Non-significant differences in other sites were likely caused by the lower numbers of plants analysed at other sites (cf. Table 1), and in some cases also by the absence of the densest vegetation classes (4 and 5). Importantly, the occurrence of 4x plants was strongly associated with human-induced disturbance (see details to each site in Table 1, Fig. S1). Specifically, at SAND the 4x plants were most abundant on a sand pile – a remnant from ancient sand exploitation. At WEIT the 4x plants occurred in crevices of a limestone quarry wall and at disturbed sites in the close vicinity. At KOP the tetraploids were concentrated on and along a dirt road at the entrance to a Natural Reserve and in small gravel pits exploited in the 1970s. Furthermore, tetraploids were never found in non-disturbed parts of steppe meadows, where only diploids were present. At TLM the tetraploids were found exclusively along the road and railway tracks leading to an andezite quarry, but were completely missing in semi-natural steppes and natural andezite rock outcrops situated above the road and rails, where diploids were abundant. Similarly, tetraploids at MAR were found along a dirt road near to a gravel pit. Finally, at GLA three tetraploids were found at the start of a dirt road under a recently built hill. Lower soil moisture values measured around the 4x plants as compared to 2x plants at WEIT and SAND (only significant at WEIT) furthermore indicates an association of

tetraploid plants with drier micro-sites (Table 2, Fig. S3). At KOP no such pattern was found, probably because of the high level of groundwater on this Danube island (Table 2, Fig. S3).

Persistence, competitive and fitness traits

The probability to produce accessory rosette(s) in flowering plants and thus to reproduce polycarpically was significantly higher in tetraploid than in diploid plants (34.4% of flowering tetraploids *versus* 2.6% of flowering diploids produced accessory rosettes) (Table 2, Fig. S4). Moreover, tetraploids produced on average significantly more shoots (4.45) and more accessory rosettes (1.18) per flowering plant than diploids (1.93 and 0.04, respectively) (Table 2, Figs. S5 and S6). The number of shoots per plant in diploids was negatively correlated with vegetation density (Spearman test for whole data set, $r = -0.23$, $p < 0.001$), indicating a possible environmental effect on this trait, but no such correlation was observed in tetraploids (Spearman test for whole data set, $r = 0.02$, $p = 0.8$). Diploids produced significantly more seeds not only per plant, but also per shoot and per flower head (Wilcoxon test, $p < 0.001$ for both traits) at each of the study sites (Table 2, Fig. 5). However, the seed production of tetraploid plants was dependant on the frequency of tetraploids in the population; two rare tetraploids from GLA produced significantly fewer seeds, than the tetraploids from other mixed ploidy populations, where they were more common (linear mixed-effect model, $t = 2.62$, $p = 0.009$; Fig. 5). One triploid plant from SAND produced only 20 mature seeds of unusual rounded shape and they did not germinate with exception of one seed. The diploids and tetraploids from SAND did not differ in average germination rate per plant (57.5% and 54.2%, respectively; t-test, $t = 0.45$, $p = 0.7$; Fig. S7), nor in the time of seedling emergence (median: 8 and 7 days, respectively; Wilcoxon test, $p = 0.5$; Fig. S7). At KOP many more tetraploids then diploids were still flowering when visited on 9 August 2009

(93.5% and 54.8%, respectively; Chi-square test = 18.22, $p < 0.001$; Fig. S8), while almost half of the 2x plants had already withered.

Haplotype distribution among cytotypes

In total, twelve cpDNA haplotypes were found in the 69 plants studied (Table 3, Table S2). In diploids, five haplotypes with low frequency (0.015 – 0.07) were unique for 2x, while four haplotypes were shared with tetraploids. Of these shared haplotypes three were very frequent (H1, H2, H12; Fig. 6). In tetraploids, three haplotypes of seven were unique for 4x (H9, H10 and H11), with one of them being frequent (H9, 0.15). No shared haplotypes between cytotypes were found at KOP and MAR. At the remaining sites some of the 2x and 4x plants shared one of the most common haplotypes (H1, H2, H12), but with different proportions (Table 3). The triploid plant from MAR (Ma-134) had the same haplotype (H2) as the majority of co-occurring diploids, while the triploid plant from SAND (DK-293) shared its H9 haplotype with co-occurring tetraploid plants (Table 3, Table S2).

Pattern of microsatellite variation

In total, 48 alleles were found among 247 plants in SAND, with 33 alleles shared between diploids and tetraploids (Table 4). Seven private alleles were found in diploids, of them two with relatively high frequency (0.52 and 0.23). Similarly, tetraploids harboured eight private alleles, with one of them being very frequent (0.76) and two alleles were moderately frequent (0.18 and 0.14) (Table 4). One triploid plant (DK-293) was highly heterozygous, showing at each locus three different alleles (Table 4). Interestingly, of the three alleles found in this triploid at the CM-8337 locus, two alleles were private for tetraploids and one was unique for

diploids, thus strongly suggesting a hybridogeneous origin of this triploid (Table 4). Principal component analyses revealed two slightly overlapping clusters of plants corresponding to diploids and tetraploids (Fig. 7). None of the 24 tetraploid offspring from the two rare tetraploid mother plants sampled at GLA arose from self-pollination, as all analyzed offspring exhibited some non-mother alleles at least at one locus (Table S3).

DISCUSSION

Evidence for secondary origin of narrow contact zones

In agreement with expectation, both molecular markers showed a certain level of genetic differentiation between cytotypes and hence corroborated a secondary contact zone. Some fraction of variation was however shared between cytotypes. This shared polymorphism might be simply explained by the close relationship between the diploid progenitor and its allotetraploid descendent (Mráz *et al.*, 2012) although further additional non-exclusive processes like incomplete lineage sorting or occurrence of homoplasies are possible too (Font *et al.*, 2009; Löser *et al.*, 2009; Mráz *et al.*, 2012).

The role of anthropogenic disturbances and other non-adaptive processes in microspatial and microhabitat segregation of cytotypes

We found strong microspatial cytotype segregation at every mixed ploidy population of *C. stoebe* studied. Our data thus confirm the results of a handful of recently published studies focused on microspatial cytotype distribution showing non-random patterns (Baack, 2004;

Kolář *et al.*, 2009; Hülber *et al.*, 2009; Kao and Parker 2010; Šafářová and Duchoslav, 2010; Trávniček *et al.*, 2011*a*, *b*). Only rarely no cytotype segregation was reported (Halverson *et al*., 2008). Such non-random distribution of cytotypes at fine spatial scale can results from several non-exclusive processes: (i) ecological differentiation between cytotypes, (ii) frequency-dependent exclusion at small spatial scale, (iii) limiting seed dispersal and (iv) historical processes.

Our data clearly indicate ecological differentiation (thus process (i)) of cytotypes of *C. stoebe* with tetraploids occurring more frequently in open and drier micro-sites, while they were less common than diploids in more dense grassland communities. Microhabitat segregation has been reported only in few diploid-polyploid complexes, e.g. in *Anthoxanthum alpinum* (Felber-Girard *et al.*, 1996), *Senecio carniolicus* (Hülber *et al.*, 2009), the *Dactylorhiza maculata* group (Stahlberg, 2009) or *Solidago altissimma* (Richardson and Hanks, 2011), and these patterns were explained as differences in competitive ability and / or tolerance to stress (but see Hülber *et al.*, 2011). Therefore, the pattern observed in mixed ploidy populations of *C. stoebe* might suggest better adaptation of tetraploids to water stress. However, microhabitat segregation of cytotypes was spatially correlated with local anthropogenic disturbances at all studied sites. Because tetraploids preferentially (at SAND) or solely (at KOP, TLM, WEIT) occurred at ruderal microhabitats and were rare or even absent in more natural microsites, such a pattern indicates close association with recent anthropogenic activities carried out at the scale of several metres or tens of metres (see Table 1). Furthermore, the cytotypes did not respond differentially to water stress treatments in a greenhouse experiment (Mráz *et al.*, in prep.). Thus, a non-adaptive scenario (hypotheses ii-iv above) might be more plausible to explain the observed cytotype segregation.

Based on distributional data and historical land use of sites (Table 1), we suggest that such a pattern is likely a result of relatively recent immigration of tetraploids into already

established diploid populations, where human activities not only created new open niches suitable for colonization by the tetraploid newcomers but also mediated, likely through transport of material (see Table 1), the introduction of tetraploid propagules over the last 100 years (see below). Thus, an unintentional introduction of tetraploids to locally disturbed sites had to contribute to the non-random distribution of cytotypes. Such scenario is also supported by following facts. Firstly, mixed ploidy populations were found only on sites with recent human impacts (quarries, gravel pits, transport routes; Table 1, Fig. S1), while pure diploid populations were more frequent and found also in more natural sites in the region studied (Španiel *et al.*, 2008 and this paper). Secondly, within mixed ploidy populations diploids were more numerous and exhibited a wider ecological niche than tetraploids by occupying not only ruderal microsites but also natural or semi-natural open stands like dry meadows, pastures, steppes or even natural rocky outcrops (like at TLM). Thirdly, based on the inspection of herbaria covering the study area (BRA, SAV, SLO – herbaria acronyms according to Thiers, 2011; Mráz, unpubl.) the occurrence of the diploid cytotype in Bratislava and surrounding is recorded through herbarium specimens since 1871 and onwards, while the earliest undoubtedly tetraploid collection dates to 1919. Similarly, at TLM the earliest documented occurrence of diploids is from 1938, while tetraploids were collected here for the first time only in 2005 (Španiel *et al.*, 2008). Altogether, these data suggest very recent (may be less than 100 years) introductions of tetraploid cytotype in the mixed-ploidy populations studied. Such a pattern is in an agreement with the documented recent spread of the tetraploid cytotype in Western and Central Europe, by following the main transport corridors (Ochsmann, 2000; Korneck, 2004; Wells *et al.*, 2008). Fourthly, although assuming better persistence and increased interspecific competition ability through perenniality and multi-stem formation in tetraploids in denser grassland communities, they were less frequent in such vegetation than monocarpic diploids suggesting limited time available for tetraploids to

spread wider into the more closed grasslands. Importantly, absence of long distance dispersal mediated by grazing domestic animals (goats, sheep) over the fifty years (see Table 1; D Senko, Institute of Botany, Bratislava, Slovakia, 'pers. comm.') may also have contributed to the restricted distribution of tetraploids at fine scale. We suggest that these spatiotemporal changes considerably contributed to the present distributional pattern of cytotypes in mixed ploidy sites. Historical, non-adaptive processes probably played an important role in habitat segregation also in two other diploid-polyploid complexes, namely in *Deschampsia caespitosa* in the British Isles and *Taraxacum* sect. *Ruderalia* in Switzerland, where polyploids have colonized recently disturbed sites while diploids are mainly found on more relict or semi-natural habitats (Rothera and Davy, 1986; Meirmans *et al.*, 1999).

In addition to historical introductions of tetraploid cytotypes, other non-adaptive processes have been likely involved in the formation and the maintenance of microspatial segregation between the *C. stoebe* cytotypes. Firstly, prevailing short-distance dispersal in *Centaurea* spp. (see Material and Methods) will lead to cytotype clustering at a small scale, which in turn will enhance intraploid pollination and ultimately favour cytotype coexistence (see also Baack, 2005). Secondly, minority cytotype exclusion might indeed have reduced the fitness of the less frequent cytotype as observed at GLA (Fig. 5) and thus contribute to cytotype homogenization over small distances. Thirdly, we found a significant shift in flowering time between ploidies at the KOP site. Such flowering asynchrony may also substantially limit interploid gene flow. Interestingly, no obvious shift in flowering was observed at other sites, suggesting an influence of specific site conditions, different local genotypes, or both.

Intercytotype interactions and evidence for a strong postzygotic reproduction barrier

The frequency of the intermediate cytotype can provide good evidence for the level of effective gene flow between cytotypes. Of 1307 cytometrically analysed plants and seedlings from six mixed ploidy populations, only three triploids were found. Although triploids could arise also among diploid plants through unreduced gamete formation (Ramsey and Schemske 1998), our combined molecular data (SSRs, cpDNA and ITS; present data and Mráz *et al.*, 2012) and the absence of triploids in pure diploid populations clearly indicate their origin through intercytotype crosses. Moreover, maternally inherited cpDNA data show that these crosses were bidirectional, i.e. both diploid and tetraploid plants could serve as mother or pollen parents.

Although the rare occurrence of interploid hybrid could be explained by fine spatial segregation of diploids and tetraploids, many plants of both cytotypes grew intermingled or in very close proximity (several centimetres or decimetres) (Fig. 3). Therefore, intensive intercytotype pollen transfer can be expected in these plants as (i) foraging pollinators usually move short distances between flowers, often visiting neighbouring plants (Mitchell *et al.*, 2009), (ii) the generally observed synchrony in flowering between diploids and tetraploids of *C. stoebe*, and (iii) the observation that pollinators readily move between the cytotypes of *C. stoebe* in common garden experiment and in the field (Min Hahn, University of Fribourg, Switzerland, 'pers. comm.'; P Mráz, observ.). Thus, together with the difficulties to obtain viable triploid progeny from artificial crosses (Mráz *et al.*, unpubl.), our field and experimental data indicate strong postzygotic isolation likely caused by the early abortion of embryos ('triploid block' mechanism; Marks, 1966). Strong postzygotic isolation between diploids and tetraploids has been frequently observed in other diploid-polyploid complexes, including *Centaurea* species (Hardy *et al.*, 2001; Koutecký *et al.*, 2011), hence supporting the largely accepted view that polyploidization is among the most important speciation

mechanisms in vascular plants (Levin, 2002). The only progeny of the triploid plant from SAND was aneuploid and likely arose from fusion of an aneuploid ovule (n = 12) of a triploid mother with strongly irregular meiosis and haploid pollen (n = 9) likely from a diploid donor.

Differences in competitive and fitness traits

In concordance with common garden experiments (Henery *et al.*, 2011), tetraploids in mixed ploidy populations produced significantly more shoots, but less seeds per plant than diploids. Overall lower seed production in tetraploids can be explained by structural differences between cytotypes, namely by their significantly lower number of florets per capitulum and capitula per stem (Mráz *et al.*, 2011). Importantly, the reduction in seed set in tetraploids was the most pronounced in GLA, the site with the lowest proportion of tetraploids among the mixed ploidy sites. This strongly suggests frequency-dependent mating disadvantage of the minority cytotype, thus confirming experimental data (Husband, 2000). Furthermore, our field data corroborate earlier findings that tetraploids more frequently produced accessory rosettes resulting in a predominantly polycarpic life cycle in contrast to diploids (*Mráz et al.*, 2011). This difference was, however, smaller in this field study (c. 34% of 4x *versus* ca 2% of 2x plants exhibited polycarpy) than in a previous greenhouse experiment (c. 65% of 4x *versus* ca 3% of 2x; Mráz *et al.*, 2011). This discrepancy is most probably due to the fact that the field data were gathered during the first half of August when most of the shoots are still green, while the formation of accessory rosettes is stimulated by withering that happens under field conditions in late summer and autumn (Mráz *et al.*, 2011). Thus later observations are expected to provide much better estimates of polycarpy in tetraploids.

CONCLUSIONS

Our study demonstrates that anthropogenic disturbance and later immigration of the tetraploid cytotype into established diploid populations are the main causes in creating the present spatial segregation of cytotypes and along with this, habitat differentiation even at a very small spatial scale. Thus, habitat cytotype segregation at different spatial scales might not necessarily be associated with differences in the adaptive potential of the cytotypes (e.g. Ramsey, 2011; Manzaneda *et al.*, 2012), but may result from various spatio-historical processes, their timing and interactions (different centres of speciation or refugia, colonization routes, anthropogenic disturbance; Stebbins, 1985; van Dijk and Bakx-Schotman, 1997; Mandáková and Münzbergová, 2006; Mráz *et al.*, 2008; Cosendai and Hörandl, 2010). Besides spatio-temporal processes, our results highlight additional non-adaptive mechanisms contributing to the establishment and maintenance of spatial segregation of cytotypes of *C. stoebe* at the small spatial scale. Most important are differences in the life cycle, limited seed dispersal and asynchronous flowering (at one site). Furthermore, our study is, to our knowledge, the first one demonstrating a relationship between the frequency of the less common cytotype and its fitness (seed set) in natural mixed ploidy population. Frequency-dependent mating thus could further contribute to the spatial aggregation of cytotypes at the small spatial scale. Given the higher persistence ability of the tetraploid cytotype as compared to the diploid cytotype, these natural contact zones of *C. stoebe* offer most rewarding study sites to follow spatio-temporal dynamics of the cytotypes also over a longer time scale.

SUPPLEMENTARY DATA

Supplementary data consist of: Table S1: details about the four microsatellite loci used in present study; Table S2: list of sequenced diploid, triploid and tetraploid plants of *Centaurea stoebe* and their haplotypes and GenBank accession numbers; Table S3: allelic composition at

four microsatellite loci of two tetraploid plants sampled at the GLA site and their progeny; Fig. S1: habitats of six mixed ploidy populations of *Centaurea stoebe*; Fig. S2: Cytotype distribution at SAND across heterogeneous microhabitats with different vegetation density; Fig. S3: averaged soil moisture around randomly selected diploid and tetraploid plants in three mixed ploidy populations; Fig. S4: estimated mean proportions of plants forming accessory rosettes for diploids and tetraploids; Fig. S5: number of shoots in diploid and tetraploid plants; Fig. S6: number of accessory rosettes in diploid and tetraploid plants; Fig. S7: germination rate per plant and germination speed in diploid and tetraploid plants at SAND; Fig. S8: proportions of plants that were still flowering and that had finished flowering at the mixed ploidy KOP site on 14 August 2009.

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FIG. 1. Distribution of mixed ploidy populations and pure diploid populations of *Centaurea stoebe* in Central Europe included in the present study. Red colour represents the diploid and blue colour the tetraploid cytotype. Population codes and exact proportion of cytotypes are given in Table 1.

FIG. 2. Histograms of absolute DNA content of propidium-iodide-stained nuclei of (A) a diploid plant ($2n = 2x = 18$) of *Centaurea stoebe*, (B) an aneuploid progeny (DK-293-P10, $2n = 21$, see embedded photograph of its mitotic chromosomes in the right corner, scale bar = 5 μm) of a triploid hybrid (DK-293) found at the SAND site, and (C) *Bellis perennis* used as an internal standard. Photo credit: P. Mráz.

FIG. 3. Spatial distribution of diploid, triploid and tetraploid plants of *Centaurea stoebe* in five mixed ploidy populations. Dirt (MARCH, KOP) and asphalt roads (TLM) are schematically drawn. For site abbreviations see Table 1.

FIG. 4. The ratio between the proportion of diploid and the proportion of tetraploid plants of *Centaurea stoebe* occurring in five vegetation density classes (1 – without vegetation, 5 – very dense vegetation; see Material and Methods) at five mixed-ploidy sites.

FIG. 5. Seed production per plant in four mixed ploidy populations of *Centaurea stobe*. The number of analysed plants per ploidy level and respective site is given above each box plot.

FIG. 6. Haplotype network of twelve haplotypes from 69 accessions of *Centaurea stoebe* based on combined *trn*T-*trn*L and *rbc*L-*atp*B sequences. Three different ploidies are marked

by different colours. The size of each pie plot is proportional to the number of accessions sharing the haplotype.

FIG. 7. Principal component analysis of 168 diploid, 79 tetraploid and one triploid plant of *Centaurea stoebe* from the SAND population based on the presence / absence of 48 SSR alleles. Confidence ellipses were defined by the gravity centre (centroid) of the cloud and 1.5 standard deviations. Outlier position of one tetraploid (DK-314) is due to one unique allele, which was, however, found to be relatively frequent in the tetraploid plants from Serbia (Mráz unpubl.), therefore this individual was kept in the analysis.

TABLE 1. *Cytotype structure, habitat description and historical and present use of mixed-cytotype sites of* Centaurea stoebe *and pure diploid populations screened in the initial transect study*

Site's acronym	N plants / cytotype composition			Coordinates (N / E)	Altitude (m)	Site description
	2x	4x	3x			
SAND	198	116	1	48.201 / 16.974	192-196	Slovakia; Devínska Nová Ves, Sandberg hill: steppe on tertiary sands, since 1964 Nature Reserve, from 1897 to 1960s exploited for sand, until 1950s intensively grazed, grazing definitely abandoned in 1964 (Klačka and Pokorný 1995).
WEIT	77	34	0	48.194 / 16.980	200-204	Slovakia; Devínska Nová Ves, Weit quarry: grasslands at the bottom of old limestone quarry and rock crevices, since 1964 Nature Reserve, intensively exploited from 1897 to 1932, surrounding intensively grazed until 1960s (Klačka and Pokorný 1995).
KOP	88	49	0	48.097 / 17.161	134-136	Slovakia; Podunajské Biskupice, Kopáč island on the river of Danube: steppe grasslands on gravel sediments, the island had been formed during a big flood around 1809, since 1976 Nature Reserve, until 1960s grazed, in 1960-1970s the gravel exploitation for building of the private houses in neighbour villages (Pišút 2002, Pišút and Timár 2007).
TLM	76	41	0	48.297 / 18.537	180-190	Slovakia; Tlmače, Kusá hora hill: rocks, steppe and shrub vegetation on steep slope on andezite background, and ruderal vegetation along the asphalt road and railway going to the still working andezite quarry open in 1930s (Breznická 2008).
MAR	40	83	1	48.273 / 16.888	140	Austria; Marchegg: ruderal vegetation along the gravel road near the large gravel pit filled by water, quaternary sediments of Morava river.
GLA[1]	27 112	3 0	0	48.204 / 16.986	160-162	Slovakia; Devínska Nová Ves, Glavica: ruderal vegetation at the foot of artificially created hill built in 2003-2005 from the exploited material from the highway tunel 'Sitina', recultivation in 2005-2006, the site was completely destroyed in 2009-2010 by building of residential complex.
MER	30	0	0	48.182 / 16.984	230	Slovakia; Devín, 'Merice': steppe grasslands on calcareous bedrock, in the past grazed.
STOC1	30	0	0	48.202 / 17.006	217-220	Slovakia; Bratislava, Dúbravka: steppe grasslands on calcareous bedrock, until 1950-1960s grazed.
STOC2	30	0	0	48.204 / 17.003	181-191	Slovakia; Bratislava, Dúbravka, Stockerau quarry: rock crevices and ruderal vegetation at the bottom of old limestone quarry (active from 1891 to 1970s) (Klačka and Pokorný 1995).
DEV	30	0	0	48.167 / 17.003	225-260	Slovakia; Devín, 'Mokrý jarok': ruderal vegetation along the gravel road going among the vineyards and gardens, and abandoned vineyards in late succession stage, granite bedrock.

[1]Number of plants in the first line refers to the transect sampling in 2008; the number in the second line refers to the sampling performed in 2009, when we took the exact position of 112 diploids, but the micro-site with rare co-occurring 4x plants found in 2008 was already destroyed (see Fig. S1b).

TABLE 2. *Tests with significance values (p) for spatial and habitat segregation, and differences in competitive, persistence and fitness traits between diploid and tetraploid plants from the mixed-ploidy populations of* Centaurea stoebe.

Site	Spatial distribution	Vegetation density 2*x* vs 4*x*	Soil moisture 2*x* vs 4*x*	*P* of rosettes formation 2*x* vs 4*x*	rosettes # per plant 2*x* vs 4*x*	shoot # per plant 2*x* vs 4*x*	seed # per plant 2*x* vs 4*x*
	Mantel test	χ^2 test	t-test	linear mixed effect model	Wilcoxon test	Wilcoxon test	Wilcoxon test
SAND	**<0.001**	**0.004**	0.07	NT	**<0.001**	**<0.001**	**<0.001**
WEIT	**0.015**	0.212	**<0.001**	NT	**<0.001**	**0.013**	**<0.001**
KOP	**<0.001**	0.521	0.1	NT	**0.01**	**0.026**	NM
TLM	**<0.001**	0.479	NM	NT	0.053	**<0.001**	**0.04**
MAR	**<0.001**	NM	NM	NM	**0.01**	**0.031**	NM
GLA	NM	NM	NM	NM	NM	NM	**0.02**
Total	NT	**<0.001**	NT	**<0.05**	**<0.001**	**<0.001**	**<0.001**

NM – not measured, NT – not tested.

TABLE 3. *Chloroplast haplotype distribution of* Centaurea stoebe *per site and ploidy*

Site and ploidy	N	N_H	Haplotype											
			H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
SAND 2x	6	3	3	0	0	0	1	0	0	2	0	0	0	0
SAND 4x	6	2	1	0	0	0	0	0	0	0	5	0	0	0
SAND 3x	2[1]	2	1	0	0	0	0	0	0	0	1	0	0	0
GLA 2x	6	2	2	0	0	0	0	0	0	0	0	0	0	4
GLA 4x	2	2	1	0	0	0	0	0	0	0	0	0	0	1
WEIT 2x	6	4	0	2	0	0	0	1	1	0	0	0	0	2
WEIT 4x	6	2	0	2	0	0	0	0	0	0	4	0	0	0
KOP 2x	6	2	5	0	0	0	0	1	0	0	0	0	0	0
KOP 4x	4	1	0	0	0	0	0	0	0	0	0	4	0	0
TLM 2x	6	2	0	0	0	5	0	0	1	0	0	0	0	0
TLM 4x	6	2	5	0	0	0	0	0	1	0	0	0	0	0
MAR 2x	6	2	0	5	1	0	0	0	0	0	0	0	0	0
MAR 4x	6	3	2	0	0	0	0	0	0	0	0	0	3	1
MAR 3x	1	1	0	1	0	0	0	0	0	0	0	0	0	0

N – number of plants per site and cytotype. N_H – total number of cpDNA haplotypes per site and cytotype

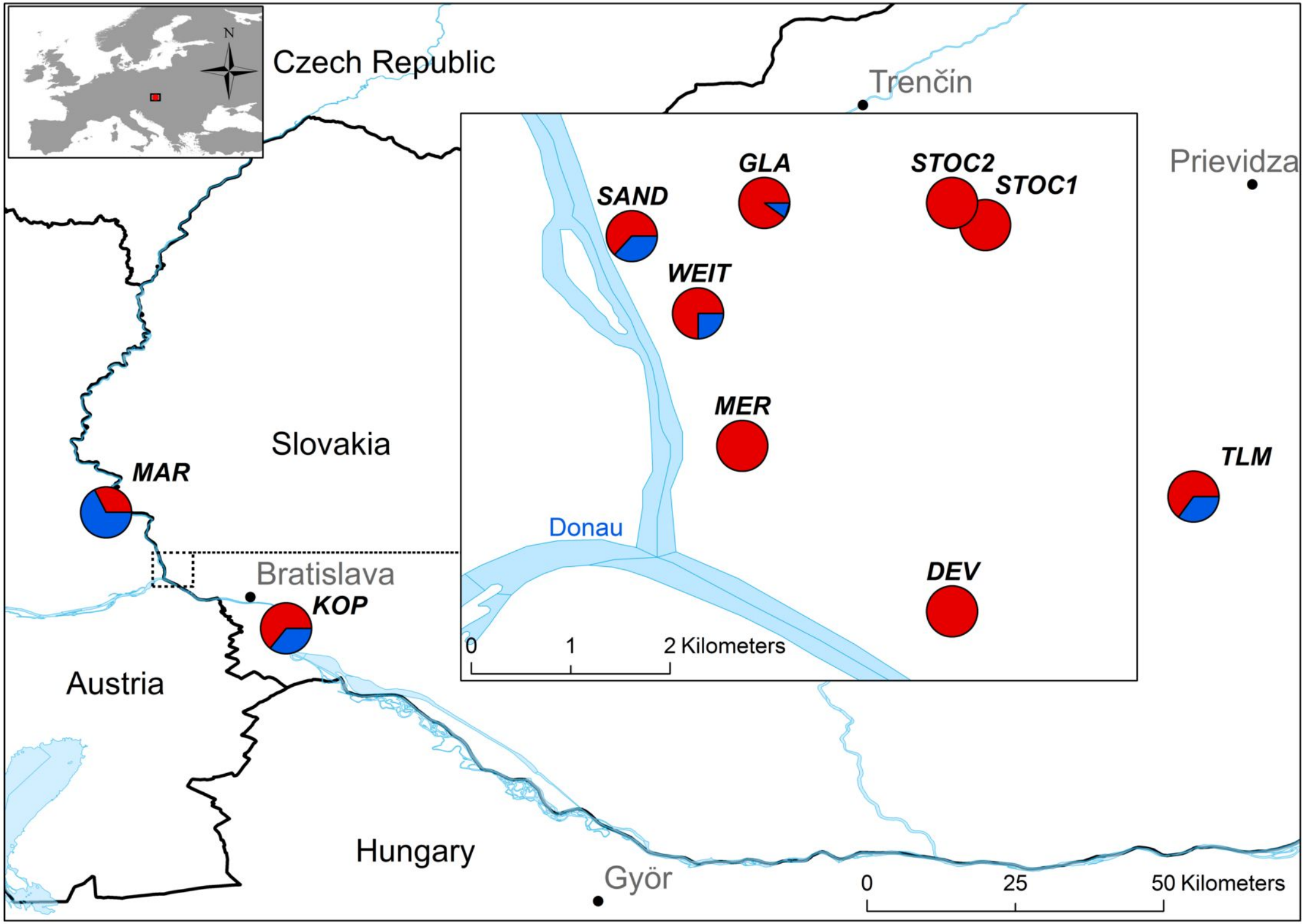
[1]In addition to one triploid adult plant (DK-293) found at the SAND site, another triploid (DK-189-P4), the progeny of a tetraploid mother plant (DK-189), found in the germination experiment was included in the analysis.

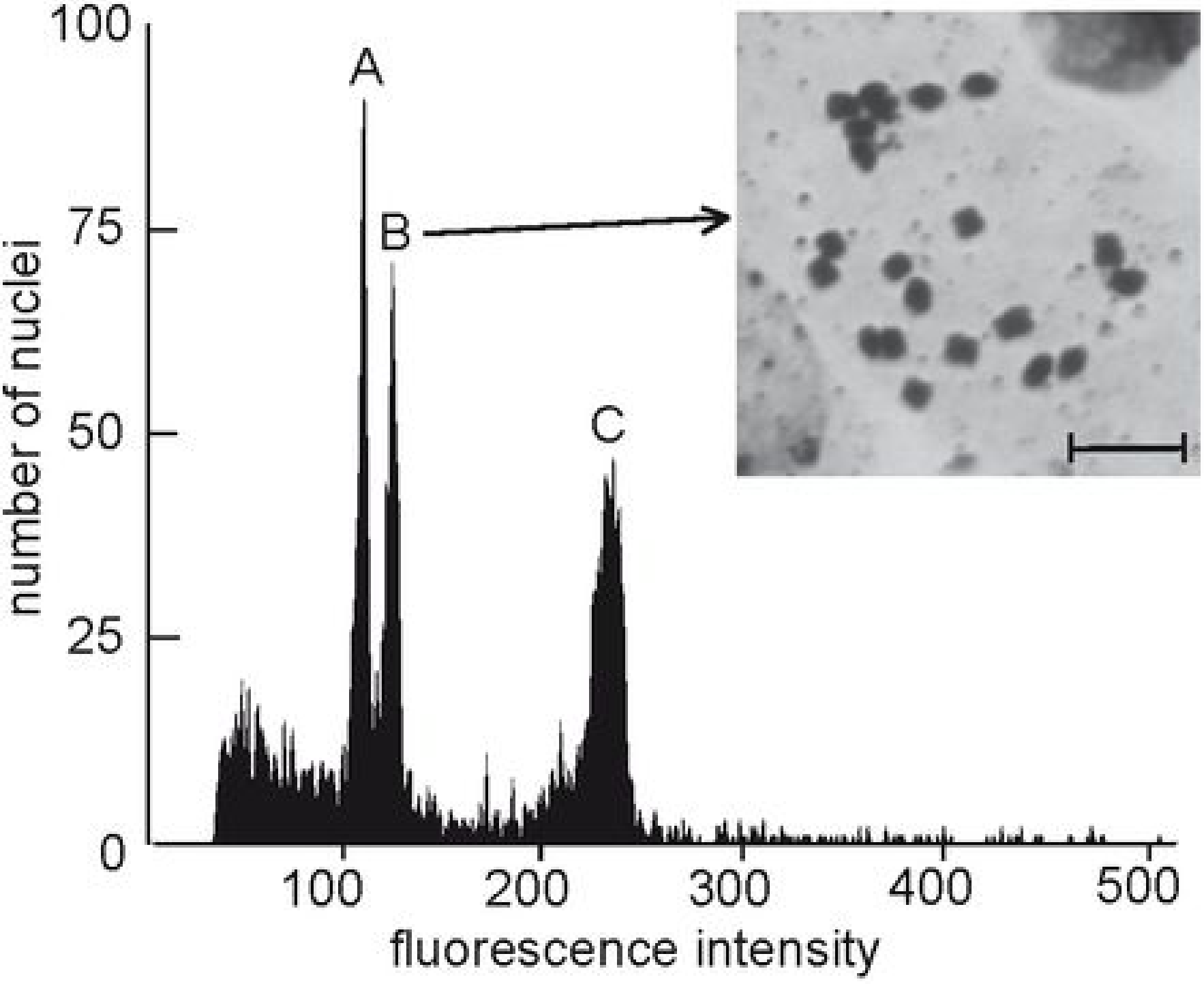
TABLE 4. *Allele frequencies computed from the presence or absence of alleles at four microsatellite loci in diploid, triploid and tetraploid plants of* Centaurea stoebe *in the Sandberg population (SAND).*

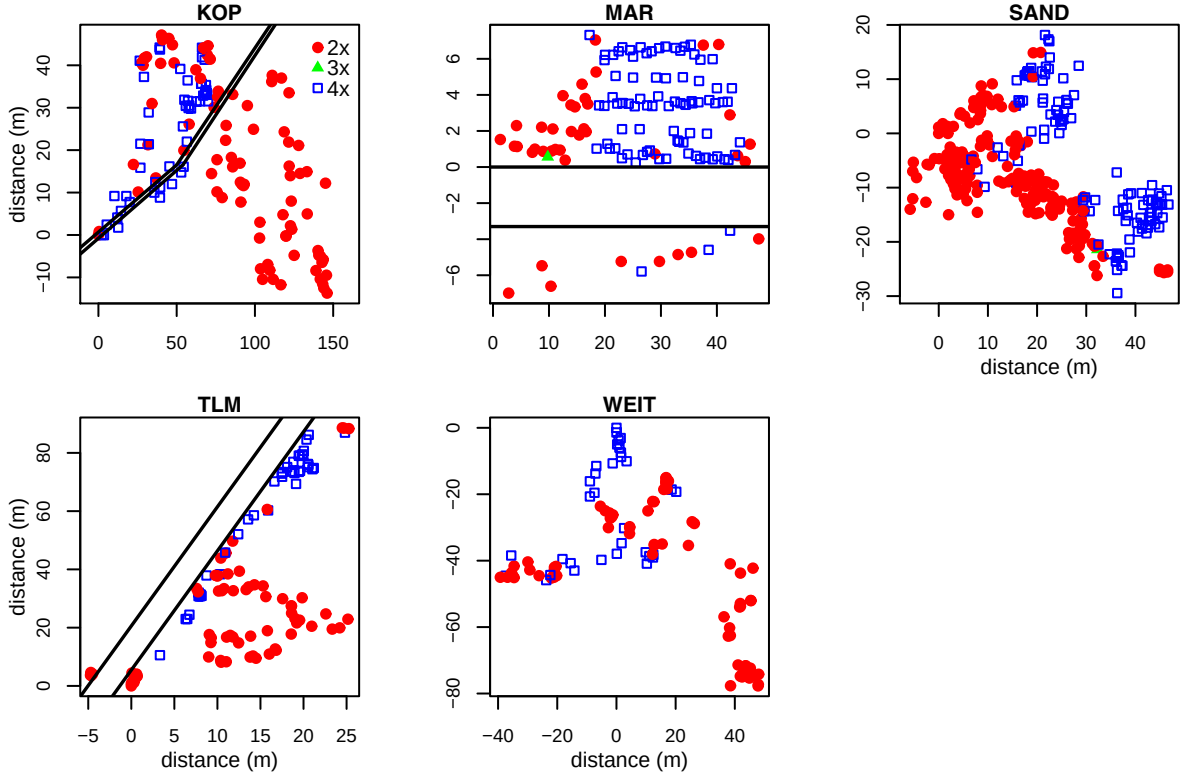
locus	CM-730											CM-1922											
Allele size	140	143	147	150	154	157	163	169	173	176	197	195	198	201	204	207	210	213	215	219	222	225	228
2x (*n*=168)	.11	.36	.24	.70	.01	.11	0	**.01**	.01	0	0	.10	.20	.39	.05	.37	.01	.39	**.01**	.01	.02	0	**.01**
4x (*n*=79)	.11	.09	.54	.89	.25	.54	**.03**	0	.03	**.01**	**.11**	.44	.01	.41	.11	.46	.54	.35	0	.04	.03	**.18**	0
3x (*n*=1)		1	1	1										1			1	1					

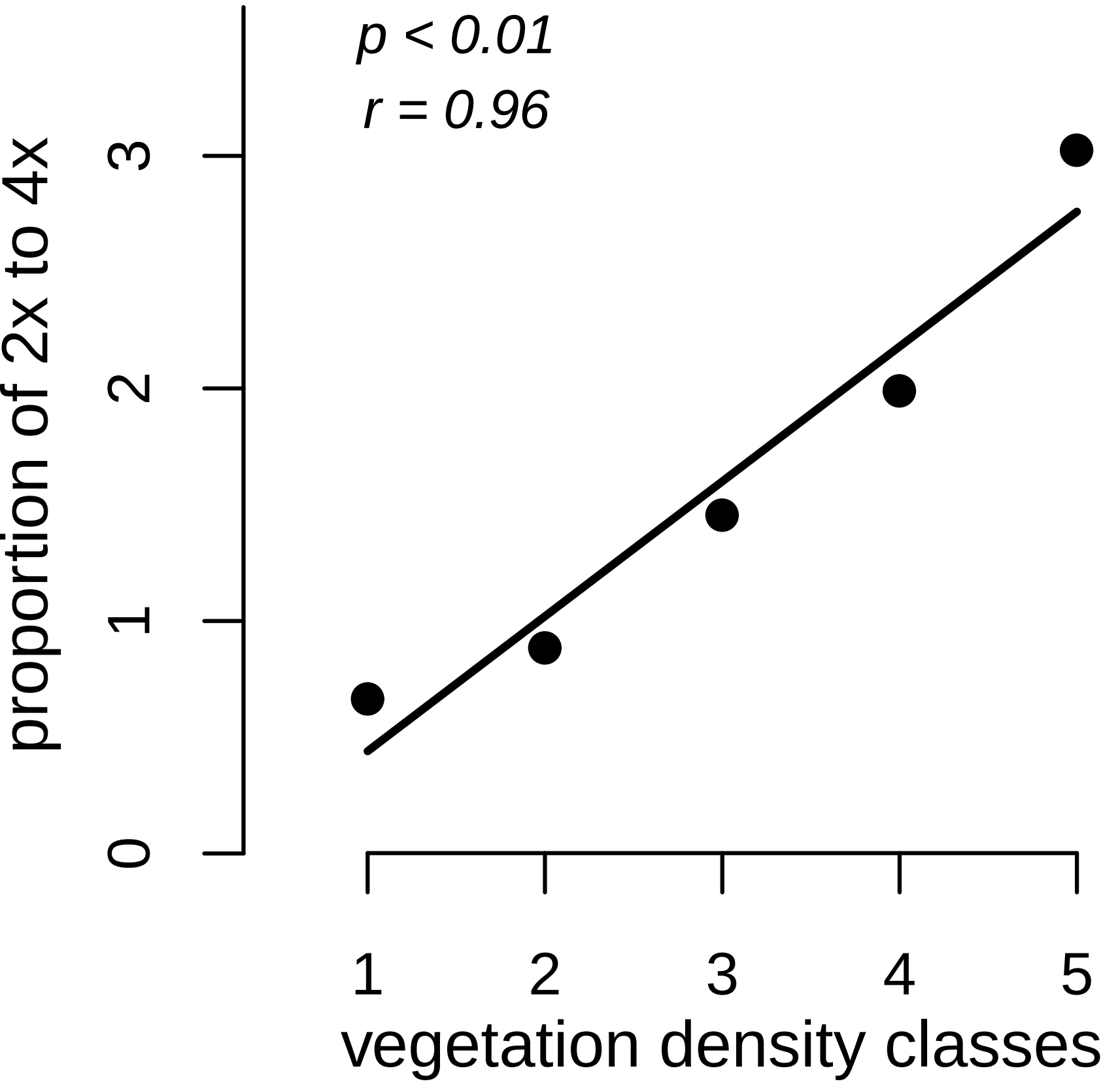
locus	CM-8337									CM-10060															
Allele size	231	234	237	240	243	246	249	255	258	206	209	212	215	218	221	224	227	230	233	237	240	242	245	248	250
2x (168)	0	**.52**	.11	0	.51	.29	.14	.05	0	.02	.33	.12	.05	.18	.01	.28	0	.1	**.23**	.11	.02	**.02**	.30	**.03**	.01
4x (79)	**.01**	0	.13	**.76**	.86	.13	.20	.15	**.04**	.46	.39	.39	.10	.05	.10	.11	**.14**	.37	0	.67	.23	0	.15	0	.03
3x (1)	1	1		1							1						1			1					

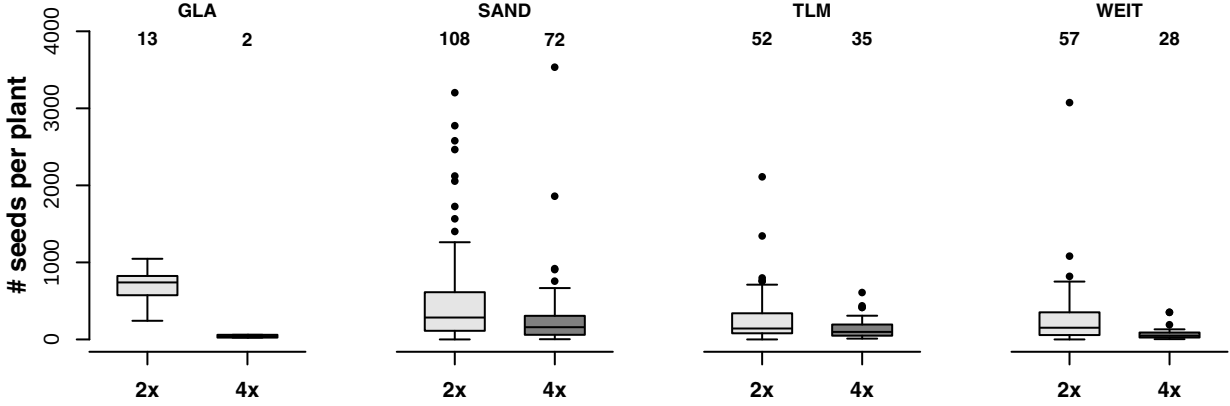
After the cytotype abbreviation, the number of plants is given in parentheses. Frequencies in bold denote private alleles for either diploid or tetraploid plants.

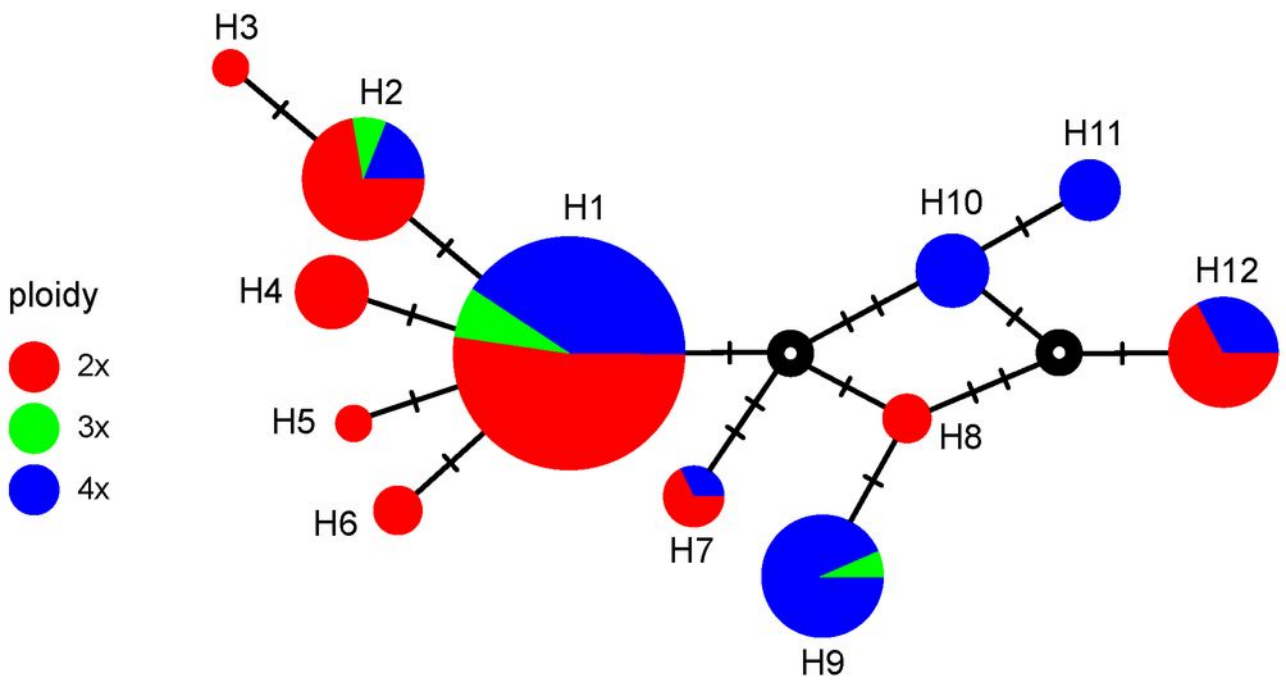

N
Czech Republic
Trenčin
Prievidza
SAND
GLA
STOC2
STOC1
WEIT
MER
TLM
DEV
Donau
Slovakia
MAR
Bratislava
KOP
Austria
Hungary
Györ
0
1
2 Kilometers
0
25
50 Kilometers

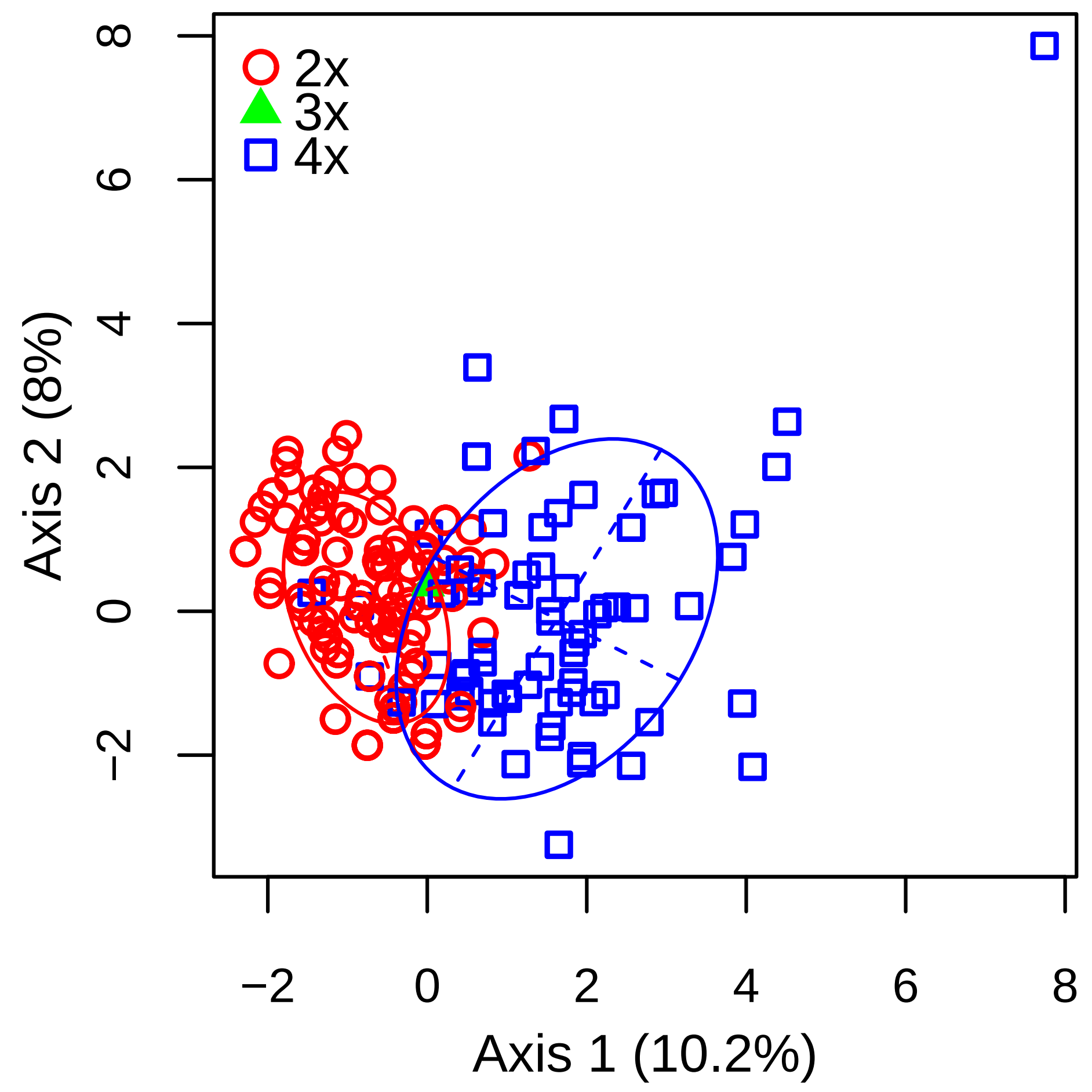

100
75
50
25
0
number of nuclei
A
B
C
100
200
300
400
500
fluorescence intensity


KOP
MAR
SAND
TLM
WEIT
distance (m)
2x
3x
4x


p < 0.01
r = 0.96
proportion of 2x to 4x
0
1
2
3
1
2
3
4
5
vegetation density classes


GLA
SAND
TLM
WEIT
13
2
108
72
52
35
57
28
seeds per plant
0
1000
2000
3000
4000
2x
4x
2x
4x
2x
4x
2x
4x


H3
H2
H1
H11
H10
H12
H4
H5
H6
H7
H8
H9
ploidy
2x
3x
4x


2x
3x
4x
Axis 2 (8%)
Axis 1 (10.2%)
−2
0
2
4
6
8

Príloha B

Mráz P, Garcia-Jacas N, Gex-Fabry E, Susanna A, Barres L, Müller-Schärer H

Allopolyploid origin of highly invasive *Centaurea stoebe* s.l. (Asteraceae)

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Allopolyploid origin of highly invasive *Centaurea stoebe* s.l. (Asteraceae)

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ABSTRACT

Spotted knapweed (*Centaurea stoebe*) occurs from Western Asia to Western Europe both as diploid and tetraploid cytotypes, predominantly in single-cytotype populations with higher frequency of diploid populations. Interestingly, only tetraploids have been recorded so far from its introduced range in North America where they became highly invasive.

We performed phylogenetic and network analyses of more than 40 accessions of the *C. stoebe* and *C. paniculata* groups and other related taxa using cloned internal transcribed spacer (ITS) and sequences of the chloroplast *trn*T–*trn*L and *atp*B*rbc*L regions to (i) assess the evolutionary origin of tetraploid *C. stoebe* s.l., and (ii) uncover the phylogeny of the *C. stoebe* group. Both issues have not been studied so far and thus remained controversial.

Cloned ITS sequences showed the presence of two slightly divergent ribotypes occurring in tetraploid cytotype, while only one major ribotype was present in diploid *C. stoebe* s.str. This pattern suggests an allopolyploid origin of tetraploids with contribution of the diploid *C. stoebe* s.str. genome. Although we were not able to detect the second parental taxon, we hypothesize that hybridization might have triggered important changes in morphology and life history traits, which in turn may explain the colonization success of the tetraploid taxon. Bayesian relaxed clock estimations indicate a relatively recent – Pleistocene origin of the tetraploid *C. stoebe* s.l. Furthermore, our analyses showed a deep split between the *C. paniculata* and *C. stoebe* groups, and a young diversification of the taxa within the *C. stoebe* group. In contrast to nrDNA analyses, the observed pattern based on two cpDNA regions was inconclusive with respect to the origin and phylogeny of the studied taxa, most likely due to shared ancient polymorphism and frequent homoplasies.

1. Introduction

Successful biological invasions are generally based on both ecological and evolutionary processes, but the latter ones have been studied to a lesser extent (Vanderhoeven et al., 2010). Ellstrand and Schierenbeck (2000) stressed the importance of hybridization as an evolutionary stimulus of invasiveness. In fact, hybridization between species or between divergent populations within the same species leads to the formations of new genotypic and phenotypic combinations, which may allow the colonization of new ecological niches (Stebbins, 1950; Anderson and Stebbins, 1954). Newly formed plant hybrids are often stabilized by polyploidization (genome doubling) alleviating the problems of chromosome pairing during meiosis and thus sterility (Burke and Arnold, 2001; Abbott et al., 2010). Besides the reproductive assurance of otherwise sterile hybrids, polyploidization leads to fixation of heterotic genotypes and increases genetic variation through a higher number of allelic variants per locus (Comai, 2005). Thus, hybridization and polyploidization either alone, or in concert, may considerably increase the adaptive potential as compared to their ancestors. Many polyploids are successful colonizers of naturally or artificially disturbed habitats (Stebbins, 1985; Ehrendorfer, 1980; Thompson, 1991; Brochmann et al., 2004) and thus polyploidy has been listed in several comprehensive studies focusing on putative determinants of invasiveness in plants. (e.g. Verlaque et al., 2002; Küster et al., 2008; Pyšek et al., 2009). However, these meta-analyses have not distinguished autopolyploids, arising within populations of single species, from allopolyploids, in which interspecific hybridization was involved, most likely because of lack of this information. Nevertheless, knowledge of the evolutionary history of invasive species is crucial for understanding underlying mechanisms of their invasion success.

Several invasive polyploids have recently been found to be of hybridogeneous origin due to progress in molecular biology (Gray et al., 1990; Baumel et al., 2002; Ainouche et al., 2004; Vilatersana et al., 2007; Kim et al., 2008; Jacob and Blattner, 2010). Interspecific hybridization is usually inferred by biparentally inherited

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nuclear DNA markers, most often using the internal transcribed spacer (ITS). Wide-spread use of ITS1–5.8S–ITS2 stems from their easy amplification with universal primers and relatively high level of interspecific polymorphism (Baldwin et al., 1995). On the other hand, some constraints might hamper its application for phylogenetic reconstructions. More specifically, ITS nrDNA shows higher level of homoplasy than other nuclear markers, the nucleotide position is not independent due to conservative secondary structure, paralogues/ortologues can be frequent and they can be subject to various level of intra-or inter-array homogenization (Álvarez and Wendel, 2003). However, in the case of suppression of the last mentioned mechanism, and thus retention of two (or more) divergent ITS copies within one genome, this marker may prove to be highly informative with respect to the hybridization history. Indeed, many case-studies documented a hybridogeneous origin of polyploid taxa using multicopy ITS or ETS (external transcribed spacer) nrDNA markers (Soltis and Soltis, 1991; Sang et al., 1995; Campbell et al., 1997; Andreasen and Baldwin, 2003; Fehrer et al., 2009). In addition to biparentally inherited polymorphism assessed by nuclear markers, incongruencies between nuclear and plastid phylogenies may indicate reticulation event(s) (Rieseberg and Soltis, 1991; Soltis and Kuzoff, 1995).

Frequent hybridization and polyplodization considerably shaped the evolutionary pattern in the species-rich genus *Centaurea* L. (Hellwig, 2004). Interspecific homoploid hybridization is frequent and single hybrids or hybrid populations are often easily recognized due to their intermediate morphology (e.g. Kummer, 1977; Fernández Casas and Susanna, 1986; Garcia-Jacas and Susanna, 1994; Ochsmann, 2000; Koutecký, 2007; Blair and Huffbauer, 2010; Pisanu et al., 2011). The situation is however more complicated in widespread polyploid taxa. Given their high frequency, surprisingly little is known about their auto- or allopolyploid origin. Based on polysomic segregation at two allozymic loci, an autopolyploid origin has been suggested for the tetraploid cytotype of *C. phrygia* L. (Hardy et al., 2000). On the contrary, a quite complex scenario involving several diploid and tetraploid species in several steps has been proposed for the west Mediterranean tetraploid and hexaploid *C. toletana* Boiss. cytotypes (Garcia-Jacas et al., 2009). Similarly, the presence of different ITS paralogues in *C. boissieri* subsp. *atlantica* (Font Quer) Blanca and *C. debdouensis* Breitw. and Podlech has been explained by recent hybridization (Suárez-Santiago et al., 2007).

The *Centaurea stoebe* group (*Centaurea* sect. *Centaurea*, formerly sect. *Acrolophus*) is represented by diploid ($2n = 2\times = 18$) and tetraploid ($2n = 4\times = 36$) populations occurring sympatrically in the native European range (Ochsmann, 2000; Španiel et al., 2008; Treier et al., 2009). The taxonomic position of both cytotypes is still debated. While Ochsmann (2000) proposed to distinguish diploid (*C. stoebe* subsp. *stoebe*; *C. stoebe* s.str. thereafter) from tetraploid cytotype at the subspecies level [*C. stoebe* subsp. *micranthos* (Gugler) Hayek; *C. stoebe* s.l. thereafter], Španiel et al. (2008) suggested a single species concept with no recognition of intraspecific units. In addition to diploid and tetraploid *C. stoebe* L. several other morphologically similar species to *C. stoebe* have been described [e.g. *C. corymbosa* Pourr., *C. reichenbachii* DC., *C. triniifolia* Heuff., *C. vallesiaca* (DC.) Jord.], but their phylogenetic relationship to *C. stoebe* remains obscure (Ochsmann, 2000).

Centaurea stoebe has been introduced to North America at the end of 20th century and since that became highly invasive (Sheley et al., 1998). More importantly, only the tetraploid cytotype has been recorded so far in the introduced range (Treier et al., 2009; Mráz et al., 2011). This pronounced shift in cytotype composition between the native and introduced range could either be the result of the stochastic introduction of only the tetraploid cytotype, or tetraploids might have a demographic advantage over the diploids, in the case if the diploids had been also introduced (Treier et al., 2009). Both cytotypes differ in their life cycle and this trait could tentatively explain the invasion success of the polyploid cytotype. In fact, tetraploids are short-lived perennials and polycarpic, while diploids are predominantly annual or biennial monocarpic plants (Boggs and Story, 1987; Müller, 1989; Ochsmann, 2000; Story et al., 2001; Henery et al., 2010; Mráz et al., 2011). In addition to the different life cycle, recent multivariate morphometric study based on plants grown under uniform conditions from more than 60 populations from both the native and introduced range showed that the cytotypes also differ in other morphological traits, thus supporting the distinct taxonomic status of both cytotypes (Mráz et al., 2011). Differences in phenotypic and life-cycle traits between cytotypes could be the results of direct polyploidization (autopolyploidization), as whole genome duplication might induce morphological and physiological changes (Müntzing, 1936; Blakeslee, 1941; Maherali et al., 2009), or alternatively, hybridization associated with polyploidization (allo-polyploidization). In the latter case, greater phenotypic and genetic differences between diploid progenitors and their polyploid derivates might be expected than under autopolyploidy, although the extent of differentiation depends on the divergence between the parental taxa (Stelkens et al., 2009).

The aim of the present study was (i) to determine the origin (auto- vs. allopolyploid) of the tetraploid cytotype of *C. stoebe* using biparentally inherited nrDNA ITS marker and two cpDNA loci and (ii) to infer their relationship with closely related taxa belonging to the *C. stoebe* group.

2. Material and methods

2.1. Plant material and ploidy level determination

Forty-two accessions of *Centaurea stoebe* s.l. originating from 38 populations sampled across the native European and introduced North American range and representing all known cytotypes (i.e. $2\times$ and $4\times$ as major cytotypes, and $3\times$ and $6\times$ as rare ones; cf. Mráz et al., 2011) were included in the present study (Table 1). Within *C. stoebe* s.l. we included also the accessions recognized by Ochsmann as separate taxa (e.g. *C. reichenbachii*, *C. tauscherii* A. Kern., *C. triniifolia*, *C. vallesiaca*) to cover variation as large as possible within the group (Ochsmann, 2000). In addition, nine other species were added: *C. cuneifolia* Sm., a species morphologically similar to *C. stoebe* from the Balkans; three species from the *C. paniculata* group, namely *C. aplolepa* Moretti, *C. leucophaea* Jord., and *C. paniculata* L.; and three species showing similar ITS sequences based on a previous study (*C. donetzica* Klokov, *C. sarandinakiae* N.B. Illar., and *C. vankovii* Klokov; see Garcia-Jacas et al., 2006). All taxa belong to the sect. *Centaurea*, except of *C. donetzica*, *C. sarandinakae* and *C. vankovii* which are members of sect. *Phalolepis* (Cass.) DC. (Wagenitz and Hellwig, 1996). Leaf material for DNA extraction was collected either from the plants in the field or from seed-derived plants cultivated in the greenhouse, dried in silica-gel and stored at room temperature. Rarely, herbarium specimens were used. Details on vouchers, population codes and GenBank accession numbers are given in Table 1.

Ploidy level estimations and chromosome counts were determined on seed-derived plants cultivated in the greenhouse, or in rare cases on silica-gel dried material. Details for sample preparation and analyses using flow cytometry and chromosome counting are given in Mráz et al. (2011). Most of the ploidy estimations presented here are new (see Table 1), although some are from our previous publications (Treier et al., 2009; Mráz et al., 2011). For some taxa for which we used already published ITS sequences, ploidy level information were taken from Ochsmann (2000).

Table 1
Origin of the plant material used for the present study, total number of ITS clones sequenced per sample/number of clones used for phylogenetic analyses. A dash indicates failure of cloning (ITS) or not analysed (cpDNA).

Taxon and ploidy[a]	Sampling code	Code used for analyses	Country code, locality, altitude, collector(s), date and herbarium	Total #ITS clones/retained for plots	GenBank accession ITS	cpDNA haplotype	GenBank accession cpDNA (*trnL–trnT*/*atpB–rbcL*)
C. aplolepa 2×	IT2-12	A	IT, Capo Berta, 20 m, Müller-Schärer, 1.6.2008 (NHMR)	5/2	JF913981–JF913982	H2	JF960874/JF960915
C. corymbosa 2×*		CO	FR, Narbonne, La Clappe, M. Riba, 1995 (BC).	Not cloned		–	–
C. cuneifolia 2×	BG7-1	CU	BG, Belovo, 443 m, P. Mráz and Mrázová, 3.8.2008 (NHMR)	8/3	JF913983–JF913985	–	–
C. diffusa 2×*	DIF	DI	ARM, Talin, between villages Pokr Arthik and Bagravan, Susanna 1589 et al., 26.8.1995 (BC).	Not cloned	–	–	–
C. donetzica 2×	DON	DO	UA, Donetzk region, Krasny Liman, Romaschenko, 13.07.2009 (BC).	5/3	JF913986–JF913988	–	–
C. leucophea 2×	FRE-4	L	FR, Drôme, Allan, 283 m, Treier and Broenniman, 4.8.2005 (NHMR)	4/1	JF913989	H3	JF960872/JF960913
C. paniculata 2×	FRA15-2	P	FR, Ain, St-Maurice de Gourdans, 190 m, P. Mráz and Priestman, 2.5.2008 (NHMR)	5/2	JF913990–JF91399	H9	JF960869/JF960910
C. sarandinakiae 4×	SARAN	SA	UA, Crimea, Kara-Dag, Futorna and Romaschenko, 13.07.2009 (BC).	4/3	JF913992–JF913994	–	–
C. stoebe 2×	BG1-3	S1	BG, Bosnek, 875 m, P. Mráz and Mrázová, 1.8.2008 (NHMR)	8/2	JF913995–JF913996	H1	JF960856/JF960897
C. stoebe 2×	BG4-4	S2	BG, Dagonovo, 806 m, P. Mráz and Mrázová, 2.8.2008 (NHMR)	7/–	–	H10	JF960859/JF960890
C. stoebe 2×	BG5-5	S3	BG, Yagoruda, Mt. Granchar, 2150 m, Mrázová, 2.8.2008 (NHMR)	8/1	JF913997	H2	JF960860/JF960891
C. stoebe 2×*[b]	SW4-6	S4	CH, Wallis, Ausserberg, 924 m, Thébault, 8.9.2005 (NHMR)	8/1	JF913998	H1	JF960891/JF960932
C. stoebe 2×*	SCHA-10	S5	CH, Graubünden, Ramosch, 1237 m, Treier and Normand, 18.8.2005 (NHMR)	12/2	JF913999–JF914000	H2	JF960880/JF960921
C. stoebe 2×*	DE6-14	S6	DE, Sachsen-Anhalt, Halle, 85 m, Thébault and Broennimann, 21.8.2005 (NHMR)	8/1	JF914001	H4	JF960862/JF960903
C. stoebe 2×*	DE10-3	S7	DE, Sachsen-Anhalt, Zadel, 145 m, Thébault and Broennimann, 22.8.2005 (NHMR)	7/2	JF914002–JF914003	H1	JF960863/JF960904
C. stoebe 2×*[c]	Albida-1	S8	FR, Gard, Anduze, Tisson, 160 m, 5.1995 (NHMR)	8/2	JF914004–JF914005	H8	JF960855/JF960896
C. stoebe 2×[d]	FRA11-3	S9	FR, Haute Loire, Leotoing, 612 m, P. Mráz and Priestman, 1.5.2008 (NHMR)	5/2	JF914006–JF914007	H2	JF960865/JF960906
C. stoebe 2×[d]	FRA13-4	S10	FR, Haute Loire, Espoly-St-Marcel de l'Ermitage, 786 m, P. Mráz and Priestman, 1.5.2008 (NHMR)	5/1	JF914008	H2	JF960867/JF960908
C. stoebe 2×[d]	FRA14-5	S11	FR, Puy de Dôme, Mt. Puy de Crouël, 394 m, P. Mráz and Priestman, 1.5.2008 (NHMR)	6/1	JF914009	H2	JF960868/JF960909
C. stoebe 2×	FRA16-21	S12	FR, Savoie, Termignon, 1398 m, P. Mráz and S. Mráz, 10.9.2008 (NHMR)	7/2	JF914010–JF914011	H3	JF960870/JF960911
C. stoebe 2×	FRA17-1	S13	FR, Savoie, Modane, 1258 m, P. Mráz and S. Mráz, 10.9.2008 (NHMR)	9/2	JF914012–JF914013	H3	JF960871/JF960912
C. stoebe 2×	IT3-16	S14	IT, Piemont, Casellete, 442 m, P. Mráz and S. Mráz, 11.9.2008 (NHMR)	–/–		H5	JF960875/JF960916
C. stoebe 2×[e]	RO14-1	S15	RO, Cheile Turzei, 564 m, P. Mráz and Mrázová, 7.8.2008 (NHMR)	12/3	JF914014–JF914016	H2	JF960877/JF960918
C. stoebe 2×	SER5-2	S16	RS, Vranje, 450 m, P. Mráz and Mrázová, 30.7.2008 (NHMR)	6/3	JF914017–JF914019	H2	JF960883/JF960924
C. stoebe 2×	SER8-3	S17	RS, Mt. Pirot, 1335 m, P. Mráz and Mrázová, 30.7.2008 (NHMR)	14/2	JF914020–JF914021	H1	JF960885/JF960926
C. stoebe 2×	SER9-1	S18	RS, Basara, 952 m, P. Mráz and Mrázová, 30.7.2008 (NHMR)	4/1	JF914022	H12	JF960886/JF960927
C. stoebe 2×*	SRUG-4	S19	RU, Samara, Perevoloki, 78 m, Naumoff, 24.9.2006 (NHMR)	6/2	JF914023–JF914024	H1	JF960887/JF960928
C. stoebe 2×*	SRUG-12	S20	RU, Samara, Perevoloki, 78 m, Naumoff, 24.9.2006 (NHMR)	9/2	JF914025–JF914026	H4	JF960888/JF960929
C. stoebe 2×*	SRUO-2	S21	RU, Dagestan Republic, Karabudokhkentskiy district, 970 m, Nikolaeva, 20.7.2006 (without voucher)	11/3	JF914027–JF914029	H4	JF960889/JF960930
C. stoebe 2×	DK2-421	S22	SK, Devínska Nová Ves, 191 m, P. Mráz and Mrázová, 12.8.2008 (NHMR)	3/1	JF914030	–	–
C. stoebe 2×*	SUAI-2	S23	UA, Poltava, Chutove, 131 m, Treier and Broennimann, 15.9.2005 (NHMR)	8/2	JF914031–JF914032	–	–
C. stoebe 3×	Ma-134	S24	AT, Marchegg, 35 m, Bowman and Farkas, 2007 (NHMR)	5/2	JF914033–JF914034	H1	JF960879/JF960920
C. stoebe 3×	DK2-293	S25	SK, Devínska Nová Ves, 206 m, P. Mráz and Procházka, 15.8.2008 (NHMR)	–/–	–	H6	JF960864/JF960905
C. stoebe 4×	BG1-5	S26	BG, Bosnek, 875 m, P. Mráz and Mrázová, 1.8.2008 (NHMR)	13/3	JF914035–JF914037	H2	JF960857/JF960898
C. stoebe 4×	BG2-2	S27	BG, Topolnitsa, 650 m, P. Mráz and Mrázová, 1.8.2008 (NHMR)	6/2	JF914038–JF914039	H7	JF960858/JF960899
C. stoebe 4×	BG6-1	S28	BG, Yundola saddle, 1638 m, P. Mráz and Mrázová,	7/3	JF914040–	H1	JF960861/JF960902

Table 1 (*continued*)

Taxon and ploidy[a]	Sampling code	Code used for analyses	Country code, locality, altitude, collector(s), date and herbarium	Total #ITS clones/retained for plots	GenBank accession ITS	cpDNA haplotype	GenBank accession cpDNA (*trnL–trnT*/*atpB–rbcL*)
			2.8.2008 (NHMR)		JF914042		
C. stoebe 4×	FRA12-1	S29	FR, Allier, Moulins, 207 m, P. Mráz and Priestman, 31.4.2008 (NHMR)	7/2	JF914043–JF914044	H1	JF960866/JF960907
C. stoebe 4×[f]	HU11-8	S30	HU, Tököl, 107 m, P. Mráz and Mrázová, 27.7.2008 (NHMR)	12/2	JF914045–JF914046	H1	JF960873/JF960914
C. stoebe 4×[g]	RO11-5	S31	RO, Băile Herculeane, 229 m, P. Mráz and Mrázová, 5.8.2008 (NHMR)	13/2	JF914047–JF914048	H1	JF960876/JF960917
C. stoebe 4×	RO14-8	S32	RO, Cheile Turzei, 564 m, P. Mráz and Mrázová, 7.8.2008 (NHMR)	5/3	JF914049–JF914051	H1	JF960878/JF960919
C. stoebe 4×	SER2-3	S33	RS, Bogutovac, 245 m, P. Mráz and Mrázová, 29.7.2008 (NHMR)	7/2	JF914052–JF914053	H2	JF960881/JF960922
C. stoebe 4×	SER4a	S34	RS, Massif of Kopaonik, 1460 m, P. Mráz and Mrázová, 29.7.2008	–/–	–	H1	JF960882/JF960923
C. stoebe 4×	SER7-5	S35	RS, Grkinja, 279 m, P. Mráz and Mrázová, 30.7.2008 (NHMR)	13/3	JF914054–JF914056	H1	JF960884/JF960925
C. stoebe 4×*	SUAD-10	S36	UA, Chernivtsi, 313 m, Treier and Broennimann, 9.9.2005 (NHMR)	6/3	JF914057–JF914059	H1	JF960890/JF960931
C. stoebe 4×	DK-347	S37	SK, Devínska Nová Ves, 232 m, P. Mráz and Procházka, 12.8.2008 (NHMR)	7/2	JF914060–JF914061	–	–
C. stoebe 4×*	USMT10-4	S39	US, Montana, Missoula, 1146 m, Treier and Broennimann, 13.10.2005 (NHMR)	14/2	JF914062–JF914063	H1	JF960893/JF960934
C. stoebe 4×*	USOR10-1	S40	US, Oregon, Klamath Falls, 1263 m, Treier and Broennimann, 13.10.2005 (NHMR)	6/3	JF914064–JF914066	H2	JF960894/JF960935
C. stoebe 4×*	USWI1-3	S41	US, Wisconsin, Necedah, 277 m, Hufbauer, 13.10.2005 (NHMR)	6/3	JF914067–JF914069	H11	JF960895/JF960936
C. stoebe 6×*	URS3	S42	CA, British Columbia, Elko, 920 m, Bourchier, 17.10.2007 (NHMR)	7/3	JF914070–JF914072	H1	JF960892/JF960933
C. vankovii 2×	VAN	V	UA, Crimea, Mt. Demerdji, 1200 m, Futorna and Romaschenko, 15.07.2009 (BC).	4/3	JF914073–JF914074	–	–

ARM – Armenia, AT – Austria, BG – Bulgaria, CA – Canada, CH – Switzerland, DE – Germany, FR – France, HU – Hungary, IT – Italy, RO – Romania, RS – Serbia, RU – Russia, SK – Slovakia, UA – Ukraine, US – United States.

[a] Ploidy level estimations of the plants/populations marked by asterisk (*) are based on previously published data (see Section 2), those without asterisk are new records.

[b] Morphologically corresponds to *C. vallesiaca* DC.

[c] Morphologically corresponds to *C. maculosa* subsp. *albida* (Lecoq and Lamotte) Dostál, det. J.M. Tison.

[d] Morphologically corresponds to *C. maculosa* Lam.

[e] From this site both *C. stoebe* s.l. and *C. reichenbachii* has been reported (cf. Ochsmann, 2000).

[f] Locus classicus of *C. tauscheri* A. Kern.

[g] Locus classicus of *C. triniifolia* Heuf.

2.2. DNA extraction, amplification, cloning, and sequencing

Total DNA was extracted from 10 to 15 mg of silica-dried leaf tissue with the DNeasy 96 Plant Kit (Qiagen Inc., Valencia, CA, USA), or in some cases using CTAB method (Doyle and Doyle, 1987). The ITS region was amplified using the primers 17SE and 26SE (Sun et al., 1994) in a 25 µl reaction volume containing 3 µl of diluted genomic DNA, 10× AmpliTaq buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of each primer, 0.5 µl of DMSO (Sigma–Aldrich, St. Luis, MO, USA) and 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA). The cycling profile included an initial denaturation step at 94 °C/2 min followed by 35 cycles of 94 °C/1 min 30 s, 57 °C/2 min, 72 °C/3 min, and ended with 72 °C/15 min and 4 °C thereafter.

The *trnT–trnL* locus was amplified using "a" and "b" primers of Taberlet et al. (1991) and the *atpB–rbcL* locus with the primers proposed by Chiang et al. (1998). The PCRs were performed in 25 µl volume containing 5 µl of genomic DNA (4 ng µl^{-1}), 10× PCR Buffer, 1 mM MgCl2, 0.2 mM dNTPs, 0.4 µM of each primer, 0.25 µM BSA and 1 U of Taq polymerase (Qiagen). The cycle profile included the initial denaturation at 94 °C/3 min followed by 36 cycles of 94 °C/30 s, 50 °C/30 s, 72 °C/1 min, and ended with 72 °C/5 min and 4 °C thereafter.

The cloning of ITS1–ITS2 regions was performed using TOPO TA Cloning ® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. When possible, at least eight positive colonies from each reaction were screened with direct PCR using T7 and M13 universal primers and following reaction conditions: initial denaturation at 94 °C/10 min followed by 30 cycles of 94 °C/30 s, 55 °C/1 min, and ended with 72 °C/10 min and 4 °C thereafter. Five to fifteen clones per accession were selected for sequencing. Direct sequencing was performed using BigDye Terminator Cycle Sequencing v3.1 (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol on an ABI 3730xl capillary sequencer (Applied Biosystems) at the University of Florida ICBR Core Facility. Sequences were edited manually using BioEdit 7.0.5.3 (Hall, 1999) and assembled using Mega 4.01 (Tamura et al., 2007).

2.3. Phylogenetic and network analyses

We used Bayesian and parsimony analyses to infer ITS phylogeny and a distance network analysis (split graphs) from cloned ITS sequences, to which we added previously published sequences of two closely related taxa, *C. corymbosa* and *C. diffusa* Lam. (Garcia-Jacas et al., 2006). The same clones of the same accession, and the clones showing unique substitutions within and between accessions were excluded from the analyses, as they may represent random PCR errors (Cline et al., 1996; Popp and Oxelman, 2001). In some cases, we regrouped slightly different clones obtained from the same accession (differing usually by one substitution) to obtain one consensual sequence and thus to reduce the number of clones for analyses. The data matrix is available on request from the corresponding author. Bayesian posterior probabilities were estimated using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The evolutionary model was selected using jModeltest (Posada, 2008) and maximum likelihood parameters were specified

according to the Akaike Information Criterion (AIC: TM3ef + g). Bayesian analysis was initiated with random starting trees and continued until the value of standard deviation of split sequences droped below the 0.01 as convergence diagnostic value. Log-output file was monitored with Tracer 4.1 to ensure that all parameters achieved sufficient sampling size (>200). The fraction of the sampled values discarded as burn-in was set at 0.25. Posterior probabilities (PP) of 0.95–1.00 were considered statistically significant. For the Neighbor-Net analyses we used the Neighbor-Net (NN) algorithm (Bryant and Moulton, 2004) as implemented in SplitsTree 4.10 software (Huson and Bryant, 2006) with the criterion set to uncorrected pair-wise (p) distances, excluding both constant and non-informative characters. The haplotype network based on substitution polymorphisms in two assembled cpDNA loci (*trnT–trnL* and *atpB–rbcL*) was constructed using the median-joining algorithm implemented in Network 4.6.0.0 (www.fluxus-engineering.com, Bandelt et al., 1999). The same weight (10) was attributed to all variables sites.

2.4. Divergence time estimation

To estimate the divergence time between different ITS clades, we applied two methods. (i) A molecular clock approach using two slightly different substitution rates. The first one corresponds to 2.51×10^{-9} substitution per site and year based on independently calibrated ITS data of herbaceous representatives of the genus *Eupatorium* (Asteraceae, subtribe Eupatorieae; cf. Schmidt and Schilling, 2000; Kay et al., 2006). The second one corresponds to 3×10^{-9} substitution derived from the tribe Madieae (Baldwin and Sanderson, 1998) and used by Suárez-Santiago et al. (2007) to estimate divergence time between sections *Willkommia* and *Centaurea* (syn. *Acrolophus*). Although the likelihood ratio test (baseml package in PAML; Yang, 2007) rejected the assumption of constancy rate of ITS evolution in our data ($p = 0.01$), this was not the case for data set of Suárez-Santiago et al. (2007) probably due to larger number of taxa used in latter study. The Tamura-Nei substitution model with gamma distribution was chosen using online FindModel server (www.hiv.lanl.gov/content/sequence/findmodel/findmodel) and was applied to calculate mean genetic distances between the major clades using MEGA 4.01. (ii) Dating analyses were also performed with BEAST 1.6.1 (Drummond and Rambaut, 2007) using a Bayesian method based on a relaxed molecular clock hypotheses, implying that the evolutionary rate is not constant over time. Because of the lack of *C. stoebe* fossils, we used two age estimations for a Cardueae tribe phylogeny calibrated with five fossils (Barres et al., unpubl. data) as calibration points. *Centaurea lingulata* Lag. from *Centaurea* subgen. *Cyanus* and *Centaurea behen* L. from subgen. *Centaurea* were added as external calibration points to the *C. stoebe* dataset. *Rhaponticoides hajastana* (Tzvel.) M. V. Agab. & Greuter and *Psephellus persicus* (DC.) Wagenitz from the Centaureinae were also included as most external groups and coded as outgroups. The split of subgen. *Cyanus* from all other Cenatureinae was estimated at c. 13.06 mya (Barres et al., unpubl. data) and was used to calibrate the split of *C. lingulata*. The split of subgen. *Centaurea* was estimated in 10.16 mya (Barres et al., unpublished data) and was used to calibrate the split of *C. behen* from the *C. paniculata–C. stoebe* clade. We used a normal distribution prior with ±1 SD for both calibration points, as they were published age estimations. For the datation dataset, model selection was performed with MrModelTest 2.3 (Nylander, 2004) following the Akaike criteria. The best-fit model selected for the estimation of divergence times was SYM + G. We assumed a Constant Size Coalescent Model for the tree prior and an uncorrelated lognormal distribution for the molecular clock model (Drummond et al., 2006; Ho, 2007). For all other parameters we used the default prior distributions. We ran MCMC chains for 50 million generations. The 10% of the first sampled trees were removed as burn-in, and the posterior probability density was summarized using TreeAnnotator 1.6.1 (Drummond and Rambaut, 2007). Parameter estimates and their 95% highest posterior density intervals (HPDs) are shown in Table 3.

3. Results

3.1. Phylogenetic and Neighbor-Net analyses of the cloned ITS sequences

In total, we sequenced 342 clones, from which 96 clones representing 45 accessions were retained for further analyses. The cloning failed in three accessions (see Table 1). Furthermore, one accession (S2; BG4-4) was excluded from the analyses as we obtained completely different sequences containing many autoapomorphic changes not shared with any other population of *C. stoebe* or other *Centaurea* species. Total alignment of ITS1–5.8 rDNA–ITS2 was 634 bp and ranged from 632 to 634 bp per accession. The number of different ITS clones found in an individual plant varied from 1 to 3. Bayesian phylogenetic analysis revealed obvious separation of the clones of three accessions belonging to the *C. paniculata* group from the remaining clones/sequences (Fig. 1). Within the *C. paniculata* branch, we found two closely related ITS copies consistently differentiated by two substitutions (Figs. 1 and 2). While two sequenced species of *C. paniculata* and *C. aplolepa* showed intra-individual polymorphism by sharing these two ribotypes, only one paniculata ribotype was found in *C. leucophaea*. The clones of the *C. stoebe* group, as well as of other taxa formed one well supported, but highly polytomic clade. Within this major clade another one supported subclade emerged. The major clade and subclade were representing by two divergent ITS sequences here referred to ribotypes (copies) A and B (Fig. 1). The clones belonging to A and B ribotypes differed, with some exceptions, consistently in three substitutions at positions 77, 199 and 577 (Table 2). Three clones (S19b, S21a, S23b) from three diploid *C. stoebe* s.str. accessions, which exhibited A ribotype had at position 199 C instead of T. Furthermore, one clone of tetraploid *C. sarandinakiae* (SAa) belonging to the ribotype B had G and T at positions 77 and 199 respectively, like the ribotype A. Finally, the clone S35c from tetraploid *C. stoebe* s.l. belonging to the B ribotype had at position 77 G instead of A (Table 2). In addition to three diagnostic sites, approximately half of the clones of the B ribotype showed further substitution (C → T) at position 499. This substitution occurred also in one S5a clone (diploid *C. stoebe* s.str. from Switzerland) belonging to the ribotype A. Ribotype A was found in all sequenced diploid, triploid, tetraploid and hexaploid accessions of *C. stoebe* s.l., and in all other diploid taxa, except one tetraploid accession of *C. stoebe* s.l. from Serbia (S35), where only B ribotype and one putatively recombinant clone was found (Fig. 2). Some further subclades showed fairly high support within clade A. Three clones of three diploid *C. stoebe* s.str. accessions (S19, S21, S23) originating from the same region (Russia and Ukraine) were clustered together, and further three clones of accessions S5, S16 and S17 formed another well-supported subclade, however with no clear geographic affinities (two accessions from Serbia, one from Switzerland). Within the A clade the clones of diploid *C. donetzica* and *C. vankovii* formed a further subclade, but with lower support. In contrast to ribotype A, the B ribotype was found in all sequenced polyploid accessions of *C. stoebe* s.l. (i.e. tri-, tetra- and hexa-) and tetraploid C. *sarandinikiae* but was absent in all diploid *C. stoebe* s.str.

The topology of the parsimony strict consensus tree (not shown; RI = 0.8537, CI = 0.6104) was coincident with the Bayesian tree, but bootstrap support values were very low. The

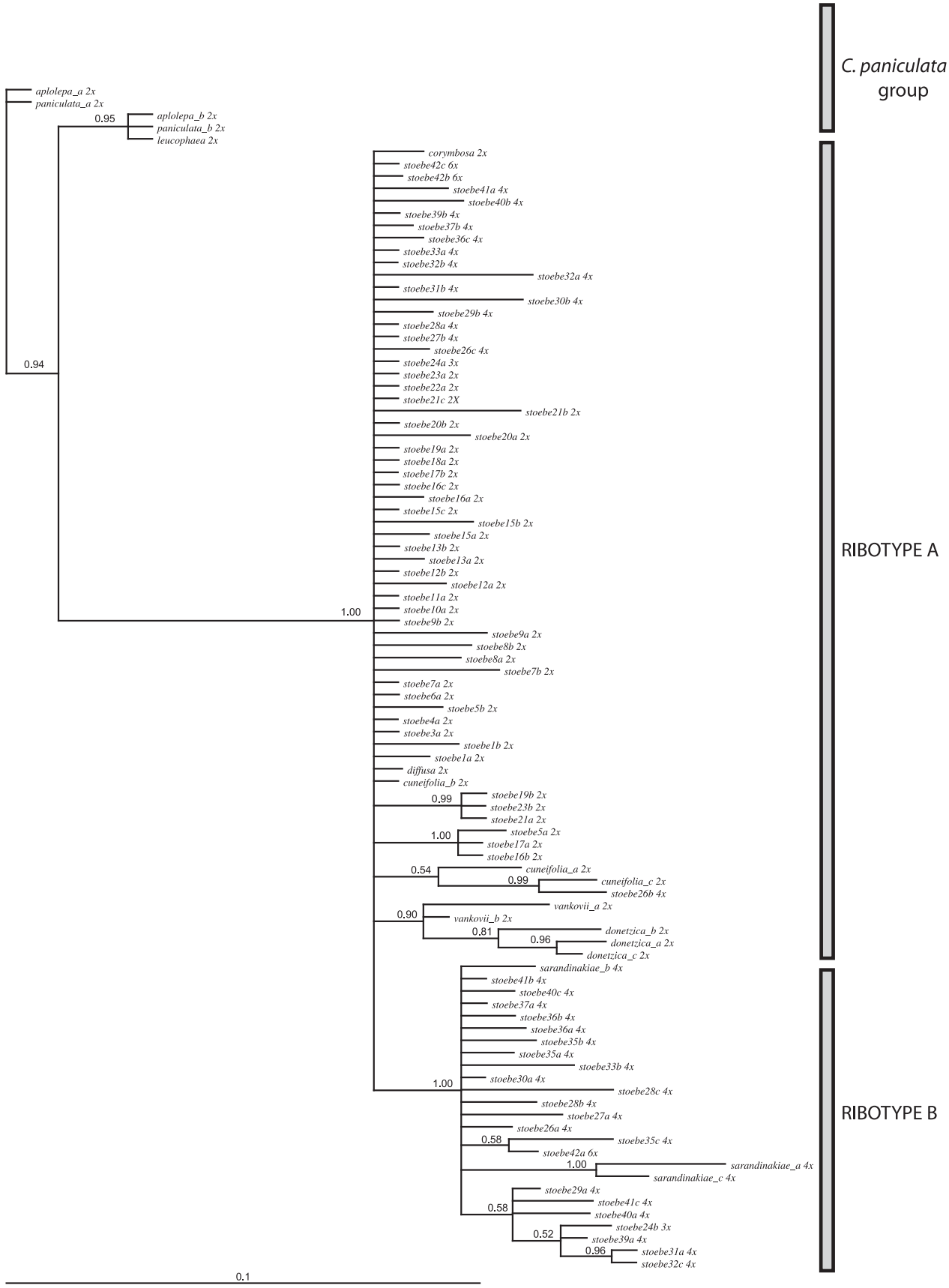

Fig. 1. Bayesian majority rule consensus tree from the ITS data using *Centaurea aplolepa*, *C. leucophea* and *C. paniculata* as outgroup species. Numbers above branches indicate Bayesian-credibility values (PP). Plant codes are as follow: A – *C. aplolepa*; CO – *C. corymbosa*; CU – *C. cuneifolia*; DI – *C. diffusa*; DO – *C. donetzica*; L – *C. leucophea*; P – *C. paniculata*; SA – *C. sarandinakiae*; S1 to S42 – *C. stoebe*; V – *C. vankovii* (see Table 1).

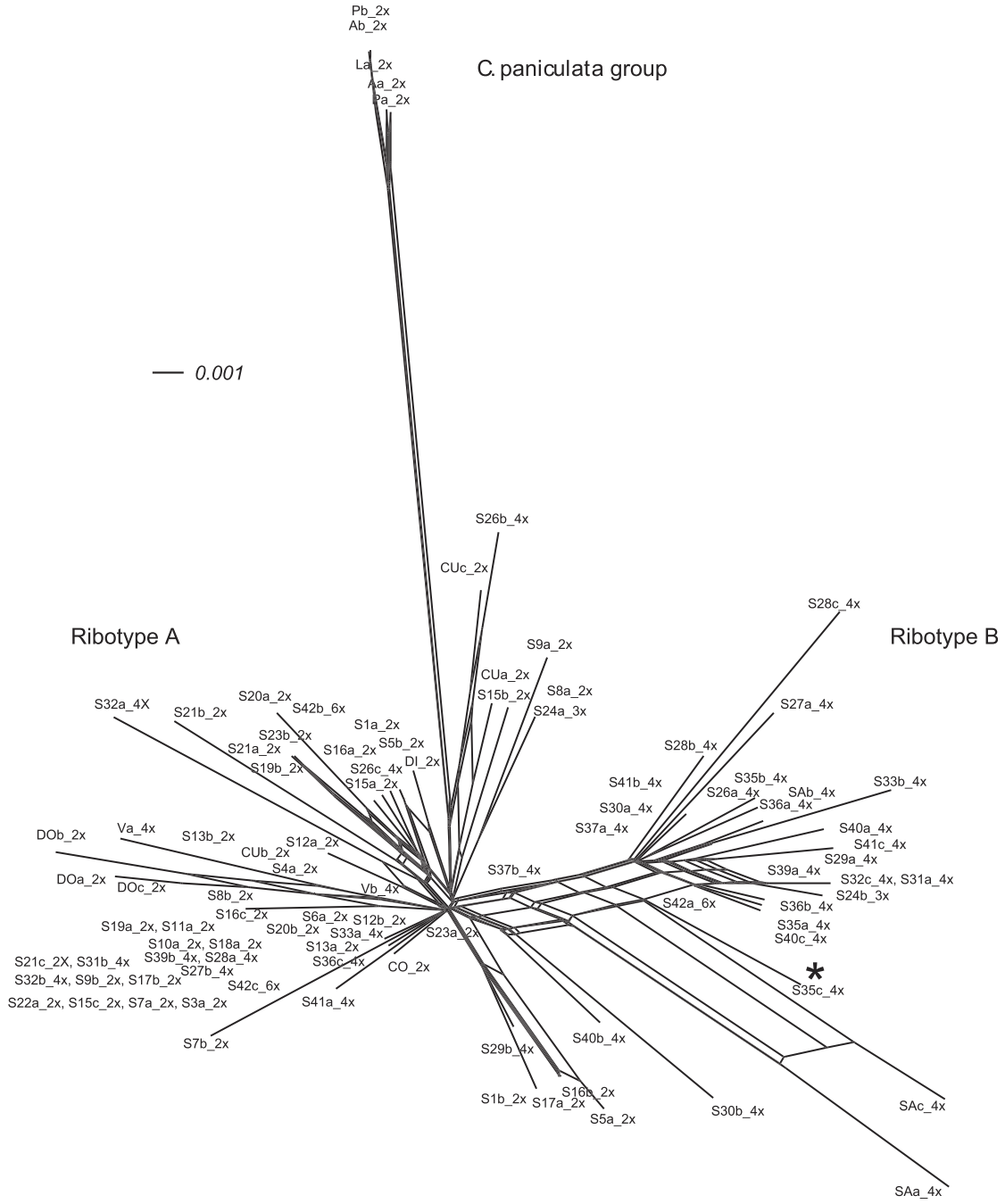

Fig. 2. NN split graphs based on uncorrected *p*-distances of the ITS matrix (non-informative and constant characters excluded). ITS copies A and B are tentatively indicated, and one putatively recombinant clone (S35c) is marked by asterisk. Plant codes are as follow: A – *C. aplolepa*; CO – *C. corymbosa*; CU – *C. cuneifolia*; DI – *C. diffusa*; DO – *C. donetzica*; L – *C. leucophea*; P – *C. paniculata*; SA – *C. sarandinakiae*; S1 to S42 – *C. stoebe*; V – *C. vankovii* (see Table 1).

Table 2
Diagnostic substitutions for A and B ribotypes found in the *Centaurea stoebe* group. Intermediate clones among both ribotypes are given below, the ploidy level and taxon's names are given in parentheses.

	77	199	577
Ribotype A	G	T	T
Ribotype B	A	C	G
A (S19b, S21a, S23b – all 2× *C. stoebe* s.str.)	G	C	T
B (SAa – *C. sarandinakiae* – 4×)	G	T	G
B (S35c – *C. stoebe* s.l. – 4×)	G	C	G

Neighbor-Net (NN) unrooted analysis confirmed the pattern obtained by the Bayesian and parsimony approach and revealed three main networks: one corresponding to the *C. paniculata* group, the second one to ribotype A, and the third one to ribotype B (Fig. 2). One ITS clone from a tetraploid individual (S35c) was placed in NN analysis between A and B networks together with two clones from *C. sarandinakiae* (SAa and SAc), suggesting its putative recombinant character (Fig. 2). In fact, the S35c clone exhibits G at site 77 as in ribotype A, while at sites 199 and 577 it has C and G respectively as in ribotype B (see above and Table 2).

3.2. Divergence time estimations based on ITS sequences

Mean genetic distance between the *Paniculata* clade and *Stoebe* clade using Tamura-Nei substitution model with gamma

Table 3
Split and diversification age estimations (in mya) calculated by a strict molecular clock approach and Bayesian analyses using relaxed molecular clock model (BEAST).

Method	Split of *C. paniculata* and *C. stoebe* clades	Origin of *C. stoebe* clade diversification	Origin of B ribotype diversification
Molecular clock (*Eupatorium* ITS substitution rate, 2.51×10^{-9})	8	–	–
Molecular clock (Madieae ITS substitution rate, 3×10^{-9})	9.6	–	
BEAST (95% HPD interval)	9.53 (7.73–11.37)	2.37 (1.41–5.21)	1.94 (0.62–2.46)

distribution was 0.024. When two slightly different calibrated substitution rates were used (see Section 2 and Table 3), the divergence time between the *C. paniculata* and the *stoebe* groups based on strict molecular clock approach was estimated to be c. 8 and 9.6 mya, respectively.

Bayesian estimations assuming relaxed ITS evolution over time provided slightly different age estimations than strict molecular clock method (cf. Table 3 and Fig. 3). Specifically, the split between the *C. paniculata* and C. *stoebe* clades was estimated to be c. 9.53 mya. The diversification of the *Stoebe* clade was estimated at 2.37 mya, and the origin of B ribotype diversification was estimated to be c. 1.94 mya (Table 3).

3.3. Haplotype diversity analyses

Combined *atpB–rbcL* and *trnT–trnL* sequences of 38 accessions resulted in 1303 bp long alignment. From 14 variable sites in total, six were parsimoniously informative. In addition to single nucleotide substitutions, 17 insertion–deletion polymorphisms were found, which were however excluded from subsequent analyses. Based on single nucleotide polymorphisms we constructed a haplotype network resulting in 12 different haplotypes (Fig. 4 and Table 1). No clear structure and largely shared cpDNA haplotype diversity in respect of analysed taxa and ploidies were found. The same pattern, i.e. no resolution was obtained using Maximum likelihood analysis (results not shown). In haplotype network the H1 and H2 haplotypes were the most frequent and included most of the diploids and tetraploids of the *C. stoebe* group, but in different proportions (Fig. 4). Three accessions belonging to three species from the *C. paniculata* group each belong to different haplotypes. While *C. paniculata* s.str. showed a unique haplotype (H9), two other species, *C. aplolepa* and *C. leucophaea*, shared their haplotypes (H2 and H3, respectively) with other accessions of *C. stoebe*. Three mutation steps present each twice on different branches may indicate frequent homoplasies (Fig. 4).

4. Discussion

4.1. Pattern of individual nrDNA polymorphism and hybridogeneous origin of tetraploid C. stoebe s.l.

Most of the accessions showed multiple ITS copies within each individual genome suggesting absence or very slow pace of concerted evolution. This pattern agrees with recent findings (reviewed in Bailey et al., 2003) that intra-individual polymorphism is much more frequent than previously thought (Baldwin et al., 1995). Generally, three main sources of intra-individual polymorphisms are recognized: hybridization (Kaplan and Fehrer, 2007; Záveská Drábková et al., 2009; Hirschegger et al., 2010; Jacob and Blattner, 2010; Šingliarová et al., 2011), ancestral polymorphism (Pamilo and Nei, 1988; Muir and Schlötterer, 2005) or presence of pseudogenes (Buckler and Holtsford, 1996; Kita and Ito, 2000; Mayol and Rosselló, 2001). In our case, the presence of pseudogene(s) can be ruled out, as cloned sequences showed no long indels, mutations in coding 5.8 S rDNA were extremely rare, the G–C content was in the range 56.1–57.4% and the ribotypes showed normal secondary structure (data not shown). Distinguishing between ancestral polymorphism and hybridization is more difficult. Ancestral polymorphism implies occurrence of all divergent ribotypes in the ancestral taxon and their various combinations in descendent heterozygous individuals. For this reason one may expect the presence of B ribotype also in some diploid *C. stoebe* s.str. accessions, which however was not the case and all diploid plants showed A ribotype (Figs. 1 and 2). Therefore, we propose a hybridization hypothesis to explain the co-occurrence of two divergent A and B ribotypes in almost all tetraploid individuals of *C. stoebe* s.l. One could argue, however, that such a pattern can reflect directional homogenization towards the A ribotype and loss of the B ribotype in diploid *C. stoebe* s.str. This hypothesis seems less plausible because a lack of concerted evolution and conservation of different ribotypes is the rule in the groups of *Centaurea* investigated to date (present data; Suárez-Santiago et al., 2007; Garcia-Jacas et al., 2009; Boršić et al., 2011; Hilpold, pers. comm.). Based on these results, we propose that one of the putative parental species of tetraploid *C. stoebe* s.l. is in fact diploid *C. stoebe* s.str., as both cytotypes share the A ribotype and are morphologically similar (Mráz et al., 2011). The donor of the B ribotype remains currently unknown. Although the B ribotype was found in tetraploid *C. sarandinakiae*, this species is morphologically clearly distinct from *C. stoebe* s.l. and very similar to *C. donetzica* and *C. vankovii*, which makes *C. sarandinakiae* as second parent very unlikely. Thus, the B progenitor could be either already extinct, as it was revealed in many polyploid complexes with exhausted sampling effort (Jacob and Blattner, 2010; Brokaw and Hufford, 2010) or the second parental species has not yet been sampled. Considering the fact that still new taxa from *Centaurea-Phalolepis* are being described each year mostly from East Mediterranean and Black Sea areas (Trigas et al., 2008; Doğan and Duran, 2009) we cannot exclude the possibility that the second parental taxon may still exist. The potential cradle of tetraploid *C. stoebe* s.l. may well be located in this area as SE Europe represents the diversity center of *Centaurea-Phalolepis* and the B ribotype was found in *C. sarandinakiae*, which is endemic to this region. Similarly, Ochsmann (2000) suggested that the tetraploid cytotype originated most likely in SE Europe, where the tetraploids are most frequent.

Molecular clock and Bayesian approach placed the split between the *C. paniculata* and *C. stoebe* clades within the late Miocene. On the other hand, diversification of the *Stoebe* group took place in a range from the late Miocene to the Pliocene (Bayesian estimation 1.41–5.21 mya, Fig. 3 and Table 3) and was probably favored by the climatic changes associated to the abrupt increase of the Mediterranean Sea level (Duggen et al., 2003). Interspecific hybridization(s) giving rise to tetraploid *C. stoebe* s.l. is expected to have occurred within the Pleistocene (Bayesian estimation 0.62–2.46 mya, Fig. 3 and Table 3), characterized by the alternation of glacial and interglacial cycles, pointing out its very young age.

Besides the tetraploid cytotype, co-occurrence of A and B ribotypes was found in one triploid individual collected in a mixed-ploidy population, and in one hexaploid plant found in one tetraploid population from the introduced range (Mráz et al., 2011). Since this triploid is from a mixed-ploidy site (Mráz et al., unpubl.),

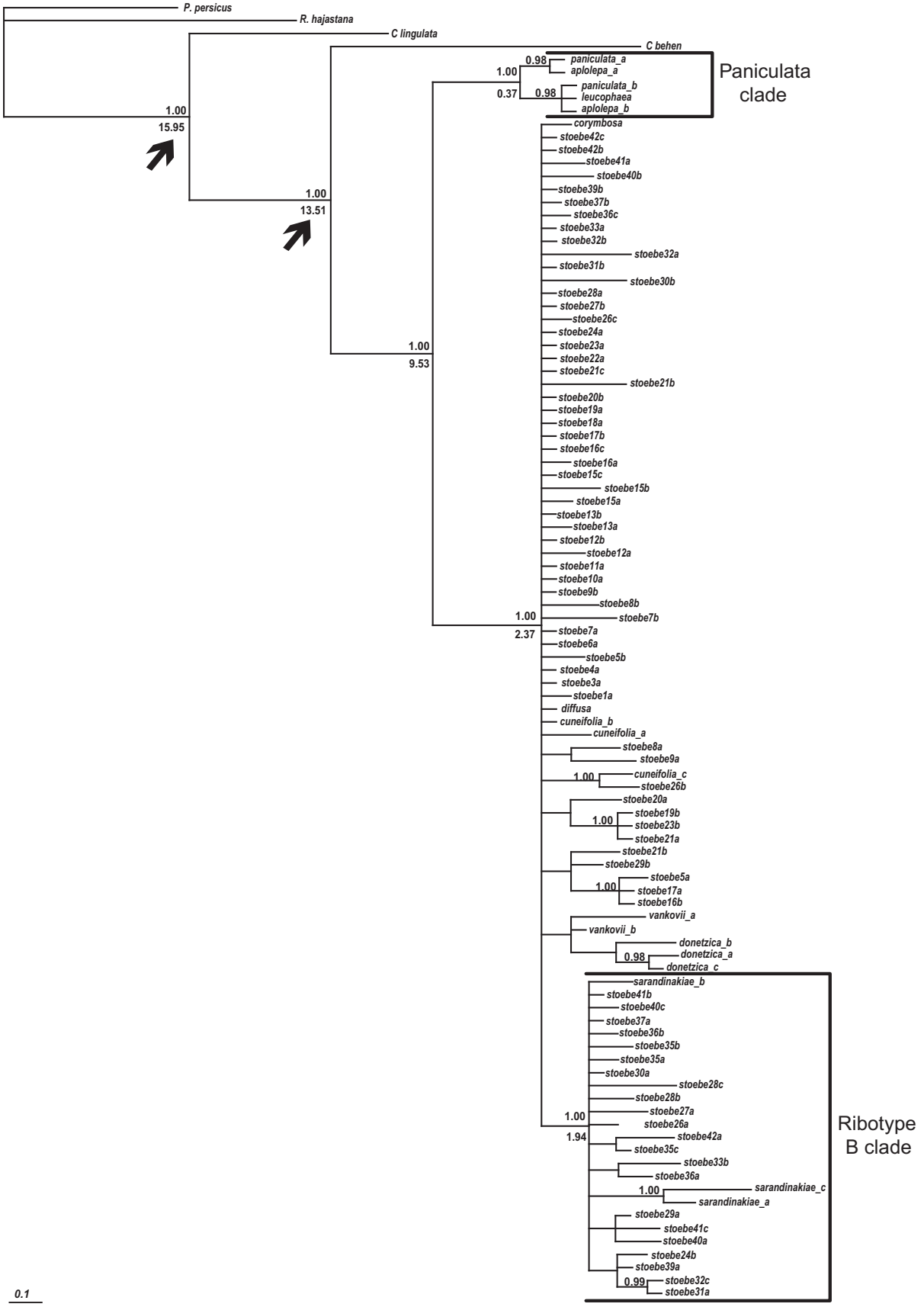

Fig. 3. Bayesian majority rule consensus tree from the ITS datation dataset. Numbers above branches indicate Bayesian-credibility values (PP) and numbers under branches indicate estimated ages of the main supported clades using BEAST. Two calibration data points labeled by arrows were used (see Section 2).

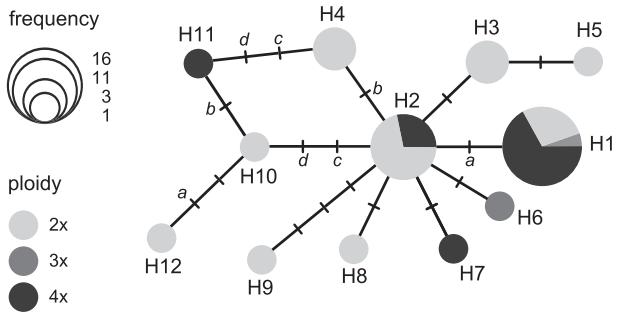

Fig. 4. Haplotype network of twelve haplotypes from 38 accessions of the *Centaurea stoebe* and *C. paniculata* groups based on combined *trn*T–*trn*L and *rbc*L–*atp*B sequences. Three different ploidies are marked by different shading, one hexaploid accession of *C. stoebe* s.l. (URS3) belonging to H1 haplotype is not distinguished. Putatively homoplasious mutations are labeled by italic lowercase letters.

its hybridogenous origin between a 2× and 4× plant is most likely. Such a scenario is corroborated not only by the ITS pattern, but also by microsatelite analyses (Mráz et al., unpubl.). The origin of the single hexaploid plant found in the introduced range in Northern America has been explained through fusion of reduced ($n = 18$) and unreduced gametes of tetraploid plants ($n = 36$, cf. Mráz et al., 2011) and therefore the sharing of A and B ribotypes in this hexaploid is logic.

The allotetraploid origin of *C. stoebe* s.l. further questions the single species concept proposed by Španiel et al. (2008) and rather supports the taxonomic recognition of the tetraploid cytotype as a separate taxon (Ochsmann, 2000; Mráz et al., 2011). Our molecular data are further supported by strong reproductive barriers between both cytotypes (Mráz et al., unpubl.), differences in morphology and life-cycle (Mráz et al., 2011) and the slightly lower homoploid genome size found in tetraploids (Bancheva and Greilhuber, 2006; Mráz and Keller, unpubl.).

Although the second parental species of tetraploid *C. stoebe* s.l. remains so far unknown, we assume that hybridization stimulated the phenotypic and life-history change between both cytotypes, as interspecific hybridization is considered a prominent and instant mechanism of creating new variation (Arnold, 1992; Rieseberg, 1997). The change from the annual/biennial monocarpic life cycle to a perennial one could explain the colonization success of polycarpic tetraploids not only in the introduced range, but also in Central and Western Europe, where the massive spread of 4× cytotype has been recently recorded (e.g. Ochsmann, 2000). Indeed, a perennial life cycle assuring greater persistence and extended seed production may constitute a more efficient strategy for colonizing mesophilous climates like those found in Central Europe. This is in a striking contrast with the conditions in the Mediterranean region, where most successful colonizers among Cardueae are biennial monocarps (Garcia-Jacas et al., 2008). Although polyploidy is often assumed to be an important trait explaining invasion or colonization success (e.g. Ehrendorfer, 1980; Stebbins, 1985; Verlaque et al., 2002; Brochmann et al., 2004; Küster et al., 2008; Pyšek et al., 2009; Pandit et al., 2011), in many cases it is only the consequence of interspecific hybridization (Paun et al., 2009). In this view, interspecific hybridization seems to be a more efficient speciation mechanism as compared to polyploidization *per se*, which in turn acts mostly as a "stabilizing" mechanism of the breeding behavior in newly created hybrids and hybridogeneous species (Grant, 1981). It is thus necessary to distinguish between auto- and allopolyploids in studies focusing on causes of plant invasiveness, as hybridization could be of greater importance for the formation of "evolutionary novelty" leading, in our case, to better colonization and persistence than polyploidization alone.

4.2. ITS variation and phylogenetic relationships within and between the *C. stoebe* and *C. paniculata* groups

The A ribotype was the most common ribotype and was found in all taxa and cytotypes of *C. stoebe*, including the taxa which were considered by Ochsmann (2000) to be morphologically either closely related to (e.g. *C. reichenbachii*, *C. triniifolia*) or distinct from *C. stoebe* (*C. corymbosa*, *C. diffusa*, *C. vallesiaca*). *Centaurea reichenbachii* and *C. triniifolia* are morphologically indistinguishable from *C. stoebe* and accordingly, they do not deserve taxonomic recognition (cf. Mráz et al., 2011; Mráz, unpubl.). On the other hand, *C. corymbosa*, *C. cuneifolia*, *C. diffusa* and *C. vallesiaca* are phenotypically well differentiated in spite of their similar ITS sequences. Such pattern may indicate very recent diversification of this group probably associated with range isolation as in the case of *C. corymbosa* and *C. vallesiaca*. Both of these species are endemics of small regions outside of the continuous *C. stoebe* range. Furthermore, adaptation to specific habitats, such as crevices of calcareous cliffs in the case of the Mediterranean *C. corymbosa* (Colas et al., 1997), or extremely dry steppes in the Black Sea region in *C. diffusa*, could further accelerate morphological differentiation of these taxa.

Our study confirms the results of Ochsmann (2000) and Suárez-Santiago et al. (2007) that the *C. paniculata* group is well separated from the *C. stoebe* group. The distinct position of the *C. paniculata* group is furthermore supported by morphological differences (Ochsmann, 2000), and by c. 1.5 higher homoploid genome size at diploid level in the *C. paniculata* group (Mráz, unpubl.). These finding thus challenge the suggested relationships of the *C. paniculata* group and the bulk of section *Centaurea* (syn. *Acrolophus*, cf. Wagenitz and Hellwig, 1996) and relate it rather to the West Mediterranean section *Willkommia*. The estimated divergence time between the *C. stoebe* and *C. paniculata* groups (c. 8–11 mya) largely overlaps with the Tortonian stage of Miocene characterized by northwards shift of bioms due to increased precipitations and temperature (Pound et al., 2011). This phenomenon could contribute to the fragmentation of the range of common ancestors, and geographically separated populations could diverge.

4.3. Shared plastid DNA diversity

In contrast to ITS sequences, two combined cpDNA loci did not reveal any clear structure with respect to the taxonomic position or ploidy level of the studied accessions. Sharing of different cpDNA haplotypes could be explained by (i) interspecific gene flow, (ii) ancestral polymorphism and (iii) an independent origin of haplotypes. Based on present and still unpublished data from more than 900 accessions (Treier et al., unpubl.) it seems that all three mutually non-exclusive explanations may act in concert. Firstly, extensive gene flow between closely related species of *Centaurea* is a largely accepted fact (see Section 1) and is obvious also in the sect. *Centaurea* (Ochsmann, 2000). Ancestral cpDNA polymorphism and slow mutational rate of this marker could further, at least partly, explain the observed shared cpDNA haplotypes between the phylogenetically divergent *C. stoebe* and *C. paniculata* taxa. Finally, occurrences of homoplasious mutations could further blur the cpDNA pattern (see Fig. 4). Our results thus suggest that cpDNA cannot be used to infer species relationships in *Centaurea* and thus confirm findings from other *Centaurea* sections and groups (Font et al., 2009; Garcia-Jacas et al., 2009; Löser et al., 2009).

4.4. Conclusions

Cloning of nrDNA revealed evidence for a hybridogeneous origin of tetraploid *Centaurea stoebe* s.l. Together with differences in morphology, life cycle and homoploid genome size, allopolyploidization provides a further argument for taxonomic recognition of

the tetraploid cytotype as a different species. Although we do not know the second parental taxon, hybridization could have triggered important changes in the phenotype and life-cycle of the newly formed allotetraploid taxon. Such changes could contribute to better colonization abilities and thus invasion success of the tetraploid cytotype as compared to one of its progenitors – the diploid *C. stoebe* s.str. Our data furthermore indicate very recent diversification of the highly variable *C. stoebe* group, where some morphologically distinct taxa diverged in geographical allopatry. On the basis of cloned ITS sequences and in congruence with other studies (Ochsmann, 2000; Garcia-Jacas et al., 2006; Suárez-Santiago et al., 2007), the *C. paniculata* group is clearly distinct from most of the rest of species of sect. *Centaurea* (syn. *Acrolophus*), but closely related to sect. *Willkommia*, which consists of West Mediterranean taxa.

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Príloha C

Šingliarová B, Hodálová I, Mráz P

Biosystematic study of the diploid-polyploid *Pilosella alpicola* group with variation in breeding system: patterns and processes

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Biosystematic study of the diploid-polyploid *Pilosella alpicola* group with variation in breeding system: Patterns and processes

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Abstract Members of the *Pilosella alpicola* species group (Asteraceae) are distributed throughout the alpine region of the European mountains (Alps, Carpathians, Balkan mountains). Like other *Pilosella* species groups (*Hieracium* subg. *Pilosella*), the taxonomy and species' relationships are poorly understood mostly due to widespread facultative apomixis, frequent hybridization and polyploidization—the most important phenomena substantially involved in the evolutionary history of the genus. We assessed morphology, ploidy level, variation in breeding system and molecular variation within the *P. alpicola* group to provide a new taxonomic concept and to clarify evolutionary relationships among species and origin of polyploids. Multivariate morphometric analyses (UPGMA, CDA, PCA) applied on 324 plants originated from 21 populations revealed existence of four well-separated clusters corresponding to four allopatric taxa: *P. alpicola* s.str. (Alps), *P. rhodopea* (Balkan Peninsula, Southern Carpathians), *P. serbica* (Serbia and Montenegro) and *P. ullepitschii* (Carpathians). In total, four ploidy levels (2*x*, 3*x*, 4*x*, 5*x*) were detected among 557 plants analysed from 19 populations by classical chromosome counting and flow cytometric analysis. The tetra- and pentaploids of *P. alpicola* s.str. have an allopatric distribution (Wallis Alps vs. Dolomites, respectively). Four ploidy levels with complex cytogeographic pattern and high frequency of mixed ploidy populations (75%) indicating a primary contact zone were recorded in *P. rhodopea*. *Pilosella ullepitschii* and *P. serbica* are exclusively diploid and are both sexually reproducing. In spite of a clear morphological separation, a molecular analysis (ITS and cpDNA sequences) suggests close relationships and rather recent origin of all studied taxa, except *P. alpicola* s.str. The latter taxon is an agamospermic allopolyploid that likely originated polytopically from a hybridization between *P. rhodopea* from the Balkans and *P. glacialis* from the Alps. In contrast to *P. alpicola* s.str., our data strongly support an autopolyploid origin of *P. rhodopea* polyploids which reproduced strictly sexually. *Pilosella petraea*, sometimes treated as a member of the *P. alpicola* group, differs from the remaining taxa by its conspicuous morphology, ecology and ITS polymorphism and should be removed from the group. Range shifts and extinctions were likely involved in shaping the evolutionary and modern distributional pattern of the group. Our combined methodological approach enabled us to propose a new taxonomic circumscription for the *P. alpicola* group and revealed auto- and allopolyploidization events.

Keywords allopatric speciation; allopolyploidy; apomixis; autopolyploidy; biogeography; flow cytometric seed screening analysis; *Hieracium*; ITS; multivariate morphometrics

Supplementary Material Tables S1 and S2, and Figures S1 and S2 are available in the free Electronic Supplement to the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

■ INTRODUCTION

The genus *Pilosella*, often treated as a subgenus of *Hieracium* L., belongs to the taxonomically most intricate vascular genera of the Northern Hemisphere. Hybridization, polyploidization and versatile breeding systems have all been considered key evolutionary mechanisms in the genus (e.g., Krahulcová & al., 2000; Fehrer & al., 2007b). These phenomena have largely contributed to the taxonomic complexity and thus have so far obscured species relationships in *Pilosella*. Thus, detailed biosystematics studies are needed to improve taxonomic understanding of the genus. We performed a detailed study of the *Pilosella alpicola* group using various methodological approaches with the aim to solve the taxonomic problems and to clarify the role of polyploidization and hybridization in the evolution of this group.

The reticulate pattern of morphological variation in the genus *Pilosella* has resulted in several, in some cases contradictory, taxonomic concepts (Nägeli & Peter, 1885; Zahn, 1922–1930; Sell & West, 1976; Tyler, 2001; Schuhwerk, 2002; Krahulec & Krahulcová, 2006; Bräutigam & Greuter, 2007). The most common species concept is based on the recognition of so-called "basic" and "intermediate" species (Nägeli & Peter, 1885). Species are characterized as basic (*species principales*) if they have unique phenotypic traits and intermediate (*species intermediae*) if they have a combination of characters from two or more basic species. This classification system has resulted in the identification of ca. 160 species, and many infraspecific taxa within both basic and intermediate species (Zahn, 1922–1930). Large ploidy level variation is characteristic for the genus; the ploidy ranges from diploid (2*x*) to octoploid (8*x*), with basic chromosome number $x = 9$

(Schuhwerk, 1996). In general, basic species are either diploid or polyploid, while intermediate species are usually polyploid (Schuhwerk, 1996); however, many taxa exhibit infraspecific cytotype variation. The asexual formation of the seeds was recorded in polyploids as early as 1907 (Rosenberg, 1907) and was later characterized as aposporous apomixis of the *Hieracium* type with autonomous endosperm formation (Pogan & Wcisło, 1995). Diploids are strictly self-incompatible, however an induced autogamy was occasionally recorded in interspecific crosses (Krahulcová & al., 1999). Polyploids are usually (facultatively) apomictic, but even they (4*x*, 6*x*) often reproduce sexually. However, both breeding systems may operate even within one inflorescence (capitulum). The genus is notorious for widespread interspecific and interploidal hybridization (Krahulcová & al., 2000; Fehrer & al., 2007b).

Morphologically, the *Pilosella alpicola* group (= *P.* sect. *Alpicolinae* (Nägeli & Peter) Szeląg) is characterized by a dwarf habit, the absence of above-ground stolons, and small numbers of inflorescences of relatively large size, which are covered by long and dense indumentum. Ecologically, the members of the group are confined to the primarily alpine and subalpine meadows and screes on acid bedrock and occupy very fragmented ranges in the Pyrenees, Alps, Carpathians and Balkan mountains (Nägeli & Peter, 1885; Zahn, 1922–1930; Bräutigam, 1992). Two different species concepts have been applied for this group. The broader one comprises two species—a Pyrenean *P. breviscapa* DC. (Soják) (= *P. candollei* Monn., nom. illeg.) and *P. alpicola* F.W. Schultz & Sch. Bip. (Zahn, 1922–1930; Sell & West, 1976). Within the latter species, Zahn (1922–1930) distinguished further six subspecies (*Hieracium alpicola* subsp. *alpicola* from the Alps; subsp. *ullepitschii* (Błocki) Zahn and subsp. *furcotae* Degen & Zahn from the Carpathians; subsp. *rhodopeum* (Griseb.) Zahn, subsp. *micromegas* (Fr.) Nägeli & Peter and subsp. *glandulifolium* Nägeli & Peter from the Balkans) and some other taxa at lower ranks. In spite of the existence of several taxa described from different parts of the range, many national floras followed this very broad species concept usually referring to only one taxon—*Pilosella* (*Hieracium*) *alpicola* (Jordanov, 1960; Hess & al., 1972; Zángheri, 1976; Pignatti, 1982; Pawłowski, 1988; Buttler, 1991; Gottschlich & Pujatti, 2002; Szeląg, 2002; Aeschimann & al., 2004). Recently, Szeląg (2008) proposed a narrow species concept and included six taxa at species rank in *P.* sect. *Alpicolinae*: *P. breviscapa*, *P. petraea* F.W. Schultz & Sch. Bip. (= *Hieracium heuffelii* Janka), *P. alpicola* F.W. Schultz & Sch. Bip., *P. rhodopea* (Griseb.) Szeląg, *P. serbica* (F.W. Schultz & Sch. Bip.) Szeląg and *P. ullepitschii* (Błocki) Szeląg. Phylogenetic analysis of the genus *Pilosella* based on ITS sequences showed that at least *P. ullepitschii* (as *P. alpicola* subsp. *ullepitschii*) and *P. breviscapa* are not closely related (Fehrer & al., 2007a). Moreover, our preliminary observations suggested that *P. petraea* does not belong to the *P. alpicola* group. Under these aspects, Zahn's and Szeląg's concepts, based exclusively on morphological observations, deserve a critical revision.

With the exception of the Carpathian populations (Šingliarová & Mráz, 2009), very little is known about karyological and reproductive variation of the *P. alpicola* group. This information, however, is fundamental for the interpretation of morphological and genetic variation, especially in polyploid apomictic complexes. A recent detailed study revealed that 15 Carpathian populations belonging to *P. ullepitschii*, *P. rhodopea* and *P. petraea* were uniformly diploid ($2n = 2x = 18$) (Murín & al., 1999; Šingliarová & Mráz, 2009; Mráz & Šingliarová, 2009). Three cytotypes have been reported so far from only three localities from the remaining part of the range (Alps, Balkans). The tetraploid cytotype ($2n = 4x = 36$) of *Hieracium alpicola* was recorded in the Swiss Alps (Favarger, 1959), the diploids and triploids under the name *H.* resp. *P. alpicola* ($2n = 2x = 18$ and $2n = 3x = 27$) were found in the Pirin Mts (Bulgaria) (Vladimirov & Szeląg, 2001; Krahulcová & al., 2009b) and finally a diploid chromosome number ($2n = 18$) was published for *P. serbica* (Szeląg & al., 2007) from its type locality in Serbia. Concerning the breeding system, sexuality was experimentally ascertained for diploid *P. ullepitschii* (Šingliarová & Mráz, 2009).

The main aim of this study is a taxonomic revision of the *Pilosella alpicola* group. We carried out a large-scale ploidy level survey, analysis of breeding system using flow cytometry seed screening (FCSS), and multivariate morphometric and molecular analyses. More specifically, we aim to answer the following questions: (1) How variable are morphology, ploidy level and breeding system within the *Pilosella alpicola* group and how can we explain this variation? (2) What are the phylogenetic relationships within the *Pilosella alpicola* group? (3) How did the polyploid cytotypes originate?

■ MATERIALS AND METHODS

Sampling. — Plants were collected randomly from their natural habitats in 2005–2009 throughout their distributional range and transplanted into an experimental garden at the Institute of Botany in Bratislava. In addition, we also used herbarium specimens, deposited in the herbarium of the Institute of Botany in Bratislava (SAV) for further morphometric study. The list of localities is given in Table 1. The populations of *P. breviscapa* were not included in our study, as this taxon is phylogenetically not related to *P. alpicola* s.l. (Fehrer & al., 2007a).

Chromosome number and ploidy level determination. — Chromosome counts were performed on somatic mitosis in root-tip meristems of selected cultivated plants using colchicine as a pre-treatment medium (for further details see Mráz & al., 2008). Selected permanent slides were deposited at the Institute of Botany in Bratislava.

Flow cytometry, a rapid and reliable method for accurate estimation of DNA-ploidy level based on differential intensity of fluorescence of cell nuclei in fluid stream (Doležel, 1991), was used to detect ploidy level for most of the plants. Ploidy level was assessed in 370 cultivated plants originating from 19 localities (Table 1). For eleven plants both the exact chromosome number and the ploidy level estimation were determined. Because the analyses revealed a high frequency of populations with mixed cytotypes in the Balkans, we performed additional flow cytometric measurements of 108 herbarized plants (air-dried, 1–2 years old) that originated from mixed-ploidy

Table 1. List of analyzed populations of the *Pilosella alpicola* group.

Code	Taxon / Locality	MF[a]	PL[b] 2*x*	3*x*	4*x*	5*x*	Total/new
	***P. alpicola* s.str.**						
MOR	CH, Wallis Alps, Mt. Monte Moro, N 46°00′57″, E 07°57′58″, 2306 m	15	–	–	28	–	28
SIM1	CH, Wallis Alps, Simplon Pass, N 46°15′00″, E 08°00′50″, 2000–2400 m	9	–	–	51	–	51
SIM2	CH, Wallis Alps, Simplon Pass, Mt. Hübschhorn, 2100–2400 m	–	–	–	27	–	27
PUF	IT, Dolomites, Mt. Puflatsch, N 46°33′07″, E 11°36′47″, 2160 m	10	–	–	–	34	34
SCH	IT, Dolomites, Mt. Schlern, N 46°29′56″, E 11°34′54″, 2019 m	–	–	–	–	16	16
DUR	IT, Dolomites, Val Duron valley, N 46°29′52″, E 11°39′35″, 2236 m	–	–	–	–	17	17
Total		**34**					**173/173**
	P. ullepitschii						
BAR*	SK, Západné Tatry Mts, Trnovecká valley, N 49°09′46.5″, E 19°44′04″, 1885 m	–	21	–	–	–	21
LAL*	SK / PL, Západné Tatry Mts, Ľaliové saddle, N 49°13′35″, E 19°59′30″, 1952 m	4	22	–	–	–	22
KR2*	SK, Vysoké Tatry Mts, Mt. Kriváň, SW, N 49°09′27″, E 19°59′25″, 1900 m	12	20	–	–	–	20
KR5*	SK, Vysoké Tatry Mts, Mt. Kriváň, SE, N 49°09′02″, E 19°59′55″, 1890–1900 m	10	22	–	–	–	22
FUR *	SK, Vysoké Tatry Mts, Furkotská valley, N 49°09′12″, E 20°01′43″, 1910 m	19	37	–	–	–	37
MLY*	SK, Vysoké Tatry Mts, Mlynická valley, N 49°09′30″, E 20°02′30″, 1675–2017 m	8	27	–	–	–	27
MEN*	SK, Vysoké Tatry Mts, Mengusovská valley, N 49°09′57″, E 20°03′40″, 1800–1875 m	19	21	–	–	–	21
OST*	SK, Vysoké Tatry Mts, Mt. Ostrva, N 49°08′58″, E 20°05′22″, 1959 m	4	20	–	–	–	20
BUC*	RO, Bucegi Mts, Cabana Babele, N 45°24′24″, E 25°28′30″, 2160–2204 m	19	41	–	–	–	41
NMA*	RO, Nemira Mts, Mt. Nemira Mare, N 46°15′21.5″, E 26°19′25.5″, 1641 m	–	18	–	–	–	18
NMI*	RO, Nemira Mts, Mt. Nemira Mica, N 46°13′59″, E 26°19′55″, 1619 m	–	6	–	–	–	6
SMA*	RO, Nemira Mts, Mt. Sandru Mare, N 46°11′57″, E 26°20′21″, 1590–1640 m	–	48	–	–	–	50
Total		**95**					**305/0**
	P. rhodopea						
KOR	AL, Korab Mts, Mt. Maja e Korabit, N 41°48′00″, E 20°31′38″, 1920 m	20	13	11	11	–	35
MUS	BG, Rila Mts, Mt. Jastrebets, N 42°13′29″, E 23°34′46″, 2359 m	19	–	6	23	7	36
DOD	BG, Rila Mts, Mt. Dodov vrah, N 42°09′59″, E 23°20′23″, 2540 m	20	25	2	–	–	27
MAL	BG, Rila Mts, Maljovitsa hut, N 42°11′22″, E 23°22′28″, 1985 m	–	–	4	–	–	4
GRA	BG, Rila Mts, Granchar valley, N 42°07′16″, E 23°35′29″, 2200 m	20	–	25	4	–	29
GRA-W	BG, Rila Mts, Granchar valley, Dzhanka ridge, N 42°07′16″, E 23°35′29″, 2342 m	–	8	31	15	–	54
VEZ	BG, Stara planina Mts, Mt. Vezhen, N 42°45′35″, E 24°23′45″, 2130 m	18	15	–	–	–	15
BOT	BG, Stara planina Mts, Mt. Botev, N 42°42′54″, E 24°55′01″, 2352 m	20	7	16	16	1	40
TAZ	BG, Stara planina Mts, above the Taza chalet, N 42°41′30″, E 24°40′00″, 2050 m	–	3	5	4	–	12
VICH	BG, Pirin Mts, Mt. Vichren, N 41°45′39″, E 23°24′27″, 2331 m	20	–	31	3	–	34
BEZ	BG, Pirin Mts, Mt. Bezbog, N 41°43′35″, E 23°30′57″, 2414 m	20	–	34	1	–	35
COZ*	RO, Cozia Mts, Mt. Cozia, N 45°19′04″, E 24°20′17″, 1592 m	20	14	–	–	–	14
Total		**177**					**335/321**
	P. serbica						
KOP	SR, Kopaonik Mts, Mt. Suvo Rudishte, N 43°16′28″, E 20°48′55″, 1917 m	**18**	**63**	**–**	**–**	**–**	**63/63**
Total		**324**					**876/557**

Abbreviations for countries: AL, Albania; BG, Bulgaria; CH, Switzerland; IT, Italy; PL, Poland; RO, Romania; SK, Slovakia; SR, Serbia.
Symbol "*" denotes the populations for which the DNA-ploidy level and/or chromosome numbers were published by Šingliarová & Mráz (2009).
[a] MF, number of plants used for morphometric study.
[b] PL, number of plants studied karyologically and/or measured for the DNA-ploidy level (total/new).

populations. Ploidy level estimations failed in ca 20% of cases most likely due to the disintegration of plant tissues during drying process and subsequent storing of herbarium vouchers. In addition, we estimated the ploidy level for 46 herbarized plants not used in the morphometric analyses. Ploidy level was also estimated for 33 mother plants and 181 seeds in order to detect the mode of reproduction using flow cytometric seed screen analysis (FCSS, see below). In total, we detected the ploidy level for 557 plants and 181 seeds.

Samples from living plants and herbarium vouchers were prepared using a two-step procedure (Otto, 1990; Doležel & Göhde, 1995). For details on sample preparations see Mráz & al. (2008). The clones of previously cytologically analysed diploid plants of *Pilosella lactucella* (Wallr.) P.D. Sell & C. West ($2n = 2x = 18$) were used as an internal reference standard for ploidy level estimations of living individuals, while the DNA-ploidy levels of herbarium specimens were assessed using *Bellis perennis* L. (2C = 3.38 pg, Schönswetter & al., 2007b). Flow cytometric analyses of cultivated plants were performed with a FACSCalibur instrument (Becton Dickinson) equipped with an argon-ion laser exciting at 488 nm at the Institute of Biology and Ecology, P.J. Šafárik University in Košice. Propidium iodide (PI) was used as a stain. The ploidy level of herbarium specimens was detected by a Partec Cyflow instrument equipped with a HBO lamp at the Institute of Botany, Slovak Academy of Sciences, Bratislava with 4,6-diamino-2-phenylindole (DAPI) as a stain. Histograms were accumulated at flow rate of about 20–50 particles per second for a total count of 3000–5000 nuclei. Only measurements with coefficients of variation up to 5% for fresh material (PI staining) or 10% for herbarium vouchers (DAPI staining) were used for analysis.

Breeding system detection. — The breeding system in Lactuceae genera with co-occurring sexual and asexual reproduction can be easily inferred from a combination of isolation, castration and open pollination experiment (Richards, 1997). However, the number of plants of the alpine *P. alpicola* group flowering at lowland experimental conditions was very low. Therefore, we performed flow cytometry seed screening analysis (FCSS, cf. Matzk & al., 2000) for study of reproductive pathways in *P. alpicola* s.str., *P. rhodopea* and *P. serbica*. This method is based on the comparison of the ploidy of embryo and corresponding endosperm within one seed. In the case of sexually formed seed (gamete fusion), the ratio between relative fluorescence of endosperm and embryo peaks is expected to vary around 1.5 because of a diploid embryo and a triploid endosperm (depending on the ploidies of ovule and pollen involved), while in the case of autonomous apomixis (e.g., Koltunow, 1993; Noyes, 2007) in the genus *Pilosella* this ratio is exactly 2.0. Moreover, the FCSS method allows the identification of the ploidy of gametes that participated on embryo formation when the ploidy level of the maternal (seed) plant is known. FCSS analysis was applied on individual seeds to accurately discriminate both embryo and endosperm peaks. Ripe achenes were collected from the plants from following natural populations: GRA-W, KOP, MOR, PUF and SIM1 (see Table 1). The ploidy of the mother plant was estimated from the dried-up part of involucral bracts and/or stems. The seed samples and the samples from the maternal plant were prepared in a two-step procedure (see above). Each seed was measured separately and twice—without standard and after addition of standard. We used the leaves of diploid *P. lactucella* as a reference standard. Relative fluorescence intensity of DAPI stained nuclei (1500–5000 particles) was measured by Partec Cyflow instrument equipped with a HBO lamp.

Morphometric analysis. — Multivariate morphometrics (Sneath & Sokal, 1973) has been successfully used in several taxonomically difficult genera with widespread apomictic reproduction (e.g., *Crataegus*: Smith & Phipps, 1988; *Alchemilla*: Sepp & Paal, 2000; *Ranunculus*: Hörandl & al., 2009; *Sorbus*: Lepší & al., 2009), including the closely related genus *Hieracium* (Chrtek & al., 2007; Tyler, 2006). Surprisingly, as far as we know this approach has never been applied in the genus *Pilosella*, except still unpublished work of Urfus (Urfus, 2006).

For the purpose of morphometric comparison we measured selected morphological characters on 324 herbarized plants from 21 populations (Table 1). A population sample usually included 10–20 flowering individuals. In some cases the sample size, however, was lower than ten plants per population because of a small number of flowering plants. Samples from known type localities of traditionally recognized taxa were also included in our study. Morphological characters measured or scored are those used for identifying taxa of the *P. alpicola* group (Zahn, 1922–1930; Szeląg, 2008) and some new potentially useful were added. Forty-three characters (26 quantitative, 12 semiquantitative, one binary, and four ratios derived (Table 2; Fig. 1; Fig. S1) were measured or scored on herbarized plant material. Ligules and involucral bracts were attached to paper by adhesive tape immediately in the field, then scanned and measured by CARNOY v.2.0 (Schols & al., 2002).

Five datasets were used in the analyses:

1. Twenty-one population samples of the *P. alpicola* group from the whole area characterized by the mean values of all 43 characters as OTUs (operational taxonomic units) (matrix A).
2. A complete, pooled dataset including all 43 characters and 324 individual plants from 21 populations covering the whole area as OTUs (matrix B).
3. A dataset consisting of 139 plants from the Balkans and Cozia Mts with detected ploidy level (61, 2*x*; 54, 3*x*; 22, 4*x*; 2, 5*x*) as OTUs and 42 characters (excluding LibDS—invariable across the group) (matrix C).
4. A dataset consisting of 34 plants from the Alps as OTUs and all 43 characters (matrix D).
5. A data matrix including all 324 plants from the whole area as OTUs and 26 quantitative and 4 ratio characters only (matrix E).

Pearson (parametric) and Spearman (non-parametric) correlation coefficients were computed for all data matrices to study the relationships between particular variables. As no character pair showed strong correlation potentially distorting further computations, all 43 (matrices A, B, D), 42 (matrix C) and 30 (matrix E) characters were used in further analyses. We performed both hierarchical and non-hierarchical multivariate evaluation of the morphological data in the following steps:

Table 2. Morphologic characters used for morphometric analyses of the *Pilosella alpicola* group.

Characters[a]
LNo – number of rosette leaves
LoL – length of leaves from outer part of rosette (hereafter outer leaves) (cm)[b]
LoW – maximum width of outer leaves (cm)[b]
LiL – length of leaves from inner part of rosette (hereafter inner leaves) (cm)[b]
LiW – maximum width of inner leaves (cm)[b]
LoDT – density of simple eglandular trichomes on adaxial side of outer leaves[c]
LiDT – density of simple eglandular trichomes on adaxial side of inner leaves[c]
LoLT – length of simple eglandular trichomes on adaxial side of outer leaves (mm)[d]
LiLT – length of simple eglandular trichomes on adaxial side of inner leaves (mm)[d]
LoDS – density of stellate trichomes on adaxial side of outer leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LiDS – density of stellate trichomes on adaxial side of inner leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LobDS – density of stellate trichomes on abaxial side of outer leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LibDS – density of stellate trichomes on abaxial side of inner leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LbDG – density of glandular trichomes on abaxial side of leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LmDG – glandular trichomes at the margins of leaves (0 – a few, 1 – sparse, 2 – dense)[e]
LSNo – number of cauline leaves
LSL1 – length of the lowest stem leaf (cm)
LSL2 – length of the second lowest stem leaf (cm)
SL – stem length (from basis to terminal capitulum) (cm)
SLL – stem length up to the lowest stem leaf (cm)
SDT – density of simple eglandular trichomes on peduncle (0 – a few, 1 – numerous, 2 – dense)[e]
STL – length of simple eglandular trichomes on peduncle (mm)
STC – colour of simple eglandular trichomes on peduncle (0 – dark up to the middle, 1 – dark more than to the middle)[f]
SDS – density of stellate trichomes on peduncle (0 – a few, 1 – sparse, 2 – dense)[e]
SDG – density of glandular trichomes on peduncle (0 – a few, 1 – sparse, 2 – dense)[e]
CNo – number of well-developed capitula (capitula with flowers)
CaNo – number of undeveloped (aborted) capitula
AL – length of acladium of terminal capitulum (cm)
INo – number of involucral bracts of terminal capitulum
IL – length of involucral bracts of terminal capitulum (mm)[g]
IW – width of involucral bracts of terminal capitulum (mm)[g]
ITL – length of simple eglandular trichomes on involucral bracts (mm)[g]
ITC – colour of simple eglandular trichomes on involucral bracts (0 – predominantly pale, in some cases with dark basis, 1 – dark approximately to the middle, 2 – predominantly dark, eventually with pale apex)[f]
IC – colour of involucral bracts (0 – silver grey, 1 – grey, 2 – grey-black)
IDT – density of simple eglandular trichomes on involucrum (0 – a few, 1 – sparse, 2 – dense)
FNo – number of ligules in terminal capitulum
FL – length of ligule in terminal capitulum (mm)[g]
FW – width of ligule in terminal capitulum (mm)[g]
StL – length of styles in terminal capitulum (mm)[g]
LoL/LoW
LiL/LiW
FW/FL
StyL/FL

[a] See also Fig. 1 and Fig. S1.
[b] Mean values of the two largest leaves.
[c] Mean number of eglandular trichomes on the two largest leaves, counted on 0.25 cm² in the central part using paper template.
[d] Mean value of three randomly selected trichomes from one leaf.
[e] Estimated, see Fig. S1.
[f] pale – white, ivory, yellowish, silver grey; dark – smoky grey, grey-black, dark brown-grey
[g] Mean values from the three measurements per individual.

1. **Cluster analyses** (Everitt, 1986) with different algorithms, UPGMA (unweighed pair-group method using arithmetic averages), complete linkage cluster analysis and Ward's method of clustering (minimization of the increase of error sum of squares), were performed on **matrix A** to get first insight into the phenetic relationships among all studied populations of the *P. alpicola* group. The characters in the primary matrix were standardized by zero mean and unit standard deviation and the Euclidean coefficient was used to compute the secondary distance matrix.

2. Differentiation suggested by cluster analyses (four entities, see Results) was tested by canonical **(CDA1)** and nonparametric classificatory discriminate **(NCD)** analyses (Klecka, 1980; Krzanowski, 1990) based on the **matrix B.** To reveal correlation of characters with the canonical axes, total canonical structure was computed. The NCD was used to assess the percentage of plants correctly assigned to the predetermined groups.

3. **PCA** based on **matrix C** was used to infer potential morphological differentiation among different cytotypes of plants originated from the Balkans and Cozia Mts in Romania.

4. Morphological consistency of two cytotypes of *P. alpicola* s.str. from two disjunct ranges (tetraploids from Walliser Alps and pentaploids from Dolomites) was tested by principal coordinate analysis **PCoA** using Gower's coefficient for mixed data (Podani, 2001) because the number of OTUs exceeds the number of characters, based on **matrix D.**

5. Because Zahn (1922–1930) and Szeląg (2008) used mostly semi-quantitative or qualitative characters to distinguish relevant taxa from the *Pilosella alpicola* group, we ran **CDA2** on **matrix E** to assess a taxonomic significance of quantitative characters for distinguishing morphological entities revealed by cluster analyses and CDA1.

6. Descriptive data analysis was used to obtain basic statistics of quantitative characters and ratio (mean, percentile 10% and 90%, and standard deviation) for each taxon revealed. For semi-quantitative and binary characters the frequencies of particular states are presented.

All analyses were carried out using SAS v.9.2; only cluster analyses and PCoA were computed in SYN-TAX 2000 (Podani, 2001).

DNA extraction and sequencing. — We selected the internal transcribed spacer (ITS) of ribosomal nuclear DNA to test monophyly and relationships within the *Pilosella alpicola* group as ITS sequences were more informative in the genus *Pilosella* than plastid *matK* and *trnL-trnF* introns (Fehrer & al., 2007a). Moreover, ITS is a suitable marker to unravel

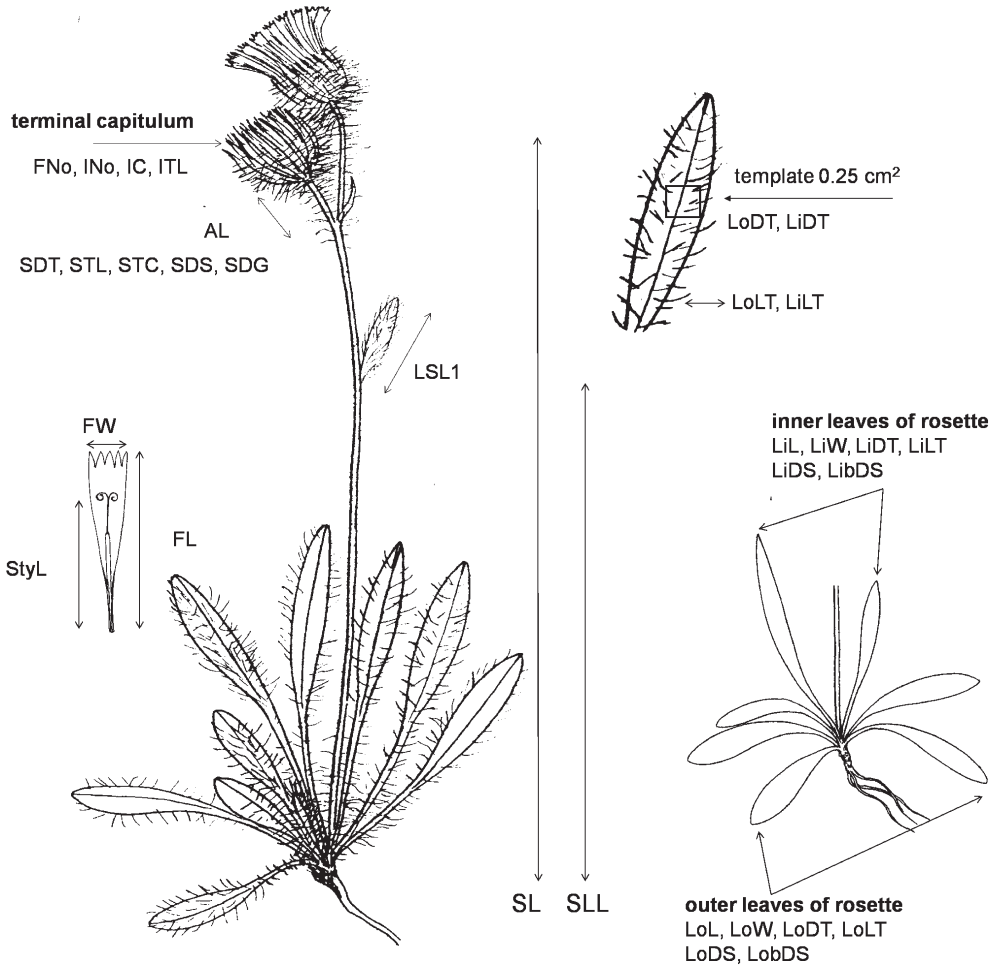

Fig. 1. Illustrations of selected morphological characters measured in the *Pilosella alpicola* group.

hybridization and allopolyploidization events, if the process of homogenisation is very slow or completely absent (e.g., Fehrer & al., 2009). In addition, we tested the plastid *rps16-trnK* spacer for polymorphism. Timme & al. (2007) recently reported that this locus belongs to the most divergent in Asteraceae and that it might be potentially suitable for species-level phylogeny. Moreover, according to M. Ronikier (pers. comm.) this marker showed intraspecific polymorphism in the closely related *Hypochaeris uniflora*.

In total, we sequenced 21 plants of *P. alpicola* s.l. from 15 populations (see Tables 1 and 6; Table S1) for ITS1–2 (GenBank accession numbers HM627292–627314, except of HM627297 and HM627306). We included also one plant of *P. petraea* from its type locality (accession number HM627306, Ciclova Montană in Banat Mts, Romania), because this taxon was considered a member of *Pilosella* sect. *Alpicolinae* by Szeląg (2008) (see Introduction), and one diploid accession of *P. cymosa* (accession number HM627297, Primovce, Slovakia, cf. Mráz & Šingliarová, 2009), as this species was considered by Zahn (1922–1930) as one of the putative parental taxa of *P. petraea*. The leaves were sampled from cultivated plants with known ploidy level, dried in silica gel and stored at room temperature. Total DNA was extracted from 10–15 mg of silica-dried leaf tissue with the DNeasy 96 Plant Kit (Qiagen), following the manufacturers protocol. The ITS1 and ITS2 spacers were amplified using the primers ITSA (GGAAGGAGAAGTCGTAACAAGG) and ITSB (CTTTTCCTCCGCTTATTGATATG) (Blattner, 1999) in a 25 µl volume containing 1 µl of genomic DNA (10–35 ng), 2.5 µM buffer (10× Buffer II, Applied Biosystems), 1.5 mM $MgCl_2$, 0.2 µM of each dNTP, 0.2 µM of each primer, 0.25 µM BSA and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). The cycle profile included the initial denaturation at 95°C/10 min followed by 28 cycles of 95°C/30 s, 94°C/30 s, 52°C/30 s, 72°C/1 min, and ended with 72°C/10 min and 4°C thereafter. PCR products were purified using QIAquick Gel Extraction Kit. Sequencing was performed in both directions using BigDye Terminator v.3.1 (Applied Biosystems). In total, three plants from geographically distant localities (SIM1, DOD, KOP; see Table 1), were tested for polymorphism in the *rps16* gene and the *rps16-trnK* intergenic spacer. We used the same PCR conditions and specific primers as given in Timme & al. (2007). The samples were run on an ABI PRISM 3100 Genetic Analyser. Sequences were assembled and edited using Seqscape v.2.5.0 (Applied Biosystems).

Heterozygous sites (intra-individual polymorphic nucleotide site) in ITS1 and ITS2 were scored on both forward and reverse strains according to Aguilar & Feliner (2003), with exception of "at least 25% of strength of the weakest signal criteria". In some cases we accepted a lower threshold in one of two strands. In order to compare our sequenced material with other taxa from *Pilosella*, we conducted an alignment with MEGA v.4 (Tamura & al., 2007) adding 16 *Pilosella* species with already known ITS sequences (GenBank accessions AJ633390, AY879161, AJ633406, AJ633504, AJ633389, AJ633396, AJ633397, AJ633398, AJ633402, AJ633403, AJ633399, AJ633405, AJ633400, AJ633393, AJ633394, all from Fehrer & al., 2007a, and AY879158 from Schuhwerk, unpub.).

■ RESULTS

Ploidy level variation and cytotype pattern. — Four ploidy levels, varying from diploid to pentaploid, were found in the *Pilosella alpicola* group (Table 1; Figs. 2 and 3).

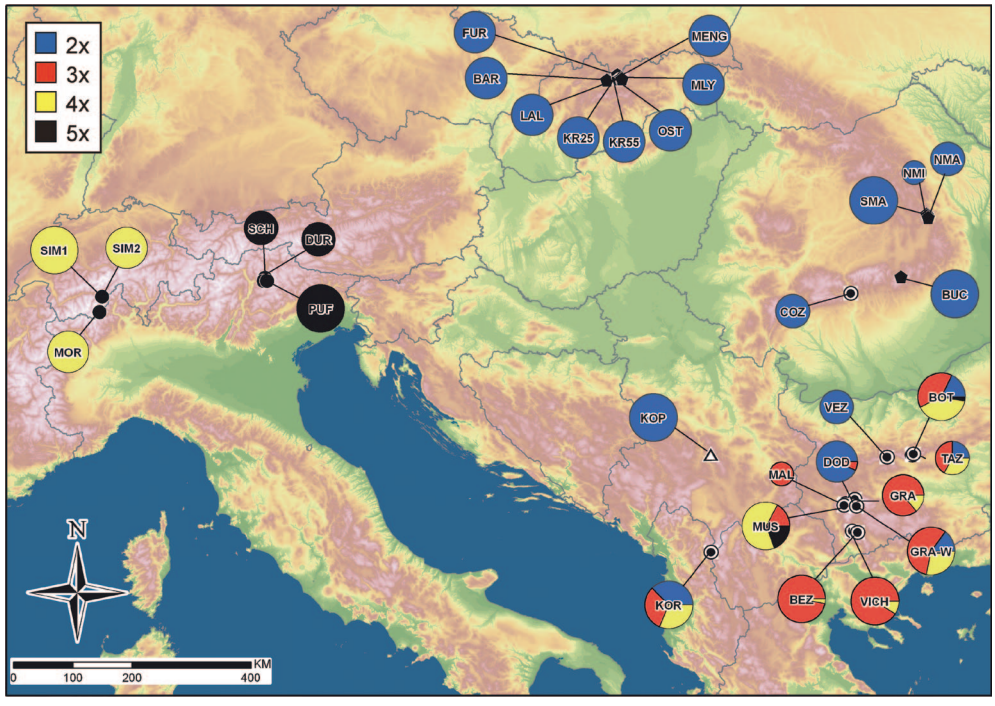

Fig. 2. Cytogeographic pattern in the *Pilosella alpicola* group. *Pilosella alpicola* s.str. (black circles), *P. ullepitchii* (black pentagons, data based on the results published by Šingliarová & Mráz 2009), *P. rhodopea* (double circles, DNA-ploidy level of COZ population published by Šingliarová & Mráz 2009), *P. serbica* (open triangle). Population codes are given in Table 1. Circle size represents four classes of sample size: 1–9, 10–19, 20–30 and more than 30 individuals analysed per population.

The nominate taxon *P. alpicola* s.str. included allopatrically distributed tetra- (4*x*) and pentaploid populations (5*x*). While solely tetraploid cytotypes occured in the Swiss Alps (106 plants analysed from three populations—SIM1, SIM2 and MOR), the pentaploids represented the only cytotype detected in the Italian Alps (67 plants from SCH, PUF and DUR). In total, four ploidy levels (2*x*, 3*x*, 4*x*, 5*x*) were detected in *P. rhodopea*. From 335 plants originating from 12 populations (including the published data on 14 plants from the Cozia population, Šingliarová & Mráz, 2009), 85 were diploid (25.4%), 165 were triploid (49.3%), 77 were tetraploid (23%) and 8 were pentaploid (2.4%). Coexistence of two or more cytotypes was found in 9 out of 12 analysed populations. In one population (BOT) we found all four ploidies. The diploids of *P. rhodopea* were more frequent (52.2%) in the northern part of the species range (Cozia Mts, Stara planina Mts), but southwardly they were either less common (Korab Mts, 37.1%), or completely missing, as in the Pirin Mts. In the latter mountain range (BEZ, VICH), the triploid cytotype completely predominated (94%), while tetraploids constituted a minor cytotype. The populations from the Rila Mts are very variable in cytotype composition (Table 1; Fig. 2). In *P. serbica* only diploids were found (63 plants).

Breeding system. — Castration performed in two tetraploid plants of *P. alpicola* s.str. revealed an apomictic seed formation with 75.6% of ripe achenes from four capitula. An apomictic mode of reproduction in this taxon was further confirmed by FCSS in six other tetraploid plants from Switzerland (SIM1, MOR) and two pentaploid plants from Italy (PUF) (Table 3; Fig. 4). In both cases the ratio of DAPI fluorescence intensity of endosperm and embryo nuclei was 2.0.

In contrast, FCSS of 16 maternal plants of *P. rhodopea* (GRA-W) of three different ploidies (2*x*, 3*x*, 4*x*) showed that all plants reproduced sexually (Table 3; Fig. 4). The DAPI fluorescence intensity ratio of the endosperm and embryo was in the range of 1.33–1.75, depending on the ploidy of gametes involved in fecundation. Three analysed triploid plants produced exclusively tetraploid embryos that arose by fusion of unreduced triploid egg cells and haploid pollen (3*x* + *x*) as evidenced by presence of heptaploid endosperm (6*x* + *x*). Diploids and tetraploids usually produced the embryos of the same ploidy level as the corresponding maternal plant, but very rarely triploid embryos were also detected in both cases (Table 3). In this case, the reduced haploid ovule of the diploid mother was fertilized by diploid pollen (*x* + 2*x*), because the ploidy of endosperm was tetraploid (2*x* + 2*x*). In the tetraploid mother plant, the reduced diploid ovule was fertilized by haploid pollen (2*x* + *x*), because the endosperm was pentaploid (4*x* + *x*).

Seed progeny originating from ten diploid maternal plants of *P. serbica* was formed by the sexual pathway having diploid embryos and triploid endosperms (Table 3; Fig. 4).

Morphological variation. — The morphometric study of the *Pilosella alpicola* group revealed four morphologically separated groups corresponding to four taxa: *P. alpicola* s.str., *P. ullepitschii*, *P. rhodopea* and *P. serbica* (Fig. 5).

Cluster analyses performed on 21 populations of the *P. alpicola* group consistently proved the existence of four clusters

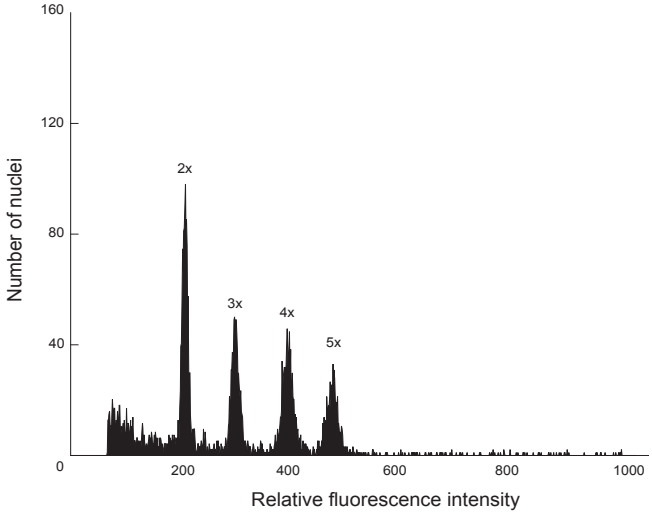

Fig. 3. Histogram of the relative DNA content of DAPI-stained nuclei in simultaneous analysis of diploid (2*x*, *P. ullepitschii*), triploid (3*x*, *P. rhodopea*), tetraploid (4*x*, *P. alpicola* s.str.) and pentaploid (5*x*, *P. alpicola* s.str.) plants of the *Pilosella alpicola* group.

Table 3. Reproduction pathways in three taxa of the *Pilosella alpicola* group detected by flow cytometric seed screen analysis.

Taxon	Locality	PLmat/N	Nseed	PLem/PLen	Fluorescence intensity endosperm/embryo ratio	Ploidy egg cell	Ploidy pollen	Embryo origin
P. alpicola s.str.	SIM1	4*x*/4	19	4*x*/8*x*	2.00	4*x*	–	Autonomous apomixis
P. alpicola s.str.	MOR	4*x*/2	3	4*x*/8*x*	2.00	4*x*	–	Autonomous apomixis
P. alpicola s.str.	PUF	5*x*/1	3	5*x*/10*x*	2.00	5*x*	–	Autonomous apomixis
P. rhodopea	GRA-W	2*x*/5	35 1	2*x*/3*x* 3*x*/4*x*	1.50 1.33	1*x* 1*x*	1*x* 2*x*	Sexual Sexual
P. rhodopea	GRA-W	3*x*/3	29	4*x*/7*x*	1.75	3*x*	1*x*	Sexual
P. rhodopea	GRA-W	4*x*/8	1 48	3*x*/5*x* 4*x*/6*x*	1.67 1.50	2*x* 2*x*	1*x* 2*x*	Sexual Sexual
P. serbica	KOP	2*x*/10	42	2*x*/3*x*	1.50	1*x*	1*x*	Sexual

Abbreviated column headers are as follows: PLmat/N, mother plant ploidy level/number of maternal plants analysed; Nseed, number of seeds; PLem/PLen, ploidy of embryo/endosperm.

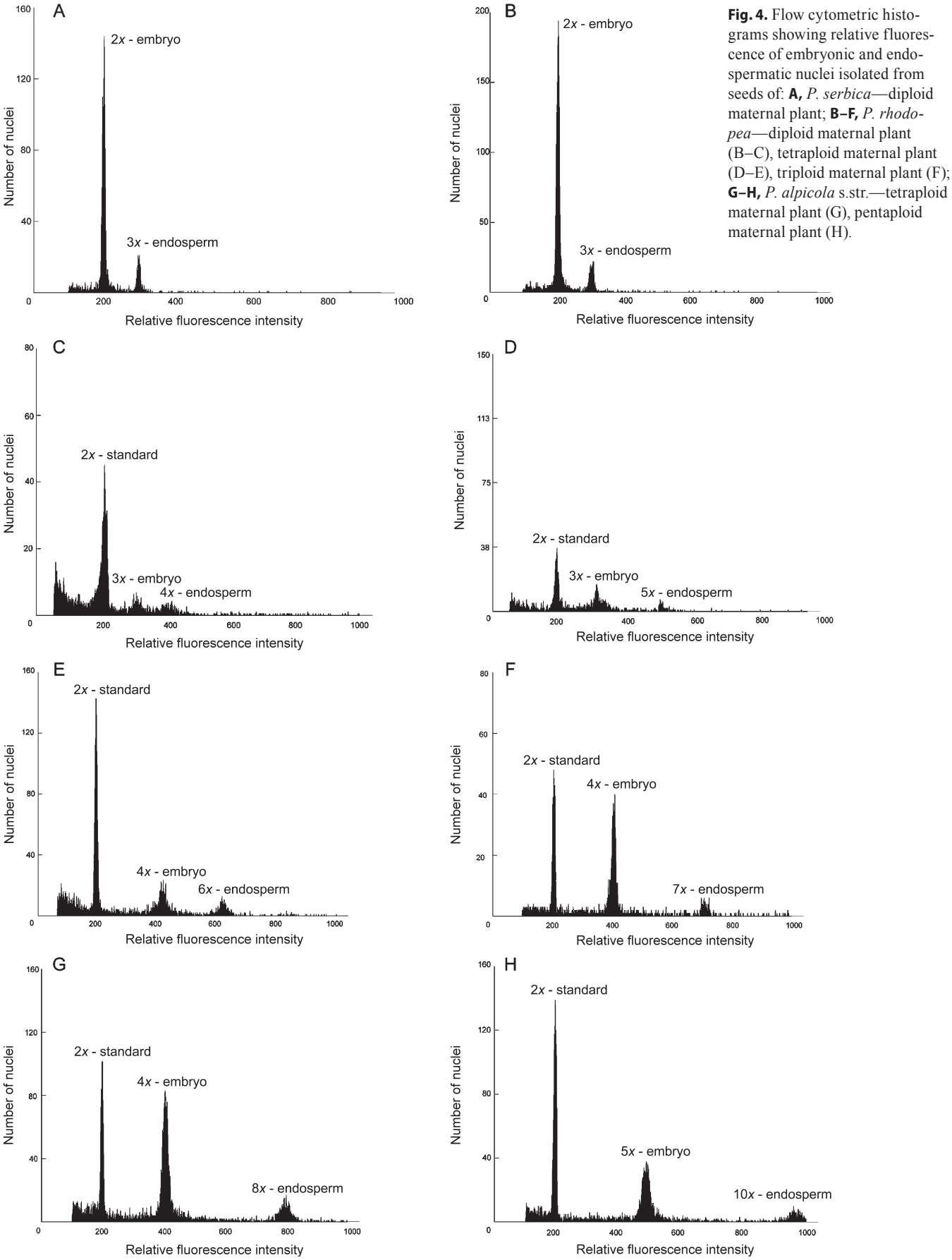

Fig. 4. Flow cytometric histograms showing relative fluorescence of embryonic and endospermatic nuclei isolated from seeds of: **A,** *P. serbica*—diploid maternal plant; **B–F,** *P. rhodopea*—diploid maternal plant (B–C), tetraploid maternal plant (D–E), triploid maternal plant (F); **G–H,** *P. alpicola* s.str.—tetraploid maternal plant (G), pentaploid maternal plant (H).

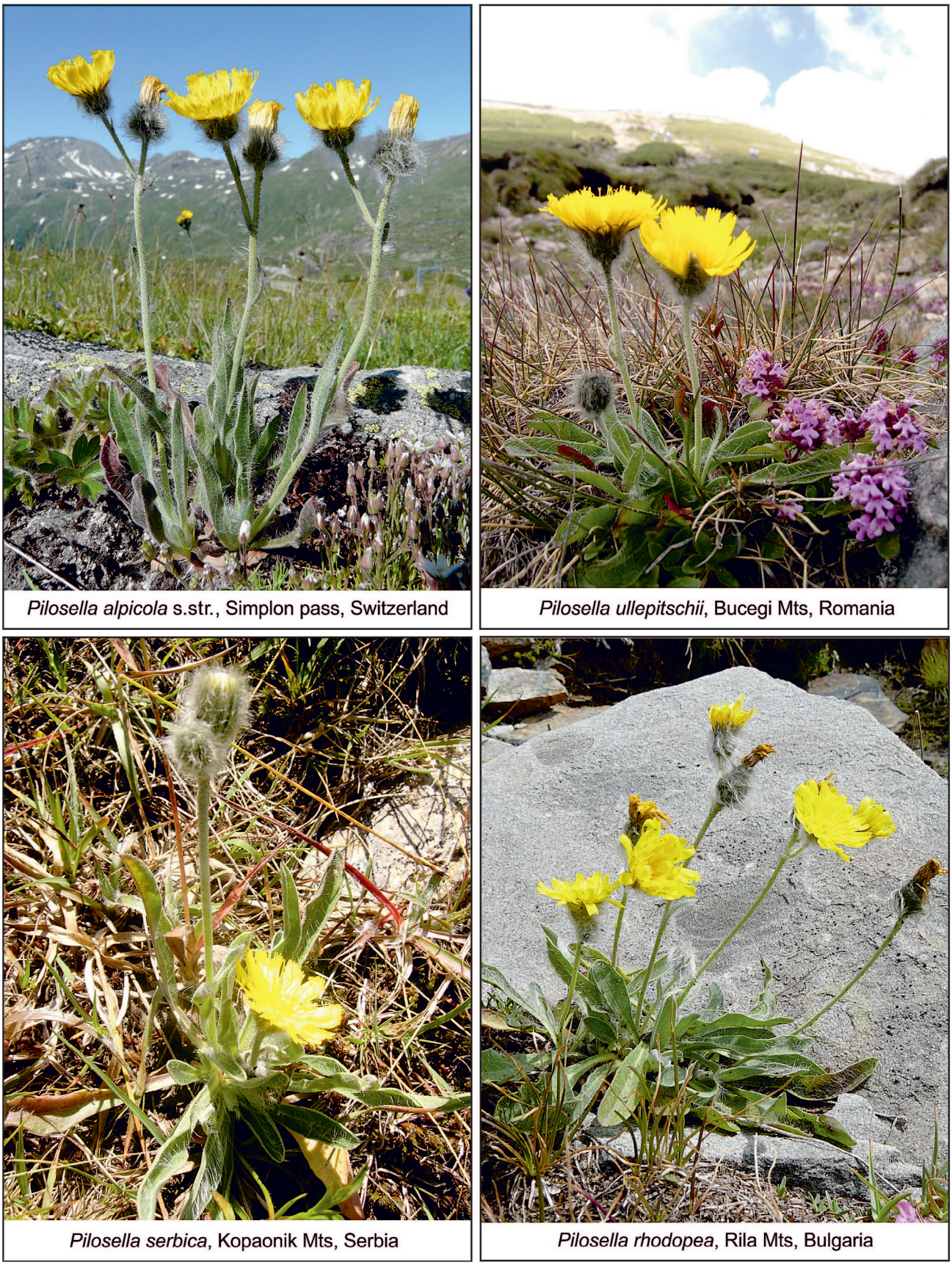

Fig. 5. Four species of the *Pilosella apicola* group. Photos by B. Šingliarová and P. Mráz.

Table 4. Total canonical structure (CDA1: can1, can2, can3) showing correlation of the characters[a] measured in the *Pilosella alpicola* group with the axes (highest values in bold).

Character	can 1	can 2	can 3
LNo	0.2472	0.0909	0.2074
LoL	–0.3120	0.4793	0.1478
LoW	0.0000	–0.0248	–0.0026
LiL	0.1046	0.4878	0.2171
LiW	–0.4113	0.1760	0.1520
LoDT	0.1616	0.5787	0.0042
LiDT	–0.1617	0.3527	0.0275
LoLT	–0.1251	**0.7225**	–0.0158
LiLT	–0.2724	0.4963	–0.0048
LoDS	0.7768	–0.0526	–0.1190
LiDS	**0.9158**	–0.0736	–0.0175
LobDS	**0.9376**	–0.0143	–0.0506
LibDS	**0.9754**	–0.0979	–0.0172
LbDG	0.1803	–0.1668	**0.7240**
LmDG	–0.0822	–0.2201	**0.8406**
LSNo	–0.3735	–0.1908	0.0714
LSL1	–0.2861	0.1733	0.3085
LSL2	–0.2586	–0.1300	0.2970
SL	–0.2370	0.5840	0.2443
SLL	–0.2403	0.0762	–0.0690
SDT	0.1804	–0.0087	0.4883
STL	–0.0244	0.3161	–0.0989
STC	–0.1644	0.4701	–0.1087
SDS	0.8341	0.0780	0.0445
SDG	–0.5554	0.4569	0.0048
CNo	0.1616	**0.7930**	0.1983
CaNo	0.0542	0.2306	**0.6325**
AL	0.1628	0.0075	0.1688
INo	–0.3090	–0.0172	0.4803
IL	–0.1060	0.0629	0.4570
IW	–0.1744	–0.1576	0.1103
ITL	–0.1834	0.0173	–0.3422
ITC	–0.2042	0.0676	0.0611
IC	**–0.9809**	–0.0925	0.0148
IDT	–0.7809	0.0004	0.0246
FNo	–0.2259	0.5760	–0.0753
FL	–0.4312	0.2979	–0.2291
FW	–0.1148	0.3824	–0.1714
StyL	–0.4848	0.3450	–0.0893
LoL/LoW	–0.1046	0.0865	0.1754
LiL/LiW	0.2897	0.1049	0.0452
FW/FL	–0.1444	0.5812	0.0438
StyL/FL	0.3138	0.2278	0.0296

[a] For character codes see Table 2.

(UPGMA plot presented here, Fig. 6). The population from the type locality of *P. serbica* (Kopaonik Mts, Serbia) was separated at the highest level of dissimilarity. The second cluster consisted of three sub-clusters. The first sub-cluster included all Carpathian populations (*P. ullepitschii*), but one—the southernmost Carpathian COZ population which fell into the second sub-cluster merging all populations from the Balkans (*P. rhodopea*). Three populations from the Alps formed the third sub-cluster (*P. alpicola* s.str.).

CDA1 (Fig. 7; Table 4) was performed to test for morphological differentiation of the *Pilosella alpicola* group and at the same time to identify morphological characters that are most suitable for distinguishing the four groups revealed by cluster analyses. The analysis shows delimitation of the individuals into four well-separated groups corresponding to four different geographical regions—the Alps, the Balkan mountains, Serbia and the Carpathians (Fig. 7). The plants from the Carpathian COZ population, however, were grouped together with the Balkan plants. The Carpathian plants without COZ population were separated along the first canonical axis and differ from the plants from other regions by less numerous stellate trichomes on leaves (LiDS, LobDS, LibDS) and dark colour involucres (IC). Along the second axis there is an isolated group of individuals from the Alps with a higher number of capitula (CNo) and longer trichomes on the outer leaves (LoLT). The last group, plants from the Kopaonik Mts, was differentiated from the remaining Balkan populations and COZ population along a third axis correlated with density of the glandular trichomes on the leaves (LmDG, LbDG) and the number of aborted capitula (CaNo).

Classificatory discriminant analysis (both parametric and non-parametric, data not shown) correctly classified plants into four groups with the highest percentage possible—100%.

Karyological and flow cytometric study revealed four different ploidy levels within *P. rhodopea*. Therefore, we performed **PCA** in order to test possible morphological differentiation between the cytotypes. The analysis showed no clear pattern correlated with ploidy level within *P. rhodopea* (Fig. 8). On the other hand, according to **PCoA** (Fig. S2), slight differentiation was found in the group of Alpine plants of *P. alpicola* s.str. Exclusively pentaploid populations from PUF (Italy) tend to be separated from the two other Swiss tetraploid populations along the second axis. This separation was caused by differences in length and width of the leaves and the plant height (LoL, LiL, LoW, LiW, SL).

We also tested the importance of quantitative and ratio characters only, excluding all semiquantitative characters. The **CDA2** differentiated the plants from the Alps from the rest along the first axis (Fig. 9). Alpine plants had deeper branched stems (ALL) and more dense simple trichomes on their leaves (LiDT, LoDT). Individuals from the Balkans and the Carpathians were partially intermingled. Carpathian plants were separated from the plants that originated from the Balkan's along a second axis due to the longer styles (StL) and flowers (FL), while plants from the Kopaonik Mts have higher numbers of capitula (CNo), involucral bracts (INo) and flowers (FNo) than plants from remaining Balkan mountain ranges.

The results of exploratory data analysis (means, standard deviation, minimum, maximum, 10% and 90% percentiles, frequencies of semiquantitative and binary characters) showed that recognised taxa differ mainly in semiquantitative characters (e.g., LoDS, LiDS, LibDG, IC; see Table 5 and Table S2). This is in accordance with the results from CDA2 suggesting that the quantitative characters alone are insufficient for correct identification of the *Pilosella alpicola* taxa.

Based on the detailed morphometric study, we provide the determination key for taxa recognised in this study (Appendix).

Variation in *rps16-trnK*, and ITS sequences. — The length of the aligned matrix of the *rps16* gene and the *rps16-trnK* intergenic spacer was 903 bp. Three sequenced plants belonging to three species had identical cpDNA sequences (GenBank accession numbers HM627315–17).

Only a limited amount of variation was recorded in nuclear ITS1-5.8S-ITS2. Therefore, we did not perform any phylogenetic analyses, but a comparison of poly-

morphic sites is provided in Table 6. The total length of the ITS1-5.8S-ITS2 region was 639 bp. We found one substitution at position 583 (ITS2) in one sample of *P. ullepitschii* exhibited C, other *P. ullepitschii* accessions showed additive polymorphism—Y (C+T, with much stronger C signal in all samples), and all remaining taxa of the group had T. *Pilosella petraea* showed at positions 74 (ITS1) and 612 (ITS2) two other substitutions—C and T respectively, both unique among all so far sequenced *Pilosella* species (Table 6; Table S1). *Pilosella cymosa* had one unique substitution among all so-far sequenced *Pilosella* taxa (including another *P. cymosa* accession) at site 559 having T instead of C. In addition, in *P. petraea* we recorded one deletion (G) in one ITS copy at position 70 and in *P. cymosa* another deletion (C) in one ITS copy at position 88. In both cases, the deletions resulted in one nucleotide shift and overlapping peaks (only sequences without deletions are given in Table 6 and Table S1).

In total, we recorded 23 different intra-individual polymorphic nucleotide sites in the *P. alpicola* group, 11 in ITS1 and 12 in ITS2. None substitution or nucleotide polymorphism was found in the highly conservative 5.8S region. Twelve of

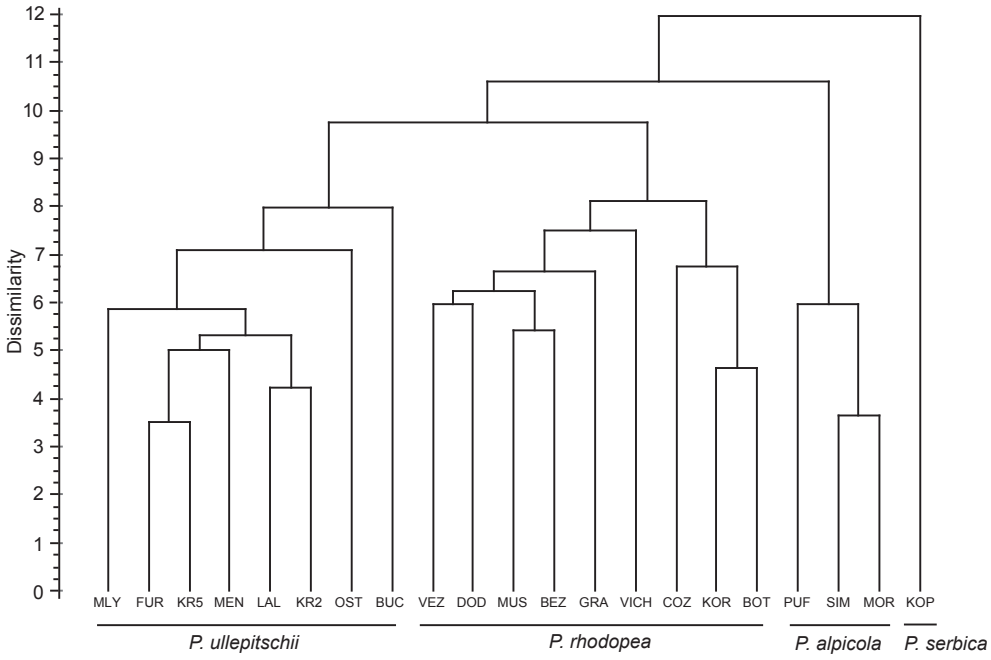

Fig. 6. Cluster analysis (UPGMA) of 21 populations of the *Pilosella alpicola* group. Population codes are given in Table 1.

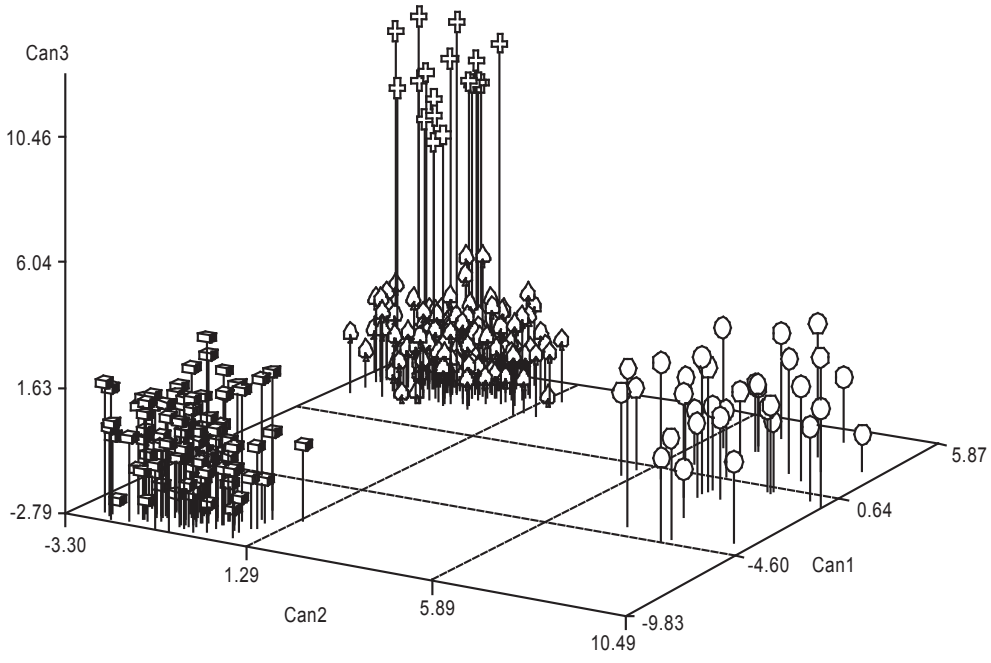

Fig. 7. Canonical discriminant analysis (CDA1) based on 43 morphological characters of individuals of the *Pilosella alpicola* group from the Alps (34 plants, circles), Carpathians (95 plants, cubes), Bulgaria, Albania, Cozia Mts in Romania (177 plants, spades) and Serbia (18 plants, crosses). The first three axes explain 83.5%, 10.2% and 6.3% of total variation, respectively.

twenty-three polymorphisms in the *P. alpicola* group were present in at least two samples and 11 were unique to individual plants. Two polymorphisms at positions 131—Y (C+T, ITS1) and 600—M (A+C, ITS2) were shared by all samples sequenced, except for one belonging to *P. petraea* and *P. cymosa*. The number of intra-individual polymorphic nucleotide sites varied between 2 to 11 per sequence (Table 6) and it was not correlated with ploidy level (Spearman rank correlation coefficient, $\rho = 0.301$, $P = 0.189$). Conversely, a significant association between this parameter and taxon was found (Kruskal-Wallis test, df = 3, $P = 0.008$; 1 accession of *P. petraea* and 1 accession of *P. cymosa* were not included in comparison). While in *P. ullepitschii*, *P. rhodopea* and *P. serbica* the number of intra-individual polymorphic sites ranged from 2 to 4 per sequence, in *P. alpicola* s.str. it was more than two-fold higher (10–11). *Pilosella petraea* had 7 and *P. cymosa* 6 polymorphic sites, each of them different from those found in the *P. alpicola* group (Table 6). Most of the intra-individual polymorphisms found in *P. ullepitschii*, *P. serbica* and *P. rhodopea* had no complementary base to other so far sequenced *Pilosella* taxa (Table 6; Table S1).

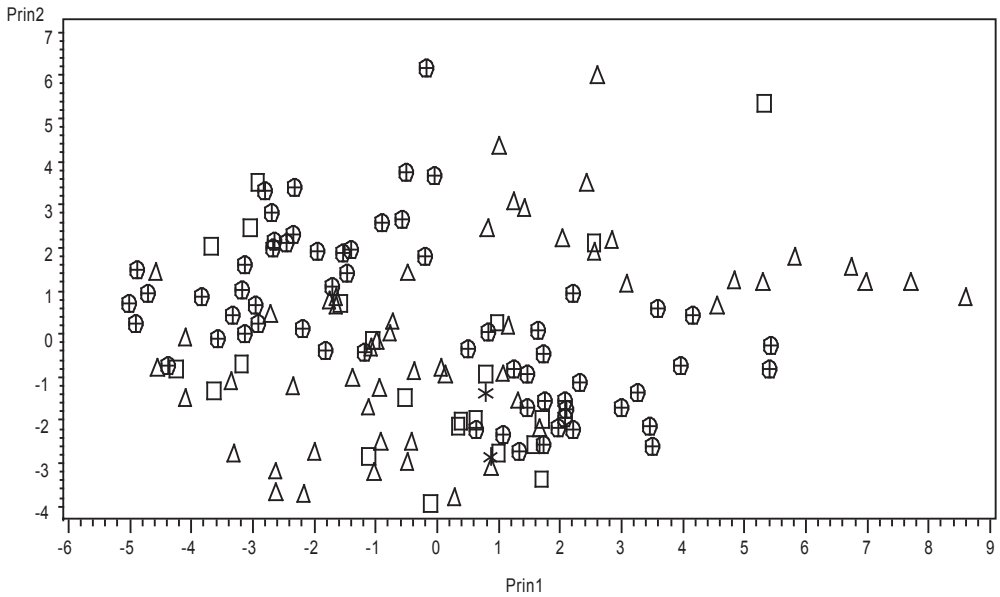

Fig. 8. Principal component analysis (PCA) based on 42 morphological characters of different cytotypes of *P. rhodopea* from Bulgaria, Albania and Cozia Mts in Romania. Diploid, circle; triploid, triangle; tetraploid, square; pentaploid, pentagon. The first two axes explain 18.9% and 10.5% of total variation, respectively.

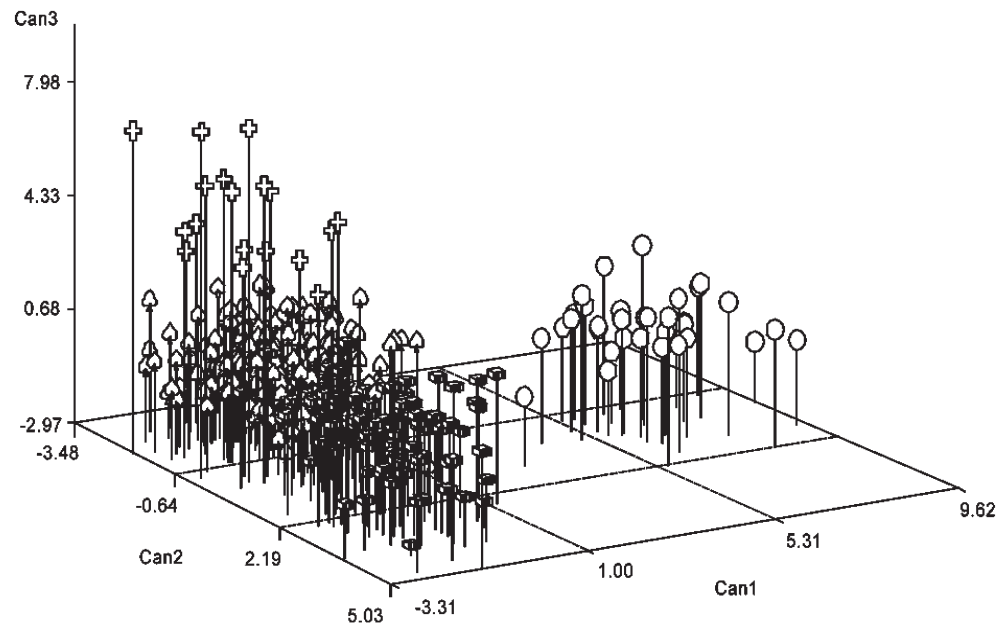

Fig. 9. Canonical discriminant analysis (CDA2) based on 26 quantitative and 4 derived ratio morphological characters of individuals of the *Pilosella alpicola* group from the Alps (34 plants, circles), Serbia (18 plants, crosses), Balkan Peninsula and Cozia Mts (177 plants, triangles) and Carpathians (95 plants, squares). The first three axes explain 56.7%, 27.1% and 16.2% of total variation, respectively.

Table 5. Frequencies (in %) of particular states of semiquantitative and binary characters[a] in different taxa of the *Pilosella alpicola* group.

	P. alpicola s.str.			*P. ullepitschii*			*P. rhodopea*			*P. serbica*		
State	0	1	2	0	1	2	0	1	2	0	1	2
LoDS	15	79	6	100	0	0	7	46	47	6	83	11
LobDS	6	85	9	100	0	0	0	21	79	0	22	78
LiDS	6	47	47	100	0	0	0	12	88	0	28	72
LibDS	12	47	41	100	0	0	0	0	100	0	0	100
LbDG	79	21	0	84	16	0	77	23	0	0	11	89
LmDG	94	6	0	68	32	0	94	6	0	0	11	89
SDG	24	76	0	53	47	0	46	47	7	0	17	83
SDT	6	32	62	19	61	20	21	60	19	39	61	0
SDS	0	18	82	27	72	1	0	10	90	0	0	100
STC	9	91	–	26	74	–	89	11	–	100	0	–
ITC	74	26	0	0	0	100	97	3	0	89	11	0
IC	24	61	15	0	0	100	47	46	7	30	70	0
IDT	0	26	74	24	60	16	44	66	0	83	17	0

[a] For character codes see Table 2.

■ DISCUSSION

Ploidy level variation, cytotype pattern and breeding system. — Including present and published karyological data, the *Pilosella alpicola* group shows a clear geographic and taxon-specific pattern (Fig. 2). *Pilosella ullepitschii* is an exclusively diploid and a strictly outcrossing Carpathian taxon (Murín & al., 1999; Šingliarová & Mráz, 2009). The only published tetraploid record from the Vysoké Tatry Mts (Uhríková & Dúbravcová, 2000) has never been confirmed in spite of a large number of individuals analysed from 13 localities (Šingliarová & Mráz, 2009) and we consider this record as questionable. Based on the analyses of the plants from the type locality (Mt. Kopaonik, Serbia), *Pilosella serbica* is also a diploid sexually reproducing species (Szeląg & al., 2007 and this paper). Further, a karyologically uninvestigated population was found in the Prokletije Mts in Montenegro (Szeląg, 2008; Šingliarová & al., in prep.).

Our results confirm that plants of *P. alpicola* s.str. in the Wallis Alps are tetraploid (cf. Favarger, 1959), but also reveal a new pentaploid cytotype. Both cytotypes have non-overlapping distributions: tetraploids occur in the Wallis Alps only, while pentaploids were ascertained only from the Italian Dolomites. Such allopatric distribution of tetra- and pentaploids suggests polytopic origin of polyploids. Tetraploid and pentaploid plants of *P. alpicola* s.str. reproduce apomictically, which is mirrored in low allozyme variation (Šingliarová & al., in press). However, the tetraploids are more variable than the almost genetically uniform pentaploids (Šingliarová & al., in press). Higher level of genetic variation found in the Wallis Alps might suggest some level of facultative sexual reproduction retaining in tetraploids. Facultative apomixis is indeed quite common in the *Pilosella* polyploids (e.g., Gadella, 1987; Pogan & Wcisło, 1995; Skalińska, 1971, 1973; Krahulcová & al., 2000, 2009a).

The Balkan subendemic *P. rhodopea* was cytologically the most variable taxon. We confirmed the existence of diploid and triploid cytotypes already reported by Šingliarová & Mráz (2009), Vladimirov & Szeląg (2001) and Krahulcová & al. (2009b). In addition, we found two new ploidies—4*x* and 5*x*. Interestingly, we found a high proportion of mixed-ploidy populations (75%) in *P. rhodopea*. A substantially lower proportion of mixed-cytotype populations was recorded in other *Pilosella* taxa studied in detail: *P. bauhini* (8.3%; Rotreklová, 2004), *P. officinarum* (12%; Mráz & al., 2008) and *P. echioides* (24%; Trávníček & al., 2011). However, proportion of ploidy-mixed populations revealed in two former studies could be influenced by lower number of analysed plants. Moreover, a significant impact of sampling strategy on cytotype composition has recently been shown (Duchoslav & al., 2010; Šafářová & Duchoslav, 2010). Here, we underline the importance of hybridization between cytotypes and participation of reduced and unreduced gametes in formation, maintenance of ploidy level variation and frequent occurrence of mixed ploidy populations in *P. rhodopea*. Furthermore, our data suggest a lack of strong reproductive barriers between cytotypes of *P. rhodopea*. Although 2*x* and 4*x* plants mostly produced offspring of identical ploidy (Table 3), rare triploid progeny were detected too. Triploids arose through hybridization between 2*x* and 4*x* (in both directions) (Table 3; Fig. 4). The triploid mother plants included in this study produced only a tetraploid progeny resulting from the fusion of an unreduced triploid ovule (formed via apomeiosis) and haploid sperm, although our unpublished data (Šingliarová & Mráz, unpub.) as well as the data from other genera (Cifuentes & al., 2010; Cosendai & Hörandl, 2010) revealed the formation of ovules with more variable ploidy level (including aneuploid one). Similar results were obtained from inter- and intraploidal experimental crosses in *P. echioides* (Peckert & Chrtek, 2006), where triploids originated mostly from 2*x* × 4*x* (both directions) and from 4*x* (mother plant) × 3*x* (pollen donor) crosses. Furthermore, if triploid mother plants of *P. echioides* were crossed with diploid pollen donors, mostly tetraploid progeny was obtained (Peckert & Chrtek, 2006), which agrees with our field data. Our results show that all studied *P. rhodopea* cytotypes are, at least to a certain level, fertile, including the triploids, which moreover substantially contribute to the formation of tetraploids, in accordance to the triploid bridge hypothesis (Harlan & de Wet, 1975).

The overall high frequency of triploid *P. rhodopea* in the Balkans (almost 50% of all plants analysed) is rather surprising as extremely low frequency of triploid embryos was detected in seed progeny analyses of 2*x*, 3*x* and 4*x* plants (Table 3). At GRA-W the cytotype proportion of adults plants was 2*x*—14.8%, 3*x*—57.4% and 4*x*—27.8% (Table 1), but the ploidy proportion in their seed progeny was quite different: 2*x*—30.7%, 3*x*—1.8% and 4*x*—67.5% (Table 3). Thus, there were less triploids in seed samples than expected from the cytotype frequency in the field. Although our results might be biased by a low number of plants used in FCSS (n = 16), Trávníček & al. (2011) observed the same shift in *P. echioides*.

Table 6. Variation in ITS region (ITS1-5.8S-ITS2) of the *Pilosella alpicola* group, *P. cymosa* and *P. petraea* sequenced in the present study and of *P. breviscapa*, *P. glacialis* (= *Hieracium angustifolium*) and *P. lactucella* based on ITS sequences by the other authors (source GenBank). Intraindividual polymorphisms are marked by grey background.

			Alignment position																																			
Taxon-accesion[a]	PL[b]	N[c]	39	46	55	70	74	75	76	100	104	114	116	118	131	206	208	224	231	445	449	451	463	479	485	494	512	526	536	537	547	557	559	562	573	583	596	600	602	612
u-MLY14-HM627304	2*x*	3	C	T	A	S	A	T	A	G	A	A	C	G	Y	T	T	G	C	T	G	G	C	C	T	T	G	T	T	T	T	C	C	C	C	C	C	M	A	G
u-OST17-HM627307	2*x*	4	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	Y	·	M	·	·
u-BAR6-HM627292, *u*-BUC7-HM627294	2*x*	3	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	Y	·	M	·	·
u-SMA19-HM627312	2*x*	4	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	Y	·	·	·	·	Y	·	M	·	·
r-VEZ11-HM627313	2*x*	4	·	·	·	G	·	·	·	·	R	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	K	·	·	·	·	·	T	·	M	·	·
r-VEZ18-HM627314	2*x*	3	·	·	·	G	·	·	·	·	R	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	M	·	·
r-COZ1-HM627295, *r*-COZ2-HM627296	2*x*	2	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	M	·	·
r-BOT3-HM627293	2*x*	3	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	Y	T	·	·	·	·	·	·	·	·	·	T	·	M	·	·
r-GRA12-HM627300, *r*-GRA14-HM627301, *r*-MUS7-HM627305, *s*-KOP17-HM627303	3*x*, 4*x*, 5*x*, 2*x*	2	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	M	·	·
r-DOD14-HM627299	3*x*	4	·	·	·	G	·	·	·	·	R	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	Y	·	·	T	·	M	·	·
r-DOD7-HM627298, *s*-KOP7-HM627302	2*x*	3	·	·	·	G	·	·	·	·	·	·	·	·	Y	·	Y	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	M	·	·
a-SIM3-HM627310	4*x*	11	·	·	·	G	·	·	R	·	R	M	Y	R	Y	·	Y	S	·	Y	·	·	·	·	·	T	·	·	·	·	·	·	·	Y	·	T	·	M	·	·
a-SIM4-HM627311	4*x*	11	·	Y	R	G	·	·	·	·	R	M	Y	R	Y	·	Y	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	Y	T	·	M	R	·
a-SCH13-HM627309, *a*-PUF6-HM627308	5*x*	10	·	Y	R	G	·	·	·	·	R	M	Y	R	Y	·	·	·	·	Y	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	M	R	·
p-O1-HM627306	2*x*	7	Y	·	·	G	C	·	·	·	·	·	·	·	T	·	·	·	Y	·	K	·	Y	·	·	T	R	Y	·	·	·	·	·	·	·	T	Y	A	·	T
c-CYM1-HM627297	2*x*	6	·	·	·	G	·	·	·	R	·	·	·	·	T	Y	·	·	·	·	·	R	·	Y	·	T	·	·	Y	·	·	Y	T	·	·	T	·	A	·	·
H. breviscapum-AJ633393			·	C	G	G	·	G	·	·	·	C	T	A	T	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	A	G	·
H. angustifolium-AJ633407			·	C	G	G	·	·	·	·	·	C	T	A	T	·	·	·	·	C	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	A	G	·
H. lactucella-AJ633389			·	C	G	G	·	·	·	·	·	C	T	A	T	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	T	·	A	G	·

[a] Initial letter in sample code indicates species studied: *a*, *P. alpicola* s.str.; *c*, *P. cymosa*; *p*, *P. petraea*; *r*, *P. rhodopea*; *s*, *P. serbica*; *u*, *P. ullepitschii*. For population codes see Table 1.
[b] PL, ploidy level.
[c] N, total number of intraindividual polymorphisms per accession.

It seems that the predominant triploids in *P. rhodopea* may have some advantage over other cytotypes. However, the underlying mechanisms of selection for triploid plants are not yet known and deserve further experimental approach to infer the differences and/or patterns in intercytotype pollen competition, seed set, seed dispersal, germination rate, seedling survival, tolerance to competition, etc. Since spatial and habitat differentiation might also play a role in maintaining the high frequency of triploids, a detailed study of populations with mixed cytotypes might shed more light on cytotype coexistence and frequency at microspatial scales. For instance, only tri- and tetraploid plants were found on intensively grazed pastures in the GRA population, while on the ridge situated westerly (GRA-W) besides 3*x* and 4*x* also 2*x* plants were recorded. Because the latter locality was less affected by grazing and because there were also naturally disturbed, competition-free sites (scree), this finding may suggest niche differentiation among cytotypes (see Felber-Girard & al., 1996; Hardy & al., 2000; Schönswetter & al., 2007a; Raabová & al., 2008) and lower competitive ability of diploid cytotype in dense vegetation.

Sympatric cytotype pattern with predominating mixed-ploidy populations and no obvious reproductive barriers between the *P. rhodopea* cytotypes strongly suggest a primary origin of polyploids within diploid populations. This suggestion is further supported by morphometric analysis from a recent study and from allozyme pattern (Šingliarová & al., in press) indicating no phenetic and genetic differentiation between diploids and polyploids. Of course, we cannot exclude the possibility that *P. rhodopea* polyploids might be the immigrants into diploid populations and that frequent hybridization between cytotypes could completely blur original genetic differences. Primary polyploid formation in diploid populations of *P. echioides* was suggested already by Peckert & Chrtek (2006). Both *P. alpicola* and *P. echioides* seem to be very rare documented cases of in situ origin of polyploids, as Petit & al. (1999) considered that most (if not all) of so-far studied contact zones of diploid and polyploid cytotypes are of secondary origin.

Morphological differentiation. — Morphometric study of the *Pilosella alpicola* group revealed the existence of four well-separated groups of plants corresponding to four taxa—*P. alpicola* s.str., *P. rhodopea, P. ullepitschii* and *P. serbica* (Fig. 5). They differ mainly in semiquantitative traits like density, length and colour of indumentum on the leaves, stems and inflorescences. These characters are considered to be involved in evapotranspiration and water-stress adaptation in plants (e.g., Ehleringer, 1982; Sandquist & Ehleringer, 1997; Ackerly & al., 2000). The density of stellate trichomes is higher and simple trichomes are brighter in *P. rhodopea* and *P. serbica*, which originate from southern ranges, than in *P. ullepitschii* occurring in more northern latitudes with more than 40% higher precipitation during the growing season in comparison to southern latitudes. These morphological differences are stable in cultivation and thus suggest a genetic basis of such variation. Interestingly, phenotypic differentiation indicating an adaptation to different moisture conditions is strongly supported also by ecophysiological traits involved in water-regime, like water-use efficiency (measured through carbon isotope discrimination—$\Delta^{13}C$), specific leaf area and percentage of leaf carbon. All these data likely suggest an adaptive evolution in allopatry of Carpathian and Balkan taxa (Mráz & al., in prep).

The results of morphometric analyses confirm the already suggested conspecific status of *Hieracium alpicola* subsp. *micromegas* with *Pilosella rhodopea* and *P. alpicola* subsp. *furcotae* with *P. ullepitschii* (cf. Szeląg, 2008). When analysed separately, slight morphological differences were observed between vicariant Alpine tetra- and pentaploids of *P. alpicola* s.str. (PCoA, Fig. S2). This differentiation might be the result of either a polytopic origin and/or long-term vicariancy. Multiple origins of *P. alpicola* s.str. are supported not only by cytogeographic and ITS patterns revealed in this study, but also by small differences in allozyme profiles (Šingliarová & al., in press). Indeed, polytopic formation of polyploid lineages is a common phenomenon in vascular plants (e.g., Soltis & Soltis, 1999), and it was suggested also for the polyploid *Pilosella officinarum* complex on the basis of cytogeographic pattern (Mráz & al., 2008).

ITS variation and origin of polyploid taxa. — The whole *Pilosella alpicola* group shows very low interspecific ITS variation and complete uniformity in plastid DNA sequences. This is in concordance with overall phylogenetic pattern observed in the genus *Pilosella* (Fehrer & al., 2007a) suggesting rather young age of this genus. Our nrDNA data confirmed the close relationships between the members of the *Pilosella alpicola* group, with exception of the nominate species, *P. alpicola* s.str. In spite of very limited interspecific variation, our analyses revealed frequent intra-individual ITS polymorphism. In apomictic *P. alpicola* s.str. this polymorphism can be partially explained by its allopolyploid origin and apomictic reproduction. Both phenomena could substantially retard or even completely suppress homogenisation processes of different ITS repeats found in many plant taxa (cf. Sang & al., 1995; Wendel & al., 1995; Aguilar & al., 1999) due to reduced/lacking recombination rate and meiotic paring in the case of non-homologous ITS regions (cf. Campbell & al., 1997; Fehrer & al., 2009; Hörandl & al., 2009; Záveská-Drábková & al., 2009). However, most of the intra-individual polymorphic sites found in the *P. alpicola* group (excluding *P. alpicola* s.str.) do not show any additive pattern, and therefore they cannot be interpreted by recent introgression from other taxa that have been sequenced so far (Table 6; Table S1). It means that standing intra-individual ITS variation might be due to the past hybridization events involving already extinct lineages, as it was suggested for the closely related genus *Hieracium* (Fehrer & al., 2009), and/or due to very high mutation and slow homogenisation rates. At least two nucleotide polymorphisms at positions 131 and 600 were found in all accessions irrespective of their taxonomic, geographic and ploidy level status (Table 6). This fact suggests that these polymorphisms had to precede the diversification and polyploidization events of the *Pilosella alpicola* group. Similar pattern was observed in diploid and polyploid *Hieracium* taxa (Fehrer & al., 2009).

The high number of polymorphic nucleotide sites found in *P. alpicola* s.str. by direct sequencing indicates the existence of at least two divergent ITS copies, and thus suggests its

allopolyploid origin. Based on morphological, molecular and ecological data we hypothesize that *P. alpicola* s.str. is a hybridogeneous species between *P. glacialis* (Reyn. ex Lachen.) F.W. Schultz & Sch.Bip. (syn. *Hieracium angustifolium* Schur.) (or some closely related so far not sequenced taxon) from the Alps and *P. rhodopea* from the Balkans. In total, the ITS sequences of *P. rhodopea* (and *P. serbica*) and *P. glacialis* differed in eight nucleotide positions, and in seven of them we recorded an additive pattern in *P. alpicola* s.str. More important, at position 445 three from four sequenced accessions of *P. alpicola* s.str. had Y (C+T), while the other samples of *P. alpicola* s.l., including all remaining *Pilosella* species had T, but *P. glacialis* having C. This suggests that the second parental species could be *P. glacialis* (Table 6). The complementary characters of ITS copies, however, were not consistent in all sequenced samples of *P. alpicola* s.str. (Table 6). Interestingly, all sequenced individuals of *P. alpicola* s.str. showed at position 104 R (A+G), similar to two diploid (VEZ) and one triploid plant (DOD) of *P. rhodopea* (Table 6). This polymorphism was not detected in all remaining samples exhibiting adenin at the same position, like other *Pilosella* taxa. This likely suggests that the second putative parent might be *P. rhodopea*. *Pilosella alpicola* s.str. shows some intermediate morphological characters which might originate from introgression with *P. glacialis* (higher number of capitula, longer acladia and taller stems). Nevertheless, *P. alpicola* s.str. is still morphologically closer to *P. rhodopea* than to *P. glacialis*. This might be due to different levels of genomic dosage of both putative parental taxa, with greater influence of *P. rhodopea*. Although the ratio of divergent ITS copies should be estimated by rigorous quantitative methods, the higher signal of nucleotides originated from *P. rhodopea* at all polymorphic sites might indeed suggest a larger amount of *P. rhodopea* genome in *P. alpicola* s.str. *Pilosella glacialis* is, like *P. alpicola* s.str., an endemic species of the Alps with similar ecological demands. Although the current range of *P. rhodopea* does not overlap with those of *P. glacialis*, both taxa could have hybridized in the past, either in periglacial Alpine refugia or in the area between the Alps and the Balkan mountains. We hypothesize that the first putative parent subsequently went extinct and left only his progeny—*P. alpicola* s.str.

It is not known if apomictic reproduction of *P. alpicola* s.str. arose as a consequence of interspecific hybridization between two divergent taxa reproducing sexually (cf. Asker & Jerling, 1992; Carman, 2001), or if the hybridization already involved at least one parental species with apomictic reproduction. Few studies have reported *P. glacialis* as a diploid species (Favarger, 1969a,b), and as a rule in the genus *Pilosella* the diploid taxa reproduce exclusively sexually (Krahulcová & al., 2000). Because all tested cytotypes of *P. rhodopea* are sexual too, the first hypothesis might be correct. However, we have only limited information on cytotype variation in *P. glacialis* (see above) and therefore we cannot exclude the possibility that some polyploid apomictic cytotypes might be found in this taxon.

In contrast to *P. alpicola* s.str., our morphological, cytogeographical and ITS data strongly support an autopolyploid origin of *P. rhodopea* polyploids. Moreover, sharing of the same allele suite and presence of both balanced and unbalanced heterozygotes in *P. rhodopea* are also consistent with this assumption (Šingliarová & al., in press).

Biogeographic pattern. — The modern range of the *Pilosella alpicola* group is restricted to disjunct mountain ranges (Fig. 2). However, during long glacial periods one might expect that the *P. alpicola* group had a more continuous distribution because the ice ages provided more favourable conditions for many alpine and mountain taxa than the short and warm interglacial periods (e.g., Schmitt 2007). During cold periods alpine and mountain taxa might expand their ranges and overcome potential dispersal barriers (e.g., lowlands, large river valleys, etc.) that separate their modern ranges. Because our molecular data indicate a monophyletic origin of the *P. alpicola* group (with the exception of *P. alpicola* s.str.), we assume that the progenitor of this group, either an extinct or extant species, occupied a larger and more continuous range than the group does currently. Unfavourable warm climatic conditions during the Pleistocene have subsequently induced range fragmentation leading to the formation of new, morphologically distinct taxa adapted to local conditions. Thus, we suggest that the range expansion and contraction played a significant role in shaping the modern distributional and evolutionary pattern in the group.

In this way we could explain the hybridogenous origin of *P. alpicola* s.str., an endemic to the Alps. We suppose that under favourable conditions *P. rhodopea*, one of its putative parental species, underwent range expansion from its core area in the Balkans, with subsequent hybridization to *P. glacialis* (or a closely related taxon) in the Alpine periglacial refugium/refugia. Indeed, molecular and distributional patterns of animal and plant taxa support the biogeographic link between the Alps and the Balkans (e.g., Szeląg, 2006; Schmitt, 2009; Stevanović & al., 2009). Furthermore, the existence of two isolated populations of *P. rhodopea* in the Southern Carpathians provide further evidence for former broader distribution of *P. rhodopea*, and underline floristic and faunistic affinities between the Southern Carpathians and the Balkans (e.g., Szeląg, 2006; Schmitt, 2009).

In spite of an efficient mechanism for colonisation and long-distance seed dispersal via achenes with pappus, the modern range of the *P. alpicola* group is very restricted. This might suggest a high extinction rate either during or more likely after range expansions. Generally, the *Pilosella alpicola* taxa are week competitors, and in comparison to other *Pilosella* taxa, vegetative reproduction via stolons is highly restricted. We suggest that the altered environment during unfavourably warm and humid periods (e.g., Atlantic period) led to the considerable loss of suitable habitats. Furthermore, given the weak competitive ability of *Pilosella alpicola* taxa, this may also have led to decreasing of population sizes.

Interestingly, an apomictic mode of reproduction which is considered to be very advantageous for colonising new habitats (Vandel, 1928; Bierzychudek, 1985; Asker & Jerling, 1992; Hörandl, 2006), has not contributed to the range expansion of *P. alpicola* s.str. as was observed in many apomictic taxa (e.g.,

Antennaria rosea complex: Bayer, 1990; *Paspalum simplex*: Urbani, 2002; *Taraxacum* and *Chondrilla*: Van Dijk, 2003; *Townsendia hookeri*: Thompson & Whitton, 2006; *Pilosella officinarum*: Mráz & al., 2008; *Hieracium alpinum*: Mráz & al., 2009; *Ranunculus kuepferi*: Cosendai & Hörandl, 2010). This might suggest either the existence of some effective selection mechanisms that prevent the range expansion of *P. alpicola* s.str., at least in its Swiss range (in Italian Dolomites the spreading is strongly limited due to unsuitable geological and soil conditions; Šingliarová & al., in prep.), and/or the taxon might be too young and has not had sufficient time for further spread. A similar pattern was observed in hybridogenous and agamospermous species *Erigeron trifidus*, which forms only restricted and disjunct populations (Burke & Bain, 2008).

Taxonomic position of *Pilosella petraea*. — Unfortunately we did not have suitable samples of *P. petraea* for morphometric studies due to missing data for flower traits. Nevertheless, literature data (Heuffel, 1853; Zahn, 1922–1930; Nyárády, 1965; Szeląg, 2008) as well as our measurements of leaf traits of five cultivated plants from the type locality (Šingliarová, unpub.) indicate strong morphological differentiation of this taxon from the remaining taxa of the *Pilosella alpicola* group. First, leaves were without small glandular trichomes that are characteristic of all other *P. alpicola* taxa. Furthermore, leaves had substantially fewer dense stellate trichomes on both leaf sides (with exception of leaf margins) when compared to *P. alpicola* s.l. Moreover, *P. petraea* had thicker [0.08(–0.09) mm] and setaceous-like simple eglandular trichomes, while the hairs in *P. alpicola* s.l. were thinner [(0.03–)0.04–0.05(–0.06) mm] (Šingliarová, unpub.). *Pilosella petraea* also had a higher number of capitula in synflorescence (2–15) that were smaller in size (6–8 mm, cf. Zahn, 1922–1930) than *P. alpicola* taxa having typically 1–3 larger flower heads [(6–)7–11(–13)mm] (Zahn, 1922–1930; Šingliarová, unpub.). Moreover, *Pilosella petraea* substantially differs from other taxa by its ecological demands. It grows in the crevices of calcareous rocks in mountain belt, while the representatives of the *Pilosella alpicola* group typically occur in subalpine/alpine belt on acid bedrock. ITS pattern of *P. petraea* likely suggests different evolutionary origin of this species and alongside with morphological and ecological data supports exclusion of this taxon from the *Pilosella alpicola* group. The high number of intraindividual ITS polymorphisms found in this diploid species might indicate its hybridogenous origin. Zahn (1922–1930) already pointed out that *P. petraea* is a taxon morphologically between *P. alpicola* s.l. and *P. cymosa*, suggesting its hybrid history. The ITS pattern obtained in this study (Table 6), however, does not support the hypothesis on this parental combination, although the limited number of *P. cymosa* accessions should be taken into consideration.

■ CONCLUSIONS

Our combined methodological approach enabled us to provide a new circumscription of the *P. alpicola* group and revealed auto- and allopolyploidization events operating within it. The group consists of four morphologically well-differentiated and geographically vicariant species with contrasting cytotype patterns. Interestingly, the mode of reproduction of polyploid cytotypes reflects their origin: *P. rhodopea* autopolyploids reproduce strictly sexually, while allopolyploid cytotypes of *P. alpicola* s.str. reproduce apomictically. Our data also revealed a rare primary contact zone between diploid and polyploid cytotypes of *P. rhodopea*. In spite of clear morphological separation the molecular data suggest recent diversification of the group. We hypothesize that fragmentation of more continuous ranges, range shifts connected with interspecific hybridization (as in the case *P. alpicola* s.str.) and adaptation to different climates were the main drivers of speciation in the group. Our data suggest that the *Pilosella alpicola* group is a promising model for studying plant speciation, adaptation and recent polyploidization.

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Appendix. Identification key for the *Pilosella alpicola* group.

Characters (values) are expressed as (minimum–)10th percentile–90th percentile(–maximum).
Pale: white, ivory, yellowish, silver grey.
Dark: smoky grey, grey-black, brown-grey.

1. Plant (8–)10–24(–29) cm high; acladium if present (1.0–)2.2–5.0(–5.5) cm long; simple hairs on leaves and peduncle dense; simple hairs on involucrum usually pale with dark base or occasionaly dark up to the middle. – Inflorescences (1–)2–3 (Alps: Switzerland, Italy) . . . . . ***P. alpicola* s.str. F.W. Schultz & Sch. Bip.**

1. Plant (2–)5–14(–19) cm high; acladium if present (0.1–)0.3–2.5(–3.5) cm long; simple hairs on leaves and peduncle sparse; simple hairs on involucrum pale occasionally with dark base or dark up to the middle or completely dark **2**

2. Simple hairs on involucrum consistently dark; stellate hairs on leaves rare; on peduncle sparse. – Inflorescences 1–2(–3); acladium if present (0.2–)0.3–1.0(–1.2) cm long (Carpathians: Slovakia, Poland, Romania) . . . . . ***P. ullepitschii* (Błocki) Szeląg**

2. Simple hairs on involucrum pale, occasionaly with dark base or dark up to the middle; stellate hairs on leaves and peduncle dense **3**

3. Glandular hairs on leaves and peduncle rare to sparse, inflorescences 1–2(–3); acladium if present (0.1–)0.3–3.4(–3.5) cm long (Carpathians: Romania; Balkans: Bulgaria, Albania, Macedonia, Greece) . . . . . ***P. rhodopea* (Griseb.) Szeląg**

3. Glandular hairs on leaves and peduncle dense, inflorescences 2–4; acladium (0.3–)0.4–1.5(–1.9) cm long (Balkans: Serbia, Montenegro) . . . . . ***P. serbica* (F.W. Schultz & Sch. Bip.) Szeląg**

Príloha D

Šingliarová B, Chrtek J, Plačková I, Mráz P

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Allozyme Variation in Diploid, Polyploid and Mixed-Ploidy Populations of the *Pilosella alpicola* Group (Asteraceae): Relation to Morphology, Origin of Polyploids and Breeding System

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Abstract The *Pilosella alpicola* group includes four species (*P. alpicola* s.str., *P. ullepitschii, P. rhodopea* and *P. serbica*) with allopatric distributions (Alps, Balkans, Carpathians) and contrasting cytotype patterns (diploid, diploid-polyploid and polyploid species). Whereas diploid taxa (*P. ullepitschii* and *P. serbica*) reproduce sexually, the mode of reproduction of polyploid cytotypes reflects their origin: autopolyploids of *P. rhodopea* reproduce sexually, while allopolyploid cytotypes of *P. alpicola* s.str. apomictically. We used allozymes to elucidate overall genetic variation within the group and to test their utility for taxon discrimination, assessment of polyploid origin and possible correlations with breeding systems. Variation of five allozyme systems encoded by eight polymorphic loci and 29 alleles was studied in 20 populations and 298 plants representing all taxa. Allozymes were proved to be only of limited usefulness for the taxonomic classification within the *P. alpicola* group. The Western Carpathian populations of *P. ullepitschii* formed the only genetically well-differentiated group. The same

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allele suite shared by all cytotypes of *P. rhodopea* and presence of both balanced and unbalanced heterozygotes in tetraploids was consistent with autopolyploid origins of polyploids and provided further evidence for a primary contact zone. An isolated relic population of *P. rhodopea* from the Southern Carpathians exhibited lowered values of genetic diversity when compared to the core area. Pronounced fixed heterozygosity was found in *P. alpicola* s.str., supporting its allopolyploid origin. In accordance with assumptions, genotypic variability was significantly higher in sexually reproducing diploid and diploid-polyploid taxa than in apomictic *P. alpicola* s.str.

Keywords Allopolyploidy · Alps · Apomixis · Autopolyploidy · Balkans · Carpathians · Genetic diversity · *Hieracium* · Primary contact zone

Introduction

Members of the genus *Pilosella* Vaill. (syn. *Hieracium* subgenus *Pilosella* (Vaill.) Gray, Asteraceae, Lactuceae) are characterized by the presence of several ploidy levels in natural populations (2*x*–8*x*, based on *x*=9), diverse breeding systems (self-incompatibility, facultative/obligate apomixis, vegetative reproduction via stolons) and widespread interspecific hybridization (reviewed in Krahulcová et al. 2000). The joint effect of these processes has led to a reticulate pattern of diversity reflected in contradictory taxonomic concepts (Nägeli and Peter 1885; Zahn 1922–1930; Tyler 2001; Bräutigam and Greuter 2007).

The *Pilosella alpicola* group encompasses four closely related, but morphologically distinct taxa distributed allopatrically (Šingliarová et al. 2011). Formerly, they were treated as subspecies of the *Pilosella alpicola* (Steud. & Hochst.) F.W. Schultz & Sch. Bip. (Zahn 1930). A cytogeographical survey of the group (Šingliarová and Mráz 2009; Šingliarová et al. 2011) revealed a taxon-specific pattern. *Pilosella alpicola* s.str. is a tetra- and pentaploid species (2*n*=4*x*=36 and 5*x*=45) with an allopatric cytotype distribution in the Swiss and Italian Alps, respectively. The Carpathian endemics *P. ullepitschii* (Błocki) Szeląg and *P. serbica* (F.W. Schultz & Sch. Bip.) Szeląg from Serbia are exclusively diploid (2*n*=2*x*=18) and strictly self-incompatible (Šingliarová and Mráz 2009; Šingliarová et al. 2011). Populations of the Balkan subendemic *P. rhodopea* (Griseb.) Szeląg are diploid, diploid-polyploid or consist of several polyploid cytotypes (2*n*=2*x*, 3*x*, 4*x*, 5*x*; Šingliarová and Mráz 2009; Šingliarová et al. 2011).

Our previous biosystematic study confirmed the close relatedness of the four recognized species of the *P. alpicola* group and indicated an autopolyploid as well as an allopolyploid origin of the polyploids (Šingliarová et al. 2011). Sexual reproduction has been proved in diploid-polyploid *P. rhodopea*, which, coupled with the absence of morphological and molecular (internal transcribed spacer) differentiation between cytotypes, suggests an autopolyploid origin of polyploids. On the contrary, an additive pattern of ITS polymorphism suggested hybridogeneous origin of polyploid *P. alpicola* s.str., putatively arising from *P. rhodopea* and Alpine *P. glacialis* (Reyn. ex Lachen.) F.W. Schultz & Sch. Bip. (= *Hieracium angustifolium* Hoppe). Moreover, an

apomictic mode of reproduction was proved in this taxon (Šingliarová et al. 2011). Although the ITS sequences provided valuable data on the 'rough' phylogenetic relationship of the *P. alpicola* group to the other members of the genus (Fehrer et al. 2007; Šingliarová et al. 2011), as well as on the origin of polyploids (Šingliarová et al. 2011), there is no information about genetic differentiation within this group, and consequences of breeding systems and origins of polyploids for standing genetic variation.

Allozymes have been proved useful for establishing relationships at low systematic levels (Gottlieb 1984). As codominant markers, they can help elucidate origins of polyploids (Roose and Gottlieb 1976; Soltis and Rieseberg 1986; Soltis and Soltis 1989; Mahy et al. 2000; Rosquist and Prentice 2002; Crawford et al. 2006) or hybrids (Brochmann et al. 1992; Gauthier et al. 1998; Mráz et al. 2005) and indirectly estimate the breeding system within a population (Sipes and Wolf 1997; Sydes and Peakall 1998). Allozymes are especially suitable for detection of differences in population genetic structure between sexual and apomictic taxa (Bayer and Crawford 1986; Hughes and Richards 1988; Yahara 1990; Bayer 1991; Noyes and Soltis 1996; Hörandl et al. 2000, 2001; Hörandl and Greilhuber 2002; Kashin et al. 2005) due to different levels and patterns of heterozygosity and genotypic diversity. Allozymes have been used successfully also in several population genetic studies in the genus *Pilosella* (Krahulec et al. 2004; Kashin et al. 2005; Peckert et al. 2005; Tyler 2005; Bruun et al. 2007; Krahulcová et al. 2009a). In our previous paper, we used allozymes to compare the genetic diversity between the populations of *P. ullepitschii* from the core area (Western Carpathians) and isolated populations originated from Romanian Eastern and Southern Carpathians (Šingliarová et al. 2008).

The present study aimed to elucidate the amount and pattern of genetic variation within the *Pilosella alpicola* group using allozyme markers. The main objectives were to establish:

i) the degree of genetic differentiation among four recognized species. Considering the clear morphological separation of species (Šingliarová et al. 2011), we would expect a similar pattern using allozymes.

ii) molecular evidence for auto- and allopolyploid origin of polyploids. Different criteria of genetic diversity are predicted for these two types of polyploids (Soltis and Soltis 2000; Ramsey and Schemske 2002). In the case of autopolyploids (*P. rhodopea*), polysomic inheritance resulting in multiallelic loci and the presence of both balanced and unbalanced heterozygotes is expected. Allopolyploid *P. alpicola* s.str., however, should be characterized by disomic inheritance mirrored in fixed heterozygosity. The distinction can be, however, imperfect in many instances, as auto- and allopolyploidy represent only opposite ends of a wide spectrum of intergenome differentiation (Stebbins 1980; Soltis and Soltis 2000; Obbard et al. 2006).

iii) correlation between genetic diversity and breeding systems operating within the group. Breeding system was proved to crucially affect the patterns of genetic variation (Hamrick and Godt 1989; Karron 1991; Sun and Wong 2001). In general, sexual taxa (diploid *P. serbica* and *P. ullepitschii* and diploid-polyploid *P. rhodopea*) are expected to be more variable than their apomictic counterpart polyploid *P. alpicola* s.str.

Material and Methods

Sampling

The plants were collected from their natural populations in 2005–2007 during extensive field survey across the whole range of the group (Table 1, Fig. 1) and transferred into the experimental garden of the Institute of Botany, Slovak Academy of Sciences in Bratislava. If possible, the plants were collected in at least 4 m intervals. Altogether 298 plants originated from 20 populations from Switzerland, Italy, Slovakia, Romania, Serbia and Bulgaria were sampled (Table 1, Fig. 1). Eleven populations (166 plants) of *P. ullepitschii* were allozymatically studied by Šingliarová et al. (2008), but only four systems (except LAP, see below) and six loci were scored. For the purposes of the present study we included these populations into the data set. Moreover, six plants of *P. ullepitschii* from population SMA were additionally analyzed for the first time. The number of plants analyzed per population ranged from 11 to 24 (Tables 1 and 2; Electronic Supplementary Material 1), except for population KOP (type locality of *P.*

Table 1 List of populations of four taxa of the *Pilosella alpicola* group analyzed in the present study and their geographic origin. Initial letters in population codes indicate species studied: A – *P. alpicola,* R – *P. rhodopea*, S – *P. serbica*, U – *P. ullepitschii*. Allozyme variation of populations marked by an asterisk was published in Šingliarová et al. (2008), see paragraph Sampling

Code	*N*	Locality
A-SIM	24	CH, Walliser Alps, Simplon Pass, N 46°15′00″, E 08°00′50″, 2,000–2,300 m
A-DUR	14	I, Dolomites, Val de Duron valley, N 46°29′52″, E 11°39′35″, 2,236 m
R-COZ	11	RO, Cozia Mts, N 45°19′04″, E 24°20′17″, 1,592 m
R-BOT	17	BG, Stara planina Mts, Mt. Botev, N 42°42′54″, E 24°55′01″, 2,352 m
R-DOD	14	BG, Rila Mts, Mt. Dodov vrah, N 42°09′59″, E 23°20′23″, 2,540 m
R-MUS	14	BG, Rila Mts, Mt. Yastrebets, N 42°13′29″, E 23°34′46″, 2,359 m
R-BEZ	14	BG, Pirin Mts, Mt. Bezbog, N 41°43′35″, E 23°30′57″, 2,414 m
R-VICH	15	BG, Pirin Mts, Mt. Vichren, N 41°45′39″, E 23°24′27″, 2,331 m
S-KOP	4	SR, Kopaonik Mts, Mt. Suvo Rudishte, N 43°16′28″, E 20°48′55″, 1,917 m
U-LAL*	17	SK/PL, Západné Tatry Mts, Ľaliové saddle, N 49°13′35″, E 19°59′30″, 1,952 m
U-BAR*	14	SK, Západné Tatry Mts, Trnovecká valley, N 49°09′46.5″, E 19°44′04″, 1,885 m
U-MLY*	16	SK, Vysoké Tatry Mts, Mlynická valley, N 49°09′30″, E 20°02′30″, 1,675 m
U-FUR*	16	SK, Vysoké Tatry Mts, Furkotská valley , N 49°09′12″, E 20°01′43″, 1,910 m
U-KR25*	16	SK, Vysoké Tatry Mts, slope of Mt. Kriváň, N 49°09′27″, E 19°59′25″, 1,900 m
U-MEN*	16	SK, Vysoké Tatry Mts, Mengusovská valley, N 49°09′57″, E 20°03′40″, 1,800–1,875 m
U-KR55*	16	SK, Vysoké Tatry Mts, Mt. Kriváň, SE ridge, N 49°09′02″, E 19°59′55″, 1,900 m
U-OST*	15	SK, Vysoké Tatry Mts, Mt. Ostrva, N 49°08′58″, E 20°05′22″, 1,959 m
U-SMA*	20	RO, Nemira Mts, Mt. Sandru Mare, N 46°11′57″, E 26°20′21″, 1,590–1,640 m
U-NMA*	11	RO, Nemira Mts, Mt. Nemira Mare, N 46°15′21.5″, E 26°19′25.5″, 1,641 m
U-BUC*	14	RO, Bucegi Mts, near the chalet Cabana Babele, N 45°24′24″, E 25°28′30″, 2,204 m
Total	298	

Abbreviations used: BG – Bulgaria, CH – Switzerland, I – Italy, PL – Poland, RO – Romania, SK – Slovakia, SR – Serbia. *N* – sample size.

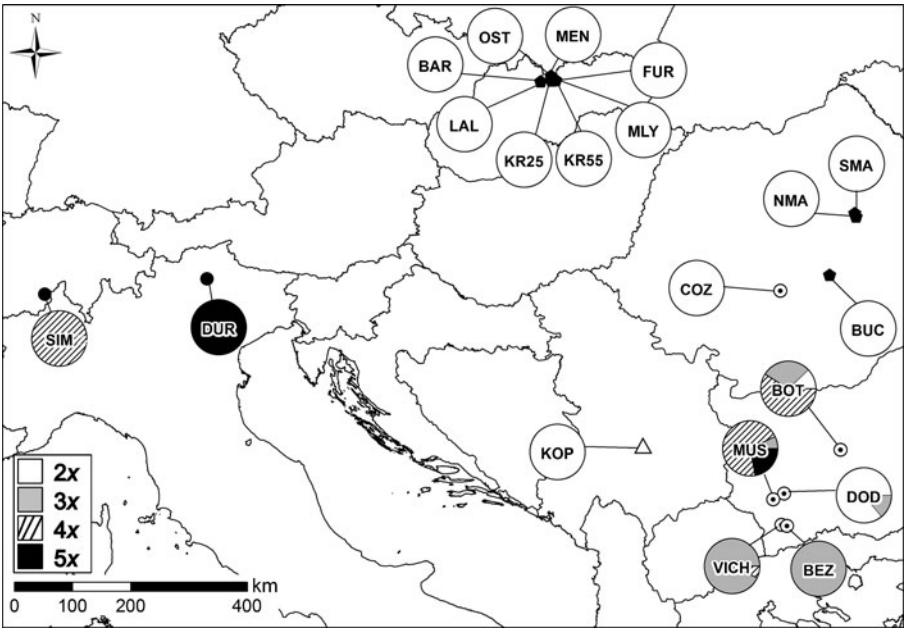

Fig. 1 Distribution of populations of the four species of the *Pilosella alpicola* group included in the present study and their cytotype composition. Species symbols: black circles – *P. alpicola* s.str., white circles with dot – *P. rhodopea*, black pentagons – *P. ullepitschii,* white triangle – *P. serbica*

serbica) from which only four plants were studied because the remaining collected plants died during transport or in early stages of cultivation.

The ploidy level of all analyzed plants was assessed during a large-scale cytogeographical survey (Šingliarová et al. 2011), and populations were assigned to particular species according to the results of multivariate morphometric analyses (Šingliarová et al. 2011).

Electrophoresis

Young leaves were sampled from living cultivated plants and immediately used for allozyme extraction. Approximately 1 cm^2 (ca. 40 mg) of fresh leaf tissue was ground in an ice-cold extraction buffer following Kato (1987): 0.1 M Tris-HCl (pH=8.0), 70 mM mercaptoethanol, 26 mM sodium metabisulphite, 11 mM L-ascorbic acid, 4% (w/v) soluble PVP-40, the pH adjusted after the addition of the ascorbate, with Dowex Cl (1-8X). Crude homogenates were centrifuged at 15,000 rpm for 10 min. Supernatants were stored at –75°C. Polyacrylamid gel electrophoresis (PAGE) was carried out using 8.16% separation polyacrylamid gel with the buffer 1.82 M Tris-HCl, pH 8.9; 4% stacking gel with the buffer (0.069 M Tris-HCl, pH 6.9) and electrode buffer (0.02 M Tris, 0.24 glycine, pH 8.3).

PAGE was initially performed on six isozyme systems of which five turned out to be polymorphic and interpretable; their abbreviations and EC numbers are in parentheses: shikimate dehydrogenase SKDH (monomeric, EC 1.1.1.25), phosphoglucomutase PGM (monomeric, EC 5.4.2.2.), 6-phosphogluconate dehydrogenase 6-PGDH (dimeric, EC 1.1.1.44), superoxide dismutase SOD (dimeric, EC 1.15.1.1),

Table 2 Summary of genetic variation for eight loci in 20 populations of the *Pilosella alpicola* group. First letters in population code indicate species: U – *P. ullepitschii*, R – *P. rhodopea*, A – *P. alpicola* s.str., S – *P. serbica*

Code	*PL*	*N*	*P99*	*P95*	Σ*A*	*A*
A-SIM	4*x*	24	87.5	62.5	18	2.25
A-DUR	5*x*	14	62.5	50	14	1.75
alpicola	**4*x*, 5*x***	**38**	**75**	**56.3**	**16 (18)**	**2**
R-COZ	2*x*	11	50	50	13	1.625
R-BOT	2*x*, 3*x*, 4*x*	17 (2/5/10)	75 (50/62.5/62.5)	75 (50/62.5/50)	19 (13/17/17)	2.375 (1.625/2.125/2.125)
R-DOD	2*x*, 3*x*	14 (12/2)	87.5 (87.5/50)	75 (75/50)	18 (18/12)	2.25 (2.25/1.5)
R-MUS	3*x*, 4*x*, 5*x*	14 (1/10/3)	75 (25/75/62.5)	62.5 (25/62.5/62.5)	16 (10/16/13)	2 (1.25/2/1.625)
R-BEZ	3*x*	14	87.5	62.5	19	2.375
R-VICH	3*x*, 4*x*	15 (14/1)	50 (50/50)	50 (50/50)	12 (12/12)	1.5 (1.5/1.5)
rhodopea	**2*x*, 3*x*, 4*x*, 5*x***	**85**	**70.8**	**62.5**	**16.2 (25)**	**2**
serbica S-KOP	**2*x***	**4**	**87.5**	**87.5**	**17**	**2.125**
U-LAL*	2*x*	17	62.5	62.5	14	1.75
U-BAR*	2*x*	14	62.5	62.5	15	1.875
U-MLY*	2*x*	16	87.5	87.5	17	2.125
U-FUR*	2*x*	16	75	75	15	1.875
U-KR25*	2*x*	16	75	75	16	2
U-MEN*	2*x*	16	62.5	62.5	15	1.875
U-KR55*	2*x*	16	62.5	62.5	15	1.875
U-OST*	2*x*	15	62.5	50	14	1.75
U-SMA*	2*x*	20	50	50	14	1.75
U-NMA*	2*x*	11	50	50	12	1.5
U-BUC*	2*x*	14	37.5	37.5	11	1.375
ullepitschii	**2*x***	**171**	**57.3**	**56.5**	**13.7 (22)**	**1.72**

Abbreviations used: *PL* – ploidy level, *N* – sample size, *P99* and *P95* percentages of polymorphic loci, Σ*A* – sum of alleles, *A* – mean number of alleles per locus, H_o – observed heterozygosity, H_e – expected heterozygosity (calculated only for diploids and tetraploids), *G/N* – proportion of distinguishable genotypes, G_{uni} – number of unique genotypes, *D* – genotype diversity.

leucine aminopeptidase LAP (monomeric, EC 3.4.11.1). Dihydrolipoamiddehydrogenase DIA (EC 1.6.4.3) was not interpretable.

Systems 6-PGDH and PGM displayed two zones of activity, and the LAP system showed three, interpreted as two or three putative loci respectively (e.g., *6-Pgdh-1*, *6-Pgdh-2*, with '1' coding for the faster locus). Alleles were designated sequentially with the fastest one coded as 'a'.

In total, nine polymorphic loci representing five enzyme systems were scored. The fastest locus of LAP (*Lap1*) had a clear and interpretable pattern in all plants

H_o	H_e	G/N	G_{uni}	D	Code
0.55	0.540	0.417	10	0.79	A-SIM
0.509	n.a.	0.143	2	0.143	A-DUR
0.53	**-**	**0.292**	**-**	**0.467**	***alpicola***
0.205	0.212	0.818	8	0.945	R-COZ
0.301 (0.186/0.275/0.325)	n.a./n.a./0.419	1 (1/1/1)	2/5/10	1	R-BOT
0.330 (0.333/0.326)	.349/n.a.	0.929 (0.917/1)	10/1	0.989	R-DOD
0.491 (0.25/0.588/0.625)	n.a./0.571/n.a.	0.429 (1/0.5/0.333)	1/5/1	0.835	R-MUS
0.344	n.a.	0.643	7	0.901	R-BEZ
0.108 (0.069/0.5)	n.a./n.a.	0.4 (0.357/1)	4/1	0.571	R-VICH
0.294	**-**	**0.703**	**-**	**0.874**	***rhodopea***
0.438	**0.371**	**0.75**	**3**	**0.833**	***serbica*** S-KOP
0.228	0.213	0.706	11	0.956	U-LAL*
0.295	0.298	0.929	12	0.989	U-BAR*
0.367	0.350	0.75	9	0.958	U-MLY*
0.289	0.304	0.938	11	0.992	U-FUR*
0.289	0.326	0.938	11	0.992	U-KR25*
0.195	0.280	0.938	14	0.992	U-MEN*
0.289	0.318	1	15	1	U-KR55*
0.267	0.266	0.867	9	0.981	U-OST*
0.175	0.181	0.45	8	0.895	U-SMA*
0.205	0.199	0.818	8	0.964	U-NMA*
0.17	0.158	0.643	7	0.934	U-BUC*
0.232	**0.263**	**0.76**	**-**	**0.957**	***ullepitschii***

Values of genetic parameters for enzyme systems SHDH, PGM, PGDH and SOD were previously published in Šingliarová et al. (2008).

from the Western Carpathians (*P. ullepitschii*), from the Alps (*P. alpicola* s.str.) and in 85% of *P. rhodopea* from the Balkans. Conversely, only a strong and consistent smear was observed in samples from the Eastern and Southern Carpathians (*P. ullepitschii*, *P. rhodopea*), Serbia (*P. serbica*) and in some polyploid plants from Bulgaria (*P. rhodopea*). This locus was therefore omitted, and only eight loci were subsequently used for evaluation of genetic diversity. Banding patterns of particular enzyme systems were interpreted with regard to their quaternary structure following Weeden and Wendel (1989). No deviations from expected patterns have been observed. Despite the generally complicated genetic interpretation in polyploids

(Gottlieb 1981; Weeden and Wendel 1989; Obbard et al. 2006), the resolution of gels and staining intensity enable us to take relative band intensity into account. Phenotypes were compared to banding patterns of related diploids and interpreted as corresponding to genotypes of different allelic dosages (see e.g., Arft and Ranker 1998; Hörandl et al. 2000; Hardy and Vekemans 2001).

Genetic Analyses

To provide a measure of the level of genetic variation within populations of the *Pilosella alpicola* group, the following population genetics parameters were computed: P – proportion of polymorphic loci, where the frequency of the most common allele is less than or equal to 0.99 and 0.95 (including monomorphic loci with fixed heterozygotes), ΣA – sum of alleles, A – mean number of alleles per locus, H_o – observed heterozygosity, H_e – expected heterozygosity (computed for diploids and tetraploids only). The parameters were computed using POPGENE version 1.32 (Yeh et al. 1999) for diploids; for polyploids they were calculated by hand. Genotypic variation was estimated using standard parameters for clonal populations (Ellstrand and Roose 1987; Eckert and Barrett 1993): number of genotypes (G), proportion of distinguishable genotypes (i.e., number of distinguishable genotypes to the number of samples, G/N) and number of unique genotypes (G_{uni}). Multilocus genotype diversity D (modified Simpson diversity index) was calculated as $D = 1 - \sum_i [n_i(n_i - 1)]/N(N - 1)]$, where n_i is the number of individuals of genotype i (Pielou 1969; but see also Noyes and Soltis 1996; Hörandl et al. 2000; Liston et al. 2003). The D value ranges from 0 to 1, 0 meaning that all individuals represent the same multilocus genotype and 1 meaning that each individual has a unique multilocus genotype. All parameters were generated for *i*) each population, *ii*) each of the four species analyzed and *iii*) particular cytotypes of *P. rhodopea*. Wright's (1951) deficit or excess of heterozygotes (F_{IS}) in sexual populations (for diploid taxa and for diploids or tetraploids in *P. rhodopea* populations, where diploid or tetraploid plants prevailed) was calculated for each polymorphic locus and for overall loci as Weir and Cockerham's (1984) estimates using the FSTAT software (Goudet 1995) for diploids and by hand for tetraploids.

Hierarchical clustering (UPGMA), principal component (PCA) and principal coordinate analyses (PCoA) were performed to gain insight into the genetic relationships within the *P. alpicola* group. UPGMA was based on Nei's standard genetic distances calculated from allele frequencies (Nei 1972), PCA was based on allele frequencies, and PCoA was based on Jaccard's coefficient of dissimilarity computed from presence/absence of alleles. Based on Nei's standard genetic distance, we computed genetic identity $(I = 1/\exp(D_a))$. Karyologically uniform populations and each ploidy level found in mixed-ploidy populations were either considered as separate OTUs (operational taxonomic units; PCA) or different ploidy levels from the same population were merged together (PCoA, UPGMA). Differences in genetic parameters (*P95*, *P99*, A, $\sum A$, H_o, G and G/N) among three taxa (*P. alpicola* s.str., *P. rhodopea* and *P. ullepitschii*) were tested using the TukeyHSD *post hoc* comparison test. Because *P. alpicola* was represented by only two populations, we also performed separate two-sample *t*-tests with Welch approximation to test for the difference between *P. rhodopea* and *P. ullepitschii* with a higher number of populations analyzed. Genetic parameters

violating the normality assumption (assessed by Shapiro-Wilk test) were tested using non-parametric Wilcoxon (two species) or Kruskal-Wallis sum rank tests when comparing three species. The correlation between pairwise Nei genetic distances and geographical distances was inferred using Mantel test with 999 permutations. All analyses were performed using various functions implemented in the 'ade4' and 'adegenet' packages (Chessel et al. 2004; Jombart 2008) and basic 'stats' package within R environment (R Development Core Team 2009).

Results

Eight loci yielded a total of 29 alleles. The allele frequencies are shown in the Electronic Supplementary Material **1**. Fifteen alleles were common to all taxa; however, they were not always present in all populations. Two alleles were shared by *P. ullepitschii* and *P. rhodopea*, two by *P. ullepitschii* and *P. alpicola* s.str., and one each by the *P. alpicola* s. str. and *P. rhodopea* pair, and *P. rhodopea* and *P. serbica* pair. Four alleles (*Pgm2-c*, *6Pgdh1-d*, *6Pgdh2-b*, *6Pgdh2-d*) were unique to *P. rhodopea,* and one species-specific allele was detected in each of the diploid taxa *P. ullepitschii* (*6Pgdh2-e*) and *P. serbica* (*Pgm1-c*). Unique alleles were not evenly distributed in populations of particular species, but they were always found in plants of a single population (Electronic Supplementary Material **1**). Only *P. alpicola* s.str. lacks any private alleles at either of the eight loci scored. However, if we included the *Lap1* locus, which was absent in many individuals of other species (see Material and Methods), *P. alpicola* s.str. exhibited three unique alleles at this locus. Specifically, *Lap1-a* was moderately frequent in tetraploid plants from the Simplon Pass (SIM), and alleles *Lap1-b* and *Lap1-c* were present in all pentaploid plants studied from the Val de Duron valley (DUR).

In most of the plants, at most one or two alleles per locus were recorded. Only in five polyploid plants of *P. rhodopea*, all from the BOT population (representing 8% of all *P. rhodopea* polyploids), were found three alleles at the *Pgm2* locus. Similarly, loci *Lap1* and *Lap2* in pentaploid *P. alpicola* s.str. (DUR) consistently possessed three alleles.

Within particular species, the highest genetic identity ($I = 0.944$) was found between two *P. alpicola* s.str. populations (Table **3**). Slightly lower values of genetic identity were observed between *P. rhodopea* populations (mean = 0.894, ranging from 0.808 to 0.975). The lowest values (mean = 0.852) of the parameter along with the widest range (from 0.646 to 0.989) were detected between *P. ullepitschii* populations. As for the genetic identities between taxa, the highest values were observed between *P. alpicola* s. str. and *P. rhodopea* (from 0.809 to 0.965, mean = 0.903), while pair of taxa *P. serbica* and *P. ullepitschii* displays the lowest values (from 0.678 to 0.837, mean = 0.781). Remaining pairs of taxa show moderate values of genetic identity.

Cluster and ordination analyses based on allele frequencies (UPGMA, PCA) and presence/absence of alleles (PCoA) were fairly congruent. All analyses revealed clear separation of the Western Carpathian populations of *P. ullepitschii* from the rest (Figs. **2**, **3** and **4**, Table **3**). In UPGMA (Fig. **2**), the populations of *P. ullepitschii* from the Western Carpathians were separated at the highest level of dissimilarity. The second cluster consisted of two sub-clusters. The first subcluster was formed by populations of *P. ullepitschii* from the Eastern Carpathians (NMA, SMA) and *P.*

rhodopea from the Southern Carpathians (COZ), indicating a rather geographical grouping. The second subcluster contained a mixture of *P. alpicola* s.str., *P. rhodopea*, *P. serbica* and *P. ullepitschii* populations from the Southern Carpathians (BUC).

In the ordination analyses (Figs. 3 and 4), only populations of the Western Carpathian *P. ullepitschii* (PCA, PCoA) and population of *P. serbica* (KOP) (PCA) can be distinguished, while the Romanian populations of *P. ullepitschii* were only slightly differentiated (PCoA) or intermingled (PCA) with the populations of *P. alpicola* s.str., *P. rhodopea* and *P. serbica*.

There was no significant association between Nei's genetic distances and geographical distances (Mantel test, r=0.152, P=0.064). However, when two *P. alpicola* s.str. populations were removed from the analysis, the correlation was highly significant (Mantel test, r=0.657, P<0.001; Fig. 5).

Genetic diversity values at both species and population level are given in Table 2. The highest values for most of genetic diversity parameters (*P99*, *P95*, *A,* ΣA) were found in diploid *P. serbica;* however, the small sample size (four plants) could significantly bias the values. Therefore, the values of genetic parameters for this species are given in all tables but they are not further discussed. Mean genetic diversity estimates per population and species of *P. rhodopea* were slightly higher (*P99*=70.8, *P95*=62.5, ΣA=25, *A*=2) than those of *P. alpicola* s.str. (*P99*=75, *P95*=56.3, ΣA=18, *A*=2) and *P. ullepitschii* (*P99*=57.3, *P95*=56.5, ΣA=22, *A*=1.72). Values of observed heterozygosity (H_o) averaged per population were similar in *P. ullepitschii* (0.232) and *P. rhodopea* (0.294), while those observed in *P. alpicola* s.str. were approximately two fold higher (0.53). Similarly, the highest levels of expected heterozygosity (H_e) was recorded in tetraploid populations of *P. alpicola* s.str. (H_e=0.540) and *P. rhodopea* (H_e= 0.571 and 0.419), while values observed in diploid populations were considerably lower − *P. ullepitschii* (H_e=0.263), *P. serbica* (H_e=0.371), *P. rhodopea* (H_e=0.212 and 0.349). In *P. rhodopea*, H_o and H_e of particular populations increased from pure diploid (COZ, H_o=0.205, H_e=0.212) through diploid-polyploid (BOT, H_o= 0.301, H_e for tetraploids = 0.419 and DOD, H_o=0.330, H_e of diploids = 0.349) to polyploid populations (BEZ, H_o=0.344 and MUS, H_o=0.491, H_e of tetraploids = 0.571). The only exception was polyploid population VICH ($3x$+$4x$) with the lowest proportion of heterozygotes among all analyzed populations (H_o=0.108). In sexual taxa (*P. ullepitschii*, *P. serbica* and all cytotypes of *P. rhodopea*), values of observed heterozygosity averaged for particular loci covered a whole spectrum between zero and one (Table 4). On the contrary, mean observed heterozygosity for particular loci in apomictic *P. alpicola* s.str. was equal or close to zero (homozygous) or one (heterozygous, see Table 4). No significant differences in parameters *P95*, *P99*, *A*, $\sum A$, H_o, *G* and *G/N* were found among tested taxa (TukeyHDS test when comparing *P. alpicola* s.str., *P. rhodopea* and *P. ullepitschii*; or two-sample *t*-test, when comparing *P. rhodopea* and *P. ullepitschii*; results not shown).

The most striking difference between sexual taxa (*P. ullepitschii*, *P. rhodopea*) and apomictic *P. alpicola* s.str. was found in genotypic variability (*G*, *G/N* and *D*, see Table 2). *P. alpicola* str. showed significantly reduced clonal variation when compared to both sexual taxa (*G*: TukeyHSD test, P<0.05 for both comparisons; *G/N*: Kruskal-Wallis test, P=0.064; *D*: Kruskal-Wallis test, P=0.048).

Pooled values of genetic and genotypic diversity for particular cytotypes of *P. rhodopea* (Table 5) revealed that both genetic diversity (*D*) and genotypic variation

Table 3 Pairwise Nei's genetic distances – D_a (below diagonal) and genetic identities – I (above diagonal) between 20 populations of the *Pilosella alpicola* group

		P. alpicola		*P. rhodopea*						*P. serbica*	*P. ullepitschii*												
		SIM	DUR	COZ	BOT	DOD	MUS	BEZ	VICH	KOP	LAL	BAR	MLY	FUR	KR25	MEN	KR55	OST	SMA	NMA	BUC		
P. alpicola	SIM		0.944	0.921	0.940	0.965	0.924	0.946	0.937	0.834	0.766	0.842	0.813	0.857	0.829	0.852	0.841	0.831	0.915	0.919	0.926	SIM	*P. alpicola*
	DUR	0.058		0.833	0.913	0.894	0.838	0.937	0.947	0.809	0.715	0.757	0.803	0.839	0.789	0.800	0.773	0.788	0.922	0.937	0.958	DUR	
P. rhodopea	COZ	0.082	0.183		0.825	0.869	0.915	0.808	0.824	0.787	0.711	0.904	0.824	0.831	0.795	0.868	0.844	0.829	0.912	0.896	0.795	COZ	*P. rhodopea*
	BOT	0.062	0.091	0.192		0.969	0.888	0.959	0.914	0.845	0.827	0.791	0.818	0.879	0.864	0.857	0.856	0.858	0.843	0.854	0.946	BOT	
	DOD	0.036	0.112	0.140	0.031		0.909	0.946	0.900	0.845	0.823	0.824	0.813	0.870	0.861	0.862	0.856	0.861	0.856	0.863	0.924	DOD	
	MUS	0.079	0.177	0.089	0.119	0.095		0.863	0.847	0.905	0.742	0.837	0.789	0.811	0.811	0.824	0.827	0.803	0.802	0.823	0.822	MUS	
	BEZ	0.055	0.065	0.213	0.042	0.062	0.147		0.975	0.833	0.766	0.777	0.780	0.808	0.793	0.815	0.792	0.784	0.851	0.885	0.973	BEZ	
	VICH	0.065	0.054	0.194	0.090	0.105	0.166	0.025		0.779	0.694	0.750	0.715	0.786	0.739	0.754	0.739	0.743	0.887	0.940	0.969	VICH	
P. serbica	KOP	0.181	0.212	0.240	0.169	0.169	0.100	0.183	0.250		0.803	0.824	0.799	0.787	0.837	0.814	0.753	0.799	0.678	0.694	0.799	KOP	*P. serbica*
P. ullepitschii	LAL	0.266	0.335	0.341	0.190	0.195	0.299	0.266	0.365	0.219		0.884	0.791	0.941	0.981	0.882	0.845	0.968	0.696	0.646	0.781	LAL	*P. ullepitschii*
	BAR	0.172	0.278	0.101	0.235	0.194	0.178	0.252	0.288	0.194	0.123		0.875	0.885	0.908	0.938	0.889	0.918	0.822	0.770	0.761	BAR	
	MLY	0.207	0.220	0.194	0.201	0.207	0.237	0.248	0.336	0.224	0.235	0.133		0.828	0.842	0.963	0.934	0.834	0.792	0.733	0.745	MLY	
	FUR	0.154	0.175	0.185	0.129	0.139	0.209	0.213	0.241	0.240	0.061	0.122	0.189		0.978	0.899	0.886	0.989	0.855	0.815	0.854	FUR	
	KR25	0.187	0.237	0.230	0.146	0.150	0.210	0.232	0.302	0.178	0.019	0.096	0.172	0.022		0.909	0.894	0.987	0.774	0.727	0.812	KR25	
	MEN	0.160	0.223	0.142	0.154	0.148	0.193	0.204	0.287	0.206	0.125	0.064	0.038	0.106	0.095		0.953	0.918	0.825	0.768	0.791	MEN	
	KR55	0.173	0.258	0.170	0.155	0.155	0.190	0.233	0.302	0.284	0.169	0.118	0.068	0.121	0.112	0.048		0.884	0.779	0.754	0.751	KR55	
	OST	0.185	0.238	0.188	0.150	0.150	0.219	0.247	0.297	0.224	0.033	0.086	0.181	0.011	0.013	0.086	0.123		0.820	0.766	0.812	OST	
	SMA	0.089	0.081	0.092	0.171	0.156	0.221	0.161	0.120	0.388	0.363	0.196	0.233	0.157	0.256	0.192	0.225	0.199		0.973	0.896	SMA	
	NMA	0.084	0.065	0.110	0.158	0.147	0.195	0.122	0.062	0.366	0.437	0.261	0.310	0.205	0.319	0.264	0.287	0.266	0.027		0.919	NMA	
	BUC	0.077	0.043	0.229	0.056	0.079	0.196	0.027	0.031	0.224	0.247	0.273	0.294	0.158	0.208	0.235	0.287	0.197	0.110	0.085		BUC	

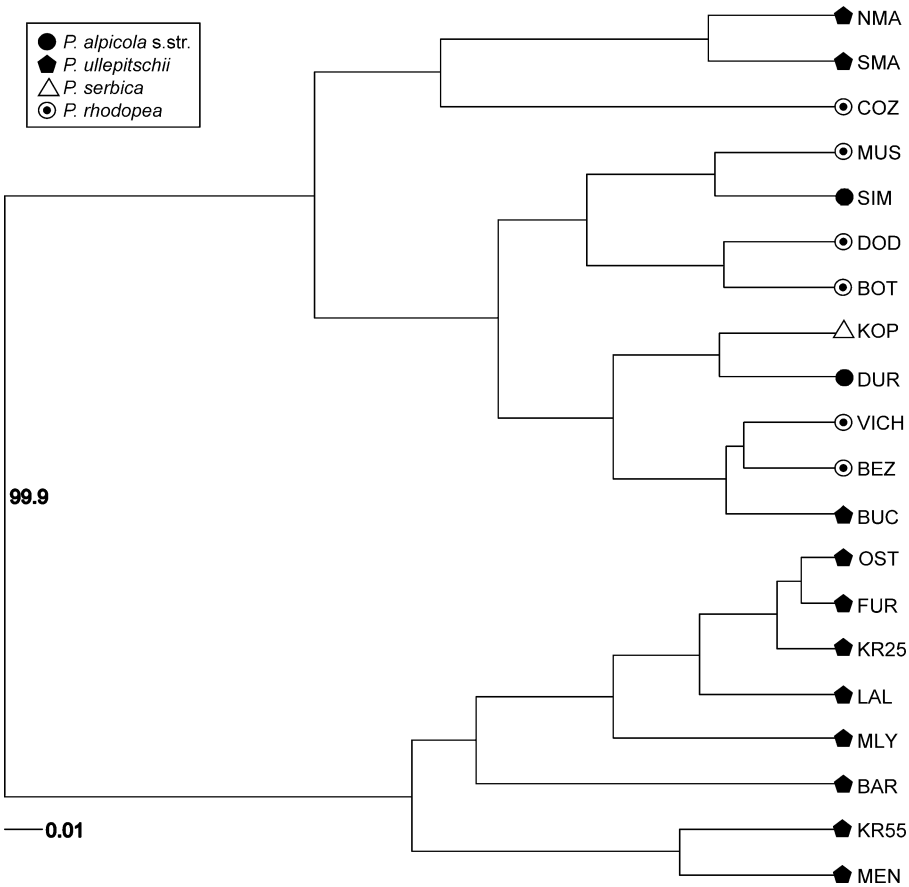

Fig. 2 Cluster analysis (UPGMA) of 20 populations of the *Pilosella alpicola* group based on Nei's (1972) genetic distance. Population codes are given in Table 1. *Pilosella alpicola* s.str. (N= 2, black circles), *P. rhodopea* (N= 6, white circles with dot), *P. serbica* (N= 1, open triangle), *P. ullepitschii* (N= 11, black pentagons). Only bootstrap values (based on 999 permutations) above 50% are shown

(*G/N*) were highest in diploids and tetraploids, while in triploids they were lower. The mean heterozygosity of *P. rhodopea* tetraploids (H_o=0.458) was nearly double that of diploids and triploids (H_o=0.255 and 0.240, respectively).

Inbreeding coefficients calculated for sexual taxa (Table 6) markedly varied among loci and populations of particular taxa. Except *P. serbica*, populations of all taxa displayed both excess and deficit of heterozygotes of rather moderate character.

Discussion

Correspondence between Allozyme and Morphological Differentiation

Although many studies showed allozymes to be useful taxonomic marker (e.g., Samuel et al. 1990; Bayer 1991; Hörandl 2004; Ruiz et al. 2004), our allozymic

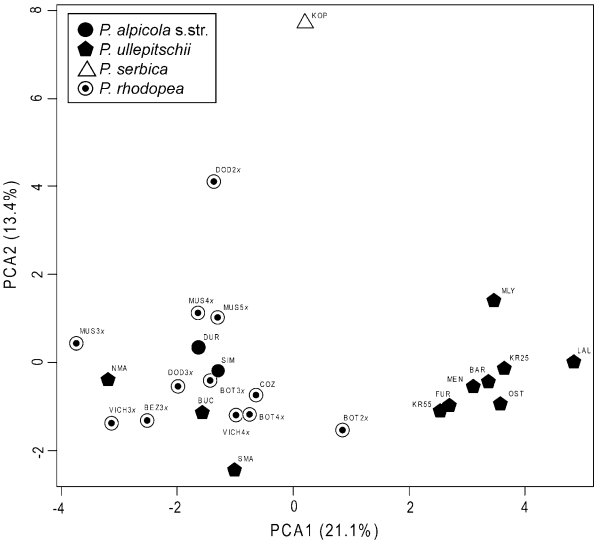

Fig. 3 Principal component analysis (PCA) of populations of the *Pilosella alpicola* group based on allelic frequencies of karyologically uniform populations and particular cytotypes from ploidy-mixed populations. *Pilosella alpicola* s.str. (*N*= 2, black circles), *P. rhodopea* (*N*= 12, circles with dot), *P. serbica* (*N*= 1, open triangle), *P. ullepitschii* (*N*= 11, black pentagons) as OTUs. For population codes see Table 1

results were rather incongruent with previously detected morphological variation of the *Pilosella alpicola* group (Šingliarová et al. 2011). We propose two non-exclusive explanations for the low resolution of allozymes in the *P. alpicola* group: *i*) the group has diversified quite recently and the morphologically well differentiated taxa had insufficient time to accumulate taxon-specific mutations at the protein level. The low number of taxon-specific alleles detected in the present study supports this hypothesis. Furthermore, no parsimoniously informative ITS site was found in 21 sequenced accessions and this supports a very recent origin of the *P. alpicola* member species (Šingliarová et al. 2011). In such a situation, low allozymic variation and high level of shared ancestral polymorphism is not surprising. *ii*) As suggested by Tyler (2005), frequent interspecific hybridization observed in the genus

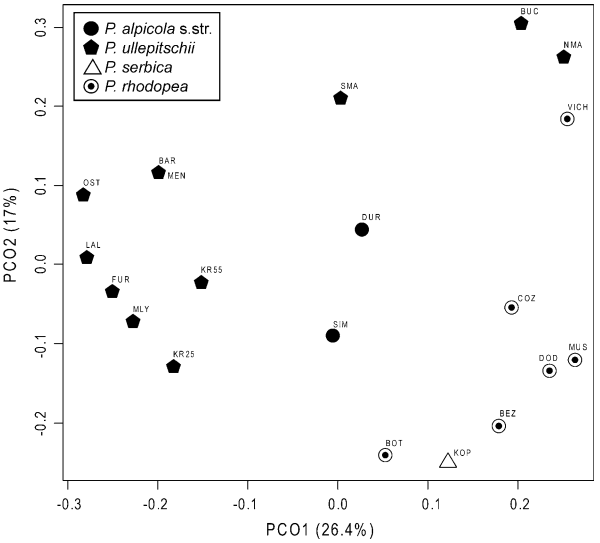

Fig. 4 Principal coordinates analysis (PCoA) of populations of the *Pilosella alpicola* group based on Jaccard's coefficient of dissimilarity. *Pilosella alpicola* s.str. (*N*= 2, black circles), *P. rhodopea* (*N*= 6, white circles with dot), *P. serbica* (*N*= 1, open triangle), *P. ullepitschii* (*N*= 11, black pentagons) as OTUs. For population codes see Table 1

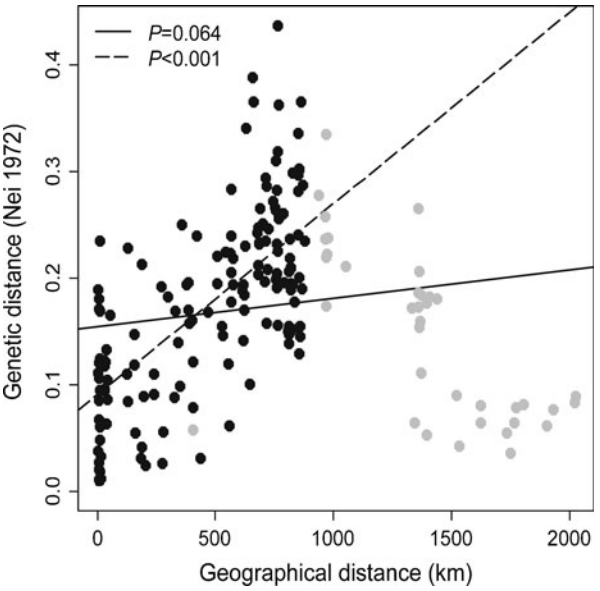

Fig. 5 Correlations between pairwise genetic distances (according to Nei 1972) among populations of the *Pilosella alpicola* group and their pairwise geographical distances. Regression line including all populations is represented by the full line, while the dashed line shows the correlation after removing two *P. alpicola* s.str. populations. Black dots denote pairwise comparisons between populations of *P. ullepitschii, P. serbica* and *P. rhodopea*, while gray dots denote comparisons between populations of *P. alpicola* s.str. and populations of three remaining taxa

Pilosella could potentially blur genetic differentiation between species. Indeed, we observed some interspecific hybrids between the members of the *P. alpicola* group and other *Pilosella* species, but they were less frequent when compared to the lowland taxa. In addition to their intermediate morphology, these interspecific hybrids showed an additive pattern of ITS polymorphism corroborating their hybrid origin (Šingliarová and Mráz, unpubl.). However, no such ITS pattern, neither conspicuous morphology (indicating hybrid origin), nor introgression were recorded in the *P. alpicola* plants included in our study. Based on this, interspecific hybridization seems not to be a primary reason of weak allozymic differentiation of the *P. alpicola* group.

Table 4 Averaged observed heterozygosity (H_o) for individual loci of four species of the *Pilosella alpicola* group and their cytotypes

	P. alpicola		*P. rhodopea*			*P. serbica*	*P. ullepitschii*
Locus/ploidy	4*x*	5*x*	2*x*	3*x*	4*x*	2*x*	2*x*
Skdh1	0.04	0	0.04	0	0	0.25	0.34
Pgm1	0	0	0.12	0	0.05	0.25	0.02
Pgm2	0.96	1	0.72	0.64	0.9	0.75	0.47
6Pgdh1	0.88	1	0.12	0.11	0.52	0.75	0.19
6Pgdh2	0.04	0	0.04	0.06	0	0	0.04
Sod1	1	1	0.2	0.14	0.81	0.25	0.43
Lap2	0.5	1	0.44	0.53	0.48	1	0.21
Lap3	0.96	0.07	0.4	0.39	0.9	0.25	0.35
Mean	0.55	0.51	0.26	0.23	0.46	0.44	0.26

Table 5 Genetic diversity parameters of four cytotypes of *Pilosella rhodopea*

	Ploidy	*N*	*P99*	*P95*	$\sum A$	*A*	H_o	*G*	*G/N*
P. rhodopea	2*x*	25	100	87.5	22	2.75	0.255	22	0.880
	3*x*	36	75	75	22	2.75	0.240	18	0.5
	4*x*	21	62.5	62.5	19	2.375	0.458	16	0.762
	5*x*	3	62.5	62.5	13	1.625	0.625	1	0.333

N – sample size, *P99* and *P95* percentages of polymorphic loci, $\sum A$ – sum of alleles, *A* – mean number of alleles per locus, H_o – observed heterozygosity, *G* – number of genotypes, *G/N* – proportion of distinguishable genotypes.

The Western Carpathian populations of *P. ullepitschii* formed the only well-differentiated group, while the Romanian populations of *P. ullepitschii* were clustered with the populations of *P. alpicola* s.str. from the Alps and *P. rhodopea* from the Balkan Peninsula (Figs. **2**, **3** and **4**). Such pattern could result from longer geographical separation of the Western Carpathian populations from the remaining populations. Indeed, several phylogeographic studies of high mountain plants confirmed the existence of a strong genetic barrier between the Western Carpathians and Southeastern Carpathians (e.g., Mráz et al. 2007; Ronikier et al. 2008; Těšitel et al. 2009). Alternatively, such separation could be caused by other processes such as genetic drift due to population size reduction or recent founder event(s) of isolated Romanian populations (*cf.* Šingliarová et al. 2008).

Genetic relatedness of the Romanian *P. ullepitschii* and *P. rhodopea* populations could be tentatively explained by recent contact of both taxa in this area. Although extremely rare and allopatrically distributed, both taxa are still present in the Southern Carpathians (see above). Indeed, the distributional and genetic pattern of many mountain species suggested biogeographical connections between the Bulgarian mountains and the Southern Carpathians (Reed et al. 2004; Puşcaş et al. 2008; Varga and Schmitt 2008; Schmitt 2009). It has been demonstrated that, despite the assumed selective neutrality of allozyme markers (e.g., Kimura 1968), environmental factors (temperature, humidity) can promote a shift in allele frequencies in populations through directional selection mechanisms (Koehn and Hilbish 1987; Karl and Avise 1992; Prentice et al. 2000; Karl et al. 2009). Thus, more similar environmental conditions between the southeastern Carpathians and the Balkans might be another reason of grouping of southeastern Carpathian *P. ullepitschii* and *P. rhodopea* populations.

The intermingled position of *P. alpicola* s.str. populations within populations of other species (Figs. **2** and **3**) might be surprising considering the geographical distance, striking morphological differences and allopolyploid origin (Šingliarová et al. 2011). The grouping with *P. rhodopea* populations can nonetheless be explained because this species was suggested to be one of the putative parental species of the allopolyploid *P. alpicola* s.str. (Šingliarová et al. 2011). A different level of genomic dosage of both putative parental taxa, with putatively greater influence of *P. rhodopea* (*P. alpicola* str. is morphologically more similar to *P. rhodopea* than to *P. glacialis*), could have shaped the allozyme pattern of *P. alpicola*

Table 6 Inbreeding coefficients (F_{IS}) for diploid and tetraploid sexual populations. Dashes indicate monomorphic loci, positive values (maximum = 1) an excess of homozygotes, negative ones (maximum = –1) an excess of heterozygotes, zero = Hardy-Weinberg-equilibrium

	P. rhodopea				*P. serbica*	*P. ullepitschii*									
	2*x*	2*x*	4*x*	4*x*	2*x*	all 2*x*									
	COZ	DOD	BOT	MUS	KOP	LAL	BAR	MLY	FUR	KR25	MEN	KR55	OST	SMA	NMA	BUC
SHDH-1	–	0	–	–	0	-0.486	-0.696	-0.25	0.125	0.153	0.477	0.231	-0.189	0.269	–	–
PGM-1	–	0.283	–	-0.038	0	–	–	–	–	–	–	–	–	–	-0.176	–
PGM-2	-0.667	-0.203	0.062	-0.087	-0.500	-0.067	-0.238	-0.304	-0.098	-0.132	-0.154	-0.352	0.239	-0.307	-0.176	-0.106
PGDH-1	0	-0.023	-0.038	-0.115	0	–	–	-0.304	-0.071	-0.154	–	–	–	–	–	–
PGDH-2	–	–	–	–	–	–	–	-0.2	–	–	–	–	–	–	–	–
SOD-1	–	0.154	0.117	-0.145	0	0.273	0.316	-0.429	-0.134	0.016	-0.429	-0.216	-0.077	1	-0.132	-0.13
LAP-2	0	0.114	0.853	0.123	-0.600	1	-0.083	1	1	1	0.894	0.695	0	0.054	0.259	-0.011
LAP-3	0.589	0.125	0.04	0.062	0	-0.185	0.829	0.277	0.1	0.135	0.381	0.174	0.031	–	–	–
Mean	0.037	0.078	0.207	-0.034	-0.217	-0.074	0.012	-0.052	0.05	0.118	0.309	0.093	-0.004	0.036	-0.029	-0.074

s.str. In spite of its hybrid origin, no unique alleles were found in *P. alpicola* s.str. in eight loci that were interpretable for all taxa and regions. If the three unique alleles in the *Lap1* locus, however, had been included in the analyses, they would have contributed to better separation of *P. alpicola* s.str. from the Balkan populations. Moreover, different alleles recorded in two subareas (*Lap1-a* in the tetraploids from SIM and *Lap1-b* and *c* in the pentaploids from DUR) indicate a polytopic origin of allopatric cytotypes.

Allozyme Variation in Diploid-Polyploid Pilosella rhodopea

Allozyme variation ascertained for diploid-polyploid *P. rhodopea* in the present study is consistent with the hypothesis of an autopolyploid origin (Šingliarová et al. 2011). All cytotypes possessed almost identical suites of alleles (Electronic Supplementary Material **1**). The only two unique alleles in *P. rhodopea* missing in diploids (*6Pgdh2-b*, *6Pgdh2-d*) were present in one triploid plant from the BEZ population, where we found only 3*x* and 4*x* plants (Šingliarová et al. 2011). Considering that also diploids were previously detected from the same locality by Krahulcová et al. (2009b), these unique alleles could be a part of common allele suite of unsampled diploids. The third unique allele found in *P. rhodopea* (*Pgm2-c*) was frequent in all cytotypes at the BOT locality (2*x*, 3*x*, 4*x*), the only population from the Stara planina Mts analyzed. Because all cytotypes shared this unique allele, we consider it evidence of *in situ* origin of polyploid cytotypes. Alternatively, the triploid and tetraploid cytotypes might still be later immigrants to the already established diploid population and could have acquired this allele through frequent intercytotype gene-flow. Independent autopolyploid origin of polyploids within diploid populations is supported also by previously established commonness of mixed ploidy populations (75%) and absence of morphological and ITS differentiation (Šingliarová et al. 2011). While there are numerous examples of secondary contact zones of previously allopatric cytotypes (e.g., Thompson and Lumaret 1992; Hardy et al. 2000; Husband and Sabara 2004; Schlaepfer et al. 2008; Kolář et al. 2009), evidence for primary contact zones is still quite sparse (Soltis and Soltis 1989; Weiss et al. 2002; Stuessy et al. 2004; Kolář et al. 2009; Trávníček et al. 2011).

Observed heterozygosity in polyploids of *P. rhodopea* is in agreement with the expectation that polyploids exhibit higher heterozygosity when compared to diploid relatives (Soltis and Rieseberg 1986; Soltis and Soltis 1989, 1993). Values detected in tetraploids (0.458, Table **5**) resembled those published for other autopolyploid taxa (e.g., 0.43 for *Dactylis*, Soltis and Soltis 1993; 0.474 for *Lotus*, Gauthier et al. 1998; 0.54 for *Centaurea*, Hardy and Vekemans 2001; 0.472 for *Thymus*, López-Pujol et al. 2004). Heterozygous tetraploids of *P. rhodopea* exhibited both balanced and unbalanced allele dosages, H_o across loci was variable (Table **4**), and there is no evidence for fixed heterozygozity. These findings indicate polysomic inheritance and thus an autopolyploid origin of *P. rhodopea* polyploids (Soltis and Soltis 1993). Conversely, estimates of genetic diversity (*P*, *A*) are lower for polyploids of *P. rhodopea* when compared to the diploids.

Although the polyploid state allows for the presence of three or more alleles at a single locus (Soltis and Rieseberg 1986; Soltis and Soltis 1989; Mahy et al. 2000), more than two alleles per locus was recorded only in small portion of *P. rhodopea*

polyploids (8%) when compared to other autopolyploids (e.g., 37% for *Thymus loscosii*, López-Pujol et al. 2004). Nevertheless, similar values were also reported in the literature (e.g., 12% for *Vaccinium oxycoccos*, Mahy et al. 2000).

Significant loss of genetic diversity was found in isolated Romanian populations of *P. ullepitschii* (Šingliarová et al. 2008). Because of secondarily formed habitats, we assumed that these populations experienced a strong bottleneck probably due to a founder effect mediated by human activities. A similar pattern was found in the remote diploid *P. rhodopea* population in the Cozia Mts (Southern Carpathians). When compared with diploids from the main range in the Balkans (DOD population), COZ population displayed markedly lower values in all genetic parameters (Table **2**). Considering relic habitat of *P. rhodopea* in the Cozia Mts (crevices in steep rock cliffs) and presence of several endemic and relic taxa (Nyárády 1955; Popescu et al. 1970), long-term vicariance scenario with genetic depauperation of small isolated population appears the most plausible.

Allozyme Variation in Allopolyploid Pilosella alpicola *s.str.*

Contrary to the previous assumption of a combination of two divergent genomes (Šingliarová et al. 2011), allozyme phenotypes of exclusively polyploid *P. alpicola* s. str. lacked unique alleles in eight shared loci. Three unique alleles, however, were found at the locus *Lap1*, which has not been scorable for all analyzed plants of the *P. alpicola* group (see Material and Methods). These unique alleles could originate from the second parental taxon – *P. glacialis*; unfortunately this species has not been included in the analyses. Nevertheless, the pronounced fixed heterozygosity observed at several loci (three for tetraploids and four for pentaploids, see Table **4** and Electronic Supplementary Material **1**) is convincing evidence of allopolyploid origin of the species. The values of observed heterozygosity of *P. alpicola* s.str. at individual loci were either close to zero or close to one, as usual in allopolyploids with disomic inheritance (Roose and Gottlieb 1976; Soltis and Soltis 1990; Arft and Ranker 1998).

Allozyme Variation and Mode of Reproduction

The patterns of allozyme variation within the *Pilosella alpicola* group agree with the mode of reproduction detected by Šingliarová and Mráz (2009) and Šingliarová et al. (2011). The populations of sexually reproducing species were genetically more variable than apomictic populations of *P. alpicola* s.str. (Table **2**).

Despite significant loss of genetic diversity in isolated Romanian populations of *P. ullepitschii* (Table **2**, and Šingliarová et al. 2008), mean genetic variation found in diploid *P. ullepitschii* was even higher than average values published for other sexual plants (*P* and *A*, Hamrick and Godt 1989). Values of averaged observed heterozygosity were similar or slightly exceeding those in other diploid sexual taxa (*Tolmiea*, Soltis and Rieseberg 1986; *Taraxacum*, Hughes and Richards 1988; *Lotus*, Gauthier et al. 1998; *Hemerocallis*, Kang and Chung 2000; *Ranunculus*, Hörandl et al. 2000). However, even higher values are known for sexual diploid taxa from apomictic complexes (e.g., Kashin et al. 2005 for *Taraxacum* and *Pilosella*; Hoebee et al. 2006 for *Sorbus*). Multilocus genotypic measures (*G/N* and *D*, Table **2**) reached

values close to maximum, which fits well the assumption for self-incompatible species (Ellstrand and Roose 1987).

Diploid and diploid-polyploid populations of sexual *P. rhodopea* (COZ, DOD, BOT) displayed genotypic diversity comparable to diploid *P. ullepitschii*. Decreased genotype variability, however, was found in polyploid populations (BEZ, VICH, MUS). Though the lower values of *G/N* for the MUS population were mainly due to genotypically uniform pentaploids, in two populations from the Pirin Mts, they were apparently caused by higher clonality of triploids (Tables **2** and **5**). The triploid cytotype, not frequent in the genus *Pilosella* (Schuhwerk 1996 and references therein), predominates in most mixed-ploidy populations of *P. rhodopea* (Šingliarová et al. 2011). A similar shift of proportion of cytotypes in the field in favour of triploids was observed also in *Pilosella echioides* (Peckert and Chrtek 2006; Trávníček et al. 2011). All populations with decreased genotype variability were formed only by polyploids (Šingliarová et al. 2011 and this study). We therefore hypothesize that these populations could have been founded by a small number of polyploid plants representing only a fraction of variability present in the source population(s). Moreover, the significantly decreased value of mean observed heterozygosity in the VICH population indicated a higher level of inbreeding. According to *G/N* values, vegetative reproduction could also play a role in the success of triploids. However, we did not observe increased formation of accessory rosettes in triploids, which could form independent ramets (Šingliarová, unpubl.). Alternatively, it cannot be ruled out that lower genotypic diversity may be a result of apomixis, but no asexually formed seeds were detected in polyploids of *P. rhodopea* so far (Šingliarová et al. 2011).

Averaged values of genotypic diversity (*G/N*, *D*) of apomictic *P. alpicola* s.str. (*G/N*=0.292, *D*=0.467, Table **2**) are consistent with values detected in other apomictic taxa (*Pilosella echioides* *G/N*=0.05–0.5, *D*=0–0.55, Peckert et al. 2005; *Hieracium alpinum* *G/N*=0.1–0.133, *D*=0.453–0.66, Štorchová et al. 2002; Chrtek and Plačková 2005; *G/N*=0.06, *D*=0.87 in *Erigeron annuus*, Ellstrand and Roose 1987; *G/N*=0.1, *D*=0.49 in *E. compositus*, Noyes and Soltis 1996; *G/N*=0.12–0.13, *D*=0.2–0.35 in *Ranunculus variabilis*, Hörandl et al. 2001).

When considering two *P. alpicola* s.str. populations separately, the tetraploid population (SIM) showed a lower value of genotypic diversity when compared to the other taxa (see above) but much higher when compared to the pentaploid DUR population, where only two multilocus genotypes were detected. This might indicate relatively frequent sexual recombination and thus facultative character of apomixis in tetraploids. Indeed, facultative apomixis is quite common in polyploid *Pilosella* taxa, especially with even ploidies, whereas it is more obligate in odd-ploid *Pilosella* including pentaploids (Koltunow et al. 1998; Krahulcová et al. 2000, 2004; Krahulec et al. 2004; Fehrer et al. 2005; for rare exceptions see Pogan and Wcisło 1995; Rotreklová et al. 2002).

Conclusion

Our study showed that genetic differentiation among morphologically well-defined species of the *P. alpicola* group inferred by allozymes was rather low. We assume that recent diversification of the group shaped this pattern and more discriminative markers

should be used in the future to achieve possibly better taxonomic separation. The type of polyploidy (auto- *vs* allo-) and reproduction modes, however, were proved to have significant impact on genetic and genotypic diversity observed in particular taxa. Close genetic relatedness of cytotypes found in ploidy-mixed populations of the *P. rhodopea* suggests the existence of primary contact zones of diploid and polyploid cytotypes. In this light, the *P. rhodopea* populations represent a promising system to explore evolutionary processes involved in coexistence of conspecific cytotypes.

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Príloha E

Mráz P, Chrtek J, Šingliarová B

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Geographical parthenogenesis, genome size variation and pollen production in the arctic-alpine species *Hieracium alpinum*

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Abstract *Hieracium alpinum* L. (Asteraceae) is an arctic-alpine species distributed throughout Europe with both diploid and triploid cytotypes. We determined the ploidy levels of plants from 23 populations from Austria, Bosnia and Herzegovina, Finland, Italy, Norway, Romania, Slovakia, Switzerland and Ukraine. Data showed a non-overlapping pattern of cytotype distribution: sexually reproducing diploids ($2n = 2x = 18$) occur solely in the Eastern and Southern Carpathians, while apomictic triploids ($2n = 3x = 27$) cover the rest of the range. Such clear-cut allopatry is rather rare in vascular plants with geographical parthenogenesis. Comparison of absolute genome size indicates genome downsizing (by on average 3.7%) of haploid DNA amount in triploids relative to diploids. Genome size further correlated with longitude and latitude in the Alps, with decreasing absolute DNA content from west to east, and from south to north. While previously published data indicated complete male sterility of triploid plants, we found that plants from the Alps and Bosnia and Herzegovina commonly produced some pollen, whereas populations from the Western Carpathians and Scandinavia seemed to be almost completely pollen sterile. Scenarios about the evolution of geographical parthenogenesis in *H. alpinum* are discussed.

Résumé *Hieracium alpinum* L. (au sens strict) est une espèce arctique-alpine d'aire de répartition très large, comprenant les régions nordiques (le Groenland, l'Islande, l'Ecosse, la Scandinavie et le nord de la Russie) et les montagnes de l'Europe continentale (les Alpes, les Carpates, les Sudètes, les Vosges et le plateau de Vranica). Dans cette étude, nous avons compté le nombre chromosomique et estimé la ploïdie par cytométrie de flux de plantes provenant de 23 populations échantillonnées en Autriche, Bosnie et Herzégovine, Finlande, Italie, Norvège, Roumanie, Slovaquie, Suisse et Ukraine. Ces données et celles de la littérature montrent une nette séparation spatiale entre deux cytotypes différents: Les populations diploïdes sexuées sont réparties uniquement dans les Carpates orientales et occidentales (Roumanie et Ukraine), tandis que les populations triploïdes apomictiques occupent l'aire de répartition restante. Ce type d'allopatrie stricte est rare chez les plantes avec parthénogenèse géographique. En comparant la taille du génome haploïde (1Cx) des

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plantes triploïdes avec celui des plantes diploïdes, nous avons identifié une sensible réduction de taille du génome polyploïde (la divergence moyenne est 3.7%). Parmi les plantes triploïdes, les individus du plateau de Vranica (Bosnie et Herzégovine) ont significativement moins d'ADN que les triploïdes provenant des Alpes ou des Carpates occidentales (2C = 10.28 pg d'ADN contre 11.02 et 10.93 pg, respectivement). Une corrélation significative entre la taille du génome et la longitude et la latitude a été révélée dans les Alpes, avec des valeurs décroissantes d'ouest en est, et du sud vers le nord. Tandis que les données publiées indiquaient une stérilité mâle complète chez les triploïdes, nous avons trouvé des plantes triploïdes provenant des Alpes et du plateau de Vranica produisant du pollen, bien qu'en faible quantité et de taille hétérogène. Divers scénarios sur l'évolution de la parthénogénèse géographique chez *H. alpinum* sont discutés à la lumière de ces nouveaux résultats.

Keywords Apomixis · Chromosome numbers · Compositae · Flow cytometry · Genome downsizing · Male sterility · Polyploidy

Introduction

Sexual and asexual organisms belonging to closely related taxa often differ in spatial distribution. This geographical differentiation of sexuals and asexuals is called "geographical parthenogenesis" (Vandel 1928), and is one of the most challenging topics in evolutionary ecology. In plant species with geographical parthenogenesis, the apomictic (=asexual) lineages usually have larger ranges than their sexual relatives, are often shifted to higher latitudes or altitudes, and tend to occupy previously glaciated areas (Bierzychudek 1985; Asker and Jerling 1992). Several non-exclusive hypotheses have been proposed to explain the widespread distribution of apomictic groups. As almost all apomictic plants are polyploid, and many of them are of allopolyploid origin, multiple gene copies might provide greater physiological tolerance to apomicts than to their sexual diploid progenitors. Furthermore, uniparental reproduction is a safer pathway than sexual mating for the establishment of new populations during colonisation. Further potential mechanisms putatively involved in geographical parthenogenesis include biotic interactions or niche partitioning among closely related clones (Hörandl 2006).

The large polyploid genus *Hieracium* s. str.—hawkweed (excluding the genus/subgenus *Pilosella*) exhibits a geographical pattern of parthenogenesis. The great majority of karyologically analysed taxa are either tri- or tetraploid, reproduce asexually and occupy a large holarctic range (Gustafsson 1946; Schuhwerk 1996). Conversely, sexual diploid taxa are rare and mostly confined to restricted ranges in southern parts of Europe (Merxmüller 1975; Vladimirov 2000; Castro et al. 2007; Chrtek et al. 2007). In *Hieracium* s.str., species typically contain only one ploidy level. One of the rare hawkweeds showing intraspecific variation in ploidy level and breeding system is *H. alpinum*.

Hieracium alpinum L. (s. str.) is an arctic-alpine species with two main areas of distribution: northern Europe (Scandinavia, Scotland, Iceland, Greenland, northwest Siberia and northern Ural) and high mountain ranges in Central Europe (Alps, Carpathians, Sudetes). Isolated populations were also recorded in the Harz (Germany), the Vosges (France), the Vranica planina (Bosnia and Herzegovina) and Central Italy (Gottschlich 1987; Bräutigam 1992). *H. alpinum* inhabits the alpine tundra and only rarely occurs in the subalpine belt. Published chromosome data on *H. alpinum* indicate the presence of at least two different ploidy levels: $2n = 3x = 27$ and $2n = 2x = 18$ (Table 1). There is only one tetraploid record ($2n = 4x = 36$) from the Western Carpathians (Szeląg and Jankun 1997). Available data suggest that triploids are widely distributed while diploids occur only in the Carpathians (Table 1). However, ploidy levels in some regions, notably in the Alps have not yet been studied in sufficient detail to exclude the possibility that diploids also occur in this large mountain range, considered to be an important evolutionary centre and refugium of high mountain flora (e.g. Pawłowski 1970; Ozenda 1985; Schönswetter et al. 2005).

Diploid plants of *H. alpinum* are sexual and strictly outcrossing (Chrtek 1997; Mráz 2003). In contrast, triploid *H. alpinum* reproduces *via* agamospermy (apomixis), like other polyploid *Hieracium* taxa. Based on the embryological study of female meiosis, Skawińska (1963) determined a diplospory of *Antennaria* type in triploid *H. alpinum*: an unreduced embryo sac is formed from the mother cell through two mitotic divisions, with further parthenogenetic development into a mature embryo sac before the opening of flower heads. *Hieracium* s.str. polyploids are believed to be obligate apomicts, as no direct embryological or molecular evidence has provided evidence for sexual reproduction. Furthermore, male meiosis is partially or completely disturbed, mainly due to difficulties in chromosome pairing, resulting in a low production of pollen of heterogeneous size or in complete male sterility (Rosenberg 1927; Gentcheff and Gustafsson 1940; Aparicio 1994; Mráz et al. 2002). Only few data about the variation in pollen production and its geographic pattern are available for *H. alpinum*. Complete male sterility was observed in triploid plants from the Western Carpathians and Sudetes (Chrtek 1997), as well as from Scotland (Slade and Rich 2007). Only Rosenberg (1927)

Table 1 List of published chromosome counts for *Hieracium alpinum* L.

Ploidy and region	N[a]	Published source
Diploid ($2n = 2x = 18$)		
Eastern Carpathians	17	Chrtek 1997 [b]; Mráz 2001, 2003; Mráz and Szeląg 2004; Mráz et al. 2005
Southern Carpathians	4	Mráz 2003; Mráz and Szeląg 2004
Triploid ($2n = 3x = 27$)		
Alps	2	Huber and Baltisberger 1992; Schuhwerk and Lippert 1999
Greenland	4	Böcher and Larsen 1950; Jorgensen et al. 1958; Gadella and Kliphuis 1971
Murmansk region	1	Sokolovskaya and Strelkova 1960[c]
Scandinavia	1	Engelskjøn and Knaben 1971[d]
Scotland	2	Stace et al. 1995
Sudetes	7	Měsíček and Jarolímová 1992; Chrtek 1994; Chrtek and Plačková 2005
Ural	2	Lavrenko et al. 1988, 1989
Western Carpathians	21	Skalińska 1959; Uhríková and Murín 1970; Murín and Májovský 1992; Chrtek 1997; Mráz 2001; Štorchová et al. 2002; Chrtek et al. 2004
Tetraploid ($2n = 4x = 36$)		
Western Carpathians	1	Szeląg and Jankun 1997

Data from the present study are shown in Fig. 1

[a] Number of chromosome records for particular geographical region (if two chromosome counts from the same locality were counted by different persons or counted on different plants later, they are considered as two different number records)

[b] Including *H. augusti-bayeri* (Zlatník) Chrtek

[c] Aneuploid chromosome number ($2n = 26$)

[d] Published as "*H. alpinum* L. coll."

reported a case of male meiosis, albeit highly disturbed, in triploid *H. alpinum* of unknown origin. The possibility of male meiosis deserves additional investigation as viable pollen can considerably enhance evolutionary potential by allowing gene flow between apomictic triploids and sexual diploids.

The main aims of our study are (1) to verify the geographic distribution patterns of diploids and triploids across Europe, (2) to look for intraspecific variation in genome size across the range of the cytotypes, and finally (3) to assess geographic variation in pollen production in triploid plants.

Materials and methods

Ploidy level and genome size estimation

Plants were collected at flowering time between 2003 and 2007 from their natural habitats (Appendix 1) and transplanted into experimental gardens in Košice (Slovakia) or Grenoble (France). Chromosome counts were made on four plants (Appendix 1) using root-tip meristems of pot-grown plants. Root tips were pre-treated with 0.5% solution of colchicine for 1.5–3 h at room temperature, fixed in a mixture of ethanol and glacial acetic acid (3:1) for at least 1 h and stored in 70% ethanol at 4°C until use. Hydrolysis was done in 1 N HCl at 60°C for 7–10 min. The "squash and smear" method replacing the glass covers with cellophane followed Murín (1960). Giemsa solution in phosphate buffer was used as a stain.

Nuclear DNA content was analysed for 56 plants (Appendix 1) in the Laboratory of Flow Cytometry at P.J. Šafárik University, Košice, using leaves of *Zea mays* CE-777 (2C = 5.43 pg) as internal reference standard (Lysák and Doležel 1998). Samples were prepared using a two-step procedure (Otto 1990; Doležel and Göhde 1995). Approximately 1 cm^2 of leaf tissues of both the sample and the reference internal standard were chopped together for about 30 s in a Petri dish containing 1 ml of ice-cold Otto I buffer (0.1 M citric acid monohydrate + 1 ml 0.5% Tween 20 adjusted to 200 ml and filtered through a 42 µm filter). Filtration through 42 µm nylon mesh was followed by centrifugation at 150 g for 5 min. The supernatant was removed and 100 µl of fresh Otto I buffer was added. The nuclei in the pellet were resuspended and incubated for 30 min at room temperature. DNA was stained with 1 ml of modified Otto II buffer (0.4 M disodium hydrogenphosphate dodecahydrate) including 50 µl of propidium iodid (PI), 50 µl ribonuclease (A R5000, Sigma), and 2 µl mercaptoethanol.

Flow cytometry was carried out with a FACSCalibur instrument (Becton Dickinson, USA) equipped with an argon-ion laser exciting at 488 nm. For comparison, the exact position of peaks of previously counted di-, tri- and tetraploid *Hieracium* taxa relative to the peak of the internal standard was measured (Chrtek et al. 2007). Coefficients of variation (CV) of the peaks of internal standard adjusted at channel 100 ranged from 3.5 to 6.9%, with an average value of 5.4%, and CV of peaks of measured samples varied between 2.9 and 5.1%, with an average value of 3.9%. Genome size was determined in at

least three independent runs per plant, and the mean was used. Measurements differing by more than 2% were discarded, and the sample was re-analysed.

Mean genome size of each population (=locality) was used in data analysis. Differences in mean monoploid genome size (1Cx) were tested by Tukey HSD test (1) between all populations comprising at least three individuals, and (2) between four different regions (Alps, Western Carpathians, Southern Carpathians and Vranica planina). Two-sample *t* test with Welch approximation (due to unequal variances) was used to test the differences in mean monoploid genome size (1Cx) between diploid and triploid plants. Correlations between the genome size and the geographical position of populations (altitude, latitude and longitude) in the Alps were tested by Spearman rank tests. All tests were done using the basic packages of R software (R Development Core Team 2006).

Pollen production

Pollen measurements were carried out on herbarium samples collected as vouchers for a phylogeographic study of *H. alpinum* (Appendix 2). Five flowers per plant (both inner and outer flowers) in the stage before anthesis were broken up with tweezers to release the pollen from the anthers. Pollen was stained by Alexander's stain (Alexander 1969). The staining pattern proved unsuitable to assess pollen viability because some clearly deformed pollen grains were stained as "viable" (red cytoplasm and green cell wall) while many pollen grains of regular shape and

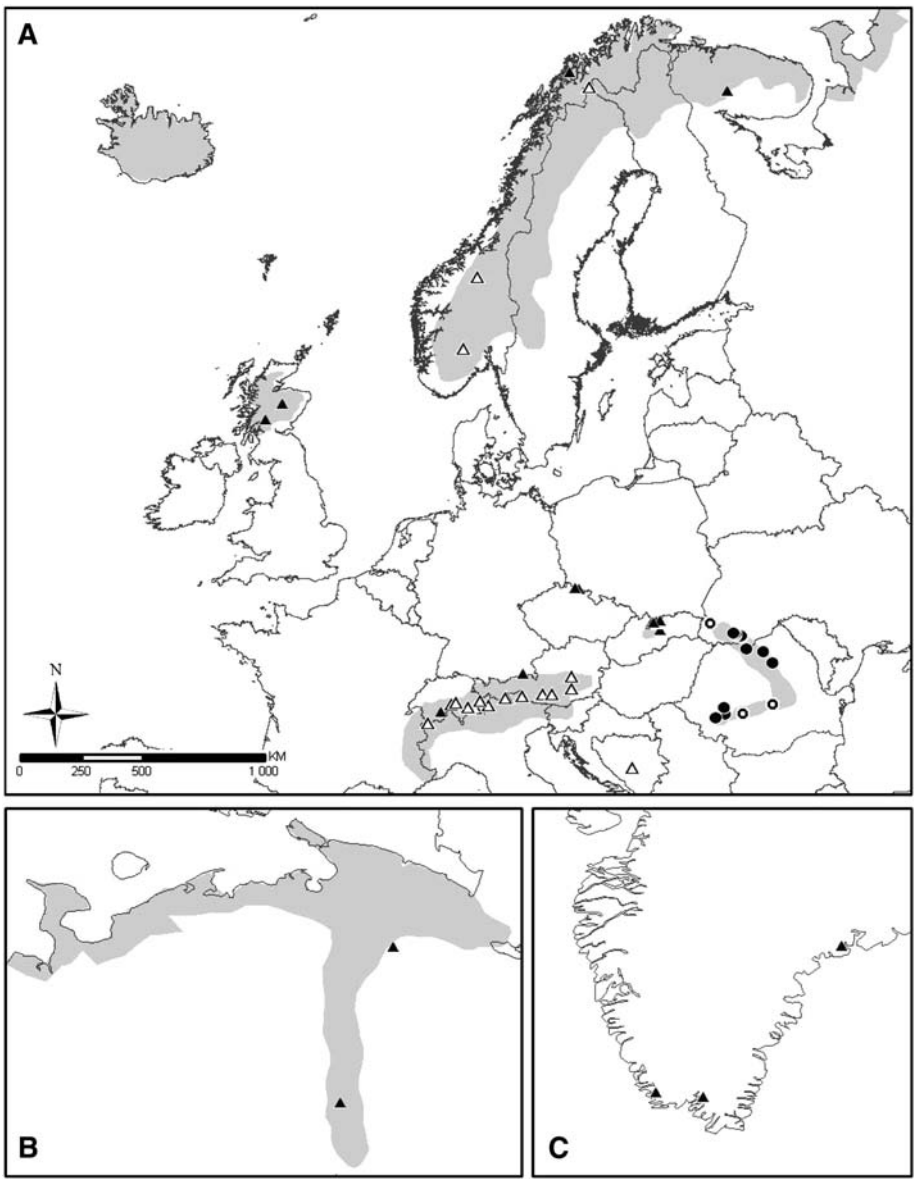

Fig. 1 Distribution of karyologically and/or flow cytometrically analysed populations of *Hieracium alpinum* L. Total range of the species is marked by *grey shading* (**a** Europe, **b** Ural and Northern Russia, **c** Greenland). Symbols used: *filled circle*/*open circle* diploid populations (published/new data), *filled triangle*/*open triangle* triploid populations (published/new data)

Table 2 Absolute genome size estimation in diploid and triploid populations of *Hieracium alpinum* L.

Ploidy level and population[a]	*N*	Mean 2C-value (pg DNA)	2C value range (pg DNA)	Variation max/min (%)	Mean 1Cx-value[b]
Diploid populations (2*x*)					
ROM, Nedea	3	7.57 ± 0.03	7.55–7.61	0.7	3.79a
ROM, Bucegi	4	7.56 ± 0.07	7.45–7.61	2.1	3.78a
Triploid populations (3*x*)					
AUT, Turracher Höhe	1	11.01			3.67
AUT, Reißeckhütte	2	10.88 ± 0.08	10.82–10.93	1.0	3.62
AUT, Leobnerhütte	3	11.01 ± 0.02	10.99–11.02	0.2	3.67b, d
AUT, Stallersattel	1	10.89			3.63
AUT, Altes Almhaus	1	10.96			3.65
BIH, Vranica	3	10.28 ± 0.06	10.22–10.33	1.1	3.43c
CHE, Julier pass	1	11.23			3.74
CHE, Passo dello Spluga	2	11.04 ± 0.09	10.97–11.10	1.2	3.68
CHE, Furkapass	1	11.10			3.70
CHE, Oberalppass	1	11.02			3.67
CHE, Grand St. Bernard	4	11.07 ± 0.09	10.95–11.14	1.7	3.69b
ITA, Passo di Giovo	2	11.09 ± 0.00	11.09–11.10	0.2	3.70
ITA, Passo di Pennes	2	10.89 ± 0.17	10.77–11.01	2.2	3.63
ITA, Passo dello Stelvio	3	11.11 ± 0.09	11.01–11.23	2.0	3.70b
SVK, Furkotská dolina	2	11.01 ± 0.07	10.96–11.06	0.9	3.67
SVK, Baranec	4	10.89 ± 0.04	10.85–10.94	0.8	3.63d

[a] Country codes: *AUT* Austria, *BIH* Bosnia and Herzegovina, *CHE* Switzerland, *ITA* Italy, *ROM* Romania, *SVK* Slovakia

[b] 1Cx-value (mean monoploid genome size) is the mean 2C-value divided by ploidy level. Multiple comparison test (Tukey HSD, at $P < 0.05$) was performed on 1Cx values for populations comprising at least three analysed plants. Mean monoploid values (1Cx) sharing the same letter are not significantly different

size were not stained at all. Therefore, pollen production was only estimated semi-quantitatively on a six-point scale ranging from "−" no pollen, or only sporopolenin remnants present, to "+++++" pollen very abundant, as in the diploid cytotype.

Results

Geographical pattern of the cytotype distribution

Diploids were found only in the Eastern and Southern Carpathians (Romania and Ukraine), while a triploid level was ascertained for plants originating from the Western Carpathians, the Alps, Scandinavia and the Vranica planina (Bosnia and Herzegovina). For the last region, as well as for the accessions from Austria, Italy and Finland, these are the first DNA-ploidy level estimations reported. The cytogeographic pattern based on present and published records clearly shows a spatial split of diploid populations and triploid populations. The sexually reproducing diploid cytotype occurs exclusively in the Eastern and Southern Carpathians (Romania and Ukraine), while apomictic triploids occupy the remaining range of the species (Fig. 1; Table 1).

Genome size variation

Absolute genome size was quantified for 40 plants of *H. alpinum* originating from the diploid (the Southern Carpathians) and triploid range (the Alps, the Vranica planina and the Western Carpathians). Averaged absolute genome size values (2C) for each population, as well as ranges for 2C and 1Cx (monoploid genome size) are given in Table 2. The individual 2C values within diploid cytotype range from 7.45 to 7.61 pg DNA (2.1% divergence within diploid ploidy level), with mean value of 7.56 pg. Between-individual variation in 2C value within triploid ploidy level was higher compared to diploid plants, and ranged from 10.22 to 11.23 pg, representing almost 10% divergence. This differentiation within triploids, however, arises primarily from low DNA content found in all analysed plants from the geographically isolated Vranica population. Because triploid plants from the Vranica planina showed considerably lower DNA content than triploids from the Alps and the Western Carpathians in first repetitive analyses, these accessions were re-analysed (again with three replications *per* plant) 1 month later, but the initial result was confirmed. Average divergence in mean 2C values between the Vranica population and the four tested triploid

Table 3 Comparison of the absolute genome size of *Hieracium alpinum* L. belonging to different cytotypes and geographical origins

Region (ploidy level)	N^a	Mean 2C value (pg DNA)	2C value range (pg DNA)	% min-max	Mean 1Cx value (pg DNA)[b]
S Carpathians (2*x*)	7	7.56	7.45–7.61	2.1	3.78a
Alps (3*x*)	23	11.02	10.77–11.23	4.3	3.67b
W Carpathians (3*x*)	6	10.93	10.85–11.06	1.9	3.64b
Vranica, Balkan (3*x*)	3	10.28	10.22–10.33	1.1	3.43c

[a] Number of analysed plants per region

[b] Mean monoploid values (1Cx) sharing the same letter are not significantly different (Tukey HSD test, $P < 0.05$)

populations is 7.2%. The maximal inter-individual divergence within one mountain range was recorded among the plants from the Alps (4.3%).

Mean monoploid genome size (1Cx) differed significantly between the Vranica and all other tested populations, as well as between diploid populations and all tested triploid populations (Tukey HSD tests, $P < 0.05$). No significant differences were recorded between triploid populations from the Alps and the Western Carpathians, with exception of the Baranec population in the Western Carpathians (Table 2). When data obtained from individual plants were grouped by geographical origin, statistically significant differences were found between: (1) the triploids from the Alps/Western Carpathians and the Vranica planina, (2) the triploids from the Alps/Western Carpathians and the diploids from the Southern Carpathians, and (3) the triploid plants from the Vranica planina and the diploids from the Southern Carpathians (Table 3). A significant monoploid genome downsizing was recorded in diploid plants when compared to all triploids with mean divergence of 3.7% (Welch two sample *t* test, $t = 7.93$, $df = 27.5$, $P < 0.001$). When plants from the Vranica planina were excluded from the analysis the divergence was smaller (3.1%), but still highly significant (two sample *t* test, $t = 9.23$, $df = 35$, $P < 0.001$).

Significant negative correlations between genome size and latitude and longitude were revealed for accessions from the Alps ($r_s = -0.629$, $P = 0.0214$, and $r_s = -0.576$, $P = 0.0395$, respectively). No significant correlation was found between genome size and altitude ($r_s = 0.517$, $P = 0.0719$).

Variation in pollen production

The only plants included in our observations that originated from the range occupied solely by diploids are those from the Horhany ridge (Ukrainian Eastern Carpathians). In this population, we found a high amount of pollen of homogeneous size (Fig. 2a, b). Most of the triploid plants did not produce pollen at all (85% of all triploids studied, cf. Table 4; Fig. 2c). However, some pollen production was observed in 10 plants from the Alps (27% of plants studied from this range), in both plants analysed from the Vranica planina and in one plant from the Western Carpathians (2.6%) and Scandinavia (6.2%) (Table 4). These male-fertile triploids produced only a small amount of pollen in comparison with diploids and this pollen was always of heterogeneous size (Fig. 2d). Moreover, the pollen of triploid plants frequently showed poorly developed exine structure.

Discussion

Cytogeographic pattern and geographical parthenogenesis

Hieracium alpinum can be considered a clear-cut example of geographical parthenogenesis: diploids occupy only a restricted area at the low latitude range margin, whereas apomictic triploids cover a much larger area, including polar latitudes and previously extensively glaciated areas like the Alps and northern Europe (Fig. 1). A surprising pattern is the completely non-overlapping distribution of sexual and asexual plants. The recent closest localities of diploid (Mount Pikuï in the Eastern Carpathians, Ukraine) and triploid (the Belianske Tatry mountains in the Western Carpathians, Slovakia) cytotypes are separated by c. 200 km. In other species showing geographical parthenogenesis, both reproduction modes co-occur, at least in some areas (Asker and Jerling 1992; Hörandl 2006). Assuming a high colonisation potential of triploid *H. alpinum* (based on its present range), it is interesting that no triploid plant (either of in situ origin or as immigrant from triploid range) have been detected in the diploid range so far. This might suggest that (1) recent diploids are not able to produce stable triploid progeny, or (2) effective dispersal of triploids into the diploid range is prevented, or (3) there is some selection mechanism precluding their successful establishment. The first two hypotheses seem plausible. Indeed, the production of unreduced gametes, considered the most important pathway to polyploidy (Ramsey and

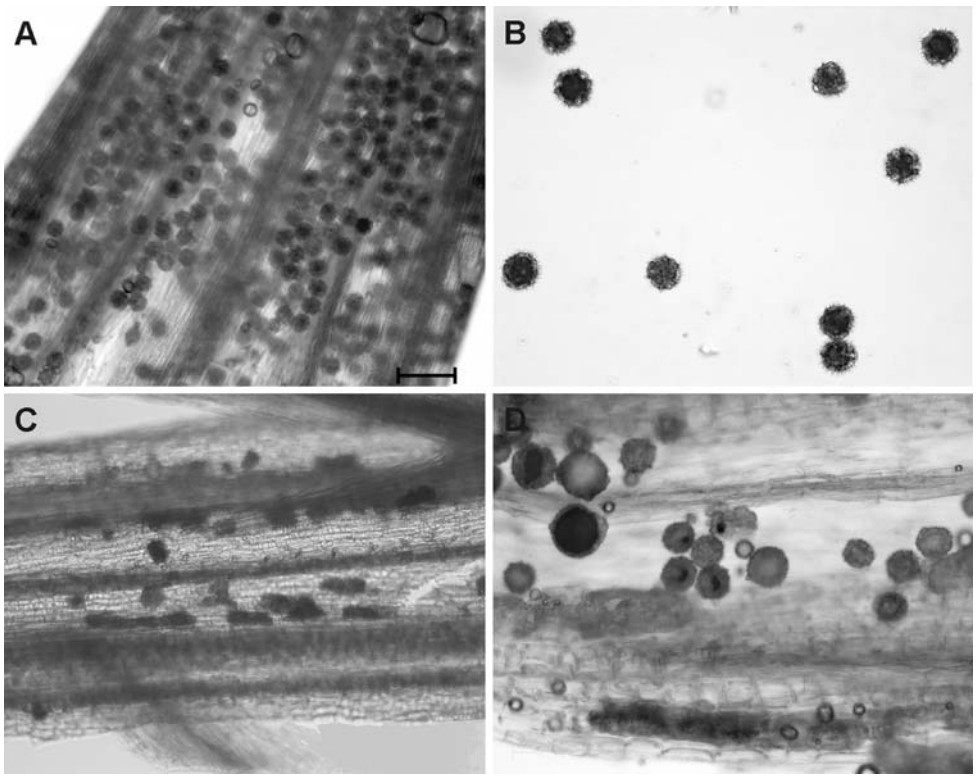

Fig. 2 Pollen in *Hieracium alpinum* L. **a** anthers with a lot of pollen in diploid plant, **b** homogeneous sized pollen in diploid plant (both plants from the population Alp-56, Ukraine Mount Mala Syvulya), **c** anthers without pollen in triploid plant (population Alp-26, Austria, Sölkpass), some remnants of degenerated tapetum layer are visible, **d** few pollen grains of heterogenous size in the anther of triploid plant (population Alp-23, Austria, Mount Seekareck). *Scale bar* = 100 μm (**a** and **c**), 50 μm (**b** and **d**)

Table 4 Semi-quantitative estimation of pollen production in *Hieracium alpinum* L.

Region/population code (Appendix 2)	N^a	Pollen quantity[b]
Alps (3*x*)		
Alp-2, 4, 11, 12, 15, 18, 20, 22, 24, 25	18	− (all plants)
Alp-5	2	−, ++
Alp-14	2	−, +
Alp-16	1	++++
Alp-17	3	−, +, +++
Alp-21	2	−, +
Alp-23	4	−, ++, +++, +++
Alp-26	3	−, −, +++
Eastern Carpathians (2*x*)		
Alp-56	2	+++++ (both plants)
Scandinavia (3*x*)		
Alp-93, 94, 95, 96, 97, 98, 101, 103	13	− (all plants)
Alp-102	4	−, −, −, ++
Yamal Peninsula (3*x*)		
Alp-Yam	2	− (both plants)
Vranica (3*x*)		
Alp-87	2	++, +++
Western Carpathians (3*x*)		
Alp-32, 34, 35, 36, 37, 38, 39, 40, 41, 45, 46, 47, 48	37	− (all plants)
Alp-44	3	−, −, +++

[a] Total number of plants analysed

[b] Relative pollen abundance from "−" (no pollen) to "+++++" (pollen very abundant as in diploid cytotype) for each of the plants analysed

Schemske 1998), seems to be rare in diploid *Hieracium* taxa including *H. alpinum*. No larger pollen grains indicating larger genome content were observed during a detailed study of pollen production and size in diploid hawkweeds (Kovalčiková 2004). Furthermore, long-distance dispersal is stochastic and usually involves singular

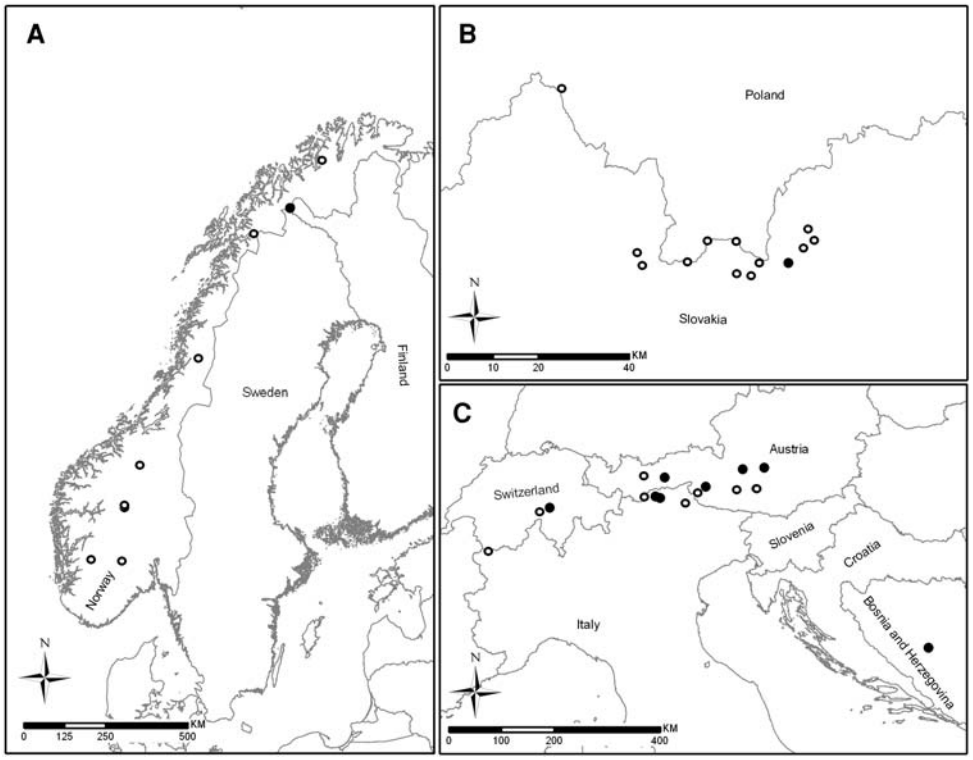

Fig. 3 Distribution of triploid apomictic populations of *Hieracium alpinum* L. where pollen production was examined (**a** Scandinavia, **b** the Western Carpathians, **c** the Alps and Vranica planina). Symbol used: "*filled circle*" at least one plant from analysed population produced some pollen; "*open circle*" none of analysed plants from population produced pollen (for details see "Material and methods", supplementary material Appendix 2 and Table 4). The remnant locality from the Yamal Peninsula (NE Russia) was not included in the map

events only (Nathan 2006, but see Alsos et al. 2007), maybe restricting the invasion of the diploid range by triploids. Nevertheless, the strict allopatry of *H. alpinum* cytotypes would probably be better explained by restricted effective dispersal involving adaptive mechanisms that further prevent recruitment. It has been hypothesised that sexual lineages are better competitors than asexual ones because sexual recombination can sustain selection, while apomicts might be more successful on disturbed places or extreme types of habitats with fewer biotic interactions (Asker and Jerling 1992, but see de Kovel and de Jong 2001). In *H. alpinum*, however, there are no striking differences between the habitats occupied by both cytotypes in their respective ranges. Diploids and triploids both grow in open as well as in more dense vegetation communities, suggesting that selection does not play a key role in maintaining cytotype separation in *H. alpinum*.

Results from a large-scale molecular screening of *H. alpinum* populations (Mráz et al., unpublished data) indicate a polytopic origin of triploid apomicts and only a loose genetic relatedness with the recent diploids. Together with the disjunct ranges of diploids and triploids, and the occurrence of many closely related microspecies within the triploid range, this suggests that triploid plants of *H. alpinum* are probably remnants of extinct diploid lineages rather than descendants of contemporary diploid populations.

Genome size variation and its geographical pattern

In our study, we confirmed a general trend of genome downsizing in polyploids with respect to their diploid progenitors (Leitch and Bennett 2004). This contrasts with the closely related genus *Pilosella,* where downsizing was not apparent in triploids, but only at higher ploidy levels (Suda et al. 2007). Several mechanisms may lead to genome size reduction in polyploids: (1) unequal homologous recombination, (2) elimination of specific DNA sequences (including sequences in low- and high copy number from both coding and non-coding regions), and (3) change in transposons activity (Leitch and Bennett 2004).

In *H. alpinum*, a significant genome size reduction (in average ca 7%) was detected in the isolated population of the Vranica planina. Because the exact chromosome numbers of plants from the Vranica population were not determined, we could not exclude the possibility that this intraspecific variation in DNA content is due to aneuploidy. In fact, aneuploidy ($2n = 26$) was previously reported in individuals of *H. alpinum* from the Murmansk region (Sokolovskaya and Strelkova 1960), but aneuploidy is extremely rare in the genus *Hieracium* (Schuhwerk 1996). The loss of DNA observed in *H. alpinum* from the isolated population of Vranica planina could be attributed to other phenomena like a selection for smaller genome size. Indeed, Knight and Beaulieu

(2008) showed a correlation between plant genome size and some traits involved in evapotranspiration, such as length of the guard cells, epidermal cell area and stomatal density. Taking into account the particular climatic conditions at Vranica planina (the southernmost known population of *H. alpinum*), we cannot exclude an adaptive scenario in this case. An alternative explanation for genome size reduction in Balkan plants might be the different evolutionary origin, because the Vranica population is genetically distinct from the other triploid populations (Mráz et al., unpublished data).

With the exception of the Vranica plants, between-individual or inter-population (or interregional) variation in genome size of *H. alpinum* was moderate. The maximal divergence between two triploid plants (excluding the Vranica population) was 4.3%. The plants from the Alps were more variable (expressed as min and max range of 1Cx) than the plants from the Western Carpathians, in concordance with higher genetic variation found in triploid plants from the former region (Mráz et al. unpublished data). However, we analysed more plants from the larger area of the Alps than from the Carpathians, and possible sampling bias should be taken into consideration. Significant negative correlations between genome size of triploid *H. alpinum* and latitude and longitude were recorded in the Alps. Clinal variation in genome size remains a contentious issue. It has been tentatively proposed to be an adaptation to local climatic conditions (e.g. Temsch and Greilhuber 2001; Schmuths et al. 2004; Bancheva and Greilhuber 2006). Therefore we also examined whether some relationship could be found between the genome size and mean annual precipitation and temperature as derived from the WorldClim model (Hijmans et al. 2005). We found no correlation (data not shown), but the results should be interpreted with caution because the WorldClim model is spatially very coarse.

Variation in pollen production

A large quantity of pollen of homogeneous size is characteristic for diploid *H. alpinum* (Chrtek 1997; Mráz et al. 2002; Kovalčiková 2004; Chrtek et al. 2006; Slade and Rich 2007). In contrast, most plants from the triploid range did not produce pollen at all (Table 4). This finding concurs with those of Chrtek (1997), Kovalčiková (2004) and Slade and Rich (2007) who observed no pollen in triploid cytotype in the Western Carpathians and Scotland. However, in the present study, we detected some level of pollen production in triploids from the Alps, the Vranica planina, and very rarely from the Western Carpathians and Scandinavia (Table 4, Fig. 3). The most noteworthy is the high proportion (27%) of partially male fertile plants from the Alps, and mainly from the eastern part. From present and published data (see above), it is obvious that triploids in northern latitudes (Scandinavia, Scotland, Sudetes, Yamal Peninsula, the Western Carpathians) are mostly male sterile, while triploids from southerly situated Alps or the Vranica planina can more frequently produce some pollen. This indicates that triploid *H. alpinum* is not completely male sterile, as previously suggested. Intraspecific polymorphism in pollen production has also been reported in other polyploid *Hieracium* taxa (Mráz 2002; Kovalčiková 2004; Slade and Rich 2007; Rich et al. 2008). Interestingly, in some cases we recorded variation in pollen production within one flower head, or even within one flower (some anthers with pollen, some anthers completely empty). Similarly, variation in pollen production within the same head or the same flower was observed in triploid *H. villosum* (Urbanska 1991). Slade and Rich (2007) reported that cultivated plants of some polyploid taxa produced pollen more often than wild plants, suggesting that environmental factors might have an influence on pollen production in apomictic *Hieracium* species.

Pollen production is considered as a significant reproductive cost in plants. For instance, Meirmans et al. (2006) found that male-sterile apomictic dandelions (*Taraxacum* sect. *Ruderalia*) produce more flower heads *per* plant, and thus more seeds, than pollen-producing apomicts. Pollen production thus seems implausible in apomictic *H. alpinum*, because successful production of seeds is completely independent of pollination and fertilisation. Maynard Smith (1978) proposed a non-adaptive hypothesis for the retention of male function, suggesting that apomicts producing pollen are phylogenetically too recent to have accumulated enough mutations for male sterility. Alternatively, male apomicts could be advantageous if they are able to mate with diploids, thus creating new clones or reducing the fitness of co-occurring sexual competitors (Mogie 1992). Although we have no indication of present-day sympatric occurrence of diploids and triploids, such a situation might have been possible in the past, when new triploid clones arose within diploid populations.

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Appendix

Appendix 1. New chromosome counts and estimations of DNA-ploidy level in *Hieracium alpinum* s.str.

Appendix 2. Geographic origin of the plants of *Hieracium alpinum* L. used for pollen observations.

This Appendix can be downloaded freely from http://www.birkhauser.ch/BH.

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Cytogeography of *Pilosella officinarum* (Compositae): Altitudinal and Longitudinal Differences in Ploidy Level Distribution in the Czech Republic and Slovakia and the General Pattern in Europe

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• *Background and Aims Pilosella officinarum* (syn. *Hieracium pilosella*) is a highly structured species with respect to the ploidy level, with obvious cytogeographic trends. Previous non-collated data indicated a possible differentiation in the frequency of particular ploidy levels in the Czech Republic and Slovakia. Therefore, detailed sampling and ploidy level analyses were assessed to reveal a boundary of common occurrence of tetraploids on one hand and higher ploids on the other. For a better understanding of cytogeographic differentiation of *P. officinarum* in central Europe, a search was made for a general cytogeographic pattern in Europe based on published data.
• *Methods* DNA-ploidy level and/or chromosome number were identified for 1059 plants using flow cytometry and/or chromosome counting on root meristem preparations. Samples were collected from 336 localities in the Czech Republic, Slovakia and north-eastern Hungary. In addition, ploidy levels were determined for plants from 18 localities in Bulgaria, Georgia, Ireland, Italy, Romania and Ukraine.
• *Key Results* Four ploidy levels were found in the studied area with a contrasting pattern of distribution. The most widespread cytotype in the western part of the Czech Republic is tetraploid ($4x$) reproducing sexually, while the apomictic pentaploids and mostly apomictic hexaploids ($5x$ and $6x$, respectively) clearly prevail in Slovakia and the eastern part of the Czech Republic. The boundary between common occurrence of tetraploids and higher ploids is very obvious and represents the geomorphologic boundary between the Bohemian Massif and the Western Carpathians with the adjacent part of Pannonia. Mixed populations consisting of two different ploidy levels were recorded in nearly 11% of localities. A statistically significant difference in a vertical distribution of penta- and hexaploids was observed in the Western Carpathians and the adjacent Pannonian Plain. Hexaploid populations tend to occur at lower elevations (usually below 500 m), while the pentaploid level is more or less evenly distributed up to 1000 m a.s.l. For the first time the heptaploid level ($7x$) was found on one site in Slovakia. In Europe, the sexual tetraploid level has clearly a sub-Atlantic character of distribution. The plants of higher ploidy level (penta- and hexa-) with mostly apomictic reproduction prevail in the northern part of Scandinavia and the British Isles, the Alps and the Western Carpathians with the adjacent part of Pannonia. A detailed overview of published data shows that extremely rare records on existence of diploid populations in the south-west Alps are with high probability erroneous and most probably refer to the closely related diploid species *P. peleteriana*.
• *Conclusions* The recent distribution of *P. officinarum* in Europe is complex and probably reflects the climatic changes during the Pleistocene and consequent postglacial migrations. Probably both penta- and hexaploids arose independently in central Europe (Alps and Carpathian Mountains) and in northern Europe (Scandinavia, Great Britain, Ireland), where the apomictic plants colonized deglaciated areas. We suggest that *P. officinarum* is in fact an amphidiploid species with a basic tetraploid level, which probably originated from hybridizations of diploid taxa from the section *Pilosellina*.

Key words: Amphidiploidy, apomixis, Asteraceae, flow cytometry, geographical parthenogenesis, *Hieracium*, postglacial migration, polyploidy.

INTRODUCTION

The genus *Pilosella* Hill., often treated as a subgenus of *Hieracium* L. [syn. *Hieracium* subgen. *Pilosella* (Hill) Gray], is one of the taxonomically most intricate vascular plant groups of the temperate flora. The reticulate pattern of morphological variation reflected in several thousands of taxa described from the species level to the form (Zahn, 1921–1923) complicates taxonomic treatement. Widespread polyploidy, various modes of reproduction (sexuality, obligate and facultative apomixis of aposporous type, haploid parthenogenesis, vegetative propagation), inter- and intraspecific hybridization within the same and across different ploidy levels are the most important processes involved in microevolution of the genus (Krahulcová *et al.*, 2000). The ploidy level occurring in natural populations varies from diploid ($2n = 2x = 18$) to octoploid ($2n = 8x = 72$). The most frequent cytotypes are tetraploids, pentaploids and hexaploids. Diploids are less frequent, and triploids, heptaploids, octoploids and aneuploids are rather rare (Schuhwerk and Lippert, 1997;

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Krahulcová *et al.*, 2000). Higher ploidy levels up to dodecaploid ($2n = 12x = 108$) were found in plants obtained by experimental hybridization (Skalińska, 1976). In approximately half of the taxa of the genus *Pilosella* analysed more than one ploidy level was found, even in plants growing together in one locality (Schuhwerk, 1996 and references therein).

Sell and West (1976) recognize 63 'numbered' species (including nothospecies) of *Pilosella* (treated as subgenus of *Hieracium*) in Europe. Six species having only one capitulum per stem (with the exception of hybrids) are members of the section *Pilosellina* Zahn. All but one are diploids occurring mainly in western, southern and central parts of Europe: *P. argyrocoma* (Fries) (southern and central Spain), *P. castellana* (Boiss. & Reuter) F. W. Schultz & Sch. Bip. (Spain and northern Portugal), *P. hoppeana* (Schult.) F. W. Schultz & Sch. Bip. (central and eastern Alps), *P. pseudopilosella* (Ten.) F. W. Schultz & Sch. Bip. (southern Europe, from Portugal and Spain to Bulgaria, Turkey and Romania), *P. peleteriana* (Mérat) F. W. Schultz & Sch. Bip. (northern and western Europe and the western part of central Europe). In addition, some authors distinguish a lowland form of *P. hoppeana* as a distinct diploid taxon – *P. macrantha* (Ten.) F. W. Schultz & Sch. Bip. (central and southern Europe) (e.g. Holub, 1986; Chrtek, 1998, 2002; Gottschlich, 1998; Schuhwerk and Fischer, 2003). The only almost exclusively polyploid species of section *Pilosellina* is *Pilosella officinarum* F. W. Schultz & Sch. Bip. (syn. *Hieracium pilosella* L.). It is distributed much wider than the diploid taxa mentioned above (cf. Hultén and Fries, 1986; Bräutigam, 1992). It extends from the British Isles across the whole of Europe, except the Arctic parts, to western Asia and north-western Siberia. Moreover, it has been introduced into New Zealand, North America and Patagonia, and has become an invasive and troublesome weed (Hultén and Fries, 1986; Chapman *et al.*, 2000; Cárdenas Vergara, 2005; Wilson *et al.*, 2006). *Pillosella officinarum* usually grows on dry, permeable and nutrient-poor soils from sea level to the sub-Alpine belt. The obligate heliofilous species occurs in tussock grassland communities usually with regular disturbance (grazing, mowing). Due to its low competitive ability, it tends to establish itself on open, sparsely vegetated, sites (roadside dykes, eroded slopes, landslides, etc.). Morphologically, *P. officinarum* may be distinguished from other representatives of *Pilosellina* section by long and slender stolons bearing small distant leaves decreasing in size towards the apex, involucral bracts (0·5–)1–2 mm wide, covered by stellate, glandular and eglandular trichomes (Sell and West, 1976). Despite its more or less easy identification in the field, a great phenotypic plasticity has been recorded within the species (Turesson and Turesson, 1960; Gadella, 1987, 1991). A high level of morphological variation is reflected in numerous infraspecific taxa described from the entire distribution range (Nägeli and Peter, 1885; Zahn, 1921–1923). The correlation between some phenotypic characters on one hand (e.g. rosette size, the number and length of stolons) and ploidy level on the other was revealed by Gadella (1991). In total, five cytotypes (2*x*, 4*x*, 5*x*, 6*x* and 7*x*) have been recorded in natural populations of *P. officinarum* (cf. Table 1). The mode of seed reproduction of each particular cytotype depends on the ploidy level. Diploids are sexual. Tetraploid plants reproduce sexually, but several apomictic populations deviate from this general rule (Gadella, 1984, 1987). Pentaploid *P. officinarum* is almost exclusively

TABLE 1. *Ploidy levels found in* Pilosella officinarum *in Europe according to the literature and present records*

Country	Ploidy level	References
Armenia	4*x*	Nazarova and Ghukasyan, 2004
Austria	4*x*, 5*x*, 6*x*	Turesson and Turesson, 1960; Gadella, 1972, 1984; Schuhwerk and Lippert, 1997
Belarus	4*x*, 5*x*	Dmitrieva, 1987; Parfenov and Dmitrieva, 1988
Belgium	4*x*	Gadella, 1972, 1984
Bulgaria	4*x*, 5*x*, 6*x*	Mráz *et al.*, this study
Croatia	6*x*	Gadella, 1984
Czech Republic	4*x*, 5*x*, 6*x*, 7*x*	Měsíček and Jarolímová, 1992; Krahulcová and Krahulec, 1999; Krahulcová *et al.*, 2001; Rotreklová *et al.*, 2002, 2005; Košťálová, 2004; Mráz *et al.*, this study
Denmark	4*x*	Turesson and Turesson, 1960; Gadella, 1972, 1984
Finland	4*x*, 5*x*	Turesson and Turesson, 1960; Jalas and Pellinen, 1985
France	2*x*, 4*x*, 5*x*, 6*x*	Delcourt, 1972; Auquier and Renard, 1979; Natarajan, 1981, 1988; Gadella, 1972, 1984
Georgia	4*x*, 5*x*	Mráz *et al.*, this study
Germany	4*x*	Turesson and Turesson, 1960; Gadella, 1972; Bräutigam and Bräutigam, 1996; Schuhwerk and Lippert, 1997, 2002; Albers and Pröbsting, 1998; Rotreklová *et al.*, 2005
Hungary	5*x*, 6*x*	Mráz *et al.*, this study
Ireland	4*x*, 5*x*, 6*x*	Gadella, 1972, 1984; Finch, 2005; Watson, 2005; Mráz *et al.*, this study
Italy	2*x*, 4*x*, 5*x*, 6*x*	Gadella, 1972, 1984; Mráz *et al.*, this study
Luxembourg	4*x*	Gadella, 1972, 1984
Macedonia	6*x*	Gadella, 1972
Netherlands	4*x*, 5*x*, 7*x*	Gadella and Kliphuis, 1963; Gadella, 1972, 1984
Norway	4*x*, 5*x*	Gadella, 1972
Poland	4*x*, 5*x*, 6*x*	Skalińska, 1967; Skalińska *et al.*, 1971; Gadella, 1972; Pogan *et al.*, 1987; Pogan and Wcisło, 1989; Rotreklová *et al.*, 2005
Portugal	4*x*	Fernades and Queirós, 1971; Gadella, 1972
Romania	5*x*, 6*x*	Gadella, 1972; Mráz *et al.*, this study
Russia	6*x*	Lavrenko and Sereditov, 1991
Slovakia	4*x*, 5*x*, 6*x*, 7*x*	Májovský *et al.*, 1970; Uhríková and Feráková, 1977; Mičieta, 1982; Murín, 1986; Píšťanský and Mičieta, 2000; Rotreklová *et al.*, 2002, 2005; Mráz *et al.*, this study
Spain	4*x*	Gadella, 1984
Sweden	4*x*, 5*x*, 6*x*, 7*x*	Turesson and Turesson, 1960; Turesson, 1972; Lövkvist and Hultgård, 1999
Switzerland	4*x*, 5*x*, 6*x*	Gadella, 1972, 1984
United Kingdom	4*x*, 5*x*, 6*x*	Turesson and Turesson, 1960; Gadella, 1972, 1984; Morton, 1974; Moore, 1982; Edmonds *et al.*, 2005; Finch, 2005; Grime *et al.*, 2005; Watson, 2005
Ukraine	4*x*, 6*x*	Pashuk, 1987; Mráz *et al.*, this study

apomictic, although a rare sexual seed production was also reported (Turesson and Turesson, 1960; Turesson, 1972; Gadella, 1984). Facultative apomixis in pentaploids was later confirmed embryologically by Pogan and Wcisło (1995). Recently, two accessions of fully sexual pentaploids have been found in the Czech Republic (Krahulcová *et al.*, 2000; Rotreklová *et al.*, 2002). Hexaploids are either sexual or apomictic, while very rare heptaploids are either apomictic or sterile (Gadella, 1984, 1991). Vegetative reproduction by means of over-ground stolons is common for all cytotypes and, together with apomixis, it might contribute to the uniclonal structure of populations.

Diploid plants of *P. officinarum* are rare and their distribution is considered to be of a relict character (e.g. Gadella, 1984). They were reported from the Valley of Aosta (Italy) (Gadella, 1972) and south-eastern France (Delcourt, 1972), respectively (but see the Discussion below). In most of Europe, the tetraploid and pentaploid populations of *P. officinarum* are by far the most common cytotypes (Gadella, 1984). Tetraploids are widespread in the lowlands of west and central Europe (e.g. Turesson and Turesson, 1960; Gadella, 1972, 1984; Pogan and Wcisło, 1989; Schuhwerk and Lippert, 1997, 2002; Krahulcová and Krahulec, 1999), while the pentaploids occur chiefly in regions that were covered by the Pleistocene glaciation – Scandinavia, the British Isles (Turesson and Turesson, 1960; Gadella, 1972, 1984, 1987; Finch, 2005; Watson, 2005). Several hexaploid populations of *P. officinarum* were found mainly in the Alps, Scandinavia, Balkan Peninsula (e.g. Turesson and Turesson, 1960, Gadella, 1972, 1984, 1991; Lavrenko and Sereditov, 1991; Schuhwerk and Lippert, 1997) and the Western Carpathians (see below). The rare occurrence of heptaploids was reported from only three localities in Sweden (Turesson and Turesson, 1960), one site in the Netherlands (Gadella, 1984) and one population in the Czech Republic (Košťálová, 2004).

Four ploidy levels (tetra-, penta-, hexa- and heptaploid) have been recorded in the Czech Republic and Slovakia (Májovský *et al.*, 1970; Uhríková and Feráková, 1977; Mičieta, 1982; Murín, 1986; Měsíček and Jarolímová, 1992; Krahulcová and Krahulec, 1999; Píšťanský and Mičieta, 2000; Krahulcová *et al.*, 2001; Rotreklová *et al.*, 2002, 2005; Košťálová, 2004). Recently, Píšťanský and Mičieta (2000) recorded tetraploids in approx. 30 localities mainly in southern and western Slovakia, while other authors reported pentaploid and hexaploid plants mostly from eastern, northern and central Slovakia. Most of the chromosome counts coming from the Czech Republic that had been published indicated that the plants analysed were tetraploids.

Almost all published data on ploidy level of *P. officinarum* are based on classical chromosome counting. This precise method is, however, considerably time-consuming. Since routine introduction of the flow cytometry in plant science in the nineties of the last century (Doležal, 1991), this approach has rapidly became popular for estimating DNA-ploidy level (Doležel, 1991). This is mainly due to the very easy sample preparation and the possibility of screening large numbers of individuals in a very short time. Here, the search which was carried out for a boundary between the area of distribution of the tetraploid cytotype and the range of penta- and hexaploids of *P. officinarum* in the territory of Slovakia and the Czech Republic, using mostly a flow cytometric approach, is reported. Moreover, an attempt was made to find out if there was a correlation between the distribution of particular ploidy levels on one hand and the altitude on the other. To understand better the cytogeographic differentiation of *P. officinarum* in central Europe, a search, based on published data, was made for a general cytogeographic pattern in Europe.

The area studied

Research has been carried out in the area of the Czech and Slovak Republics with an adjacent part of north-eastern Hungary. The area studied belongs to two different biogeographic regions, the mountain range of the Western Carpathians and the Bohemian Massif. The border between both regions is situated in the eastern part of the Czech Republic, lying north-north-east to south-south west. These two regions differ in a variety of environmental and historical parameters. In this respect, differences in the cytotype distribution cannot be explained in any easy way. On the other hand, this area covering their border can show that the pattern in cytotype distribution can be very contrasting even across a very narrow zone.

The Bohemian Massif has an old Paleogenic relief, younger areas being only canyons, those areas with Tertiary volcanism in the northern part of Bohemia, and glacial cirques in the Sudetes and the Šumava Mountains. The highest point is Mt Sněžka (1602 m), the lowest is the valley of the River Elbe on the German border at 115 m. Mostly acid Varisian parts were later covered with Permian-Carboniferous or Mesozoic sediments. Base-rich bedrocks are concentrated at lower altitudes. Vegetation cover has a coarser grain (homogeneous on a larger scale) in comparison with the Carpathian Mountains.

The Western Carpathians, including the Intra-Carpathian (Pannonian) Basin, represent the north-west part of the Carpathian arc extending from north-east Austria and south-east Czech Republic to north-east Slovakia and south-east Poland. The relief is young, of Tertiary age, similar to the Alps. The highest point is Gerlach Peak (2655 m). The bedrock is more complicated, mostly of Mesozoic and Tertiary ages. Calcium-rich substrates occur from lowland to the high mountains; e.g. in central Slovakia almost consistant limestone substrates can be found from the xerothermic Slovak Karst to the highest altitudes of the Belaer Tatra with altitudes above 2000 m. Some areas are very continental, with climatic conditions which do not allow the growth of *Fagus sylvatica* as in the area between the High and Low Tatra Mountains. On the other hand, some not distant areas are more oceanic, as in north-west Slovakia. For all these reasons, the vegetation cover is fine-grained (homogeneous in small areas but, on a larger scale, heterogeneous). Large regions with homogeneous vegetation are rare.

The area of the Czech Republic has a rather uniform climate; the warmest month is July and it is also the

month of highest rainfall. This contrasts with Slovakia, where the same condition applies only at higher altitudes. At lower altitudes, the warmest month is also July, but the highest rainfall is distributed from May to September, depending on the exact geographic position (Vesecký, 1961). In this way, the same area is rather oceanic in May–June and more continental in September and vice versa. Slovakia (the Carpathian Mountains with the Pannonian Plain) is therefore fine-grained and more diverse with respect to relief, bedrock and climate.

MATERIALS AND METHODS

Material collection

Plants of *Pilosella officinarum* F. W. Schultz & Sch. Bip. (syn. *Hieracium pilosella* L.) for the present study were collected in 2003–2006 in their natural habitats throughout Slovakia and the Czech Republic, to a lesser extent also in the north-eastern part of Hungary to cover all geographic regions. They were cultivated in pots in the Botanical Garden of P. J. Šafárik University, Košice and in the experimental field of the Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice. For the complete list of localities see Supplementary Information 1 (available online). Besides the plants from the region above mentioned, some plants from a further 18 localities from different parts of Europe have also been analysed (Supplementary Information 4, available online).

As a rule, three or five plants from each population were sampled, three from the pure populations, five in the case of co-occurrence of other potentially hybridizing species of the genus *Pilosella*. Efforts were made to avoid collecting samples originating from one clone. If it was apparent that plants at the collecting site did originate from one plant, they belonged to one clone (usually plants growing very close together in a very small area of several square cm), only one individual plant *per* locality (population) was dug up. In some cases however, several cultivated plants died before analysis. For both these reasons, some populations are represented by only one plant. To determine the proportion of mixed cytotypes in populations, only those populations with two and more plants analysed were involved. Despite the fact that the ploidy level of only one plant had been estimated by us, some localities (marked in Supplementary Information 1, available online) can be considered as collecting sites with two or more analysed plants because the chromosome number of other plant/plants from the same locality was published earlier (see Supplementary Information 2, available online). Therefore, in addition to the data collected for this research, a few previous accounts from the literature (Rotreklová *et al.*, 2005) were used to search for some localities consisting of two different ploidy levels. These plants were not included in the total number of plants analysed in this present study. The voucher specimens have been deposited recently in the herbarium of Patrik Mráz, at the Institute of Biology and Ecology, P. J. Šafárik University, Košice and in the herbarium of the Institute of Botany, Průhonice (PRA).

Chromosome counts

The chromosome counts are based on the somatic mitosis in the root-tip cuttings of pot-cultivated plants. The material was pre-treated at room temperature with a 0·5% solution of colchicine for 1·5–3 h and then fixed in a cold mixture of ethanol and acetic acid (3 : 1) for at least 1 h. The fixed material was stored in 70 % ethanol at 4 °C until processed. The root tips were macerated in 1 N HCl at 60 °C for 7–10 min. The squash and smear method with cellophane replacing the glass covers (Murín, 1960) and with Giemsa solution in a phosphate buffer was used. Selected permanent slides are deposited at the Institute of Biology and Ecology, P. J. Šafárik University in Košice.

Estimation of ploidy level

Flow cytometry was used to detect the DNA-ploidy level (Suda *et al.*, 2006) for most of the plants. An analysis of relative DNA content was performed with a PA II ploidy analyser (Partec GmbH, Münster, Germany) equipped with an HBO-100 mercury arc lamp in the Flow Cytometry Laboratory, Institute of Botany, Academy of Sciences, Průhonice, Czech Republic and FACSCalibur instrument (Becton Dickinson, USA) equipped with an argon-ion laser excitation at 488 nm in the Flow Cytometry Laboratory, Institute of Biology and Ecology, P. J. Šafárik University, Košice. Sample preparations were carried out in a two-step procedure (Otto, 1990; Doležel and Göhde, 1995). Approximately 1 cm^2 of leaf tissues from both the sample and the reference internal standard were ground together for about 30 s in a Petri dish containing 1 ml of ice-cold Otto I buffer (4·2 g citric acid monohydrate + 1 mL 0·5 % Tween 20 adjusted to 200 mL and filtered through a 42-μm filter). Filtration through a 42-μm nylon mesh was followed by centrifugation at 150 *g* for 5 min. The supernatant was removed and 100 μL of fresh Otto I buffer was added. The nuclei in the pellet were resuspended and stored for 30 min at room temperature for incubation. For DNA staining 1 mL of Otto II buffer (0·4 M disodium hydrogenphosphate dodekahydrate) including 50 μL of propidium iodide, 50 μL ribonuclease, 2 μL mercaptoethanol (FACSCalibur, Becton Dickinson) or DAPI (4′,6-diamidino-2-phenylindole) at a concentration of 4 μg ml^{-1} (PA II flow cytometer, Partec GmbH) was used. The clones of previously cytologically studied diploid ($2n = 2x = 18$) plants of *Pilosella lactucella* (Wallr.) P. D. Sell & C. West (Rotreklová *et al.*, 2002, 2005) were used as an internal reference standard for the relative DNA content measurements. Moreover, one tetraploid and several pentaploid and hexaploid plants of *P. officinarum* with known chromosome numbers were used in separate and mixed flow cytometry analysis to determine the exact position of peaks of known polyploids in relation to the diploid standard peak (Fig. 1). Histograms were accumulated at a flow rate of about 20–50 particles per second for a total count of 3000–5000 nuclei. The resulting values were expressed as a peak ratio, which is a ratio of the mean position of the G_0/G_1 peak in the DNA histogram of the tested plant

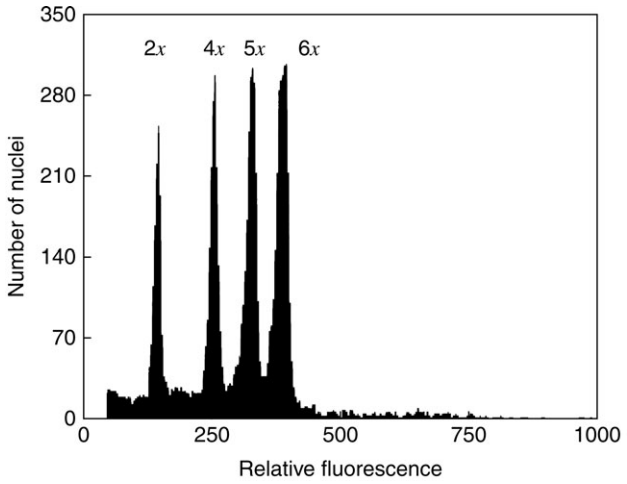

FIG. 1. Histogram of relative DNA content of DAPI-stained nuclei from a diploid plant of *Pilosella lactucella* (2*x*) used as a reference plant, with tetraploid (4*x*), pentaploid (5*x*) and hexaploid (6*x*) plants of *Pilosella officinarum*.

to the mean position of the G_0/G_1 peak in the histogram of the reference plant.

Maps

The distribution maps of cytotypes/ploidy levels in the Czech Republic and Slovakia are based on the co-ordinates determined by a GPS receiver, or found *ex post facto* from the tourist maps at a scale of 1 : 50 000 (usually old literature data). For most references from Europe for which the appropriate geographical co-ordinates were not given in original sources, the geographical position of collecting sites was estimated using *Microsoft Encarta World Atlas* (1998 Edition) and GeoNet Name Server (http://gnswww.nga.mil/geonames/GNS/index.jsp; accessed in December 2005). However, for approx. 10 % of references, estimation of co-ordinates failed (marked by an asterisk in Supplementary Information 3, available online) usually due to the absence of the name of the nearest village/town, or the existence of two or more villages/towns with the same name. Most of the chromosome numbers of the plants from the British Isles were obtained from the online version of Cytological database of the Botanical Society of the British Isles (accessed in February 2005). Distributional maps were prepared using distribution mapping software DMAP (Morton, 2004).

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's pairwise comparison (using Minitab for Windows Release 11) were applied to determine the significance ($P < 0{\cdot}05$) of the difference in the altitudinal distribution between pentaploid, hexaploid and mixed populations in the West Carpathians and adjacent Pannonia (Slovakia and the eastern part of the Czech Republic).

RESULTS

Ploidy level distribution in the Czech Republic and Slovakia

The DNA-ploidy levels and/or chromosome numbers were detected for 1059 plants sampled at 336 localities throughout the Czech Republic and Slovakia. Some plants were sampled also in north-eastern Hungary, along the Slovak–Hungarian state border. In total, 1055 plants were analysed by flow cytometric analysis. For eight plants the ploidy level was found using two approaches – by classical counting and by flow cytometry – while another four plants were counted only (cf. Supplementary Information 1, available online).

Altogether, four ploidy levels, tetra-, penta-, hexa- and heptaploid, were revealed in the area on which the study focused. The tetraploid level (4*x*; altogether 426 plants which represent 40·2 % of all plants analysed) was found to be the most common, followed by pentaploid (5*x*; 389 plants, 36·7 %) and hexaploid (6*x*; 241 plants, 22·8 %). Three heptaploid plants (7*x*) were discovered in a mixed population with one pentaploid plant at only one site in western Slovakia (Fig. 2). The record of heptaploid ploidy level is the first for *P. officinarum* in the territory of the Western Carpathians. The effort made to determine the chromosome number of heptaploid plants was not successful (the plants died), thus the new ploidy level should be considered merely as a DNA-ploidy level, i.e. not based on an exact chromosome count. Estimations of ploidy levels given for the plants from the Hungarian part of the Western Carpathians are the first records of ploidy level for *P. officinarum* for this area. In 32 localities out of 302 (10·6 %), from which at least two plants were analysed, mixed populations consisting of two different ploidies were found.

The distribution of ploidy levels in the Czech Republic is not proportional to that in Slovakia. While tetraploids are the most widespread in the Czech Republic, specifically in its western part, penta- and hexaploids predominated in Slovakia and in the eastern part of the Czech Republic (Fig. 2). The boundary between a common occurrence of the tetraploid cytotype and higher ploids is very conspicuous and corresponds well with the natural geological and geomorphological boundary between the Bohemian Massif and the Western Carpathians with the adjacent Pannonian Plain (Král, 1999). If the proportion of the particular ploidy level for each geographic region is taken into account separately, i.e. the Bohemian Massif on one hand and the Western Carpathians with Pannonia on the other, then the differences are very striking (Fig. 3).

Apart from latitudinal differentiation in ploidy level distribution in the Czech Republic and Slovakia, a statistically significant difference was also found between the proportion of pentaploids and hexaploids across the altitudes in the territory of the Western Carpathians and adjacent Pannonia (Slovakia, north-east Hungary and the eastern part of the Czech Republic) (Table 2). Generally, pure hexaploid populations tend to occur at lower elevations (usually below 500 m), while the pentaploids are very common above 500 m a.s.l. Mixed populations consisting

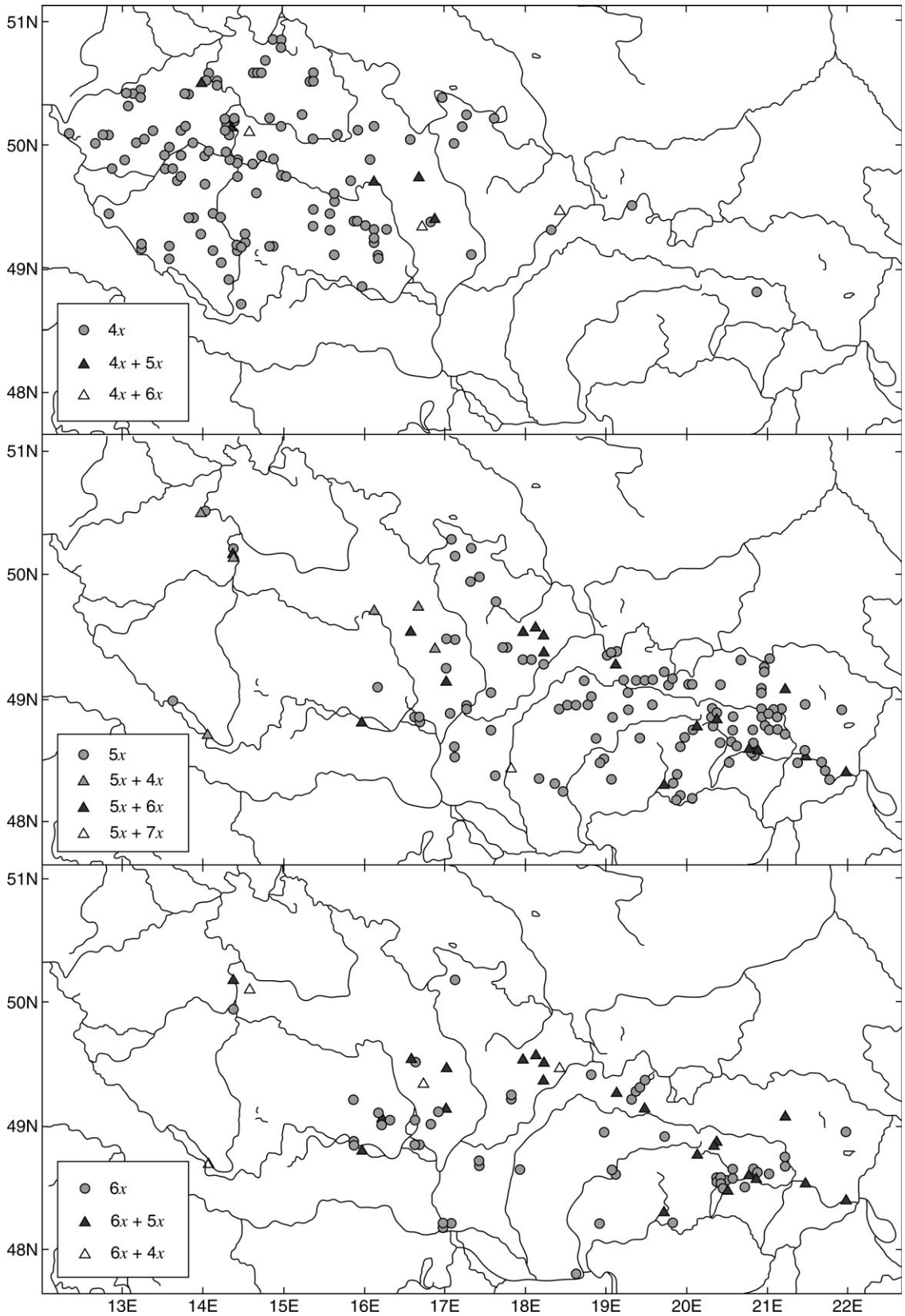

FIG. 2. Distribution of ploidy levels of *Pilosella officinarum* in the Czech Republic, Slovakia and north-east part of Hungary based on present data.

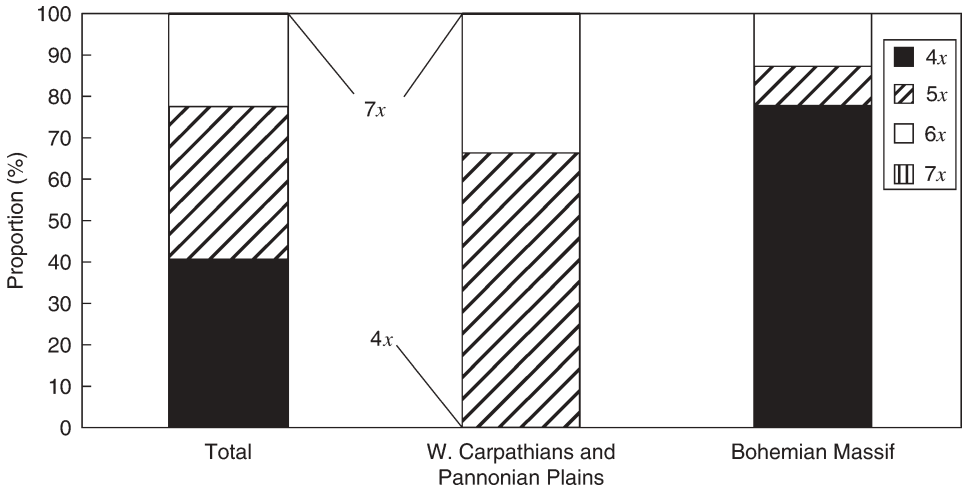

FIG. 3. Proportions of the ploidy level of *Pilosella officinarum* in the Czech Republic, north-east Hungary and Slovakia based on present data, expressed as a portion of plants of a particular ploidy level compared with the total plants analysed. The proportions over the whole area studied are given (total), together with the proportions found in two different geomorphological regions – the Bohemian Massif and the Western Carpathians with the adjacent part of Pannonia.

of two different ploidy levels were found relatively evenly along the altitudinal gradient up to 1000 m (Fig. 4).

General pattern of ploidy level distribution in Europe

Pilosella officinarum is the European taxon most examined by karyology. Chromosome numbers were counted in plants originating from 655 localities, excluding present data, across the whole continent (refer to Supplementary Information 3, available online, and Table 1). It was possible to localize geographically nearly 600 sites (Fig. 5). The most common cytotype, tetraploid (without present data), was reported in 284 localities (43 %), followed by pentaploid found at 257 collecting sites (40 %). The hexaploid ploidy level is obviously rarer, i.e. detected in 74 cases (11 %). Mixed populations consisting of two or more different cytotypes were found on 40 sites (6 %). However, in most publications the number of plants analysed per population was not given and therefore the proportion of mixed populations would be probably higher if only populations with at least two or more analysed plants per locality were taken in consideration. Tetraploids are distributed mostly in western Europe and the western part of central Europe, being the only cytotype detected in Denmark and Germany. It prevails considerably in the Netherlands (82 %), Poland (71 %) and France (65 %). The pentaploids have two main centres of distribution: at high latitudes in northern Europe (Sweden, 70 %; the British Isles, 64 %) and in major orophytic systems in Europe – the Alps (30 %) and the Carpathian Mountains with the adjacent part of Pannonia (present data for the Western Carpathians indicate 66 % of pentaploids). The predominant ploidy level in the Alps is hexaploid (59 %, in Switzerland even 84 %). The records on diploids and heptaploids are extremely scarce. The former

TABLE 2. *Means and standard deviations of altitudes of pure pentaploid (5x), pure hexaploid (6x) and mixed populations (5x + 6x) of* Pilosella officinarum *in the Western Carpathians and adjacent part of Pannonia*

Ploidy level	*N*	$X \pm$ s.d.
5*x*	86	546 ± 292^{a}
6*x*	40	370 ± 189^{b}
5*x* + 6*x*	21	513 ± 253^{ab}

Only populations with two or more analysed plants were included. The tetraploids and heptaploids were, according to their comparatively low abundance, excluded from this analysis. Altitudinal ranges and means are given in metres above sea level. *N*, Total number of populations; *X*, mean; s.d., standard deviation. Means in columns sharing the same superscript letters are not significantly different (Tukey's pairwaise comparisions, $P = 0{\cdot}003$, $F = 6{\cdot}19$).

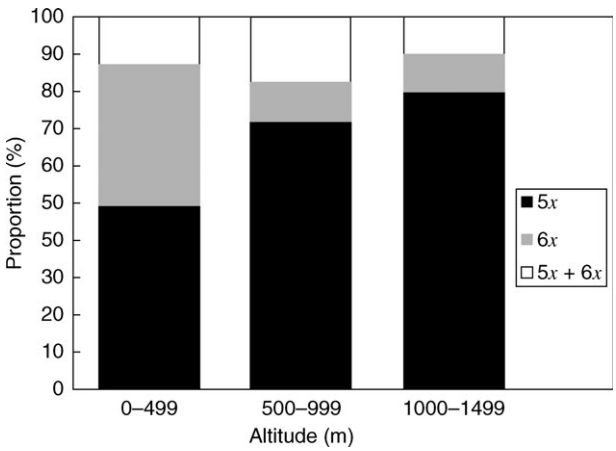

FIG. 4. Proportions of pure pentaploid (5*x*), pure hexaploid (6*x*) and mixed populations (5*x* + 6*x*) of *Pilosella officinarum* found at different altitudinal ranges in the Western Carpathians and the adjacent part of Pannonia, expressed as a number of populations of the particular ploidy level compared with the total number of populations analysed in the particular altitudinal range.

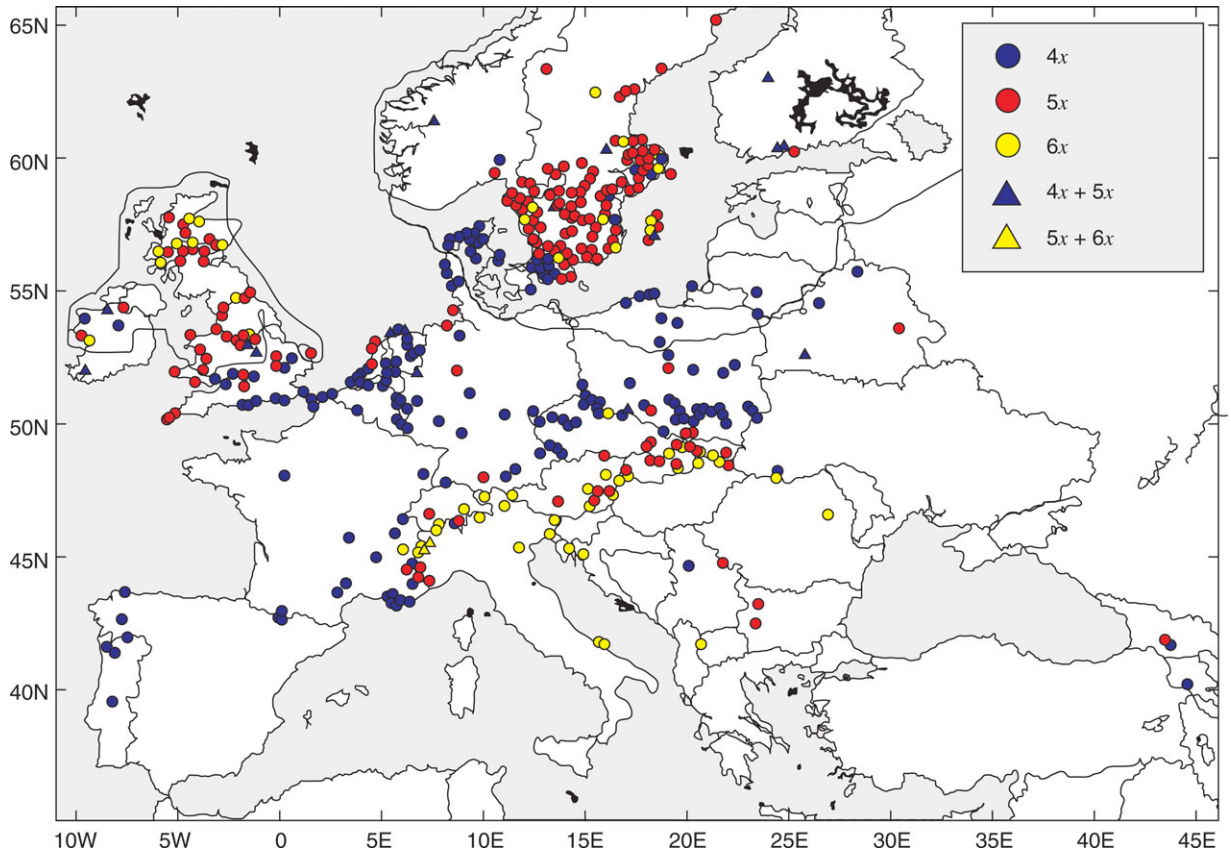

FIG. 5. Cytotype distribution of *Pilosella officinarum* in Europe (based on data from Supplementary Information 2–4, available online; present data from the Czech Republic, Slovakia and Hungary are not included). The rare heptaploid level and rare mixed cytotype combinations are not given in the map. The bold black line indicates the boundary of Last Glacial Maximum in northern Europe according to Adams (1997).

were found only in the south-west Alps (but see Discussion), the latter mostly in northern and central Europe (Sweden, Netherlands and the Czech Republic; heptaploids are not included in Fig. 5). The new data from 18 European localities confirm this general pattern: Bulgaria 4*x*, 5*x* and 6*x*; Georgia 4*x* and 5*x*; Ireland 5*x* and 6*x*; Italy 6*x*; Romania 5*x*, 6*x* and mixed $5x + 6x$; Ukraine 6*x* (cf. Table 1 and Supplementary Information 4, available online).

DISCUSSION

Ploidy level distribution in the Czech Republic and Slovakia

According to the results of the present research, the tetraploids strongly prevail in the western part of the Czech Republic, while the pentaploids and hexaploids represent two main cytotypes in Slovakia and the eastern part of the Czech Republic. This corresponds well with scattered data published in previous studies, with the exception of the tetraploids. Our results contradict the data published by Píšťanský and Mičieta (2000), who reported a significant predominance of tetraploid populations in the Western Carpathians, but only a rare occurrence of penta- and hexaploid plants (cf. Supplementary Information 2, available online). As preliminary results did not confirm any common occurrence of tetraploids in Slovakia (Šingliarová and Mráz, 2004), in 2004 five localities were visited from which the tetraploids were published by Píšťanský and Mičieta (2000). However, no tetraploid was detected in any of them. Based on these observations, all data of Píšťanský and Mičieta (2000) were considered as dubious.

The present study revealed a new heptaploid ploidy level in *P. officinarum* in the territory of the Western Carpathians. So far, the heptaploids had been detected only in three localities in Sweden (Turesson and Turesson, 1960), in one site in the Netherlands (Gadella, 1984) and one plant in a population of hexaploid plants near Prague in the Czech Republic (Košťálová, 2004). In the Western Carpathians locality, from four analysed plants three were heptaploid and only one pentaploid. Although pentaploids reproduce the most often via aposporic apomixis, there are some data on facultative apomixis (Turesson and Turesson, 1960; Turesson, 1972) or even full sexuality (Krahulcová *et al.*, 2000; Rotreklová *et al.*, 2002). Moreover, apomictic pentaploids usually produce 2*x* to 3*x* pollen grains (Gadella, 1987; Krahulcová and Krahulec, 2000). A possible explanation of an increased ploidy level may be the fusion of reduced and unreduced gametes, as was suggested in the case of a large Dutch heptaploid population situated between two localities – the first occupied by tetra- and the second by pentaploid plants (Gadella, 1988).

A sympatry of two ploidy levels within one population was confirmed in nearly 11 % of populations. The presence of cytotype mixtures is pronounced especially in the Western Carpathians (16·4 %), whereas in the Bohemian Massif a co-occurrence of different ploidy levels is rarer (5·9 %) and confined only to the warmest regions in relict river valleys or in the zone adjacent to the Western Carpathians and the Pannonian Plain. Higher numbers of mixed populations in the Western Carpathians might be explained by a high presence of two different ploidy levels (5*x* and 6*x*) in this territory. However, this is not the case for the Bohemian Massif which has only one completely dominating tetraploid cytotype. Whether the presence of mixted cytotypes is mainly due to the more or less stochastic co-existence of different clones with different ploidy levels or to the local formation from one dominant ploidy level is yet unknown. Undoubtedly, the production of fully or partially reduced or unreduced gametes and gene flow between plants in the locality may contribute to the presence of a cytotype mixture in populations (cf. Krahulcová *et al.*, 2000). Mixed cytotype populations were found previously also in other parts of Europe in 37 localities (cf. Supplementary Information 3, available online), as well as in the Western Carpathians (Skalińska, 1967).

Surprisingly, the boundary between a common occurrence of tetraploids and higher ploids of *P. officinaum* is rather sharp and corresponds well to the natural boundary of two geomorphological units: the Western Carpathians with the adjacent Pannonian Plain and the Bohemian Massif. From the cytological point of view, a similar boundary between two cytotypes of *P. bauhini* was recorded by Rotreklová (2004), albeit, with the reverse pattern in comparison with *P. officinarum*. The tetraploids of *P. bauhini* are more frequent in Slovakia and Hungary and rare in the Czech Republic, Poland or Germany. On the other hand, pentaploid populations prevail in the Czech Republic and Germany. The border between Hercynian (including the Bohemian Massif) and Carpathian regions seems to be an important biogeographic boundary in central Europe as is also seen in the distribution patterns of many plant species. There is a whole set of species reaching this border from the east, from the Carpathian Mountains, e.g. *Cardamine glandulosa, Dianthus latifolius, Euphorbia serrulata, Galium rivale, Luzula luzulina, Salvia glutinosa etc*. (Hendrych, 1987). On the contrary, some species have migrated eastwards but did not enter, or only rarely, the Carpathian Mountains, like *Campanula rotundifolia s.str*. (Kovanda, 1977) and *Cirsium heterophyllum*. Such strong cytological and chorological differentiation between neighbouring geomorphological regions is difficult to explain. Differences in floristic composition suggest that historical processes, such as migration and expansion from different refugial areas using various migration corridors, might have played an important role (see also below). Moreover, the mountainous areas of the Bohemian Massif and the Western Carpathians are separated from each other by the north-west part of the Pannonian Plain (the Intra-Carpathian Basin) and so-called rather narrow Moravia gate, connecting the Pannonian lowland with lowlands of Silesia, Poland. This natural geographic and climatic barrier might have contributed to the different floristic and cytological patterns of these regions.

A significant difference in the proportion of penta- and hexaploid populations across altitude was found within the Western Carpathians and adjacent Pannonia. While pentaploids are more or less evenly present up to 1000 m, the hexaploids usually grow in regions with a warmer climate, usually below 500 m a.s.l. However, there are several regional deviations. Prevailing populations of the pentaploid level are present in the Zemplínske vrchy Mountains (south-east Slovakia) belonging to the warmest region of the Western Carpathians situated in the neighbouring zone with the Pannonian Plain. On the other hand, several hexaploid populations were recorded at a high elevation with a cold and humid climate in the Oravská Magura Mountains. In the Bohemian Massif, rare penta- and hexaploids or mixed populations are confined mostly to the warm, low-elevated, regions, such as river valleys, or to the adjacent zone with the Pannonian Plain (Fig. 2). One hexaploid population was found on the top of the Hrubý Jeseník Mountains (eastern part of the Sudetes range, Czech Republic). It seems that there are at least two hexaploid types in the area studied, differing in distribution and breeding systems (T. Urfus, unpubl. res.). The first one is confined mostly to thermophilous vegetation in the Carpathian Mountains and has an apomictic breeding system; it is probably related to apomictic hexaploids occurring throughout the Carpathian Mountains to the Balkan. The second hexaploid type is confined to relict river valleys in the Bohemian Massif and is sexual; this type is probably related to sexual hexaploids of the Alps (Gadella, 1984).

Amphidiploid origin of Pilosella officinarum*?*

Diploids of *P. officinarum* that are considered to have a relict distribution were found by Delcourt (1972) and by Gadella (1972) only in the south-western Alps. However, 16 records of data from the French Alps published by the former author were doubted later due to mis-identification as a closely related but different diploid species from the section *Pilosellina* – *P. peleteriana* (Gadella, 1984). Nevertheless, two diploid plants of *P. officinarum* counted by Gadella might belong to this species, as it is obvious from the photograph of these plants (Gadella, 1972: 362). These diploids originating from a very widely defined locality ‘the valley of Aosta’ (north-west Italy) have long stolons with decreasing leaf size towards the stolon apex. On the other hand, these plants could also represent the hybrids between true *P. officinarum* and some diploid taxon from *P.* section *Pilosellina*. These questionable data on existence of diploids of *P. officinarum* may suggest that a well-established diploid cytotype within *P. officinarum* does not really exist in nature. Another fact supports this hypothesis: most of the diploid species of *Pilosella* that had been counted up to the present, including the closely related taxa from the section *Pilosellina*, have been found usually in several if not many localities and occupied much wider ranges (e.g. Zahn, 1921–1923; Bräutigam, 1992; Schuhwerk, 1996). Moreover, polyploidy

in diploid taxa of section *Pilosellina* is either unknown or very scattered records of polyploids might be regarded as mis-identifications with *P. officinarum* or interspecific hybrids. The almost exclusive presence of polyploid populations with the tetraploid ones being the commonest leads to the hypothesis that *P. officinarum* is likely to be an amphidiploid species originated from one or more crosses between diploid members of section *Pilosellina*. Both place and time of this hybridization are difficult to estimate. Analysis of ITS sequences showed low differentiation between diploid taxa (Fehrer *et al.*, 2007*a*), which suggests a relatively low age of the particular members of this group. With respect to chloroplast haplotypes, diploid members of the section *Pilosellina* share both main types. *Pilosella hoppeana*, *P. macrantha* and *P. peleteriana* share the haplotype typical of steppe and mountain species such as *P. onegensis*, *P. alpicola*, *P. glacialis, P. echioides*, etc., i.e. those species, which occurred together during the Glacial Period in steppic and tundra-like habitats in the area of central Europe. The other diploids confined to southern Europe (*P. castellana*, *P. argyrocoma* and *P. pseudopilosella*) share the haplotype with *P. lactucella*, *P. vahlii* and *P. breviscapa* (Fehrer *et al.*, 2007*a*). Central European populations of *P. officinarum* exhibit the same haplotype as *P. hoppeana* and *P. macrantha* (Krahulec *et al.*, 2004, Fehrer *et al.*, 2007*a, b*). The close relationship of diploid *P. peleteriana* and *P. officinarum* has been proven by an allozyme pattern observed in the plants originating from Scandinavia (Tyler, 2005). The possible polyphyletic and polytopic origin of polyploid populations of *P. officinarum* is supported by its enormous morphological variation. Zahn, a monographer of the genus, distinguished about 600 subspecies (Zahn, 1921–1923, 1922–1930). Morphologically, the tetraploid plants of *P. officinarum* found recently in Bulgaria resemble hybrids between hexaploid *P. officinarum* and diploid *P. macrantha*. To understand the origin of *P. officinarum* it is necessary to know more about the detailed distribution of haplotypes in the whole distribution area of *P. officinarum* and its diploid relatives. Recently Trewick *et al.* (2004) included several plants from their natural European range of distribution in their study on the origin of the introduction of *P. officinarum* into New Zealand. They found a mixed distribution of two common chloroplast haplotypes with no clear geographic pattern. However, three rare haplotypes were distributed mainly in the Alps, Sudeten Mountains, the Carpathian Mountains and Finland.

General pattern of ploidy level distribution in Europe and its relationship with polyploidy and apomixis

Gadella (1984, 1987, 1991) studied the distribution of particular cytotypes of *P. officinarum* in Europe and tried to explain its pattern. The revision presented in this paper (Fig. 5) showed that this pattern is more complex and fine grained (as was shown in the area of the Slovak and Czech Republics) than that suggested by Gadella. Despite the fact that *P. officinarum* is the most karyologically studied vascular plant species, it is realized that the published data cover only some parts of its natural distribution range sufficiently (western, central and northern Europe). Large areas in southern, south-eastern and eastern Europe have scarce or almost no data.

In total, four different cytotypes of *P. officinarum* were found in Europe (see Table 1 and Fig. 5). The records on diploids are highly questionable (see above). The most common ploidy levels are 4*x*, 5*x* and 6*x*. The range of sexual tetraploid cytotypes clearly separates the higher ploidy levels (5*x* and 6*x*) into two groups occurring in geographically different regions – into northern Europe and the mountains of central and south-east Europe (the Alps and the Carpathian Mountains, mountains in Bulgaria). Such a pattern of ploidy level distribution suggests an independent origin of penta- and hexaploids. Concerning the results from the Czech Republic and Slovakia, it seems that they match the general pattern in central Europe. While the prevailing tetraploid cytotype in the Bohemian Massif shows linkage to the tetraploid populations in the western part of central Europe, penta- and hexaploid populations in the Western Carpathians and the Pannonian Plain are likely to be related to the high ploids found in the Alps and in the Balkans (cf. Fig. 5).

It was hypothesized that the prevailing occurrence of high ploids (5*x* and 6*x*) correlates with either high latitudes or high altitudes and that their common distribution in northern Europe and in the Alps may be the result of the last Pleistocene glaciation (Gadella, 1984, 1987, 1991). The detailed map given in Fig. 5 shows that the tetraploid level is confined to western Europe and the western part of central Europe and that it has a sub-Atlantic distribution character. Northwards, sexual tetraploids are rare or completely missing and they are replaced by apomictically reproducing penta- and hexaploids (cf. Turesson and Turesson, 1960). The boundary of tetraploids and high ploids matches well with the border of the ice-sheet during the Last Glacial Maximum (cf. Adams, 1997) in the British Isles and it is very close to this geographic position in Scandinavia. Interestingly, the same pattern of cytotype distribution was found in *Parnassia palustris* L. (Parnassiaceae), where the boundary between diploids and tetraploids more or less correlates with the limit of Last Glacial Maximum (Gornall and Wentworth, 1993; Borgen and Hultgård, 2003). It therefore seems that the relationship between the presence of high ploid apomictic plants (5*x* and 6*x*) in northern Europe and glaciations might have a real basis and suggests evolutionary advantages of polyploidy associated with apomixis in the colonization of deglaciated areas in Scandinavia (cf. Asker and Jerling 1992). Merxmüller (1975) pointed out that diploid, sexually reproducing taxa of the closely related genera *Hieracium* and *Pilosella* are mostly confined to the southern latitudes, while there was a tendency for polyploids, mostly apomictic species, to prevail in northerly situated regions. Such geographically limited parthenogenesis is known also in other sexual–apomictic genera and was summarized by Bierzychudek (1985) (for thorough recent revision on complex causality of geographical parthenogenesis, see Hörandl, 2006). The diploid members of section *Pilosellina* have a more restrained range of distribution in

comparison with polyploid *P. officinarum* and are confined mostly to southern and central Europe (see Introduction). This recalls the situation of several other groups of polyploid vascular plants associated with apomixis, e.g. *Antennaria* L. (Bayer and Stebbins 1987), *Ranunculus auricomus* group (Hörandl, 2006) and *Taraxacum* (den Nijs *et al.* 1990), where polyploid apomicts tend to have larger ranges than sexuals. In the present case, the colonizing success of *P. officinarum* might be attributed to the combinations of different factors, such as its probable allopolyploid origin (see above), increased heterozygosity and the existence of a high number of genetically different clones, the presence of an apomictic mode of reproduction in high ploids (5*x* and 6*x*) with the occurrence of residual sexuality, vegetative reproduction via above-ground stolons, the possibility of long-distance dispersal via achenes with a pappus, and the opportunity of recurrent formation of novel genotypes via hybridization. It is possible that all these factors have played an important role in shaping the present cytogeographic patterns of *P. officinarum*.

SUPPLEMENTARY INFORMATION

Supplementary information is available online at www.aob.oxfordjournals.org and contains the lists of *Pilosella officinarum* localities accompanied by geographical co-ordinates, ploidy levels and/or chromosome numbers (and references) for data (1) presented in this study for the areas of the Czech Republic, Slovakia and Hungary; (2) for previously reported data from the Czech Republic and Slovakia; (3) for previously published data from the rest of European area; and (4) for new data from Europe outside of Slovakia, Czech Republic and Hungary.

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Príloha G

Mráz P, Szeląg Z

Chromosome numbers and reproductive systems in selected species of the genera *Hieracium* L and *Pilosella* Hill (*Asteraceae*) from Romania

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Chromosome numbers and reproductive systems in selected species of *Hieracium* and *Pilosella* (Asteraceae) from Romania

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Mráz, P. & Szeląg, Z. 2004: Chromosome numbers and reproductive systems in selected species of *Hieracium* and *Pilosella* (Asteraceae) from Romania. — *Ann. Bot. Fennici* 41: 405–414.

Chromosome numbers are given for 17 taxa of the genus *Hieracium* and 4 taxa of the genus *Pilosella* originating from Romanian Eastern and Southern Carpathians: *Hieracium alpinum* (2n = 18), *H. atratiforme s. lato* (2n = 36), *H. bifidum s. lato* (2n = 27, 36), *H. inuloides* (2n = 27), *H. jankae* (2n = 27), *H. lachenalii s. lato* (2n = 27), *H. lubricicaule* (2n = 27), *H. kotschyanum* (2n = 27), *H. magocsyanum* (2n = 27), *H. ostii-bucurae* (2n = 27), *H. praecurrens s. lato* (2n = 27), *H. ratezaticum* (2n = 36), *H. sabaudum s. lato* (2n = 27), *H. telekianum* (2n = 27), *H. tomiasae* (2n = 27), *H. tubulare* (2n = 27), *H. transylvanicum* (2n = 18), *Pilosella aurantiaca* (2n = 36, 45), *P. bauhinii* (2n = 45), *P. cymosa* (2n = 36) and *P. pavichii* (2n = 18). For the first time the chromosome numbers are reported for the following taxa: *H. atratiforme s. lato*, *H. jankae*, *H. lubricicaule*, *H. kotschyanum*, *H. magocsyanum*, *H. ostii-bucurae*, *H. telekianum*, *H. tomiasae* and *H. tubulare*. Apomictic mode of reproduction was proved in triploids *H. jankae*, *H. lubricicaule*, *H. kotschyanum, H. magocsyanum*, *H. ostii-bucurae*, *H. telekianum*, *H. tomiasae* and *H. tubulare* by emasculation experiments. Diploid populations of *Hieracium alpinum* were confirmed in the Southern and Romanian Eastern Carpathians. Because diploid *H. alpinum* occurs just in the Eastern and Southern Carpathians and nowhere else, these parts of its area of distribution may be considered as glacial refugia for this species. The Skhidni Beskidi Mts. (the most western part of the Eastern Carpathians) probably represented a strong barrier of postglacial migrations of some diploid *Hieracium* taxa from the Eastern Carpathians towards the Western Carpathians.

Key words: apomixis, breeding system, cytotaxonomy, glacial refugia, *Hieracium*, *Pilosella*, ploidy level

Introduction

Hieracium and *Pilosella* are among the most taxonomically complicated genera in the plant kingdom. Although it is a much debated point, in this paper the two entities will be treated as distinct genera. It seems that their complexity is caused by a reticulate pattern of morphological and molecular variation. Probably, in the past, immense hybridization events, including introgression, were the most powerful mechanism of speciation resulting in thousands of taxa in the genus *Hieracium*. This history contrasts with the present situation, when hybridization is very rare (Mráz *et al*. 2003). The situation in *Pilosella* is different; its taxa are usually highly outcrossing under current natural conditions (Krahulcová *et al*. 2000). Polyploidy connected with an apomictic mode of reproduction is another typical feature of both genera. In *Hieracium* sexual diploids are very rare, while apomictically reproducing polyploids (diplospory of *Antennaria* type) are much more common (Merxmüller 1977). Conversely, facultative apomixis (apospory of *Hieracium* type) and even full sexuality in polyploids are known in the genus *Pilosella* (Krahulcová *et al*. 2000). In *Hieracium* triploids and tetraploids (2n = 27, resp. 36; pentaploids are very rare, cf. Stace *et al*. 1995, Chrtek 1996) are believed to reproduce by obligate apomictic formation of the seeds. However, some irregularities observed in the development of the apomictic megaspore mother sac led us to suggest that at least some traces of residual sexuality could exist in *Hieracium* polyploids. Skawińska (1963) observed that ca. 50% of ovules in the triploid *Hieracium alpinum* were early degenerated probably due to true meiotic division leading to an unviable macrospore tetrad.

Knowledge of ploidy level is one of the most important sources of information indicating the mode of reproduction and forms the basis for taxonomic evaluation. Although many chromosome counts for many taxa were published in both genera (for review *see* Schuhwerk 1996), some regions are still poorly investigated and represent *terra incognita* from the karyological point of view. Romania is a country with a very high diversity of these two genera. Nyárády (1965) reported 55 species in *Pilosella* and 165 species in *Hieracium*. In each genus there are several hundreds (if not thousands) of infraspecific taxa (subspecies, varieties and forms). Moreover, the Munţii Retezatului (Retezat Mts.) in the Southern Carpathians are considered to be one of the most important centres of evolution of the genus *Hieracium*, with many endemic taxa described from the area (Nyárády 1930).

To the best of our knowledge, chromosome numbers for *Hieracium* and *Pilosella* species from Romania have been presented in four sources only. Christoff and Popoff (1933) published chromosome counts for several taxa from the seed collection of the Botanical Garden in Cluj, but with no indications of localities, thus the origin of plant material is unkown. Ştefureac and Tăcină (1979) reported the relict diploid species *Hieracium pojoritense*, endemic of calcareous moutains in NE Romania. Later, Mráz (2003b) confirmed the diploid counts for this species. The diploid level of *H. transylvanicum* and *H. alpinum* was reported for the first time from the Romanian Carpathians by Mráz (2003b). The triploid level (2n = 27) was reported for *H. borsanum*, *H. brevipiliferum* and an unnamed taxon from *Hieracium* sect. *Alpina*; the tetraploid level (2n = 36) for *H. ratezaticum* and *H. pietroszense* (Mráz 2001, 2003a, 2003b). *Pilosella bauhinii* was counted as tetra- and hexaploid in one Romanian population (Rotreklová 2002).

Material and methods

Chromosome counts were made in pot-grown plants by P. Mráz. Root tip cuttings were pretreated with 0.5% solution of colchicine for 1.5–3 hours at room temperature. Subsequently fixative (absolute ethanol and glacial acetic acid, 3:1) replaced colchicine. Roots were stored in 70% ethanol and hydrolysed for 10 minutes in 1 N HCl at 60 °C. The squash and smear method with cellophane replacing the glass covers followed Murín (1960). Giemsa solution in phosphate buffer was used as a stain. Voucher specimens of the analysed plants or gatherings from the field are deposited in Herbarium P. Mráz and Herbarium Z. Szeląg. The number given in

parentheses after each locality is the cultivation number of plants cultivated in the Botanical Garden of the P. J. Šafárik University.

The mode of reproduction for polyploid species from *Hieracium* sect. *Cernua* and *H. jankae* (cultivated by the second author) was determined by emasculation experiments. The emasculation was carried out by cutting off the whole upper half of the capitulum together with the styles (five flower heads per species). Those flower heads producing fully developed seeds after emasculation were recognized as apomictic.

The chromosome counts published here are the results of several expeditions of the first author in Romania (in 2000 with V. Jurkovičová *et al*., in 2001 with Ph. Choler, and in 2002 with Z. Szeląg).

Taxonomic concept

The analysed taxa of the genus *Hieracium s. stricto* are arranged into sections recognized by Stace (1998), whereas the species of *Pilosella* are given in alphabetical order.

For polyploid taxa, i.e. triploids and tetraploids, we have usually adopted a narrow concept of species. However, in several cases the taxa are treated as *s. lato*, because we are not sure of the application of correct names (lack of knowledge of whole range of variability, type material and locality, etc.).

Results

Genus *Hieracium*

Hieracium sect. *Alpina*

Hieracium alpinum L. — 2n = 18

Localities: **1**. Munţii Retezatului [Retezat Mts.] on the ridge between Mt. Bărlea and Mt. Seşele Mari, ca. 2300 m, 8.VII.2001 *P. Mráz* (1 plant, no. 1018). **2**. Munţii Retezatului [Retezat Mts.], on the ridge Culmea Lolaia, Saua Ciurila saddle, ca. 1800 m, 7.VII.2001 *P. Mráz* (2 plants, nos. 1054, 1055). **3**. Munţii Retezatului [Retezat Mts.], Valea Pietrele, ca. 1950 m, 8.VII.2001 *P. Mráz* (1 plant, no. 1028). **4**. Munţii Rodnei [Rodna Mts.], on the path from Borşa to Staţia Meteo below Mt. Pietrosul Mare, 1500–1700 m, 5.VII.2001 *P. Mráz* (1 plant, no. 1061). **5**. Munţii Bistriţei Mts., the Massif Ceahlău, northern slopes of Mt. Toaca, 1545 m, 18.VII.2000 *P. Mráz & V. Jurkovičová* (1 plant, no. 807). **6**. Munţii Bistriţei Mts., Mt. Pietrosul Bogolin, in the Massif of Mt. Pietrosul Broşteni lor, 1650–1700 m, 20.VII.2000 *P. Mráz* (1 plant, no. 828).

We confirm the presence of diploid populations in the Ukrainian (Chrtek 1997, Mráz 2001) and Romanian Eastern and Southern Carpathians (Mráz 2003b).

Because in the rest of the area of distribution (including the Western Carpathians) *H. alpinum* is represented by triploids (e.g. Schuhwerk 1996, Chrtek 1997) we suppose that glacial refugia of diploid *H. alpinum* have been in the territory of the Eastern and Southern Carpathians or very close to these mountain ranges. The diploid state is usually assumed to be ancestral to higher ploidy levels. It seems that triploid populations of *H. alpinum* have arisen outside the Eastern and Southern Carpathians. The glacial refugia of this species in the territory of the Eastern Carpathians (at that time no counts were available from the Southern Carpathians) were deduced also by Štorchová *et al*. (2002). The tetraploid level for *H. alpinum* was reported only once (Szeląg & Jankun 1997) from the Polish part of the Tatra Mts.

Hieracium sect. *Vulgata*

Hieracium lachenalii s. lato — 2n = 27

Locality: **1**. Munţii Apuseni [Bihor Mts.], Pietroasa, SE slopes of Mt. Cornul Munţilor, 46°38´17.0´´N, 22°40´19.2´´E, 1453 m, 3.VIII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1284).

Hieracium lachenalii s. lato is a very complex taxonomic entity. For most of the distribution area *H. lachenalii s. lato* has been found to be triploid (cf. Schuhwerk 1996, Schuhwerk & Lippert 1998). In Central Europe the chromosome number 2n = 27 was also reported by Májovský *et al*. (1974) from the Slovak part of the Western Carpathians, and by Krahulcová (1990) from the Bohemian Karst. The tetraploid level (2n = 36) was reported by Lavrenko and Serditov (1987), and a hypertriploid level (2n = 28) by Rostovtseva (1979) (as *H. tilingii*).

Hieracium transylvanicum Heuff. — 2n = 18

Localities: **1**. Munţii Hargithei [Hargitha Mts.], Băile Tuşnad, Mt. Piatra Şoimului, beech-fir forest below the top, 46°08´55.1´´N, 25°50´57.9´´E, 750 m, 29.VII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1240/T). **2**. Munţii Apuseni [Bihor Mts.], Pietroasa, ridge of Dl. Pălţinetu, 46°37´37.1´´N, 22°39´42.7´´N, 1266 m, 3.VIII.2002 *P. Mráz & Z. Szeląg* (2 plants, nos. 1279, 1282). **3**. Munţii Hăghimaşului [Hăghimaş Mts.], Bălan, spruce forest by the path from Bălan to Mt. Ecem, ca. 1200–1300 m, 16.VII.2000 *P. Mráz & V. Jurkovičová* (1 plant, no. 783).

This diploid species is distributed across the Eastern and Southern Carpathians to the mountains in the Balkan Peninsula. The western-most localities are situated in Steier in Austria (Zahn 1922–1939). Some localities were given also from the Western Carpathians, but these data are highly doubtful. For the first time the diploid chromosome number for *H. transylvanicum* was reported by Rosenberg (1927), but without indication of the exact locality of the studied material. Later, Chrtek (1996) reported the diploid level for plants from the Ukrainian Eastern Carpathians and Vladimirov (2000) for plants coming from the Stara Planina Mts. in Bulgaria. Mráz (2003b) confirmed diploid counts in plants from the Romanian Eastern Carpathians.

Hieracium sect. ***Subalpina***

Hieracium atratiforme s. lato — 2n = 36

Locality: **1**. Munţii Godeanului [Godeanu Mts.], Mt. Tomeasa, spruce forest by the path from Gura Apei, 45°21´15.7´´N, 22°42´35.4´´E, 1480 m, 2.VIII.2002 *P. Mráz & Z. Szeląg* (2 plants, nos. 1248, 1249).

This is the first chromosome number report for this taxon, which is morphologically between *H. transylvanicum* and *H. kotschyanum*.

Hieracium ratezaticum (Nyár. & Zahn) Mráz — 2n = 36

Locality: **1**. Munţii Retezatului [Retezat Mts.], the slopes 0.2 km W of the Zănoaga lake, exp. S–SE, 45°20´30´´N, 22°20´E, 1850–1980 m, 8.VII.2001 *P. Mráz* (1 plant, no. 1045).

Mráz (2001) reported the same chromosome number from other specimens collected in 1998 from the same locality.

Hieracium sect. ***Hieracium***

Hieracium bifidum s. lato — 2n = 27, 36

Localities: **1**. Munţii Hăghimaşului [Hăghimaş Mts.] Bălan, spruce forest by the path from Bălan to Mt. Ecem, ca. 1200 m, 16.VII.2000 *P. Mráz & V. Jurkovičová* (2 plants, nos. 784, 785, 2n = 27). **2**. Munţii Godeanului [Godeanu Mts.], Mt. Tomeasa, SE rocky slope, by the path from Gura Apei, 45°21´16.7´´N, 22°42´08.3´´E, 1796 m, 2.VIII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1254, 2n = 36). **3**. Munţii Banatului [Banat Mts.], summit area of the Mt. Treskovać, rock crevices and rocky ground, 44°33´43´´N, 22°03´08´´E, 680 m, 31.VII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1265, 2n = 36).

The *Hieracium bifidum* group represents a morphologically very polymorphic complex and is in need of a thorough taxonomic and nomenclatural revision (Mráz & Marhold 2002). Up to now, the diploid, triploid and tetraploid levels have been reported (cf. Schuhwerk 1996). From the Western Carpathians (Mt. Pilsko) the triploid number for *H. bifidum* was published by Májovský *et al*. (1970), although this count most probably refers to a taxon of the *H. lachenalii* group, not to *H. bifidum s. lato*.

The taxon counted here from the Munţii Hăghimaşului is probably related to *H. pseudobifidum* (morphological position: *bifidum–transylvanicum*). It has a similar indumentum in the basal part of its stem and partially on the leaves, and smaller capitula with scattered to numerous glandular trichomes as in *H. transylvanicum*. On the other hand, there are numerous stellate trichomes on the peduncles and involucres.

Hieracium praecurrens s. lato — 2n = 27

Locality: **1**. Munţii Hargithei [Hargitha Mts.], Băile Tuşnad, Mt. Piatra Şoimului, beach-fir forest below the top, 46°08´55.1´´N, 25°50´57.9´´E, 750 m, 29.VII.2002 *P. Mráz & Z. Szeląg* (2 plants, nos. 1239, 1240/P).

The triploid chromosome number for this

taxon with intermediate morphology between diploid *H. transylvanicum* and usually triploid *H. murorum s. lato* was found by Mráz (Chrtek *et al.* 2004). The area of distribution is more or less the same as that of *H. transylvanicum* except in the Western Carpathians (cf. Sell & West 1976), where the occurrence of *H. transylvanicum* is strongly doubtful.

Hieracium sect. ***Foliosa***

Hieracium inuloides Tausch — 2n = 27

LOCALITY: **1**. Munţii Apuseni [Bihor Mts.], Pietroasa, SE slopes of Mt. Cornul Munţilor, 46°38´17.0´´N, 22°40´19.2´´E, 1453 m, 3.VIII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1285).

This taxon is very rare not only in the Romanian Carpathians (Nyárády 1965), but also in the whole Carpathian arc (Zahn 1922–1938), and the locality given above is new. According to the first author of this contribution, the Romanian plants from the Bihor Mts. are morphologically similar to those from the Western Carpathians and the Sudeten Mts., from where the taxon was originally described.

The triploid number presented here agrees with that of Morton (1974) for *H. subcrocatum* and with two counts from the Western Carpathians (Mráz in Chrtek *et al.* 2004). Finch (in Moore 1982) also published 2n = 27 for *H. latobrigorum*.

Hieracium sect. ***Pilosissima***

Hieracium jankae R. Uechtr. — 2n = 27 (apomictic mode of reproduction)

LOCALITY: **1**. Munţii Banatului [Banat Mts.], summit area of the Mt. Treskovać, scattered *Caprinus orientalis* forest on quartzite, 44°33´35.3´´N, 22°03´36.3´´E, 680 m, 31.VII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1266).

This is the first chromosome report for this species that is based on a plant from the type locality. The triploid chromosome number was previously given for *H. jankae s. lato* from the Pirin Mts. in Bulgaria (Vladimirov & Szeląg 2001b) and for "*H. jankae* Uechtr. *cf.* subsp. *patentiramum* Rech. f. & Zahn" (Schuhwerk & Lippert 1998).

Hieracium sect. ***Sabauda***

Hieracium sabaudum s. lato — 2n = 27

LOCALITIES: **1**. Munţii Banatului [Banat Mts.], ca. 0.5 km SE from Mt. Treskovać, oak forest margin, 44°33´35.3´´N, 22°03´36.3´´E, 481 m, 31.VII.2002 *P. Mráz & Z. Szeląg* (no. 1269). **2**. Munţii Hargithei [Hargitha Mts.], Băile Tuşnad, Mt. Piatra Şoimului, beech forest margin by the andesit rocks, 46°08´55.1´´N, 25°50´57.9´´E, 850 m, 29.VII.2002 *P. Mráz & Z. Szeląg* (no. 1237).

Triploid chromosome numbers greatly prevail in the literature. However, diploid and tetraploid levels have also been reported (cf. Schuhwerk 1996). Both diploid (Feráková 1971, Májovský *et al.* 1974, Uhríková & Feráková 1977, Hrušovská-Osuská 1988) and triploid chromosome numbers (Májovský *et al.* 1970, 1974, 2000) were given for populations from the Western Carpathians. However, with high probability all diploid numbers refer to *H. umbellatum* rather than to *H. sabaudum s. lato* (cf. Chrtek *et al.* 2004).

Hieracium sect. ***Cernua***

Hieracium kotschyanum Heuff. — 2n = 27 (apomictic mode of reproduction)

LOCALITIES: **1**. Munţii Godeanului [Godeanu Mts.], Mt. Tomeasa, SE rocky slope, by the path from Gura Apei, 45°21´16.7´´N, 22°42´08.3´´E, 1796 m, 2.VIII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1253). **2**. Munţii Godeanului [Godeanu Mts.], rocky slopes above the dam Gura Apei, 45°20´21.5´´N, 22°42´49.0´´E, 1085 m, 1.VIII.2002 *P. Mráz & Z. Szeląg* (2 plants, nos. 1259, 1260).

Hieracium lubricicaule (Nyár.) Borza — 2n = 27 (apomictic mode of reproduction)

LOCALITY: **1**. Munţii Godeanului [Godeanu Mts.], Mt. Tomeasa, SE rocky slope, by the path from Gura Apei, 45°21´16.7´´N, 22°42´08.3´´E, 1796 m, 2.VIII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1258).

H. magocsyanum Jáv. — 2n = 27 (apomictic mode of reproduction)

LOCALITIES: **1**. Munţii Retezatului [Retezat Mts.], on the ridge Culmea Lolaia, Saua Ciurila saddle, ca. 1800 m, 7.VII.2001 *P. Mráz* (3 plants, nos. 1034, 1037, 1058). **2**. Munţii Godeanului [Godeanu Mts.], Mt. Tomeasa, SE rocky slope, by the path from Gura Apei, 45°21´16.7´´N, 22°42´08.3´´E, 1796 m, 2.VIII.2002 *P. Mráz & Z. Szeląg* (2 plants, nos. 1256, 1257).

Hieracium ostii-bucurae Nyár. *ex* Szeląg* — 2n = 27 (apomictic mode of reproduction)

LOCALITY: **1**. Munţii Godeanului [Godeanu Mts.], rocky slopes above the dam Gura Apei, 45°20´21.5´´N, 22°42´49.0´´E, 1085 m, 1.VIII.2002 *P. Mráz & Z. Szeląg* (1 plant, nos. 1264).

Hieracium telekianum Boros & Lengyel — 2n = 27 (apomictic mode of reproduction)

LOCALITY: **1**. Munţii Hargithei [Hargitha Mts.], Băile Tuşnad, Mt. Piatra Şoimului, andesit rocks, 46°08´55.1´´N, 25°50´57.9´´E, 850 m, 29.VII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1241).

Hieracium tomiasae (Nyár. & Zahn) Nyár. — 2n = 27 (apomictic mode of reproduction)

LOCALITY: **1**. Munţii Godeanului [Godeanu Mts.], Mt. Tomeasa, SE rocky slope, by the path from Gura Apei, 45°21´16.7´´N, 22°42´08.3´´E, 1796 m, 2.VIII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1251).

Hieracium tubulare (Nyár.) Zahn — 2n = 27 (apomictic mode of reproduction)

LOCALITY: **1**. Munţii Hargithei [Hargitha Mts.], Băile Tuşnad, Mt. Piatra Şoimului, andesit rocks, 46°08´55.1´´N, 25°50´57.9´´E, 850 m, 29.VII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1243).

Hieracium sect. *Cernua* is the correct section name for the taxa of the *H. sparsum* group (Szeląg 2003b). The centre of distribution of *Hieracium* sect. *Cernua* in Europe is concentrated in the central part of the Balkan Peninsula and the Southern Carpathians. In addition, only scattered, relict stations are in the Eastern Alps, Eastern Sudeten and Western and Eastern Carpathians. In *H.* sect. *Cernua* a diploid (only *H. sparsum*), triploids and tetraploids have so far been detected (Christoff 1942, Chrtek 1996, Schuhwerk & Lippert 1998, 1999, Vladimirov & Szeląg 2001b, Chrtek *et al.* 2004). All the taxa included in our analyses are Romanian endemics and their chromosome numbers are here reported for the first time.

Genus *Pilosella*

Pilosella aurantiaca (L.) F. W. Schultz & Schultz Bip. — 2n = 36, 45

LOCALITIES: **1**. Munţii Apuseni [Bihor Mts.], Pietroasa, SE slopes of Mt. Cornul Munţilor, 46°38´17.0´´N, 22°40´19.2´´E, 1453 m, 3.VIII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1283, 2n = 36). **2**. Munţii Hargithei [Hargitha Mts.], Mt. Hargitha Ciceu, 46°23´53.5´´N, 25°37´51.0´´E, 1566 m, 28.VII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1278, 2n = ca. 36). **3**. Munţii Rodnei [Rodna Mts.], Prislop saddle, ca. 1380 m, 22.VII.2000 *P. Mráz & V. Jurkovičová* (1 plant, no. 801, 2n = 36). **4**. Munţii Godeanului [Godeanu Mts.], Mt. Tomeasa, SE slope, by the path from Gura Apei, 45°21´16.7´´N, 22°42´08.3´´E, 1796 m, 2.VII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1255, 2n = 45).

Triploid (2n = 27) to heptaploid (2n = 63) numbers have so far been published for this species. In the Polish and Slovak part of the Carpathians the tetraploid, pentaploid, hexaploid and heptaploid levels were given by Skalińska (1967, 1970), Skalińska *et al.* (1968, 1974) and Uhríková (1970). Mráz (in Rotreklová *et al.* 2002) published a tetraploid chromosome number for a plant from the Ukrainian Eastern Carpathians. Pashuk (1987) reported an aneuploid cytotype (2n = 30) from the same territory.

Pilosella bauhinii (F. W. Schultz ex Besser) Arv.-Touv. — 2n = 45

LOCALITY: **1**. Munţii Hargithei [Hargitha Mts.], Băile Tuşnad, Mt. Piatra Şoimului, andesite rocks, 46°08´55.1´´N, 25°50´57.9´´E, 850 m, 29.VII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1238).

Several ploidy levels have so far been reported in the literature (cf. Schuhwerk 1996). From the Carpathians, tetraploid, pentaploid

* This species name was recently validated by Szeląg (2003a).

and hexaploid numbers (2n = 36, 2n = 45, 2n = 54) were given from Slovakia (Uhríková 1970, Rotreklová 2002, Rotreklová *et al*. 2002). Rotreklová (2002) reported tetraploids and hexaploids from one population from Romania. Some cytogeographical trends within this species in the territory of Central and Eastern Europe are noteworthy (cf. Rotreklová 2002). The pentaploid cytotype seems to be the most common in Western Europe and in the western part of Central Europe, while in the southeastern part of the area (e.g. Slovakia, Hungary, Greece) tetraploids prevail (Gadella 1984, Papanicolaou 1984, Schuhwerk & Lippert 1997, 1998, 2002, Bräutigam & Bräutigam 1996, Krahulcová *et al*. 2001, Rotreklová 2002, Rotreklová *et al*. 2002).

Pilosella cymosa (L.) F. W. Schultz & Sch. Bip. — 2n = 36

Locality: 1. Munţii Banatului [Banat Mts.], summit area of the Mt. Treskovać, scattered *Caprinus orientalis* forest on quartzite, 44°33´35.3´´N, 22°03´36.3´´E, 680 m, 31.VII.2002 *P. Mráz & Z. Szeląg* (1 plant, no. 1265).

The tetraploid chromosome number (2n = 36) is most frequent for this taxon (Schuhwerk & Lippert 1997, 2002, Vladimirov & Szeląg 2001a), but diploids, hexaploids and heptaploids are given in the literature too (e.g. Měsíček & Javůrková-Jarolímová 1992, Schuhwerk & Lippert 1997, 1998, Šimek 2000).

Pilosella pavichii (Heuff.) Holub — 2n = 18

Locality: 1. Munţii Banatului [Banat Mts.], summit area of the Mt. Treskovać, rock crevices and rocky ground, 44°33´35.3´´N, 22°03´36.3´´E, 680 m, 31.VII.2002 *Mráz & Z. Szeląg* (1 plant, no. 1268).

This subendemic species of the Balkan Peninsula occurs in the Carpathians just in southern Romania (Zahn 1922–1939), although Nyárády (1965) reported also some localities from the surroundings of Cluj-Napoca. Previously diploid (Christoff & Popoff 1933, Strid & Franzén 1981, Vladimirov 2000) and tetraploid (Vladimirov 2003) levels are reported in the literature.

Discussion

The proportion of the three ploidy levels of the investigated taxa within the genus *Hieracium* from Romania analyzed in this paper and given in the cited literature is as follows: 67% triploids, 19% tetraploids and 14% diploids.

The distribution of the ploidy levels in the genus *Hieracium* overall is noteworthy. Schuhwerk and Lippert (1998, 1999) stated that while the triploids and tetraploids each represent about 35%–40% among the investigated taxa (the diploid level is very rare, ca. 5%) in the Balkan Peninsula, only triploids and diploids occur in the Iberian Peninsula (about 80% and 20% respectively). For Central Europe the proportion of triploids is much higher (ca. 70%) than that of tetraploids (ca. 20%), diploids are, as in the above cases, rare (8%). In the Western Carpathians the proportions of triploids and tetraploids are more or less equal, 53% and 45%, and the diploids are represented just by *H. umbellatum* (ca. 3%). It seems, however, that the tetraploid species are much more common at higher altitudes than at lower ones in the territory of Western Carpathians (Chrtek *et al*. 2004).

Up to now three diploid species have been found in Romania: *H. alpinum*, *H. pojoritense* and *H. transylvanicum*. However, also *H. umbellatum* and *H. hryniawiense* can be considered as diploid in Romania, although never counted from Romanian plants. *Hieracium umbellatum* is a diploid species in most of its range and, moreover, in Central Europe only diploid populations have been found so far (cf. Schuhwerk 1996). The second taxon is probably endemic to the Eastern Carpathians occurring in the montane and subalpine meadows. Diploid populations of *H. hryniawiense* were reported from adjacent parts of the Ukrainian Carpathians (Chrtek 1996, as *H. conicum*; Mráz 2003b). One analyzed population is situated in the Ukrainian part of the Marmarosh Mts., ca. 100 m from the state boundary with Romania (Chrtek 1996). While four diploid species are present in the Ukrainian Eastern Carpathians (*H. pojoritense* does not occur there), the widely distributed *H. umbellatum* is the single, certainly diploid taxon in the territory of the Western Carpathians (most of Slovakia, Moravian parts of Czech Republic, the

south-eastern part of Poland, northern Hungary and a very small part of north-eastern Austria) (Chrtek *et al*. 2004).

We suppose that the Skhidni Beskidi Mts. (in the literature often given as "Waldkarpaten"; the most western part of the Ukrainian Eastern Carpathians, slightly overlapping in Poland and Slovakia) represented in the past a strong barrier to the postglacial migrations of many montane and (sub-)alpine plant species from the Eastern Carpathians towards the Western Carpathians (Wołoszczak 1896, Pax 1898, Jasiewicz 1965, Malinovskiĭ 1991). The diploid taxa *H. alpinum*, *H. hryniawiense* and *H. transylvanicum* did not reach the territory of the Western Carpathians, perhaps due to the very low altitude of the Skhidni Beskidi Mts. (the highest peak is Mt. Pikuĭ, 1408 m a.s.l.), where the suitable habitats, treeless in the case of *H. alpinum* and *H. hryniawiense*, or spruce forests in the case of *H. transylvanicum*, were missing during most of the last post-glacial (with the exception of Mt. Pikuĭ). The detailed distributions of apomictic polyploid taxa of the genus *Hieracium* have recently been successfully used as phylogeographic markers to determine the migration routes and barriers in the Nordic countries (Tyler 2000).

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Príloha H

Chrtek J, Mráz P, Severa M

Chromosome numbers in selected species of *Hieracium* s.str. (*Hieracium* subgen. *Hieracium*) in the Western Carpathians

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Chromosome numbers in selected species of *Hieracium* s. str. (*Hieracium* subgen. *Hieracium*) in the Western Carpathians

Počty chromozomů vybraných druhů rodu *Hieracium* s. str. (*Hieracium* podrod *Hieracium*) ze Západních Karpat

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Chrtek J. jun., Mráz P. & Severa M. (2004): Chromosome numbers in selected species of *Hieracium* s. str. (*Hieracium* subgen. *Hieracium*) in the Western Carpathians. – Preslia, Praha, 76: 119–139.

Chromosome numbers of 23 species (including subspecies) of *Hieracium* s. str. from the Western Carpathians are presented. First chromosome numbers are reported for *Hieracium kuekenthalianum* (= *H. tephrosoma*, 2n = 36), *H. praecurrens* (2n = 27) and *H. virgicaule* (2n = 27); first counts from the Western Carpathians are given for *H. atratum* (2n = 27), *H. bifidum* (2n = 27, 36), *H. carpathicum* (2n = 36), *H. inuloides* (2n = 27), *H. jurassicum* (2n = 27), *H. macilentum* (= *H. epimedium*, 2n = 27), *H. nigritum* (2n = 36), *H. pilosum* (= *H. morisianum*, 2n = 27) and *H. silesiacum* (2n = 36). New ploidy level (tetraploid, 2n = 36) is reported for *H. bupleuroides*, hitherto published counts refer only to triploids (2n = 27). Previously published chromosome numbers were confirmed for several other species, i.e. *H. alpinum* (s.str., 2n = 27), *H. bupleuroides* (2n = 27), *H. crassipedipilum* (*H. fritzei* group, 2n = 27, 36), *H. lachenalii* (2n = 27), *H. murorum* (2n = 27), *H. prenanthoides* (2n = 27), *H. racemosum* (2n = 27), *H. sabaudum* (2n = 27), *H. slovacum* (*H. fritzei* group, 2n = 36), and *H. umbellatum* (2n = 18). Triploids and tetraploids predominate, diploids (2n = 18) were found in *H. umbellatum*. A comprehensive list of previously published chromosome numbers in *Hieracium* s. str. from the Western Carpathians is provided.

K e y w o r d s : *Asteraceae*, chromosome numbers, *Hieracium,* Slovakia

Introduction

The genus *Hieracium* L. in the narrow sense (*Hieracium* subgen. *Hieracium*) belongs to one of the taxonomically most intricate groups of vascular plants. It is well known as one in which agamospermy is widespread. Many more or less stabilized agamospermous strains (clones) have been described at specific or subspecific rank. The species concept in *Hieracium* has long been a matter of discussion (see e.g. Schuhwerk 2002). The Central European school of hieraciology (founded by Nägeli and Peter) follows a broad species definition (species are then divided into subspecies, varieties, etc.); on the other hand, Scandinavian and British botanists, together with those from the former Soviet Union follow a narrow species concept, i.e. almost every morphologically recognizable type is given specific rank ("microspecies").

Hieracium s. str. comprises an immense agamic complex with a base-number of x = 9. Triploids and tetraploids form the bulk of taxa investigated so far. Diploids (2n = 18) are

rather rare and supposed to be confined to certain geographical areas. They have been reported mostly from SW Europe, namely *H. cordifolium* Lapeyr. (Schuhwerk & Lippert 1998), *H. flocculiferum* Zahn (Schuhwerk & Lippert 1998), *H. rupicaprinum* Arv.-Touv. et Gautier (Schuhwerk & Lippert 1998), *H. eriophorum* St. Amans, *H. hispanicum* Arv.-Touv., *H. laniferum* Cav. s.l., *H. lucidum* Guss. (Merxmüller 1975), *H. cerinthoides* L. (Delay 1969), and from the Eastern and Southern Carpathians, namely *H. alpinum* L. (Chrtek 1997, Mráz 2001a, Mráz 2003c), *H. augusti-bayeri* (Zlatník) Chrtek f. (Chrtek 1997), *H. conicum* Arv.-Touv. (Chrtek 1996, Mráz 2003c ut *H. hryniawiense* Woł.), *H. pojoritense* Woł. (Ştefureac & Tăcină 1979, Mráz 2003c) and *H. transsilvanicum* Heuff. (Pashuk 1987, Chrtek 1996, Mráz 2003c; for diploid *H. transsilvanicum* see also Rosenberg 1927). A few diploids have been found in the Alps, i.e. *Hieracium porrifolium* L. (Favarger 1965), and *H. intybaceum* All. (Favarger 1997; apart from diploids, triploid and tetraploid cytotypes have also been reported in this species). Some diploids have been discovered in the Asian part of Russia, i.e. *Hieracium korshinskyi* Zahn (Rostovtseva 1983), *H. filifolium* Juxip (Krasnikov in Tupitsyna 1997), *H. narymense* Schischk. et Serg. (Krogulevič 1978), and *H. virosum* Pall. (Pulkina & Tupitsyna 2000), and in the Balkan Peninsula, i.e. *H. waldsteinii* Tausch (Schuhwerk & Lippert 1999), *H. sparsum* Friv. (Christoff 1942, Vladimirov & Szeląg 2001), and *H. kittanae* Vladimirov (Vladimirov 2003). Further diploid counts have also been reported from the geographically rather widespread and karyologically differentiated (two or three ploidy levels) species (in the broad sense), such as *H. umbellatum* L. (diploid cytotype seems to be common, apomictic triploids also reported), *H. sabaudum* L., *H. prenanthoides* Vill., *H. laevigatum* Willd. and *H. racemosum* Waldst. et Kit. ex Willd. (but polyploids most probably prevail in the last four species) (Schuhwerk 1996, Schuhwerk & Lippert 1999). Diploid cytotypes have also been found in the otherwise polyploid (3x, 4x) *Hieracium bifidum* Kit. ex. Hornem. (Rosenberg 1927), and *H. glaucinum* Jord. [Natarajan 1981, *H. jaubertianum* Timb. et Loret, *H. glaucinum* subsp. *jaubertianum* (Timb. et Loret)]. Diploid *H. speciosum* Hornem. was reported by Gentcheff & Gustafsson (1940).

Published chromosome numbers above the tetraploid level are very rare. Pentaploid counts (2n = 45) come from British Isles (Stace et al. 1995; unnamed taxon from *H.* sect. *Alpina* F. N. Williams), the Sudeten Mts, Czech Republic [Chrtek 1996; *Hieracium chrysostyloides* (Zahn) Chrtek f.], and Siberia (Pulkina & Tupitsyna 2000; *Hieracium virosum* Pall.). Hexaploids (2n = 54) and heptaploids (2n = 63) were found in *Hieracium virosum* (Pulkina & Tupitsyna 2000). Aneuploids are very rare (for references see Schuhwerk 1996).

As far as we are aware, the diploids are sexual, and the triploids, tetraploids and pentaploids agamospermous. Development of the unreduced embryo sac follows the "Antennaria type" of diplospory i.e. the female meiosis is fully omitted (e.g. Nogler 1984). All previously studied plants showed autonomous endosperm development, the plants not requiring pollination. However, irregularities were reported showing remnants of sexual processes in some *Hieracium* (s. str.) species (e.g. Bergman 1941). Irregularities were also detected in the course of microsporogenesis. While in the sexual diploid plants the reduction division of pollen mother cells is, not surprisingly, normal, in polyploid agamospermous plants a graded series of degeneration of meiosis has been observed (Rosenberg 1927, Gentcheff 1937, Gentcheff & Gustafsson 1940, Aparicio 1994). Thus, polyploid taxa differ markedly from each other in their ability to produce viable pollen grains.

As shown above, chromosome data can indicate breeding behaviour and patterns of variation in hawkweeds, and are therefore of great value. Unfortunately, our knowledge of chromosome numbers in this genus is still incomplete and much needs to be done to remedy this situation.

The genus *Hieracium* in the Western Carpathians

The Western Carpathians are in the eastern part of Central Europe. They lie mostly in the territory of Slovakia, the northernmost part is within the borders of Poland, westwards they extend to the Czech Republic and Austria, and southwards to Hungary. The highest mountain range are the Tatry Mts (highest peak Gerlachovský štít, 2654.4 m a.s.l.).

Zahn (1930–1939) in his account of Central European hawkweeds recognized 65 species (broad species concept = species groups) in the Western Carpathians. Later some groups of mountain hawkweeds were thoroughly revised and new taxonomic concepts were proposed. This concerns the *Hieracium alpinum* group (Chrtek 1997), the *H. fritzei* group (Chrtek & Marhold 1998), the *H. rohacsense* group (Mráz 2001a, 2002), and the *H. piliferum* group (Szeląg 2001, Mráz 2003a) (species groups correspond to species in a broad sense).

There have been relatively few chromosome studies on *Hieracium* in this region. Chromosome counts have been reported by Skalińska et al. (1959), Skawińska (1963), Májovský (1970a, 1970b, 1974, 1976, 1978), Uhríková & Feráková (1977), Hindáková & Májovský (1977), Mičieta (1978), Murín & Paclová (1979), Murín & Májovský (1987, 1992), Hrušovská-Osuská (1988), Chrtek (1996, 1997), Szeląg & Jankun (1997), Schuhwerk & Lippert (1999), Májovský et al. (2000) and Mráz (2001b, 2003a, 2003c). Karyotype analysis was given for several previously counted *Hieracium* taxa by Uhríková (1975). Chromosome counts are also included in the above mentioned taxonomic treatments of particular species groups.

To sum up, there have previously been counts for 16 species (in the broad sense) from the Western Carpathians. Nevertheless, most of these species (in the broad sense) can be split into morphologically more or less easily distinguishable units which often differ from each other with respect to their chromosome numbers (see e.g. Chrtek & Marhold 1998). On the other hand, there is probably little (if any) variation in chromosome number within each "unit" (subspecies, microspecies) (see also Stace et al. 1995).

In this paper we report the chromosome numbers of 23 species (in several cases subspecies are recognized) from the Western Carpathians (see also Append. 1, where both previously published and new counts from the Western Carpathians are summarized).

Material and methods

Plants

Plants were collected between 1996–2003 from their natural habitats in the Slovakian part of the Western Carpathians and transplanted into the experimental garden in Průhonice near Praha (J. Ch., M. S., Institute of Botany, Academy of Sciences of the Czech Republic)

and Košice (P. M., Botanical Garden of the P. J. Šafárik University). Pot grown plants were kept in either field conditions or in an unheated greenhouse. Voucher specimens are deposited in Herbarium of the Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice (PRA, plants counted by J. Ch.), Herbarium of the Charles University, Praha (PRC, M. S.) and Herbarium P. Mráz, now at the Institute of Biology and Ecology, P. J. Šafárik University, Košice (P. M.). The numbers given in parentheses after each locality refer to cultivation numbers.

Chromosome numbers

The studies were made on the pot-grown plants. Two different methods were used:

1. Actively growing roots were placed into pretreatment solution of saturated p-dichlorbenzene and kept for 3–4 hours at room temperature, then fixed in a mixture of ethanol and acetic acid (3:1) and stored in 70% ethanol. The squash method and staining by lacto-propionic orcein were used (Dyer 1963; method used by J. Ch. and M. S.),

2. Root tips were pre-treated with 0.5% solution of colchicine for 1.5–3 hours at room temperature, subsequently fixative (absolute ethanol and glacial acetic acid, 3:1) replaced colchicine, roots were stored in 70% ethanol and hydrolysed for 7–10 minutes in 1N HCl at 60 °C. The squash and smear method with cellophane replacing the glass covers followed Murín (1960). Giemsa solution in phosphate buffer was used as a stain (method used by P. M.).

Taxonomic treatment

The state of knowledge of particular groups of hawkweeds of the Western Carpathians is not equal. Some groups (species in a broad sense, species collectivae sensu Zahn) were recently studied taxonomically and it seems justified to distinguish well defined and recognizable taxa at the species rank within them. Therefore, we use a term "species group" in such cases. On the other hand, classification of less known species (in a broad sense) follows that of Zahn (1930–1939), i.e. the studied plants are (whenever possible) determined to subspecies. Whenever recent taxonomic treatment of a group in the Western Carpathians is not available, we follow the monograph by Zahn (1930–1939).

The species and species groups are arranged alphabetically. The number of quadrats of grid mapping project of Central Europe (Niklfeld 1971) is given in parentheses after the geographic co-ordinates of each locality.

Results and discussion

Hieracium alpinum group: *H. alpinum* L. 2n = 27

Localities: **1**. Belianske Tatry Mts, Ždiar: Monkova dolina valley, 1780–1800 m a.s.l., 49°14'16" N, 20°12'55" E (6787c), coll. P. Mráz and V. Jurkovičová, 8 VIII 2000 (1 plant no. 842, 2n = ca 27, counted by P. M.). – **2**. Vysoké Tatry Mts, Štrbské Pleso: Mengusovská dolina valley, Satanov žľab, ca 1800 m a.s.l., 49°10'00" N, 20°03'27.8" E (6886a), coll. P. Mráz and V. Mrázová, 12 VIII 2001 (1 plant no. 1126, 2n = 27, counted by P.M.). – **3**. Vysoké Tatry Mts, Štrbské Pleso: Zlomisková dolina valley, 1900 m a.s.l., 49°09'47" N, 20°06'20" E (6886b), coll. P. Mráz and V. Jurkovičová, 7 VIII 2000 (1 plant no. 850, 2n = ca 27, counted by P. M.).

The counts presented here confirm references from the Western Carpathians (Skalińska in Skalińska et al. 1959, Skawińska 1963, Uhríková & Murín in Májovský 1970b, Murín in Murín & Májovský 1992, Chrtek 1997, Mráz 2001b, Chrtek in Štorchová et al. 2002). The only tetraploid count from the area was published by Szeląg & Jankun (1997), based on plants from Mt Ornak (Tatry Zachodnie Mts, Poland). On the other hand, diploid plants of *H. alpinum* have been reported from the Eastern Carpathians (Ukraine, Romania) (Chrtek 1997; Mráz 2001b, 2003c) and from Southern Carpathians (Romania) (Mráz 2003c). In the Carpathians, the diploid and triploid cytotypes are non-overlapping (strictly confined to the eastern, southern and western parts, respectively).

Triploids have been reported from other parts of the distribution area. The counts come from the Krkonoše Mts (the Sudeten Mts, Czech Republic) (Měsíček in Měsíček & Jarolímová 1992, Chrtek 1994), the Alps (Huber & Baltisberger 1992), Scandinavia (Engelskjön & Knaben 1971), British Isles (Stace et al. 1995), Iceland (Löve 1970), Greenland (Böcher & Larsen 1950, Jørgensen et al. 1958, Gadella & Kliphuis in Löve 1971), and from the Usa river basin in the Komi Republic, NW Russia (Sokolovskaya 1970). Aneuploidy (2n = 26) derived from a triploid cytotype was reported by Sokolovskaya & Strelkova (1960) from the Khibiny Mts, NW Russia.

However, due to varied use of the name *Hieracium alpinum* (in both broad and narrow sense) some counts may refer to another closely related species (microspecies).

Hieracium atratum Fr. 2n = 27

(*H. alpinum* < *H. murorum*)

Localities: **1**. Vysoké Tatry Mts, Štrbské Pleso: Mengusovská dolina valley, by the marked path, 1600 m a.s.l., 49°09'50" N, 20°04'40" E (6886a), coll. P. Mráz and V. Jurkovičová, 6 VIII 2000 (1 plant no. 835, 2n = 27, counted by P. M.). – **2**. Západné Tatry Mts, Roháče mountain group, Zuberec: Roháčske plesá mountain lakes, near the marked path above the lowest lake, 11 km SE of the village, 1590 m a.s.l., 49°12'26" N, 19°44'31" E (6784c), coll. J. Chrtek jun., 12 VII 2003 (2 plants no. H 854/1, H 854/2, 2n = 27, counted by J. Ch.).

Hieracium atratum belongs to the taxonomically most complex groups of mountain hawkweeds. Zahn (1930–1939) recognized 8 subspecies in the Western Carpathians, 6 being endemic to this area. However, there remain many unresolved taxonomic questions and the species needs a detailed revision, not only in the Western Carpathians.

Our plants belong to grex *atratum*. The plants conform well to herbarium specimens from the Tatry Mts deposited in BP and determined by K. H. Zahn as *H. atratum* subsp. *atrellum*, *H. atratum* subsp. *atrellum* var. *furkotanum* Zahn and *H. atratum* subsp. *atrellum* var. *greineri* Korb et Zahn (the latter names seem to be synonyms) (P. Mráz, pers. observ.). The counts are the first from the Western Carpathians. Based on hitherto published counts, *H. atratum* includes both triploids and tetraploids. Triploids have been reported from Greenland (Jørgensen et al. 1958) and from the Krkonoše Mts (the Sudeten Mts, Czech Republic) (Chrtek 1994). Tetraploid counts come from the Krkonoše Mts (Chrtek 1994) and British Isles (Mills & Stace 1974; microspecies *H. chrysolorum* P. D. Sell et C. West).

Hieracium bifidum Kit. ex Hornem. 2n = 27, 36

Localities: **1**. Veľká Fatra Mts, Turecká: Malá Ramžiná valley, southern slope below the elevation 1497 m a.s.l., ca 1 km WSW of Mt. Krížna (1574), 1340 m a.s.l., 48°52'34" N, 19°04'02" E (7180a), coll. P. Mráz, 12 VII 1997 (2 plants no. 376, 377, 2n = 36, counted by P. M.). – **2**. Slovenský raj, distr. Spišská Nová Ves, Spišské Tomášovce: "Prielom Hornádu" river valley, near the confluence with the Biely potok brook, 2 km SSW of the

village, 530 m a.s.l., 48°56'42" N, 20°27'23" E (7088d), coll. J. Chrtek jun., 12 VIII 2003 (3 plants no. H 883/1–3 , 2n = 36, counted by J. Ch.). – **3**. Slovenský raj, distr. Spišská Nová Ves, Spišské Tomášovce: near the marked path between settlements of Čingov and Ďurkovec, 2 km SSE of the village, 540 m a.s.l., 48°56'40" N, 20°28'45" E (7088d), coll. J. Chrtek jun., 12 VIII 2003 (3 plants no. H 884/1–3 , 2n = 36, counted by J. Ch.). – **4**. Slovenský raj, distr. Spišská Nová Ves, Dedinky: "Glac" plateau, above the Veľký Sokol valley, 4.5 km N of the village, near marked path in a beech forest, 930 m a.s.l., 48°54'25" N, 20°22'55" E (7088c), coll. J. Chrtek jun., 5 VIII 2002 (3 plants no. H 838/1–3 , 2n = 36, counted by J. Ch.). – **5**. Západné Tatry Mts, Sivý vrch mountain group: Radové skaly calcareous rocks, 5.3 km SSE of Zuberec, 1620 m a.s.l., 49°12'43" N, 19°37'53" E (6783d), coll. J. Chrtek jun., 11 VII 2003 (3 plants no. H 856/1–3 , 2n = 27, counted by J. Ch.).

Hieracium bifidum is a morphologically extremely variable taxon. Zahn (1930–1939) reported 39 subspecies from the Western Carpathians, their taxonomic value needs confirmation. Our plants differ morphologically from each other, and can be placed into two different groups of subspecies (greges). While plants from localities 1 and 5 belong to a group of subspecies (grex) *bifidum* (Zahn 1930–1939, phyllaries and peduncles with dense stellate hairs, whitish, shortly dark-based simple eglandular hairs and only occasional glandular hairs), the remaining plants (localities 2–4) share some characters of *Hieracium murorum* and obviously belong to a group of subspecies (grex) *subcaesium* (Zahn 1930–1939, phyllaries and peduncles with scattered to numerous stellate hairs, rather dark simple eglandular hairs, and scattered, sometimes numerous glandular hairs).

A triploid count for *H. bifidum* from the subalpine part of Mt Pilsko (the Západné Beskydy Mts) was reported by Murín & Uhríková in Májovský (1970a). However, it was searched for by P. M. in 1997, 1998, and 2000 without success (P. Mráz et al., unpubl.). The report is probably based on misidentified plants of *H. lachenalii.* This taxon was recently karyologically analysed from summit part of Mt Pilsko (see this species below).

Triploid and tetraploid counts were found by P. Mráz & Z. Szeląg (unpubl.) in the Romanian Carpathians. Diploids, triploids and tetraploids are among the counts previously published from different parts of the distribution area (for counts published until 1996 see Schuhwerk 1996, for recently published ones see Schuhwerk & Lippert 1999). However, the only diploid count comes from a plant cultivated in a botanical garden (Rosenberg 1927, see also above). Among the polyploids, triploids are the most common.

Hieracium bupleuroides C.C. Gmel. 2n = 27, 36

L o c a l i t i e s : **1**. Nízke Tatry Mts, Iľanovo: Machnaté sedlo saddle, ca 2 km NW of Mt. Krakova hoľa (summit), 1473 m a.s.l., 48°59'38.8" N, 19°37'20.7" E (7083b), coll. P. Mráz and V. Mrázová, 13 VII 2001 (1 plant no. 1083 2n = 36; 1084 2n = ca 36, counted by P. M.). – **2**. Západné Tatry Mts, Sivý vrch mountain group: Biela skala, 4.8 km S of Zuberec, calcareous rocks, 1300 m a.s.l., 49°12'57" N, 19°37'00" E (6783d), coll. J. Chrtek jun., 11 VII 2003 (2 plants no. H 850/1,2, 2n = 27, counted by J. Ch.). – **3**. Slovenský raj, distr. Spišská Nová Ves, Hrabušice: Veľký Sokol valley, lower part near the marked path, 7.5 km SW of the village, 640 m a.s.l., 48°55'55" N, 20°20'08" E (7088c), coll. J. Chrtek jun. and K. Chrtková, 5 VIII 2002 (3 plants no. H 837/1–3 , 2n = 27, counted by J. Ch.). – **4.** Revúcka vrchovina highlands, distr. Rožňava, Vyšná Slaná, Mt. Veľký Radzim (998.5), calcareous rocks on the southern slopes, ca 970 m a.s.l., 48°45'30" N, 20°20'23" E (7288a), coll. P. Mráz and J. Mráz, 26 VIII 2003 (1 plant no. 1440, 2n = 27, counted by P. M.). – **5**. Muránska planina plateau, Závadka nad Hronom: Mt. Malá Stožka (1204), calcareous rocks E of the summit, 1970 m a.s.l., 48°46'30" N, 19°55'55" E (7285b), coll. M. Severa, 5 VII 2000 (3 plants no. MSB 13/1–3, 2n = 27, counted by M. S.). – **6**. Belianske Tatry Mts, Ždiar: Monkova dolina valley, near the marked path 4.2 km SW of the village, 1500 m a.s.l., 49°14'55" N, 20°13'33" E (6787c), coll. M. Severa & J. Chrtek jun., 6 VII 2000 (3 plants, MSB 17/1–3, 2n = 27, counted by M. S.).

Hieracium bupleuroides is a somewhat variable species in the Western Carpathians. Zahn (1930–1939) recognized 7 subspecies in the area; the taxonomic value of some of them requires further confirmation. Our material can be identified with at least two different sub-

species. Plants from localities 2, 5 and 6 belong to subsp. *gmelinianum* Zahn (distinct basal rosette of leaves, few to 15 (–20) stem leaves, involucral bracts with stellate hairs, scattered simple eglandular hairs and towards the top with occasional glandular hairs). On the other hand, plants from the localities 3 and 4 can be identified with subsp. *tatrae* (Griseb.) Nägeli et Peter (basal leaves often withering at the time of flowering, rather numerous stem leaves, involucral bracts with stellate hairs, without simple eglandular and glandular hairs). The tetraploid count from the locality no. 1 (the Nízke Tatry Mts) is new for the species. The plant is almost glabrous, stellate hairs are dense on peduncles and scattered on involucral bracts, the simple eglandular and glandular trichomes are missing; the leaves are almost entire.

This is the second reference on ploidy level in *H. bupleuroides* from the Western Carpathians. A triploid accession has been reported from southern Slovakia, from a relic locality at a very low altitude (Slovenský kras, Zádielska dolina valley) (Murín & Uhríková in Májovský 1970a). Outside of the Western Carpathians, triploid counts come from the Alps (Polatschek 1966, Schuhwerk & Lippert 1999); the same chromosome number has been published by Christoff & Popoff (1933, locality not given).

Hieracium carpathicum Bess. 2n = 36

(*H. caesium – H. prenanthoides*)

Locality: **1.** Vysoké Tatry Mts, Tatranská Javorina: Bielovodská dolina valley, W slopes of Mt. Holica, spruce forest, ca 1260 m a.s.l., 49°14'01.7" N, 20°06'37.0" E (6786d), coll. P. Mráz and V. Mrázová, 15 VIII 2001 (2 plants no. 1149, 1150, 2n = 36, counted by P. M.).

This is the first chromosome number record from the Western Carpathians. Mills & Stace (1974) have reported the same number in plants from Mid Perth in Central Scotland. Sell & West (1965) identified the Scottish plant, originally described as *H. perthense* F. N. Williams with those from the Western Carpathians. Thus, according to these authors, *H. carpathicum* s. str. (*H. carpathicum* subsp. *carpathicum*) occurs in two geographically remote areas, namely in the Western Carpathians and Scotland. On the other hand, Zahn (1921–1923) reported *H. carpathicum* s. str. (as subsp. *carpathicum*) from the Tatry Mts only. Later on, he enlarged the range of distribution adding the localities situated in the Nízke Tatry Mts, the Slovenské rudohorie Mts and the Veľká Fatra Mts (Zahn 1930–1939).

Hieracium fritzei group (*H. alpinum* > *H. prenanthoides*): *H. crassipedipilum* (Pawł. et Zahn) Chrtek f. 2n = 27, 36

Localities: **1**. Západné Tatry Mts, Roháče mountain group, Zuberec: Mt. Roh, ca 10.5 km ENE of Zuberec, S exp., 1550 m a.s.l., 49°14'20" N, 19°45'38" E (6784d), coll. P. Mráz and V. Jurkovičová, 22 VIII 2000 (2 plants no. 864, 2n = ca 27; 865, 2n = 27, counted by P. M.). – **2**. Nízke Tatry Mts, Iľanovo: Mt. Krakova hoľa, 1745 m a.s.l., 48°59'05.5" N, 19°37'58.5" E (7083b), coll. P. Mráz and V. Mrázová, 13 VII 2001 (1 plant no. 1072, 2n = 36, counted by P. M.). – **3**. Západné Tatry Mts, Pribylina: Gáborove sedlo saddle in the upper part of Gáborova dolina valley, 1900 m a.s.l., 49°12'05" N, 19°49'53" E (6884b), coll. P. Mráz and V. Jurkovičová, 26 VII 1999 (2 plants no. 605, 2n = ca 27; no. 610, 2n = 27, counted by P. M.).

Hieracium fritzei group (*H. alpinum* > *H. prenanthoides*): *H. slovacum* Chrtek f. 2n = 36

Locality: **1**. Belianske Tatry Mts, Ždiar: Kopské sedlo saddle, calcareous bedrock, ca 1740 m a.s.l., 49°14'23" N, 20°13'14" E (6787c), coll. P. Mráz and V. Jurkovičová, 8 VIII 2000 (1 plant no. 844, 2n = 36, counted by P. M.).

A taxonomic revision of the *H. fritzei* group was published by Chrtek & Marhold (1998). They recognized 4 taxa at the species rank in the Western Carpathians (all endemics), i.e. *H. crassipedipilum* (Pawł. et Zahn) Chrtek f., *H. krivanense* (Woł. et Zahn) Shlyakov, *H. pinetophilum* (Degen et Zahn) Chrtek f. and *H. slovacum* Chrtek f. First chromosome counts originated at the same time; Chrtek in Chrtek & Marhold (1998) found 2n = 27 in *H. pinetophilum*, and 2n = 36 in *H. crassipedipilum* and *H. slovacum*. Later on, the triploid level was confirmed in *H. pinetophilum* (Mráz 2001b, Chrtek in Štorchová et al. 2002), tetraploid in *H. slovacum* (Chrtek in Štorchová et. al. 2002) and a new chromosome number, 2n = 27, was discovered in *H. crassipedipilum* (Chrtek in Štorchová et al. 2002). The first reported chromosome number in *H. krivanense* (2n = 36) was published by Mráz (2001b), later on this ploidy level was confirmed by Chrtek (in Štorchová et al. 2002).

The chromosome numbers presented here confirm karyological differentiation in *H. crassipedipilum*, this species contains both triploids and tetraploids. Tetraploids have been found on calcareous bedrocks, while triploid on granite or calcareous bedrocks.

Apart from the Western Carpathians, members of the *Hieracium fritzei* group occur in the Eastern Carpathians and in the highest parts of the Sudeten Mts (N Czech Republic, S Poland). The *H. fritzei* group is also found in the Southern Carpathians (cf. Zahn 1930–1939, Nyárády 1965), however taxa occurring in this mountain range morphologically resemble the plants between *H. alpinum* and *H. sparsum* groups. The *H. prenanthoides* group is not very common here.

Hieracium inuloides Tausch 2n = 27 (Fig. 1)

(*H. laevigatum – H. prenanthoides*)

Localities: **1**. Nízke Tatry Mts, eastern part, Telgárt: below the saddle between Mt. Kráľova hoľa and Mt. Kráľova skala, S slopes, ca 0.5 km SE of Mt. Kráľova hoľa, ca 1600 m a.s.l., 48°53'21" N, 20°09'44" E (7186b), coll. P. Mráz, 21 VIII 1999 (1 plant no. 715, 2n = 27, counted by P. M.). – **2**. Nízke Tatry Mts, central part, Vyšná Boca: subalpine meadows W of the Kumštové sedlo saddle, *Calamagrostietum villosae*, 1650 m a.s.l., 48°55'19.1" N, 19°41'01.3" E (7084c), coll. P. Mráz and V. Mrázová, 14 VII 2001 (1 plant no. 1096, 2n = 27, counted by P. M.).

Zahn (1930–1939) recognized 6 subspecies of *H. inuloides* in the Western Carpathians, three of them were described from this area.

Our counts are the first for the Western Carpathians. The same chromosome number (2n = 27) was found by P. Mráz & Z. Szeląg (unpubl.) in plants from the Romanian Eastern Carpathians. Triploids have also been reported by Morton (1974) and Finch in Moore [1982, ut *H. latobrigorum* (Zahn) Roffey] and Jørgensen et al. (1958).

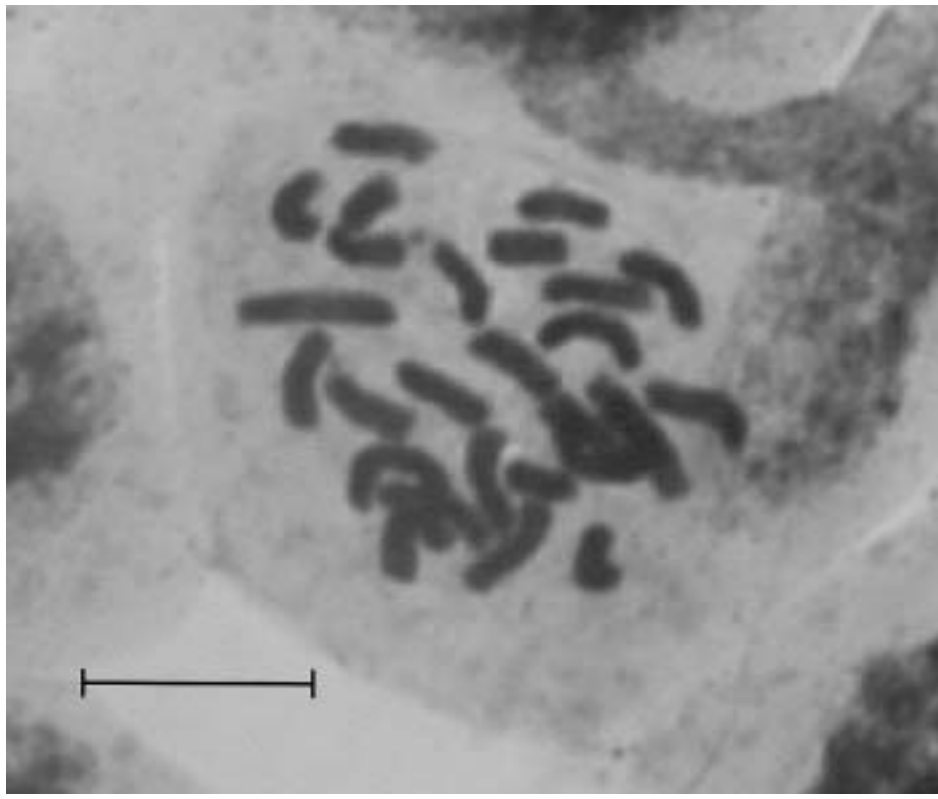

Fig. 1. – *Hieracium inuloides* Tausch, somatic metaphase, 2n = 27 (cult. no. 1096). Scale bar = 10μm.

Hieracium jurassicum Griseb. (s.l.) 2n = 27

(Syn.: *Hieracium juranum* Fr., *H. murorum* < *H. prenanthoides*)

L o c a l i t y : **1.** Nízke Tatry Mts, central part, distr. Liptovský Mikuláš: Mt. Krakova hoľa, near the marked path to the Machnaté sedlo saddle, 6.5 km S of Iľanovo, 1509 m a.s.l., 48°59'28.5" N, 19°37'23.3" E (7083b), coll. J. Chrtek jun. et al., 13 VII 2001 (3 plants no. H 780/1–3 , 2n = 27, counted by J. Ch.).

The present chromosome number is the first from the Western Carpathians. It coincides with triploids found in the Alps (Schuhwerk & Lippert 1999).

Hieracium kuekenthalianum Zahn 2n = 36

(Syn.: *Hieracium tephrosoma* Nägeli et Peter, *H. bocconei – H. villosum*)

L o c a l i t y : **1**. Nízke Tatry Mts, eastern part, Telgárt: below the saddle between Mt. Kráľova hoľa and Mt. Kráľova skala, S slopes, ca 0.5 km SE of Mt. Kráľova hoľa, ca 1600 m a.s.l., 48°53'21" N, 20°09'44" E (7186b), coll. P. Mráz, 29 VIII 1996 (1 plant no. 335, 2n = 36, counted by P. M.).

Our plants correspond to subsp. *pseudoglandulosodentatum* (Rech. f. et Zahn) (*H. tephrosoma* subsp. *pseudoglandulosodentatum* Rech. f. et Zahn), described from the Vysoké Tatry Mts. First chromosome number in this taxon.

Hieracium lachenalii Suter (s.l.) 2n = 27

Localities: **1**. Západné Beskydy Mts, Sihelné: Mt. Pilsko, rocks on the cote 1492 m, ca 1.2 km SE of the summit, 49°31'25" N, 19°19'35" E (6481d), coll. P. Mráz and V. Jurkovičová, VIII 2000 (1 plant no. 924, 2n = 27, counted by P. M.). – **2**. Volovské vrchy Mts, Rožňava: Mt. Skalisko (1293), ca 0,2 km E of the rocky summit, ca 1280 m a.s.l., 48°44'43" N, 20°34'40" E (7289c), coll. P. Mráz and V. Jurkovičová, 13 VI 2000 (2 plants: no. 760, 2n = 27; 761, 2n = ca 27, counted by P. M.).

Hieracium lachenalii represents a taxonomically very difficult entity. For most of the distribution area, it has been found to be a triploid taxon (Schuhwerk 1996, Schuhwerk & Lippert 1998). From Central Europe, the chromosome count 2n = 27 was published by Uhríková in Májovský (1974) from Slovak part of the Western Carpathians and by Krahulcová (1990) from Czech Republic. The tetraploid level (2n = 36) was reported by Lavrenko & Serditov (1987), and hypertriploid (2n = 28) by Rostovtseva (1979) (ut *H. tilingii* Juxip). The chromosome number of 2n = 27 published for allegedly *H. bifidum* from Mt Pilsko (cf. Májovský 1970a) probably relates to *H. lachenalii* s.l. (see comments on *H. bifidum* above).

Hieracium macilentum Fr. 2n = 27

(Syn.: *Hieracium epimedium* Fr., *H. bifidum* > *H. jurassicum*)

Locality: **1.** Vysoké Tatry Mts, Starý Smokovec: Veľká Studená dolina valley, near the marked path in dwarf pine stands, 3.5 km NW of the village, 1460 m a.s.l., 49°10'18" N, 20°12'00" E (6887a), coll. J. Chrtek jun. and K. Chrtková, 8VIII 2000 (3 plants no. 836/1,2,4, 2n = 27, counted by J. Ch.).

This is the first chromosome number ascertained for this species in the Western Carpathians. Our accessions are referable to subsp. *tornatoris* (Nyár. et Zahn) (*H. epimedium* subsp. *tornatoris* Nyár. et Zahn); altogether 6 subpecies were recognized by Zahn (1930–1939) in the area studied.

Previously published chromosome numbers correspond to two ploidy levels. Triplods (2n = 27) have been found in the Krkonoše Mts [*H. wimmeri* R. Uechtr., *H. epimedium* subsp. *wimmeri* (R. Uechtr.) Zahn; Chrtek 1994], in the Alps (Polatschek 1966), and in Zetland [Mills & Stace 1974, *H. zetlandicum* Beeby, *H. demissum* subsp. *zetlandicum* (Beeby) Zahn, *H. epimedium* subsp. *zetlandicum* (Beeby)].

Hieracium murorum L. (s.l.) 2n = 27

Locality: **1**. Biele Karpaty Mts, Bzince pod Javorinou: Mt. Maleník, ca 3 km NW of the village, 340 m a.s.l., 48°48'20" N, 17°45'10" E (7172d), coll. P. Mráz and V. Jurkovičová, IX 1999 (no. 724, 2n = 27, counted by P. M.).

Two ploidy levels, namely triploids and tetraploids, have been reported in this taxonomically very complex taxon (e.g. Schuhwerk 1996, and other standard reference manuals). Triploids prevail, diploids have not been found to date. Surprisingly (*H. murorum* is a widespread species), our count is the third from the Western Carpathians, the previous ones (2n = 27) were detected by Uhríková & Murín in Májovský [1970a, ut *H. silvaticum* (L.) Grufberg] and Mičieta (1978).

Hieracium nigritum R. Uechtr. 2n = 36 (Fig. 2)

(*H. fritzei – H. murorum*)

Localities: **1**. Nízke Tatry Mts, Vyšná Boca: subalpine grassland W of the Kumštové sedlo saddle, *Calamagrostietum villosae*, 1650 m a.s.l., 48°55'19.1" N, 19°41'01.3" E (7084c), coll. P. Mráz and V. Mrázová, 14 VII 2001 (1 plant no. 1097, 2n = 36, counted by P. M.). – **2**. Západné Tatry Mts, the Roháče mountain group, Zuberec: near the marked path from the Látaná dolina valley to the Zábrať saddle, open places in dwarf-pine stands, 10.5 km SEE of the village, 1520 m a.s.l., 19°45'03.5" E, 49°13'25.6" N (6784d), coll. J. Chrtek jun., 7 VII 2000 (2 plants no. H 604/1, 2, 2n = 36, counted by J. Ch.).

The first chromosome counts from the Western Carpathians. While the plants from locality 1 are identical with the nominate subspecies described from the Sudeten Mts, the plants from the Roháče Mts (loc. 2) correspond with subsp. *spalenae* Rech. f. et Zahn [peduncles and involucral bracts without simple eglandular hairs; in subsp. *nigritum* there are scattered simple eglandular hairs, both subspecies are reported to occur in the studied area, Zahn (1930–1939)]. The same chromosome number was found by Chrtek (1996) in plants of subsp. *nigritum* from the Hrubý Jeseník Mts (the Sudeten Mts, Czech Republic). In the light of the above results, the triploid count (2n = 27) of *H. nigritum* reported by Rosenberg (1926) seems to be somewhat spurious. The plant originated from a botanical garden and a misidentification cannot be excluded.

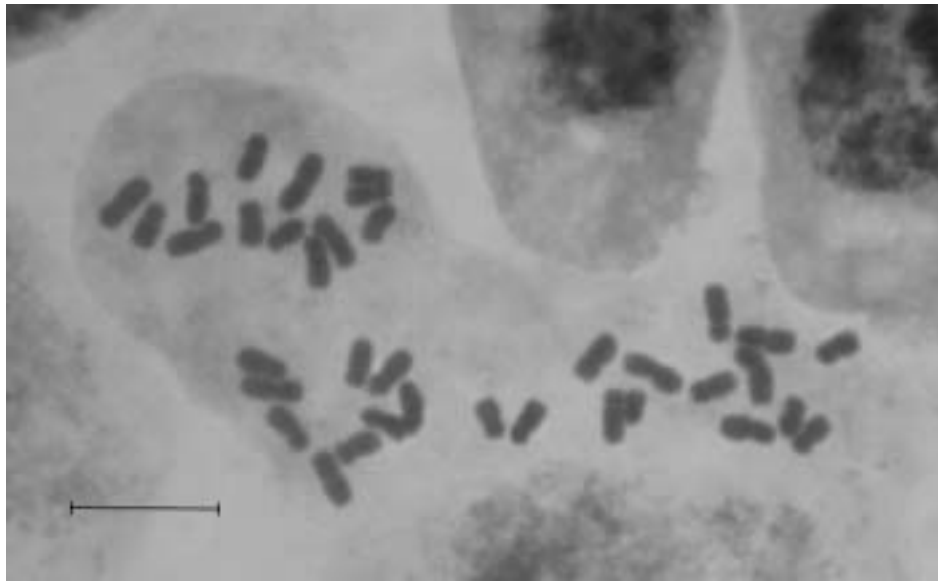

Fig. 2. – *Hieracium nigritum* R. Uechtr., somatic metaphase, 2n = 36 (cult. no. 1097). Scale bar = 10μm.

Hieracium pilosum Schleich. ex Froel. 2n = 27

(Syn.: *Hieracium morisianum* Rchb. f.)

Locality: **1**. Západné Tatry Mts, the Roháče mountain group, Zuberec: Sedlo pod Osobitou saddle, ca 0.5 km SE of Mt. Osobitá, ca 1570 m a.s.l., 49°15'30" N, 19°43'25" E (6784a), coll. P. Mráz, 23 VII 1999 (1 plant no. 579, 2n = 27, counted by P. M.).

Our report agrees with that of Moore (1982). Christoff & Popoff (1933) published a tetraploid chromosome number. Only a single locality of *H. pilosum* (ut subsp. *villosiceps* Nägeli et Peter; Mt Rozsutec in the Malá Fatra Mts) in the Western Carpathians is given by Zahn (1930–1939). Further records from subalpine belt of the Veľká Fatra Mts and the Malá Fatra Mts have been recently completed by Bernátová et al. (1995).

Hieracium praecurrens Vuk. (s.l.) 2n = 27

(*H. murorum – H. transsilvanicum*)

Locality: **1.** Vysoké Tatry Mts, Tatranská Javorina: Bielovodská dolina valley, W slopes of Mt. Holica, spruce forest, 1329 m a.s.l., 49°14'12.9" N, 20°06'42.3" E (6786d), coll. P. Mráz and V. Mrázová, 15 VIII 2001 (3 plants no. 1154, 1155, 1157, 2n = 27, counted by P. M.).

This is the first chromosome count in *H. praecurrens*, it coincides with yet unpublished report on triploids from Romania by P. Mráz & Z. Szeląg. Although five subspecies [from 27 recogized by Zahn (1930–1939)] were given from the Western Carpathians, *H. praecurren*s is a very rare species in the studied area.

Hieracium prenanthoides Vill. 2n = 27

Localities: **1**. Veľká Fatra Mts, Turecká: Mt. Krížna, S and W slopes below the top, ca 1550–1560 m a.s.l., 48°52'32" N, 19°064'40" E (7180b), coll. P. Mráz, 24 VII 1999 (1 plant no. 580, 2n = 27, counted by P. M.). – **2**. Veľká Fatra Mts, Turecká: Mt. Majerova skala, ca 1260 m a.s.l., 48°51'42" N, 19°06'25" E (7180b), coll. P. Mráz, 24 VII 1999 (1 plant no. 581, 2n = 27, counted by P. M.).

A highly critical species (species group) in the Western Carpathians. Zahn (1930–1939) distinguished 9 subspecies in the area, divided into two groups (greges), namely grex *prenanthoides* and grex *lanceolatum*. Our present plants can be placed into grex *prenanthoides*. However, we are unable to find a correct subspecific name for the plants.

Three ploidy levels, namely diploids (very rare), triploids and tetraploids, have been reported in this species (for references see e.g. Schuhwerk 1996, Chrtek 1996, and common reference manuals). Previous counts from the Western Carpathians come from the Veľká Fatra Mts (2n = 27, Uhríková in Májovský 1974), the Vysoké Tatry Mts, and the Belianske Tatry Mts (both accessions 2n = 27, Chrtek 1996).

Hieracium racemosum Waldst. et Kit. ex Willd. (s.l.) 2n = 27

Locality: **1.** Volovské vrchy Mts, Košice, Mt. Bankov, ca 4 km NW of the city centre, oak forest margin, 420 m a.s.l., 48°44'50" N, 21°13'00" E (7293c), coll. P. Mráz and V. Mrázová, 30 IX 2001 (2 plants no. 1172, 1173, 2n = 27, counted by P. M.).

The triploid chromosome number confirms the previous data from SW part of the Western Carpathians, locality Bôrik in Bratislava [firstly published by Uhríková in Májovský 1976; secondly from the same locality and with the same collector (Schwarzová) and counter (Uhríková) in Májovský 1978] and Bratislava (Hindáková in Hindáková & Májovský 1977). Ouside of the Western Carpathians, triploids were recorded in Austria (ut subsp. *leiopsis* Murr et Zahn) in a mixed population with diploids (Schuhwerk & Lippert 1999). Diploids were also reported from Italy (Selvi & Fiorini 1996). The first tetraploid chromosome count was published by Merxmüller (in Moore 1982, ut *H. crinitum* Sibth. et Sm.).

Hieracium sabaudum L. (s.l.) 2n = 27 (Fig. 3)

Localities: **1**. Volovské vrchy Mts, Košice: Mt. Bankov, ca 4 km NW of the city centre, meadow, 420 m a.s.l., 48°44'50" N, 21°13'00" E (7293c), coll. P. Mráz and V. Mrázová, 30 IX 2001 (2 plants no. 1174, 1175, 2n = 27, counted by P. M.). – **2**. Volovské vrchy Mts, Prakovce: "Walcwerk", ca 0.3 km NE of the Prakovce zastávka railway station, 395 m a.s.l., 48°53'50" N, 21°10'00" E (7191c), coll. P. Mráz, 20 X 2000 (1 plant no. 875, 2n = 27, counted by P. M.). – **3**. Volovské vrchy Mts, Jaklovce: along state route between Jaklovce and Margecany villages, ca 0.8 km SW of the Margecany catholic church, 320 m a.s.l., 48°49'50" N, 20°54'00" E (7192a), coll. P. Mráz, 16 X 2000 (1 plant no. 872, 2n = 27, counted by P. M.). – **4**. Volovské vrchy Mts, Rožňava: E slope of Mt. Sitárka, ca 4 km E of the town, 440 m a.s.l., 48°39'39" N, 20°35'14" E, coll. P. Mráz and V. Jurkovičová, 16 V 2000 (2 plants no. 738, 739, 2n = 27, counted by P. M.). – **5**. Biele Karpaty Mts, Bzince pod Javorinou: Mt. Maleník, ca 3 km NW of the village, 340 m a.s.l., 48°48'20" N, 17°45'10" E (7172d), coll. P. Mráz and V. Jurkovičová, IX 1999 (1 plant no. 723, 2n = 27, counted by P. M.).

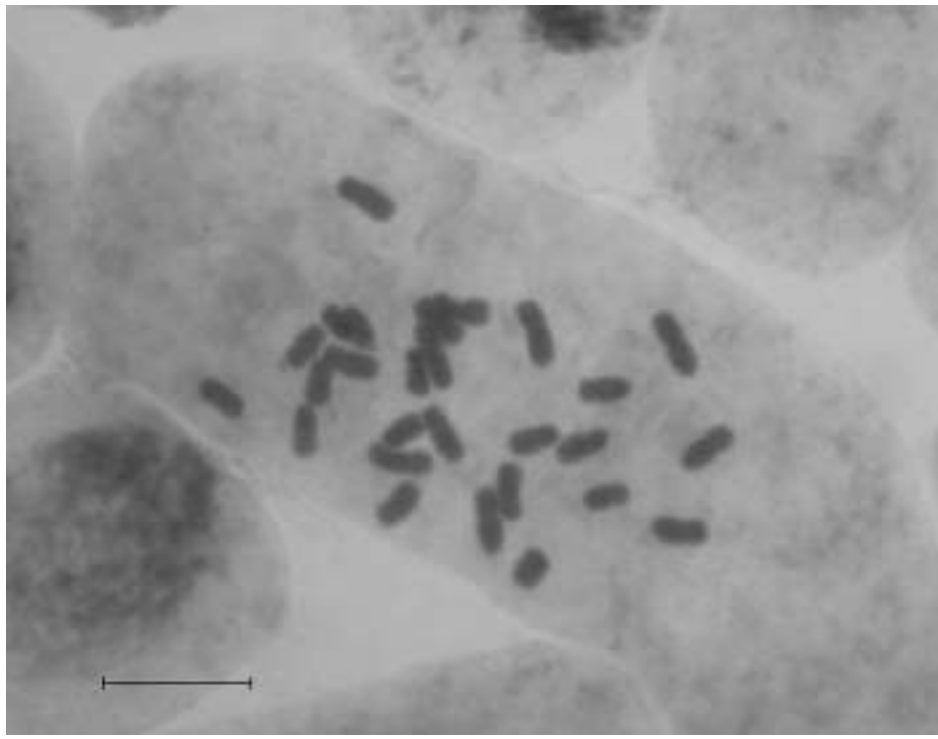

Fig. 3. – *Hieracium sabaudum* L., somatic metaphase, 2n = 27 (cult. no. 872). Scale bar = 10μm.

Morphological variation in the species (species group) is very complicated and extensive; Zahn (1930–1939) reported 11 subspecies from the Western Carpathians.

The present chromosome number (2n = 27) is in agreement with references from the Zemplínske vrchy Mts (Murín & Váchová in Májovský 1970a; the same chromosome number from the same locality, counted by the same people was also given in Májovský 1974, so we suppose that the same record was actually published twice), and the Malé Karpaty Mts (Uhríková & Králik in Májovský et al. 2000). However, diploids have also been reported from the Western Carpathians, from the SW part (Devínska Kobyla hills near Bratislava; Uhríková & Feráková 1977), and from the Považský Inovec Mts (N part, on the border with

the Strážovské vrchy Mts; Hrušovská-Osuská 1988). Májovský et al. (1987) also published the triploid counts from two mountain ranges (the Strážovské vrchy Mts and the Malé Karpaty Mts, without precise identification of the collection sites) based on reference "Feráková 1986". However, it is not given in the part References (Májovský et al. 1987).

Three ploidy levels, i.e. diploids, triploids, and tetraploids have been reported from other parts of the distribution range (for references see e.g. Schuhwerk 1996, Májovský et al. 1987, and other common chromosome number indexes). Triploids seem to prevail, geographically the nearest diploid count comes from the Pannonian lowland (Bratislava) in SW Slovakia (Feráková 1971, later treated as *H. vagum* Jordan, det. P. D. Sell, see Májovský et al. 1987), closely adjacent to the Carpathian arc.

Because the diploid level of *Hieracium sabadum* is unique within this aggregate species (see Schuhwerk 1996), the second author revised herbarium specimens deposited in SLO related to the published locality "Bratislava, Ostredky, ruderal habitat" (see above, Feráková 1971: 249). There are three specimens collected and determined by V. Feráková on 5 October 1969 as "*Hieracium sabaudum* L. subsp. *autumnale* Zahn". The one specimen with label "Bratislava, Ostredky" is morphologically undoubtedly *Hieracium umbellatum* s. str. The plant produces large amount of pollen grains of homogeneous size (observation of pollen in glycerol jelly by the second author). High amount of pollen with regular size and shape is a characteristic feature of all diploid species with normal meiosis studied so far, including *H. umbellatum* (Mráz et al. 2002). On the other hand, two other specimens belong morphologically to *H. sabaudum* s.l. Their labels read as follows "Bratislava, Ostredky, ruderálne stanovište, 2n=18" and "Bratislava, Ostredky – smetisko pri cintoríne". Both plants produce substantially lower amount of variably sized pollen typical of polyploid taxa (tri- and tetraploids studied so far, Mráz et al. 2002). Thus, just one possible and logical explanation of the published diploid chromosome number for *H. sabadum* (later treated as *H. vagum*, see above) is that *H. umbellatum* was counted instead of *H. sabaudum* s.l. (both taxa occurred at the locality). The fourth specimen was sent to P. D. Sell (V. Feráková, in lit.), who revised it as *H. vagum.* It belongs with high probability to *H. sabadum* s.l. The remaining diploid counts from Slovakia (see above: Devínská Kobyla and Považský Inovec) also probably refer to *H. umbellatum*.

Hieracium sparsum group: *H. silesiacum* Krause 2n = 36 (Fig. 4)

[Syn.: *Hieracium sparsum* subsp. *silesiacum* (Krause) Zahn

L o c a l i t i e s : **1**. Vysoké Tatry Mts, Štrbské Pleso: along the marked path on the S slopes of Mt. Kriváň, ca 1 km E of Mt. Jamy (1572.2), ca 1500–1550 m a.s.l., 49°08'10" N, 20°00'30" E (6886b), coll. P. Mráz and V. Mrázová, 16 VIII 2001 (2 plants no. 1159, 1160, 2n = 36, counted by P. M.). – **2**. Západné Tatry Mts, the Roháče mountain group, Zuberec: open place in dwarf-pine stand near a marked tourist path in the Smutná dolina valley, ca 150 m of the former Ťatliakova chata chalet, 11.5 km SE of Zuberec, 1410 m a.s.l., 49°12'45" N, 19°45'00" E (6784c), coll. J. Chrtek jun. (2 plants H 618/1, 2, 2n = 36, counted by J. Ch.). – **3**. Nízke Tatry Mts, central part: Vyšná Boca, Mt. Lajštroch (1602,1), E slope ("Rovienky"), 2,7 km SW of the village, open place in dwarf-pine stand near a marked tourist path, 1550 m a.s.l., 48°54'38" N, 19°43'12" E (7084c), 28VII 1999, coll. J. Chrtek jun. (2 plants, H 614/1,2, 2n = 36, counted by J. Ch.). – **4.** Nízke Tatry Mts, central part, Vyšná Boca: subalpine meadows W of the Kumštové sedlo saddle, *Calamagrostietum villosae*, ca 1630 m a.s.l., 48°55'19.1" N, 19°41'01.3" E (7084c), coll. P. Mráz and V. Mrázová, 14 VII 2001 (1 plant no. 1094, 2n = 36, counted by P. M.).

The investigated plants come from the both main distribution areas of *H. silesiacum* in the Western Carpathins, i.e. from the Tatry Mts (accessions no. 1, 2), and from the Nízke Tatry

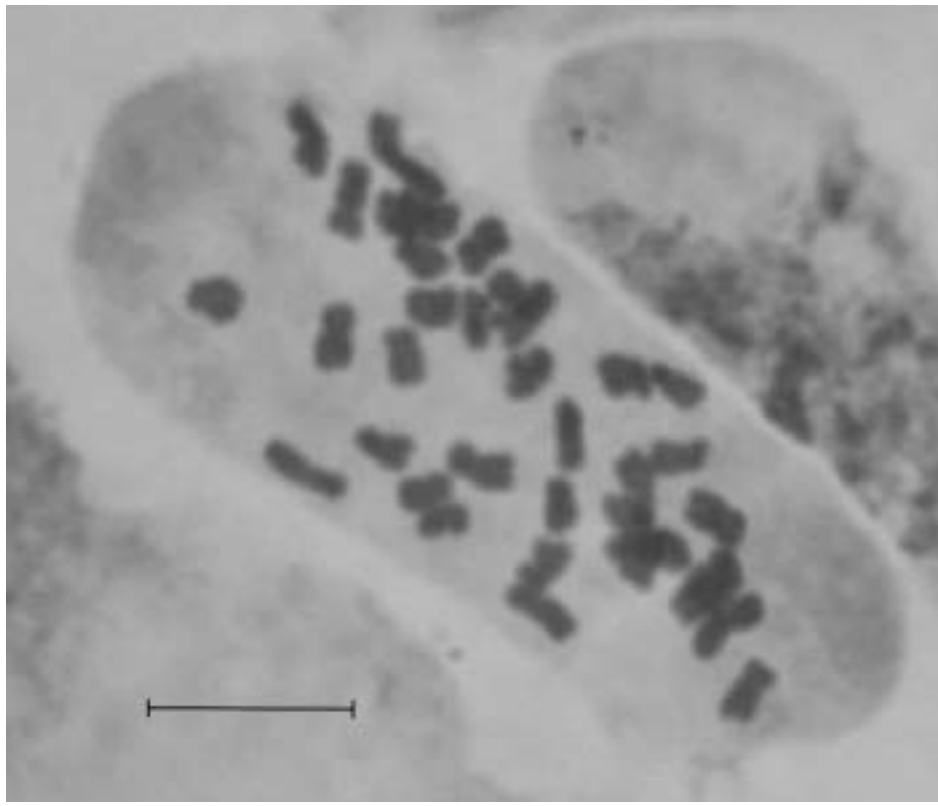

Fig. 4. – *Hieracium silesiacum* Krause, somatic metaphase, 2n = 36 (cult. no. 1160). Scale bar = 10μm.

Mts (accessions no. 3, 4). Comprehensive list of localities along with comments on ecology and morphological variation of the species in the Western Carpathians was published elsewhere (Chrtek et al. 2002).

The same chromosome number has been found in plants from the Hrubý Jeseník Mts (the Sudeten Mts, Czech Republic; Chrtek 1996). It seems that *H. silesiacum* is the only known tetraploid species from the *Hieracium sparsum* group, the remaining taxa are either diploid (*H. sparsum* s. str.) or triploid (Christoff 1942, Schuhwerk & Lippert 1999, Vladimirov & Szeląg 2001).

Hieracium umbellatum L. 2n = 18

L o c a l i t y : **1**. Volovské vrchy Mts, Prakovce: "Walcwerk", ca 0.3 km NE of the Prakovce zastávka railway station, 395 m a.s.l., 48°53'50" N, 21°10'00" E (7191c), coll. P. Mráz, 20 X 2000 (1 plant no. 873, 2n = 18, counted by P. M.).

Both sexual diploids (2n = 18) and apomictic triploids (2n = 27) are known in this species (for references see e.g. Schuhwerk 1996, Májovský al. 1987, and other standard chromosome number indexes). Surprisingly, the present accession is the only third from the Western Carpathians; it confirms the previously published diploid counts from the Devínska

Kobyla hill near Bratislava (Uhríková & Feráková 1977) and from another site at Prakovce village (Volovské vrchy Mts) (Mráz 2003c).

Hieracium villosum Jacq. 2n = 27

L o c a l i t i e s : **1**. Veľká Fatra Mts, Liptovské Revúce: Mt. Čierny kameň (1479.4), calcareous rocks on the south peak, 3 km NW of the village, 1450 m a.s.l., 48°55'55" N, 19°08'20" E (7080d), coll. M. Severa, 6 VIII 1999 (3 plants, MS 20/1–3 , 2n = 27, counted by M. S.). – **2**. Malá Fatra Mts, Terchová: Mt. Steny (between Mt. Hromové and Mt. Poľudňový grúň), calcareous rocks E and SE of the south peak, 7 km SSW of the village, 1520 m a.s.l., 49°11'45" N, 19°03'47" E (6880a), coll. M. Severa, 4 VIII 1999 (4 plants MS 2/1–4, 2n = 27, counted by M. S.). – **3**. Malá Fatra Mts: Zázrivá: Mt. Veľký Rozsutec (1610), calcareous rocks at the peak, 6.3 km SW of the village, 1590 m a.s.l., 49°13'55" N, 19°05'55" E (6780d), coll. M. Severa, 4 VIII 1999 (3 plants, MS 4/1–3 , 2n = 27, counted by M. S.). – **4**. Veľká Fatra Mts, Vyšná Revúca: Mt. Suchý vrch (1549), calcareous rocks at the peak, 5.7 km W of the village, 1540 m a.s.l., 48°54'35" N, 19°06'57" E (7080d), coll. M. Severa, 6 VIII 1999 (4 plants, MS 5/1–4, 2n = 27, counted by M. S.). – **5**. Nízke Tatry Mts, central part, Závažná Poruba: Mt. Krakova hoľa (1751), rocky outcrops (limestones) ca 1 km SSE of the peak, ca 8 km S of the village, ca 1600 m a.s.l., 48°58'37" N, 19°38'17" E (7083b), coll. M. Severa, 4 VII 2000 (3 plants, MS 14/1–3 , 2n = 27, counted by M. S.). – **6**. Nízke Tatry Mts, central part, Nižná Boca: Mt. Ohnište (1538), calcareous rocks S of the peak, 5.5 km NW of the village, 1520 m a.s.l., 48°58'28" N, 19°42'20" E (7084a), coll. M. Severa 5 VII 2000 (3 plants, MS 15/1–3 , 2n = 27, counted by M. S.). – **7**. Belianske Tatry Mts, Tatranská Kotlina: Mt. Skalné vráta (1619,8 m), calcareous rocks near a small path above the chalet Plesnivec, 3 km W of the village, ca 1450 m a.s.l., 49°13'39" N, 20°16'42" E (6787d), coll. M. Severa & J. Chrtek jun., 6 VII 2000 (11 plants, MS 16A/1–11, 2n = 27, counted by M. S.). – **8**. Belianske Tatry Mts, Ždiar: Monkova dolina valley, near the marked path 4.2 km SW of the village, 1500 m a.s.l., 49°14'55" N, 20°13'33" E (6787c), coll. M. Severa & J. Chrtek jun., 6 VII 2000 (2 plants, MS 17/1,2, 2n = 27, counted by M. S.).

Our samples refer to *H. villosum* subsp. *villosum*. *Hieracium villosum* belongs, together with its intermediate taxa, to the most interesting group of mountain hawkweeds in the Western Carpathians. Based on morphology, chromosome numbers and multilocus isozyme genotypes, 5 types were recognized within *H. villosum* in the target area (Severa 2001). Nevertheless, some of them might perhaps be placed in *H. pilosum*. The most widespread type, identical with *H. villosum* subsp. *villosum*, was shown to be an apomictic triploid (2n = 27) with aborted pollen; no genetic variation was discovered. The remaining types are apomictic tetraploids (2n = 36) with viable pollen grains, each confined to rather small geographic areas. Both intra- and interpopulation genetic variation was detected in two of them, the remainig two are homogeneous in this respect (Severa 2001). Whether they are worth recognizing taxonomically, and if so, at what ranks, are moot points.

Tetraploid *H. villosum* has been reported from several localities in the Western Carpathians (Skalińska et al. 1959, Murín in Murín & Paclová 1979, Murín in Murín & Májovský 1987). Outside of our area tetraploid counts also strongly prevail (for references see Schuhwerk 1996). Aneuploids (2n = ca 28) were reported by Polatschek (1966) from the Alps (but see notes in Dobeš & Vitek 2000).

Hieracium virgicaule Nägeli et Peter 2n = 27

(*H. bupleuroides – H. umbellatum*)

L o c a l i t y : **1.** Chočské vrchy Mts, Prosiek: Prosiecka dolina valley, 684 m a.s.l., 48°59'38.8" N, 19°37'20.7" E (6882b), coll. P. Mráz and V. Mrázová, 13 VIII 2001 (1 plant no. 1085, 2n = 27, counted by P. M.).

First karyological report for this species, which is considered to be endemic to the Western Carpathians. Its occurrence, based on a single locality in the Eastern and Southern Carpathians, respectively, is doubtful (cf. Zahn 1930–1939).

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Souhrn

Západokarpatské jestřábníky z podrodu *Hieracium* (*Hieracium* subgen. *Hieracium*) jsou zatím karyologicky málo známé. V tomto příspěvku jsou poprvé uvedeny počty chromozomů u druhů *Hieracium kuekenthalianum* (= *H. tephrosoma*, 2n = 36), *H. praecurrens* (2n = 27) a *H. virgicaule* (2n = 27); u *H. bupleuroides* byla poprvé zjištěna tetraploidní úroveň (2n = 36), všechny doposud publikované údaje se vztahují k triploidním rostlinám (2n = 27). U několika druhů jsou poprvé zveřejněny počty chromozomů ze Západních Karpat; jedná se o *H. atratum* (2n = 27), *H. bifidum* (2n = 27, 36), *H. carpathicum* (2n = 36), *H. inuloides* (2n = 27), *H. jurassicum* (2n = 27), *H. macilentum* (= *H. epimedium*, 2n = 27), *H. nigritum* (2n = 36), *H. pilosum* (= *H. morisianum*, 2n = 27) a *H. silesiacum* (2n = 36). Dříve publikované počty byly potvrzeny u *H. alpinum* (s. str., 2n = 27), *H. bupleuroides* (2n = 27), *H. crassipedipilum* (okruh *H. fritzei*, 2n = 27, 36), *H. lachenalii* (2n = 27), *H. murorum* (2n = 27), *H. prenanthoides* (2n = 27), *H. racemosum* (2n = 27), *H. sabaudum* (2n = 27), *H. slovacum* (okruh *H. fritzei*, 2n = 36), a *H. umbellatum* (2n = 18). Celkově příspěvek přináší počty chromozomů 22 druhů v širším smyslu (species collectivae sensu Zahn, okruhy). Při zahrnutí druhů v užším pojetí rozlišovaných v okruhu *H. fritzei* se údaje vztahují k 23 druhům, v některých případech jsou počty vztaženy ke spolehlivě rozlišovaným subspeciím. V současné době jsou se zahrnutím údajů z této práce známy počty chromozomů u 28 druhů v širším pojetí (okruhů) z celkového počtu 65 druhů uváděných ze Západních Karpat. Téměř všechny studované taxony jsou polyploidní (tri- a tetraploidní), diploidní počet byl zjištěn pouze u *Hieracium umbellatum*. Publikovaná data o diploidních populacích *H. sabaudum* ze Slovenska jsou zřejmě mylná a vztahují se s největší pravděpodobností k velice proměnlivému a sexuálně se rozmnožujícímu druhu *H. umbellatum*. U mnoha druhů v širším pojetí (okruhů) jsou v Západních Karpatech známy 2 ploidní úrovně (tri- a tetraploidi). Karyologická diferenciace často odpovídá taxonomicky významným morfologickým znakům a podporuje spíše užší pojetí druhů přijímané u některých okruhů.

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Appendix 1. – Chromosome numbers in the genus *Hieracium* s. str. published from the Western Carpathians (and from adjacent region of the Pannonian lowland – marked by *). Nomenclature follows original references.

Taxon	Chromosome number	Reference
H. alpinum L.	27	Skalińska et al. 1959
H. alpinum L.	27	Skawińska 1963
H. alpinum L.	27	Májovský et al. 1970b
H. alpinum L.	27	Murín & Májovský 1992
H. alpinum L.	27	Chrtek 1997
H. alpinum L.	36	Szeląg & Jankun 1997
H. alpinum L.	27	Mráz 2001b
H. alpinum L.	27	Štorchová et al. 2002
H. alpinum L.	27	this study
H. atratum Fr.	27	this study[1]
H. bifidum Kit. ex Hornem.	27	Májovský et al. 1970a
H. bifidum Kit. ex Hornem.	27, 36	this study
H. bupleuroides C. C. Gmel.	27	Májovský et al. 1970a[2]
H. bupleuroides C. C. Gmel.	27, 36	this study[3]
H. carpathicum Bess.	36	this study
H. crassipedipilum (Pawł. et Zahn) Chrtek f. (*H. fritzei* group)	36	Chrtek & Marhold 1998
H. crassipedipilum (Pawł. et Zahn) Chrtek f. (*H. fritzei* group)	27	Štorchová et al. 2002
H. crassipedipilum (Pawł. et Zahn) Chrtek f. (*H. fritzei* group)	27, 36	this study
H. halleri Vill. (*H. alpinum* group)	27	Chrtek 1997
H. halleri Vill. (*H. alpinum* group)	27	Mráz 2001b
H. halleri Vill. (*H. alpinum* group)	27	Štorchová et al. 2002
H. inuloides Tausch	27	this study
H. jurassicum Griseb. (s.l.)	27	this study
H. krivanense (Woł. et Zahn) R. N. Shlyakov (*H. fritzei* group)	36	Mráz 2001b
H. krivanense (Woł. et Zahn) R. N. Shlyakov (*H. fritzei* group)	36	Štorchová et al. 2002
H. kuekenthalianum Zahn	36	this study[4]
H. lachenalii subsp. *sciaphilum* Zahn	27	Májovský et al. 1974
H. lachenalii Suter (s.l.)	27	this study
H. lingelsheimii Pax	36	Schuhwerk & Lippert 1999
H. macilentum Fr.	27	this study[5]
H. murorum L.[6] (s.l.)	27	Mičieta 1978
H. murorum L.[6] (s.l.)	27	this study
H. nigrescens Willd. (s.l.)	36	Mráz 2001b[7]
H. nigrescens subsp. *koprovanum* Rech.f. et Zahn	36	Mráz 2001b
H. nigritum R. Uechtr.	36	this study[8]
H. pilosum Schleich. ex Froel.	27	this study
H. pinetophilum (Degen et Zahn) Chrtek f. (*H. fritzei* group)	27	Chrtek & Marhold 1998
H. pinetophilum (Degen et Zahn) Chrtek f. (*H. fritzei* group)	27	Mráz 2001b
H. pinetophilum (Degen et Zahn) Chrtek f. (*H. fritzei* group)	27	Štorchová et al. 2002
H. piliferum Hoppe	36	Mráz 2003a

H. praecurrens Vuk. (s.l.)	27	this study
H. prenanthoides subsp. *lanceolatum* (Vill.) Zahn	27	Májovský et al. 1974
H. prenanthoides Vill.	27	Chrtek 1996
H. prenanthoides Vill.	27	this study[9]
H. rohacsense Kit.	36	Mráz 2001b
H. racemosum Waldst. et Kit. ex Willd.	27	Májovský et al. 1976
H. racemosum Waldst. et Kit. ex Willd.	27	Hindáková & Májovský 1977
H. racemosum Waldst. et Kit. ex Willd. (s.l.)	27	this study
H. sabaudum L.	27	Májovský et al. 1970a
H. sabaudum L.	18	Feráková 1971*
H. sabaudum L. subsp. *sabaudum*	27	Májovský et al. 1974
H. sabaudum L.	18	Uhríková & Feráková 1977[10]
H. sabaudum agg.	18	Hrušovská-Osuská 1988[10]
H. sabaudum L.	27	Májovský et al. 2000
H. sabaudum L. (s.l.)	27	this study
H. silesiacum Krause (*H. sparsum* group)	36	this study
H. slovacum Chrtek f. (*H. fritzei* group)	36	Chrtek & Marhold 1998
H. slovacum Chrtek f. (*H. fritzei* group)	36	this study
H. stygium R. Uechtr. (*H. chlorocephalum* group)	36	Chrtek 1996
H. stygium R. Uechtr. (*H. chlorocephalum* group)	36	Mráz 2001b
H. sylvaticum (L.) Grufberg (= *H. murorum* L.)	27	Májovský et al. 1970a
H. umbellatum L.	18	Májovský et al. 1970a
H. umbellatum L.	18	Uhríková & Feráková 1977
H. umbellatum L.	18	Mráz 2003c
H. umbellatum L.	18	this study
H. valdepilosum Vill. (s.l.)	36	Mráz 2003c[11]
H. villosum Jacq.	36	Skalińska et al. 1959
H. villosum Jacq.	36	Murín & Paclová 1979
H. villosum Jacq.	36	Murín & Májovský 1987
H. villosum Jacq.	27	this study[12]
H. virgicaule Nägeli et Peter	27	this study

[1] At least two names can be accepted for plants from the Western Carpathians (both seem to be synonyms): *H. atratum* subsp. *atrellum* var. *furkotanum* Zahn and var. *greineri* Korb et Zahn. See notes in the text part.

[2] This report is related, with high probability to *H. lachenalii* s.l. See notes on *H. bifidum* in the text part.

[3] The triploid counts are referable to two subspecies: subsp. *tatrae* (Griseb.) Nägeli et Peter and subsp. *gmelinianum* Zahn. See notes in the text part.

[4] Plants from the Western Carpathians belong to subsp. *pseudoglandulosodentatum* (Rech. f. et Zahn). See notes in the text part.

[5] The counted plants belong to subsp. *tornatoris* (Nyár. et Zahn).

[6] See also *H. sylvaticum.*

[7] Two conspecific names can be used for the counted plants: *H. pietroszense* subsp. *jarzabcynum* Pawł. et Zahn (cf. Mráz 2003b) and *H. nigrescens* subsp. *mlynicae* Hruby et Zahn.

[8] The counts comprise two distinct taxa: *H. nigritum* subsp. *nigritum* and *H. nigritum* subsp. *spalenae* Rech. f. et Zahn. See notes in the text part.

[9] Our plants can be placed into grex (group of subspecies) *prenanthoides.*

[10] This report is related, with high probability to *H. umbellatum.* See notes on *H. sabaudum* in the text part.

[11] Based on herbarium revision by J. Chrtek jun., the plants belong to the *H. dentatum* group.

[12] The plants belong to subsp. *villosum.*

Príloha I

Rotreklová O, Krahulcová A, Mráz P, Mrázová V, Mártonfiová L, Peckert T, Šingliarová B

Chromosome numbers and breeding systems in some species of *Hieracium* subgen *Pilosella* from Europe

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Chromosome numbers and breeding systems of some European species of *Hieracium* subgen. *Pilosella*

Počty chromozomů a způsoby reprodukce některých evropských druhů jestřábníků (*Hieracium* subgen. *Pilosella*)

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Chromosome numbers (ploidy levels) were recorded in the following 25 taxa of *Hieracium* subgen. *Pilosella*: *H. arvicola* Nägeli et Peter (2n = 45), *H. aurantiacum* L. (2n = 36, 45), *H. bauhini* Besser (2n = 36, 45), *H. bifurcum* M. Bieb. (2n = 45), *H. brachiatum* Bertol. ex DC. (2n = 36, 45), *H. caespitosum* Dumort. (2n = 36), *H. cymosum* L. (2n ~ 4x), *H. densiflorum* Tausch (2n = 36, ~ 4x), *H. echioides* Lumn. (2n = 18, 45), *H. fallacinum* F. W. Schultz (2n = 36, 45), *H. floribundum* Wimm. et Grab. (2n = 36, ~ 4x, 45,), *H. glomeratum* Froel. in DC. (2n = 45), *H. iseranum* Uechtr. (2n = 36), *H. kalksburgense* Wiesb. (2n ~ 5x), *H. lactucella* Wallr. (2n = 18), *H. macranthum* (Ten.) Ten. (2n = 18), *H. onegense* (Norrl.) Norrl. (2n = 18), *H. pilosella* L. (2n = 36, 45, 54), *H. piloselliflorum* Nägeli et Peter (2n = 45), *H. pilosellinum* F. W. Schultz (2n = 36, 45), *H. piloselloides* Vill. (2n = 27, 36, ~ 4x, 45, ~ 5x), *H. pistoriense* Nägeli et Peter (2n = 27), *H. rothianum* Wallr. (2n ~ 3x), *H. schultesii* F. W. Schultz (2n = 36, 45, ~ 5x), *H. zizianum* Tausch (2n = 27, 36, 54), and one hybrid, *H. onegense* × *H. pilosella* (2n = 36). Besides chromosome counts in root-tip meristems, flow cytometry was used to determine the DNA ploidy level in 83 samples of 9 species. The presence of a long marker chromosome was confirmed in tetraploid *H. caespitosum* and *H. iseranum*, in pentaploid *H. glomeratum*, and in both tetraploid and pentaploid *H. floribundum*. The documented mode of reproduction is sexual (*H. densiflorum*, *H. echioides*, *H. piloselloides*) and apomictic (*H. brachiatum*, *H. floribundum*, *H. pilosellinum, H. piloselloides, H. rothianum, H. zizianum*). *Hieracium bifurcum* and *H. pistoriense* are sterile. The chromosome number and/or mode of reproduction of *H. bifurcum* (almost sterile pentaploid), *H. pilosellinum* (apomictic pentaploid), *H. piloselloides* (apomictic triploid), *H. pistoriense* (sterile triploid), *H. rothianum* (apomictic triploid) and *H. zizianum* (apomictic triploid) are presented here for the first time. The sexual reproduction recorded in the pentaploid *H. echioides* is the second recorded case of this mode of reproduction in a pentaploid cytotype of *Hieracium* subgenus *Pilosella*. A previously unknown occurrence of *H. pistoriense* (*H. macranthum* – *H. bauhini*) in Slovakia is reported.

Keywords: *Compositae*, Czech Republic, DNA ploidy level, flow cytometry, France, Germany, Hungary, Italy, karyology, Poland, reproduction mode, Slovakia, Slovenia

Introduction

Hieracium subgen. *Pilosella* shows a great variability in morphology, karyology and breeding systems. Variability in chromosome number relative to mode of reproduction is reviewed in detail by Krahulcová et al. (2000). A brief résumé is also published by Rotreklová et al. (2002). Evidently a detailed knowledge of chromosome number (ploidy) and mode of reproduction of taxa is essential for understanding speciation differentiation within both subgenera of the genus *Hieracium*, i.e. *Pilosella* (Krahulec et al. 2004) and *Hieracium* (Chrtek et al. 2004). The putative origin of the facultatively apomictic species *H. floribundum*, *H. glomeratum*, *H. iseranum* and *H. piloselliflorum* by spontaneous natural hybridization, as discussed by Fehrer et al. (2005), can serve as an example. The possible origin of these hybridogeneous species was suggested, based on molecular, karyological and breeding system data augmented by the results of crosses between the putative parental species.

Here we present data, which resulted from broader parallel studies on *Hieracium* subgen. *Pilosella,* e.g. cytogeography of *H. bauhini* and *H. pilosella* as the source of variability in *Hieracium* subgen. *Pilosella* at various geographic scales.

In addition to new karyological data for seven species, this paper includes records of the chromosome numbers and mode of reproduction of 19 species of *Hieracium* subgen. *Pilosella* published in recent years for plants from other parts of their distribution area (Vladimirov & Szeląg 2001, Rotreklová et al. 2002, Schuhwerk & Lippert 2002, Mráz & Szeląg 2004).

Materials and methods

Plants were collected in 1996–2003 from natural habitats and cultivated in pots in the Botanical Garden of Masaryk University, Brno (O. R.), the Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice (A. K., T. P.) and the Botanical Garden of P. J. Šafárik University, Košice (P. M.). Root tip cuttings of mature plants were used for chromosome counts. Methods used to count chromosomes are described in Rotreklová et al. (2002). A PA-I (Department of Botany, Masaryk University, Brno) and a PA-II ploidy analyzers (Institute of Botany, Průhonice), both produced by Partec GmbH, Münster, Germany and equipped with HBO-100 mercury arc lamps, were used for the flow-cytometric detection of DNA ploidy level (i.e. the relative DNA content) in some cases. Sample preparations were carried out in two stages (Otto 1990, Doležel & Göhde 1995). Stem tissues of a particular plant and a reference standard (0.5 cm^2 of leaf blade) were chopped with a new razor blade for about 20 s in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (4.2 g citric acid monohydrate + 1 ml 0.5% Tween 20 adjusted to 200 ml and filtered through a 0.22 μm filter), then 0.5 ml more Otto I buffer was added. The solution was filtered through nylon cloth (50 μm mesh size). For DNA staining, 2 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$) including DAPI (4´,6-diamidino-2-phenylindole; 4 μg/ml final concentration) was used. As a reference standard for DNA ploidy level analyses, the diploid *Hieracium lactucella* (O. R.) or *Zea mays* (A. K.) was used. The breeding system was determined by comparing the seed set of open-pollinated and emasculated capitula: emasculated capitula of sexual plants produce no seed while both emasculated and open-polli-

nated capitula of apomictic plants produce seed. Capitula were emasculated by cutting off the upper half of a capitulum with a razor blade before anthesis.

Voucher specimens are deposited in the herbarium of the Department of Botany, Masaryk University, Brno (BRNU, plants examined by O. R.), in the herbarium of the Institute of Botany, Průhonice (PRA, plants examined by A. K. and T. P.) and in the Herbarium of P. Mráz, recently deposited at the Institute of Biology and Ecology, Košice.

Results and discussion

It is important to stress that the chromosome numbers given here (2n = number) are based on counting chromosomes, and do not provide the same information as the DNA ploidy level (2n ~ multiple of x) determined by flow cytometry. Other findings indicate that in the subgenus *Pilosella* (A. Krahulcová et al., unpublished) aneuploidy and/or different DNA contents of the parental genomes (even at the same ploidy level) of hybrids can slightly change the relationship between the chromosome number and the DNA ploidy level of an individual. This is particularly true for high-ploidy hybrids, i.e. for those with *H. pilosella* as one of the parents.

Hieracium subgen. *Pilosella* is divided into two species groups, referred to as basic and intermediate. Members of the latter are considered to be of hybrid origin and are intermediate between putative parental taxa, names of which are indicated at the beginning of each species' entry. This position is based solely on morphological characters and follows the evaluation of Zahn (1930) and Nägeli & Peter (1885).

Although most of the analyzed taxa belong to the so called intermediate or hybridogeneous species, some of them may be declared as nothotaxa, e.g. real hybrids of recent origin (F1 or very early generations). This is indicated by the rarity of these plants in the field, seed sterility, and co-occurrence with the parents. Survey of detected chromosome numbers and reproductive systems is given in Table 1.

Hieracium arvicola Nägeli et Peter, Die Hieracien Mittel-Europas 1: 666, 1885. 2n = 45

H. bauhini / H. piloselloides – H. caespitosum

Locality: **1.** Czech Republic, distr. Brno: village of Kuřim, railway station, 290 m a.s.l., 49°18'04" N 16°32'01" E, plants growing together with pentaploid *H. bauhini* (Rotreklová 2004), coll. O. Rotreklová, 9 June 1998, det. S. Bräutigam, 2n = 45 (1 plant), counted by O. Rotreklová.

The first record of a pentaploid cytotype from the Czech Republic augments a single reference to tetraploids occurring in Bavaria, Germany (Schuhwerk & Lippert 1997).

Hieracium aurantiacum L., Sp. Pl. 801, 1753. 2n = 36, 2n = 45

Localities: **1.** Czech Republic, Krušné hory Mts, distr. Sokolov: grassy place in a former tin mine 3.5 km NNE of the village of Přebuz, 890 m a.s.l., 50°24'00" N, 12°30'00" E, coll. F. Krahulec, 8 June 2000, 2n = 36 (2 plants), counted by A. Krahulcová. **2.** Slovakia, Belianske Tatry Mts: Predné Meďodoly valley, ca 1480 m a.s.l., 49°13'30" N, 20°14'30" E, coll. P. Mráz & V. Jurkovičová, 8 August 2000, 2n = 45 (1 plant, no. 855), counted by P. Mráz.

The species *H. aurantiacum* forms a polyploid series. Tetraploid plants (2n = 36), which are the most common, are confirmed here for the Krušné hory Mts, and pentaploid plants (2n = 45) for Slovakia (e.g. Rotreklová et al. 2002 and the references therein). In Bavaria,

Table 1. – List of species of *Hieracium* subgen. *Pilosella* included in this study, their chromosome numbers and breeding systems. Abbreviations of countries, from which particular cytotypes originated: Cz = Czech Republic, Sk = Slovakia, Hu = Hungary, Ge = Germany, Pl = Poland, Slo = Slovenia, It = Italy, Fr = France.

Species	Country	2n	Breeding system
H. arvicola Nägeli et Peter	Cz	45	
H. aurantiacum L.	Cz	36	
	Sk	45	
H. bauhini Besser	Sk	36, ca 45	
	Cz	45	
H. bifurcum M. Bieb.	Cz	45	almost sterile
H. brachiatum Bertol. ex DC.	Cz	45	apomictic
	Sk	36, 45	
	Slo	45	
H. caespitosum Dumort.	Ge, Pl	36	
H. cymosum L.	Sk	36	
H. densiflorum Tausch	Cz	36	sexual
H. echioides Lumn.	Cz	18, 45	both sexual
	Sk	18	sexual
	Pl, Hu	18	sexual
H. fallacinum F. W. Schult	Cz	36, 45	
H. floribundum Wimm. et Grab.	Cz	36	apomictic
	Pl	36, 45	
H. glomeratum Froel. in DC.	Cz, Pl	45	
H. iseranum Uechtr.	Pl	36	
H. kalksburgense Wiesb.	Cz	45	
H. lactucella Wallr.	Cz, Sk	18	
H. macranthum (Ten.) Ten.	Sk	18	
H. onegense (Norrl.) Norrl.	Sk	18	
H. onegense × *H. pilosella*	Sk	36	
H. pilosella L.	Pl, Ge	36	
	Cz	36, 45	
	Sk	45, 54	
H. piloselliflorum Nägeli et Peter	Pl	45	
H. pilosellinum F. W. Schultz	Cz	36	
		45	apomictic
H. piloselloides Vill.	Cz	36	apomictic
	Ge	27, 36, 45	all apomictic
	Sk, Hu, It	36	
	Slo	36	sexual
		45	apomictic
H. pistoriense Nägeli et Peter	Sk	27	sterile
H. rothianum Wallr.	Cz	27	apomictic
H. schultesii F. W. Schultz	Cz	36	
	Sk	45	
	Pl	45	
H. zizianum Tausch	Sk	36	
	Fr	27	apomictic
		54	

Germany, two hexaploid plants of *H. aurantiacum* (2n = 54), in addition to the tetraploid cytotype (Schuhwerk & Lippert 2002), were recently recorded. Both tetraploid (2n = 36) and pentaploid (2n = 45) plants are recorded in Romania (Mráz & Szeląg 2004).

Hieracium bauhini Besser, Prim. Fl. Galic. 2: 149, 1809. 2n = 36, 2n = 45

Localities: **1.** Slovakia, Tríbeč Mts: Klátová Nová Ves village, Sádok quarter, Chríb Hill, by the 13th century Romanian-Gothic church, 250 m a.s.l., 48°33'30" N, 18°16'10" E, coll. P. Mráz, V. Mrázová & R. Mráz, 2 November 2001, 2n = ca 45 (1 plant, no. 1181), counted by P. Mráz. **2.** Slovakia, Slovenský kras Mts, Plešivecká planina plateau: Plešivec town, on the forest road ca 0.5 km NE of Zbojnícka priepasť chasm, ca 3.5 km NNE of the railway station Plešivec, 545 m a.s.l., 48°34'22" N, 20°25'09" E, coll. P. Mráz, 30 May 2001, 2n = 36 (1 plant, no. 941), counted by P. Mráz. **3.** Slovakia, Slovenský kras Mts, Silická planina plateau: Silická Brezová village, ca 0.2 km SE of elevation marker Delené (500.7), ca 490 m a.s.l., 48°33'32" N, 20°24'55" E, coll. P. Mráz, 13 June 2001, 2n = ca 36 (1 plant, no. 964), counted by L. Mártonfiová. **4**. Slovakia, Slovenský kras Mts, Silická planina plateau: Brzotín village, Brzotínska skala Mt, ca 2 km SSE of the village, 620 m a.s.l., 48°33'10" N, 20°30'35" E, coll. P. Mráz, 28 May 2002, 2n = 36 (1 plant, no. 1189), counted by P. Mráz. **5.** Slovakia, Rožňavská kotlina basin: Krásnohorské Podhradie village, W slopes of the hill Krásna Hôrka, 423 m a.s.l., 48°34'35" N, 20°35'45" E, coll. P. Mráz, 29 May 2002, 2n = 36 (1 plant, no. 1194), counted by P. Mráz. **6.** Slovakia, Čierna hora Mts, Košice city: Kavečany quarter, in the Zoological garden grounds, 461 m a.s.l., 48°47'19.7" N, 21°12'01.7" E, coll. P. Mráz & V. Mrázová, 28 July 2001, 2n = 36 (1 plant, no. 1101), counted by P. Mráz. **7.** Czech Republic, Brno: SW margin of the city, along the road between city-parts Nový Lískovec and Kohoutovice, 49°11'02" N, 16°32'38" E, 350 m a.s.l., coll. O. Rotreklová, 29 June 2004, 2n = 45 (1 plant), counted by O. Rotreklová.

Tetraploid, pentaploid and hexaploid plants are recorded in this species (see Rotreklová 2004 for detailed references). Our data confirm the distribution pattern of particular cytotypes within Central Europe published by Rotreklová (2004): tetraploids occur commonly in the eastern part (Slovakia and Hungary), and pentaploids prevail in the western part (the Czech Republic, Germany, Belgium, Netherlands). In terms of morphology, all tetraploid plants belong to the *H. magyaricum* species group and the pentaploid plant from the Czech Republic to the *H. bauhini* species group (sensu Zahn 1930).

Hieracium bifurcum M. Bieb., Fl. Taur.-Caucas. 2: 251, 1808. 2n = 45, almost sterile.

H. echioides < *H. pilosella*

Localities: **1.** Czech Republic, distr. Znojmo: Hnanice village, vineyard of Šobes ca 2 km N of village, slope above the vineyard above the red tourist path, 270 m a.s.l., 48°49'08" N 15°58'26" E, coll. T. Peckert, 16 June 2001, 2n = 45 (4 plants), almost sterile (2 plants), counted by T. Peckert. **2.** Czech Republic, distr. Znojmo: rockery in the forest along the blue tourist path 4 km SE of the church in the Podmolí village, 340 m a.s.l., 48°49'18" N 15°58'66" E, coll. T. Peckert, 16 June 2001, 2n = 45 (2 plants), almost sterile (1 plant), counted by T. Peckert.

Only pentaploids (2n = 45) are known for this species from Austria (Schuhwerk & Lippert 1997). Our records are the first for the Czech Republic. The sterility of pentaploids was not previously reported for this species.

Hieracium brachiatum Bertol. ex DC. in Lam. et DC., Fl. Franc., ed. 3, 5: 442, 1815. 2n = 36; 2n = 45, apomictic

H. pilosella > *H. bauhini/H. piloselloides*

Localities: **1.** Czech Republic, distr. Znojmo: village of Přímětice, ca 330 m a.s.l., 48°53'57" N 16°03'16" E, coll. V. Grulich, 15 May 2000, 2n = 45, apomictic (1 plant), counted by O. Rotreklová. **2.** Czech Republic, distr. Blansko: Rozseč nad Kunštátem, crossroads 0.5 km N of the church in the village, 630 m a.s.l., 49°31'41" N 16°27'50" E, coll. M. Kočí, July 2000, 2n = 45, apomictic (2 plants), counted by O. Rotreklová. **3.** Czech Republic, distr. Slavkov u Brna: Kobeřice village, meadow on the SE margin of the village, 320 m a.s.l., 49°05'22" N

16°53'25" E, coll. Z. Lososová, 2 June 2002, 2n = 45, (1 plant), counted by O. Rotreklová. **4.** Czech Republic, Brno: SW margin of the city, along the road between city-parts Nový Lískovec and Kohoutovice, 49°11'02" N, 16°32'38" E, 350 m a.s.l., coll. O. Rotreklová, 29 June 2004, 2n = 45 (2 plants), counted by O. Rotreklová. Plants grew together with *H. pilosella* and pentaploid *H. bauhini* (see above). **5.** Slovakia, Slovenský kras Mts, Silická planina plateau: Silica village, xerotherm meadow ca 0.5 km SE of the village, red tourist path, 560 m a.s.l., 48°33'27" N, 20°31'55" E, coll. P. Mráz & V. Jurkovičová, 17 May 2000, 2n = 36, (1 plant, no. 742), counted by P. Mráz. **6.** Slovakia, Rožňavská kotlina basin: Krásnohorské Podhradie village, W slopes of the hill Krásna Hôrka, 423 m a.s.l., 48°34'35" N, 20°35'45" E, coll. P. Mráz, 29 May 2002, 2n = 45 (1 plant, no. 1196), counted by P. Mráz. **7.** Slovenia, Juliske Alps Mts: pasture on the NW margin of the Stara Fužina village, 580 m a.s.l., 46°17'30" N, 13°53'25" E, coll.V. Grulich, 18 June 1998, 2n = 45 (1 plant), counted by O. Rotreklová.

Pentaploids and an apomictic mode of reproduction are most frequently reported in this taxon and confirmed by our results. Pentaploids (2n = 45) occur in Germany (Bräutigam & Bräutigam 1996, Schuhwerk & Lippert 1997), the Czech Republic and Bulgaria (Rotreklová et al. 2002). Recently, Schuhwerk & Lippert (2002) recorded a triploid (2n = 27) and a tetraploid (2n = 36) plant from Bavaria in Germany. However, the few detailed population studies reveal a great variability in chromosome number, including high ploidy levels and aneuploidy (Germany: 2n = 36, 45, 63, Bräutigam & Bräutigam 1996; the Czech Republic: 2n = 45, 48, 63, 72, Krahulcová et al. 2000, Rotreklová et al. 2002). Recently, a hybrid swarm between a pentaploid *H. bauhini* and a tetraploid *H. pilosella* was studied in Bavaria, Germany (Schuhwerk & Lippert 2002). At this locality, pentaploid *H. brachiatum*, *H. pilosellinum* (2n = 45) and heptaploid *H. leptophyton* (2n = 63) were detected. Whereas both *H. brachiatum* and *H. leptophyton* are thought to be hybrids between *H. pilosella* and *H. bauhini*, the genome of *H. pilosellinum* possibly contains a third basic species, *H. cymosum* (Schuhwerk & Fischer 2003). As only one or two plants from each population were examined in our study, the high variation in chromosome number within populations was not detected. The tetraploid (2n = 36) and pentaploid (2n = 45) plants from Slovakia and pentaploid (2n = 45) plants from Slovenia are the first chromosome counts for *H. brachiatum* from those countries.

Hieracium caespitosum Dumort., Fl. Belg. 62, 1827.
2n = 36, with a long marker chromosome

[Syn. *Hieracium pratense* Tausch]

L o c a l i t i e s : **1.** Germany, Saxonia, East Lusatian Hills: former brown coal mine area near the village Schönau-Berzdorf, in the artificial valley of the Pliessnitz River between the heaps and in the periphery of the village near a new road, 200–230 m a.s.l., 51°03' N, 14°54' E, coll. F. Krahulec & A. Krahulcová (excursion during the 4th Hieracium Workshop), 3 June 2000, 2n = 36, with a long marker chromosome (2 plants), counted by A. Krahulcová. **2.** Poland, Góry Izerskie Mts: Velká Jizerská louka meadow close to Czech – Polish border, 7 km SSE of the town of Swieradów Zdrój, 830 m a.s.l., 50°51'00" N, 15°21'40" E, coll. F. Krahulec (excursion during the 4th Hieracium Workshop), 4 June 2000, 2n = 36, with a long marker chromosome (1 plant), counted by A. Krahulcová.

A pentaploid *H. caespitosum* detected using flow cytometry is recorded for the Saxonian locality no. 1 (Bräutigam & Bräutigam 1996). Tetraploid and pentaploid plants of *H. caespitosum* commonly occur (Krahulcová & Krahulec 1999 and the references therein, Fehrer et al. 2005). In addition, a rare triploid cytotype (2n = 27) is recorded from Poland (Skalińska 1967) and Bavaria in Germany (Schuhwerk & Lippert 2002). A long marker chromosome, which is strikingly larger than other chromosomes in the karyotype, was first observed in pentaploid plants of *H. cespitosum* from New Zealand (Jenkins & Jong 1997) and latter in some other species from the Czech Republic (Krahulcová & Krahulec 1999).

Hieracium cymosum L., Sp. Pl., ed. 2, 2: 1126, 1763. 2n ~ 4x

Locality: **1.** Slovakia, distr. Banská Bystrica: meadow 1km SW of the church in the Donovaly village, 970 m a.s.l., 48°53'17" N, 19°13'01" E, coll. O. Rotreklová & Z. Lososová, 26 June 2001, 2n ~ 4x (1 plant), flow cytometry analysis done by O. Rotreklová.

Although considerable variation from diploid to heptaploid is reported in the literature, the diploid (2n = 18) and the tetraploid (2n = 36) cytotypes are the most frequent. Diploids are reported from the Czech Republic (Měsíček & Javůrková-Jarolímová 1992, Šimek 2000), Germany (Schuhwerk & Lippert 1997, 2002) and Greece (Schuhwerk & Lippert 1997). Tetraploids are reported from Armenia (Nazarova 1984), Austria (Schuhwerk & Lippert 1997), Italy (Gadella & Kliphuis 1970a), the Netherlands (Gadella 1984), Poland (Vladimirov & Szeląg 2001), Romania (Mráz & Szeląg 2004) and Greece (Grau & Erben 1988). Other ploidy levels are more rare, e.g. triploid (2n = 27, Austria: Schuhwerk & Lippert 1997) and pentaploid (2n = 45, Germany: Schuhwerk & Lippert 1997, Macedonia: Schuhwerk & Lippert 1998 and Slovakia: Májovský et al. 2000). Hexaploids (2n = 54) are reported from Greece (Strid & Anderson 1985) and Poland (Vladimirov & Szeląg 2001). Both hexaploid and heptaploid plants are recorded by Gadella & Kliphuis (1970b) from the French Alps.

Hieracium densiflorum Tausch, Flora, Regensburg, 11, Ergänzungsbl. 1: 59, 1828.
2n = 36, 2n ~ 4x, sexual

H. bauhini – H. cymosum

Localities: **1.** Czech Republic, distr. Břeclav: Růžový kopec Hill 1 km NW of the town of Mikulov, ca 260 m a.s.l., 48°49'19" N, 16°37'21" E, coll. O. Rotreklová, 6 May 2000, 2n = 36 (2 plants), counted by O. Rotreklová. **2.** Czech Republic, distr. Břeclav: slope 2 km ENE of the church in the village of Borkovany, 240 m a.s.l., 49°02'10" N, 16°49'40" E, coll. O. Rotreklová & Z. Lososová, 14 May 2001, 2n = 36 (2 plants), sexual (1 plant), counted by O. Rotreklová; 2n ~ 4x, sexual (1 plant), flow cytometry analysis done by O. Rotreklová. **3.** Czech Republic, distr. Hodonín: Čejkovické Špidláky 2 km NE of the castle in the village of Čejkovice, 230 m a.s.l., 48°55'04" N, 16°57'42.8" E, coll. T. Vymyslický, 30 May 2001, 2n = 36, sexual (1 plant), counted by O. Rotreklová (Fig. 1c). **4.** Czech Republic, distr. Vyškov: Nature reserve Visengrunty 1.3 km SSW of the church in Bošovice village, 340 m a.s.l., 49°02'33" N, 16°49'42" E, coll. O. Rotreklová & Z. Lososová, 8 June 2001, 2n = 36, sexual (1 plant), counted by O. Rotreklová. **5.** Czech Republic, distr. Břeclav, Dunajovické kopce Hills: Jánská hora Hill, 2.7 km WSW of the church in Dolní Dunajovice village, 265 m a.s.l., 48°50'46" N, 16°33'25" E, coll. O. Rotreklová & J. Danihelka, 21 May 2002, 2n ~ 4x, sexual (3 plants), flow cytometry analysis done by O. Rotreklová. **6.** Czech Republic, distr. Slavkov u Brna: slopes 0.75 km SW of the church in Rašovice village, 250 m a.s.l., 49°07'07"N, 16°56'24"E 3 June 2002, coll. O. Rotreklová, 2n ~ 4x (3 plants), flow cytometry analysis done by O. Rotreklová.

Ploidy levels from triploid (2n = 27) to hexaploid (2n = 54) are published for this species. The chromosome number 2n = 36 is recorded for Germany (Schuhwerk & Lippert 1997), the Czech Republic and Slovenia (Rotreklová et al. 2002). A pentaploid ploidy level is recorded for Germany (Schuhwerk & Lippert 1997) and hexaploid one for Greece (Schuhwerk & Lippert 1998). In Bavaria, Germany, Schuhwerk & Lippert (2002) detected triploids (2n = 27), pentaploids (2n = 45) and hexaploids (2n = 54). In the Czech Republic, this species occurs mainly on dry grasslands in southern Moravia; in Bohemia, it occurs in the vicinity of Prague and of Kadaň, as well as in the České středohoří Mts. The chromosome numbers of Bohemian plants is unknown. The tetraploid plants (2n = 36) recorded in Moravian populations (Rotreklová et al. 2002, five localities) together with the new records presented here, indicate that *H. densiflorum* in Moravia is karyologically uniform. A sexual mode of reproduction, already reported in tetraploid *H. densiflorum* (Rotreklová et al. 2002), is confirmed.

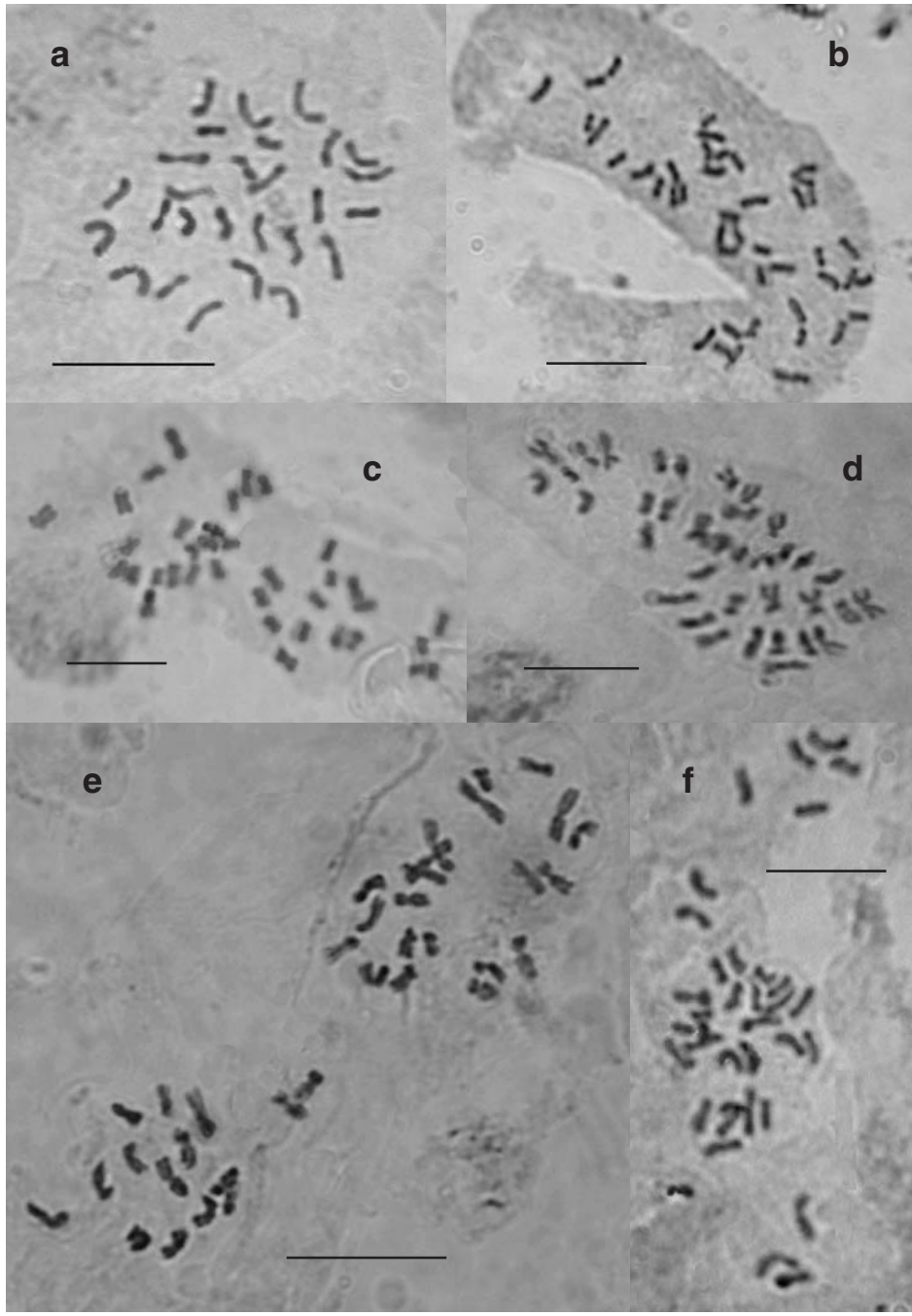

Fig. 1. – Microphotographs of somatic metaphases of six species of *Hieracium* subgen. *Pilosella*. a: *Hieracium pistoriense*, 2n = 27, Slovakia, distr. Nitra, village of Pohranice; b: *H. pilosellinum*, 2n = 45, Czech Republic, distr. Slavkov u Brna, village of Rašovice; c: *H. densiflorum*, 2n = 36, Czech Republic, distr. Hodonín, Čejkovické Špidláky; d: *H. piloselloides*, 2n = 36, Slovenia, Kranjska Gora, Planica valley; e: *H. pilosellinum*, 2n = 36, Czech Republic, distr. Břeclav, Dunajovické kopce Hills, Jánská hora Hill; f: *H. fallacinum*, 2n = 36, Czech Republic, distr. Břeclav, Dunajovické kopce Hills, Jánská hora Hill. [Scale bars = 10 μm]

Hieracium echioides Lumn., Fl. Poson. 348, 1791.
2n = 18, 2n ~ 2x; 2n = 45, both cytotypes sexual

Localities: **1.** Czech Republic, distr. Znojmo: Havranické vřesoviště heath, SW part Staré vinice, ca 1 km W of the village, 330 m a.s.l., 48°48'41" N, 14°05'44" E, coll. T. Peckert & J. Chrtek, September 2001 and May 2003, 2n = 45, sexual (3 plants), counted by T. Peckert. **2.** Czech Republic, distr. Mělník: village of Tišice, the sands along the railway Tišice – Neratovice, ca 100 m from railway station Tišice, 160 m a.s.l., 50°15'57"N, 14°33'07"E, coll. T. Peckert, 25 July 2002, 2n ~ 2x (8 plants), sexual (2 plants), flow cytometry analysis done by T. Peckert. **3.** Czech Republic, distr. Litoměřice: Radobýl Hill, 3 km W of the center of the town of Litoměřice, 350 m a.s.l., 50°13'44"N, 14°05'34"E, coll. T. Peckert &. J. Chrtek, 31 May 2003, 2n ~ 2x, sexual (2 plants), flow cytometry analysis done by T. Peckert. **4.** Slovakia, distr. Malacky: Borský Mikuláš village, a pinewood along the road from Borský Mikuláš village to Šaštín village, 200 m a.s.l., 48°37'49" N, 17°11'24" E, coll. T. Peckert & J. Chrtek, 11 June 2002, 2n ~ 2x (8 plants), sexual (2 plants), flow cytometry analysis done by T. Peckert. **5.** Slovakia, Zemplínske vrchy Mts: Streda nad Bodrogom village, ca 1 km SE from the centre of the village, loco dicto Veterné piesky in the massive of Tarbucka Hill, 140 m a.s.l., 48°22'40" N, 21°47'02" E, coll. P. Mráz & V. Mrázová, 16 April 2001, 2n = 18 (1 plant, no. 918), 2n = ca 18 (1 plant, no. 919), counted by L. Mártonfiová. **6.** Slovakia, Zemplínske vrchy Mts.: Streda nad Bodrogom village, Tarbucka Hill (277 m), the sands on the north hillside, 180 m a.s.l., 48°22'27" N, 21°46'59" E, coll. T. Peckert & J. Chrtek, 12 June 2001, 2n ~ 2x (14 plants), sexual (2 plants), flow cytometry analysis done by T. Peckert. **7.** Poland, distr. Tarnobrzeg: Skorocice village, the nature reserve near the village, 210 m a.s.l., 50°29' N, 20°50' E, coll. T. Peckert, J. Chrtek & Z. Szeląg, 11 June 2001, 2n ~ 2x, sexual (1 plant), flow cytometry analysis done by T. Peckert. **8.** Poland, town of Sandomierz: "Góry Peprzove", slope above the Wisła River, ca 200 m a.s.l., 50°42' N, 21°48' E, coll. T. Peckert, J. Chrtek & Z. Szeląg, 11 June 2001, 2n ~ 2x (4 plants), sexual (2 plants), flow cytometry analysis done by T. Peckert. **9.** Poland, town of Przemyśl, sandy ditch along the road from town of Przemyśl to the Ukrainian border, 210 m a.s.l., 49°47' N, 22°50' E, coll. T. Peckert, J. Chrtek & Z. Szeląg, 11 June 2001, 2n ~ 2x (11 plants), sexual (2 plants), flow cytometry analysis done by T. Peckert. **10.** Hungary, Bakony Mts.: Fenyőfő village, the sands on the south-west border of the village, ca 320 m a.s.l., 47°17' N, 17°45' E, coll. T. Peckert & J. Chrtek, 9 July 2002, 2n ~ 2x (16 plants), sexual (2 plants), flow cytometry analysis done by T. Peckert.

In this species the ploidy range is from diploids to pentaploids (see Rotreklová et al. 2002 for a detailed survey). In this paper, a pentaploid cytotype (2n = 45) is the first reported for the Czech Republic. This is the second case of sexuality in pentaploid plants of *Hieracium* subgen. *Pilosella* (the first was published for pentaploid *H. pilosella,* Rotreklová et al. 2002). The diploid level (2n = 18) is the first record of this cytotype in *H. echioides* from Slovakia.

Hieracium fallacinum F. W. Schultz, Arch. Fl. Fr. Allem. 1: 56, 1844.
2n = 36, 2n ~ 4x, 2n = 45

H. densiflorum ≥ *H. pilosella*

Locality: **1.** Czech Republic, distr. Břeclav: Dunajovické kopce Hills, Jánská hora Hill, 2.7 km WSW of the church in the village of Dolní Dunajovice, 265 m a.s.l., 48°50'46" N, 16°33'25" E, coll. O. Rotreklová & J. Danihelka, 21 May 2002. At this locality *H. pilosella*, tetraploid *H. densiflorum* (see above) and additional plants of likely hybrid origin coexist. In total, six plants from three intermediate morphologically different clones were collected and cultivated. While the plants from clone 1 (closer to *H. pilosella* in morphology) corresponded to *H. pilosellinum* (see below), the plants from clone 2 and 3 (closer to *H. densiflorum)* corresponded to *H. fallacinum.* Tetraploid and pentaploid were detected in clone 2 (2n ~ 4x, 1 plant; 2n = 45, 1 plant) and only tetraploids in clone 3 (2n = 36, 2 plants, Fig. 1f). The chromosome counts and flow cytometry analysis done by O. Rotreklová.

Chromosome numbers for species of 2n = 45 and 2n = 54, are reported from Germany (Schuhwerk & Lippert 1997, 2002). This is the first record of tetraploids in this taxon and first karyological records from the Czech Republic.

Hieracium floribundum Wimm. et Grab., Fl. Siles. 2/2: 204, 1829.
2n = 36, 2n ~ 4x, apomictic; 2n = 45, with a long marker chromosome in both cytotypes

H. caespitosum > *H. lactucella*

Localities: **1.** Czech Republic, distr. Blansko: Rudice village, quarry in the SW margin of the village, 490 m a.s.l., 49°20'03" N, 16°43'06" E, coll. Z. Lososová, 24 May 2002, 2n = 36, with a long marker chromosome (3 plants), 2n ~ 4x (1 plant), apomictic (3 plants). The chromosome counts and flow cytometry analysis done by O. Rotreklová. **2.** Poland, Góry Izerskie Mts: near the settlement of Orle 3.5 km W of the village of Jakuszyce, 830 m a.s.l., 50°48'45" N, 15°23'50" E, coll. F. Krahulec (excursion during the 4th Hieracium Workshop), 4 June 2000, 2n = 45, with a long marker chromosome (1 plant), counted by A. Krahulcová. **3.** Poland, Lower Silesia, Massife of Ślęza: near N border of the preserve "Łąka Sulistrowicka", 6.5 km S of the town of Sobótka, 285 m a.s.l., 50°50'40" N, 16°43'30" E, coll. P. Kwiatkowski, 6 June 2001, 2n = 36, with a long marker chromosome (2 plants), counted by A. Krahulcová.

The tetraploid cytotype is the most common in this species in Central Europe (Rotreklová et al. 2002 and the references therein). The pentaploid cytotype is recorded for the Carpathians (Slovakia, Veľká Fatra Mts: Rotreklová et al. 2002) and "Bayerischer Wald" (Bavaria in Germany: Schuhwerk & Lippert 2002). The present record of a pentaploid *H. floribundum*, together with that of a tetraploid from the Góry Izerskie Mts by Rotreklová et al. (2002), suggests that both tetraploids and pentaploids coexist in this mountain range. At least, both cytotypes occur at locality no. 1, because the tetraploid *H. floribundum*, detected by flow cytometry, was reported by Fehrer et al. (2005) from the same area and locality, named Karlstal. The occurrence of triploid plants in Scandinavia, reported by Turesson & Turesson (1963), was recently confirmed for Sweden, Öland Island (Schuhwerk & Lippert 2002). The tetraploid chromosome number (2n = 36) is recorded here for plants from the type locality (Shlyakov 1989) in Lower Silesia (locality no. 3).

Hieracium glomeratum Froel. in DC. , Prodr. 7, 1: 207, 1838.
2n = 45, with a long marker chromosome

H. caespitosum – H. cymosum

Localities: **1.** Czech Republic, Krušné hory Mts, distr. Sokolov: grassy place in a former tin mine 3.5 km NNE of the village of Přebuz, 890 m a.s.l., 50°24'00" N, 12°30'00" E, coll. F. Krahulec, 8 June 2000, 2n = 45, with a long marker chromosome (1 plant), counted by A. Krahulcová. **2.** Poland, Góry Izerskie Mts: Velká Jizerská louka meadow close to Czech – Polish border, 7 km SSE of the town of Świeradów-Zdrój, 830 m a.s.l., 50°51'00" N, 15°21'40" E, coll. F. Krahulec (excursion during the 4th Hieracium Workshop), 4 June 2000, 2n = 45, with a long marker chromosome (1 plant), counted by A. Krahulcová.

The pentaploid chromosome number recorded here confirms one of two ploidy levels (tetraploid and pentaploid) previously recorded for this species (for an overview, see e.g. Krahulcová & Krahulec 1999, additional original counts in Rotreklová et al. 2002, Fehrer et al. 2005). Both cytotypes were recently recorded in Bavaria, Germany (Schuhwerk & Lippert 2002). The occurrence of pentaploid *H. glomeratum* (detected by flow cytometry) in the Góry Izerskie mountain range is also reported by Fehrer et al. (2005).

Hieracium iseranum Uechtr. in Fiek, Fl. Schles. 261, 1881.
2n = 36, with a long marker chromosome

H. floribundum > *H. pilosella*

Localities: **1.** Poland, Góry Izerskie Mts: near the settlement of Orle 3.5 km W of the village of Jakuszyce, 830 m a.s.l., 50°48'45" N, 15°23'50" E, coll. F. Krahulec (excursion during the 4th Hieracium Workshop), 4 June 2000, 2n = 36, with a long marker chromosome (1 plant), counted by A. Krahulcová.

The first report of the chromosome number of *H. iseranum* was for populations in the Krkonoše Mts, where all the plants are tetraploid (2n = 36) (Krahulcová & Krahulec 1999) except for one pentaploid (2n = 45) (Krahulcová et al. 2001). Tetraploid *H. iseranum* is also reported by Fehrer et al. (2005) from the same locality in Poland (the Góry Izerskie Mts, Karlstal), as well as from Germany (Upper Lusatia and the Erzgebirge). However, ploidy level was determined using flow cytometry and the presence of the characteristic marker chromosome not confirmed.

Hieracium kalksburgense Wiesb., General-Doublett.-Verz. Schles. Bot. Tauschver. 21: sine pago, 1883. 2n ~ 5x

[Syn. *H. canum* Peter, *H. laschii* Zahn]
H. cymosum < *H. pilosella*

Locality: **1.** Czech Republic, distr. Znojmo: vineyard Šobes 2.4 km WNW of the church in the Hnanice village, 280 m a.s.l., coll. T. Vymyslický, June 2002, 2n ~ 5x (1 plant), flow cytometry analysis done by O. Rotreklová. The plants coexisted with both putative parents, *H. cymosum* and *H. pilosella.*

The first chromosome numbers for this taxon, i.e. diploid (2n = 18), tetraploid (2n = 36) and pentaploid (2n = 45), were published for Germany by Schuhwerk & Lippert (1997, 2002). At one of the Bavarian localities in Germany, the tetraploid *H. kalksburgense* occurred with both putative parents, *H. pilosella* and a tetraploid *H. cymosum* (Schuhwerk & Lippert 2002). Our record is the first for the Czech Republic.

Hieracium lactucella Wallr., Sched. Crit. 1: 408, 1822. 2n = 18

Localities: **1.** Czech Republic, Brdy Hills, distr. Beroun: in the periphery of the military area ca 10 km SSW of the town of Hořovice, at the roadside in the spruce forest 0.5 km S of the settlement of Dolní Kvaň, 520 m a.s.l., 49°45'10" N, 13°51'00" E, coll. J. Hadinec, R. Hlaváček & Z. Skála, 13 June 2001, 2n = 18 (1 plant), counted by A. Krahulcová. **2.** Slovakia, Volovské vrchy Mts: Úhorná village, on the forest route from the Panské sedlo saddle to Mt Biele skaly, ca 2.3 km NW of the village, 1100 m a.s.l., 48°43'19" N, 20°39'01" E, coll. P. Mráz, 14 June 2002, 2n = 18 (1 plant, no. 1212), counted by P. Mráz. **3.** Slovakia, Volovské vrchy Mts: Vyšný Klátov village, Mt Predná Holica, meadow below the Lajoška chalet, 900 m a.s.l., 48°45'47" N, 21°04'38" E, coll. P. Mráz, 9 June 2002, 2n = 18 (1 plant, no. 1205), counted by P. Mráz. **4**. Slovakia, Veľká Fatra Mts, distr. Banská Bystrica: Baník Hill 1.1 km SSW of the church in Donovaly village, 1055 m a.s.l., 48°52'50" N, 19°13'30" E, coll. O. Rotreklová & Z. Lososová, 26 June 2001, 2n = 18 (2 plants), counted by O. Rotreklová.

The diploid chromosome numbers presented here confirm the constant ploidy level previously recorded for this sexual species (see Rotreklová et al. 2002 for supplementary data and references).

Hieracium macranthum (Ten.) Ten., Fl. Napol. 5: 190, 1836. 2n = 18

Localities: **1.** Slovakia, distr. Nitra: Pohranice village, SW slope of the Kolíňanský vrch Hill, 270 m a.s.l., 48°19'00" N, 18°09'00" E, coll. O. Rotreklová & P. Eliáš jr., 6 September 2002, 2n = 18 (1 plant), counted by O. Rotreklová. **2.** Slovakia, Biele Karpaty Mts: Lubina village, part Miškech dedinka, ski course on the southern slope of Chríb Hill, 430 m a.s.l., 48°48'20" N, 17°41'20" E, coll. P. Mráz & V. Jurkovičová, 30 April 2000, 2n = 18 (1 plant no. 733), counted by V. Mrázová, 2n = 18 (1 plant no. 734), counted by B. Šingliarová. **3.** Slovakia, Malé Karpaty Mts: Nové Mesto nad Váhom, ca 1.5 km WNW of the city centre, on SE slope of Mt Rovenec, meadow in the garden of Mr. J. Jurkovič, 250 m a.s.l., 48°48'05" N, 17°49'20" E, coll. P. Mráz, 27 May 2001, 2n = 18 (1 plant, no. 929), counted by P. Mráz. **4.** Slovakia, Tribeč Mts: Klátová Nová Ves village, Sádok quarter, Chríb Hill, by the 13th century Romanian-Gothic church from, 250 m a.s.l., 48°33'30" N, 18°16'10" E, coll. P. Mráz, V. Mrázová & R. Mráz, 2 November 2001, 2n = 18 (1 plant, no. 1183), counted by P. Mráz. **5.** Slovakia, Slanské vrchy Mts: Slanská Huta village, ca 2 km SE of the village, pasture on the margin of beech forest, 552 m a.s.l., 48°35'07.5" N,

21°28'16.6" E, coll. P. Mráz & V. Mrázová, 5 August 2001, 2n = 18 (1 plant, no. 1121), counted by B. Šingliarová. **6.** Slovakia, Slanské vrchy Mts: Slanská Huta village, on the mountain ridge between Veľký Milič Mt and Malý Milič Mt, on the path in the beech forest, 770 m a.s.l., 48°34'52.2" N, 21°27'52.3" E, coll. P. Mráz and V. Mrázová, 5 August 2001, 2n = ca 18 (1 plant, no. 1122), counted by B. Šingliarová. **7.** Slovakia, Zemplínske vrchy Mts: Ladmovce village, Nature reserve Kašvár, ca 0.3 km W of the elevation marker Šomoš (215.7), ca 200 m a.s.l., 48°24'55" N, 20°46'05" E, coll. P. Mráz & V. Mrázová, 16 April 2001, 2n = 18 (1 plant, no. 908), counted by P. Mráz & L. Mártonfiová. **8.** Slovakia, Zemplínske vrchy Mts: Streda nad Bodrogom village, ca 1 km SE from the village center, *loco dicto* Veterné piesky in the massive of Tarbucka Hill, 140 m a.s.l., 48°22'40" N, 21°47'02" E, coll. P. Mráz & V. Mrázová, 16 April 2001, 2n = 18 (1 plant, no. 920), counted by B. Šingliarová.

Gadella & Kliphuis (1972) report the occurrence of diploid *H. macranthum* in Macedonia. Our results confirm those published for Slovakia by Uhríková & Májovský (1980) and Feráková (in Májovský et al. 1987). The tetraploid plant reported by Raimondo et al. (1983) probably belongs to another polyploid taxon of the *H. pilosella* agg.

Hieracium onegense (Norrl.) Norrl. in T. Sael. et al., Herb. Mus. Fenn., ed. 2, 1: 118, 1889 [cit. sec. Shlyakov 1989: 351]. 2n = 18

[Syn. *H. caespitosum* subsp. *brevipilum* (Nägeli et Peter) P. D. Sell; *H. pratense* subsp. *silvicola* Zahn]

Locality: **1.** Slovakia, Volovské vrchy Mts: Smolnícka Huta village, meadow ca 0.5 km SW from the centre of the village, 550 m a.s.l., 48°44'20" N, 20°46'50" E, coll. P. Mráz & J. Mráz, 22 October 2000, 2n = 18 (1 plant, no. 879), counted by P. Mráz & L. Mártonfiová.

Our result confirmed the occurrence of this diploid taxon in Slovakia (Rotreklová et al. 2002).

H. onegense × *H. pilosella* 2n = 36

Localities: **1.** Slovakia, Volovské vrchy Mts: Smolnícka Huta village, meadow ca 0.5 km SW from the centre of the village, 550 m a.s.l., 48°44'20" N, 20°46'50" E, coll. P. Mráz & J. Mráz, 22 October 2000, rev. by J. Chrtek jr., 2n = 36 (1 plant, no. 884), counted by P. Mráz. The plants occur at the locality together with one of the putative parents, a diploid *H. onegense* (see above).

The tetraploid count presented here is the first for Slovak populations. Up till now, only the pentaploid level (2n = 45) has been reported for the related hybridogeneous taxon, *H. flagellare* subsp. *tatrense* Nägeli et Peter, from the Tatra Mts in S Poland (Skalińska 1967).

Hieracium pilosella L., Sp. Pl. 800, 1753. 2n = 36, 2n = 45, 2n = 54

Localities: **1.** Czech Republic, Krušné hory Mts, distr. Sokolov: grassy place in a former tin mine 3.5 km NNE of the village Přebuz, 890 m a.s.l., 50°24'00" N, 12°30'00" E, coll. F. Krahulec, 8 June 2000, 2n = 36 (1 plant), counted by A. Krahulcová. **2.** Czech Republic, Ještědský hřeben Mts, distr. Liberec: in the S periphery of the village of Kryštofovo údolí ca 5.5 km SSW of the town of Chrastava, 440 m a.s.l., 50°46'04" N, 14°55'57" E, coll. F. Krahulec & S. Bräutigam, 7 July 2001, 2n = 36 (1 plant), counted by A. Krahulcová. **3**. Czech Republic, Javorníky Mts, on the main ridge near the Portáš chalet, 900 m a.s.l., 49°17'47" N, 18°14'40" E, coll. P. Mráz & V. Jurkovičová, 14 October 2000, 2n = 45 (1 plant, no. 869), counted by P. Mráz & V. Mrázová. **4.** Poland, Góry Izerskie Mts: near the settlement of Orle 3.5 km W of the village of Jakuszyce, 830 m a.s.l., 50°48'45" N, 15°23'50" E, coll. F. Krahulec (excursion during the 4th Hieracium Workshop), 4 June 2000, 2n = 36 (2 plants), counted by A. Krahulcová. **5.** Germany, Sachsen, Niederschlesischer Oberlausitz-Kreis: Niederspree village, on the margin of forest road along the lake bank, 51° 24'10" N, 14° 52'60" E, 105 m a.s.l., coll. P. Mráz, 3 June 2000, 2n = 36 (1 plant, no. 751), counted by L. Mártonfiová. **6.** Slovakia, Ostrôžky Mts: Polichno village, meadow on the top of the Mt Bralce, 817 m a.s.l., 48°25'27.23" N, 19°27'44" E, coll. P. Mráz & V. Jurkovičová, 17 April 2001, 2n = 45 (1 plant no. 725), counted by P. Mráz. **7.** Slovakia, Chočské vrchy Hills: Prosiek village, 0.5 km N of the

village, pasture, 644 m a.s.l., 49°09'29.9" N, 19°29'54.4" E, coll. P. Mráz, 15 July 2000, 2n = ca 45 (1 plant no. 1076), counted by P. Mráz. **8.** Slovakia, Západné Tatry Mts: Pribilina village, Račkova dolina valley, meadow near the Agriculture University of Nitra chalet, 940 m a.s.l., 49°07'57" N, 19°46'59" E, coll. V. Mrázová, 18 June 2001, 2n = 45 (1 plant no. 978), counted by L. Mártonfiová. **9.** Slovakia, Západné Tatry Mts: Pribilina village, Račkova dolina valley, near the crossroad with the Jamnická dolina valley, 960 m a.s.l., 49°07'57" N, 19°46'59" E, coll. V. Mrázová, 18 June 2001, 2n = 45 (1 plant no. 977), counted by L. Mártonfiová. **10.** Slovakia, Vysoké Tatry Mts: ca 1.5 km NW of Štrbské pleso tarn, 1400 m a.s.l., 49°07'48" N, 20°02' 27" E, coll. P. Mráz & V. Mrázová, 7 August 2000, 2n = 45 (1 plant no. 838), counted by P. Mráz and L. Mártonfiová. **11.** Slovakia, Vysoké Tatry Mts: on the tourist path between Štrbské pleso tarn and Jamské pleso tarn, 1450 m a.s.l., 49°07'56" N, 20°02'28" E, coll. P. Mráz & V. Mrázová, August 2001, 2n = 45 (1 plant no. 1158), counted by P. Mráz. **12.** Slovakia, Volovské vrchy Mts: Hnilčík village, on the phylite rocks near the crossroad to Hnilčík-Roztoky quarter, 640 m a.s.l., 48°51'24.4" N, 20°34'28.6" E, coll. P. Mráz and V. Jurkovičová, 3 July 2000, 2n = 45 (2 plants nos. 767, 768), counted by L. Mártonfiová. **13.** Slovakia, Volovské vrchy Mts: Čučma village, S slopes of Mt Skalisko, ca 1220 m a.s.l., 48°44'47" N, 20°34'33" E, coll. P. Mráz & V. Jurkovičová, 13 June 2000, 2n = 54 (1 plant no. 762), counted by P. Mráz. **14.** Slovakia, Volovské vrchy Mts: Čučma village, ca 200 m SW of the top of Mt Skalisko (1293 m), 1250 m a.s.l., 48°44'38.4" N, 20°34'35.4" E, coll. P. Mráz & V. Mrázová, 12 June 2001, 2n = 45 (1 plant, no. 959), counted by P. Mráz. **15.** Slovakia, Volovské vrchy Mts: Čučma village, S slopes of Mt Skalisko, *loco dicto* Doboška ca 1 km N from elevation marker 874.2, 890 m a.s.l., 48°43'50" N, 20°35'25" E, coll. P. Mráz & V. Mrázová, 12 June 2001, 2n = 45 (2 plants, nos. 962, 963), counted by P. Mráz. **16.** Slovakia, Volovské vrchy Mts: Úhorná village, on the forest route from the Panské sedlo saddle to Mt Biele skaly, NW of the village, 1100 m a.s.l., 48°43'19" N, 20°39'01" E, coll. P. Mráz, June 2002, 2n = 45 (3 plants, nos. 1213, 1215, 1216), counted by P. Mráz. **17.** Slovakia, Volovské vrchy Mts: Prakovce village, *loco dicto* Hutno on E margin of the village, near the forest road, 400 m a.s.l., 48°49'08" N, 20°55'25" E, coll. P. Mráz, September 2000, 2n = 45 (1 plant, no. 867), counted by P. Mráz. **18.** Slovakia, Volovské vrchy Mts: Kojšov village, *loco dicto* Strieborná lúka ca 2.5 km NW of the village, 700 m a.s.l., 48°50'25" N, 20°59'20" E, coll. P. Mráz, 5 July 2000, 2n = 45 (1 plant, no. 774), counted by P. Mráz. **19.** Slovakia, Volovské vrchy Mts: Vyšný Klátov village, Mt Predná Holica, meadow below the Lajoška chalet, 900 m a.s.l., 48°45'47" N, 21°04'38" E, coll. P. Mráz, June 2002, 2n = 54 (4 plants, nos. 1207, 1208, 1210, 1211), counted by P. Mráz. **20.** Slovakia, Volovské vrchy Mts: Košice city, Botanical garden grounds, 236 m a.s.l., 48°44'03" N, 21°14'15" E, coll. P. Mráz, May 2000, 2n = 54 (1 plant, no. 735), counted by P. Mráz. **21.** Slovakia, Slovenský kras Mts: Plešivec town, Plešivecká planina plateau, on the forest route ca 0.5 km NE of Zbojnícka priepasť chasm, 545 m a.s.l., 48°34'22" N, 20°25'09" E, coll. P. Mráz, 30 May 2001, 2n = 54 (1 plant, no. 934), counted by P. Mráz. **22.** Slovakia, Slovenský kras Mts: Kečovo village, Silická planina plateau, xerothermic slopes on the N margin of the village, 350 m a.s.l., 48°29'45" N, 20°29'20" E, coll. P. Mráz, 30 May 2002, 2n = 54 (1 plant, no. 1200), counted by P. Mráz. **23.** Slovakia, Slovenský kras Mts: Zádiel village, Zádielska planina plateau, ca 0.5 km NW from elevation point 591.2, 580 m a.s.l., 48°37'58.3" N, 20°41'57.4" E, coll. E. Karasová, 25 May 2001, 2n = 54 (1 plant, no. 930), counted by L. Mártonfiová. **24.** Slovakia, Vihorlat Mts: Jasenov village, ca 0.5 km NE of the village, pasture, 150 m a.s.l., 48° 54'14.7" N, 21°54'36.6" E, coll. P. Mráz & V. Mrázová, 13 May 2001, 2n = 45 (1 plant no. 926), counted by P. Mráz, 2n = ca 45 (1 plant, no. 927), counted by P. Mráz. **25.** Slovakia, Zemplínske vrchy Mts: Veľká Tŕňa village, xerothermic margins of the road 1.5 km NNW of the village, pasture, 180 m a.s.l., 48° 28' 35" N, 21°40'24.5" E, coll. P. Mráz & V. Mrázová, 14 April 2001, 2n = 45 (1 plant, no. 913), counted by P. Mráz, 2n = ca 45 (1 plant, no. 914), counted by P. Mráz. **26.** Slovakia, Zemplínske vrchy Mts: Veľká Bara village, former vineyards, ca 100 m N from Piliš Hill (277.6), ca 240 m a.s.l., 48°25'47" N, 21°42'28.3" E, coll. P. Mráz & V. Mrázová, 15 April 2001, 2n = ca 45 (1 plant, no. 909), counted by L. Mártonfiová. **27.** Slovakia, Zemplínske vrchy Mts: Streda nad Bodrogom village, ca 1 km SE of the village centre, *loco dicto* Veterné piesky on the Tarbucka Hill massive, ca 140 m a.s.l., 48°22'40.5" N, 21° 47'02.4" E, coll. P. Mráz & V. Mrázová, 16 April 2001, 2n = ca 45 (1 plant, no. 921), counted by L. Mártonfiová. **28.** Slovakia, Zemplínske vrchy Mts: Kráľovský Chlmec village, Veľký kopec Hill (263.9), 260 m a.s.l., 48°25'01.4" N, 21°57'44.7" E, coll. P. Mráz & V. Mrázová, 17 April 2001, 2n = ca 45 (1 plant, no. 904), counted by L. Mártonfiová. **29.** Slovakia, Zemplínske vrchy Mts: Luhyňa village, N slopes of Ondrejský kopec Hill, oak forest margin, 160 m a.s.l., 48°30'03.8" N, 21°38'01.3" E, coll. P. Mráz & V. Mrázová, 14 April 2001, 2n = ca 54 (1 plant, no. 912), counted by P. Mráz.

The chromosome number 2n = 36 recorded here for two localities in the Czech Republic is that commonly recorded for *H. pilosella* in this country, including plants from the mountain ranges surrounding the Czech basin (Krahulcová & Krahulec 1999, Rotreklová et al. 2002). In contrast, only pentaploids (2n = 45) and hexaploids (2n = 54) were recorded in

Slovakia. However, Píšťanský & Mičieta (2000) record tetraploids from 27, pentaploids from 4 and hexaploids from 2 localities in Slovakia, but there are no voucher specimens available at present (K. Mičieta, personal communication).

Because Šingliarová & Mráz (2004) using flow cytometry on a sample of 80 plants from different parts of Slovakia detected only pentaploids and hexaploids, the high number of tetraploids reported by Píšťanský & Mičieta (2000) is surprising. Our results (both published here and unpublished) show that pentaploid plants occur mainly in the Western Carpathians and hexaploids in the warmer regions. Hexaploids also occur at higher altitudes, usually accompanied by other *Hieracium* taxa (e.g. *H. lactucella*), as previously observed by Skalińska (1967) in the High Tatra Mts. The eastern boundary of the common occurrence of tetraploids is probably in Moravia. Surveys of the karyological variability in *H. pilosella*, covering its whole distribution area, was reported by Krahulcová & Krahulec (1999) and Rotreklová et al. (2002). Recently, plants with tetraploid chromosome numbers were recorded in Bavaria, Germany (Schuhwerk & Lippert 2002).

Hieracium piloselliflorum Nägeli et Peter, Die Hieracien Mittel-Europas 1: 707, 1885. 2n = 45

H. floribundum < *H. pilosella*

Locality: **1**. Poland, Góry Izerskie Mts: Velká Jizerská louka meadow close to Czech – Polish border, 7 km SSE of the town of Świeradów-Zdrój, 830 m a.s.l., 50°51'00" N, 15°21'40" E, coll. F. Krahulec (excursion during the 4th Hieracium Workshop), 4 June 2000, 2n = 45 (1 plant), counted by A. Krahulcová.

The hybridogeneous species *H. piloselliflorum* consists of tetraploids, pentaploids and hexaploids, and most populations have an independent origin (Fehrer et al. 2005). All three ploidy levels are recorded in the Krkonoše Mts (Krahulcová et al. 2001), and tetraploids and pentaploids in other areas of the Czech Republic (Rotreklová et al. 2002). The pentaploid chromosome number presented confirms the occurrence of this cytotype in the Góry Izerskie Mts (Fehrer et al. 2005).

Hieracium pilosellinum F. W. Schultz, Arch. Fl. Fr. Allem. 1: 57, 1844.
2n = 36; 2n = 45, apomictic.

H. pilosella > *H. densiflorum*

Localities: **1.** Czech Republic, distr. Břeclav: Dunajovické kopce Hills, Jánská hora Hill, 2.7 km WSW of the church in the village of Dolní Dunajovice, 265 m a.s.l., 48°50'46" N, 16°33'25" E, coll. O. Rotreklová & J. Danihelka, 21 May 2002. 2n = 36 (1 plant) counted by O. Rotreklová (Fig. 1e). Plants grew here together with *H. pilosella,* tetraploid *H. densiflorum* and with tetraploid *H. fallacinum* (see above). **2.** Czech Republic, distr. Slavkov u Brna: slopes 0.75 km SW of the church in the village of Rašovice, 250 m a.s.l., 49°07'07" N, 16°56'24" E, 3 June 2002, coll. O. Rotreklová, 2n = 45, apomictic (1 plant), counted by O. Rotreklová (Fig. 1b). The plant coexisted here with *H. pilosella* and tetraploid *H. densiflorum* (see above).

The first chromosome number (2n = 45) for this species was recorded from Bavaria in Germany by Schuhwerk & Lippert (2002). Our data are the first for *H. pilosellinum* from the Czech Republic. The tetraploid number (2n = 36) is reported here for *H. pilosellinum* for the first time, as is the apomictic reproduction of the pentaploid cytotype.

Hieracium piloselloides Vill., Prosp. Pl. Dauph. 34, 1779.
2n = 27, apomictic; 2n = 36, 2n ~ 4x, both apomictic and sexual; 2n = 45, 2n ~ 5x, apomictic

Localities: **1.** Germany, Thuringia, distr. Jena: Leutra village, Nature reserve Leutrathal, slope with S exposure N of the village, ca 300 m a.s.l., 50°52'30" N, 11°33'52" E, coll. M. Chytrý, 11 June 1999, 2n = 45, apomictic (1 plant), counted by O. Rotreklová. **2.** Germany, Ausburg: near the Church of the Holy Cross, 500 m a.s.l., 48°22'18" N, 10°53'30" E, coll. M. Chytrý & Z. Lososová, 1 August 2001, 2n = 36, apomictic (1 plant), counted by O. Rotreklová. **3.** Germany, Bavaria, distr. Regensburg: quarry above the Naab River 1.5 km N of Nittendorf village, ca 400 m a.s.l., 49°01'30" N, 11°58'10" E, coll. O. Rotreklová & P. Šmarda, 11 July 2000, 2n = 36, apomictic (1 plant), 2n ~ 4x (1 plant), 2n ~ 5x (1 plant). The chromosome counts and flow cytometry analysis done by O. Rotreklová. **4.** Germany, Baden-Württemberg, Müllheim village, alluvium of the Rhine 8 km NW of the village, 47°52' N, 7°33' E, coll. P. Šmarda, June 2002, 2n = 27 (2 plants), apomictic (1 plant), counted by O. Rotreklová. **5.** Czech Republic, distr. Břeclav: Dunajovické kopce Hills, Jánská hora Hill, 3.25 km W-WNW of the church in the village of Dolní Dunajovice, 260 m a.s.l., 48°51'22" N, 16°33'04" E, coll. O. Rotreklová & J. Danihelka, 21 May 2002, 2n = 36, apomictic (2 plants), counted by O. Rotreklová. **6.** Slovakia, Nízké Tatry Mts.: S slope of Mt Chopok, slope along the road 200 m of chair lift near the Srdiečko chalet, 1080 m a.s.l., 48°56'14" N, 19°37'23" E, coll. O. Rotreklová & Z. Lososová, 27 June 2001, 2n ~ 4x (1 plant), flow cytometry analysis done by O. Rotreklová. **7.** Slovakia, Volovské vrchy Mts: Prakovce village, housing estate „SNP", in the park „SNP", introduced probably with grass seeds or with building material, 445 m a.s.l., 48°48'37.4" N, 20°54'34.1" E, coll. P. Mráz, 9 June 2002, 2n = 36 (1 plant, no. 1204), counted by P. Mráz. **8.** Hungary, distr. Szentendre: 1 km S of the village of Sziget-monostor, 150 m a.s.l., 47°41'45" N, 19°05'28" E, coll. P. Šmarda & T. Vymyslický, 23 June 2000, 2n = 36 (2 plants), counted by O. Rotreklová. **9.** Slovenia, distr. Kranjska Gora: Planica valley, car-park 2 km SSE of Rateče village, 1080 m a.s.l., 46°29'17" N, 13°43'17" E, coll. O. Rotreklová & Z. Lososová, 24 June 2000, 2n = 36 (2 plants), sexual (1 plant), counted by O. Rotreklová (Fig. 1d). **10.** Slovenia, distr. Kranjska Gora, Vršič Saddle, 1600 m a.s.l., 46°26'20" N, 13°44'50" E, coll. O. Rotreklová & Z. Lososová, 24 June 2000, 2n = 36, (1 plant), counted by O. Rotreklová. **11.** Slovenia, Juliske Alps Mts.: Trenta village, 620 m a.s.l., 46°22'80" N, 13°45'10" E, the plant raised from achenes, coll. Z. Lososová, 24 June 1999, 2n = 45, apomictic (1 plant), counted by O. Rotreklová. **12.** Slovenia, Juliske Alps Mts, Mt Triglav: Dolina Vrata valley, car park 1 km NE of the Aljažev dom chalet, 1090 m a.s.l., 46°25'30" N, 13°51'00" E, coll. O. Rotreklová & Z. Lososová, 22 June 2003, 2n ~ 4x (2 plants), flow cytometry analysis done by O. Rotreklová. **13.** Italy, Liguria: Monterosso al Mare village, 1 km N of the village, 353 m a. s. l, 44°09'45.6" N, 9°38'57.6" E, coll. P. Šmarda, 28 May 2003, 2n ~ 4x (1 plant), flow cytometry analysis done by O. Rotreklová.

Ploidy levels ranging from diploid (2n = 18) to hexaploid (2n = 54) were recorded (see Rotreklová et al. 2002 for survey of published data), with tetraploid plants (2n = 36) the most common. Recently the following chromosome counts for this species from Bavaria in Germany were published by Schuhwerk & Lippert (2002): 2n = 27, 2n = 36 and 2n = 45. Chromosome numbers for *H. piloselloides* from Slovakia and Hungary and this apomictic mode of reproduction are recorded here for the first time.

Hieracium pistoriense Nägeli et Peter, Die Hieracien Mittel-Europas 1: 601, 1885.
2n = 27, sterile

H. bauhini – H. macranthum

Locality: **1.** Slovakia, distr. Nitra: Pohranice village, SW slope of the Kolíňanský vrch Hill, 270 m a.s.l., 48°19'00" N, 18°09'00" E, coll. O. Rotreklová & P. Eliáš jr., 6 September 2002, 2n = 27, sterile (1 plant), counted by O. Rotreklová (Fig. 1a). The plant grew together with putative parents, a diploid *H. macranthum* (see above) and tetraploid *H. bauhini* (Rotreklová 2004).

Nägeli & Peter (1885) described under the name of *H. pistoriense* two intermediate taxa between *H. macranthum* and *H. bauhini*: *H. pistoriense* subsp. *pistoriense* and subsp. *gracilicaule*, the second of which was described from Mt Johannisberg [János hegy] near Budapest, Hungary. Our plants from Slovakia correspond in morphology to the subsp.

gracilicaule. The occurrence of *H. pistoriense* in Slovakia is not included in the checklist of Slovak vascular plants (Chrtek 1998), nor in Zahn's last monograph (Zahn 1930). Therefore, our record of this generally very rare taxon (see Zahn 1930) seems to be the first for Slovakia, as are our data on chromosome number and mode of reproduction.

Hieracium rothianum Wallr., Sched. Crit. 417, 1822. 2n ~ 3x, apomictic

H. echioides > *H. pilosella*

Locality: **1.** Czech Republic, distr. Břeclav: Dunajovické kopce Hills, Jánská hora Hill, 2.7 km WSW of the church in the village of Dolní Dunajovice, 265 m a.s.l., 48°50'46" N, 16°33'25" E, coll. O. Rotreklová & J. Danihelka, 21 May 2002, 2n ~ 3x, apomictic (1 plant), flow cytometry analysis done by O. Rotreklová. Plant was revised by T. Peckert.

The triploid level has not been previously found in *H. rothianum*. Only the chromosome number 2n = 36 is reported from Austria (Schuhwerk & Lippert 1997), the Czech Republic, Slovakia (both Rotreklová et al. 2002) and Germany (Schuhwerk & Lippert 2002). So far, all references are to the subspecies *rothianum*. Apomictic reproduction is already recorded for the tetraploid cytotype (Rotreklová et al. 2002).

Hieracium schultesii F. W. Schultz, Arch. Fl. Fr. Allem. 1: 35, 1842. 2n = 36; 2n = 45, 2n ~ 5x

H. lactucella – H. pilosella

Localities: **1.** Czech Republic, distr. Žďár nad Sázavou: meadow in the NW part of the village of Cikháj, together with *H. pilosella* and diploid *H. lactucella* (see Rotreklová et al. 2002), 650 m a.s.l., 49°39'07" N, 15°57'54" E, coll. O. Rotreklová, 30 May 2001, 2n = 36 (2 plants), counted by O. Rotreklová. **2.** Slovakia, Nízké Tatry Mts: Čertovica saddle 13 km S of the Liptovský Hrádok, slope along the road 0.5 km SW of Čertovica chalet, 1380 m a.s.l., together with *H. pilosella* and *H. lactucella*, 48°53'40" N, 19°43'00" E, coll. O. Rotreklová & Z. Lososová, 27 June 2001, 2n = 45 (2 plants), counted by O. Rotreklová. **3.** Slovakia, distr. Banská Bystrica: Baník Hill 1.1 km SSW of the church in the village of Donovaly, 1055 m a.s.l., 48°52'50" N, 19°13'30" E, together with diploid *H. lactucella* (see above) and *H. pilosella*, coll. O. Rotreklová & Z. Lososová, 26 June 2001, 2n = 45 (1 plant), counted by O. Rotreklová. **4.** Poland, Lower Silesia, Massife of Ślęza: near N border of the preserve "Łąka Sulistrowicka", 6.5 km S of the town of Sobótka, 290 m a.s.l., 50°50'40" N, 16°43'30" E, coll. P. Kwiatkowski, 6 June 2001, 2n ~ 5x (1 plant), flow cytometry analysis done by A. Krahulcová.

Information on the ploidy variation in *H. schultesii* is scarce. We present here additional records of pentaploid *H. schultesii*: the first two from the Krkonoše Mts (Krahulcová & Krahulec 1999) and the Western Carpathians (Rotreklová et al. 2002). Two new records of the pentaploid cytotype in Slovakia confirm the relatively common occurrence of pentaploids in the Western Carpathians assumed by Rotreklová et al. (2002). In addition, triploids and tetraploids are also recorded in this species (see Rotreklová et al. 2002 for references).

Hieracium zizianum Tausch, Flora, Regensburg, 11, Ergänzungsbl. 1: 62, 1828.
2n = 27, apomictic; 2n = 36, 54

H. piloselloides – H. cymosum

Localities: **1.** Slovakia, Volovské vrchy Mts: Prakovce village, *loco dicto* Ortvaň, by the road to the housing estate „SNP", introduced probably with building material, 405 m a.s.l., 48°48'55.6" N, 20°54'15.1" E, coll. P. Mráz, 9 June 2002, 2n = 36 (one plant, no. 1203), counted by P. Mráz. Plants from the same locality were determined as *H. zizianum* by S. Bräutigam on the basis of collections from 1996. **2.** France, Maritime Alps Mts, Mercantour National Park: Col de Tende, 8 km NNW of the village of Tende, 44°08'50" N, 7°33'50" E, coll. M. Kočí, 9 June 2000, 2n = 27 (1 plant), apomictic, 2n = 54 (1 plant), counted by O. Rotreklová. Plants were revised by S. Bräutigam.

The first chromosome numbers for this taxon were from Bavaria in Germany (2n = 36, 45) and Italy (2n = 36) (Schuhwerk & Lippert 2002). Our data are new for Slovakia and France, and for this species the triploid (2n = 27) and hexaploid (2n = 54) chromosome numbers are also new. The breeding system was also previously unknown.

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Souhrn

V práci jsou uvedeny výsledky studia počtů chromozomů (popřípadě ploidní úrovně) a reprodukčních systémů jestřábníků z podrodu *Pilosella* za uplynulé dva roky. Jsou zde uvedeny počty chromozomů následujících 26 taxonů včetně jednoho hybrida, pro část z nich i reprodukční systémy: *H. arvicola* Nägeli et Peter (2n = 45), *H. aurantiacum* L. (2n = 36, 45), *H. bauhini* Besser (2n = 36, 45), *H. bifurcum* M. Bieb. (2n = 45, téměř sterilní), *H. brachiatum* Bertol. ex DC. (2n = 36; 45, apomiktický), *H. caespitosum* Dumort. (2n = 36), *H. cymosum* L. (2n ~ 4x), *H. densiflorum* Tausch (2n = 36, ~ 4x, sexuální), *H. echioides* Lumn. (2n = 18, ~ 2x, 45, obě ploidní úrovně sexuální), *H. fallacinum* F. W. Schultz (2n = 36, 45), *H. floribundum* Wimm. et Grab. (2n = 36, ~ 4x, apomiktický; 2n = 45), *H. glomeratum* Froel. in DC. (2n = 45), *H. iseranum* Uechtr. (2n = 36), *H. kalksburgense* Wiesb. (2n ~ 5x), *H. lactucella* Wallr. (2n = 18), *H. macranthum* (Ten.) Ten. (2n = 18), *H. onegense* (Norrl.) Norrl. (2n = 18), *H. onegense* × *H. pilosella* (2n = 36), *H. pilosella* L. (2n = 36, 45, 54), *H. pilosselliflorum* Nägeli et Peter (2n = 45), *H. pilosellinum* F. W. Schultz (2n = 36, 45), *H. piloselloides* Vill. (2n = 27, apomiktický; 2n = 36, ~ 4x, apomiktický i sexuální; 45, ~ 5x, apomiktický), *H. pistoriense* Nägeli et Peter (2n = 27, sterilní). *H. rothianum* Wallr. (2n ~ 3x, apomiktický), *H. schultesii* F. W. Schultz (2n = 36, 45, ~ 5x) a *H. zizianum* Tausch (2n = 27, apomiktický; 2n = 36, 54). Vedle stanovení chromozomových počtů z meristémů kořenových špiček u většiny druhů byla ke zjištění ploidie u 10 druhů (86 rostlin) použita průtoková cytometrie. Dlouhý signální chromozom byl pozorován v karyotypu následujících taxonů: *H. caespitosum*, *H. floribundum*, *H. glomeratum* a *H. iseranum*. Chromozomové počty, případně též reprodukční mechanismy jsou poprvé publikovány pro druhy: *H. bifurcum* (téměř sterilní pentaploidní rostlina), *H. pilosellinum* (apomiktická pentaploidní rostlina), *H. piloselloides* (apomiktická triploidní rostlina), *H. pistoriense* (triploidní sterilní rostlina), *H. rothianum* (apomiktická triploidní rostlina) a *H. zizianum* (apomiktická triploidní rostlina). První nebo nový chromozomový počet byl zjištěn z následujících zemí: Česká republika: *H. arvicola* (2n = 45), *H. bifurcum* (2n = 45), *H. echioides* (2n = 45), *H. fallacinum* (2n = 36, 45), *H. floribundum* (2n = 45), *H. kalksburgense* (2n ~ 5x), *H. pilosellinum* (2n = 36, 45) a *H. rothianum* (2n = 27); Maďarsko: *H. piloselloides* (2n = 36); Slovensko: *H. brachiatum* (2n = 36, 45), *H. cymosum* (2n = 36), *H. echioides* (2n = 18), *H. piloselloides* (2n = 36) a *H. zizianum* (2n = 36); Slovinsko: *H. brachiatum* (2n = 45) a *H. piloselloides* (2n = 45); a Francie: *H. zizianum* (2n = 27, 54). V podrodu *Pilosella* je podruhé prezentován sexuální způsob reprodukce pentaploidního cytotypu (*H. echioides*). Dříve publikované (Rotreklová et al. 2002) a nové údaje o počtu chromozomů *H. densiflorum* z České republiky naznačují, že tento druh je na jižní Moravě pravděpodobně pouze tetraploidní. Nález velmi vzácného taxonu *H. pistoriense* (*H. bauhini – H. macranthum*) nedaleko Nitry je prvním údajem pro území Slovenska.

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Chromosome numbers and taxonomic-chorological notes on selected species of *Hieracium* s. str. (*Asteraceae*) from Montenegro

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Abstract. The chromosome numbers for 15 species of *Hieracium* s.str. from Durmitor Mt (Montenegro) are reported, supplied by taxonomic and chorological notes. Eleven taxa (*H. blecicii* nom. prov., *H. calophyllum* s.l., *H. expallens*, *H. gymnocephalum*, *H. flexuosum, H. mirificissimum*, *H. naegelianum*, *H. paratrichum* nom. prov., *H. scheppigianum*, *H. spirocaule* nom. prov., *H. valdepilosum* s.l.) were found to be triploid, with $2n = 3x = 27$, and four taxa (*H. durmitoricum*, *H. pallescens*, *H. pseudoshenkii*, *H. coloriscapum* s.l.) were tetraploid, with $2n = 4x = 36$. For six species the chromosome number has not been reported so far. For the first time *H. mirificissimum* is reported for the flora of Montenegro and *H. expallens* for the floras of Serbia and Montenegro.

Key words: chorology, chromosome numbers, Durmitor Mt, *Hieracium*, Montenegro, ploidy levels, Serbia, taxonomy

Introduction

The genus *Hieracium* L. s.str. is notorious for its taxonomic complexity. According to Zahn (1921-23, 1922-38), within the territory of Serbia and Montenegro there are 79 recorded species and 213 subspecies. Afterwards, some 65 new taxa have been recorded or described. The greatest species diversity has been registered on the limestone terrains of the Southeast Dinarides (Durmitor Mt, Prokletije Mts). In the area of Durmitor Mt over 60 agamospecies have been recorded (Zahn 1921-23, 1922-38; Rohlena 1942). Particularly rich in species are the endemic *H.* sect. *Pannosa* (Zahn) Zahn and *H.* sect. *Glauciformia* (Freyn) Zahn. Other important centres of diversity are Mt Šar and the Balkan Range (= Stara Planina), especially concerning the representatives of *H.* sect. *Cernua* R. Uechtr. (= *H.* sect. *Pseudostenotheca* (Fr.) Juxip, sensu Stace 1998), which usually grow on a silicate substrate.

The taxonomic complexity of *Hieracium* is due to the prevailing polyploidy and apomixis (Gustafsson 1946, 1947a, b). In comparison with the closely related genus *Pilosella* Hill, a recent natural hybridization in *Hieracium* seems to be strongly restricted (Mráz et al. 2005). While diploid taxa are sexual (Gustafsson 1946, 1947a, b; Chrtek 1997; Mráz 2003), polyploids reproduce apomictically (e.g. Gustafsson 1946, 1947a, b).

Several approaches have been used to classify the diversity of the genus. The taxonomic concept of Nägeli and Peter (1885), later largely expanded by Zahn (1921-23, 1922-38), accepts the so-called '*species principales collectivae et species intermediae collectivae*,' with a huge number of infraspecific taxa. This approach was held mostly by the authors in Central and Southeast Europe, including Serbia and Montenegro (Rohlena 1942; Gajić 1975). The other approach held by the botanists in Great Britain, Western Europe, Scandinavia and Russia, accepts the 'narrow' species. In most cases these species correspond to the subspecies of the Central European school and are arranged in larger groups, many of which without valid taxonomic status.

Whenever possible, the 'narrow' species concept has been used in this paper. Some very complex groups, in which the taxonomic relations have not been acceptably resolved yet, were treated on aggregate level (s.l.). Stace's classification of sections was followed (Stace 1998), with the exception of *H.* sect. *Naegeliana* Zahn ex Szeląg, which has been recently described, and *H.* sect. *Cernua* (Szeląg 2003).

The chromosome number is a useful character for inferring the mode of reproduction of the studied taxa and, hence, for solving taxonomic problems. The basic chromosome number in the genus is $x=9$. So far all ploidy levels, from the diploid to the heptaploid, have been detected (e.g. see Schuhwerk 1996; Pulkina & Tupitzsyna 2000), though with a very different frequency of occurrence. Most common are the tri- and tetraploids, whereas penta-, hexa- and heptaploids have been seldom reported (Stace & al. 1995; Chrtek 1996; Pulkina & Tupitzsyna 2000). Diploids in *Hieracium* s.str. are not common, but the Balkan Peninsula has proved to be among the richest European areas in the number of diploid *Hieracium* species (Schuhwerk & Lippert 1998; Vladimirov 2000, 2003; Vladimirov & Szeląg 2001, 2006). Karyological studies of material from Serbia and Montenegro are very few (Schuhwerk & Lippert 1998; Niketić & al. 2003), which has encouraged us to carry out this study.

Material and methods

The herbarium material, seeds and living plants were collected from a single locality: Durmitor Mt, the slopes of Veliki Međed peak with southern exposition, on limestone screes within the zone of *Pinus mug*o, 1800–2000 m, 23.08.2001, coll. *M. Niketić* & *G. Tomović*. The voucher specimens are deposited in the herbarium of the Natural History Museum in Belgrade (BEO).

Two approaches to the determination of chromosome numbers were used.

(i) The living plants were brought into cultivation in the vegetation house of the Institute of Botany in Sofia. Root tips were cut, pretreated with 0.01 % colchicine solution for 90 min, fixed in 3:1 absolute ethanol-glacial acetic acid for at least 2 h at room temperature, and stored in 96 % alcohol at –18 °C until needed. Then the root tips were hydrolysed in 1N HCl for 20 min at 60 °C, stained with Gomori's haematoxylin (Melander & Wingstrand 1953) for 30 min at 60 °C, and finally squashed in 45 % acetic acid. Counting of the chromosomes was done on permanent slides. Usually 2–4 plants of each gathering were used for chromosome counting (method used by V.V.).

(ii) Achenes were germinated in Petri dishes. Root tips of the seedlings were cut and pretreated with 0.5 % colchicine solution for 1.5–3 h at room temperature. Subsequently, root tips were fixed in a mixture of absolute ethanol and acetic acid (3:1) for at least 1 h and then hydrolysed for 5 min in 1N HCl at 60 °C. The squash and smear method with cellophane replacing the glass covers followed Murín (1960). Giemsa solution in phosphate buffer was used as a stain. Selected permanent slides have been stored at the Department of Botany, Institute of Biology & Ecology, P.J. Šafárik University, Košice. The chromosome number was counted in 1–4 germinated achenes from each species (method used by P.M.).

Taxonomic and chorological notes are based on the opinion of M.N. Herbarium specimens from the following herbaria have been studied: B, BM, BEO, BEOU, BP, BRNM, C, G, LJU, LY, PR, PRC, S, SKO, SO, SOM, W, WU, Z. The material was revised and compared with own collections from the Dinarides (BEO) and the living plants in the garden. For the representatives of *H.* sect. *Pannosa* morphometric analyses have been done (including canonical and correspondent analyses – unpublished data).

Results and discussion

Hieracium* sect. *Drepanoidea Monnier

H. pseudoshenkii (Rohlena & Zahn) Niketić (Fig. 1)

Syn.: *H. bupleuroides* subsp. *pseudoshenkii* Rohlena & Zahn

$2n = 4x = 36$ (counted by P.M. & V.V.; Fig. 3A)

The species is endemic to the Southeast Dinarides: Durmitor Mt and the vicinity of Šavnik in Montenegro, and Prenj Mt in Herzegovina (Niketić & al. 2003). It grows in rocky habitats of the subalpine zone, in the openings of *Pinus mugo* communities. The chromosome number confirms an earlier count from Durmitor Mt (Niketić & al. 2003). The taxon belongs to the *H. bupleuroides* group (sensu Sell & West 1976) for which triploid (Christoff & Popoff 1933; Polatschek 1966; Murín & Uhríková 1970; Chrtek & al. 2004) and tetraploid (Chrtek & al. 2004; Szeląg & Vladimirov 2005) counts have been reported so far.

H.* sect. *Hieracium

H. pallescens Waldst. & Kit. (Fig. 2)

$2n = 4x = 36$ (counted by P.M. & V.V.)

The species is distributed in the mountains of Central Europe, extending eastwards to Romania and south-eastwards to Bosnia, Montenegro and Southwest Serbia (Zahn 1935; Behr & al. 1937). According to Zahn, this taxon belongs to the *H. incisum* group (*H. bifidum-dentatum*), whereas Sell & West (1976) place it into the *H. bifidum* group. The chromosome number is reported here for the first time.

H.* sect. *Naegeliana Zahn ex Szeląg

H. naegelianum Pančić (Fig. 4)

$2n = 3x = 27$ (counted by V.V.; Fig. 3B)

The species is distributed in the high mountains of the Balkan Peninsula and Mt Abruzzo in Italy. It is characteristic for the limestone screes in the subalpine and alpine belts, at 1800–2500 m. The species was described from Komovi Mt in Montenegro (Pančić 1875). Several subspecies are known, but the collected specimens, as well as all the populations in Montenegro, belong to the nominal taxon.

The chromosome number confirms the previous counts in specimens originating from Greece (Merxmüller 1975; Grau & Erben 1988; Franzén in Buttler 1991: 639) and Bulgaria (Vladimirov & Szeląg 2001).

Fig. 1. *H. pseudoschenkii* – herbarium specimen.

Fig. 2. *H. pallescens* – herbarium specimen.

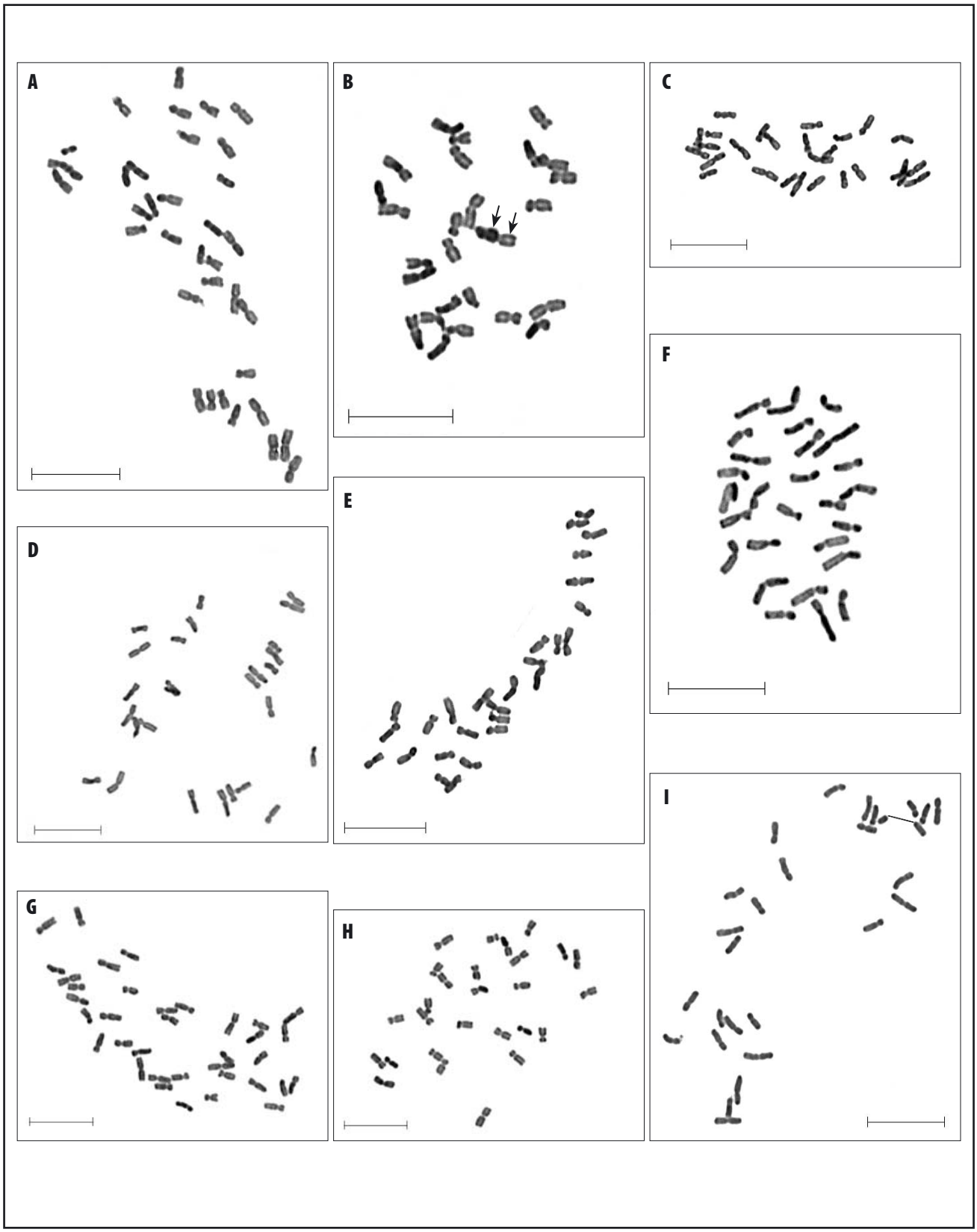

Fig. 3. Microphotographs of metaphase plates of: **A,** *H. pseudoschenkii*, $2n = 36$; **B,** *H. naegelianum*, $2n = 27$; **C,** *H. blecicii*, $2n = 27$; **D,** *H. gymnocephalum*, $2n = 27$; **E,** *H. paratrichum*, $2n = 27$; **F,** *H. calophyllum*, $2n = 27$; **G,** *H. durmitoricum*, $2n = 36$; **H,** *H. scheppigianum*, $2n = 27$; **I,** *H. valdepilosum*, $2n = 27$. Scale bar – 10 µm.

H.* sect. *Pannosa (Zahn) Zahn

H. blecicii Niketić, nom. in lit. 2002 (*H. gymnocephalum* s.l.)

$2n = 3x = 27$ (counted by P.M. & V.V.; Fig. 3C)

This yet undescribed species (cf. Niketić 2002) is endemic to the mountains of West Montenegro and a small part of Southeast Bosnia. The analysis of the distribution of phenolic compounds pointed to very similar flavonoid and phenolic acid patterns with *H. gymnocephalum* s.str. However, some significant differences have been noted concerning the presence of certain compounds (Petrović & al. 1999). *H. blecicii* is characterized by relatively broad leaves and very thick plumose hairs with short branches. In the analyzed micropopulation only individuals with tubulose flowers were found.

This is the first record of the chromosome number of the species.

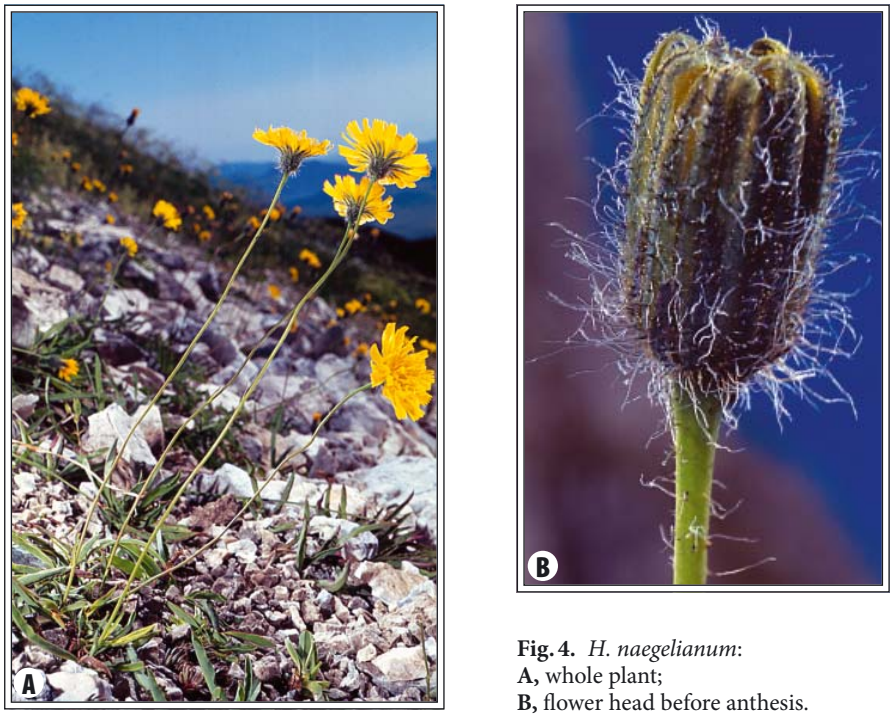

Fig. 4. *H. naegelianum*:
A, whole plant;
B, flower head before anthesis.

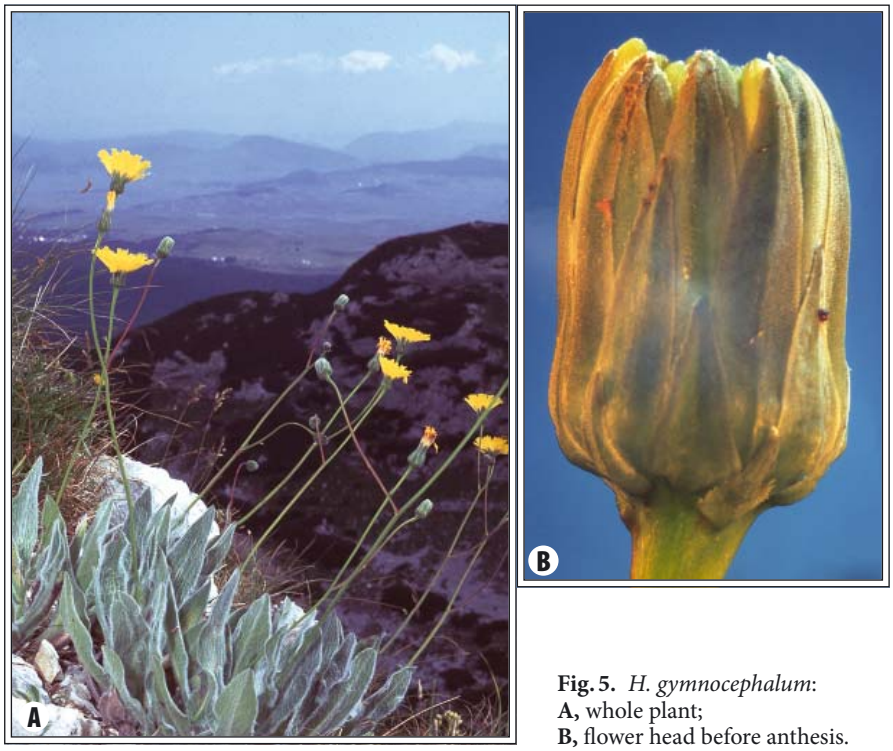

Fig. 5. *H. gymnocephalum*:
A, whole plant;
B, flower head before anthesis.

H. gymnocephalum Griseb. ex Pant. (Fig. 5)

$2n = 3x = 27$ (counted by P.M. & V.V.; Fig. 3D)

The species is an Illyrian-Scardo-Pindic element confined to the mountains of the Western Balkan Peninsula, ranging northwards from Herzegovina to Northwest Greece in the south. The most numerous populations were recorded in Montenegro. This species is a type representative of a triploid aggregate with the same name, and it is characterized by glabrous to very sparsely hairy acladium and involucre (in contrast to representatives of the other aggregates of *H.* sect. *Pannosa*). It is a very variable taxon, especially considering the hairiness of leaves and involucre and thus several infraspecific taxa have been described. The plants collected from the population on Durmitor Mt are very similar to the type specimens from Komovi Mt (60 km south-eastwards) in terms of their morphological characters.

The chromosome number of the species is reported here for the first time.

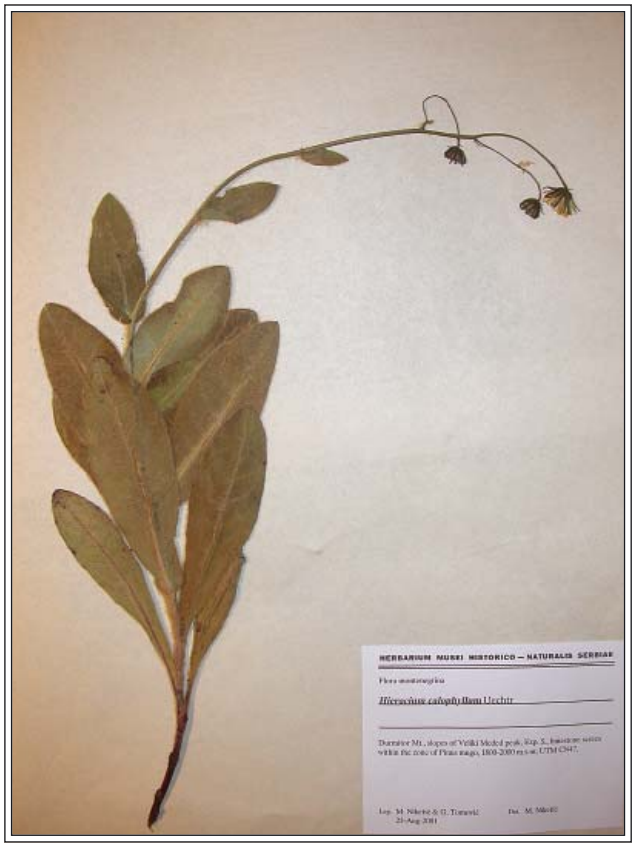

Fig. 6. *H. calophyllum* – herbarium specimen.

H. paratrichum Niketić, nom. in lit. 2003 (*H. gymnocephalum* s.l.)
2*n* = 3*x* = 27 (counted by V.V.; Fig. 3E)

This yet undescribed species is a member of the *H. gymnocephalum* group. It is distributed in some mountains of Montenegro and Herzegovina (Niketić & al. 2003). The species is characterized by scattered plumose hairs on the involucre and pedicels and by very thick plumose hairs with long branches on the leaves. The present chromosome count confirms an earlier report by Niketić & al. (2003) from Durmitor Mt.

H. spirocaule Niketić, nom. in lit. 2002 (*H. gymnocephalum* s.l.)
2*n* = 3*x* = 27 (counted by V.V.)

This yet undescribed species (cf. Niketić 2002) also belongs to the *H. gymnocephalum* complex. It is endemic to West Montenegro and a small part of Southeast Bosnia. The species is characterized by a very crowded, false basal rosette of narrow, acute bluish-green leaves with undulate margin and whitish to gray hairs throughout, and by 1–2(6) capitula.

The chromosome number is reported here for the first time.

Hieracium sect. *Pannosa* comprises remarkable, hairy-leaved taxa distributed in the Balkan Peninsula and Asia Minor, as well as on some East Mediterranean islands (Zahn 1921-23). Zahn (1921-23) designated three *species principales* in this section: *H. gymnocephalum* Griseb. ex Pant., *H. waldsteinii* Tausch and *H. pannosum* Boiss. The four species from this section that we have studied karyologically belong to the *H. gymnocephalum* group, whose centre of diversity is in the mountains of Montenegro (Niketić & al. 2003). So far only triploids have been found within this species group. In the *H. pannosum* and *H. waldsteinii* groups more ploidy levels have been detected, ranging from diploids (Schuhwerk & Lippert 1998; Vladimirov & Szeląg 2006) to tri- and tetraploids (Christoff & Popoff 1933; Papanicolaou 1984; Schuhwerk & Lippert 1998; Vladimirov & Szeląg 2001).

H.* sect. *Pilosissima Stace & P.D. Sell

H. calophyllum R. Uechtr. s.l. (Fig. 6)
2*n* = 3*x* = 27 (counted by V.V.; Fig. 3F)

H. calophyllum group is endemic to the Western Balkan Peninsula with a centre of diversity in the mountains of Montenegro (Szeląg 2002; Niketić & al. 2003). The present count confirms an earlier report by Niketić & al. (2003).

H. coloriscapum Rohlena & Zahn s.l. (Fig. 7)
2*n* = 4*x* = 36 (counted by V.V.)

The species is an Illyrian-Scardo-Pindic element, inhabiting limestone screes in the subalpine and alpine mountain zones. It has probably derived from interbreeding of *H. gymnocephalum* and *H. naegelianum*. The plant has been recently recorded in Durmitor Mt. Beyond the territory of Montenegro, several distinct subspecies have also been described (Zahn 1936; Behr & al. 1939a, b).

The present count confirms an earlier report by Niketić & al. (2003).

H. durmitoricum (Rohlena & Zahn) Niketić (Fig. 8)
Syn.: *H. scheppigianum* subsp. *durmitoricum* Rohlena & Zahn
2*n* = 4*x* = 36 (counted by P.M. & V.V.; Fig. 3G)

The taxon is endemic to Durmitor Mt and several adjacent high mountains in the Southeast Dinarides (Niketić & al. 2003). The same chromosome number has already been reported for the species from Durmitor Mt (Niketić & al. 2003). Besides *H. gymno-*

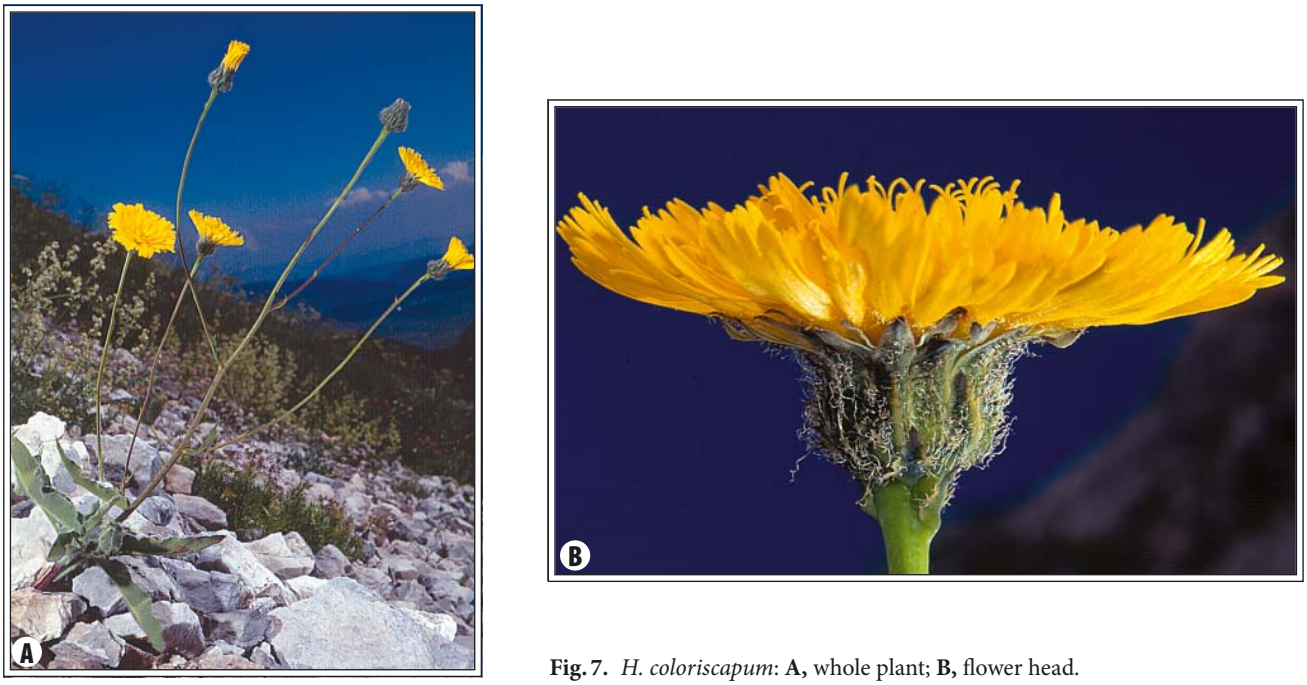

Fig. 7. *H. coloriscapum*: **A,** whole plant; **B,** flower head.

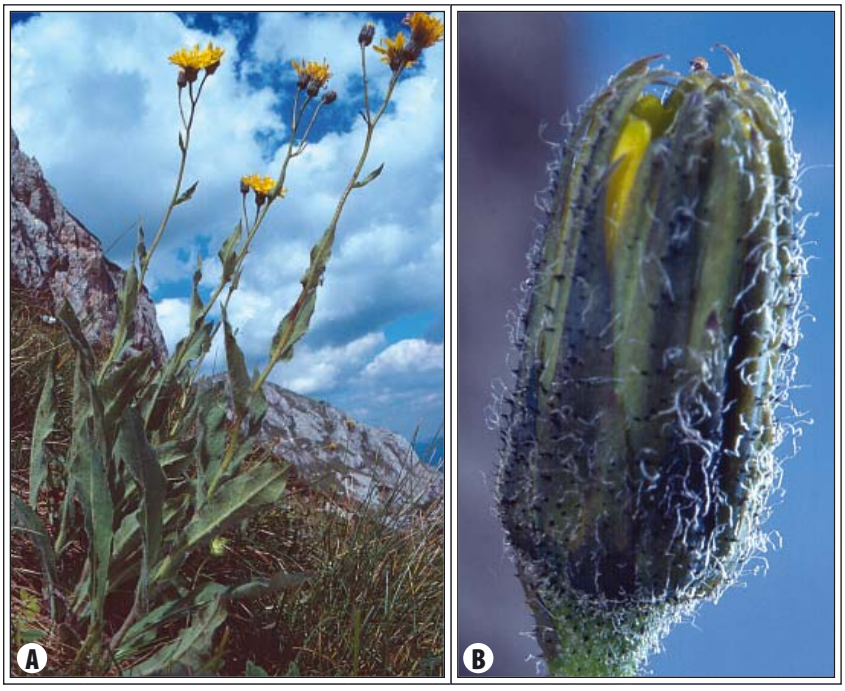

Fig. 8. *H. durmitoricum*: **A,** whole plant; **B,** flower head before anthesis.

cephalum, another possible ancestor of this hybridogenous taxon is *H. willdenowianum* (Zahn) P.D. Sell & C. West from the *H. valdepilosum* group.

H. mirificissimum Rohlena & Zahn (Fig. 9)

Syn.: *H. flexicaule* Freyn & Vandas, nom. illeg., non Tausch; *H. guentheri-beckii* subsp. *portentosum* Hayek & Zahn.

$2n = 3x = 27$ (counted by P.M. & V.V.)

The species is endemic to Southeast Dinarides – Montenegro, Bosnia and Herzegovina, and to Central and Southwest Serbia (Niketić 2003). It is considered a hybridogenous species, probably originating from interbreeding between *H. gymnocephalum* and *H. scorzonerifolium* Vill. Morphologically, it is very similar to the related species *H. guentheri-beckii* Zahn, with which it often forms mixed populations. The species is also very similar to *H. durmitoricum* and

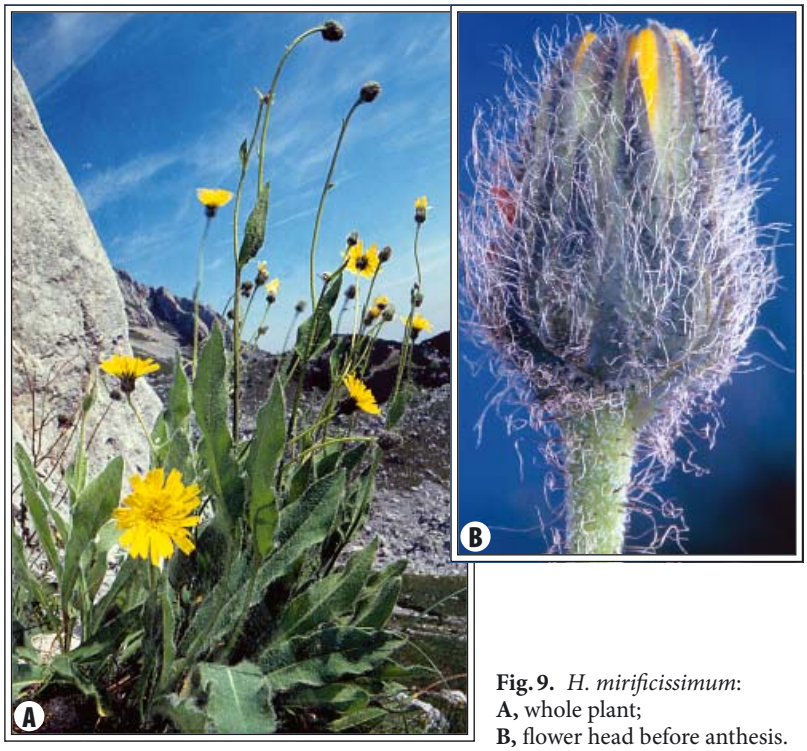

Fig. 9. *H. mirificissimum*:
A, whole plant;
B, flower head before anthesis.

H. scheppigianum Freyn. It is recorded for the first time for the flora of Serbia in the following localities:

DN-98 **Serbia,** Mt Kopaonik, "In gramin. m. Treska", July 1903, coll. *O. Bierbach*, det. *L. Adamović* sub *H. calvescens* Adamović (BM); 1600 m, on limestone, 14.08.2004, coll. *M. Niketić* & *G. Tomović* (BEO);

DN-89 **Serbia,** Mt Kopaonik, Bele Stene peak, 1600 m, on limestone, 12.09.1991, coll. *M. Niketić* (BEO);

DM-67 **Serbia,** Mt Paštrik, "An felsen der Gipfelregion", 1700 m, 27.07.1918, coll. *I. Dörfler*, sub *H. guentheri-beckii* subsp. *portentosum* (no. 877) (BP, W, WU, Z).

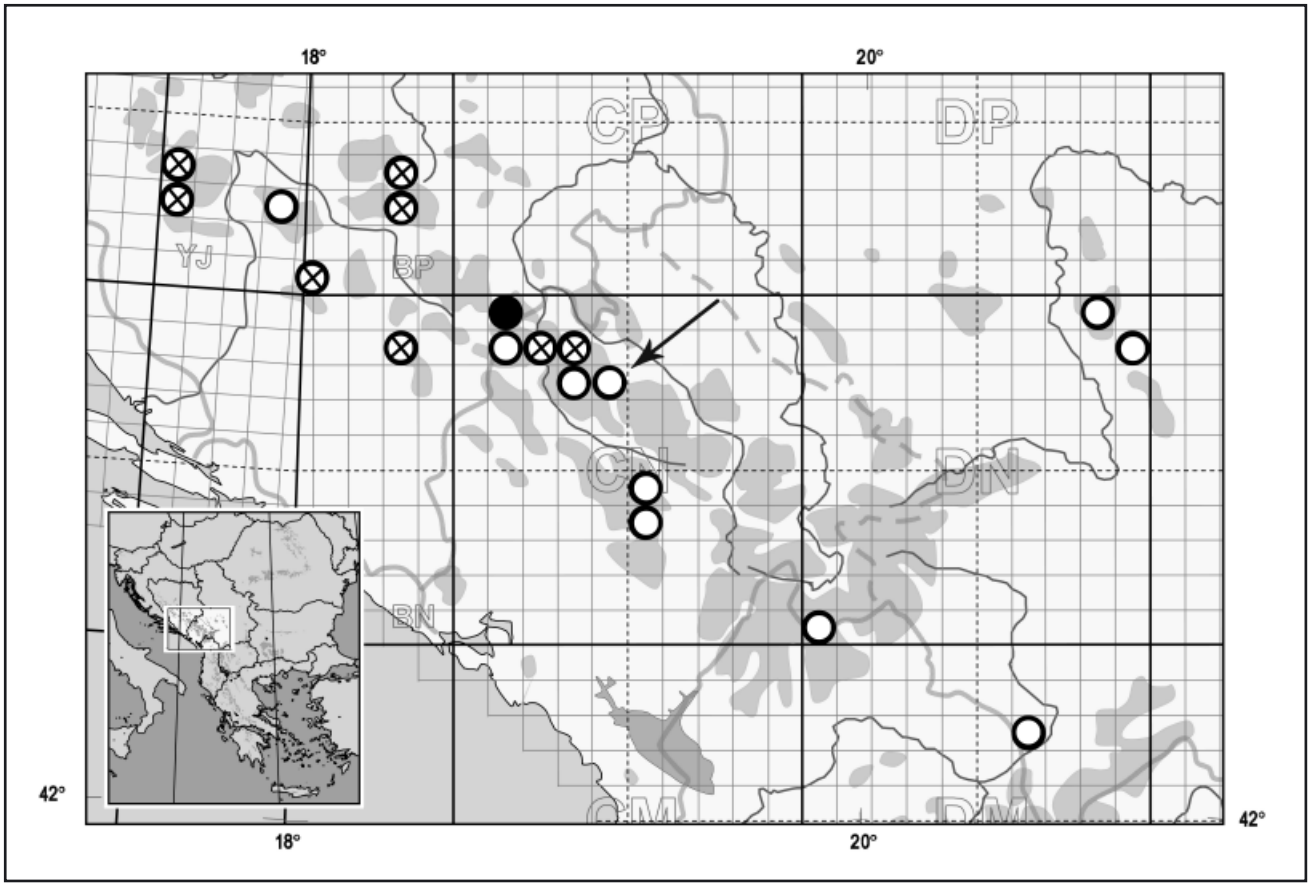

Fig. 10. *H. mirificissimum*: total distribution range, ○ – new herbarium data; ⊗ – literature data; ● – both literature and herbarium data. Arrow points to *locus classicus* of the species, Durmitor Mt, from where the plants for the karyological studies have been collected.

New localities of the species have been found on the territory of Montenegro and Herzegovina during the field research in the past few years (Fig. 10):

CN-18 **Montenegro,** Mt Volujak, Vilište peak (1960 m), 17.08.2000, coll. *M. Niketić* (BEO);

CN-37 **Montenegro,** Durmitor Mt, Planinica peak – Škrka lakes, 1800–1900 m, 17.08.1993, coll. *M. Niketić* (BEO);

CN-47 **Montenegro,** Durmitor Mt, Minin Bogaz peak, 2200–2300 m, 13.08.1994, coll. *M. Niketić* (BEO);

CN-53 **Montenegro,** Mt Maganik, Petrov peak, 1800–2100 m, 09.08.2000, coll. *M. Niketić* (BEO);

CN-54 **Montenegro,** Mt Moračke Planine, Mt Stožac, Jablanovac peak, 1900 m, 07.08.2000, coll. *M. Niketić* (BEO),

DN-00 **Montenegro,** Mt Prokletije, Bjelič, on rocky ground, limestone, 1800–1900 m, 09.07.1994, coll. *M. Niketić* (BEO),

YJ-32 **Bosnia & Hercegovina,** Mt Prenj, Pod Sivadijom – Jezera, 1650–1700 m, 07.08.2001, coll. *M. Niketić* (BEO).

After an examination of the above-mentioned herbarium material stored in BM, it was concluded that the literature report of the Apennine hybridogenous species *H. portanum* Belli (*H. gymnocephalum-heterogynum*, Zahn 1921-23) for Kopaonik Mt (det. *Adamović* sub *H. calvescens*) was incorrect and actually represents *H. mirificissimum*. At the same locality (Treska), only individuals of *H. mirificissimum* (coll. *M. Niketić* & *G. Tomović*, BEO) were recorded. The indumentum of the leaves and involucre at first glance was similar to that of *H. portanum* from Calabria, but the latter species probably originated from interbreeding between *H. gymnocephalum* and *H. heterogynum* (Froel.) Gutermann. Thus, *H. portanum* is endemic to South Italy and has never been recorded in the flora of the Balkan Peninsula.

Furthermore, examination of the herbarium specimens determined as *H. guentheri-beckii* subsp. *portentosum* Hayek & Zahn from the Paštrik Mt in Kosovo (BP, W, WU, Z) has shown no significant morphological differences from *H. mirificissimum*. This was further supported by their original descriptions (Zahn 1909, 1921-23), which were essentially very similar. Therefore, *H. guentheri-beckii* subsp. *portentosum* can be considered as a synonym of *H. mirificissimum*.

This chromosome number is reported for the first time for the species.

H. scheppigianum Freyn (Fig. 11)

Syn.: *H. agastum* Rech. f. & Zahn

$2n = 3x = 27$ (counted by P.M & V.V.; Fig. 3H)

The species is confined to the limestone areas in some mountains of Bosnia, Montenegro and Kosovo (Niketić & al. 2003). According to Zahn's classification, *H. scheppigianum* s.l. was divided into several subspecies (Zahn 1936). Niketić & al. (2003) concluded that it was a polyphyletic aggregate, and its type representative probably originated from interbreeding between *H. gymnocephalum* and a representative of *H. bupleuroides* aggregate.

The present chromosome number confirms an earlier report by Niketić & al. (2003), also from Durmitor Mt.

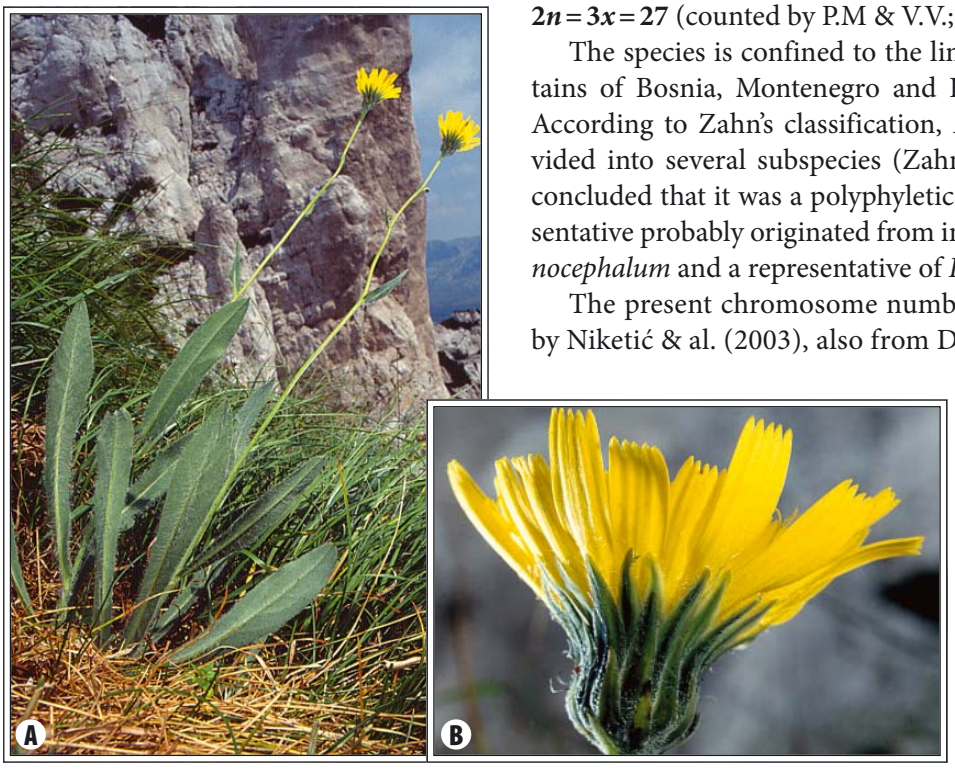

Fig. 11. *H. scheppigianum*: **A,** whole plant; **B,** flower head.

H.* sect. *Villosa (Griseb.) Gremli

H. expallens (Fr.) Arv.-Touv. (Fig. 12)

Syn.: *H. dentatum* var. *expallens* Fr.; *H. dentatum* subsp. *subexpallens* Zahn, nom. illeg.

$2n = 2x = 27$ (counted by V.V.)

The *Hieracium dentatum* group comprises taxa that are morphologically intermediate and have possibly derived from hybridization between the taxa of *H. bifidum* and *H. villosum* groups (sensu Sell & West 1976). It is distributed in the Jura, Alps, Carpathians, Central Apennines and mountains of the Northwestern Balkan Peninsula. So far about 50 subspecies have been described (Zahn 1930), all very similar morphologically. Sell & West (1976) have recognized certain taxa as distinct microspecies. Only one literary source (Behr & al. 1937) refers to *H. expallens* on the border of Albania and Serbia (Mt Paštrik). Representatives of the same taxon were recorded for the first time in the floras of Serbia and Montenegro in the following localities (Fig. 13):

CN-19 **Montenegro,** Mt Maglić, 1800–2000 m, 19.08.2000, coll. *M. Niketić* (BEO);

CN-47 **Montenegro,** Durmitor Mt, the slopes of Veliki Međed peak, 1850 m, 14.08.1992, coll. *M. Niketić* (BEO);

CN-48 **Montenegro,** Durmitor Mt, Crvena Greda peak, 1800–1900 m, 20.08.1996, coll. *M. Niketić* (BEO);

BN-91 **Montenegro,** Mt Orjen, peak, 1800 m, 22.08.1998, coll. *M. Niketić* (BEO);

CN-53 **Montenegro,** Mt Maganik, Petrov Vrh peak, 1800–2100 m, 09.08.2000, coll. *M. Niketić* (BEO);

CN-54 **Montenegro,** Mt Moračke Planine, Vragodol valley, 2100 m, 26.08.1998, coll. *M. Niketić* (BEO);

DM-74 **Serbia,** Mt Šar Planina, Brod village, Gradski Kamen hill, 1450–1650 m, 30.09.1991, coll. *M. Niketić* (BEO);

DN-22 **Serbia,** Mt Prokletije, Nedžinat lakes, 1500–1600 m, 02.09.1997, coll. *M. Niketić* (BEO).

The chromosome number for the species is reported here for the first time.

H. flexuosum Waldst. & Kit. ex Willd. (Fig. 14)

Syn.: *H. scorzonerifolium* subsp. *flexuosum* (Waldst. & Kit. ex Willd.) Nägeli & Peter

$2n = 3x = 27$ (counted by P.M.)

This hybridogenous species is distributed in the Alps and the Dinaric Alps, up to the Prokletije Mts in Montenegro and Kosovo. It is very closely related to *H. scorzonerifolium* Vill.

The present count confirms an earlier report by Niketić & al. (2003).

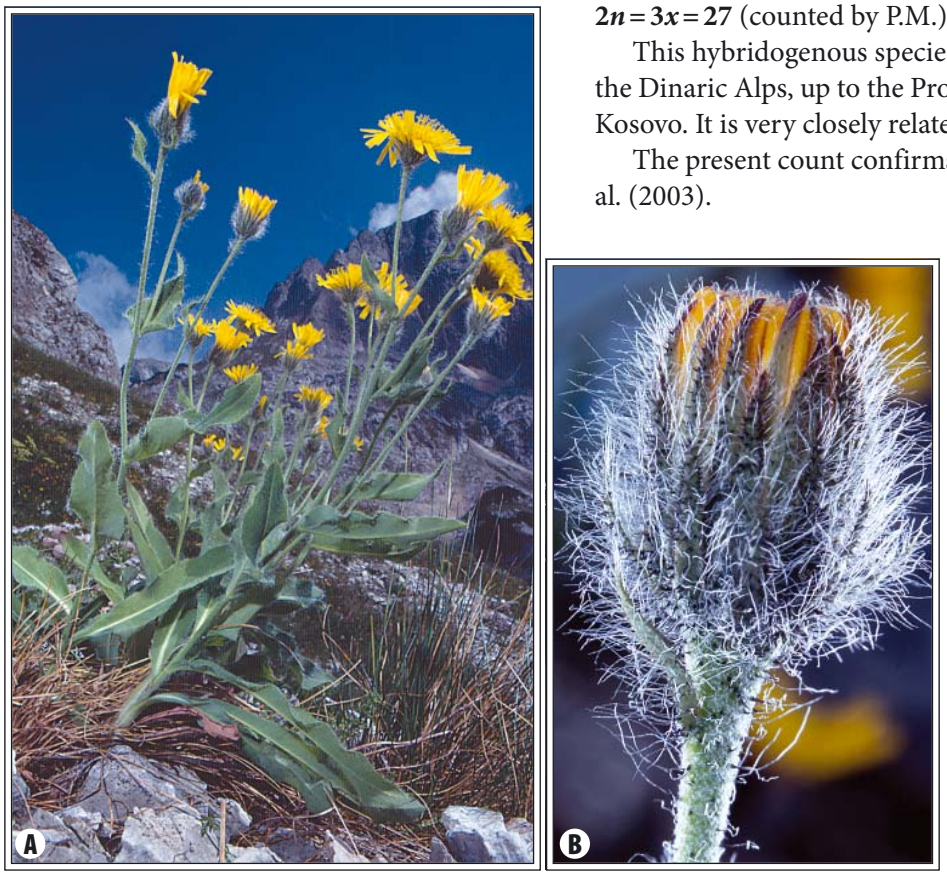

Fig. 12. *H. expallens*:
A, whole plant;
B, flower head before anthesis.

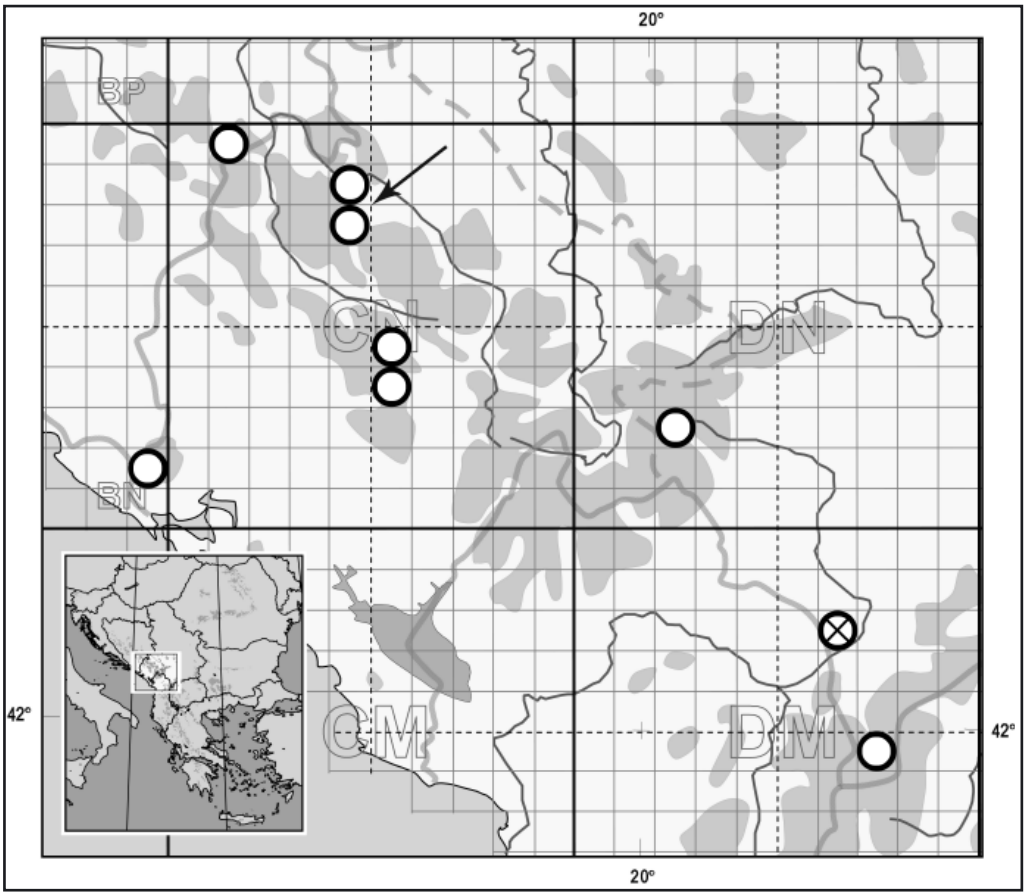

Fig. 13. *H. expallens*: part of the distribution range in Serbia and Montenegro, ○ – new herbarium data; ⊗ – literature data. Arrow points to the karyologically studied population from Durmitor Mt.

Fig. 14. *H. flexuosum*:
A, whole plant;
B, flower head.

H. valdepilosum Vill. s.l. (Fig. 15)

$2n = 3x = 27$ (counted by P.M. & V.V.; Fig. 3I)

The main taxonomic diversity of this species group in the sense of *Flora Europaea* (Sell & West 1976) is confined to the Alps, whereas the Balkan Peninsula shelters only a few isolated taxa. The studied microspecies definitely belongs to the *H. valdepilosum* group, but we were unable to determine its exact name. It has been recently discovered and reported for the first time for Serbia and Montenegro (Niketić & al. 2003). The triploid chromosome number has already been reported by Niketić & al. (2003) for the same taxon and is congruent with a count published earlier for a possibly different microspecies from the same species group (Auquier & Renard 1979).

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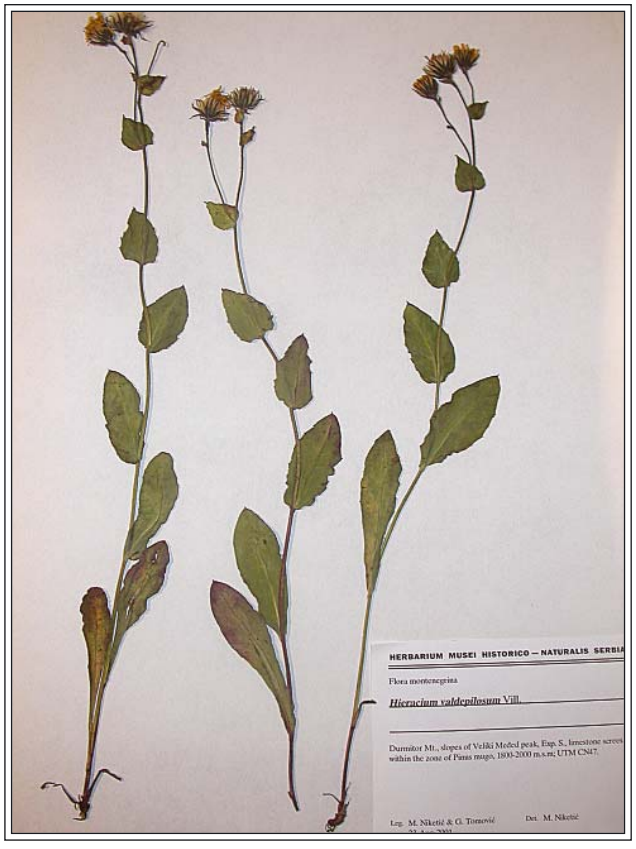

Fig. 15. *H. valdepilosum* – herbarium specimen.

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Príloha K

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Chromosome numbers and DNA-ploidy levels of selected species of *Hieracium* s.str.

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CHROMOSOME NUMBERS AND DNA PLOIDY LEVELS OF SELECTED SPECIES OF *HIERACIUM* S.STR. (*ASTERACEAE*)

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Abstract: Chromosome numbers and /or ploidy levels are reported for 44 species and subspecies of *Hieracium* s.str. from the following European countries: Andorra, Austria, Bulgaria, Czech Republic, France, Italy, Montenegro, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland and Ukraine. The chromosome numbers/DNA ploidy levels of *H. bocconei* (2n ~ 4x), *H. bupleuroides* subsp. *leviceps* (2n = 27), *H. caesioides* subsp. *caesioides* (2n = 27), *H. basifolium* (*H. caesium* agg., 2n = 36), *H. plumbeum* (*H. caesium* agg., 2n = 36), *H. glaucum* subsp. *nipholepium* (2n = 27), *H. gouanii* (2n = 18), *H. gymnocerinthe* (2n = 27), *H. ramondii* (2n = 27), *H. recoderi* (2n = 18), *H. stelligerum* (2n = 18), and *H. tomentosum* (2n = 18, 2n ~ 2x, 2n ~ 3x) were determined for the first time. New ploidy levels are reported for *H. cerinthoides* s.str. (2n = 27), *H. humile* (2n = 36), and *H. tommasinianum* (2n = 27).

Keywords: *Compositae*, Europe, Flow cytometry, Polyploidy

INTRODUCTION

Hieracium L. subgen. *Hieracium* (*Hieracium* s.str.) is one of largest and taxonomically most difficult groups of the *Asteraceae*. It comprises perennial herbs distributed in temperate regions of Europe, Asia, northernmost Mediterranean Africa, and North America (and introduced to several other regions, e.g. to New Zealand). *Hieracium* subgen. *Hieracium* forms an immense agamic complex with a base chromosome number of x = 9. Generally, polyploid taxa (triploids, tetraploids, and very rarely pentaploids) prevail in this subgenus (SCHUHWERK 1996, CHRTEK et al. 2004). Diploid species (or diploid cytotypes of otherwise polyploid species) are much less frequent and mostly confined to certain geographical areas. They have been mostly reported from SW Europe, the Eastern Carpathians and Balkan Peninsula (e.g. MERXMÜLLER 1975, CHRTEK 1996, SCHUHWERK & LIPPERT 1998, MRÁZ 2003b, VLADIMIROV 2003, VLADIMIROV & SZELĄG 2006, CASTRO et al. 2007, SZELĄG et

al. 2007). Recently, hexaploids (2n = 54) and heptaploids (2n = 63) were found in *Hieracium virosum* PALL. (PULKINA & TUPITSYNA 2000).

Until now all examined diploid species are sexual and self-incompatible (SI). However, the SI system can fail under the influence of heterospecific pollen on the stigma (mentor effect; MRÁZ 2003b, MRÁZ & TOMČÍKOVÁ 2004, MRÁZ & PAULE 2006). In contrast, triploids, tetraploids and pentaploids are agamospermous. Development of unreduced embryo sac follows the "*Antennaria* type" of diplospory, i.e., the female meiosis is fully omitted (e.g. NOGLER 1984). All hitherto studied plants showed autonomous endosperm development, i.e., the plants are not dependent on pollination. However, some irregularities have been reported showing remnants of sexual processes in the female meiosis and development of the female gametophyte (BERGMAN 1941, SKAWIŃSKA 1963).

Although chromosome counts for many *Hieracium* species have been published (see e.g. SCHUHWERK 1996), there are still considerable gaps in our knowledge of karyological diversity and its geographic pattern. As ploidy level is well correlated with the mode of reproduction (see above), detailed knowledge of chromosome numbers in particular species plays an important role in forming hypotheses about evolutionary potential of the species and evolutionary processes in the genus. In this paper we report chromosome numbers and/or ploidy levels for 111 accessions (91 accessions for chromosome counts, 19 for flow cytometry, and one for both of them) from 45 taxa from Europe. In most cases we adopted the taxonomic concept proposed by ZAHN (1921–1923). In *Hieracium alpinum* L., *H. caesium* FR., *H. nigrescens* WILLD., *H. rohacsense* KIT. and *H. waldsteinii* TAUSCH (all s.l.) the narrow species concept is accepted (microspecies grouped in an aggregate species/group). Species concept of the section *Cerinthoidea* follows MATEO (2005).

MATERIAL AND METHODS

Plants

Plants were collected between 1996 and 2006 from their natural habitats and transplanted in the experimental gardens in Průhonice near Praha and in Košice, except of *H. eriophorum* ST.-AMANS, which was grown from seeds collected in the field. Pot-grown plants were kept in either field conditions or in an unheated greenhouse. Voucher specimens are deposited in the Herbarium of the Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice (PRA, plants counted by JC and JZ) and Herbarium of the Institute of Biology and Ecology, P.J. Šafárik University, Košice (KO, plants counted or measured by flow cytometry by PM). The numbers given in parentheses after each locality refer to cultivation numbers.

Chromosome numbers

The studies were made on the root-tip meristems of pot-grown plants. Two different methods were used:

(i) actively growing roots were placed into pretreatment solution of saturated p-dichlorbenzene and kept for 3–4 hours at room temperature, then fixed in a mixture of ethanol and glacial acetic acid (3:1) overnight at 4 °C and stored in cold 70% ethanol at 4 °C

until used. The squash method and staining by lacto-propionic orceine were used (DYER 1963). (Method used by JC and JZ).

(ii) root tip cuttings were pre-treated with 0.5% solution of colchicine for 1.5–3 hours at room temperature, fixed as above, stored in 70% ethanol at 4 °C and hydrolyzed for 7–10 minutes in 1N HCl at 60 °C. The squash and smear method with cellophane replacing the glass covers followed MURÍN (1960). Giemsa solution in phosphate buffer was used as a stain. (Method used by PM).

DNA ploidy level estimation

Analysis of relative nuclear DNA content was performed with a FACSCalibur instrument (Becton Dickinson, USA) equipped with an argon-ion laser exciting at 488 nm in the Laboratory of Flow Cytometry, Institute of Biology and Ecology, P.J. Šafárik University, Košice. Sample preparations were carried out in a two-step procedure (OTTO 1990, DOLEŽEL & GÖHDE 1995). Approximately 1 cm^2 of leaf tissues of both the sample and the reference internal standard were chopped together for about 30 s in a Petri dish containing 1 ml of ice-cold Otto I buffer (0.1 M citric acid monohydrate + 1 ml 0.5% Tween 20 adjusted to 200 ml and filtered through a 42 µm filter). The filtration of chopped sample through 42 µm nylon mesh was followed by centrifugation at 150 g for 5 min. The supernatant was removed and 100 µl of fresh Otto I buffer was added. The nuclei in the pellet were resuspended and stored for 30 min at room temperature for incubation. For DNA staining 1 ml of Otto II buffer (0.4 M disodium hydrogenphosphate dodecahydrate) including propidium iodid (PI, final concentration 50 µg/ml), ribonuclease (Ribonuclease A R5000, Sigma, final concentration 50 µg/ml), and 2 µl mercaptoethanol was used. As an internal reference standard we used leaves of *Zea mays* CE-777 (2C = 5.43 pg). To estimate DNA ploidy level of analyzed plants, the exact position of peaks of previously counted di-, tri- and tetraploid *Hieracium* taxa to the peak of standard was measured. As diploid taxa we used *H. umbellatum* L. (cult. no. UMB12JP, chromosome number published in MRÁZ 2003b, and further ca. 10 plants of this species cf. TOMČÍKOVÁ 2006 and unpubl.) and *H. tomentosum* (this paper), as triploid *H. sabaudum* s.l. (cult. no. 872, CHRTEK et al. 2004) and tetraploid *H. carpathicum* BESS. (cult. no. 1150, CHRTEK et al. 2004). The ratios between nuclei fluorescence intensity of internal reference (*Zea mays*) and above mentioned *Hieracium* taxa were as follows: *H. umbellatum* (2x) 1.4–1.5, *H. tomentosum* (2x) 1.33–1.37, *H. sabaudum* s.l. (3x) 1.95–2.0 and *H. carpathicum* (4x) 2.5. Coefficient of variations (CV) of the peaks of internal standard adjusted at channel 100 ranged from 3.5% to 6.9% (with the most frequent value about 5.5%), and CV of peaks of measured samples varied between 3.0% and 5.6%. Only plants with clear peak positions corresponding to distinct ploidy level were included in the present study. DNA ploidy level estimations are given by the following formula "2n ~".

RESULTS AND DISCUSSION

***Hieracium alpinum* agg.**

***Hieracium halleri* VILL.** **2n ~ 3x**

Localities

1. Switzerland, cant. Valais, Alpes valaisannes: Col du Grand Saint Bernard, E of the col, 7°10′19″ E, 45°52′02″ N, 2560 m, 25 August 2005, leg. P. MRÁZ (H1823, analyzed by PM, peak ratio 2.05).
2. Switzerland, cant. Wallis, Walliser Alpen: Furkapass, ca. 200 m S of the col, exposed slopes with *Salix herbacea*, 2457 m, 8°24′49″ E, 46°34′22″ N, 24 August 2005, leg. P. MRÁZ (H1839, analyzed by PM, peak ratio 2.03).
3. Switzerland, cant. Graubünden, Albula Alpen: Davos, Flüelapass col, 2354 m, 9°57′54″ E, 46°44′36″ N, 22 August 2005, leg. P. MRÁZ (H1829, analyzed by PM, peak ratio 1.94).

Triploid chromosome counts were reported from the Western Carpathians (CHRTEK 1997b, MRÁZ 2001, ŠTORCHOVÁ et al. 2002) and by SCHUHWERK & LIPPERT (1999) from the Austrian Alps.

***Hieracium amplexicaule* L.** **2n = 36**

Locality

1. Italy, Trentino-Alto Adige, Gruppo dell′ Adamello: Passo del Tonale, Mt. Monticello, E slope, near the road, 2 km SE of the col (monument), 2180 m, 29 August 2005, leg. J. CHRTEK & P. MRÁZ (H1073/1b, H1073/2, counted by JC).

Hieracium amplexicaule* subsp. *amplexicaule **2n = 27**

Localities

1. Austria, Carinthia, Hohe Tauern, Goldberggruppe: Innerfragant, near the old (not marked) path to the Fraganter Hütte, ca. 1 km SW of the village, rocks above the brook, 1233 m, 28 July 2005, leg. J. CHRTEK & P. MRÁZ (H 1050/3a, H1050/4, counted by JC).
2. Austria, Carinthia, Hohe Tauern, Ankogelgruppe: Stappitz near Mallnitz, Seebachtal valley, Zelenigleiten, 4 km NE of the village, wet rocks, 1360 m, 13°10′45″ E, 47°01′12″ N, 29 July 2005, leg. J. CHRTEK (H1055/2, counted by JC).
3. Spain, Catalunya, prov. Gerona, Pirineos Mts.: Queralbs, along the road to Casa dels Plaus, near the brook of Torrent dels Plaus, 1480 m, 27 August 1996, leg. K. MARHOLD (H346, H347, counted by PM).
4. Spain, Catalunya, prov. Gerona, Pirineos Mts.: Queralbs, along the tourist path from Daió de Baix to Refugi Manelic, near the brook of Freser, 1750–1950 m, 24 August 1996, leg. K. MARHOLD (H352, counted by PM; H354, counted by JZ).

Three chromosome numbers, i.e., 2n = 18, 2n = 27 and 2n = 36 have been reported in this collective species. Triploids (2n = 27) referred to subsp. *amplexicaule* were found in the Pyrenees (SCHUHWERK & LIPPERT 1998) and in the Sierra de Baza Mts. in southern Spain (CUETO ROMERO & BLANCA LÓPEZ 1986), triploid counts without an exact identification of subspecies were reported from the Alps (GADELLA & KLIPHUIS 1970), Morocco (cf. subsp. *olivicolor* JAHAND. et ZAHN; VOGT & OBERPRIELER 1994), Spain and Balearic Islands

(CASTRO et al. 2007). Chromosome number 2n = 27 was also found in plants of subsp. *berardianum* (ARV.-TOUV.) ZAHN from the Austrian Alps (SCHUHWERK & LIPPERT 1999) and of subsp. *speluncarum* (ARV.-TOUV.) ZAHN from the Serra do Geres Mts. in Portugal (FERNANDES & QUIERÓS 1971). Tetraploids (2n = 36) of *H. amplexicaule* were reported by GENTCHEFF & GUSTAFSSON (1940) and QUÉZEL (1957). The same chromosome number has been found in plants referred to *H. pulmonarioides* VILL. from British Isles (MILLS & STACE 1974) and from botanical gardens (ROSENBERG 1927, GENTCHEFF 1937). Recently, CASTRO et al. (2007) revealed for the first time a diploid cytotype (2n = 18) in plants from Spain.

***Hieracium bocconei* GRISEB.** **2n ~ 4x**

Locality

1. Italy, Alto Adige, Deferegger Alpen: Passo Stalle (Staller Sattel), S slopes, 1954 m, 12°11′46″ E, 46°53′24″ N, 30 July 2005, leg. P. MRÁZ & J. CHRTEK (H1816, analyzed by PM, peak ratio 2.41).

To our best knowledge this is the first record on ploidy level for this species.

Hieracium bupleuroides* C.C. GMEL. subsp. *bupleuroides **2n = 27, 2n ~ 3x**

Localities

1. Austria, Vorarlberg, Allgäuer Alpen: Baad, between Starzel Joch (1867 m) and Hochstarzel (1974 m), 20 July 2002, leg. P. MRÁZ (H1235, counted by PM).
2. Slovakia, distr. Ilava, Biele Karpaty Mts.: Vršatské Podhradie, castle ruin of Vršatec, 770 m, 15 June 2005, leg. J. CHRTEK (H1033/2, H1033/3, 2n = 27, counted by JC).
3. Slovakia, distr. Tvrdošín, Chočské vrchy Mts.: Kvačianska dolina valley, upper part, 2 km SW of the church in the village of Huty, limestone rocks, 765 m, 19°32′58″ E, 49°12′16″ N, 17 August 2006, leg. J. CHRTEK & S. CHRTKOVÁ (H1209/2, 2n = 27, counted by JC).
4. Slovakia, distr. Poprad, Slovenský raj region: Stratená, calcareous rocks and small screes above the road near the upper end of the road tunnel, ca. 840 m, 9 July 2005, leg. P. MRÁZ (H1755, 2n ~ 3x, analyzed by PM, peak ratio 2.0).
5. Slovakia, distr. Gelnica, Volovské vrchy Mts.: Kojšov, Turniská in the massif of Murovaná skala, 1 July 2005, leg. P. MRÁZ & V. MRÁZOVÁ (H1752, 2n = 27, counted by PM).

***Hieracium bupleuroides* subsp. *leviceps* NÄGELI & A. PETER** **2n = 27**

Locality

1. Austria, Oberösterreich, Dachstein massif: Vorderer Gosausee mountain lake, rocks on NW bank, 6 km SSW of the village of Gosau, 940 m, 13°29′55″ E, 47°31′53″ N, 13 August 2005, leg. J. CHRTEK, det. F. SCHUHWERK (H1063/2, counted by JC).

***Hieracium bupleuroides* subsp. *tatrae* (GRISEB.) NÄGELI & A. PETER** **2n = 27, 2n ~ 3x**

Localities

1. Slovakia, distr. Poprad, Slovenský raj region: Vernár, S part of the village, limestone slopes above the road, 780 m, 20°16′07″ E, 48°54′50″ N, 5 August 2005, leg. J. CHRTEK & K. CHRTKOVÁ (H1062/1, 2n = 27, counted by JC).

2. Slovakia, distr. Poprad, Slovenský raj region: Stratená, calcareous rocks and small screes above the road near the upper end of the road tunnel, ca. 840 m, 9 July 2005, leg. P. MRÁZ (H1754, 2n ~ 3x, analyzed by PM, peak ratio 1.95).

Triploids seem to prevail in this rather variable taxon. CHRTEK et al. (2004) reported 2n = 27 for plants identified as subsp. *gmelinianum* (= subsp. *bupleuroides*) and subsp. *tatrae*. The same number was found in *H. bupleuroides* from the Slovenský kras region (southern Slovakia) by Murín and Uhríková (in MÁJOVSKÝ 1970). Further triploid counts come from the Bavarian Alps (SCHUHWERK & LIPPERT 1999), the Austrian Alps (POLATSCHEK 1966, SCHUHWERK & LIPPERT 1999 – plant referred to subsp. *bupleuroides*) and from northern Bavaria (SCHUHWERK & LIPPERT 1999). CHRISTOFF & POPOFF (1933) also published the same chromosome number, but the locality was not given. A tetraploid chromosome number was given for plants from Montenegro (NIKETIĆ et al. 2003, 2006; *H. bupleuroides* subsp. *pseudoschenkii* ROHLENA & ZAHN), Slovakia (CHRTEK et al. 2004), and southern Poland (SZELĄG & VLADIMIROV 2005).

Hieracium caesioides* ARV.-TOUV. subsp. *caesioides **2n = 27**

Localities

1. France, dépt. Alpes maritimes, valley of Roya: Tende, along the old road to the Col de Tende, ca. 0.5 km above the tunnel, 6 km NNW of the village, 1331 m, 07°33′57″ E, 44°08′19″ N, 28 August 2005, leg. J. CHRTEK & P. MRÁZ (H1067/4, H1067/5, H1067/6, H1067/10, counted by JC and JZ).
2. France, dépt. Alpes maritimes, valley of Roya: Tende, Mt. Cime de Salante, S slopes near the marked path, 8 km NW of the village, 2080 m, 28 August 2005, leg. J. CHRTEK & P. MRÁZ (H1068/2, counted by JC).

This is the first chromosome number record for *H. caesioides*.

***Hieracium caesium* agg.**

***Hieracium basifolium* (FR. ex ALMQ.) LÖNNR.** **2n = 36**

(*H. caesium* subsp. *basifolium* sensu ZAHN)

Locality

1. Sweden, prov. Gästrikland, par. Hille: dry road/forest margin at Oslättfors, ca. 15 km NW of Gävle, July 2006, leg. et det. T. TYLER (H1227/2, counted by JC).

***Hieracium plumbeum* FR.** **2n = 36**

(*H. caesium* subsp. *caesium* s. str. sensu ZAHN)

Locality

1. Sweden, prov. Gotland, par. Hall: open limestone scree by the sea 1.3 km SE of Hallshuk (close to the NW point of the island of Gotland ca. 40 km NNE of Visby), July 2006, leg. et det. T. TYLER & A. SENNIKOV (H1231/3, counted by JC).

The presented numbers are the first for the respective taxa. Chromosome number 2n = 36 has been reported by SCHUHWERK & LIPPERT (1999) for subsp. *caesium* from Bavaria (Germany) and subsp. *carnosum* (WIESB. ex DICHTL) ZAHN from Austria.

***Hieracium cerinthoides* L. s.str.** **2n = 27**

(*H. cerinthoides* subsp. *cerinthoides*)

Locality

1. Spain, Catalunya, prov. Lérida: Os de Civís, 1 km WSW of the village, margin of a pasture, 1720 m, 21 July 2006, leg. J. CHRTEK, G. MATEO & J. A. ROSSELLÓ, det. G. MATEO (H1176/1, counted by JC).

DELAY (1969) found 2n = 18 in *Hieracium cerinthoides*. However, taxonomic identity of the counted plants is not clear; they might belong to another taxon within the *H. cerinthoides* species group.

***Hieracium cordifolium* LAPEYR. s.str.** **2n = 18**

Locality

1. Andorra, Pirineos Mts.: near Bixessarri (NW of Sant Julia de Loria), valley of Torrent dels Llimois, rocks and margins of a path ca. 100 m from the street, 1.5 km NW of the village, 1305 m, 21 July 2006, leg. J. CHRTEK, G. MATEO & J. A. ROSSELLÓ, det. G. MATEO (H1177/2, counted by JC).

CASTRO et al. (2007) determined diploid and triploid cytotypes in this species. Previously, SCHUHWERK & LIPPERT (1998) reported 2n = 18 for *H. cordifolium* subsp. *neocerinthe* (FR.) ZAHN and 2n = 27 for *H. c.* subsp. *eriocerinthe* (FR.) ZAHN.

***Hieracium eriophorum* ST.-AMANS** **2n = 18**

Localities

1. France, dépt. Landes, Labenne: plage de Labenne Océan Sud, 27 September 2006, leg. E. FOREY (H1221, counted by JC).
2. France, dépt. Landes, Seignosse-le-Penon: plage de Estagnols Seignosse, 27 September 2006, leg. E. FOREY (H1222, counted by JC).
3. France, dépt. Landes, Vieux-Boucau-les-Bains: plage de Vieux-Boucau, 27 September 2006, leg. E. FOREY (H1223, counted by JC).

The same number was published by MERXMÜLLER (1975).

***Hieracium glaucum* ALL.** **2n ~ 3x**

Locality

1. Austria, Carinthia, the Karawanken Mts.: Bad Eisenkappel, limestone rocks and pine forest (alliance *Erico-Pinion*) near the road to Bad Vellach, 4.5 km SSW of the town, 658 m, 14°34′20″ E, 43°27′07″ N, 26 July 2005, leg. J. CHRTEK & P. MRÁZ (H1757, analyzed by PM, peak ratio 1.96).

***Hieracium glaucum* subsp. *nipholepium* NÄGELI & A. PETER** **2n = 27**

Localities

1. Slovenia, Primorska region, Julijske Alpe Mts.: Trenta valley, ca. 1 km W of the village of Podklanec, near the road Vršič – Bovec, 425 m, 9 November 2005, leg. B. VREŠ & J. CHRTEK (H1081/2, counted by JC).
2. Slovenia, Primorska region, Julijske Alpe Mts.: near the road from the col of Vršič to the Trenta valley, 1020 m, 9 November 2005, leg. B. VREŠ & J. CHRTEK (H1083/3, counted by JC).
3. Slovenia, Primorska region, Julijske Alpe Mts.: Zadnjica valley, along the marked path to the Luknja col, 4 km E of the village of Trenta, ca. 1100 m, 8 August 2005, leg. V. ZAVADIL (H1232/2, counted by JZ).

The same chromosome number, corresponding to the triploid level, has been recorded in plants of *H. glaucum* from Styria (Austria) (POLATSCHEK 1966), Italy (SCANNERINI 1971) and Germany (SCHUHWERK & LIPPERT 1999, plant corresponding to subsp. *isaricum* (NÄGELI ex J. HOFM.) NÄGELI & A. PETER).

***Hieracium gouanii* ARV.-TOUV.** **2n = 18**

(*H. cordifolium* subsp. *gouani* (ARV.-TOUV.) ZAHN)

Locality

1. Spain, Catalunya, prov. Gerona: rocks at the road between Ripoll and Ribes de Fresser, 24 July 2006, leg. J. CHRTEK (H1171/2, H1171/5, H1171/6, counted by JC).

The first karyological record for the species, treated by most authors at the subspecific level as *H. cordifolium* subsp. *gouanii* (ARV.-TOUV.) ZAHN. It clearly differs from *H. cordifolium* s.str. from the Central Pyrenees mainly by the taller stem and longer glabrescent leaves (MATEO 2005). It is confined to Catalunya and eastern part of the Pyrenees Mts. (NE Spain, S France).

***Hieracium gymnocerinthe* ARV.-TOUV. & GAUT.** **2n = 27**

Locality

1. Spain, Catalunya, prov. Lérida, distr. La Seu d´Urgell: Adraén, Serra del Cadí mountain ridge, NW slopes, 1 km SE of the village, 1600 m, road margin in a pine forest with dominating *Arctostaphylos uva-ursi*, 1°30′34″ E, 42°16′15″ N, 23 July 2006, leg. J. CHRTEK, G. MATEO & J. A. ROSSELLÓ, det. G. MATEO (H1172/4, counted by JC).

It differs from morphologically similar *H. ramondii* mainly in the indumentum of the phyllaries (numerous simple eglandular hairs in *H. ramondii* and numerous glandular hairs in *H. gymnocerinthe*); leaves are glabrous. It has been often treated as a subspecies of *H. cerinthoides*, which possesses (regarding the indumentum of the phyllaries) an intermediate position between *H. ramondii* and *H. gymnocerinthe*.

***Hieracium humile* JACQ.** **2n = 36**

Locality

1. Austria, Oberösterreich, Dachstein massif: Vorderer Gosausee mountain lake, rocks on NW bank, 6 km SSW of the village of Gosau, 940 m, 13°29′55″ E, 47°31′53″ N, 13 August 2005, leg. J. CHRTEK (H1064/2, H1064/3, counted by JC and JZ).

This is the first tetraploid (2n = 36) chromosome number ascertained for this species. SCHUHWERK & LIPPERT (1999) reported 2n = 27 for *H. humile* subsp. *pseudocottetii* (ZAHN) ZAHN from the Bavarian Alps (Karwendelgebirge).

***Hieracium intybaceum* ALL.** **2n = 18**

Locality

1. Italy, Trentino-Alto Adige, Gruppo dell´ Adamello: Passo del Tonale, valley of the Presena rivulet, glacial cirque above the Lago Presena mountain lake, 3 km SSE of the col (monument), 2270 m, 10°35′49″ E, 46°13′42″ N, 29 August 2005, leg. J. CHRTEK & P. MRÁZ (H1069/1, counted by JC).

Three ploidy levels, namely diploid, triploid and tetraploid have been reported in this species. The first published count (2n = 27) comes from a plant cultivated by C.H. Ostenfeld in the Botanical Garden in Copenhagen (ROSENBERG 1927). Later LARSEN (1954) reported the same chromosome number in plants from the Swiss Alps (Schynige Platte, Oberland Bernois). Nevertheless, the most common ploidy level seems to be the diploid one, although it remained unrevealed until the 1990s (DOBEŠ et al. 1997, FAVARGER 1997). Only two tetraploid populations have been published until now, namely from the Alpes valaisannes (FAVARGER 1997).

***Hieracium kittaniae* VLADIMIR.** **2n = 18**

Locality

1. Bulgaria, Central Rhodope Mts.: Trigrad gorge, limestone rocks near the natural entrance to Dyavolskoto garlo cave, September 2005, leg. P. IGNATOVA (H1228/2, counted by JC).

Hieracium kittanae is a distinct relict species restricted to crevices of limestone rock in the Central Rhodope Mts. in southern Bulgaria. Our counts coincide with previously published data (VLADIMIROV 2003).

***Hieracium lachenalii* SUTER** **2n = 27**

Localities

1. Czech Republic, Bohemia, distr. Rakovník: forest between the villages of Roztoky and Křivoklát, 350 m, 29 May 2006, leg. J. ZAHRADNÍČEK (H1150/4, counted by JC).
2. Czech Republic, Bohemia, distr. Praha-východ: Tehov, forest margin 1.8 km NE of the village, 430 m, 14°42′36″ E, 49°59′01″ N, 25 July 2002, leg. J. CHRTEK & H. CHAPMAN (H831/1, counted by JC).
3. Czech Republic, Moravia, distr. Znojmo: Lukov, forest 1.3 km SSW of the village, 410 m, 15°54′27″ E, 48°51′04″ N, June 2006, leg. J. ZAHRADNÍČEK (H1160/4, counted by JC).

Hieracium lachenalii represents a taxonomically very difficult entity. Our counts well match most of the previously published data (2n = 27; cf. CHRTEK et al. 2004). LAVRENKO & SERDITOV (1987) reported the tetraploid level (2n = 36) and ROSTOVTSEVA (1983; ut *H. tilingii* JUXIP) the hypertriploid one (2n = 28) .

Hieracium laevigatum* WILLD. subspecies group *laevigatum **2n = 27**

Localities

1. Czech Republic, Bohemia, distr. Rokycany: Strašice, N part of the village, margin of a forest, 550 m, 13°45′07″ E, 49°44′51″ N, 29 June 2005, leg. J. CHRTEK (H1031/5, H1031/11, counted by JC).
2. Czech Republic, Bohemia, distr. Hradec Králové: Hradec Králové, forest 1.2 km SE of the church in Nový Hradec Králové, 270 m, 15°52′16″ E, 50°10′17″ N, 1 July 2006, leg. J. CHRTEK & K. CHRTKOVÁ (H 1165/1, counted by JC).

Triploids (2n = 27) seem to be most frequent in this collective species. In addition, diploid counts (2n = 18) were published (e.g. SCHUHWERK 1996 and other standard reference manuals, e.g. Missouri Botanical Garden (2007)).

***Hieracium murorum* L.** **2n = 27**

Localities

1. Czech Republic, Bohemia, distr. Plzeň: Plzeň, village of Koterov, the street "V závrtku", 0.6 km SSW of the railway station "Plzeň-Koterov", slopes along the street, ca. 320 m, 13°25′07″ E, 49°43′02″ N, 12 August 2003, leg. M. KRÁL (H875/4, H875/6, H875/8, H875/16, counted by JC and JZ).
2. Czech Republic, Bohemia, distr. Beroun: Svatý Jan pod Skalou, oak-hornbeam forest 0.8 ENE of the village, 350 m, 14°08′30″ E, 49°58′15″ N, 28 May 2005, leg. J. CHRTEK (H1030/1, H1030/2, counted by JC).
3. Czech Republic, NW Bohemia, Doupovské hory Mts.: Stružná, 2 km N of the village, along the road, 650 m, 13°02′ E, 50°18′ N, 1 June 2006, leg. J. ZAHRADNÍČEK (H1152/2, counted by JC).
4. Czech Republic, Bohemia, distr. Domažlice: Kdyně, forest margin near a parking place 2.4 km NE of the town, 640 m, 13°04′08″ E, 49°24′35″ N, 12 June 2006; leg. J. CHRTEK (H1156/3, counted by JC).

The chromosome number of 2n = 27 is the most common one among the karyological data on *H. murorum*. Tetraploids (2n = 36) seem to be rare (e.g. SCHUHWERK 1996 and other standard reference manuals).

Hieracium naegelianum* PANČIĆ subsp. *naegelianum **2n = 27**

Locality

1. Montenegro, Durmitor Mts.: Mt. Veliki Međed, alpine grassland on limestone, 2050 m, 19°04′13″ E, 43°03′31″ N, 1 August 2006, leg. and det. Z. SZELĄG (H1208/1, H1208/3, counted by JC).

This chromosome number confirms the previous counts from the Durmitor Mts. (NIKETIĆ et al. 2003, 2006) and from other Balkan localities (MERXMÜLLER 1975, GRAU & ERBEN 1988, VLADIMIROV & SZELĄG 2001).

***Hieracium nigrescens* agg.**

***Hieracium decipientiforme* (WOŁ. & ZAHN) SCHLJAKOV** **2n = 36**

Locality

1. Ukraine, Oblast´ Zakarpatska, Marmaros´ki Al´py Mts.: Dilove, at the foot of Mt. Berlebashka, SE exposition, ca. 1600 m, 30 July 1996, leg. P. MRÁZ (H81, counted by PM).

CHRTEK (1997a) reported the same chromosome number for this morphologically very distinct species.

Hieracium olympicum* BOISS. subsp. *olympicum **2n = 27**

Locality

Bulgaria, Stara Planina Mts., Kaloferska Planina Mts.: Valley of Vidima River, 2 km NE of the Kaloferski Monastyr, eroded slope in the *Carpinus orientalis* forest, 870 m, 24°58´42″ E, 42°40´36″ N, 9 August 2006, leg. and det. Z. SZELĄG (H1206/3, counted by JC).

VLADIMIROV & SZELĄG (2001) reported a triploid chromosome number for this species.

Hieracium pannosum* BOISS. subsp. *pannosum **2n = 27**

Locality

1. Bulgaria, Stara Planina Mts., Trojanska Planina plateau: Mt. Kozja stena, grassy slope on limestone, 1570 m, 24°34′06″ E, 42°47′27″ N, 8 August 2006, leg. and det. Z. SZELĄG (H1205/1, counted by JZ).

This is the first triploid chromosome count from Bulgaria. Earlier, the tetraploid count for *H. pannosum* was reported from Greece (PAPANICOLAOU 1984) and Bulgaria (VLADIMIROV & SZELĄG 2001). The triploid chromosome number was reported from Greece (STRID & FRANZÉN 1981, PAPANICOLAOU 1984, SCHUHWERK & LIPPERT 1998).

***Hieracium petrovae* VLADIMIR. & SZELĄG** **2n = 18**

Locality

1. Bulgaria, Central Rhodope Mts.: Trigrad gorge, crevices of limestone rock (locus classicus), 750–800 m, 24°21′50″ E, 41°39′55″ N, 15 October 2005, leg. V. VLADIMIROV (H1229, counted by JC).

Hieracium petrovae is the only known diploid representative of the *H. pannosum* agg. It is closely related to a number of presumably descendent taxa in the *H. pannosum*, *H. pilosissimum* and *H. heldreichii* collective species. It is a calciphilous chasmophyte confined to several localities in the Central Rhodope Mts. in southern Bulgaria (VLADIMIROV

& SZELĄG 2006). Our count confirms the previously published data (VLADIMIROV & SZELĄG 2006).

***Hieracium piliferum* HOPPE** **2n ~ 3x**

Localities

1. Italy, Alpi lepontine, Spluga: Passo dello Spluga (Splügenpass), 2120 m, 9°1′54″ E, 46°30′21″ N, 23 August 2005, leg. P. MRÁZ (H1845, H1846, analyzed by PM, peak ratio 1.93).
2. Austria, Kärnten, Hohe Tauern: Kobnitz, Reißeck Hütte, S of the tunnel, 2225 m, 13°21′41″E, 46°55′59.8 ″N, 29 July 2005, leg. P. MRÁZ (H1802, analyzed by PM, peak ratio 1.98).

Hieracium piliferum* subspecies group *piliferum **2n = 27, 2n ~ 3x**

Localities

1. France, dépt. Hautes Alpes: SE of Col du Galibier, 2570 m, 6°24′09″ E, 45°04′59.8″ N, 4 July 2003, leg. P. MRÁZ (H1344, 2n = 27, counted by PM).
2. Switzerland, cant. Bern, Berner Alpen: Interlaken, ca. 1 km NE of Mt. Schillthorn, 2230 m, 7°52′35″ E, 46°34′05″ N, 19 July 2006, leg. P. MRÁZ (1 plant without no., 2n ~ 3x, analyzed by PM, peak ratio 1.93).

Hieracium piliferum* subspecies group *glanduliferum **2n ~ 3x, 4x**

Localities

1. Switzerland, cant. Valais, Alpes valaisannes: Col du Grand Saint Bernard, ca. 2600 m, 25 August 2005, leg. J. KOŠÚT (H1856, 2n ~ 3x, analyzed by PM, peak ratio 1.99).
2. France, dépt. Puy de Dome, Massif Central Mts.: Mt. Puy Sancy, very steep slope below the top exposed to E, 1875 m, 27 June 2006, leg. P. MRÁZ (H06/23, 2n ~ 4x , analyzed by PM, peak ratio 2.69).

The triploid level seems to prevail in *Hieracium piliferum*. It was first published by SCHOLTE (1977) from Switzerland. Later on, SCHUHWERK & LIPPERT (1999) found tetraploid plants in the same country. Isolated Western Carpathian populations of *H. piliferum* were also revealed to be tetraploid (MRÁZ 2003a). Tetraploid plants from the Massif Central were collected in the early stage (rosette leaves) and determined as *H. piliferum* s.l. only. However, according to local specialist F. Billy, only *H. glanduliferum* subsp. *glanduliferum* occurs at the locality (BILLY 1977).

***Hieracium porrifolium* L.** **2n = 18, 2n ~ 2x**

Localities

1. Italy, Trentino-Alto Adige: Villini dell´ Alpe, calcareous rocks along the road to Pianizza di Sopra (direction to Bolzano), 2 km E of the village, 30 August 2005, leg. J. CHRTEK & P. MRÁZ (H1075/2, H1075/5, 2n = 18, counted by JC).
2. Austria, Carinthia, the Karawanken Mts.: Bad Eisenkappel, limestone rocks and pine forests (alliance *Erico-Pinion*) near the road to Bad Vellach, 4.5 km SSW of the town,

658 m, 14°34′20″ E, 43°27′07″ N, 26 July 2005, leg. J. CHRTEK & P. MRÁZ (H1052/6, H1052/9, 2n = 18, counted by JC; H1756, 2n ~ 2x, analyzed by PM, peak ratio 1.39).
3. Slovenia, Primorska region, Julijske Alpe Mts.: Trenta valley, Trnovo ob Soči, near the road to Kobarid, 335 m, 9 November 2005, leg. B. VREŠ & J. CHRTEK (H1080/1, H1080/2, 2n = 18, counted by JC).

The species appears to be invariable in chromosome number. Diploids (2n = 18) have been reported by FAVARGER (1965) from the Julijske Alpe Mts. (Julian Alps), and by MARCUCCI & TORNADORE (1999) from the Treviso region in NE Italy.

Hieracium prenanthoides* VILL. subspecies group *prenanthoides **2n = 27**

Locality

1. Andorra, Canillo, SE margin of the village, 1530 m, 22 July 2006, leg. J. CHRTEK, G. MATEO & J. A. ROSSELLÓ (H 1187/2, counted by JC).

This is only the second reference on ploidy level in *H. prenanthoides* from the Pyrenees and it confirms the previous count stated on plants originating from the same region (cf. CASTRO et al. 2007). Three ploidy levels, namely diploids (2n = 18), triploids (2n = 27), and tetraploids (2n = 36) have been reported in this collective species. However, triploids strongly prevail among the examined plants (cf. CHRTEK 1996, SCHUHWERK 1996, and other chromosome number indexes). Diploids were only reported from the French Alps (Hautes Alpes; FAVARGER 1969a, FAVARGER 1969b); tetraploids were found by CHRISTOFF & POPOFF (1933, cultivated plant of unknown origin) and by LÖVE (1970) in plants from Iceland.

***Hieracium ramondii* GRISEB.** **2n = 27**

Locality

1. Andorra, Pirineos Mts.: Encamp, valley of Riu de les Deveses, NW slopes of Mt. Alt del Griu, 3.8 km E of the town, rocky outcrops in a light mountain forest, 2040 m alt., 1°37′52″ E, 42°32′07″ N, 22 July 2006, leg. J. CHRTEK, G. MATEO & J. A. ROSSELLÓ, det. G. MATEO (H 1173/4, H 1173/5, counted by JC).

This is the first chromosome number record for the species.

***Hieracium rohacsense* agg.**

***Hieracium rauzense* MURR** **2n ~ 3x**

Locality

1. Austria, Osttirol, distr. Lienz: Staller col, E of the Obersee glacial lake, 7.5 km WSW of Mariahilf, along the road, 2043 m, 12°12′33″ E, 46°53′23″ N, 29 July 2005, leg. P. MRÁZ & J. CHRTEK (H1759, H1760, H1761, analyzed by PM, peak ratio 1.83–1.89).

The same ploidy level based on chromosome counts was found by MRÁZ (2001) in plants originating from Vorarlberg (the Austrian Alps). Due to some level of morphological convergence, *H. rauzense* is traditionally treated within the *H. rohacsense* group, or even it is

given as a synonym of *H. rohacsense* s.str. However, the taxa are rather different with respect to their morphology, ploidy level, distribution range and allozyme pattern (MRÁZ & MARHOLD 1999, MRÁZ 2001, MRÁZ et al., unpubl.). Both taxa likely have different origins.

***Hieracium recoderi* DE RETZ** **2n = 18**

Locality

1. Spain, Catalunya, prov. Barcelona: Berga, monastery of Queralt, rocks ca. 200 m below the parking place, 24 July 2006, leg. J. CHRTEK (H1174/4, counted by JC).

This is the first karyological record for this species. The accession comes from the locus classicus (DE RETZ 1978). The species is only known from several localities in north-central Catalunya.

***Hieracium schmidtii* TAUSCH** **2n = 27**

Localities

1. Czech Republic, Bohemia, distr. Litoměřice: Boreč, the Boreč hill, NW slope, 800 m NW of the village, 350 m, 13°59′15″ E, 50°30′54″ N, 15 May 2005, leg. J. CHRTEK (H1024/6, H1024/7, counted by JC).
2. Czech Republic, Bohemia, distr. Litoměřice: Boreč, the Boreč hill, E slope, 500 m N of the village, 370 m, 13°59′25″ E, 50°30′53″ N, 15 May 2005, leg. J. CHRTEK (H1025/3, H1025/5, counted by JC).

Our chromosome counts well match those published by CHRTEK (1996) from the Krkonoše Mts. (Czech Republic), by CASTRO et al. (2007) from Spain and by SCHUHWERK & LIPPERT (1999) for *H. schmidtii* subsp. *comatulum* (BOREAU) GOTTSCHL. and *H. schmidtii* subsp. *kalmutinum* (ZAHN) GOTTSCHL. from Bavaria. Chromosome number 2n = 36 was reported for plants referred to as *H. schmidtii* agg. from Central Bohemia, Czech Republic (KIRSCHNER & ŠTĚPÁNEK in MĚSÍČEK & JAROLÍMOVÁ 1992) and for plants of *H. schmidtii* from Spain (CASTRO et al. 2007).

***Hieracium stelligerum* FROEL.** **2n = 18**

Locality

1. France, dépt. Ardeche, Vallon Pont d'Arc: crevices of the calcareous rocks along the road D 390, just on the opposite side of the "le Pont d'Arc", ca. 3.5 km SE of the village, October 2006, leg. P. MRÁZ (H06/38, 39, counted by PM).

This is the first chromosome number record for *H. stelligerum*.

***Hieracium tomentosum* L.** **2n ~ 3x**

Locality

1. France, dépt. Hautes Alpes: Briançon, 1300 m, 2005, leg. R. DOUZET (plant without no., 2n ~ 3x, analyzed by PM, peak ratio 1.98).

Hieracium tomentosum* subsp. *tomentosum **2n = 18, 2n ~ 2x**

Locality

1. France, dépt. Alpes maritimes, valley of Roya: Tende, along the old road to the Col de Tende, ca. 0.5 km above the tunnel, 6 km NNW of the village, 1331 m, 7°33′57″ E, 44°08′19″ N, 28 August 2005, leg. J. CHRTEK & P. MRÁZ (H1066/1, H1066/2, H1066/6, H1066/8, 2n = 18, counted by JC and JZ; H1852, 2n = 18, counted by PM; H1851, H1852, H1853, 2n ~ 2x, analyzed by PM, peak ratio 1.33–1.37).

Surprisingly, these are the first chromosome counts in *H. tomentosum*. While in the Alpes Maritimes we found a diploid cytotype, one plant from a northerly situated population from Briançon was triploid.

***Hieracium tommasinianum* MALY** **2n = 27**

(*H. tommasinii* RCHB. f., nom. illeg.)

Locality

1. Serbia, SW part, distr. Zlatibor: Mileševka river gorge, 10 km SW of Prijepolje, 19°44′53″ E, 43°21′31″ N, 30 April 2006, leg. M. NIKETIĆ (H1224, counted by J.Z.)

SCHUHWERK & LIPPERT (1998) found 2n = 36 in plants from Montenegro.

***Hieracium transylvanicum* HEUFF.** **2n = 18**

Locality

1. Ukraine, Oblast´ Zakarpatska, Marmaros´ki Al´py Mts.: Mt. Berlebashka (1480 m), NW slope along the trail (red marked), E of the village of Dilove, 19 September 2005, leg. J. ZAHRADNÍČEK (H1077/2, H1077/10, counted by JC and JZ).

The species appears to be invariable in chromosome number; only diploids have been reported so far. The published counts come from the Ukrainian Eastern Carpathians (PASHUK 1987, CHRTEK 1996, MRÁZ et al. 2005), Romanian Eastern Carpathians (MRÁZ 2003b, MRÁZ & SZELĄG 2004, MRÁZ et al. 2005), Durmitor Mts. in Montenegro (SZELĄG et al. 2007) and from the Stara Planina Mts. in Bulgaria (VLADIMIROV 2000, YURUKOVA-GRANCHAROVA et al. 2006). The first chromosome number in this species was reported by ROSENBERG (1927) but without indication of the exact locality (garden plant).

***Hieracium umbellatum* L.** **2n = 18**

Localities

1. Czech Republic, Bohemia, Praha: Praha-Troja, "Pustá vinice", heathland along a path, 240 m, 14°24′14″ E, 50°07′18″ N; 28 June 2006, leg. J. CHRTEK (H1162/3, H1162/4, counted by JC).
2. Ukraine, Oblast´ Zakarpatska, Skhidni Beskidi Mts.: Scherbovets, southern slopes of Mt. Pikui, 1300–1400 m, 22°59′45″ E, 48°49′52″ N, 21 July 2003, leg. P. MRÁZ & J. CHRTEK (H1420, H1422, H1427, H1430, counted by PM).

Table 1. Summary of the analyzed taxa and their chromosome numbers/DNA ploidy levels. DNA ploidy level estimations are given by the following formula "~". First record(s) for a taxon is/are marked in bold.

Taxon	Chromosome number/DNA ploidy level
H. alpinum agg.	
H. halleri	~ 3x
H. amplexicaule	36
H. amplexicaule subsp. *amplexicaule*	27
H. bocconei	**~ 4x**
H. bupleuroides subsp. *bupleuroides*	27, ~ 3x
H. bupleuroides subsp. *leviceps*	**27**
H. bupleuroides subsp. *tatrae*	27, ~ 3x
H. caesioides subsp. *caesioides*	**27**
H. caesium agg.	
H. basifolium	**36**
H. plumbeum	**36**
H. cerinthoides s.str.	27
H. cordifolium s.str.	18
H. eriophorum	18
H. glaucum	~ 3x
H. glaucum subsp. *nipholepium*	**27**
H. gouanii	**18**
H. gymnocerinthe	**27**
H. humile	36
H. intybaceum	18
H. kittaniae	18
H. lachenalii	27
H. laevigatum subspecies group *laevigatum*	27
H. murorum	27
H. naegelianum subsp. *naegelianum*	27
H. nigrescens agg.	
H. decipientiforme	36
H. olympicum subsp. *olympicum*	27
H. pannosum subsp. *pannosum*	27
H. petrovae	18
H. piliferum	~ 3x
H. piliferum subspecies group *piliferum*	27, ~ 3x
H. piliferum subspecies group *glanduliferum*	~ 3x, 4x
H. porrifolium	18
H. prenanthoides	27
H. ramondii	**27**
H. rohacsense agg.	
H. rauzense	~ 3x
H. recoderi	**18**
H. schmidtii	27
H. stelligerum	**18**
H. tomentosum	**~ 3x**
H. tomentosum subsp. *tomentosum*	**18, ~ 2x**
H. tommasinianum	27
H. transylvanicum	18
H. umbellatum	18
H. waldsteinii agg.	
H. plumulosum	18

Both sexual diploids (2n = 18) and apomictic triploids (2n = 27) are known in this species (for references see e.g. MÁJOVSKÝ et al. 1987, SCHUHWERK 1996, and other standard chromosome number indexes).

***Hieracium waldsteinii* agg.**

***Hieracium plumulosum* A. KERN.** **2n = 18**

Locality

1. Montenegro, Canyon of the Mrtvica river, 35 km SW of Kolasin, halfway through the canyon, around the bridge, 1000 m, 19°48′59″ E, 42°28′40″ N, August 2006, leg. J. ZAHRADNÍČEK, det. Z. SZELĄG (H1218/1, H1218/2, counted by JC).

Three chromosome numbers have been published for *H. waldsteinii* s.l. by SCHUHWERK & LIPPERT (1998). They reported 2n = 18 for plants referred to *H. w.* subsp. *suborieni* ZAHN from Montenegro, 2n = 27 for those identified as subsp. *plumulosum* (A. KERN.) ZAHN from Serbia and Bosnia and Herzegovina and 2n = 36 for plants of subsp. *baldaccianum* (FREYN) ZAHN from Montenegro. The first diploid (2n = 18) count in *H. plumulosum* was reported by SZELĄG et al. (2007) from the Sinjajerina Mts. in Montenegro.

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Príloha L

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Abstract Although reticulation has indisputably played an important role in the evolutionary history of the genus *Hieracium* s. str. (Asteraceae), convincingly documented cases of recent interspecific hybridization are very rare. Here we report combined evidence on recent hybridization between two diploid species, *Hieracium alpinum* and *H. transsilvanicum*. The hybrid origin of the plants from the Romanian Eastern Carpathians was supported by additive patterns of nuclear ribosomal DNA polymorphism (ITS), an intermediate position of hybrid plants in principal coordinate analysis based on amplified fragment length polymorphism phenotypes (AFLP), and additivity at one allozyme locus. Flow cytometric analyses and chromosome counting showed that two hybrids were diploid (2n ~ 2x ~ 18) while one was surprisingly tetraploid (2n = 4x = 36). To our knowledge, this is the first record of spontaneous polyploidization following interspecific crossing in the genus. Allozyme data, especially the presence of unbalanced heterozygosity at one locus, suggest the origin of this tetraploid via a triploid bridge with subsequent backcrossing to *H. alpinum*. According to PCR-RFLP analyses of the *trn*T-*trn*L intergenic spacer, all *H.* ×*krasani* hybrids examined had the *H. alpinum* haplotype while *H. transsilvanicum* served as a pollen donor. The hybrids occurred at the locality with abundant *H. alpinum* plants where paternal *H. transsilvanicum* was missing. Previously reported instances of interspecific hybridization between the same parental taxa showed an opposite direction of crossing and relative abundance of parental taxa. This suggests that the direction of hybridization might be influenced by the frequency of parental taxa at the locality.

Keywords Additive polymorphism · AFLP · Allozymes · Asteraceae · Chromosome number · nrITS · Flow cytometry · Hybridization · Polyploidization

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Introduction

Hieracium L. s.str. (i.e., without *Pilosella* Hill) is a species-rich Holarctic hawkweed genus. Most of the so far analyzed *Hieracium* taxa are tri- or tetraploid apomicts (2n = 3x/4x = 27/36, based on x = 9), while sexual diploid species (2n = 2x = 18) are rare and most of them are confined to southern latitudes (Merxmüller 1975; Schuhwerk 1996; Chrtek et al. 2004, 2007; Tyler and Jönsson 2009). Morphological (Zahn 1921–1923) and molecular patterns of variation (Fehrer et al. 2009) suggest very extensive interspecific hybridization. However, the reticulation has mostly taken place in the past (Fehrer et al. 2009), and there is almost no evidence of recent interspecific gene flow (Mráz et al. 2005). Thus, any documented case of recent hybridization is of high interest because it

might help to better understand ancient hybridization processes in this genus.

Only two incidences of natural recent hybridization have been documented so far in the genus *Hieracium* (Mráz et al. 2005; Chrtek et al. 2006). In both cases, only diploid parental taxa were involved, although crossing between diploid and polyploid cytotypes is possible as was demonstrated by experimental crossing (Mráz 2003; Mráz and Tomčíková 2004). There are several reasons why recent interspecific hybridization is rare in the genus: (1) The few diploid species are usually geographically and/or ecologically allopatric, and thus possible interspecific gene flow is strongly limited. Moreover, even in the rare cases of sympatric occurrence of diploid species, the frequency of hybridization might be decreased due to induced autogamy (so-called mentor effect) of otherwise strictly self-incompatible taxa (Mráz 2003; Mráz and Paule 2006). (2) In polyploid taxa, hybridization is strongly constrained due to agamospermic reproduction (autonomous diplospory of the *Antennaria* type, Gustafsson 1946). This breeding system is considered as obligate in *Hieracium* polyploids. Moreover, it seems that fertilization is highly improbable in polyploids because embryo formation already starts before flower opening (so-called precocious embryony, Skawińska 1963; Nogler 1984). Thus, interspecific pollen has literally no chance to fecundate an unreduced egg cell in polyploid plants. According to this, *Hieracium* polyploids have only limited possibility, if any, to play some role in hybridization as maternal (seed) plants. Nevertheless, many *Hieracium* polyploids are still able to produce some amount of pollen and might therefore contribute to gene flow as pollen donors (Mráz et al. 2002, 2009; Slade and Rich 2007). Indeed, some viable hybrid progeny have been obtained during experimental crosses between diploid taxa serving as maternal plants and apomictic polyploids as pollen donors (Mráz 2003; Mráz and Tomčíková 2004). However, also in this type of cross, the mentor effect reduced the hybridization rate substantially (Mráz 2003; Mráz and Tomčíková 2004).

Hieracium ×*krasani* Woł. (*H. alpinum* L. × *H. transsilvanicum* Heuff.) and *H.* × *grofae* Woł. (*H. alpinum* × *H. umbellatum* L.) are the only convincingly documented recent hybrids in the genus (Mráz et al. 2005; Chrtek et al. 2006). These rare diploid hybrids were found in the Romanian and Ukrainian Eastern Carpathians where their parental diploid taxa, though ecologically vicariant, co-occur. Although the hybrid plants did not differ in pollen size and pollen production from their parents, they were completely seed sterile (Mráz et al. 2005; Chrtek et al. 2006). Analyses of plastid DNA in the diploid nothotaxon *H.* ×*krasani* revealed that the maternal plant was *H. transsilvanicum* and the pollen donor was *H. alpinum* in both populations analyzed (Mráz et al. 2005). At both localities, single *H.* ×*krasani* plants were found at places where *H. transsilvanicum* was abundant while *H. alpinum* was either extremely rare or completely absent. In such a situation, one might have expected that *H. alpinum* was the seed parent because of a putative excess of *H. transsilvanicum* pollen. In 2004, during a botanical excursion to Mt. Bogolin (Mţii Bistriţei) in the Romanian Eastern Carpathians, we found several plants morphologically similar to *H.* ×*krasani*. Interestingly, the putative hybrids were intermingled with hundreds of *H. alpinum* plants, but the closest *H. transsilvanicum* plants were ca. 200–300 m away. Therefore, we considered it interesting to test not only the putative hybrid origin of these plants, but also to infer the direction of the hybridization. Moreover, we wanted to test the suitability of several molecular markers that had not been used in our previous study (Mráz et al. 2005), namely amplified fragment length polymorphism (AFLP) and nuclear internal transcribed spacer (ITS) sequencing, for inferring putative hybridization. In addition, allozyme analysis was used as it proved to be a suitable tool for testing the hybrid origin of *H.* ×*krasani* (Mráz et al. 2005).

Materials and methods

Plant material

Putative hybrid plants as well as the supposed parental taxa were collected on Mt. Bogolin (Romania) and other localities in the Ukraine and Romania (Table 1). The putative hybrids were cultivated in the experimental field of the Botanical Garden of the P. J. Šafárik University in Košice and in the greenhouse at the Institute of Botany, Academy of Sciences of the Czech Republic, in Průhonice to infer ploidy level and allozyme variation. Voucher specimens are deposited in the herbarium P. Mráz and at the Institute of Botany, Průhonice (PRA). We also included two previously corroborated natural and two artificial *H.* ×*krasani* hybrids arising from control crosses between *H. alpinum* and *H. transsilvanicum* in the analyses (cf. Mráz 2003; Mráz et al. 2005). Details about all plants studied are given in Table 1.

DNA-ploidy level and chromosome number

DNA-ploidy level was estimated by flow cytometry (FCM) for three putative hybrids from Mt. Bogolin. These analyses and chromosome counting were performed according to the methods given in Mráz et al. (2009) with the exception of the cytometrical standard for which diploid *Hieracium umbellatum* (cultivation number X18/3) instead of *Zea mays* was used. Because the peak of the putative hybrid PM

Table 1 Origin of *Hieracium* plant material and number of plants per population used for the respective analyses

Taxon	Locality/note	AFLP	ITS	cpDNA	Allozymes	FCM/ karyology
H. alpinum	Romania, Munţii Bistriţei, Mt. Pietrosul Bogolin, 1,720 m a.s.l., 47°23.1′ N, 25°32.16′ E, coll. P. Mráz and J. Chrtek, 16 July 2004 & A. Oprea, 2006	10				
	Ukraine, Chornohora Mts, Polonina Pozhyzhevska, 48°09.19′ N, 24°32.07′ E, 1,430 m a.s.l., coll. R. Letz, 18 July 2004	5				
	Ukraine, Chornohora Mts, Polonina Breskulska, the saddle between Mt. Hoverla and Mt. Breskul, 48°09′10″ N, 24°30′15″ E, 1,800 m a.s.l., coll. P. Mráz and J. Chrtek, 23 July 2003			1[a]	10[a]	
H. ×*krasani*	Romania, Munţii Bistriţei, Mt. Pietrosul Bogolin, 1,720 m a.s.l., 47°23.1′ N, 25°32.16′ E, coll. P. Mráz and J. Chrtek, 16 July 2004	3[b]	3[b]	3[b]	3[b]	3[b]
	Ukraine, Chornohora Mts, Polonina Breskulska ridge, 1,410 m a.s.l., 48°08′35″ N, 24°28′56.7″ E, coll. P. Mráz and J. Chrtek, 23 July 2003	2[c]	1[d]	2[a]	3[a]	
	Artificial hybrids[e]	2[f]	2[g]		1[h]	
H. transsilvanicum	Romania, Munţii Rodnei Mts, Mt. Pietrosul Mare, spruce forest, 1,300–1,400 m a.s.l., 47°39′ N, 24°′39″ E, coll. P. Mráz, 5 July 2001	1				
	Ukraine, Chornohora Mts, Polonina Breskulska ridge, 1,410 m a.s.l., 48°08′35.0″ N, 24°28′56.7″ E, coll. P. Mráz and J. Chrtek, 23 July 2003	1[i]			12[a]	
	Ukraine, Marmarosh Mts, Mt. Berlebashka, 1,200 m a.s.l., 47° 56′13″ N, 24° 21′31″ E, coll. J. Zahradníček, 19 September 2005	2[j]		1[k]		

[a] Published data from Mráz et al. (2005)

[b] Plant codes: PM 1711 (2x), PM 1712 (2x), PM 1713 (4x)

[c] Plant codes: PM 1399, PM 1400

[d] Plant code: PM 1399

[e] For details, see Mráz (2003)

[f] Plant codes: X5/5, X5/6

[g] Plant codes: X5/5, X5/10

[h] Plant code: X5/6

[i] Plant code: PM 1406

[j] Plant codes: H 1077/7, H 1077/10

[k] Plant code: H 1077/10

1713 suggested that the plant was tetraploid, the measurement was repeated and also confirmed by chromosome counting.

Allozyme analysis

Three plants of *H.* ×*krasani* from Mt. Bogolin (Romania) and one artificial hybrid from control crosses (cf. Mráz 2003) were analyzed; further data from Mráz et al. (2005) were included for comparison (see Tables 1, 2). Extraction, electrophoresis, and staining followed the methods described in Štorchová et al. (2002). The following enzyme systems were examined and allelically interpreted: AAT (aspartate aminotransferase, EC 2.6.1.1, dimeric), LAP (leucine aminopeptidase, EC 3.4.11.1, monomeric), MDH (malate dehydrogenase, EC 1.1.1.37, dimeric), 6PGD (6-phosphogluconate dehydrogenase, EC 1.1.1.44, dimeric), PGM (phosphoglucomutase, EC 5.4.2.2, monomeric), and SKD (shikimic acid dehydrogenase, EC 1.1.1.25, monomeric).

DNA extraction

The leaves of cultivated plants or plants from the field were dried in silica gel and stored at room temperature. In rare cases, herbarium specimens were used. Total DNA was extracted from 10–15 mg of silica-dried leaf tissue with the DNeasy 96 Plant Kit (Qiagen), following the manufacturer's protocol or by the method of Štorchová et al. (2000).

AFLPs

The AFLP procedure followed Mráz et al. (2007) except for the choice of selective primers; combinations *Eco*RI-AAG/*Mse*I-CAG and *Eco*RI-ACC/*Mse*I-CTG were used.

Table 2 Allozyme genotypes and their frequencies of two parental taxa *Hieracium alpinum* and *H. transsilvanicum* and their hybrid *H. ×krasani*

Taxon (locality/no. of plants)	*Aat-2*	*Adh-1*	*Lap-1*	*Lap-2*	*Mdh*	*6-Pgdh-1*	*6-Pgdh-2*	*Pgm-1*	*Skd*
H. alpinum (Chornohora Mts/10)	bb 1.00	ab 0.10	ab 0.10	aa 0.40	–	aa 1.00	bb 1.00	ac 1.00	aa 1.00
		bb 0.90	bb 0.60	ab 0.60					
			bc 0.20						
			cc 0.10						
H. transsilvanicum (Chornohora Mts/12)	aa 0.08	bb 1.00		aa 0.92	–	bb 0.58	aa 0.08	ab 1.00	bb 0.25
	ab 0.58			ab 0.08		bc 0.42	bb 0.92		bc 0.42
	bb 0.34								cc 0.33
H. ×krasani (Chornohora Mts/3)	bb 1.00	bb 1.00	bb 1.00	ab 1.00	–	bc 1.00	ab 1.00	ac 1.00	ac 1.00
H. ×krasani (2x) (Munţii Bistriţei/2)	ab 0.50	–	aa 0.50	–	ab 1.00	bc 1.00	bb 1.00	aa 1.00	ab 0.50
	bb 0.50		bb 0.50						ac 0.50
H. ×krasani (4x) (Munţii Bistriţei/1)	aabb	–	aaaa	–	aabb	bccc	bbbb	aaaa	aaac
H. ×krasani (artificial hybrid/1)	ab	–	bb	–	ab	bc	bb	aa	ac

Number of plants analyzed are given *in parentheses*. Missing data are indicated by '–'. Bands of *Lap-1* were not present in *H. transsilvanicum*

Fragments of a size range of 50–500 bp were manually scored with GENMAPPER version 3.1 (Applied Biosystems) for their presence/absence. Two samples, one artificial hybrid and one *H. alpinum*, from a total of 26 were analyzed twice to test for reproducibility of the AFLP analysis. For a list of samples, see Table 1. For further analysis, 70 polymorphic markers were kept; monomorphic and non-reproducible AFLP bands were removed from the dataset. The relationships between the putative parental taxa and their hybrid were inferred by principal coordinate analysis (PCO) implemented in the ade4 package (Chessel et al. 2004) in the Rcran environment (R Development Core Team 2006). The distance matrix was based on the Jaccard coefficient of similarity.

Nuclear DNA sequencing

We chose the internal transcribed spacer (ITS) of ribosomal nuclear DNA to test the hybridogeneous origin of the putative hybrid plants as this marker is inherited biparentally, and, in the case of absence of sequence homogenization, it is suitable for inferring hybridization (Sang et al. 1995; Campbell et al. 1997). The ITS region was amplified using the primers ITS A and ITS B (Blattner 1999) in a 25 µl reaction volume containing 1 µl of genomic DNA (10–35 ng), 2.5 µM buffer (10× Buffer II, Applied Biosystems), 1.5 mM $MgCl_2$, 0.2 µM of each dNTP, 0.2 µM of each primer, 0.25 µM BSA, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). The cycling profile included an initial denaturation step at 95°C/10 min followed by 28 cycles of 95°C/30 s, 52°C/30 s, 72°C/1 min, and ended with 72°C/10 min and 4°C thereafter. PCR products were purified using the QIAquick Gel Extraction Kit. Sequencing was performed in both directions using BigDye Terminator V 3.1 (Applied Biosystems). The samples were run on an ABI PRISM®3100 Genetic Analyzer. Sequences were assembled and edited using Seqscape 2.5.0 (Applied Biosystems). The presence of heterozygous sites (intra-individual polymorphisms) in ITS1 and ITS2 was assessed in most cases on both forward and reverse strands according to Aguilar and Feliner (2003). Some polymorphic sites could only be determined from one strand, especially at the beginning where saturated peaks hampered the correct reading of the sequences. In total we sequenced three hybrids previously corroborated by other methods (Mráz et al. 2005) and the three new putative hybrids (Table 1). Their ITS sequences were compared to published ITS sequences of the parental taxa from the same area (Fehrer et al. 2007).

Chloroplast DNA

The *trn*T-L intergenic spacer of chloroplast DNA was used to distinguish between *H. alpinum* and *H. transsilvanicum* and to identify the chloroplast donor of the putative hybrid plants. Two accessions of each *H. alpinum* (GenBank acc. no. AY512556, EU867711) and *H. transsilvanicum* (AY512557, EU867743) from Mráz et al. (2005) and Fehrer et al. (2009) had identical sequences. Based on these sequences, *Eco*RI was chosen as a restriction enzyme that produced discriminating patterns, and the hybrid accessions were subjected to PCR-RFLPs along with the parental species for comparison (see Table 1). PCRs, sequencing, and restriction digests were done as described in Mráz et al. (2005). Chloroplast DNA had been shown

previously to be maternally transmitted in these two species based on the examination of artificial hybrids from reciprocal crosses (Mráz et al. 2005).

Results

Ploidy level and chromosome number

The DNA-ploidy level of two putative hybrids from Mt. Bogolin (PM 1711 and PM 1712) was diploid (2n ~ 2x ~ 18). The third putative hybrid PM 1713 was cytometrically determined to be a tetraploid (relative peak position of sample to standard plant, diploid *H. umbellatum*, was 1.8), which was later confirmed by chromosome counting (2n = 4x = 36).

Allozyme analysis

Six enzyme systems with ten loci were investigated in four wild plants of *H.* ×*krasani* and one artificial hybrid between *H. alpinum* and *H. transsilvanicum*. Three loci were excluded from further analyses: *Aat-1* (monomorphic in all plants studied), *Pgm-2*, and *Lap-2* (both with low enzyme activity). Genotype frequencies of each locus for both parental species (data from Mráz et al. 2005) and both natural and artificial hybrid plants (*H.* ×*krasani*, current data and data from Mráz et al. 2005) are given in Table 2. In total, five different multilocus genotypes were detected in plants of *H.* ×*krasani*: while the three plants from Romania differed from each other in their genotypes, all plants from the Ukraine belonged to one genotype (Mráz et al. 2005), and a further genotype was detected in the artificial hybrid. The hybrid origin of the Romanian plants was clearly indicated by a unique additive pattern in *Skd*: both alleles a and c were recorded in *H.* ×*krasani*, while only allele a was present in *H. alpinum* and alleles b and c were found in *H. transsilvanicum*. Moreover, the contribution of *H. alpinum* was indicated by the band of *Lap-1* (in *H. transsilvanicum* the bands were consistently lacking, cf. Mráz et al. 2005).

AFLPs

The average number of AFLP fragments per individual ranged from 33 to 42 out of 70 polymorphic ones in total (Table 3), with the highest value recorded in an accession of *H.* ×*krasani* obtained by artificial crossing. While the mean number of AFLP bands per individual was similar in the two putative parental taxa and their putative hybrid, the total number of bands per (notho) taxon was different. *Hieracium* ×*krasani* showed many more bands than its parental taxa (59 vs. 45 and 47 fragments, respectively). This is because the hybrids also displayed bands that were exclusive to each of their parents (Table 3).

The principal coordinate analysis revealed the existence of two well-separated groups of plants belonging to the putative parental taxa. Three putative hybrid plants from Mt. Bogolin as well as four previously corroborated *H.* ×*krasani* hybrids (two natural and two artificial ones) occupied an intermediate position (Fig. 1). They exhibited a higher diversity than either parent. Moreover, hybrid plants showed a tendency to cluster according to their geographic (natural hybrids) or experimental origin (artificial hybrids) (see Fig. 1).

ITS polymorphism

Three sequenced accessions of putative hybrid origin from Mt. Bogolin showed the same additive pattern of ITS polymorphism. No indels occurred between the parental species so that the sequences could be read in both directions. Additive characters inferred from superimposed peaks represented those 12 positions that differ between the parental species *H. alpinum* and *H. transsilvanicum* (Table 4). The same pattern was observed in ITS sequences of one already corroborated natural hybrid of *H.* ×*krasani* from the Ukraine (Mráz et al. 2005), as well as in two artificial hybrids.

Chloroplast DNA analyses

In contrast to the previously investigated material of *H.* ×*krasani* (Mráz et al. 2005) that had *H. transsilvanicum* as a maternal parent, PCR-RFLPs of the *trn*T-L intergenic spacer of chloroplast DNA showed that all three hybrid plants from Mt. Bogolin investigated here had obtained their cpDNA from *H. alpinum*, including the tetraploid accession (Fig. 2).

Table 3 Number of AFLP fragments scored in the parental taxa and their hybrid

Taxon	N	TNF	MNF	EF	SF
H. alpinum	15	47	35.7 (33–38)	23	24
H. ×*krasani*	7	59	37.7 (34–42)	2	–
H. transsilvanicum	4	45	33.5 (33–35)	21	24

N Number of plants analyzed per taxon, *TNF* total number of AFLP fragments per taxon, *MNF* mean number of AFLP fragments per individual plant (range), *EF* total number of exclusive AFLP fragments per taxon, *SF* total number of shared AFLP fragments between a particular parental taxon and *H.* ×*krasani*

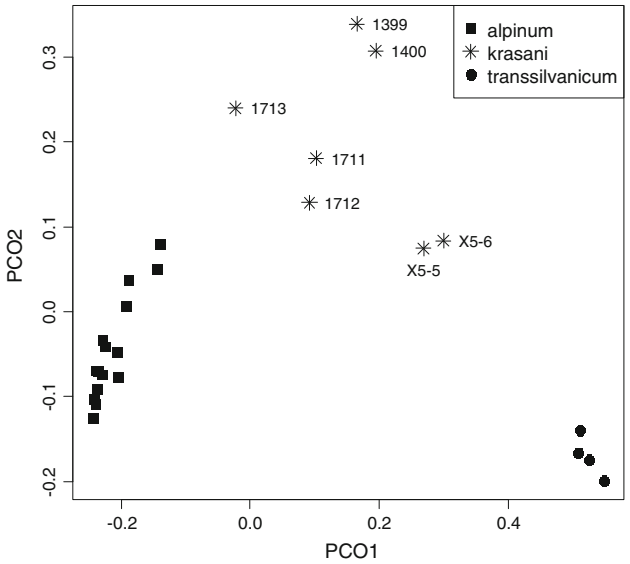

Fig. 1 Principal coordinate analysis (PCO) plot of 26 individuals of hybrid *Hieracium* ×*krasani* and its two parental taxa *H. alpinum* and *H. transsilvanicum* based on Jaccard distances calculated on amplified fragment length polymorphism (AFLP) multilocus phenotypes. Hybrid plants are labelled (see Table 1) to show their tendency to cluster according their geographic origin or their origin from the same experimental cross

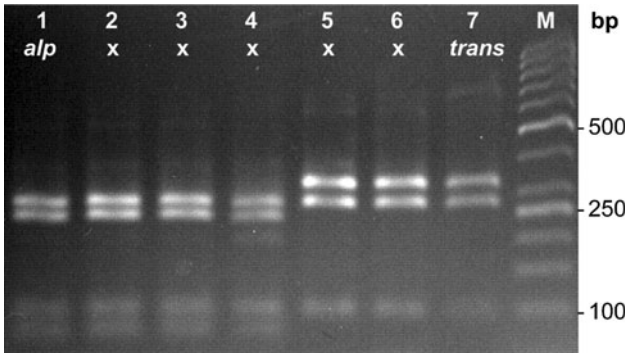

Fig. 2 PCR-RFLP of the chloroplast *trn*T-L intergenic spacer with *Eco*RI. *M* DNA size standard, *alp H. alpinum*, *x H.* ×*krasani*, *trans H. transsilvanicum*. All hybrid accessions from Mt Bogolin (lanes 2–4) show the same pattern as *H. alpinum* indicating that this species was their maternal parent. Hybrids from the Ukraine (lanes *5–6*) from Mráz et al. (2005) with *H. transsilvanicum* as their maternal parent are included for comparison

Discussion

Molecular evidence for hybrid origin

Our combined molecular approach (nuclear ITS, AFLPs, and allozymes) clearly supports the hybrid origin of three plants from Mt. Bogolin from *Hieracium alpinum* and *H. transsilvanicum*. Complete character additivity of ITS polymorphism and no sign of homogenization towards the one or other parental copy were revealed by direct sequencing. Therefore, this marker seems to be very reliable for the assessment of recent hybridization in the genus *Hieracium*. The lack of concerted evolution in natural and artificial *H.* ×*krasani* might be explained by the very recent origin and sterility of putatively F_1 hybrids (cf. Mráz et al. 2005; Mráz and Paule 2006). However, a similar pattern—retention of intra-individual external transcribed spacer (ETS) polymorphic sites—has been observed not only in old polyploid *Hieracium*, in which apomictic reproduction might efficiently prevent concerted evolution of multicopy genes as has been observed also in other apomictic genera (cf. Campbell et al. 1997; Záveská Drábková et al. 2009), but also in some diploid sexual *Hieracium* taxa that are fully fertile, but apparently of ancient hybrid origin (Fehrer et al. 2009). This might suggest that concerted evolution is not a very common and efficient mechanism in *Hieracium* s.str. In any case, early generation hybrids (most probably F_1 hybrids in our case) can be expected to have unskewed ratios of parental ITS variants as reflected by their sequencing profiles.

With respect to AFLPs and allozymes, the three hybrid plants from Mt. Bogolin were detected to be genetically different and thus to have originated from independent crosses of the parental taxa. This is not surprising given

Table 4 Additive pattern of ITS polymorphism in natural and artificial hybrid plants of *Hieracium* ×*krasani* in comparison to the parental taxa

Taxon	Alignment position											
	46	61	73	74	103	131	136	228	494	562	580	615
H. alpinum[a]	C	C	C	C	C	C	C/Y[d]	A	A	T	A	T
H. ×*krasani*[b]	Y	Y	M	Y	M	Y	Y	W	W	Y	W	Y
H. transsilvanicum[c]	T	T	A	T	A	T	T	T	T	C	T	C

[a] Gen Bank accession no AJ633429 (Fehrer et al. 2007)

[b] Gen Bank accession no HM627291 (this study, PM1712, the sequence of this accession was submitted to the Gen Bank only, as two remaining sequenced samples had the same sequence)

[c] Gen Bank accession no AJ633427 (Fehrer et al. 2007)

[d] One published ITS sequence of *Hieracium alpinum* (AJ633429) showed C at this position, but several accessions from the Eastern Carpathians displayed an intra-individual polymorphism C + T (Y) (Mráz et al., unpubl. data)

that these plants were sampled from different places at the locality and one of them even had a different ploidy level. In our previous paper (Mráz et al. 2005) we reported three plants of *H.* ×*krasani* from the Ukraine that shared the same multilocus allozyme pattern. All plants were collected at the same microsite (30 × 30 cm) and therefore we originally thought that they might represent a single clone (Mráz et al. 2005). In the present study, we re-analyzed these plants (PM 1399 and PM 1400, see Table 1) using AFLPs, and they also turned out to be genetically different from each other, suggesting a more discriminative power for the AFLPs in comparison with allozymes. The utility of AFLP markers for inferring the phylogeographic structure in some mountain taxa belonging to the *Hieracium* sect. *Cernua* has been shown by Ronikier and Szeląg (2008). The AFLPs have been applied also in rare *H. cyathis* and closely related taxa in order to test the genetic integrity of this species (Rich et al. 2008). Here we demonstrate the usefulness of amplified fragment length polymorphism also for the detection of interspecific *Hieracium* hybrids.

Evidence for bidirectional gene flow

Despite the independent origin of several hybrid plants at one locality (Mt. Bogolin, Romania), all shared the same chloroplast haplotype, which was obtained from *H. alpinum* as their maternal parent. In contrast, three hybrid plants from two localities from the Romanian (Munţii Rodnei) and Ukrainian Eastern Carpathians had *H. transsilvanicum* as their maternal parent (Mráz et al. 2005). In the latter case, many *H. transsilvanicum* plants were found at both sites while the second parental species, *H. alpinum*, was either completely absent or extremely rare. On the contrary, the hybrid individuals from Mt. Bogolin investigated here exhibited the *H. alpinum* haplotype and co-occurred with hundreds of *H. alpinum* plants. This might suggest that the direction of the hybridization could be influenced by the dominance of the maternal taxon at a given site. In our previous paper (Mráz et al. 2005) we discussed in detail this unexpected pattern, where we originally assumed putatively longer distance dispersal by seeds than by pollen. However, our previous and present data contradict this assumption. Pollen movement might be in fact more efficient than we previously thought, and many of the hybrid seeds probably drop to the ground near the maternal parents without long dispersal. A similar pattern has been observed in the genus *Asclepias*, which is also adapted for long distance seed dispersal (Broyles 2002). Furthermore, we cannot exclude a potential advantageous effect the maternal parent might have on the hybrid's adaptation to the same habitat. Indeed, artificial F_1 hybrids from reciprocal crosses as well as natural *Hieracium* hybrids showed—despite a high degree of morphological intermediacy between both parental taxa—tendencies to be more similar to the maternal species (Mráz et al. 2005; Mráz and Paule 2006). Given the strong differences in light preferences and thus ecophysiological tolerance between the two parental taxa (*H. alpinum* is an alpine strict heliophyte species while *H. transsilvanicum* is a sciophyte taxon growing in forests and forest clearings), the hybrid seedlings having the "wrong" maternal taxon might be selected against when growing in the "wrong" habitat. The two hypotheses are not mutually exclusive and can explain the observed pattern.

Spontaneous polyploidization

One hybrid plant from Mt. Bogolin was tetraploid. Given that both parental taxa are diploid in this region (Pashuk 1987; Chrtek 1996, 1997; Mráz 2001, 2003; Mráz and Szeląg 2004; Mráz et al. 2005, 2009; Chrtek et al. 2006), and that the other two hybrid plants as well as other natural and artificial *Hieracium* hybrids originating from diploid × diploid crosses were also diploid (Mráz et al. 2005; Mráz and Paule 2006; Chrtek et al. 2006), this was a quite surprising finding. Generally, two main pathways leading to polyploidy are distinguished: direct somatic doubling acting at the zygote or young embryo stages, and—more frequent and thus more important—polyploidization through the production of unreduced 2n gametes (Ramsey and Schemske 1998). In this case, a new polyploid cytotype can arise through the fusion of two unreduced gametes (2n × 2n) via a one-step model, or—likely more frequently—through a so-called "triploid bridge" (2n × n, cf. Harlan and de Wet 1975). As triploid plants usually have low fertility due to an unbalanced chromosome pairing during meiosis and may encounter problems with endosperm formation (triploid block), a second step, namely a backcross to one of the parental taxa or a cross with another hybrid plant or selfing is needed, resulting in a more stable tetraploid cytotype (Ramsey and Schemske 1998). Our allozyme data suggest that the "unreduced gametes" pathway was involved in the origin of the tetraploid hybrid (PM 1713) with subsequent backcrossing to *H. alpinum* (or to another hybrid plant) rather than direct somatic duplication. Such a scenario is supported by unbalanced heterozygosity observed at the *Skd* locus in the tetraploid plant (allelic composition aaac). The allele *Skd-a* is known only in *H. alpinum* while *Skd-c* and *Skd-b* alleles are specific to *H. transsilvanicum* (see also Mráz et al. 2005). In the case of direct somatic doubling, the allopolyploid hybrid should exhibit only homozygous and/or balanced heterozygous loci. Although the ITS electropherogram from direct sequencing of the tetraploid plant did not show a higher contribution (higher peaks) of *H. alpinum* ITS copies, this

may be due to locus loss following hybridization or else as a result of PCR drift (Wagner et al. 1994). Therefore, the allozyme data can be considered as more reliable for the inference of the tetraploid's genomic composition.

A triploid intermediate step implies the participation of unreduced gametes. Unreduced gametes can be formed in the parental diploid taxa, as has been shown in many angiosperms (e.g., Ortiz 1997; Bretagnolle 2001; Grant 2002) or in primary diploid hybrids. In the latter case, however, the production of unreduced gametes is ca. 50 times higher than in nonhybrids (Ramsey and Schemske 1998). Hence, interspecific homoploid hybrids seem to play a crucial role in polyploid evolution. To the best of our knowledge, we have no indication of the presence of unreduced pollen either in diploid *Hieracium* species or in their diploid interspecific hybrids, as they formed only a high quantity of homogeneously sized pollen, and no irregular pollen suggesting a different amount of genome has been recorded as yet (Mráz et al. 2002, 2009; Chrtek et al. 2006; Mráz and Paule 2006). On the other hand, there is no information on the frequency of unreduced ovules in diploid *Hieracium*, which might be more important than unreduced pollen as it was reported in closely related polyploid *Pilosella* (cf. Peckert and Chrtek 2006).

Polyploidization is traditionally considered as an important step following interspecific hybridization in order to restore the chromosome pairing and thus providing the hybrids with reproductive assurance (Ramsey and Schemske 1998). According to this, one would expect a higher fertility in our tetraploid hybrid than in natural and artificial diploid hybrids, which proved to be either completely seed sterile or produced only a few seeds (Mráz et al. 2005; Mráz and Paule 2006; Chrtek et al. 2006). Unfortunately, it was neither possible to verify the seed set of the tetraploid hybrid nor pollen formation as the flower heads aborted soon after transfer to the experimental field in the botanical garden and the plant did not survive the winter 2004/2005. Consequently, we could not test even its breeding system, which could offer very important information, as all natural *Hieracium* polyploids studied so far reproduced solely apomictically (e.g., Chrtek 1997; Mráz and Szeląg 2004; Chrtek et al. 2009). In contrast, experimental hybridization between sexual diploid *Hieracium pojoritense* Woł. and apomictic tetraploid *H. dentatum* s.l. as a pollen donor produced a triploid, morphologically intermediate hybrid that was completely seed sterile (Mráz 2003; Mráz unpubl. data). Thus, neither the polyploid status of the hybrid plant nor the involvement of one apomictically reproducing parent assured independent seed formation in this triploid. The possible reasons for seed set failure in this triploid hybrid might involve problems with endosperm formation (Vinkenoog et al. 2003) and, maybe to a lesser extent, genomic incompatibilities between divergent parental taxa. In this light, it is possible that the tetraploid *H.* ×*krasani* plant was sterile, too.

In conclusion, our study showed further evidence of recent interspecific hybridization in the genus *Hieracium* at the diploid level. Moreover, combined methodological approaches (karyology, allozymes, AFLPs, and nuclear and plastid sequences) allowed us to prove not only the hybrid origin of the plants studied, but also to determine the direction of hybridization and to suggest a possible origin of a rare allotetraploid plant. To our best knowledge, this is the first case of spontaneous polyploidization following interspecific hybridization between two divergent taxa in the genus *Hieracium*. Given that most *Hieracium* taxa are polyploids reproducing asexually, our findings might represent an important step towards a better understanding of polyploid evolution in *Hieracium*.

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POLYPLOIDY IN PHENOTYPIC SPACE AND INVASION CONTEXT: A MORPHOMETRIC STUDY OF *CENTAUREA STOEBE* S.L.

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The taxonomy of the *Centaurea stoebe* complex is controversial. Diploid and tetraploid plants occur in its native European range, but to date only tetraploids have been recorded from its introduced range in North America. We examined morphological differentiation of *C. stoebe* using multivariate and univariate approaches to clarify the taxonomic status of the known cytotypes. We measured more than 40 morphological traits on plants originating from 78 populations, grown from seed under uniform glasshouse conditions. The ploidy of almost 300 plants from 2 native and 20 introduced populations from Canada was assessed to test for the absence of diploids from North America. Finally, we explored whether postintroduction processes have resulted in phenotypic changes in introduced plants which may have contributed to the invasion success of *C. stoebe*. Morphometric analyses showed a clear separation of 2x and 4x plants and thus supported recognition of both cytotypes as separate taxa. Differences in the life cycle, the number of florets, the shape of capitula, and the shape of young rosette leaves were the best discriminant characters. Only minor differences were found between native and introduced tetraploids. All plants from the introduced range except for one hexaploid were found to be tetraploid. Rare diploids from Canada were identified as *Centaurea diffusa* or *Centaurea psamogenna*.

Keywords: Asteraceae, biological invasion, flow cytometry, karyology, multivariate morphometrics, polyploidy, spotted knapweed.

Online enhancement: supplemental table.

Introduction

Polyploidy, a state when an organism has more than two complete sets of chromosomes, is considered a major evolutionary mechanism in flowering plants (Müntzing 1936; Stebbins 1950; De Wet 1971; Grant 1981; Levin 1983; Otto and Whitton 2000). Genome duplication can lead to instantaneous multiple changes in organisms that are manifested at different structural, developmental, and functional levels, from the genes to phenotypic traits interacting directly with the surrounding environment. Polyploid cytotypes are often morphologically different from their diploid ancestors, but it is often difficult to separate the direct effect of polyploidization on morphology from other factors such as hybridization and/or postpolyploidization processes (Levin 1983). Two main groups of polyploids are commonly recognized on the basis of their origin; autopolyploids arise within populations of a single species, and allopolyploids arise from interspecific hybridization (Ramsey and Schemske 1998). With the exception of differences in chromosome behavior in meiosis, segregation ratios, and fertility, autopolyploids are usually morphologically more similar to their diploid progenitors than allopolyploids (Grant 1981; Soltis et al. 2007). Morphological differences between cytotypes have traditionally formed the primary basis for appropriate taxonomic characterization. However, additional characteristics such as ecological and/or distributional shifts, genetic differentiation, and the presence and strength of reproductive barriers have also been used as supporting criteria (Soltis et al. 2007). Given the frequent coexistence of different cytotypes in nature, it is surprising that there are relatively few studies using rigorous multivariate techniques that examine morphological variation within diploid-polyploid complexes (Lihová et al. 2004; Koutecký 2007; Mandáková and Münzbergová 2008). A detailed knowledge of the morphological variation within and between polyploid taxa and their diploid ancestors is important for taxonomy, but it is also critical for identification of characters and life-history traits that could potentially be involved in adaptive evolution.

Centaurea L. is a species-rich and taxonomically intricate genus with a high proportion of polyploids. The genus is distributed across large parts of Eurasia and northern Africa, and phenotypic and cytotype variation contribute significantly to its taxonomic complexity (Hellwig 2004). The European and Mediterranean regions encompass more than 700 species and subspecies of *Centaurea* s.str. (Greuter 2006–2009). In spite of the recent progress in understanding intrageneric variation of

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Centaurea (Wagenitz and Hellwig 1996; Garcia-Jacas et al. 2006; Font et al. 2009), the low-level taxonomy of the genus remains largely unresolved. This taxonomic uncertainty is also present for the diploid-polyploid complex of *Centaurea stoebe* L. (spotted knapweed). Several intraspecific taxa within *C. stoebe* have been described from different parts of the distributional range (Španiel et al. 2008 and references therein) as well as some closely related species (*Centaurea reichenbachii* DC., *Centaurea triniifolia* Heuff.; Ochsmann 2000). Using the most recent taxonomic revision (Ochsmann 2000), *C. stoebe* includes three subspecies that differ in their morphology, ploidy level, and life cycle. The nominate subspecies *stoebe* (hereafter, *C. stoebe* s.str.) is diploid (2n=2x=18) and predominantly monocarpic, whereas the subspecies *micranthos* (Gugler) Hayek (2n=4x=36, hereafter, *C. stoebe* s.l.) is tetraploid and polycarpic. The third subspecies, subsp. *serbica* (Prodan) Ochsmann, is not well known; it is a diploid taxon distributed in the Balkan Peninsula with uncertain life cycle (Ochsmann 2000). Both subspecies *stoebe* and *micranthos* are distributed across Europe and the western part of Asia, with the tetraploid *micranthos* being more frequent in southern latitudes and almost absent in Western Europe (see Ochsmann 2000; Španiel et al. 2008; Treier et al. 2009). All three subspecies are reported to differ in overlapping morphological traits such as color, the width of involucres, pappus length, and the number of lateral fimbriae on involucral bracts (Ochsmann 2000). Recently Španiel et al. (2008) studied the morphological differentiation of diploids and tetraploids and their distribution in central Europe, using multivariate morphometrics and flow cytometry. In contrast to Ochsmann (2000), they proposed a single species concept with no recognition of intraspecific units because they observed a lack of morphological discrimination, a largely sympatric distribution of the two common cytotypes, and the presence of mixed-ploidy populations (Španiel et al. 2008).

Centaurea stoebe s.l. was introduced into North America more than 120 years ago as an alfalfa contaminant. It has subsequently become a highly successful invasive plant, especially in western North America (Sheley et al. 1998). To date only the tetraploid cytotype has been confirmed in the introduced range, even though the diploid cytotype dominates in Europe and there is an overlapping distribution of two cytotypes in the native range (table 1). Such a pronounced cytotype shift may be the result of stochastic founder event(s) or may be the result of possible postintroduction selection that favored the tetraploid cytotype (Treier et al. 2009). Niche modeling indicated a higher level of niche differentiation between tetraploids from the native and introduced range than between native diploids and tetraploids (Broennimann et al. 2007; Treier et al. 2009). However, native tetraploids still showed a small but significant shift in climatic niche toward a drier climate when compared to native diploids (Treier et al. 2009). Thus, a preadaptation of tetraploids to a drier and warmer climate in North America could represent a possible advantage over diploids if both cytotypes have been introduced.

Although several studies have compared life-history traits of *C. stoebe* cytotypes from widely distributed populations from both the native and introduced range (Müller 1989; Broz et al. 2009; Henery et al. 2010), none of them have considered morphological characters in detail. In this article we present a multivariate and univariate morphometric comparison between cytotypes that addresses the following questions: (i) Do diploids and tetraploids differ morphologically, and what are the taxonomic consequences? (ii) If they differ, which are the best morphological characters for their discrimination? (iii) Are there morphological differences between tetraploids from the native and introduced range? We hypothesize a more pronounced differentiation between cytotypes than between native and invasive tetraploids, as polyploidization is expected to have a stronger impact on morphology than founder events and other processes promoting evolutionary changes following a relatively recent introduction (Schlaepfer et al. 2010). However, even small phenotypic differences between invasive and native tetraploids are of interest, as these characters might be important for the successful invasion in North America. Finally, we assess the occurrence of a rare diploid cytotype that was recently found in the introduced range of British Columbia, Canada (Treier et al. 2009), by analyzing samples from an additional 20 populations from this province.

Table 1

Published Chromosome Counts/DNA-Ploidy Level Estimations of *Centaurea stoebe* from Its Introduced Range in North America

Country (state/province)	*N*	Source
2n=2x=18:[a]		
CAN (BC)	1	Treier et al. 2009
2n=4x=36:		
CAN (BC), USA (WA)	2	Moore and Frankton 1954
CAN (BC), USA (MT)	2	Powell et al. 1974
CAN (BC)	2	Taylor and Taylor 1977
USA (AZ)	1	Morefield and Schaack 1985
USA (VA)	?[b]	Hill 1995
USA (MT)	1	Ochsmann 1999
CAN (BC, OT), USA (AZ, CA, CO, CT, ID, MD, MN, MT, NV, NY, VA, VT, WI, WY)	48	Treier et al. 2009
CAN (BC)	20	Mráz et al., this article
2n=6x=54:		
CAN (BC)	1[c]	Mráz et al., this article

Note. CAN = Canada, USA = United States; state/province names are shown as abbreviations. *N* = number of sites where *C. stoebe* was analyzed.

[a] Diploid ploidy level estimation refers with high probability to either *Centaurea diffusa* or to *Centaurea psamogenna* (*C. stoebe* × *C. diffusa*); see "Discussion."

[b] Hill (1995) did not publish exact locality(ies), but only accession(s) originated from Virginia; therefore, this record is not mapped (fig. 1).

[c] Site with 2 analyzed plants of which 1 was hexaploid and 1 was tetraploid.

Material and Methods

Material and Morphological Measurements

We used pot-grown plants originating primarily from seeds collected during a 2005 field survey across both the native

Table 2

Details of Sampled *Centaurea stoebe* and *Centaurea vallesiaca* Populations with Number of Plants Used for Morphometric and/or Ploidy Analyses

Population code	Country	Locality	Coordinates	Collector	N_{tot}	N_{leaf}	FCM
Native range, diploid (2xEU)					202	352	0
A2	AT	Niederösterreich, Neckenmarkt	47.595°N, 16.515°E	Tr, No	12	16	…
A3	AT	Niederösterreich, Hainburg	48.153°N, 16.955°E	Br, Th	9	16	…
SAF-2x	AT	Niederösterreich, Marchegg	48.273°N, 16.890°E	Tr, Br	3	6	…
CH1b	CH	Basel, Basel	47.552°N, 7.642°E	Br, Th	3	16	…
SW4[a]	CH	Wallis, Ausserberg	46.312°N, 7.845°E	Th	10	16	…
D1	DE	Bayern, Simbach am Inn	47.657°N, 7.545°E	Tr, No	3	15	…
DE10	DE	Sachsen-Anhalt, Meissen	51.195°N, 13.431°E	Br, Th	8	11	…
DE11	DE	Bayern, Kallmünz	49.171°N, 11.966°E	Br, Th	8	15	…
DE2	DE	Baden-Württemberg, Istein	47.662°N, 7.530°E	Br, Th	14	16	…
DE6	DE	Sachsen-Anhalt, Halle	51.509°N, 11.956°E	Br, Th	11	16	…
DE8	DE	Brandenburg, Ziesar	52.268°N, 12.299°E	Br, Th	6	15	…
H1	HU	Somogy, Visz	46.722°N, 17.769°E	Tr, No	13	16	…
H3	HU	Veszprém, Tapolca	46.914°N, 17.335°E	Tr, Br	6	13	…
H5	HU	Bács Kiskun, Batmonostor	46.109°N, 18.925°E	Tr, Br	7	14	…
H6	HU	Bács Kiskun, Kiskunfélegyháza	46.706°N, 19.896°E	Tr, Br	6	8	…
SHG	HU	Pest, Isaszeg	47.528°N, 19.384°E	Tr, Br	10	13	…
SRUD	RU	Moscow, Schurovo	55.052°N, 38.822°E	Sh	3	11	…
SRUG	RU	Samara, Perevoloki	53.253°N, 49.188°E	Sh	6	9	…
SUAG	UA	Zhytomyr	50.275°N, 28.911°E	Tr, Br	2	12	…
SUAH	UA	Poltava, Khorol	49.671°N, 33.701°E	Tr, Br	8	15	…
SUAI	UA	Poltava, Chutove	49.668°N, 34.948°E	Tr, Br	8	11	…
SUAJ	UA	Krivohrad, Novoarkhanhel'sk	48.646°N, 30.776°E	Tr, Br	4	13	…
UA1	UA	L'viv, Zolochiv	49.798°N, 24.711°E	Tr, Br	10	15	…
UA2	UA	L'viv, Olesko	49.930°N, 24.836°E	Tr, Br	9	15	…
UA3–2x	UA	Ivanofrankivsk, Czortova	49.400°N, 24.664°E	Tr, Br	12	13	…
UA5	UA	Khmelmitsky, Starokostyantyniv	49.772°N, 27.291°E	Tr, Br	11	16	…
Native range, tetraploid (4xEU)					188	254	30
A1	AU	Niederösterreich, Dürnstein	48.393°N, 15.532°E	Tr, No	5	10	
BIE	CH	Vaud, Bière	46.526°N, 6.33°E	Bow	10	15	15
CH1	CH	Aarau, Gontenschwil-Zetwill	47.552°N, 7.642°E	Tr, No	14	16	…
DE3	DE	Bayern, Nürnberg	49.417°N, 11.086°E	Br, Th	8	14	…
DE4	DE	Bayern, Steinbach	49.994°N, 10.631°E	Br, Th	13	16	…
DE5	DE	Bayern, Coburg	50.298°N, 10.658°E	Br, Th	11	14	…
H2	HU	Veszprém, Devecser	47.117°N, 17.443°E	Tr, Br	13	16	…
H4	HU	Somogy, Barcs	45.965°N, 17.500°E	Tr, Br	6	11	…
SHE	HU	Somogy, Böhömye	46.402°N, 17.473°E	Tr, Br	16	16	…
SHF	HU	Baranya, Pécs	46.098°N, 18.220°E	Tr, Br	11	15	…
RO9	RO	Miercurea Ciuc	46.357°N, 25.797°E	Hä	8	11	15
PH2	RO	Sucaeva, Radaseni	47.475°N, 26.268°E	Hä	9	15	…
PH3	RO	Neamt, Moldova	47.233°N, 26.516°E	Hä	15	16	…
PH4	RO	Alba, Buru	46.509°N, 23.604°E	Hä	14	16	…
SAF-4x	AT	Niederösterreich, Marchegg	48.273°N, 16.890°E	Tr, Br	6	9	…
SUAA	UA	Transkarpatia, Vynohradiv	48.138°N, 23.077°E	Tr, Br	8	14	…
SUAD	UA	Chernivtsy, Laovanka	48.250°N, 25.896°E	Tr, Br	7	13	…
UA3–4x	UA	Ivanofrankivsk, Czortova	49.400°N, 24.664°E	Tr, Br	1	1	…
UA4	UA	Khmelmitsky, Khotyn	48.516°N, 26.466°E	Tr, Br	13	16	…
Introduced range, tetraploid (4xNA)					276	421	263
CW	CA	British Columbia, Clearwater	51.640°N, −120.077°W	Bo	16	20	19
Nakusp	CA	British Columbia, Nakusp	50.223°N, −117.787°W	Bo	0	0	16
LL	CA	British Columbia, Pemberton	50.133°N, −122.515°W	Bo	16	18	12
202	CA	British Columbia, Burton Lake	49.307°N, −115.155°W	Bo	3	3	3
61PT	CA	British Columbia, Chasm	51.247°N, −121.488°W	Bo	0	0	16
MS	CA	British Columbia, Courtenay	49.640°N, −125.002°W	Bo	10	19	19
HR	CA	British Columbia, Courtenay	49.799°N, −125.063°W	Bo	0	0	9
RavenPit	CA	British Columbia, Courtenay	49.696°N, −125.093°W	Bo	0	0	16
440	CA	British Columbia, Hope	49.377°N, −121.347°W	Bo	13	20	17
CS001	CA	British Columbia, Kamloops	50.661°N, −120.409°W	Bo	14	17	16
CS002	CA	British Columbia, Merritt	50.079°N, −120.650°W	Bo	0	0	16
CS003	CA	British Columbia, Nicola	50.169°N, −120.549°W	Bo	15	19	18

Table 2

(***Continued***)

Population code	Country	Locality	Coordinates	Collector(s)	N_{tot}	N_{leaf}	FCM
CS005	CA	British Columbia, Nicola	50.318°N, −120.375°W	Bo	0	0	16
ROSN	CA	British Columbia, Rosebery	50.045°N, −117.430°W	Bo	9	16	10
147	CA	British Columbia, Rosebud Lake	49.040°N, −117.273°W	Bo	0	1	1
CS004	CA	British Columbia, Savona	50.705°N, −120.880°W	Bo	0	0	16
153	CA	British Columbia, Wyndel	49.186°N, −116.567°W	Bo	6	8	8
380	CA	British Columbia, Yale	49.667°N, −121.403°W	Bo	0	0	16
411	CA	British Columbia, Yale	49.730°N, −121.367°W	Bo	12	16	17
URS	CA	British Columbia, Elko	49.292°N, −115.121°W	Bo	1	1	2
USCA1	US	California, Long Jam	41.010°N, −121.952°W	Hu	0	4	...
USCO2	US	Colorado, Breen	37.190°N, −108.080°W	Hu	0	2	...
USID2	US	Idaho, Coeur d'Alene	47.670°N, −116.680°W	Hu	0	2	...
USMT9	US	Montana, Simms	47.301°N, −112.126°W	Tr, Br	6	10	...
USMT4	US	Montana, Alder	45.324°N, −112.081°W	Tr, Br	13	16	...
USMT8	US	Montana, Big Timber	46.016°N, −110.088°W	Tr, Br	10	14	...
USMT11	US	Montana, Dixon	47.308°N, −114.300°W	Tr, Br	8	16	...
USMT2	US	Montana, Florence	46.584°N, −114.141°W	Tr, Br	8	16	...
USMT1	US	Montana, Missoula	46.820°N, −114.101°W	Tr, Br	6	14	...
USMT10	US	Montana, Missoula	46.999°N, −113.383°W	Tr, Br	8	14	...
USMT3	US	Montana, Ross Hole	45.835°N, −113.975°W	Tr, Br	9	15	...
USNY1	US	New York, West Point	44.278°N, −73.531°W	Hu	0	1	...
USOR8	US	Oregon, Bend	44.055°N, −121.244°W	Tr, Br	11	14	...
USOR11	US	Oregon, Cougar Reservoir	44.157°N, −122.262°W	Co	10	13	...
USOR3	US	Oregon, Dee Flat	45.590°N, −121.629°W	Tr, Br	10	16	...
USOR2	US	Oregon, Hood River	45.698°N, −121.506°W	Tr, Br	9	14	...
USOR10	US	Oregon, Klamath Falls	42.238°N, −121.796°W	Tr, Br	12	16	...
USOR4	US	Oregon, La Grande	45.323°N, −118.259°W	Tr, Br	13	14	...
USOR6	US	Oregon, Mt. Vermont	44.516°N, −118.990°W	Tr, Br	8	14	...
USOR1	US	Oregon, Portland	45.618°N, −122.770°W	Tr, Br	8	15	...
USVA1	US	Virginia, Middletown	38.900°N, −78.020°W	Hu	0	3	...
USWI1	US	Wisconsin, Necedah	44.020°N, −90.070°W	Hu	12	17	...
USWY2	US	Wyoming, Casper	46.810°N, −90.820°W	Hu	0	3	...

Note. Four diploid plants of *Centaurea diffusa* and one of *Centaurea psamogenna* found in three tetraploid populations from introduced range were not included in the list of plants analyzed for ploidy level. AT = Austria, CA = Canada, CH = Switzerland, DE = Germany, HU = Hungary, RO = Romania, RU = Russia, UA = Ukraine, US = United States; Bo = R. Bourchier, Bow = G. Bowmann, Br = O. Broenniman, Co = E. Coombs, Hä = P. Häfliger, Hu = R. Hufbauer, No = S. Normand, Sh = A. Shipunov, Tr = U. Treier. N_{tot} = number of plants/population with all traits measured and used for multivariate analyses; N_{leaf} = number of plants/population for which leaf traits were measured; FCM = number of plants/population analyzed with flow cytometry in our study.

[a] Diploid *Centaurea vallesiaca* population.

and introduced range of *Centaurea stoebe* (for collection methods see Treier et al. 2009). Additional populations from Switzerland, Romania, and Canada were collected in 2006 and 2007 using similar methods. Plant populations included multiple representatives of the two cytotypes classified by geographic origin (hereafter referred to as geocytotypes: European diploids, 2xEU; European tetraploids, 4xEU; and North American tetraploids, 4xNA) and one population of *Centaurea vallesiaca* DC., a diploid species endemic to the Swiss Valais and the Italian Aosta Valley and closely related to the diploid *C. stoebe* s.str. (Ochsmann 2000). Details of sampled populations are given in table 2. At the beginning of October 2007, 5 seeds each, if available, from up to 16 sampled maternal plants per population, were directly sown in 1-L pots filled with sterilized and sieved compost. After germination, seedlings were reduced to 1 plant per pot. Plants were grown in a heated glasshouse, with 16 h artificial light per day and average temperatures of 23°C daytime and 15°C at night. Pots were watered approximately every 3–4 d. For morphological comparisons, 31 quantitative, 4 binary, and 10 derived ratio characters of leaves, stems, and reproductive organs were measured, scored, or computed (table 3). In addition to the attributes traditionally used for taxonomic or cytotype identification (Ochsmann 2000: Španiel et al. 2008), we used several novel characters that we thought could be potentially important for cytotype-level determination (table 3).

Leaf measurements (table 3) were taken 2 mo after sowing because leaves emerging later were much more extensively dissected and thus could not be accurately scanned. All leaves were assessed over a 10-d period starting December 10, 2007. To standardize measurements between plants, the sixth true leaf (not counting cotyledons) including petiole was cut from an individual rosette. In some rare cases where the sixth true leaf was damaged by fungi or insects, the fifth, seventh, or eighth true leaf was substituted. Immediately after cutting, a binary image (black for leaf and white for background, 300 dpi) of each leaf was taken using a flatbed scanner. The leaves were then dried for a minimum of 48 h at 60°C, and the leaf

dry weight was recorded. Leaf images were analyzed with the software ImageJ (Rasband 1997–2009). The number of plants sampled for each population varied between 1 and 21 individuals because of limited availability of seeds and variable germination. The total sample size was large, consisting of more than 1000 plants originating from 78 populations, including two populations with mixed ploidy (table 2).

Measurements of reproductive plant parts were taken on the start date of flowering, which was defined as the day when three fully open capitula were observed. The number of accessory rosettes of flowering plants was also assessed on this day; however, this trait was checked again in mid-June 2008 because the formation of accessory rosettes continued after flowering. Outer and inner florets including ovaries with developing pappus and involucral bracts from the middle part of inflorescence were dissected and attached to paper using transparent tape. These plant parts were later scanned at high resolution (1200 dpi) and analyzed using ImageJ. Mean data for inflorescense traits (LOF, LIF, LP, LB, WB, WAP, LAP, LDP, LAM, LF, MEANOF, MEANIN, WCAP, LCAP; see table 3) were based on the three (sometimes two) largest capitula per plant. Stem height and the length of branches and peduncles were measured using a standard ruler with 1-mm precision; stem diameter was measured using a manual caliper with 0.1-mm precision. Further details on measured characters are given in table 3. The glasshouse experiment was finished at the end of June 2008. The plants that had flowered were mounted and kept as herbarium specimens. Plants that were still at the rosette stage were transferred from the glasshouse to field plots. When these plants flowered, additional measurements were taken as detailed above. Final measurements were taken at the beginning of September 2008. The total number of plants with data for both stem and reproductive organs was lower than the number of those with data for leaf traits because only a subset of cultivated plants flowered (table 2). In addition, 39 plants were removed from the final data set for the multivariate analyses because their inner and outer florets were damaged by thrips.

Ploidy Level Analysis and Chromosome Number Determination

Ploidy levels of more than 2000 seed families from most of the populations used in this study were taken from Treier et al. (2009). An additional 293 plants from 2 native (Switzerland and Romania) and 20 introduced (Canada) populations collected in 2006 and 2007 (table 2) were analyzed using flow cytometry. Bulk samples from 4–8 greenhouse-grown plants were prepared in a two-step procedure using Partec nuclei isolation and staining buffers following the manufacturer's protocol (for more details, see Treier et al. 2009). If more than one ploidy level was detected in the bulk sample, plants were reanalyzed individually. Flow cytometric analyses were performed with a CyFlow SL flow cytometer (Partec) equipped with a green laser functioning at 532 nm. Propidium iodide was used as a stain. Histograms were accumulated at a flow rate of ~20–50 particles per second for a total count of 1000–3000 nuclei. We used a previously counted diploid plant of *C. stoebe* (2x) as an external standard. *Glycine max* cv. Polanka was used as internal standard with a known genome size (2C = 2.5 pg of DNA; Doležel et al. 1994) for precise estimation of the genome size of the putative hexaploid plant.

Additional chromosome counts of one putative hexaploid plant were made on the root-tip meristems of pot-grown plants. Root-tip cuttings were pretreated with a 0.5% solution of colchicine for 1.5–3 h at room temperature, fixed in a mixture of ethanol and glacial acetic acid (3 : 1) for at least 1 h, and stored in 70% ethanol at 4°C until used. Hydrolysis was done in 1N HCl at 60°C for 7–10 min followed by the squash-and-smear method (Murín 1960) with cellophane replacing the glass cover. Giemsa solution in phosphate buffer was used as a stain.

Multivariate and Univariate Morphometric and Statistical Analyses

Several of the recorded traits were not included in morphometric analyses because they had extremely low levels of variation (NSTEM, FCOL) or because there were missing values for many tetraploid plants (ASL and ASW were not present in entire leaves). Thus, of the 45 measured characters, 40 were used in the multivariate analyses (table 3). Some characters were closely related (e.g., level of leaf dissection: NSEG, LPER, LSHAP; or polycarpy: ROS, NOROS). The removal of closely related characters (e.g., LSHAP, NSEG, ROS) did not change the pattern revealed by the principal component analyses (PCA; results not shown). Strong correlations might substantially distort the results of discriminant analyses; however, all characters used for multivariate analyses (table 3) had pairwise correlations <0.95 (Pearson or Spearman correlation coefficient), and thus none were omitted.

To visualize the relationships among individual plants and populations, we performed PCAs based on correlation matrices of measured characters standardized to 0 means and unit standard deviations. Populations with fewer than 5 available plants were excluded from the population-level multivariate analyses. Populations were represented by mean values of their characters. In the case of mixed-ploidy populations, both cytotypes were considered as different population units. To show the phenotypic space occupied by the different geocytotypes, we constructed confidence ellipses defined by the gravity center (centroid) of the cloud and 1.5 times the standard deviation.

While PCA extracts most of the overall variation along the first few components, canonical discriminant analysis (CDA) maximizes between-group differences and minimizes within-group differentiation. We used canonical discriminant analysis to reveal the most important characters contributing to the separation of diploid and tetraploid plants and tetraploids from the native and introduced range. Correct assignment of plants to predefined groups (either cytotypes or geocytotypes) was assessed using classificatory discriminant analyses (linear, quadratic, and a nonparametric cross-validation *k*-nearest-neighbor approach).

Using different algorithms (UPGMA, Ward method, complete linkage), we performed cluster analyses based on population means to assess hierarchical clustering at the population level. A Mantel test was used to infer correlation between spatial distribution and phenotypic differentiation of populations

Table 3

List of Characters Measured in *Centaurea stoebe*

Continuous quantitative characters:	
LOF	Length of outer florets (mm) from the base to the apex of the longest tip of floret
LIF	Length of inner florets (mm) from the base to the apex of the corolla tip
LP	Length of pappi (mm) measured on ovaries of inner flowers
LB	Length of middle involucral bracts (mm) from base to the apex
WB	Maximal width of middle involucral bracts (mm)
WAP	Width of appendages of middle involucral bracts (mm) measured at the base of the dark part, excluding lateral fimbriae
LAP	Length of appendages of middle involucral bracts (mm) excluding lateral fimbriae and apical mucro (mm)
LDP	Length of the dark part of appendages of middle involucral bracts (mm) measured from the apex of appendages (excluding apical mucro) to the color transition (from black or brown to the green color of involucral bract)
LAM	Length of apical mucros of appendages of middle involucral bracts (mm)
LF	Length of longest lateral fimbria of appendages of middle involucral bracts (mm)
MEANOF	Mean no. outer florets per capitulum
MEANIF	Mean no. inner florets per capitulum
WCAP	Width of capitulum (mm) measured just before or at the beginning of flowering
LCAP	Length of capitulum (mm) measured just before or at the beginning of flowering
DST	Stem diameter (mm) measured at the base of stem
HST	Stem height (cm) measured from the base to the basal part of the principal capitulum
LACL	Length of stalk of principal capitulum (akladium; cm),
LLBRA	Length of the longest lateral branch (cm) measured from ramification to the base of the principal capitulum of the longest lateral branch
LWEI	Dry weight of the sixth rosette leaf (mg)
LARE	Leaf area of the sixth rosette leaf (mm^2)
LL	Length of the sixth rosette leaf (mm)
LW	Width of the sixth rosette leaf (mm)
ASL[a]	Length of the apical segment of the sixth rosette leaf (mm) in the case of divided or pinatiffid leaves
ASW[a]	Width of the apical segment of the sixth rosette leaf (mm) in the case of divided or pinatiffid leaves
LPER	Leaf perimeter of the sixth rosette leaf (mm)
Discrete quantitative characters:	
NF	No. lateral fimbriae of appendages of middle involucral bracts measured on one side of appendages and only the highest value of three measured bracts was kept
NCAP	Total no. capitula counted on herbarium specimens, including only buds >3 mm
NSTEM[a]	No. stems per plant
NOROS	No. accessory rosettes
NDFLOW	No. days from sowing to flowering
NSEG	Total number of segments (lobes or leaflets) on the sixth rosette leaf measured on both sides
Binary characters:	
ROS	Accessory rosettes: present (1) or absent (0)
FCOL[a]	Flower color: violet/purple/dark red (0) or white/whitish (1)
FLOW[a]	Flowering in the first year: yes (1) or no (0)
LSHAP	Shape of the sixth rosette leaf: dissected/pinnattifid (1) or entire (0)
Ratio characters	LB/WB, LAP/LDP, LAP/WAP, LAM/LB, LCAP/WCAP, HST/LLBRA, NCAP/HST, LWEI/LARE (=SLA [specific leaf area]), LPER/LL, ASL/ASWa, LL/NSEGa, LL/LW

[a] Characters were not included in multivariate morphometric analyses.

within each geocytotype separately. The robustness of the test was assessed using 9999 permutations.

Univariate statistics of quantitative characters (mean, standard deviation, minimum, maximum, and fifth and ninety-fifth percentiles) were computed for each geocytotype separately (appendix in the online edition of the *International Journal of Plant Sciences*). We assessed differences between cytotypes and tetraploid geocytotypes in selected characters related to putative reproduction success (MEANIN, NCAP), branching form (LLBRA), shape of florets and capitula (LOF, LCAP/WCAP), and leaf biomass accumulation (LWEI, LARE), using linear mixed effect models (LMM). The analyses were performed in two steps: (1) comparison of diploid and tetraploid cytotypes and (2) comparison between tetraploids from the native and introduced range. Dependent variables were transformed as required, to address normality assumptions. Populations nested within cytotype or geocytotype were considered as a random factor. Differences in the probability of forming accessory rosettes (ROS) for each geocytotype were assessed using a generalized linear mixed effect model with a binomial distribution and a logit link function with geocytotype as a main factor and population nested in geocytotype as a random factor. We used nonparametric Wilcoxon rank sum test based on population means for the character NDTOFLOW due to a strong violation of the normality assumption when applying LMM. Analyses and plotting were done within the

R statistical environment (R Development Core Team 2009) using the packages ade4, class, nlme, sp, and stats.

Results

Cytotype Distribution

All but one of the flow cytometrically analyzed samples of *Centaurea stoebe* s.l. from the introduced and native ranges were tetraploid (2n~4x) (introduced range: 263 plants from 20 Canadian populations; fig. 1, table 2; native range: 30 plants of two populations Romania and Switzerland). One plant (population URS, British Columbia) was hexaploid (2n~6x), with 5.27 pg of DNA per genome (measured against *Glycine max* as an internal standard). Hexaploidy was confirmed by chromosome counts (2n=6x=54; fig. 2). In addition, 5 diploid plants (2n~2x) were found as an admixture in otherwise tetraploid *C. stoebe* s.l. populations from Canada. However, they were identified as either *Centaurea diffusa* (2 plants from population URS, 1 plant from 153, and 1 plant from LL) or *Centaurea psamogenna*, a stable hybridogeneous taxon between *C. stoebe* and *C. diffusa* (1 plant found in population 411; see table 2 for population codes).

Morphological Variation and Morphometric Analyses

The PCA analysis based on individual plants (fig. 3*A*) showed a fairly good separation of diploid and tetraploid plants along the first PCA axis but did not distinguish between the two ranges within the cloud of tetraploid plants. The separation of both cytotypes was also clear at the population level (fig. 3*B*), but again, 4xNA populations did not separate from 4xEU populations. The characters that contributed most to the separation of the two cytotypes for the individual plant PCA were mean number of inner florets (MEANIF), presence/absence of accessory rosettes (ROS), and traits associated with leaf shape and its dissection level (NSEG, LSHAP, LW, LPER, LPER/LL, LL/LW; table 4, best correlations with the first PCA axes). Additional characters that were important for the discrimination at the population level were mean number of outer florets (MEANOF), starting day of flowering (NDTOFLOW), the density of flower heads on the stem (NCAP/HST), and number of accessory rosettes (NOROS; table 4). No obvious grouping of *Centaurea vallesiaca* plants within the diploids was found using a PCA based on individual plant characters (results not shown), although the SW4 population occupied a marginal position within the cloud of 2x populations (fig. 3*B*). This population had on average slightly larger values for LAP/WAP, LDP, LAM/LB, LF, NCAP/HST, and LPER and smaller values for LAP/LDP and MEANOF compared with other diploid *C. stoebe* populations (data not shown). Average population values for other traits of the SW4 population, including those contributing most to the cytotype differentiation (see above), were in the range of the values for diploid *C. stoebe* populations.

CDA confirmed the results of the PCA; there was good separation of the two cytotypes with only a weak overlap (fig. 4*A*) but no separation between tetraploid geocytotypes (fig. 4*B*). The best characters for cytotype discrimination were mean number of inner and outer florets (MEANIF, MEANOF), presence/absence and total number of accessory rosettes (ROS, NOROS), starting day of flowering (NDTOFLOW),

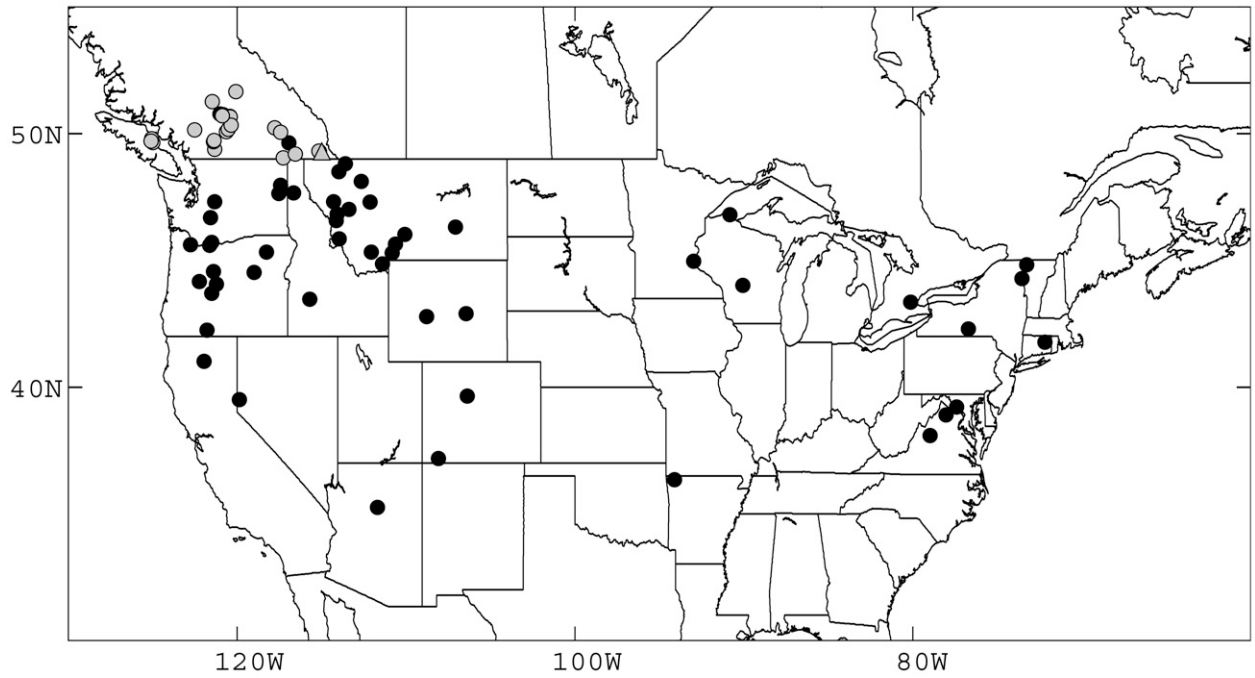

Fig. 1 Locations of tetraploid (2n=4x=36) populations of *Centaurea stoebe* s.l. in the introduced range in North America used for flow cytometry and/or karyological analyses. Black circles = literature data (table 1), gray circles = new data (table 2), gray triangle = population with a hexaploid plant (2n=6x=54; table 2). The map was created using DMAP software (Morton 2004).

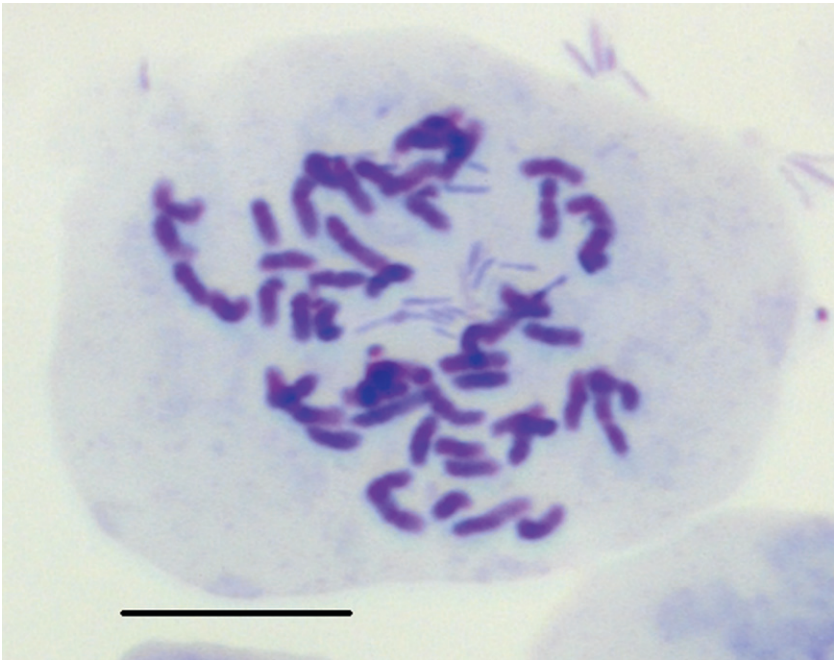

Fig. 2 Mitotic metaphase chromosomes of a hexaploid (2n=6x=54) *Centaurea stoebe* s.l. plant (plant 3 of population URS, British Columbia, Canada; 49.292°N, −115.121°W). Scale bar = 10 μm.

and several leaf traits (NSEG, LSHAP, LW, LPER, LPER/LL, LL/LW; table 4; best correlation with canonical axis). All characters had lower correlations with the canonical axis when the analysis was limited to tetraploid plants and geographic range was used as a discriminant criterion (table 4). The number of capitula (NCAP) showed the highest correlation (table 4). To validate discrimination between cytotypes and tetraploid geocytotypes, we performed three types of classification analyses that showed essentially the same results. A highly successful assignment to the corresponding ploidy was achieved

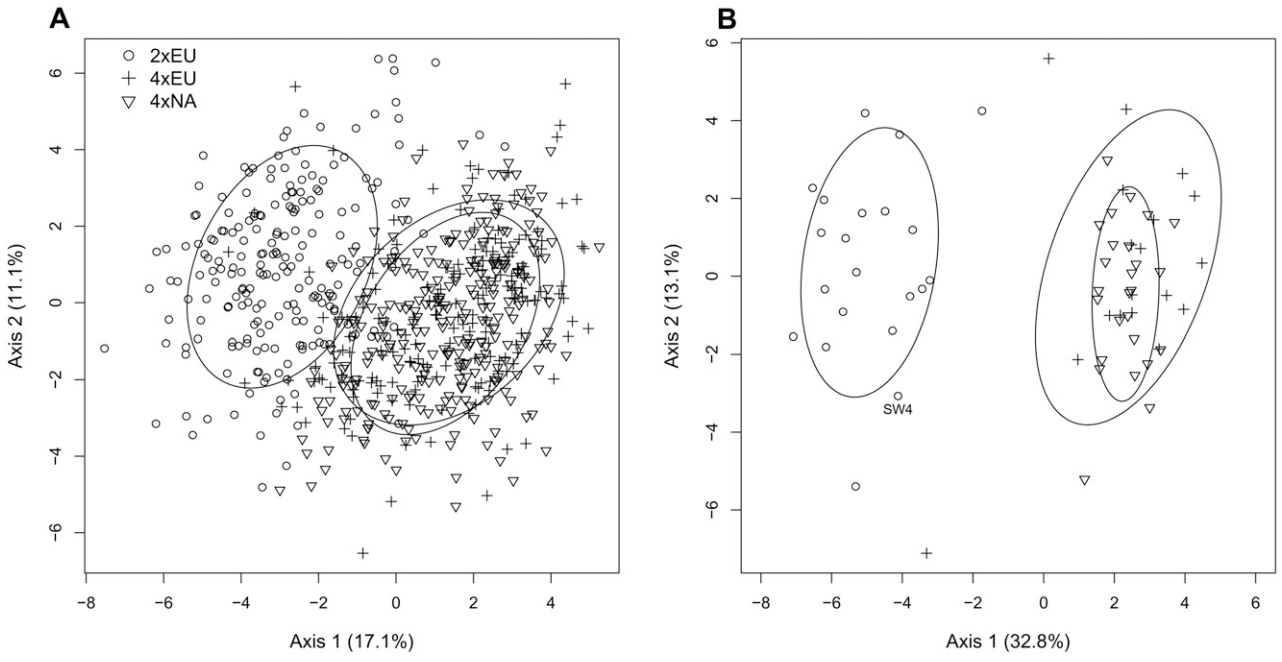

Fig. 3 Principal component analysis plots based on 40 morphological traits given for 678 plants (*A*) and 65 populations of *Centaurea stoebe* s.l. (*B*). Symbols represent the three geocytotypes: circles = native European diploids (2xEU), crosses = native European tetraploids (4xEU), triangles = invasive North American tetraploids (4xNA). The diploid *Centaurea vallesiaca* population (SW4) is indicated in *B*.

Table 4

Eigenvectors Showing Correlations of Characters with the First Two Principal Components for Analysis, PCA A and PCA B, and Correlations of Characters with the Canonical Axis from Canonical Discriminant Analysis, CAN A and CAN B

	PCA A		PCA B		CAN A	CAN B
Character	Axis 1	Axis 2	Axis 1	Axis 2	Can 1	Can 1
LOF	.006	−.284	−.105	−.244	.238	−.063
LIF	−.076	−.254	−.010	−.343	.034	−.113
LP	−.138	−.118	.144	−.212	−.290	−.048
LB	−.013	−.357	−.100	−.286	.150	.198
WB	−.186	−.073	.156	−.170	−.335	.292
LB/WB	.143	−.256	−.198	−.072	.403	−.066
WAP	−.166	.044	.156	−.065	−.295	−.128
LAP	−.060	−.286	−.003	−.290	.058	.200
LAP/WAP	.102	−.279	−.167	−.171	.308	.284
LDP	−.069	−.259	.020	−.256	−.031	.240
LAP/LDP	.020	.020	−.034	.037	.102	−.093
LAM	−.013	−.133	−.051	−.250	.085	.007
LAM/LB	−.013	.021	.006	−.125	.007	−.074
LF	−.070	−.268	.011	−.235	−.058	.262
NF	−.117	−.042	.146	−.127	−.181	−.005
MEANOF	−.184	.077	.215	.040	−.548	.121
MEANIF	−.233	−.007	.242	.045	−.688	−.045
WCAP	−.199	−.079	.190	−.035	−.390	.244
LCAP	−.059	−.191	.024	−.293	−.024	−.021
LCAP/WCAP	.135	−.048	−.157	−.135	.311	−.190
DST	−.022	−.128	.003	.107	−.098	.079
HST	.079	−.189	−.150	.102	.227	−.037
NCAP	−.086	−.008	.143	.145	−.374	−.381
LACL	−.044	−.043	.013	−.001	−.065	−.019
LLBRA	.079	−.220	−.172	.029	.340	−.016
HST/LLBRA	−.036	.070	.102	−.088	−.162	−.077
NCAP/HST	−.131	.114	.208	−.009	−.447	−.196
NOROS	.184	−.072	−.232	−.004	.538	−.260
ROS	.210	−.100	−.250	−.027	.654	−.206
NDTOFLOW	−.145	.256	.206	−.002	−.564	−.286
LWEI	−.012	−.151	−.122	−.186	.326	−.049
SLA	−.074	.012	.120	.125	−.239	.147
NSEG	−.321	.006	.250	−.014	−.718	−.042
LSHAP	−.289	−.019	.237	−.030	−.566	.010
LL	−.079	−.114	−.013	−.197	.035	−.294
LW	−.306	−.071	.222	−.149	−.511	−.095
LARE	−.047	−.158	−.098	−.171	.243	.030
LPER	−.296	−.051	.211	−.136	−.531	−.213
LPER/LL	−.315	−.004	.242	−.062	−.674	−.083
LL/LW	.289	.029	−.234	.053	.532	−.200

Note. PCA A based on individual plants, $n = 678$; PCA B based on populations, $N = 66$. Correlations of characters with the canonical axis from the canonical discriminant analysis CAN A (based on individual plants, ploidy level selected as dicriminant factor: 2x vs. 4x) and CAN B (based on individual tetraploid plants, geographic origin selected as discriminant factor: native European vs. introduced North American range) of *Centaurea stoebe*. Values above 0.2 for PCA and 0.5 levels for CDA are underlined; for definitions of abbreviations, see table 1.

using (1) parametric linear discriminant functions (95.2% diploids and 98.7% tetraploids), (2) quadratic discriminant functions (92.1% diploids and 95.1% tetraploids), and (3) a nonparametric k-nearest-neighbor cross-validation procedure with training set based on $n - 1$ individuals and with $k = 1$–31 (68.9%–80.9% of diploid and 91.4%–92.5% of tetraploid). The same tests were applied on the tetraploid geocytotypes although with lower success of correct predictions: 58.7% of 4xEU and 78.5% of 4xNA (linear discriminate function), 47.1% of 4xEU and 73.5% of 4xNA (quadratic discriminate function), and 19.8%–45% of 4xEU and 63.9%–90% of 4xNA (k-nearest-neighbor cross-validation with $k = 131$).

Different cluster algorithms grouped populations according to their ploidy level at high levels of similarity (fig. 5; different clustering algorithms gave similar results; thus, only the dendrogram based on UPGMA is shown). At low levels of similarity, however, separation of diploid and tetraploid population clusters was not as clear (fig. 5).

There was a positive relationship between geographic location and Euclidean phenotypic distance based on mean characters' values of diploid European populations (Mantel test, $r = 0.2$, $P = 0.037$). This correlation remained statistically significant when the *C. vallesiaca* population was omitted ($r = 0.19$, $P = 0.0494$). However, after removing the five pop-

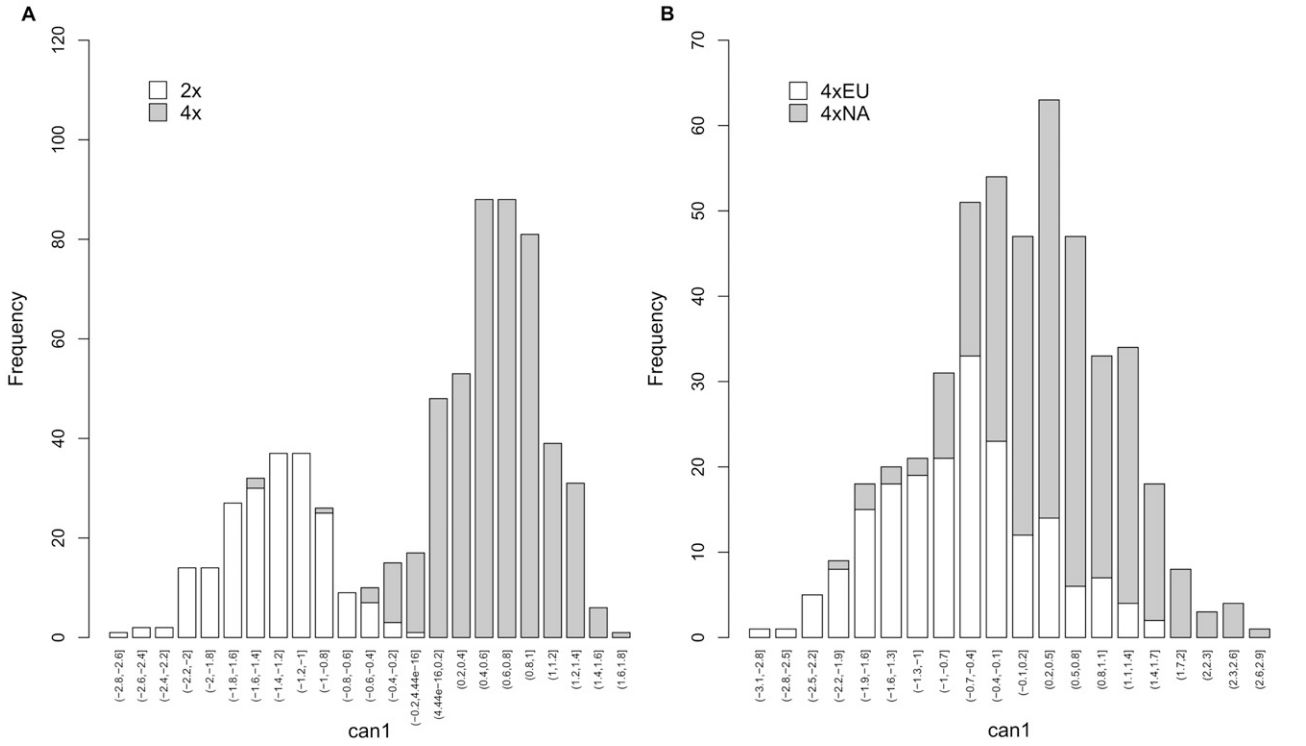

Fig. 4 Frequency histogram of linear discriminant analyses of diploid (2x) and tetraploid (4x) plants of *Centaurea stoebe* s.l. (*A*) and native European tetraploids (4xEU) and invasive North American tetraploids (4xNA; *B*).

ulations that had fewer than 5 available plants, the correlation was still positive but not significant ($r = 0.15$, $P = 0.1$). In tetraploid populations (including also those with fewer than 5 individuals), no correlation between morphological differentiation and geographical distance was found for either the native ($r = -0.08$, $P = 0.85$) or the introduced ($r = -0.02$, $P = 0.45$) range.

The proportion of tetraploid plants that flowered in the glasshouse was higher than that of the diploid plants (tetraploid: $n = 683$, 86% flowered, 87% 4xEU, 85% 4xNA; diploid: $n = 352$, 75% flowered). In the first year of cultivation tetraploid populations started flowering earlier than diploids (Wilcoxon nonparametric test based on populations means, $W = 1162$, $P < 0.001$). North American tetraploid plants flowered earlier than European tetraploids, although the difference was smaller than for the cytotype difference ($W = 359$, $P = 0.044$). Fewer than 3% of diploid plants formed accessory rosettes after flowering, indicating a fairly strict monocarpic life cycle for this cytotype. In contrast, the presence of accessory rosettes was much more frequent in tetraploid plants (65% on average, 74% for 4xEU, and 59% for 4xNA), indicating a predominantly polycarpic life cycle (fig. 6). Almost all of the diploids had their sixth rosette leaf dissected (97%), whereas less than half of the tetraploids showed this characteristic (42%; see appendix). All seven characters that were compared for diploid and tetraploid plants were significantly different (table 5; fig. 7). Specifically, diploid plants had a higher number of inner florets (MEANIN; fig. 7*A*) and capitula (NCAP; fig. 7*B*) but significantly fewer elongated capitula (LCAP/WCAP), shorter outer florets and branches (LOF, LLBRA), and smaller and lighter leaves than tetraploids (LARE, LWEI; see table 5 and appendix). In addition to these statistical differences, there were also subtle but consistent differences in coloration between the cytotypes, observed by P. Mráz. Tetraploids frequently had darker (dark green with shades of violet) involucral bracts than did diploids (bright green). The same trend was observed in the color of florets, being darker in tetraploids than in diploids. Moreover, diploid plants more frequently had paler inner flowers (white or pinkish to violet) whereas tetraploids usually had darker inner flowers (dark violet). European tetraploids had significantly more capitula (NCAP) than North American tetraploids (table 5; fig. 7).

Discussion

Cytogeographic Pattern in Introduced Range

Our data confirm a considerable shift in ploidy level distribution of *Centaurea stoebe* between the native and introduced range (Španiel et al. 2008; Treier et al. 2009). All *C. stoebe* plants from North America analyzed in this study, except for one originating from British Columbia, were found to be tetraploid. Based on our analyses, published counts, and flow cytometric estimations, ploidy level has been determined for almost 1000 plants sampled from 77 populations across the entire introduced range in North America (table 1; fig. 1). In the introduced range, tetraploid *C. stoebe* s.l. sometimes co-occurs with two other highly invasive, but diploid,

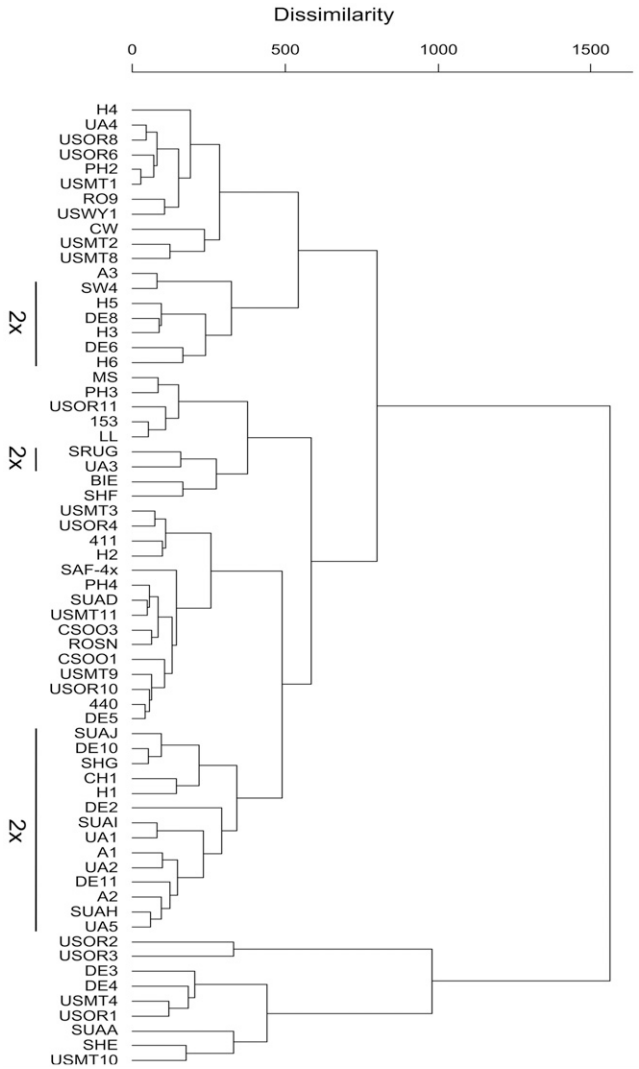

Fig. 5 Cluster analysis of 21 diploid (underlined) and 44 tetraploid populations of *Centaurea stoebe* s.l. using UPGMA method. For population codes see table 2.

European taxa, *Centaurea diffusa* and *Centaurea psamogenna* (Forcella and Harvey 1980). Our sample also contained five diploid plants from British Columbia that have been identified as *C. diffusa* or *C. psamogenna* (see "Results"). Treier et al. (2009) reported two *C. stoebe* diploids from one mixed-ploidy population in British Columbia; however, these two diploid plants were analyzed at an early rosette stage (U. A. Treier, personal communication). It is possible that these plants were misidentified because it is difficult to consistently distinguish diploid *C. stoebe* s.str. from *C. diffusa* or *C. psamogenna* at the rosette stage. This would explain this rare record of diploid *C. stoebe* from North America. Although we cannot completely exclude either historical or recent occurrence(s) of diploid *C. stoebe* s.str. in the introduced range, to date we have no convincing evidence for the presence of the diploid cytotype in North America.

Cytotype depletion in the introduced range has been reported for other invasive polyploid taxa such as *Lythrum salicaria* (Kubátová et al. 2008), *Senecio inaequidens* (Lafuma et al. 2003), and *Solidago gigantea* (Schlaepfer et al. 2008). Based on allopatric distribution and the frequency of particular cytotypes in the native range, an introduction of solely one polyploid cytotype has been suggested for two of these species (Lafuma et al. 2003; Kubátová et al. 2008). Even though diploid and tetraploid populations of *C. stoebe* s.l., are largely sympatric in their native range (Treier et al. 2009), several authors (Hufbauer and Sforza 2008; Mars et al. 2008) have suggested that the possible source area for North American populations of *C. stoebe* s.l. is the southeastern part of the native European range (the Balkans, Ukraine, and southeastern Russia). This is an area where tetraploid populations are more common than diploids (Treier et al. 2009; P. Mráz, unpublished data). Thus, even with a general situation of sympatric occurrence of diploids and tetraploids in Europe, a higher frequency of the tetraploid cytotype from the proposed source area could have increased the probability of its introduction into the new range, when compared to the diploid cytotype.

The single hexaploid plant that was found in population URS from British Columbia is the first record of hexaploidy within the complex of *C. stoebe* (Ochsmann 2000). While tetraploidy is a very common phenomenon in the genus *Centaurea*, hexaploidy is extremely rare (Phitos and Constantidi-

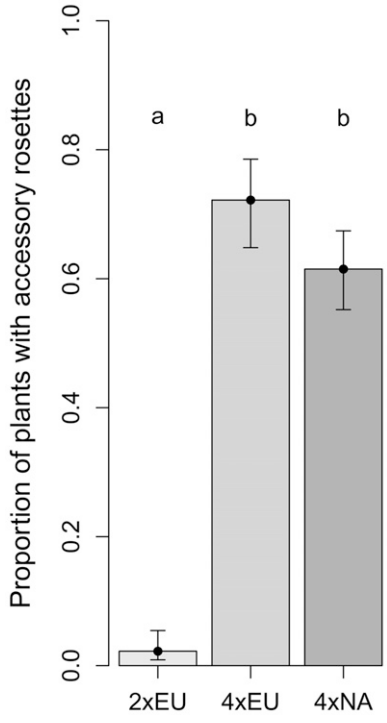

Fig. 6 Estimated mean proportions (with confidence intervals) of plants forming accessory rosettes for the three geocytotypes of *Centaurea stoebe* s.l.; European diploids (2xEU; excluding *Centaurea valesiaca*), European tetraploids (4xEU), and North American tetraploids (4xNA). Different letters above bar plots indicate significant difference between geocytotypes at $P < 0.05$.

Table 5

ANOVA Table of Linear Mixed Effect Models (LMM) for Seven Selected Variables of *Centaurea stoebe*

Traits/comparisons	2x vs. 4x	4xEU vs. 4xNA
Length of outer florets (LOF)	$F_{1, 70} = 21.3$***	$F_{1, 45} = .19$
Mean no. inner florets (MEANIF)	$F_{1, 70} = 193.98$***	$F_{1, 45} = .38$
Length/width of capitula (LCAP/WCAP)	$F_{1, 70} = 24.8$***	$F_{1, 45} = 2.77$
No. capitula (NCAP)	$F_{1, 70} = 33.01$***	$F_{1, 45} = 7.65$**
Length of longest branch (LLBRA)	$F_{1, 70} = 33.47$***	$F_{1, 45} = .06$
Leaf weight (LWEI)	$F_{1, 71} = 32.07$***	$F_{1, 46} = .01$
Leaf area (LARE)	$F_{1, 71} = 19.52$***	$F_{1, 46} = .03$

Note. Analyses were performed separately for diploid (2x; excluding *Centaurea valesiaca*) and tetraploid (4x) plants, and for European (4xEU) and North American tetraploids (4xNA). Populations nested within ploidy or geocytotype were included in the models as a random factor.

** $P < 0.01$.

*** $P < 0.001$.

nis 1993; Trigas et al. 2008; Garcia-Jacas et al. 2009). As the hexaploid plant was morphologically and genetically (nrDNA sequences and SSRs; P. Mráz, N. Garcia-Jacas, E. Gex-Fabry, A. Susanna, and H. Müller-Schärer, unpublished manuscript) indistinguishable from tetraploids of *C. stoebe* s.l., we suggest that it originated via fusion of reduced and unreduced gametes (2x + 4x) produced by tetraploid plants. This pathway is considered to be the most common in polyploid evolution of vascular plants (Ramsey and Schemske 1998).

Morphological Differentiation between the Diploid and Tetraploid Cytotype

Our multivariate morphometric data revealed strong morphological differentiation between the diploid and tetraploid cytotype of *C. stoebe*. This pattern was most clear at the population level (fig. 3*B*) but also was obvious for individual plants (fig. 3*A*). Španiel et al. (2008), looking at central European populations of *C. stoebe*, also found better separation of populations than individuals, but with a larger overlap of individual diploid and tetraploid plants than observed in our study. The reduced variation in our study may have resulted from our use of plants grown from the seeds under uniform conditions in a glasshouse whereas Španiel et al. (2008) measured traits on field-collected specimens. By growing plants from seed to maturity, we were able to include additional characters in our analysis, such as shape and size of young rosette leaves and onset of flowering in the first year of growth, that may have improved our discrimination between the two cytotypes. However, in spite of the clear separation by PCA, even the best discriminant morphological characters were still partially overlapping (see appendix). The observed large morphological variation within this complex was emphasized in the hierarchical clustering analyses (fig. 5), with the populations of the same ploidy level clustering together only at a high level of similarity. Thus, a combination of several characters rather than a single character is required for accurate determination of *C. stoebe* cytotypes. Similar high levels of phenotypic variation have been reported from other morphometric studies of closely related *Centaurea* taxa, suggesting their relatively young origin and ongoing differentiation (Hardy et al. 2000; Vanderhoeven et al. 2002; Guarino and Rampone 2006; Koutecký 2007; Olšavská et al. 2009). In addition to postploidization processes, morphological differentiation observed between diploid and tetraploid cytotypes should result principally from either

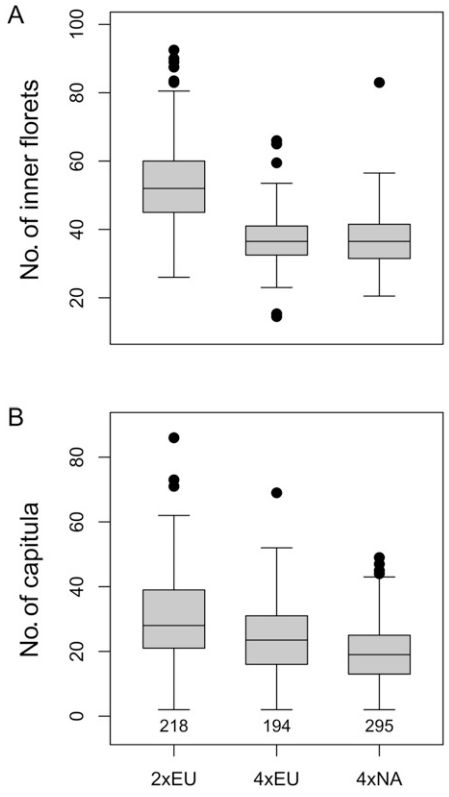

Fig. 7 Box plots of mean number of inner florets (MEANIF; *A*), number of capitula (NCAP; *B*) for European diploids (2xEU; excluding *Centaurea valesiaca*), European tetraploids (4xEU), and invasive North American tetraploids (4xNA) of *Centaurea stoebe* s.l. Number of plants per geocytotype are given above the horizontal axis in *B*.

direct autopolyploidization of the diploid cytotype or from allopolyploidization. Our preliminary results based on cloning and sequencing of two nuclear DNA regions favor the allopolyploid hypothesis (P. Mráz, N. Garcia-Jacas, E. Gex-Fabry, A. Susanna, and H. Müller-Schärer, unpublished manuscript). However, at this stage we cannot verify whether critical morphological changes manifested in tetraploids, such as a polycarpic life cycle, are the result of hybridization, because we have yet to find the second parental taxon (P. Mráz, N. Garcia-Jacas, E. Gex-Fabry, A. Susanna, and H. Müller-Schärer, unpublished manuscript).

In accordance with the literature (Dostál 1976; Boggs and Story 1987; Müller 1989; Ochsmann 2000; Story et al. 2001; Španiel et al. 2008; Treier et al. 2009; Henery et al. 2010) we have confirmed a pronounced shift in life cycle between cytotypes of *C. stoebe*. Most of the tetraploids were found to be polycarpic, as they formed accessory rosettes for bolting in the next season. In contrast, accessory rosettes were observed in fewer than 3% of diploids, indicating the prevalence of monocarpy. This character is thus the most reliable for discriminating between cytotypes and may also explain the invasion success of tetraploids in their introduced range (Müller-Schärer et al. 2004; Treier et al. 2009; Henery et al. 2010). For correct assignment of the cytotypes in the field, the presence/absence of renewing accessory rosettes on flowering plants needs to be assessed in late autumn, as the formation of accessory rosettes is stimulated by shoot withering. Eventually, this trait can be checked in spring of the subsequent year, if the withered shoots from the previous season are still present.

On average, diploid plants started flowering later than tetraploids. This shift, however, was primarily the result of a delayed onset of flowering for about one-quarter of diploid plants. We suspect that the observed late flowering peak in some of the diploid plants may have resulted from a prolonged growing season under unnatural but favorable glasshouse conditions. This may also explain the higher proportion of flowering diploids in the first year of our study, as compared to results from the common garden experiment reported by Henery et al. (2010).

Our data confirmed the previous observation (Ochsmann (2000) that diploid *C. stoebe* s.str. had a broader capitula than the tetraploid subsp. *micranthos*. Flower heads of diploids were generally more rounded (LCAP/WCAP) than those of tetraploids. This is likely associated with the increased number of both inner (MEANIF) and outer florets (MEANOF) in diploid plants (table 5; fig. 7*A*; Španiel et al. 2008). Diploids also produced more capitula than tetraploids in agreement with the results from common garden experiments (Henery et al. 2010). Considered together, the higher number of capitula and the higher number of inner florets per capitulum suggest a greater investment in seed reproduction in a given year for diploids than for tetraploids. Indeed, Henery et al. (2010) observed that diploids produced significantly more seeds per plant than tetraploids in a common garden experiment. However, with a polycarpic life cycle, the total lifetime reproductive output of tetraploids may be higher than that of diploid plants. Over the long term and during fluctuating conditions, a perennial life cycle might be more advantageous than monocarpy, as it assures greater local persistence through repeated, albeit lower, annual seed production. Such a trade-off between a higher level of perenniality and lower annual seed production could have favored the establishment and persistence of the tetraploid cytotype in the introduced range, particularly if the risk of postflowering mortality is reduced due to a lack of natural enemies (Klinkhamer et al. 1997).

In contrast to previous data (Ochsmann 2000; Španiel et al. 2008), pappus length (LP) and the number of fimbriae (NF) were not different between cytotypes (results not shown). Homogeniety of pappus lengths among cytotypes may have resulted because plants were not pollinated under glasshouse conditions and we measured this trait on immature ovules. We also found this character to be extremely variable, especially in tetraploid plants, ranging from no pappus to very long ones, even within the same population (P. Mráz, unpublished data). Such phenotypic variation under uniform conditions suggests that this character should not be used to separate small endemic taxa within the *C. stoebe* group, such as *C. triniifolia* Heuff. or *C. reichenbachii* DC. (cf. Ochsmann 2000).

Leaves on young diploid rosettes were more dissected than on tetraploid rosettes (LSHAP, NSEG) because of differential timing of heteroblastic development of leaf shape (cf. Ashby 1948; Lynn and Waldren 2001). While the first 2–3 rosette leaves in diploids were completely entire, successive leaves (usually starting from the fourth leaf) became increasingly dissected until the mature stage of the plant (approximately the tenth to the eleventh leaf stage). This sequence was also observed in tetraploids, but with dissection starting usually only from the sixth to eighth leaf. These results indicate a genetic basis for faster leaf development in diploids than in tetraploids. Although we did not include mature leaves in our measurements because of the difficulties associated with accurate scanning, we observed that the segments/lobes of highly dissected mature leaves of diploids were usually narrower than those in tetraploids.

Diploid populations showed a positive correlation between geographic and phenotypic distances suggesting an isolation-by-distance pattern for population differentiation, possibly due to local adaptation. In contrast, no such correlations were found for tetraploid populations from either Europe or North America. The lack of a correlation within the introduced range can be explained using multiple stochastic introductions, mixing of plants from different sources, and a relatively short time since introductions (Hufbauer and Sforza 2008; Mars et al. 2008). An explanation for the lack of a correlation in native European tetraploid populations is not as clear. Floristic data indicate a recent and massive spread of tetraploids in Europe facilitated by increasing human disturbance (Ochsmann 2000; Korneck 2004; Welss et al. 2008; our unpublished observations). Such recent range expansion may have limited morphological and genetic differentiation and thus population structure of native tetraploids.

Morphological Differentiation of Tetraploids from the Native and Introduced Range

As hypothesized, there was very little differentiation in the morphology of tetraploids from the native and intro-

duced ranges. The time since the first introduction(s) into North America, ~120 years ago, has probably been too short for the accumulation of any significant phenotypic changes that would result in pronounced morphological differentiation between native and introduced tetraploid populations. However, we observed small but significant differences in some single traits between the two groups of tetraploids. Similar to results from a common garden experiment (Henery et al. 2010; see also Ridenour et al. 2008), introduced tetraploids produced a lower number of capitula than native tetraploids. North American tetraploid populations flowered earlier than European tetraploid populations. Early flowering may result from a greater accumulation of biomass during early growth in North American tetraploids (Henery et al. 2010). Cross-continental comparisons of native and introduced populations of many plant species have demonstrated shifts towards higher biomass production in introduced populations (Bossdorf et al. 2005). In contrast to Treier et al. (2009) and Henery et al. (2010), when we measured the proportion of plants forming accessory rosettes, we did not find a higher level of polycarpy in North American tetraploids when compared to the native European tetraploids.

Taxonomic Consequences and Nomenclatural Notes

The results of our morphometric study support separation of the two cytotypes into different taxonomic entities as proposed by Ochsmann (2000), and they challenge the single taxon concept adopted by some authors (Štěpánek and Koutecký 2005; Španiel et al. 2008). Španiel et al. (2008) argued that the combination of (1) the weak morphological differentiation at the individual level and (2) the existence of mixed-ploidy populations and the largely sympatric distribution of the cytotypes in Europe favor recognition of only one taxon, without further taxonomic treatment of cytotypes (either as species or subspecies). First, our data demonstrate a clear morphological discrimination of the two cytotypes. This may have resulted from using plants grown under a uniform environment rather than studying field and herbarium samples (Španiel et al. 2008). Secondly, a distinct distributional pattern is often considered as an additional argument for taxonomic separation when evaluating diploid-polyploid complexes (Marhold 1999; Soltis et al. 2007). Allopatric distribution providing a prezygotic reproductive barrier between cytotypes might result from either different evolutionary histories (stochastic range fragmentation, colonization, or extinctions) or from different ecological requirements of cytotypes, or from both processes. However, polyploid speciation regardless if auto- or allopolyploid is principally a sympatric process (Schemske 2000). Thus, some level of distributional overlap between parental diploid cytotype(s) and polyploid progeny should be expected at least during the first stages after polyploid formation. Furthermore, if the diploid and polyploid cytotypes are well reproductively isolated from each other by barriers other than geography, coexistence within the same range might be expected. Recent meta-analyses of diploid-polyploid congeners at a continental scale showed that there is no evidence for consistent range shifts following genome duplication (Martin and Husband 2009), and thus, the distribution of closely related diploid and polyploid taxa can overlap.

Ochsmann (2000) suggested that the tetraploid cytotype arose in southeastern Europe and later colonized the current range. Recent observations have supported his hypothesis. There is increasing evidence that the largely sympatric distribution of both cytotypes in Europe is at least partially the result of a relatively recent spread of tetraploids, preferentially colonizing man-made habitats (railways, quarries, roadsides), as observed in the Czech Republic (P. Koutecký, personal communication), Germany (Ochsmann 2000; Korneck 2004; Welss et al. 2008); Switzerland (Ochsmann 2000); France and Slovakia (P. Mráz, personal observations). Such a pattern indicates that the tetraploid cytotype might not be a native floristic element for those countries (see also Ochsmann 2000; Greuter 2006–2009). Our recent ecological and genetic data from mixed-ploidy populations in central Europe (P. Mráz, unpublished data) indicate later arrival of tetraploids to the sites with established diploid populations resulting in secondary contact. This is the most common pattern observed in mixed-ploidy populations (Petit et al. 1999). In combination, these data suggest a recent increase in the level of range overlap between the two *C. stoebe* cytotypes compared to the past.

Once successfully established, polyploids are usually reproductively isolated from their diploid progenitor because of a ploidy barrier that causes seed abortion due to a so-called triploid block or because intercytotype hybrids that may arise are sterile (Marks 1966; Vinkenoog et al. 2003). Thus, effective gene-flow between cytotypes becomes substantially reduced and the cytotypes can diverge even under sympatric situations. We have recently observed a very strong reproductive barrier between the cytotypes of *C. stoebe*, using experimental crosses (P. Mráz and G. Bowman, unpublished data). As reproductive isolation is a prerequisite of speciation (Rieseberg and Willis 2007), it is considered an important criterion for taxon delimitation (Soltis et al. 2007). Thus, the existence of strong reproductive isolation of *C. stoebe* s.l. adds additional support for the recognition of the diploid and tetraploid cytotypes as separate taxa.

Based on these combined sources of evidence we propose to treat the two cytotypes as different species. While *C. stoebe* L. seems to be the appropriate name for the diploid cytotype (Greuter 2003; Španiel et al. 2008), the appropriate nomenclature for the tetraploid cytotype is not clear. Greuter (2003) argued that when treating the cytotypes as different subspecies, the name "*C. stoebe* subsp. *australis* (Pančić ex A. Kern.) Greuter" should be applied for the tetraploid cytotype as this name has priority over *C. stoebe* subsp. *micranthos*. However, as discussed by Španiel et al. (2008), one of two Hungarian populations of *Centaurea australis* mentioned in Kerner's protologue and for which relevant syntype material exists was diploid (Španiel et al. 2008), and the ploidy level of the second population to date has not been checked. Thus, the interpretation of the name *C. australis* (or *C. stoebe* subsp. *australis*) depends on the choice of the syntype material. If the name is based on the diploid syntype, then it should not be used for the tetraploid cytotype. Alternatively, if the name *C. australis* is typified using the syntype from the

second locality and this turns out to be a tetraploid cytotype, this name can be used for the tetraploid taxon (either as species or subspecies). Because the name *Centaurea biebersteinii* DC. was validly published in 1838 (de Candolle 1838) and has been frequently applied to the tetraploid *C. stoebe* s.l. cytotype (see Greuter 2003, 2006–2009; and many papers focused on spotted knapweed in North America), it could have a priority over any use of *C. australis* that was described later (Kerner 1872). However, we believe that the name *C. biebersteinii* refers to diploid plants because original specimens in the herbarium of Genève (G) have very rounded capitula typical for diploid plants (inspection of the first author), and de Candolle noted that the plant is annual (=monocarpic; de Candolle 1838). This suggests that the name *C. biebersteinii* DC. is inappropriate for the tetraploid cytotype at any rank. Ongoing studies of both field and herbarium material hopefully will resolve the nomenclature of the tetraploid cytotype of *C. stoebe* s.l.

Acknowledgments

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Appendix

Discrimination Key for Diploid and Tetraploid *Centaurea stoebe* in the Field

Based on the results of this study, we propose the following key to discriminate between the diploid and tetraploid cytotypes of *C. stoebe* (values of number of inner florets are expressed as [minimum–]fifth percentile to ninety-fifth percentile[–maximum]):

A. Plants annual or biannual without formation of overwintering accessory rosettes after withering of the shoot(s) (monocarpic life cycle), usually one- to few-stemmed, number of inner florets per capitulum (26–)35 to 76(–93), capitula before anthesis more rounded (length/width ratio 1.2 on average), color of involucral bracts green to bright green: *C. stoebe* s.str. (diploid cytotype).

B. Plants short-lived perennial, forming overwintering accessory rosettes after withering of the shoot(s) (polycarpic life cycle), usually few- to many-stemmed, number of inner florets per capitulum (15–)25 to 50(–83), capitula before anthesis more elongated (length/width ratio 1.35 on average), color of involucral bracts dark green often with shades of violet: *C. stoebe* s.l. (tetraploid cytotype).

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Príloha N

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Evidence for a combination of pre-adapted traits and rapid adaptive change in the invasive plant *Centaurea stoebe*

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Summary

1. Introduced plants have the potential to rapidly evolve traits of ecological importance that may add to their innate potential to become invasive. During invasions, selection may favour genotypes that are already pre-adapted to conditions in the new habitat and, over time, alter the characteristics of subsequent generations.

2. Spotted knapweed (*Centaurea stoebe*) occurs in two predominantly spatially separated cytotypes in its native range (Europe–Western Asia), but currently only the tetraploid form has been confirmed in the introduced range (North America), where it is invasive. We used several common garden experiments to examine, across multiple populations, whether tetraploids and diploids from the native range differ in life cycle, leaf traits and reproductive capacity and if such differences would explain the predominance of tetraploids and their advance into new habitats in the introduced range. We also compared the same traits in tetraploids from the native and introduced range to determine whether any rapid adaptive changes had occurred since introduction that may have enhanced invasive potential of the species in North America.

3. We found tetraploids had lower specific leaf area, less lamina dissection and fewer, narrower leaves than diploids. Diploids exhibited a monocarpic life cycle and produced few if any accessory rosettes. Diploids produced significantly more seeds per capitulum and had more capitula per plant than tetraploids. In contrast, the vast majority of European tetraploids continued to flower in both seasons by regenerating from multiple secondary rosettes, demonstrating a predominantly polycarpic life cycle.

4. During early growth tetraploids from North America achieved greater biomass than both tetraploids and diploids from the native range but this did not manifest as larger above-ground biomass at maturity. In North American tetraploids there was also evidence of a shift towards a more strictly polycarpic life cycle, less leaf dissection, greater carbon investment per leaf, and greater seed production per capitulum.

5. *Synthesis.* Our results suggest that the characteristics of tetraploid *C. stoebe* pre-adapted them (compared to diploid conspecifics) for spread and persistence of the species into habitats in North America characterized by a more continental climate. After the species' introduction, small but potentially important shifts in tetraploid biology have occurred that may have contributed significantly to successful invasion.

Key-words: biological invasion, *Centaurea stoebe,* plant life cycle, plant traits, ploidy, rapid adaptive change, reproductive potential

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Introduction

The invasiveness of a plant species may be derived from a combination of pre-adapted traits and rapid adaptive changes in the species following introduction (Müller-Schärer & Steinger 2004). Interspecific studies examining traits of invasive plants have identified that traits associated with reproductive capacity and leaf traits associated with rapid carbon capture, such as high specific leaf area (SLA), leaf area ratio, photosynthetic rate, and net assimilation rate, may pre-adapt species to become invasive (Pyšek & Richardson 2007). Once introduced to a new habitat and released from co-evolved natural enemies (Liu & Stiling 2006) these species may be able to exploit resources to a greater extent than co-occurring native species (Blumenthal 2006; Blumenthal *et al.* 2009). Recent studies have highlighted the importance of co-determinants such as residence time, range size and propagule pressure that interact with plant traits in the invasion process and show that the relative importance of traits is context-dependent and differs between species (Pyšek, Křivánek & Jarošík 2009b; Pyšek *et al.* 2009a). This partly explains the difficulty in generalizing across species about the importance of plant traits in the process leading from a species' introduction to invasion.

In addition, an increasing number of studies comparing various traits of species from their native and invasive ranges have shown that evolutionary adaptive changes can occur that alter traits, which may contribute to these species becoming invasive (Ellstrand & Schierenbeck 2000; Lee 2002; Müller-Schärer, Schaffner & Steinger 2004; Bossdorf *et al.* 2005; Barrett, Colautti & Eckert 2008; Whitney & Gabler 2008). Such trait shifts can increase the relative fitness of invasive species over native species in the same plant communities. These changes, however, may occur over several generations which may partly explain the lag time commonly seen between the first records of a species in a new region and its subsequent development into a highly successful invasive species (Sakai *et al.* 2001).

The most commonly tested hypothesis related to evolutionary change is whether plants in the introduced range, once released from their co-evolved natural enemies, have been able to reallocate resources previously used on defence, to achieve increased growth, the so-called Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey & Nötzold 1995). Whilst increased growth and/or reduced defence has commonly been detected in invasive conspecifics (Siemann & Rogers 2003a,b; Jakobs, Weber & Edwards 2004; Wolfe, Elzinga & Biere 2004; Agrawal *et al.* 2005; Blumenthal & Hufbauer 2007; Zou, Rogers & Siemann 2008a; Zou *et al.* 2008b; Abhilasha & Joshi 2009), such reallocation of resources may be minor and not always manifest as increased above-ground vegetative biomass (Maron *et al.* 2004a; Genton *et al.* 2005; Barney, Whitlow & DiTommaso 2009). Changes in traits other than size, e.g. numbers or size of propagules and SLA, may be equally important for promoting invasiveness (Bossdorf *et al.* 2005). Higher SLA and associated increased rates of carbon capture could be allocated to a more extensive root system or more propagules per plant rather than in greater leaf biomass or shoot size. Whilst SLA has been found to be generally higher in invasive plants relative to co-occurring native species (Leishman *et al.* 2007), a shift in this trait within a species could be the result of selection for adaptation to a new environmental niche during invasion.

Recent studies indicate that interspecific variation in ploidy level in species is associated with invasiveness (Pyšek *et al.* 2009a) and that within species, polyploids rather than diploids are the dominant invasive forms (Lafuma *et al.* 2003; Pandit, Tan & Bisht 2006; Schlaepfer *et al.* 2008; Treier *et al.* 2009). Hence, the potential for becoming invasive not only depends on the variation in ploidy of a species but also on the ploidy level of a cytotype. Although the reason for this is currently unclear, it has been proposed that a broader tolerance of environmental conditions in polyploids, their greater potential to rapidly evolve via an ability to carry greater genetic diversity and the potential for duplicated genes to evolve new functions in the longer term, may explain their success (Soltis & Soltis 2000). It is also possible that, in a similar interaction to that proposed for resource availability and release from natural enemies (Blumenthal 2006), some polyploids may possess traits that give them advantages over diploids when herbivores/pathogens are absent, such that they are able to increase their spread and maintain occupancy of colonized sites (Müller-Schärer, Schaffner & Steinger 2004).

In the invasive plant *Centaurea stoebe* L. tetraploids and diploids occur primarily in discrete populations across the native range in Europe whilst tetraploids dominate the non-native range in North America entirely (Treier *et al.* 2009). Such a pronounced shift in cytotyope frequency could be due to founder events or be derived from a larger degree of environmental niche overlap between the native and introduced ranges of tetraploids than in diploids (Treier *et al.* 2009). In the latter instance, assuming both cytotypes were introduced to North America, the combination of traits possessed by tetraploids may have made them inherently more invasive than diploids. Moreover, tetraploid *C. stoebe* have been shown to be polycarpic which, in the absence of specialist herbivores, could give them greater lifetime fecundity than monocarpic diploids thus facilitating their rapid spread (Müller 1989; Broz *et al.* 2009).

In conjunction with changes in life cycle associated with ploidy, a significant shift in the climatic niche has occurred between *C. stoebe*'s native and introduced ranges (Broennimann *et al.* 2007). Tetraploids in North America now occupy areas with drier and more continental conditions than do tetraploids in Europe, whereas the niche of native diploids and tetraploids is largely overlapping (Treier *et al.* 2009). In addition, North American tetraploid genotypes have reduced expression of genes related to constitutive defence, consistent with reduced defence costs in the absence of herbivores resulting in rapid adaptive change (Broz *et al.* 2009). Such a reduction of defence costs may have contributed to the observed increased vegetative growth and competitiveness of invasive

tetraploid genotypes relative to native genotypes in this species (Ridenour *et al.* 2008; He *et al.* 2009). The aforementioned findings indicate that *C. stoebe* may have undergone rapid evolutionary change in North America.

In this study, we sought to compare *C. stoebe* leaf traits, growth, life cycle and reproductive capacity of: (i) diploids with tetraploids from the native range in Europe and (ii) tetraploids from the native range (Europe) with tetraploids from the introduced range (North America). The first comparison is aimed at assessing what traits, possibly associated with previously identified life cycle differences between diploid and teraploid cytotypes, may potentially explain the dominance of the tetraploid cytotype in North America and the spatial separation of cytotypes in Europe. The second comparison is aimed at determining whether rapid adaptive changes, e.g. in reproduction, leaf traits or growth, have occurred in tetraploids from the introduced range relative to tetraploids from the native range. We chose to address this using a series of common garden experiments. In the absence of detailed population genetics information, our approach used a large number of populations from each cytotype and geographic region to average effects across multiple genotypes and to avoid confounding population level differences.

Materials and methods

STUDY SPECIES

Centaurea stoebe L. (syn. *Centaurea maculosa* Lam., Asteraceae) is native to Europe and western Asia. In North America, to where it was introduced 120 years ago (Maddox 1979; Roché, Piper & Talbott 1986), it has become highly invasive. Although currently considered a single species (Ochsmann 2000; Španiel *et al.* 2008), it consists of diploid and tetraploid forms with only the latter cytotype unambiguously identified as *C. stoebe sensu stricto* thus far in the introduced range (Treier *et al.* 2009). All seeds used in this study were collected in summer and autumn 2005 from over 100 populations as part of a distribution-wide field survey (for collection methods and locations, see Treier *et al.* 2009). When these seeds were germinated for experiments, any populations with individuals that exhibited morphological characters that we associated with other closely related taxa or hybrids were excluded from subsequent analyses. Using seed sourced from multiple open-pollinated families in each population and from multiple populations throughout the range of the species was intended to ensure robust comparison of the species traits at different ploidy levels and between different continents. The three geographic and cytotype combinations European diploids, European tetraploids and North American tetraploids are referred to hereafter as geo-cytotypes. As outlined below, several cohorts of plants were grown at different times over 3 years in separate experiments (see descriptions of Experiment 1, 2 and 3 below). The first cohort of plants was used to assess differences between the two geo-cytotype comparisons of interest by destructively measuring above-ground biomass in developing rosettes and mature plants and testing our ability to predict biomass using variables measured non-destructively. The second cohort was used to assess the differences in leaf traits between geo-cytotypes, and the third cohort used to compare life cycle and fecundity. Although the exact populations used in each cohort differed slightly, all plant material originated from the seed collections described above and thus any comparisons across populations identified are based on genotypes that are reasonably consistent between experiments. In addition, all plants were grown in similar conditions at the same location, in a single glasshouse and in potting media made at the site. In all experiments, the locations of plants or populations (if seedlings) were randomized to avoid confounding position effects.

EXPERIMENT 1: ALLOMETRIC GROWTH IN YOUNG ROSETTES AND FINAL PLANT BIOMASS

For each geo-cytotype, 15 populations were selected (i.e. a subset of 45 of the original 108 populations), from which 10 seeds, each of 10 different open-pollinated families (i.e. 10 mother plants) were selected for sowing. Single seeds were planted into individual 2 × 2 cm cells in 10 × 15 cell seedling trays filled with sterilized and sieved compost on 9 and 13 May 2008. The seedling trays (each tray containing the seeds for one population and half the seeds for another) were then watered and the seeds left to germinate in the glasshouse located at the University of Fribourg, Switzerland (46°47′34.5″ N/7°9′20.6″ E, 18–30 °C, natural photoperiod and humidity) for 4 weeks, after which *c.* 15 plants per population (where available) were re-potted into 11 cm diameter × 13 cm deep, 1 L plastic pots in a 1 : 2 mix of coarse sand and sterilized compost. The remaining seedlings that were not transplanted were left in the germination trays for approximately two further weeks. After this period, a subsample of 76 seedlings representing all three geo-cytotypes was removed from the trays, the number of leaves and the length of the longest leaf recorded, the soil carefully washed from the roots, the roots and shoot separated, dried for minimum 48 h at 50 °C and then weighed. This was performed to determine whether the number of leaves, the length of the longest leaf, and the SLA could be used as effective non-destructive predictive variables for plant biomass.

Two months after sowing, between 5 and 8 of the plants potted into 1 L pots from each population were selected from the 15 available populations for each geo-cytotype. The number of leaves, the length of the longest leaf, the number of accessory rosettes and the number of accessory rosette leaves (generally much smaller than main rosette leaves) were recorded for each plant. The pots remained in the glasshouse and were watered as required until 3 November 2008 at which point they were moved outside and buried to ¾ of the pot depth in sand to overwinter. On 20 February 2009, the plants were returned to an unheated glasshouse under 16:8 light-dark cycle. After initially producing some new rosette leaves, all plants started bolting but were allowed to grow until the plants were dissected to measure biomass between the 10 and 26 March 2009. Prior to dissection, all of the above plant parameters were again measured with an additional parameter, the height of the highest flowering shoot, included. Due to the particular cold winter, plant mortality resulted in slightly uneven numbers in each geo-cytotype group but in total 170 surviving plants were measured. We also took some more detailed measurements on a subset of plants (n = 28), where the flowering stem had only just started to form. In a similar manner to that described above for young rosettes, using this subgroup we investigated how closely the parameter length of the longest leaf was correlated with median or mean leaf length in a rosette and whether these variables, combined with the number of leaves and mean SLA for each plant, were able to significantly account for any variation in above-ground biomass of mature second-year rosettes. *Centaurea stoebe* is tap-rooted but in the experiment plants commonly formed many fibrous roots with the

notable absence of a large central tap root. During plant dissection, soil was removed from the roots to collect the tap root to measure root biomass, but because of the aforementioned root architecture only a few larger roots could be separated from soil sufficiently for measurement. After soil removal, the roots were separated from the above-ground biomass, both plant parts dried for a minimum of 48 h at 50 °C, and then weighed.

EXPERIMENT 2: LEAF TRAITS AND EARLY ROSETTE DEVELOPMENT

A second set of seeds was sown at the beginning of October 2007. This set comprised of seeds from 86 of the original 100 populations, and the number of seeds sown per population was variable as a result of the exhaustion of seed stocks. Usually five seeds, each from 16 maternal plants, per population were sown directly into 1 L pots filled with the soil mix as described above. After germination, only one seedling was left in each pot for further cultivation and extra seedlings were removed. The seedlings were grown in the glasshouse supplemented with artificial light for a 16 h day period, with temperature control conditions of 23 °C day and 15 °C night, and watered when needed (i.e. approximately every 3 days). Leaf measurements were taken 2 months after sowing as leaves emerging after this become much more extensively dissected and thus difficult to accurately scan for area measurements. The harvest lasted 1 week in late December 2007. Numbers of plants sampled for each population varied between 1 and 21 individuals as a result of the limited availability of seed and variable germination but the total sample size was large, comprising 957 scanned leaf images. To standardize measurements between plants, the 6th true leaf (not counting cotyledons) was cut from an individual rosette. In some rare cases, where the 6th true leaf was damaged by fungi or insects, the 5th, 7th or 8th true leaf was collected. Immediately after being cut from the plant, a binary image of each leaf was captured using a flat bed scanner. The leaves were then dried for minimum 48 h at 50 °C and the leaf dry weight recorded. Leaf scans were analysed with the software Image J (Rasband 1997–2009). For each leaf image leaf length, the maximum leaf width, leaf perimeter and leaf area were measured. SLA was calculated by dividing the scanned leaf area by the leaf dry weight (Garnier *et al.* 2001). A subset of these leaves ($n = 200$, representing 23 native diploid populations, 17 European tetraploid and 26 North American tetraploid populations with plants per population ranging between 1 and 6) were also analysed for leaf nitrogen content using Mass Spectrometry in the Isolab at the Institut für Pflanzenwissenschaften ETH, Zürich, Switzerland. We removed a second set of leaves from the plants to calculate leaf dry matter content (LDMC) as the ratio of leaf dry weight to fresh weight (Garnier *et al.* 2001). This set of leaves were also scanned for corresponding area measurements and we calculated leaf thickness as $(\text{SLA} \times \text{LDMC})^{-1}$ (Vile *et al.* 2005).

EXPERIMENT 3: REPRODUCTIVE CAPACITY AND LIFE CYCLE

In May 2006, five seeds each from 10 maternal plants from each of 100 populations were germinated in trays as described previously (Experiment 1). After 8 weeks, one plantlet per mother plant (1000 plants in total) was transferred to a 1 L pot (15 cm diameter, 13 cm height) with a soil mix as given in Experiment 1. The plants were grown in a naturally lit glasshouse supplemented with artificial light for 16 h daytime. The glasshouse was unheated but temperatures stayed above 0 °C in winter 2006/07. Plants were watered regularly, but were not given nutrient solution. An additional 60 plants (five plants from four populations of each geo-cytotype) were grown to be used for biomass measures. Only four plants flowered out of the 550 surviving into summer 2006. The length of the longest leaf and the number of leaves were assessed three times (10–14 July 2006, 2 months after sowing; 7–11 August 2006, 3 months; 27 April–3 May 2007, 12 months) before the plants started bolting. At this stage, all plants were relocated outside on a bed of coarse sand in the Botanical Garden of Fribourg, and the positions of the pots randomized. When the first flower opened (6 July–23 August 2007, *c.* 15 months), each plant was rescored for the number of flowering stems, the height of the highest flowering shoot and the number of inflorescence buds larger than 5 mm. The latter was used as an estimate of the total number of capitula (flower heads) per plant. Seed production per capitulum was determined by averaging the number of seeds for the first three capitula to mature per plant and the total number of capitula that bore seed was counted once the stem had dried out at the beginning of October 2007. At this time, plant survival and number of accessory rosettes (newly formed) were also assessed. During flowering, which was roughly synchronous in all individuals that flowered, pollinating insects were able to access all the plants. This meant that pollen from diploids and tetraploids were probably exchanged, with the likely associated compatibility problems and potential interference in fertilization. Sufficient pollen movement occurred, however, such that successful pollination was achieved. The plants remained outside for an additional year with supplemental watering and survival, number of flowering stems, number of accessory rosettes and total number of capitula assessed again in October 2008.

During the week of the 7–11 August 2006, we sampled 60 plants for biomass. These plants were removed from their pots and the soil was washed away from the roots. The roots were cut from the rosette and both parts dried for 48 h at 60 °C before being weighed. Immediately prior to harvest, the aforementioned measurements of plant size were taken.

STATISTICAL ANALYSIS

We used a multiple regression to determine if the variables number of leaves, length of the longest leaf, population mean SLA and the fixed factor geo-cytotype could be used to predict above-ground biomass at both the young rosette stage (6–7 weeks old) (Experiment 1), 3-month-old rosettes (Experiment 3) and in mature plants entering their second growing season (Experiment 1). We used step-wise removal with comparison of the variance ratio for each variable with a test criterion of one to determine significance of variables to the model.

For our data set on leaf traits, the line best describing the allometric scaling relationships between pairs of log-transformed leaf variables for each geo-cytotypes were determined using the standardized major axis (SMA) estimation and testing routines (SMATR ver 2.1) (Warton *et al.* 2006) for fitting bivariate lines in R (R Core Development Team 2005). If the separate SMA lines for each geo-cytotype were found to not differ significantly in slope then a common SMA slope was estimated and the cloud of points describing the pairwise trait values for each geo-cytotype were tested for shifts in elevation (i.e. a shift in one dimension relative to the other clouds of points).

Differences in plant growth and reproductive parameters amongst the three geo-cytotypes were analysed using a generalized linear mixed model (GLMM) with geo-cytotype as a main effect and 'plant' nested within 'population' nested within geo-cytotype as a random effect. Dependent variables in this analysis included number of leaves,

above- and below-ground biomass, number of capitula per plant, seed production per capitulum and total seed output. Length of the longest leaf, SLA, LDMC and leaf thickness were analysed using linear mixed models (LMM) with restricted maximum likelihood (REML) estimation using the same model structure as before but assuming a normal distribution. Differences between tetraploids from invasive and native ranges were examined by excluding diploid plants from the analyses. Differences in probability of flowering and mortality for each geo-cytotype were investigated using a LMM with a binomial distribution and a logit link with geo-cytotype as a main factor. In all LMM, dependent variables were transformed as to improve normality where required.

Results

GROWTH AND DEVELOPMENT OF YOUNG ROSETTES

Tetraploid *C. stoebe* from North American accumulated significantly greater biomass during early growth than both diploid and tetraploid conspecifics from the native range of the species (Experiment 1, Table 1, 1.5 months, higher intercept than both European diploids and tetraploids). The LMM explained 68.7% of variation in above-ground biomass in young rosettes with length of the longest leaf and number of leaves accounting for the majority, and differences in SLA between populations accounting for a minor component. The interactions between all significant linear variables and the fixed factor geo-cytotype were non-significant indicating equal slopes for the linear relationships of the three geo-cytopes. Subsequent analyses using LMM with geo-cytotype as a fixed effect revealed that the significantly greater biomass of North American tetraploids is not due to greater number of leaves (Poisson distribution, deviance ratio = 1.08, d.f. = 2, $P = 0.341$) or different length of the longest leaf (normal distribution, $F = 0.06$, d.f. = 2, $P = 0.940$) in any geo-cytotype.

Similarly, in 3-month-old first-year plants (Experiment 3, Table 1), the majority of variation in above-ground biomass was accounted for by a combination of number of leaves and length of the longest leaf (89.0%). There was, however, no detectable difference amongst geo-cytotypes in rosette biomass at this stage (Table 1, 3 months). The variables number of accessory rosettes and number of accessory rosette leaves were not significant explanatory variables. We did not have mean population SLA data for several of the populations used in these particular biomass measurements and thus could not assess its effect on biomass at this plant stage. As for 1.5-month-old young rosettes, there was no effect of geo-cytotype on number of leaves (GLMM, Poisson distribution, $F_{2,35} = 0.05$, $P = 0.954$) or length of the longest leaf (using the latter as a correlate of mean leaf length) (LMM, $F_{2,38} = 0.26$, $P = 0.775$).

As the plants from Experiment 3 matured further, we found significantly more leaves on diploid plants than both North American and European tetraploids (Fig. 1a), whilst initial differences in leaf length levelled out between geo-cytotypes (Fig. 1b). Similarly, in Experiment 1, 2.5-month-old diploid plants had significantly more leaves than tetraploids (Fig. 1a). Although North American tetraploids at this age tended to have more leaves than European tetraploids in this cohort, this does not indicate greater total leaf area deployed because length of the longest leaf of North American tetraploid plants was found to be significantly less than both other cytotypes (Fig. 1b). Leaf length was considerably greater in these plants (Experiment 1), relative to plants of equivalent age from Experiment 3, due to the fact that Experiment 3 plants were germinated in January in the glasshouse and thus initially grew much more slowly than plants from Experiment 1 which germinated in May.

LEAF TRAITS

We found that leaves from developing rosettes of the three geo-cytotypes differ in structure. SLA of diploid plants was significantly higher than that of North American and European tetraploids which did not differ significantly (Fig. 2a). This difference appears to be driven by an increasing trend in LDMC from diploids to invasive tetraploids (Fig. 2b). The calculated surrogate for leaf thickness $(\text{SLA} \times \text{LDMC})^{-1}$ also showed

Table 1. Generalized linear model of the effect of geo-cytotype on shoot dry weight of *Centaurea stoebe* young rosettes whilst accounting for variation in plant size with three standardized variables. Different superscript letters on estimated intercepts for different geo-cytotypes indicate groups that significantly differ based on least significant differences (5% level). Geo-cytotypes are European diploids (2×EU), European tetraploids (4×EU) and North American tetraploids (4×NA)

Plant age (months)	*n*	R^2	Independent variable	Estimated parameters	*t*	*P*
1.5	67	0.69	Length of longest leaf	0.050	7.16	< 0.001
			Number of leaves	0.034	4.87	< 0.001
			Population mean SLA	0.017	1.96	0.054
			2×EU	0.133[a]	11.71	< 0.001
			4×EU	0.153[a]	14.30	< 0.001
			4×NA	0.192[b]	14.39	< 0.001
3	41	0.66	Length of longest leaf	0.259	5.49	< 0.001
			Number of leaves	0.247	5.72	< 0.001
			2×EU	1.647	30.48	< 0.001
			4×EU	1.651	39.13	< 0.001
			4×NA	1.687	38.68	< 0.001

SLA, specific leaf area.

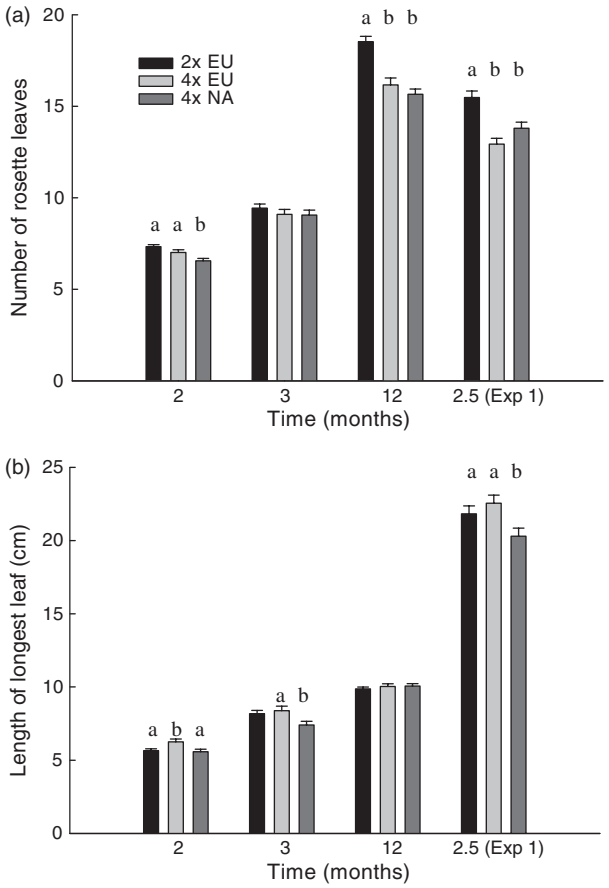

Fig. 1. Means and standard errors for: (a) total number of leaves and (b) length of the longest leaf for *Centaurea stoebe* plants from three different geo-cytotypes European diploids (2×EU), European tetraploids (4×EU), and North American tetraploids (4×NA). Measurements were made twice in the first year of growth in 2006 (after 2 and 3 months) and then again the following spring in 2007 (12 months) for Experiment 3 (see Materials and Methods). Numbers of individuals for the three measurements at different ages were: 2×EU = 344, 248, 219; 4×EU = 191, 138, 126; 4×NA = 204, 157, 140. The fourth group of bars on each chart (a & b) is the cohort of plants grown for Experiment 1 (n = 105, 104, 104 for 2×EU, 4×EU and 4×NA, respectively). Different letters above the columns indicate geo-cytotypes that differ significantly ($P < 0.05$).

European tetraploids to have significantly thicker leaves than diploids, with North American tetraploids showing intermediate values (Fig. 2c). We additionally found a small but significant positive relationship between SLA and leaf N content that accounted for 7.2% of the variation (F = 6.09, d.f. = 3, 195, $P < 0.001$). However, leaf N content did not differ between ploidy levels (F = 0.6262, d.f. = 198, P = 0.4297) or between European and North American tetraploids (F = 1.689, d.f. = 124, P = 0.1961), neither when including or excluding SLA as a covariate in a linear model.

European tetraploids had a greater increase in width for a given increase in leaf length (steeper SMA regression slope) than the other two geo-cytotypes (Fig. 3a, Table 2), which did not differ from each other. Variance in leaf width was significantly greater in European tetraploids than in the other two groups (data not shown) and the greater spread of data in this dimension may explain the steeper slope of the relationship with leaf length (Fig. 3a, Table 2). In addition, maximum leaf length of plants of equivalent developmental age was slightly but significantly longer in European tetraploid plants than the other two geo-cytotypes which again did not differ (Fig. 1b, 2 months). In contrast, for a given leaf length, leaf width was greater in leaves from European diploids than the other geo-cytotypes (Fig. 3a).

The lamina was significantly more dissected in diploids than both tetraploid geo-cytotypes (in some tetraploid genotypes, the lamina is lobed rather than finely dissected) as shown by the elevation of allometric SMA relationship between leaf perimeter and leaf area (Fig. 3b, Table 2). The regression was, however, only marginally significant indicating high variability in the extent of leaf dissection in diploids (Table 2). Lamina dissection appears further reduced in the invasive range with consequently significantly greater leaf area for given leaf length in North American tetraploids than European tetraploids, and even greater than diploids (Fig. 3b, Table 2). This pattern is consistent with the results of the analyses on the relationship between leaf length and leaf area or dry weight (Fig. 3c,d, Table 2). Invasive tetraploids with least dissected leaves had the greatest leaf area and highest leaf dry weight at a given leaf length, whilst native diploids with the most dissected leaves produced leaves with the lowest area and biomass at a given length.

MATURE PLANT BIOMASS

All surviving plants from Experiment 1 that were returned to the glasshouse after overwintering outside immediately produced new main rosette leaves, developed multiple accessory rosettes and initiated bolting within a few weeks. We wished to examine the usefulness of various plant parameters in estimating biomass of second-year rosettes in a regression model. Unfortunately, the rapid onset of bolting after revival of the plants from winter dormancy limited the number of plants we could use in this analysis. Multiple regression analysis on a subset of these plants showed that using the most representative leaf size (i.e. median or mean leaf length) accounted for more variation in above-ground biomass than using length of the longest leaf (Table 3). There was, however, moderately close correlation between length of the longest leaf and either mean or median leaf lengths (r = 0.65, $P < 0.0001$ and r = 0.69, P = 0.0002, respectively) justifying the use of length of the longest leaf as a surrogate, when maximizing efficiency of measurement for large sample sizes. In addition, we determined that number of accessory rosettes and total leaves (sum of accessory rosette leaves and main rosette leaves) were significant terms in the model but SLA was not a significant explanatory variable (Table 3). In first-year rosettes, main rosette leaves comprise most of the above-ground biomass of the plant and the leaves of accessory rosettes are small and contribute relatively little to above-ground biomass (result not shown). After resprouting in the second year, leaves of accessory rosettes were larger and more numerous. Consequently, number of accessory rosettes accounted for a significant proportion

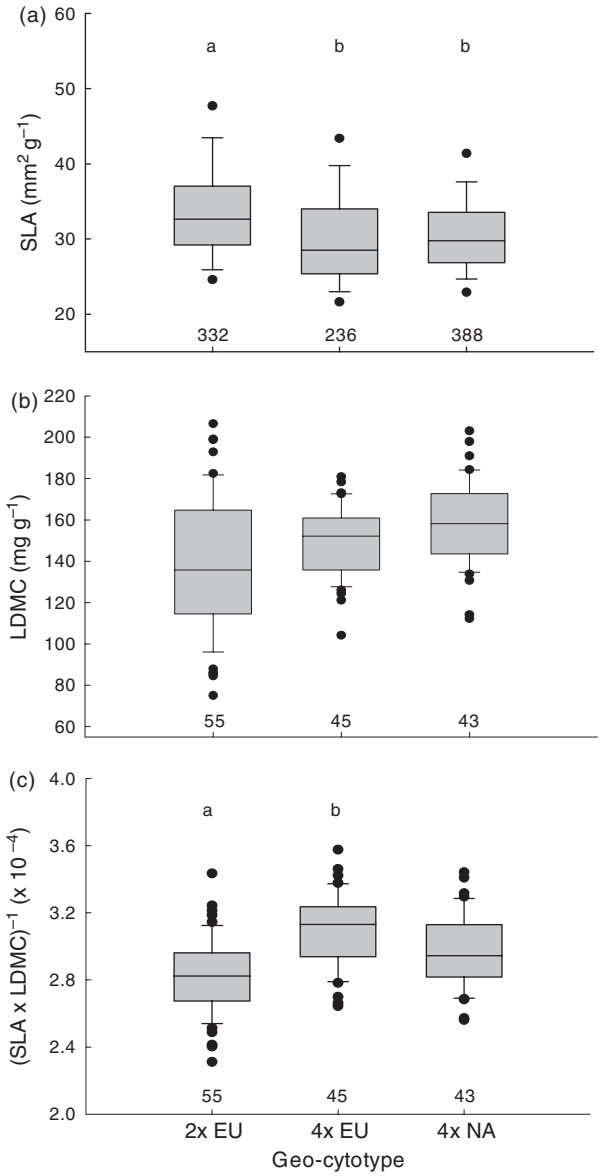

Fig. 2. Boxplots of: (a) specific leaf area (SLA), (b) leaf dry matter content (LDMC) and (c) leaf thickness (as calculated by the inverse of SLA × LDMC) for three geo-cytotypes European diploids (2×EU), European tetraploids (4×EU), and North American tetraploids (4×NA). Numbers below each boxplot indicate sample sizes. Different letters above the boxplots indicate geo-cytotypes that differ significantly ($P < 0.05$) as determined by linear mixed models using restricted maximum likelihood estimation. The upper and lower boundary of the box indicates the 75th and 25th percentile, the central line indicates the median, and whiskers above and below indicate the 90th and 10th percentiles, respectively. Outliers are shown as dots except in (a) where, for clarity, they indicate 95th and 5th percentile values due to large samples sizes and numerous outliers.

of above-ground biomass. The maximum proportion of variance accounted for by the complete model was relatively small (33.3%) compared to that in young first-year rosettes (68.7%), although small sample size is likely to have contributed to this (Table 3).

Following the previous analyses, and due to the presence of flowering shoots, we used the additional variables height of the highest flowering shoot and number of accessory rosettes in a LMM to account for variation in dry shoot weight of adult second-year plants (from Experiment 1) (Table 4). With these additional covariates, this model accounted for a similar proportion of variation in shoot weight (70.0%) as that explained for biomass of young *C. stoebe* rosettes, but there was no longer a detectable difference in shoot weight between geo-cytotypes (Table 4). Root dry weight was not found to differ between geo-cytotypes (Wald statistic = 0.93, d.f. = 2, 199, $P = 0.628$).

LIFE CYCLE AND REPRODUCTIVE CAPACITY

There were great differences in life cycle associated with ploidy level. Diploid *C. stoebe* that survived to reproductive age flowered either during the second growing season (2007, 52%, Fig. 4a), or in the third growing season (2008, 48% Fig. 4b). The majority (83%) of the diploid plants that flowered in 2007 died after flowering (Fig. 4c) with 99.5% of the surviving diploid plants flowering in the subsequent year (Fig. 4d), indicating a monocarpic life cycle in diploid *C. stoebe* plants. Consequently, in diploids there were very few individuals that were common to flowering data collected in both years and results for each season can thus be considered largely independent.

In contrast, most of the tetraploid plants flowered in the second growing season, with a significantly reduced proportional mortality in North American tetraploids than European tetraploids after flowering (11% vs. 26%, respectively, Fig. 4c). The proportion of surviving plants that flowered in the third year was again high (98% and 92%, Fig. 4d) with only 11% of the plants flowering for the first time in the third year (Fig. 4b).

In the second growing season (2007), we found no significant effect of ploidy level or geographic origin of tetraploid plants on the number of capitula or number of flowering stems produced per flowering plant (Fig. 5a,b, respectively). Also, geo-cytotypes did not differ in the proportion of capitula that were pollinated and that developed achenes (Fig. 5c). Diploids did, however, produce significantly more seeds per capitulum than tetraploid plants (Fig. 5d) resulting in a significantly higher number of seeds per plant with diploids producing, on average, 1.7 times as many seeds as European tetraploids (Fig. 5e). Although the total seed output per plant was not significantly different between European and North American tetraploids (Fig. 5e), plants from the introduced range had significantly greater seed output per capitulum than native tetraploids (Fig. 5d).

In the third growing season (2008), diploid plants produced significantly more capitula per flowering plant than both native European and North American tetraploids (Fig. 5a). This difference arose despite the production of fewer flowering stems (generally only one) per plant on diploids (Fig. 5b). Greater number of flowering stems in tetraploids was due to the production of flowering stems by multiple secondary rosettes that developed the previous year, whilst the main rosette senesced. Diploids rarely produced accessory rosettes,

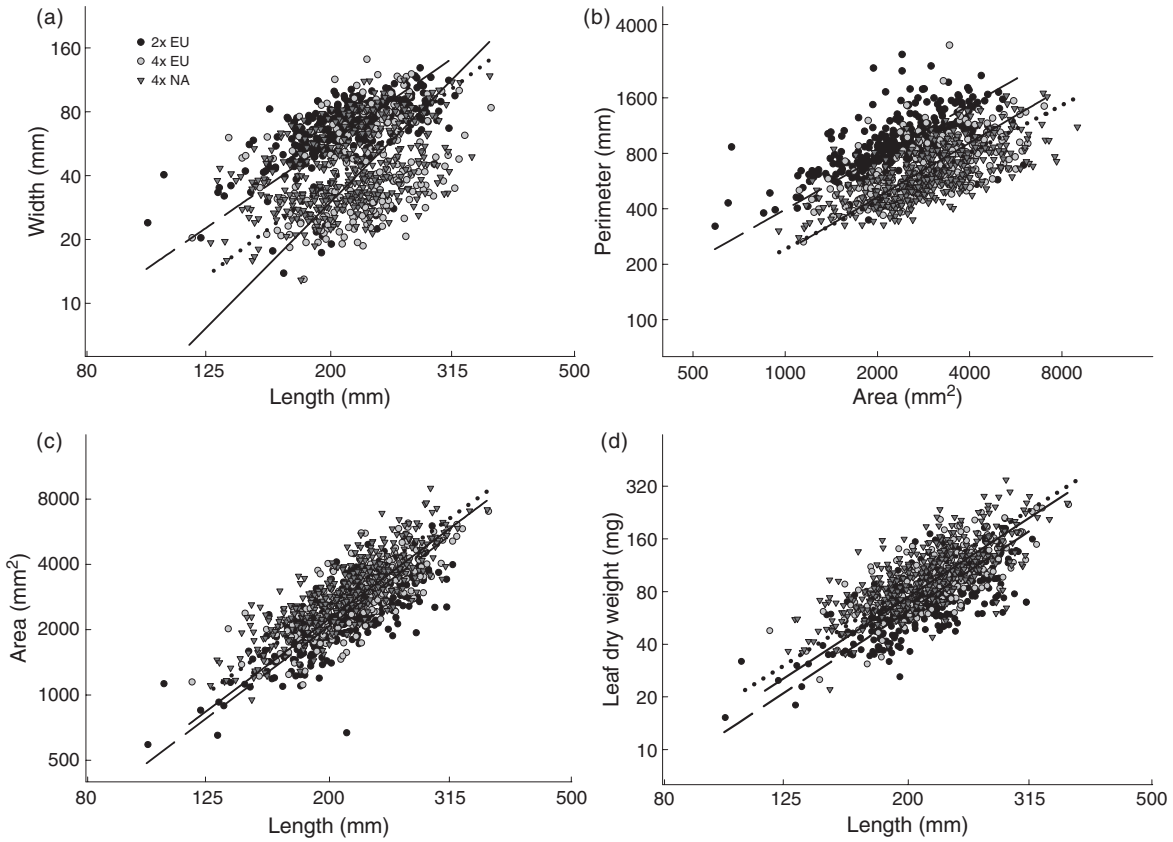

Fig. 3. Standardized major axis regression (SMA) relationships (log–log scaled) between: (a) leaf length and leaf width, (b) leaf area and leaf perimeter, (c) leaf length and leaf area and (d) leaf length and leaf dry weight measured on leaves of similar developmental age from 2-month-old *Centaurea stoebe* plants. Separate regressions are for European diploids (2×EU, circles and dashed line) European tetraploids (4×EU, grey circles and solid line) and North American tetraploids (4×NA, triangles and dotted line).

whilst invasive tetraploids tended to produce a greater number of accessory rosettes than native tetraploids (Fig. 5f).

Discussion

Previous investigations of *C. stoebe* (syn *C. maculosa*) from the native range have indicated significant life cycle differences associated with ploidy level (Broz *et al.* 2009; Treier *et al.* 2009). Directly comparing traits of native and invasive genotypes without accounting for cytotype differences could therefore lead to spurious conclusions about adaptive changes that arose after introduction to North America. In this study, we have compiled a substantial data set detailing early vegetative growth and mature reproductive stages in all geographical and cytotype combinations. The trait data for each geo-cytotype was averaged across a large number of populations in a common garden environment to minimize the effect of individual populations on the results. We found that, for several of the traits we examined in early growth, as well as in life cycle and reproduction, there were significant differences between diploids and tetraploids. In addition, within the tetraploid cytotype there were more subtle differences between plants from the native and invasive range indicating shifts in traits that may have been selected for the new environment since introduction.

LIFE CYCLE AND LEAF TRAIT DIFFERENCES OF TETRAPLOIDS AND DIPLOIDS

We found that tetraploid *C. stoebe* tended to have higher LDMC, thicker leaves with lower SLA than diploids. This was accompanied by a reduction in dissection of the lamina although the extent of dissection was variable. We also found that diploids had significantly wider leaves than tetraploids, but the higher level of dissection of the lamina in this cytotype and consequent reduction in area in combination with a higher SLA, resulted in an overall lower dry weight per leaf for an equivalent leaf length. These differences in leaf construction did not, however, result in significantly different above-ground biomass between the two cytotypes.

The lack of environmental niche differentiation between European diploids and tetraploids (Treier *et al.* 2009) suggests that differences in leaf traits are not selected by the abiotic environment but are associated with a shorter, monocarpic life history in diploids, where maximizing leaf area and carbon assimilation into stored reserves for reproduction is most advantageous. Greater leaf dissection and lower carbon construction cost per leaf in diploids are possibly associated with a shorter monocarpic life span. With less dry mass invested per leaf, diploid plants can deploy more leaves per plant (Fig. 1a). As in other species (e.g. Feng, Fu & Zheng 2008), the higher

Table 2. Results of standardized major axis regression (SMA) analysis of pairwise combinations of leaf traits from three *Centaurea stoebe* geo-cytotypes, European diploids (2×EU), European tetraploids (4×EU) and North American tetraploids (4×NA). The separate SMA regression lines for each geo-cytotype were compared for differences in slope and if found to be not significantly different were examined for shifts in elevation. Slopes or elevations of regressions that were significantly different between geo-cytotypes are shown by different letters in the final column

Traits (*X* and *Y*)	Geo-cytotype	*n*	R^2	*P*	Slope	Intercept	Homogeneity of slope	Shift in elevation	Significant difference
Length and width	2×EU	332	0.59	< 0.001	1.99	−2.81	< 0.001	–	a
	4×EU	237	0.62	< 0.001	2.92	−5.23			b
	4×NA	388	0.69	< 0.001	2.21	−3.50			a
Area and perimeter	2×EU	332	0.41	0.097	0.940	−0.223	0.101	< 0.001	a
	4×EU	236	0.36	0.002	0.980	−0.569			b
	4×NA	388	0.50	0.053	0.865	−0.210			c
Length and dry weight	2×EU	332	0.51	< 0.001	2.31	−3.52	0.690	< 0.001	a
	4×EU	237	0.50	< 0.001	2.30	−3.40			b
	4×NA	388	0.56	< 0.001	2.21	−3.16			c
Length and area	2×EU	332	0.59	< 0.001	2.10	−1.52	0.587	< 0.001	a
	4×EU	237	0.62	< 0.001	2.10	−1.49			b
	4×NA	388	0.69	< 0.001	2.02	−1.23			c

Table 3. Results of linear models examining the usefulness of standardized variables to predict above-ground biomass in mature second-year *Centaurea stoebe* plants. The variables SLA and height did not significantly account for variation in above-ground dry weight and were excluded from the models

F	Total d.f.	*P* (model)	R^2	Independent variable	Estimated parameters	*t*	*P*
3.72	27	0.025	0.23	Constant	1.169	34.20	< 0.001
				Length of longest leaf	0.069	1.96	0.061
				Number of leaves	0.151	2.95	0.007
				Number of accessory rosettes	−0.123	−2.41	0.024
5.49	27	0.005	0.33	Constant	0.704	4.22	< 0.001
				Mean leaf length	0.004	2.84	0.009
				Number of leaves	0.164	3.41	0.002
				Number of accessory rosettes	−0.121	−2.56	0.017
5.41	27	0.005	0.33	Constant	1.169	36.59	< 0.001
				Median leaf length	0.094	2.81	0.010
				Number of leaves	0.166	3.42	0.002
				Number of accessory rosettes	−0.126	−2.65	0.014

SLA, specific leaf area.

Table 4. Results from generalized linear mixed model analysis examining the effect of geo-cytotype on shoot dry weight of C*entaurea stoebe* mature second-year plants whilst accounting for variation in plant size using standardized variables as covariates. Significance of geo-cytotypes European tetraploids (4×EU) and North American tetraploids (4×NA) are in comparison to European diploids as the reference level

F	Total d.f.	*P* (model)	R^2	Independent variable	Estimated parameter	*t*	*P*
62.82	159	< 0.001	0.70	Constant	1.438	46.52	< 0.001
				Length of longest leaf	0.030	1.73	0.086
				Number of leaves	0.142	6.34	< 0.001
				Height of flowering stem	0.250	14.78	< 0.001
				Number of accessory rosettes	−0.052	−2.44	0.016
				4×EU	−0.040	−0.97	0.334
				4×NA	0.017	0.41	0.684

SLA of diploid *C. stoebe* is likely aligned with higher rates of photosynthesis, gas exchange and net assimilation relative to tetraploids.

The functional role of leaf dissection, however, is less clear. Highly dissected leaves, as seen in diploid *C. stoebe* in this study, track ambient air temperatures more closely than more

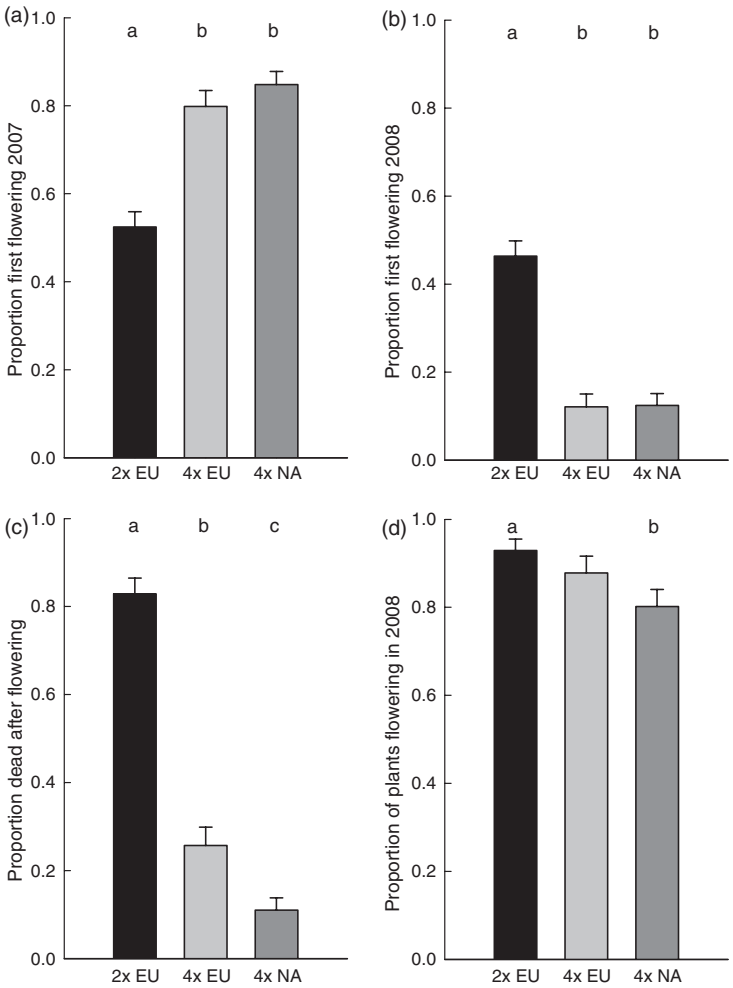

Fig. 4. Estimated mean proportions (with standard errors) of: (a) plants that first flowered in the second year, (b) plants that first flowered the third year (c) plants that died following flowering in the second year, and (d) plants that survived into the third year and then flowered, from three different *Centaurea stoebe* geo-cytotypes [European diploids (2×EU), European tetraploids (4×EU), and North American tetraploids (4×NA)]. Proportions are estimations from linear mixed models for a binomial distribution with a logit link and with geo-cytotype as a fixed factor. Different letters above the columns indicate geo-cytotypes that differ significantly ($P < 0.05$).

entire leaves (see Schuepp 1993 and references therein). This may provide more dissected leaves with potential for greater rates of photosynthesis in higher temperatures when water is available. Such effects of leaf dissection on photosynthesis have been demonstrated in studies using phylogenetically independent contrasts within the genus *Pelargonium* (Nicotra *et al.* 2008) and in other intraspecific studies comparing ecotypes (Gurevitch 1988, 1992), morphotypes (Lynn & Waldren 2002), and cultivars (Pettigrew, Heitholt & Vaughn 1993). In contrast, in North American tetraploids a shift to less dissected leaves than observed in the equivalent European cytotype might indicate both a pre-adaptation and an evolutionary shift to a leaf form that is advantageous in dryer and more continental climatic conditions east of the Rocky Mountains. Such a cline in decreased dissection of leaves with increasing altitude and concomitant decrease in ambient temperature within a species has been reported previously (Gurevitch 1992). Intriguingly, in the invasive teraploid form of *C. stoebe* a lower SLA rather than a higher SLA may have been advantageous for colonizing new environments following its introduction to North America. A higher SLA has been demonstrated in invasive vs. native conspecifics (e.g. Zou, Rogers & Siemann 2007) and in phylogenetically controlled comparisons of invasive and non-invasive species (e.g. Grotkopp, Rejmánek & Rost 2002; Grotkopp & Rejmánek 2007), and is thus commonly thought to be associated with invasiveness. In contrast, our results and others (Caño *et al.* 2008; Leishman, Thomson & Cooke 2010) suggest that rapid carbon capture via high SLA may not be always advantageous in invasive plants and highlight the importance of ecological context for each individual species in determining whether particular plant traits will confer an advantage (Pyšek, Křivánek & Jarošík 2009b; Pyšek *et al.* 2009a).

The generally parallel SMA regression lines fitted for three out of the four pairwise leaf trait combinations (Fig. 3b–d) indicate that allometric relationships did not vary over leaf size between geo-cytotypes. It seems unlikely that these relationships would differ for plants of different developmental stages but as we collected leaves of approximately the same age and only on young plants of even age we cannot verify this. A change in leaf trait relationships with plant age could partly explain why using mean population SLA or leaf dry weight per unit length values from young plants in a multiple regression did not account for a significant proportion of variation in shoot biomass of second-year plants. It seems more probable, however, that the increasing complexity in plant structure (presence of accessory rosettes and flowering shoots) and inherent variability in accumulated biomass with increasing age reduces the importance of subtle differences

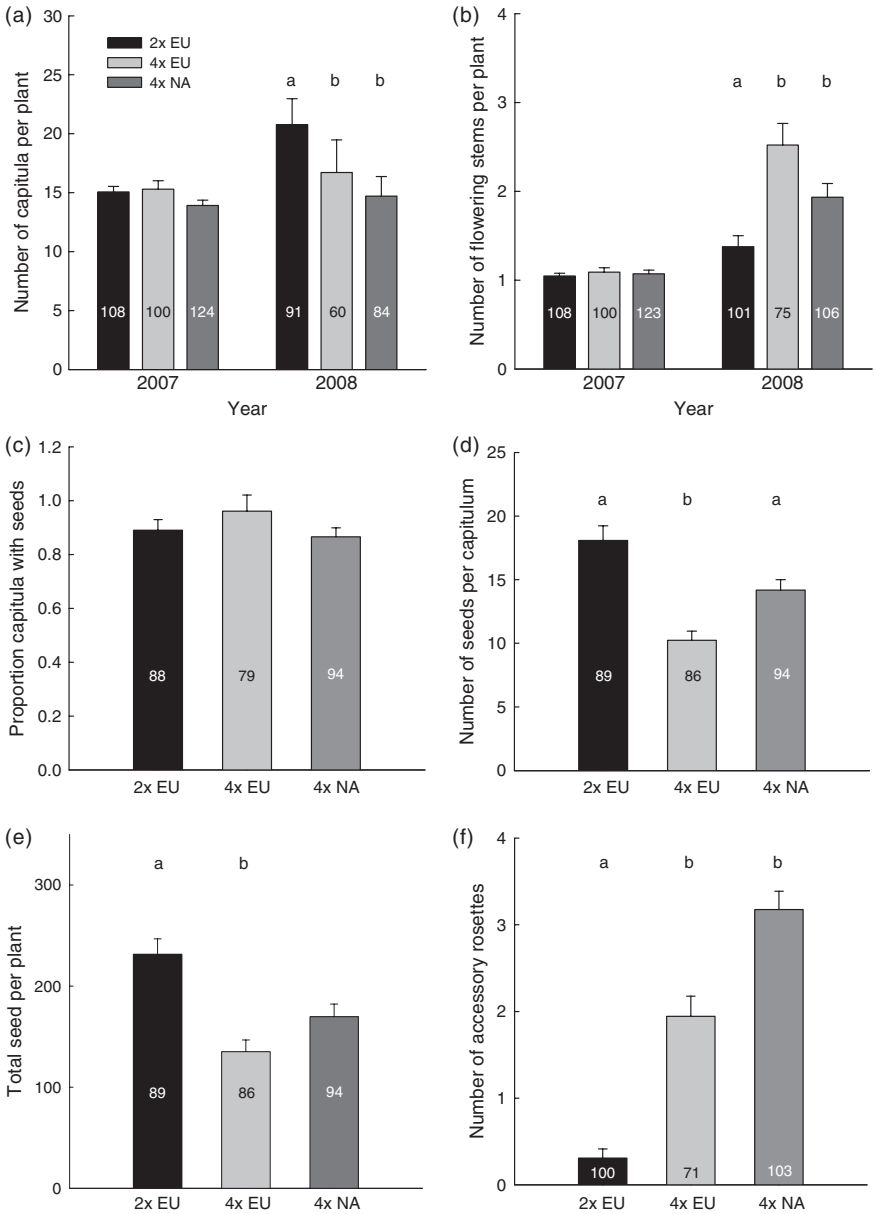

Fig. 5. Reproductive traits for three different *Centaurea stoebe* geo-cytotypes European diploids (2×EU), European tetraploids (4×EU), and North American tetraploids (4×NA) grown for three summers in a common garden experiment. Bar charts are means and standard errors for: (a) number of capitula per plant for two separate years, (b) number of flowering stems per plant for two separate years (c) proportion of capitula that were successfully pollinated and produced seeds in 2007, (d) number of seeds per capitulum in 2007 estimated from 3 to 5 dissected seed heads, (e) calculated total seed per plant in 2007, and (f) number of accessory rosettes per plant at flowering in 2008. Numbers on bars are sample sizes. Different letters above the columns indicate geo-cytotypes that differ significantly ($P < 0.05$).

in leaf construction in accounting for variation in plant weight.

In accordance with our expectations we found that, in the first year of flowering (second year of life), diploids produced significantly more seeds per flowering plant. This difference was primarily derived from a greater number of seeds produced per capitulum than tetraploids, whilst the proportion of capitula bearing seeds did not differ between the cytotypes. This finding concurs with previously published results demonstrating that diploid *C. stoebe* produce more flowers per capitulum than tetraploid conspecifics (Španiel *et al.* 2008). Roughly 50% of the diploid plants flowered in the first season with the remainder flowering the next summer. The plants flowering in the second flowering season produced larger inflorescences with significantly more capitula per plant (average of 15 capitula in 2007 vs. 20 in 2008) suggesting that diploids that had not flowered in 2007 were able to invest more in reproduction due to greater stored reserves. In tetraploids, a polycarpic life cycle dominated and investment in reproduction in the first season may potentially have reduced reproduction in the second year. Differences in propagule production between ploidy levels must be considered in light of this result. This consider-

ation aside, the average seed production in diploids would consequently be considerably greater than that estimated from the previous seasons' data alone. This would reduce differences between cytotypes in their projected population growth rates that were derived in a previous model based on number of capitula only (Broz *et al*. 2009). Whether this manifests in lifetime fecundity differences is currently a subject of further study.

EVIDENCE FOR POST-INTRODUCTION EVOLUTION IN TETRAPLOIDS

During early growth (rosettes < 3 months old), we found that North American tetraploids tend to accrue above-ground biomass faster than European tetraploids (and diploids). This extra biomass is not derived from having a greater number or longer leaves but results primarily from a combination of small differences in SLA, LDMC and area per unit leaf length that amount to greater total dry weight invested per leaf.

In North American tetraploids, greater accumulation of biomass in rapidly growing young plants may represent evidence of a trade-off between growth and defence because it compliments previous findings on lower defence-related gene expression in North American tetraploids (Broz *et al*. 2009). SLA is generally positively correlated with both leaf nitrogen and photosynthetic rate (both across (Niinemets 1999; Reich *et al*. 1999; Wright *et al*. 2004) and within (Li *et al*. 2008; Tanaka *et al*. 2008) plant species) yet North American tetraploid genotypes are able to accumulate biomass faster than diploids and European tetraploids, despite lower SLA than diploids and no significant increase in leaf N. These results suggest that genotypes in the invasive range have reallocated resources to growth via reduced expression of biochemical pathways involved in plant defence and the consequent reduction in associated energy costs, thus generally supporting the EICA hypothesis. As has been shown for other invasive species, however, evidence of reduction in defence investment does not necessarily carry over to significantly greater biomass in mature plants (Maron, Vilá & Arnason 2004b). Although it may not translate to greater final biomass, more rapid early growth may still confer a competitive advantage during the critical life stage following germination.

Our ability to detect small shifts in plant biomass and reproductive capacity between European and North American tetraploids may have been confounded by parallel changes to their respective life cycles. In a common garden, European tetraploids are less distinctly mono- or polycarpic with different populations exhibiting variation in life history and leaf traits, whilst North American tetraploids are exclusively polycarpic (Fig. 4). This matches previous field observations (Treier *et al*. 2009) and may indicate a selection towards greater persistence/longevity with concomitant increases in reproductive capacity in North America. Thus, although North American tetraploids may gain an initial growth advantage during establishment, more rapid growth and assimilation in some shorter-lived European tetraploid genotypes (and diploids) probably results in equivalent average above-ground biomass in mature second-year plants. The design of our experiment was not ideal to compare allocation to root systems between geo-cytotypes but it is possible that long term persistence in polycarpic tetraploid plants may well have selected for relatively greater allocation to an extensive root system rather than to leaves, particularly in North American genotypes where individual plants may survive for many years in drier habitats than those occupied by the species in Europe (Broennimann *et al*. 2007).

In summary, there is evidence of higher growth rate during the early vegetative stage in invasive genotypes vs. the equivalent cytotype from the native range. Whilst more rapid early growth is potentially important during establishment of seedlings, the change appears to be subtle and is not evident in older plants where increased variability with time and differences in life history between the two tetraploid groups may affect our ability to detect such a difference.

We also observed a small but significant increase in reproductive capacity in North American tetraploids. There was a trend towards higher total seed production in tetraploids from the invasive range compared with tetraploids from the native range that derived from a significant increase in the number of seeds per capitulum. This difference did not result in a significant difference in total seed production between these two groups because tetraploids in the native range tended to have more capitula per plant. As suggested above, changes in allocation to defence or traits that relate to persistence (e.g. deep woody roots, resprouting potential) may also be manifest in greater/lesser reproductive effort. Thus, differences in reproductive output may be confounded by differences in life cycle as shorter-lived genotypes (amongst European tetraploids) are likely to invest more in reproduction earlier in their life span. Therefore, it is possible that the trend towards greater seed production per plant by North American tetraploids would be greater over a longer time frame if, particularly in the absence of natural enemies, North American genotypes are better competitors (Ridenour *et al*. 2008), survive longer and continue to produce more seeds per capitulum. An increased ability to form accessory rosettes in North American tetraploids supports a hypothesized shift towards a greater ability to persist at a locality. It is clear from studies of other invasive species that shifts to greater vegetative propagation, growth and sexual reproduction are equally possible outcomes of evolutionary change in invasive genotypes (e.g. van Kleunen & Fischer 2008; Abhilasha & Joshi 2009; Hull-Sanders *et al*. 2009). Thus it is likely that measurement of just one group of traits related to one aspect of the life cycle may not detect the additive effects of potentially subtle changes to a plant's structure and function that could result in significant changes to the ecology of an invasive species.

In conclusion, evidence from this and other studies on *C. stoebe* (Broz *et al*. 2009; Treier *et al*. 2009) support the hypothesis that prominent life cycle differences and changes in leaf traits associated with an increase in ploidy may have given tetraploids both an advantage in colonizing potential via increased lifetime fecundity (due primarily to greater inherent longevity once established), and a leaf structure that may be

better adapted to the dryer and more continental habitats the species has colonized in North America. In addition, our findings suggest that tetraploids might have undergone further evolutionary changes which increased the rate of spread during colonization of North America. Given the relatively minor changes to individual traits between invasive and native forms, it will be a considerable challenge to determine the relative importance of pre-adaptation vs. rapid evolutionary change during the invasion process in this species and in others.

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Príloha O

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Rare recent natural hybridization in *Hieracium* s. str. – evidence from morphology, allozymes and chloroplast DNA

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Abstract. The first proven data on natural hybridization in the genus *Hieracium* s. str. are presented. Plants with intermediate morphological characters between the diploids *H. alpinum* and *H. transsilvanicum* were found in the Muntii Rodnei (Romanian Eastern Carpathians) in 2001 and in the Chornohora Mts (Ukrainian Eastern Carpathians) in 2003. While plants of intermediate morphology between usually so called basic species are usually tri- or tetraploid in *Hieracium* s. str., these plants were diploid (2n = 18) like both parental species in this region. The Romanian plant did not produce fertile achenes in free pollination and in control backcrosses with *H. transsilvanicum*, two hybrids from Ukraine were completly seed sterile in free pollination and reciprocal crosses. Pollen stainability as an indirect measure of male fertility was quite high in the studied Ukrainian hybrid plants and similar to the parental taxa. Evidence from allozyme analysis also confirmed the hybrid origin of the studied plants. Sequencing and PCR-RFLP analyses of the *trn*T-*trn*L intergenic spacer revealed that all hybrid plants had the *H. transsilvanicum* chloroplast DNA haplotype. Maternal inheritance of chloroplast DNA in this particular cross was proved with artificial hybrids from reciprocal experimental crosses between *H. alpinum* and *H. transsilvanicum*. In both localities, the natural hybrid plants were found in disturbed habitats, exceptionally allowing contact of the otherwise ecologically vicariate parental species. Morphologically, the hybrid plants belong to *H.* ×*krasani* Woł.

Key words: Asteraceae, chromosome numbers, hybrid zone, inheritance, allozymes, cpDNA, Carpathians

Introduction

The significance of interspecific hybridization in plant evolution has recently been stressed in many reviews (e.g. Arnold 1992, Rieseberg 1997, Rieseberg and Carney 1998). Consequently, documentation of natural hybridization is important for the description of evolutionary processes in particular taxa. Natural hybridization is unevenly distributed across taxonomic groups. Only ca 6–16% of the plant genera have one or more recorded hybrids (Ellstrand et al. 1996). Besides data on spontaneous hybridization, artificial crossing experiments have contributed much to a better understanding of microevolutionary processes like pre- and postzygotic isolation barriers, pollen competition, hybrid sterility, fertility

selection etc. (Rieseberg and Carney 1998, Lexer et al. 2003). Although morphological characters are used as a first step in the identification of hybrid plants in the field, they have limits, mainly in cases of introgression or in polyploid species complexes. Additional molecular approaches (allozymes, DNA analyses) have resulted in important insights in the role of hybridization and plant speciation in recent years (e.g. Bachmann 1994, Hedrén 1996, Morrell and Rieseberg 1998, Brochmann et al. 2000, Hedrén et al. 2000, Marhold et al. 2002, Nelson-Jones et al. 2002, Fehrer et al. 2005).

Hieracium L. s. str. belongs to the world's most species-rich plant genera (Royal Botanical Gardens Kew 1993). While most of the karyologically analyzed (micro)species are tri- or tetraploid with apomictic formation of seeds, diploid taxa are very rare and reproduce exclusively sexually, for some of them, strict allogamy has been proven (Rosenberg 1927; Bergman 1935, 1941; Battaglia 1947; Merxmüller 1975; Schuhwerk 1996; Chrtek 1997; Mráz 2003).

Hybridization probably plays a crucial role in the evolutionary processes in this genus, as revealed from the pattern of morphological characters. Many *Hieracium* species combine morphological characters of two or more basic species (''species principales'', ''Hauptarten'') and are generally suggested to be of hybrid origin (described as ''species intermediae'', ''Zwischenarten'', cf. Zahn 1921–1923). They are apomictic polyploids like most of their putative parental species (''species principales'', ''Hauptarten''), and gene flow among them is severely restricted. With respect to the current predominant apomictic mode of reproduction, recent hybridization might only be expected between diploid sexual species (Merxmüller 1975) and between pollen producing polyploids and sexual diploids. Nevertheless, there are no reliable data on recent natural hybridization in the genus *Hieracium* s. str. so far. Only a few cases of spontaneous hybridization between cultivated plants have been recorded. Merxmüller (1975: 193) briefly noted that a spontaneous hybrid arose from an interspecific cross between the diploids *H. porrifolium* L. and *H. umbellatum* L. cultivated in the Munich Botanical Garden. The supposed hybrid plants treated under the name ''*H. leiocephalum*'' Bartl. ex Griseb. had the same chromosome number ($2n = 18$) as its putative parental species according to labels of herbarium specimens deposited in Munich herbarium (M). However, there are no supplementary data that confirm true interspecific crosses (for this reason we do not use the special sign ''×'' to designate *H. leiocephalum* as a nothotaxon).

During field excursions in the Romanian (2001, P. Mráz) and the Ukrainian Eastern Carpathians (2003, P. Mráz and J. Chrtek), we found plants morphologically intermediate between *Hieracium alpinum* L. and *H. transsilvanicum* Heuff. Both species are diploid with sexual reproduction in this area (Pashuk 1987; Chrtek 1996, 1997; Mráz 2001, 2003; Mráz and Szeląg 2004). *H. alpinum* belongs to sect. *Alpina* (Griseb.) Gremli, and *H. transsilvanicum* to sect. *Vulgata* (Griseb.) Willk. & Lange (concept of sections follows Stace 1998), which are not morphologically closely related. Two taxa morphologically intermediate between *H. alpinum* and *H. transsilvanicum* have been described: *H.* ×*krasani* Woł. (according to Zahn either intermediate or closer to *H. transsilvanicum*) and *H. paxianum* Nyár. & Zahn (closer to *H. alpinum*). Both taxa are confined to the Eastern and Southern Carpathians (Zahn 1921–1923, 1930–1939; Nyárády 1965). They were, similarly to other ''intermediate species'' of *Hieracium*, originally supposed to be of hybridogenous origin (e.g. Zahn 1921–1923, 1930–1939), and have sometimes also been treated as hybrid taxa (nothospecies) (in the case of *H.* ×*krasani*; Wołoszczak 1890: 65, wrote the phrase ''inter parentes'', from which it may be suggested that Wołoszczak himself considered the newly described taxon to be a true hybrid, see Discussion). However, their hybrid origin has never been confirmed experimentally. More recently, it became a general notion that recent hybridization in *Hieracium* s. str. could be largely discounted because of

Table 1. Origin of plant material and analyses performed

Taxon	Cultvation no.	Locality	Allozyme analysis	PCR-RFLP	Sequencing (GenBank)	Pollen viability	Chromosome numbers
H. alpinum	JC H 866/ 1–10	Ukraine, Chornohora Mts, Polonina Breskulska ridge, the saddle between Mt. Hoverla and Mt. Breskul, 1800 m a.s.l., 48°09′09.8″ N, 24°30′14.6″, coll. P. Mráz & J. Chrtek jun., 23 July 2003	+	+		+	+
	PM Rodna2	Romania, Munţii Rodnei Mts, glacial cirque on the NE slopes of Mt. Pietrosul Mare, ca 0.3 km SE from Staţia Meteo, ca 1900 m a.s.l., coll. P. Mráz, 5 July 2001, 'alp.Boa.2' (sequence identical to 'alp.Ukr')			+		
	HERB JC (PRA)	Ukraine, Chornohora Mts, Mt. Pozhizhevska, SW slope, ca 1780 m a.s.l. , coll. J. Chrtek jun., J. Hadinec & J. Michálek, 22 July 1994 'alp.Ukr'			+ (AY512556)		+
H. transsilvanicum	JC H 864/ 1–12	Ukraine, Chornohora Mts, Polonina Breskulska ridge, at tourist path from the village of Hoverla to Mt. Hoverla, 1410 m a.s.l., 48°08′35.0″ N, 24°28′56.7″, coll. P. Mráz & J. Chrtek jun., 23 July 2003	+	+		+	+
	PM 1066	Romania, Munţii Rodnei Mts, border of the tourist path from the village of Borşa to Mt. Pietrosul Mare, spruce forest, 1300–1400 m a.s.l., 47°39' N, 24°39″ E, coll. P. Mráz, 5 July 2001,'tra.Boa'			+ (AY512557)		+

Table 1. Origin of plant material and analyses performed

Taxon	Cultvation no.	Locality	Allozyme analysis	PCR-RFLP	Sequencing (GenBank)	Pollen viability	Chromosome numbers
H. alpinum × *H. transsilvanicum* = *H.* ×*krasani* (natural hybrids)	JC H 863, PM 1399, PM 1400	Ukraine, Chornohora Mts, Polonina Breskulska ridge, at tourist path from the village of Hoverla to Mt. Hoverla, 1410 m a.s.l., 48°08'35.0" N, 24°28'56.7", coll. P. Mráz & J. Chrtek jun., 23 July 2003	+	+		+	+
	PM 985	Romania, Munţii Rodnei Mts, border of the tourist path from the village of Bor°a to Mt. Pietrosul Mare, spruce forest, 1350 m a.s.l., 47°39' N, 24°'39" E, coll. P. Mráz, 5 July 2001		+			+
H. alpinum × *H. transsilvanicum* (experimental hybrids)	PM X5/5, X5/6	mother plant: *H. alpinum* no. 649, pollen donor: *H. transsilvanicum* no. 1064		+			+
	PM X2/16	mother plant: *H. transsilvanicum* no. 1064, pollen donor *H. alpinum* no. 649 (for localities of both parents see Mráz 2003)		+			+

the common apomictic mode of reproduction in completely prevailing polyploid taxa (Merxmüller 1975).

The main aim of the present study was to test the hypothesis about the recent hybrid origin of the morphologically intermediate plants collected by us in the Eastern Carpathians. We performed a set of analyses including detailed morphological observations, chromosome counting, pollen fertility estimation, observation of seed production in free pollination and control hybridization, allozyme analyses, cpDNA sequencing and PCR-RFLP analyses.

Material and methods

Plants. The supposed hybrid plants from Romania and Ukraine, as well as plants of the putative parental species (*Hieracium alpinum* and *H. transsilvanicum*) from the same localities were cultivated in the experimental field of the Botanical Garden of the P. J. Šafárik University in Košice and in a glasshouse at the Institute of Botany, Academy of Sciences of the Czech Republic in Průhonice. Voucher specimens are deposited in the Herbarium P. Mráz in Košice (plants marked as PM) and at the Institute of Botany, Průhonice (PRA, plants marked as JC). Additionally, three diploid artificial hybrids from reciprocal control crosses between *H. alpinum* and *H. transsilvanicum* were included in our study to test the maternal inheritance of cpDNA (cf. Mráz 2003). Details about all plants studied are given in Table 1.

Chromosome numbers and breeding system. All cultivated plants subsequently used for molecular studies were checked for their chromosome number. Two different methods were used: (i) root tip cuttings were pretreated with 0.5% solution of colchicine for 1.5–3 hours at room temperature, subsequently fixative (ethanol and glacial acetic acid, 3:1) replaced colchicine, roots were stored in 70% ethanol and hydrolyzed for 5–7 minutes in 1N HCl at 60°C. The squash and smear method with cellophane replacing the glass covers followed Murín (1960). Giemsa solution in phosphate buffer was used for staining (method used by P. M.); (ii) actively growing roots were placed in a pretreatment solution of saturated p-dichlorbenzene for 3–4 hours, then fixed in a mixture of ethanol and acetic acid (3:1) and stored in 70% ethanol. The squash method and staining by lacto-propionic orceine were used (Dyer 1963; method used by J. C.). Altogether, chromosome numbers for 31 plants were determined (for details see Table 1).

In order to determine the breeding system of the putative hybrid plants, one capitulum of PM 985 was crossed with one of the putative parents, *Hieracium transsilvanicum* (PM 1064), which was flowering at the same time as the hybrid; and two capitula of supposed hybrids PM 1399 and PM 1400 were crossed reciprocally. The inflorescences used in control crosses were isolated by nylon bags until anthesis. Another capitulum of PM 985 and two other capitula of PM 1399 and 1400 were kept unisolated for free pollination.

Male fertility was estimated as pollen stainability using the acetocarmin method in glycerol jelly (Marks 1954). Three unopened tubular flowers per capitulum were removed and carefully cut with a razor blade in one drop of acetocarmine jelly in order to remove the pollen grains. 100–150 grains were evaluated per individual and both viable (well-stained) and non-viable (unstained) grains were scored. Two putative hybrid plants and three individual plants of both putative parental species from the Ukrainian locality were studied in this way. Because all capitula of the putative hybrid plant from Romania (PM 985) were included in the control crosses and free pollination (see above), this plant was not studied for pollen stainability.

Allozyme analyses. A total of 25 diploid plants from the Ukrainian locality were used for the analysis, i.e. 10 plants of *Hieracium alpinum* (JC H 866), 12 plants of *H. transsilvanicum* (JC H 864) and 3 plants of presumed hybrid origin (JC H 863, PM 1399 and PM 1400). Extraction, electrophoresis and staining followed the methods described in Štorchová et al. (2002). The following enzyme systems were examined: AAT (Aspartate aminotransferase, EC 2.6.1.1, dimeric), ADH (Alcohol-dehydrogenase, EC 1.1.1.1, dimeric), LAP (Leucine aminopeptidase, EC 3.4.11.1, monomeric), PGM (Phosphoglucomutase, EC 5.4.2.2, monomeric), 6PGD (6-phosphogluconate dehydrogenase, EC 1.1.1.44, dimeric), and SKD (Shikimic acid dehydrogenase, EC 1.1.1.25, monomeric). The average number of alleles per locus, percentage of polymorphic loci, observed and expected heterozygosity (Levene 1949), Shannon's diversity index, and the number of different multilocus genotypes were

calculated for *H. alpinum* and *H. transsilvanicum*, respectively. A primary data matrix based on presence / absence of alleles of eight allelically interpretable loci (see Table 2) for each studied plant was analysed by principal coordinate analysis (PCoA) using Jaccard's coefficient (SYN-TAX 2000, Podani 2001).

Chloroplast DNA analyses. DNA isolations were done as described by Štorchová et al. (2000), but fresh, silica gel-dried or herbarium material was used. In order to determine the maternal inheritance in artificial progeny from reciprocal crosses between two putative parental species and the maternal parent of the natural hybrids, a PCR-RFLP approach was designed based on a larger data set from the *trn*T-*trn*L intergenic spacer of *Hieracium* chloroplast DNA (Fehrer et al. 2005; and unpublished data). According to this, the parental taxa *H. alpinum* and *H. transsilvanicum* belong to slightly divergent cpDNA lineages discriminated by four substitutions and three small indels. One of the substitutions resulted in a loss of an *Eco*R I restriction site in *H. transsilvanicum* that was unique for the whole genus. One *H. transsilvanicum* plant from Romania and two *H. alpinum* plants were sequenced: a diploid one from the Ukraine (herbarium material) and one from a locality close to the Romanian hybrid population. The *H. alpinum* sequences were identical. One sequence per species was deposited in the GenBank database (accession numbers AY512556–AY512557). The hybrids' chloroplast haplotype

Table 2. Morphological characters of parental species (Zahn 1930–1939, Chrtek 1997, additional own observations include 20 plants per species) and hybrid plants (own observations on 4 plants)

Character	*H. transsilvanicum*	*H. ×krasani*	*H. alpinum*
Height of plant (m)	(0.2–)0.3–0.6(–0.8)	0.25–0.40	0.05–0.2(–0.3)
No. of stem leaves	(1–)2–5	2–3	0–1(–4)
No. of heads	(3–)5–20	3–6	1
Branching	upper third / quarter of stem	usually upper third of stem	stem unbranched
Length of involucral bracts (mm)	7–9	10–11	12–15
Width of involucral bracts (mm)	0.7–0.9	0.8–1.0	0.9–1.2
Glandular trichomes on the leaves	absent	scattered	scattered to numerous
Simple eglandular trichomes on peduncles and involucral bracts	absent	scattered to numerous	scattered to numerous
Stellate trichomes on involucral bracts	rare to scattered on the base and margins	rare on the base and sometimes on the margins	absent
Colour of simple eglandular trichomes of rosette leaves	reddish	white to reddish	white
Simple eglandular trichomes at the apex of ligules	absent	absent	scattered to numerous
Colour of styles	dark yellow to brown often with black scales	brown with black scales	yellow to dark yellow

was determined by PCR-RFLP. The *Eco*R I-digested fragments were separated on 3% agarose gels. PCR, RFLPs and sequencing were done as described previously (Fehrer et al. 2005).

Results

Morphology. The putative hybrids are intermediate between *Hieracium alpinum* and *H. transsilvanicum* (Fig. 1). The main distinguishing characters concern branching pattern, number of heads and indumentum of the peduncles and involucral bracts (details given in Table 2).

Chromosome numbers and breeding system. Chromosome numbers were determined for 31 plants including the four natural hybrids, three artificial hybrids from reciprocal crosses and both parental species (Table 1). For all of them, $2n = 2x = 18$ were counted (Fig. 2).

A pollen stainability of 78% and 96% was observed in the analyzed Ukrainian hybrid plants (PM 1400 and JC 863 respectively), while pollen stainability ranged from 78–93% in *Hieracium alpinum*, and from 91–99% in *H. transsilvanicum*. This suggests the hybrids have similar male fertility to both parental taxa.

In the Romanian hybrid plant (PM 985), no well-developed achenes were produced in either free pollination or in a control backcross with *Hieracium transsilvanicum*. The same results we obtained from control reciprocal crosses between the hybrid plants from Ukraine (PM 1399 and 1400) and from free pollination of both plants. All three tested putative hybrid individuals were completely seed sterile.

Allozyme analyses. Six enzyme systems with 11 loci were investigated in *Hieracium*

Fig 1. A *Hieracium alpinum* (Herb. P. Mráz *s.n.*), **B** natural hybrid plant of *H.* ×*krasani* (Herb. PRA *s.n.*) and **C** *H. transsilvanicum* (Herb. PRA *s.n.*), scale bar = 5 cm

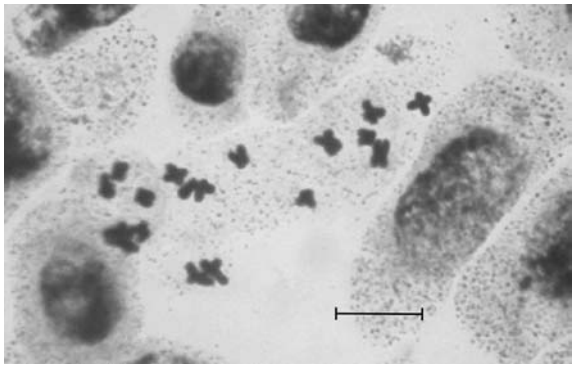

Fig 2. Chromosomes (2n = 18) in a root tip cell of the natural hybrid *H.* ×*krasani* (PM 1399), scale bar = 10 μm

alpinum, *H. transsilvanicum* and their putative hybrids. Two loci were excluded from further analyses: *Aat-1* (it turned out to be monomorphic in all studied plants) and *Pgm-2* (low enzyme activity). Altogether nine loci were therefore evaluated. Genotype frequencies of each locus are given in Table 3.

Hieracium alpinum and *H. transsilvanicum* differed consistently in five loci. The hybrid plants exhibited a unique additive genotype for *Skd*. For another two loci, they shared the genotype with *H. alpinum*, for three loci with *H. transsilvanicum*, and for three loci with both parents. Contribution of *H. alpinum* was confirmed in patterns for *Pgm-1* (allele c) and *Lap-1*. While bands of *Lap-1* are consistently lacking in *H. transsilvanicum* (besides the present plants, the same results were obtained for other *H. transsilvanicum* plants studied in 1999, Chrtek unpubl. data), the hybrid plants share these alleles with *H. alpinum*. For *H. alpinum* and *H. transsilvanicum*, the mean number of alleles per locus, percentage of polymorphic loci, observed and expected heterozygosity, number of multilocus genotypes, mean genotype diversity within loci and Shannon's diversity index are summarized in Table 4. The level of allelic variation is higher in *Hieracium transsilvanicum* than in *H. alpinum*.

The three hybrid plants shared the same multilocus allozyme phenotype. PCoA placed them between the parents along the first coordinate (Fig. 3). Allozyme analyses clearly show genetic contributions of both parental species indicating true hybrid origin.

Chloroplast DNA analyses. The Ukrainian *Hieracium transsilvanicum* was characterized by the same unique loss of an *Eco*R I restriction site as the Romanian sample of *H. transsilvanicum* from the second hybrid locality used for sequencing, allowing the distinction of the parental haplotypes by PCR-RFLP. The

Table 3. Allozyme genotypes and their frequencies. For *Adh-2*, only banding patterns were compared (not interpretable genetically). Bands of *Lap-1* were not present in *H. transsilvanicum*

Taxon (number of plants)	Locus and genotype frequencies								
	Aat-2	*Adh-1*	*Adh-2*	*Lap-1*	*Lap-2*	*Pgm-1*	*6Pgdh-1*	*6Pgdh-2*	*Skd*
H. alpinum (10)	bb 1.00	ab 0.10 bb 0.90	7 0.20 8 0.40 9 0.30 10 0.10	ab 0.10 bb 0.60 bc 0.20 cc 0.10	aa 0.40 ab 0.60	ac 1.00	aa 1.00	bb 1.00	aa 1.00
H. ×*krasani* (3)	bb 1.00	bb 1.00	2 1.00	bb1.00	ab 1.00	ac 1.00	bb 1.00	ab 1.00	ac 1.00
H. transsilvanicum (12)	aa 0.08 ab 0.58 bb 0.34	bb 1.00	1 0.08 2 0.50 3 0.08 4 0.08 5 0.08 6 0.18	–	aa 0.92 ab 0.08	ab 1.00	bb 1.00	aa 0.08 ab 0.34 bb 0.58	bb 0.25 bc 0.42 cc 0.33

Table 4. Measures of allelic and genotypic variation. N = number of individuals; A = mean number of alleles per locus; P = percentage of polymorphic loci; H_o = observed heterozygozity, H_{exp} = expected heterozygosity (computed according to Levene 1949); I = Shannon's index, G = number of different multilocus genotypes

Species	N	A	P	H_o	H_{exp}	I	G
H. alpinum	10	1.625	50.00	0.250	0.176	0.274	7
H. transsilvanicum	12	1.714	71.43	0.345	0.286	0.397	10

maternal inheritance of the plastome in *Hieracium* s. str. was confirmed by PCR-RFLP for the particular combination of taxa by examination of three artificial hybrids from reciprocal crosses between *H. transsilvanicum* and *H. alpinum*. The natural hybrids from both, Romanian and Ukrainian, populations showed the chloroplast haplotype of *H. transsilvanicum* (Fig. 4) indicating that this species was the seed parent in all cases. Attempts to do molecular analyses with the syntype specimen of *H.* ×*krasani* (see below) were unsuccessful as it did not contain usable DNA because of its age (collected in 1888).

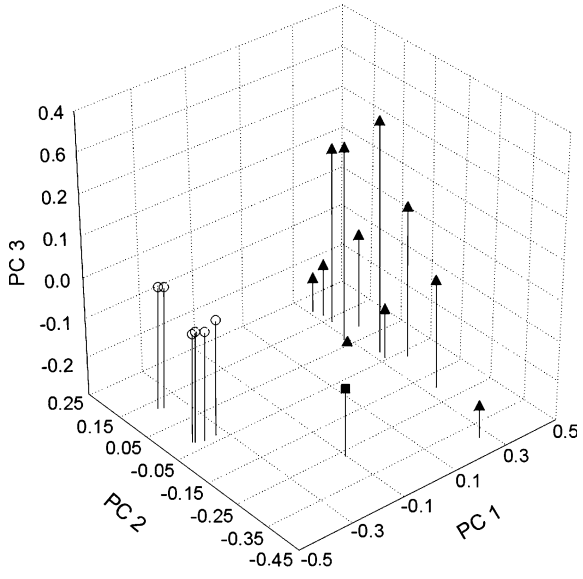

Fig 3. Principal coordinate analysis of allozyme data of *Hieracium alpinum* (circles), *H. transsilvanicum* (triangles) and hybrid plants (square)

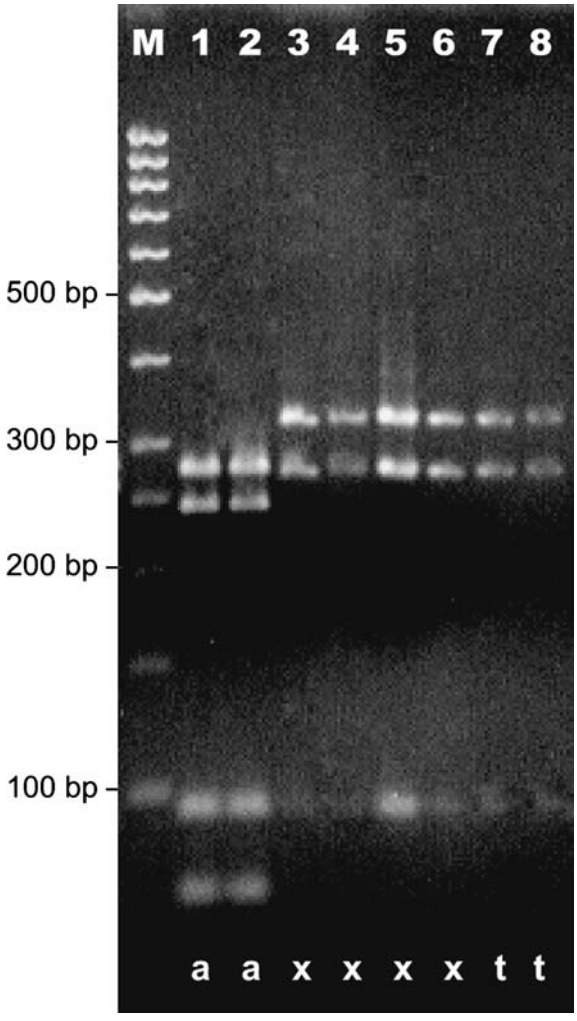

Fig 4. PCR-RFLP of the *trn*T-*trn*L intergenic spacer of chloroplast DNA. M: marker; 1 and 2: *H. alpinum* ('a'); 3–6: hybrid plants ('x'); 7–8: *H. transsilvanicum* ('t'). The hybrid in track 4 was from Romania, the three others were from the Ukrainian population

Discussion

Hybrid origin of the plants. The investigated diploid hybrid plants *Hieracium alpinum* × *H. transsilvanicum* showed a high degree of morphological intermediacy between both parental species. However, they have slightly more characters in common with the maternal parent, *H. transsilvanicum*. Similar results have been obtained from artificial reciprocal crosses between the same diploid taxa, the hybrid progeny more closely resembled the maternal plants in both directions of the cross (Paule 2004). All karyologically studied plants (*H. alpinum*, *H. transsilvanicum* and the hybrid plants from nature as well as from artificial hybridization) were shown to be diploid. Our counts are in agreement with previously published chromosome numbers in both *H. alpinum* and *H. transsilvanicum* from the Eastern Carpathians (Pashuk 1987; Chrtek 1996, 1997; Mráz 2001, 2003; Mráz and Szeląg 2004). Further hybrids from experimental reciprocal crosses were also diploid (Paule 2004). The Ukrainian hybrids produced a considerable quantity of homogeneously sized pollen with comparable stainability to the parental species, indicating regular microsporogenesis. All studied hybrid plants only produced completely empty achenes in free pollination as well as in control experimental crosses. Thus, female sexual reproduction was limited or even excluded in the hybrid plants. This finding is similar to what has been found in artificial *Hieracium* hybrids from several different diploid – diploid crosses. Megasporogenesis in artificial diploid hybrids was usually highly disturbed, and production of normally developed achenes in free and control pollination was either absent or very low (in the range of 0–12.1%) (Mráz and Paule 2003, Paule 2004, Mráz and Paule, unpubl. data). On the other hand, the hybrids might contribute to sexual reproduction as pollen donors. The artificial hybrids generally produced a rather large quantity of stainable pollen grains of homogeneous size, similar to our natural hybrid and its parents (Mráz and Paule 2003, Paule 2004, Mráz and Paule, unpubl.). Vegetative spread by means of short rhizomes is of little importance in *Hieracium* s.str. Nevertheless, just the hybrid plants from the Ukraine are probably results of such vegetative spread (they grew side by side in a spot of ca 30 × 30 cm and shared the same multilocus allozyme phenotype).

Allozyme data also supported the hybrid origin. The hybrids did not show any unique alleles, but rather exhibited an additive pattern for *Skd*; for the remaining polymorphic loci, the hybrids shared its single-locus phenotypes with either *Hieracium alpinum* or *H. transsilvanicum*. Contribution of *H. alpinum* was clearly confirmed in patterns from two loci (*Pgm-1*, *Lap-1*). Intrapopulation genetic variation of *H. alpinum* and *H. transsilvanicum* reflected their sexual mode of reproduction in the study area.

Frequency of hybridization. Recent spontaneous hybridization between *Hieracium alpinum* and *H. transsilvanicum* is undoubtedly a very rare event. Only a few plants (which are the subject of the present paper) were found during our excursions to the Ukrainian and Romanian Eastern Carpathians. This matches the very low number of what is presumably the same hybrid taxon preserved in public herbarium collections. To explain the rarity of hybridization events in *Hieracium* s.str. and between *H. alpinum* and *H. transsilvanicum* in particular, both internal and external reproduction barriers should be considered.

The parental species belong to different sections within the genus *Hieracium*, and are not morphologically closely related. Thus, chromosomal and genic incompatibilities might play an important role. Another feature that may contribute to the generally low rate of hybridization is a mentor effect, i.e. induction of self-compatibility in otherwise incompatible pollen when it is mixed with foreign pollen (Richards 1997: 223), which was recently discovered during control crosses between several diploid as well as between diploid and polyploid taxa (Mráz 2003). In many different crosses the proportion of autogamously derived progeny from diploid

mother plants reached 100%, although the diploid species are usually strictly self-incompatible (Chrtek 1997, Mráz 2003). Thus, the mentor effect may represent a very effective hybridization barrier in this diplosporic genus. In the closely related genus *Pilosella* (often treated as a subgenus of *Hieracium*), autogamy could also be stimulated by pollen from other species under certain conditions (Krahulcová et al. 1999), but its degree is substantially lower and its role in the generally self-incompatible aposporic *Pilosella* is very limited as reflected by the vast amount of recent hybridization (Krahulcová et al. 2000). A high level of selfing (90%) induced by the influence of foreign pollen was also recorded in crosses between sexual diploid (mother plants) and apomictic triploid (pollen donors) dandelions (*Taraxacum* Wigg.; Tas and van Dijk 1999). Apart from induced autogamy by heterospecific pollen, pollen competition should be taken into account. Studies on *Iris* L. and *Helianthus* L. showed the advantage of conspecific against heterospecific pollination (Carney et al. 1994, Rieseberg et al. 1995, Emms et al. 1996, Carney and Arnold 1997).

Besides internal barriers, there are some external ones preventing natural hybridization between *Hieracium alpinum* and *H. transsilvanicum*. Among the factors that are considered most critical to rates of hybridization are differences in ecological preferences. While *H. transsilvanicum* is a typical element of spruce (and fir-beech) forests of the Eastern and Southern Carpathians, *H. alpinum* is restricted to the grassland of the alpine and, extremely rarely, subalpine belts. Both taxa are clearly separated by altitudinal and ecological demands (e.g. light intensity). At the Romanian locality a single hybrid plant was found on a tourist path at 1350 m altitude in the spruce belt. It was accompanied by plants of both parental species. While *H. transsilvanicum* was abundant in its typical biotope, *H. alpinum* occurred with three individuals only ca 300 m from the hybrid site. The presence of *H. alpinum* at this place may be explained by the close proximity of the alpine belt and by the fact that the locality was strongly disturbed (and cleared) by forest machines during cutting. The biotope is thus suitable for incidental occurrence of *H. alpinum* at an atypical altitude. The small group of hybrid individuals (altogether 5 flowering plants) from the Ukraine – representing probably a single clone of plants arisen via vegetative propagation of rhizomes – grew at the border between spruce forest and secondary pasture at 1410 m altitude. As in the first case, *H. transsilvanicum* was abundant, but *H. alpinum* as pollen donor was completely absent from the locality. The next closest plants of this arcto-alpine species were observed along the disturbed margins of a tourist path at an altitude of 1600 m, ca 0.7–1 km away. In both cases, the hybrid plants were found at intermediate altitudes in biotopes disturbed by human activity, where both parental species came into (more or less close) secondary contact.

The importance of habitat disturbance providing the corridors for species movement and leading to sympatry in otherwise allopatric species as a prerequisite for hybridization was stressed e.g. by Levin et al. (1996). Similar secondary contacts may have taken place during the major climatic changes of the Pleistocene glaciation and in the short postglacial period. During this period of advancement and retreat of glaciers, new types of biotopes arose, where sympatry of many different species and thus interspecific hybridization became possible (Asker and Jerling 1992, Carman 2001). The morphological variability in the genus *Hieracium* likely reflects an immense reticulate evolution in the past. While external barriers are not obvious among all diploids found in this genus (at present, a total of no more than 25 known diploid taxa, cf. Chrtek et al. 2004), most of them are separated ecologically and/or geographically from each other. The great majority of diploid taxa belongs to the "basic" species in the sense of Zahn (1921–1923). As in the genus *Hieracium* s. str. where there are many (micro)species with intermediate morphology between different "basic" species

(which are also considered as putative parents and often have different ecological demands, however), we can assume that new hybridogenous taxa may typically arise in hybrid zones of secondary contact. Because most of the counted taxa are polyploids, hybridization processes in *Hieracium* had to be followed by a rise in ploidy level and by apomictic reproduction (but not in the case of our hybrids!) as an "escape from sterility". Hybridization in polyploid *Hieracium* species is strongly limited by their mode of reproduction. Agamospermy with full omission of female meiosis (diplospory of *Antennaria* type) has been recorded, followed by autonomous development of endosperm (e.g. Rosenberg 1927, Bergman 1941, Gustafsson 1946). Of crucial importance is precocious embryony – the unreduced egg cell develops into the embryo before the flower opens so that fertilization is impossible (Nogler 1984). Thus, the possibility of hybrid formation with *Hieracium* polyploids serving as mother plants is highly limited. Gene flow is more likely either among diploid taxa (as presented in this paper), or between diploids (as mother plants) and pollen-producing polyploids (as pollen donors).

Direction of hybridization. Apart from a few exceptions, maternal inheritance of chloroplast genes is typical in angiosperms (reviewed in Birky 1995, 2001) and was also shown recently for the closely related genus *Pilosella* (Fehrer et al. 2005). Analysis of artificial hybrid progeny from reciprocal hybridization between *Hieracium alpinum* and *H. transsilvanicum* with chloroplast DNA markers confirmed that the plastome is indeed inherited maternally in the cross in question. As the natural hybrids from Romania and Ukraine shared their chloroplast haplotype with *H. transsilvanicum*, this species apparently served as maternal parent in all cases. Recently, Mráz and Paule (2003) and Paule (2004) made a series of experimental crosses between selected diploid sexual species and between diploid sexual (mother plants) and polyploid apomictic (pollen donnors) species. Most of the F1 plants from diploid – diploid crosses proved to be true hybrids. In contrast, reciprocal crosses between *H. alpinum* and *H. transsilvanicum* seemed to depend somewhat upon the direction of the cross: While in the cross with *H. transsilvanicum* as seed parent all ten progeny plants were true hybrids, in the cross with *H. alpinum* as maternal plant, four out of 21 plants were of autogamous origin (Mráz 2003). Higher susceptibility for induced autogamy (mentor effect) in *H. alpinum* than in *H. transsilvanicum* might partially explain that the latter taxon served as maternal parent in both cases of natural hybrids. However, as this assumption is based on a single reciprocal cross only, more experimental data would be needed to draw firm conclusions about this aspect.

Initially, we assumed that *H. alpinum* was the much likelier seed parent for two reasons. (i) Due to the abundance of *H. transsilvanicum* and the absence or rarity of *H. alpinum* at both sites, a single or a few *H. alpinum* plants incidentally occurring outside their natural habitat were expected to be exposed to an excess of *H. transsilvanicum* pollen. (ii) While the plants of both parental species grew close to each other at the Romanian locality, at the Ukrainian locality the parental species were separated by a distance of at least 700 m. Pollinators are usually not expected to exceed about 30 m of flying distance (e.g. Proctor et al. 1996, Richards 1997) whereas *Hieracium* seed dispersal might be rather efficient due to possession of a hairy pappus. Therefore the result from chloroplast DNA analysis that *H. transsilvanicum* acted as seed parent was rather surprising, especially in the case of the Ukrainian hybrids. Possible explanations are: (i) individual plants of *H. alpinum* in closer vicinity to the hybrids might have been overlooked, (ii) pollinators bridged a longer distance in this case, (iii) the hybrids were present at the site for some time already and previously occurring *H. alpinum* meanwhile disappeared as community parameters do not allow its long-term survival, or (iv) the hybrids could have arisen at another locality where both parental taxa grew in closer contact and the

achenes were later wind-dispersed to where the plants were found.

Another possibility is that our hybrids already represent a backcross to *H. transsilvanicum*. In such case, the hybrid would – independent from the original direction of the cross – have acted as pollen parent (as it was highly probably seed sterile, too) so that the BC1 would, in any case, display *H. transsilvanicum* cpDNA. As F1 plants are highly heterozygous due to the pronounced differences between the parental species, products of a BC1 should be variable due to segregation and morphologically fill the whole space between the parents. It is therefore hardly possible to distinguish the result of a BC1 from a true F1. Our hybrids' closer resemblance to *H. transsilvanicum* also would not contradict the backcross scenario.

Taxonomic status of the hybrid plants. Morphologically, the studied hybrid plants belong to *Hieracium* ×*krasani* Woł. (Wołoszczak 1890, as "*H. Krašani*"). This hybrid was described from two localities (Mt. Siniak and Mt. Kukul) in the Chornohora Mts. in the Ukrainian Eastern Carpathians, where it was collected in stands of *H. alpinum* and *H. transsilvanicum* (see below). We found one herbarium specimen (syntype) corresponding to the protologue. The label reads as follows: "*Hieracium alpinum* × *pleiophyllum* (*H. Krašani* Woł.). In alpe Siniak (pr. Tatarów) Carpathorum orientalium Galiciae, ca 1600 m sm., 8/1888, legit Wołoszczak" (LWS s.n.). It is not clear if Wołoszczak himself considered *H.* ×*krasani* as a true hybrid of recent origin or a hybridogenous taxon. The first hypothesis is favoured by his words in the protologue "*H. Krašani* (*H. alpino* × *transsilvanicum*) ... inter parentes absque aliis *Hieraciis* rarissimum …" (cf. Wołoszczak 1890: 65). On the other hand, the "×" sign (or others like ≤, ≥, <, >; e.g. Zahn 1921–1923) are used as a short description of the morphological position of particular intermediate taxa between two or more "basic" species in the genus *Hieracium*. Moreover, the distinction between a hybrid taxon (nothotaxon) and a hybridogenous one was not clear at that time. In our opinion, the hybrid plants collected in Romania and Ukraine are clearly of recent origin. The plants were found at the localities as a single or only a few individuals, they are seed sterile, and they have the same diploid chromosome number as their parental species. We therefore treat them in the present paper as nothotaxon in the sense of the International Code of Botanical Nomenclature (Greuter et al. 2000). Wołoszczak also reported *H.* (×)*krasani* as a rare (notho)taxon from two original localities (see above), later on he published one additional record of just one plant from another locality of the Ukrainian Eastern Carpathians (Wołoszczak 1894: 143, "… w dziedzinie kosodrzewu w jednym okazie na Popadi" [the only one plant was found in the subalpine belt on Mt. Popadia]). In contrast, most of Zahn's "species intermediae" are actually polyploids with apomictic breeding systems and they are treated as separate taxa, i.e., not as hybrids. They usually occur in high individual numbers, and they are considered to be of hybridogenous origin.

A similar taxon of intermediate habit between *H. alpinum* and *H. transsilvanicum* from the Southern Carpathians (Munţii Retezatului, Romania) was found to be triploid (2n = 27) (Mráz unpubl.). However, this triploid species is morphologically different (in the density of indumentum, and partially in the shape of rosette leaves) from the hybrids of *H.* ×*krasani* reported in our study.

Probably *H.* ×*krasani* is the first true nothotaxon in the genus *Hieracium* s. str. to be verified by a set of different experimental approaches.

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Hieracium ×*grofae* – a rediscovered diploid hybrid from the Ukrainian Carpathians

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Abstract: Diploid hybrid plants ($2n = 18$) between sexual diploid cytotypes of *Hieracium alpinum* and *H. umbellatum* were found in the Ukrainian Eastern Carpathians. They were identified with *H.* ×*grofae* Woł., originally used for the combination *H. decipiens* ×*H. umbellatum* var. *lactaris*. As *H. decipiens* sensu Woł. (non Tausch) does not produce viable pollen grains and is most probably a polyploid apomict, it is unlikely to produce diploid hybrid plants with diploid *H. umbellatum*. Both parent species, *Hieracium alpinum* and *H. umbellatum* are also given by Wołoszczak from the original locality. Thus we conclude that *H.* ×*grofae* is result of hybridization between *H. alpinum* and high mountain form of *H. umbellatum*. Hybrid plants are morphologically intermediate between the parent species, and moreover resemble closely or they are identical with the experimental hybrids of the same parent combination. Hybrids produce rather high amount of homogeneously sized pollen grains (values of standard deviation and coefficient of variation lower then upper limits for diploids – 3μm or 7.5%, respectively), and might probably serve as male parents in further crosses; on the other hand, they are fully seed-sterile. A lectotype of *H.* ×*grofae*, a second proved nothotaxon in the genus *Hieracium* s.str., is designated. Localities of *H.* ×*grofae* are located in subalpine belt of the Marmarosh Mts, the Svydovets' Mts and the Horhany Mts (all in the Ukrainian Eastern Carpathians). Biotopes of hybrid plants usually represent secondary formed and disturbed pastures allowing close contact of altitudinally vicariant parent taxa.

Key words: Asteraceae, chromosome numbers, homoploid hybridization, lectotype

Introduction

Hieracium L. s. str. (*Hieracium* subgen. *Hieracium*, 'Archieracium' as defined by Zahn, 1921–1923) is one of the world's most species-rich plant groups (Royal Botanical Gardens Kew, 1993). It includes perennial herbs distributed predominantly in temperate regions of Europe, Asia, and North America. Polyploidy and diplosporous agamospermy seem to prevail in the genus (e.g., Gustafsson, 1946–1947; Schuhwerk, 1996). Sexuality is confined to a few diploid species, distributed mostly in South Europe (Merxmüller, 1975; Chrtek et al., 2004).

Two different species concepts appeared in the genus: i) broad species concept, utilized e.g. by Nägeli & Peter (1885, 1886) and Zahn (1921–1923, 1930–1939), and ii) narrow species concept (applied first of all by British, Scandinavian and East European authors, e.g. Pugsley, 1948; Üksip, 1960). In the former, the broadly defined species are (usually) divided into subspecies, and (if necessary) to varieties and lower taxonomic units. In the latter, binomial 'microspecies' ('agamospecies') are recognized, species groups (aggregate species, usually analogous to species in a broad sense) then accommodate one to many 'microspecies'.

Two kinds of species in the broad sense are distinguished. While the main species ('species principales collectivae') posses usually an unique set of morphological characters (although their definition is sometimes rather arbitrary), the intermediate ones ('species principales intermediae') share a morphologically intermediate position between two or more main species. The latter are usually thought to be a result of extensive past hybridization and polyploidization, nowadays there are mostly fixed by agamospermous mode of reproduction. However, their origin is still a contentious issue. Taxonomically, they are usually treated as hybridogeneous species (e.g. Zahn 1921–1923).

In contrast, recent hybridization seems to be a very rare phenomenon. It is most likely confined to crosses between diploid sexual species (Mráz et al., 2005; Mráz & Paule, 2006) or diploid sexuals (as mother parents) and pollen producing polyploids (pollen donors) (Mráz & Paule, 2003; Paule, 2004; Mráz & Tomčíková, 2004). So far, spontaneous hybridization in the wild has only been documented between diploid *Hieracium alpinum* L. and *H. transsilvanicum* Heuff. in the Ukrainian an Rumanien Eastern Carpathians (Mráz et al., 2005). Merxmüller (1975: 193) mentioned that allegedly spontaneous hybrids, morphologically intermediate between the two diploids *H. porrifolium* L. and *H. umbellatum* L., were found in his *Hieracium* garden collection at Munich Botanical Garden.

During field excursions to the Marmarosh Mts in 1996 (J.C. and P.M.) and 2005 (J. Zahradníček) and to the Svydovets' Mts in 1999 (P.M.) we found plants that superficially resembled the *Hieracium fritzei* group (*H. alpinum* > *H. prenanthoides*). This aggregate species (species group) is rather common in the highest mountains of the Sudeten range (N Czech Republic, SW Poland) and the Western Carpathians, a small area is situated in Rumanian Carpathians (Zahn, 1921–1923, 1930–1939; Chrtek & Marhold, 1998). It has also been reported from the Ukrainian Eastern Carpathians (Zahn, l.c.; Üksip, 1960; Chopyk, 1977, Malynovsk'yj, 1980; Prokudin, 1987; Shlyakov, 1989; Mosyakhin & Fedoronchuk, 1999). However, further detailed morphological studies showed affinities with *H. umbellatum*, which reaches its upper altitudinal limit on the secondary mountain grasslands ("poloniny").

Both sexual diploids (2n = 18) and apomictic triploids (2n = 27) are known in *H. umbellatum* (for references see e.g. Schuhwerk, 1996; Májovský et al., 1987 and other chromosome number indexes); plants from the Ukrainian Carpathians were proved to be diploid (see diploids counts given by Chrtek, 1996 as *H. conicum* Arv.-Touv. and Mráz, 2003 as *H. hryniawiense* Woł.; and other yet unpublished counts by J.C. and P.M.). Distinct influence of diploid sexual *Hieracium alpinum* rather widespread in the area (Chrtek, 1997; Mráz, 2001) lead us to conclusion, that our plants may represent hybrids between *H. alpinum* and high mountain morphotype of *H. umbellatum*. Several identical plants from the same region were found in herbaria PR, SAV and GLM.

A correlation between pollen size heterogeneity and ploidy level has been documented in *Hieracium* s. str. (homogeneously-sized pollen in diploids, heterogeneously-sized pollen in polyploids, Mráz et al., 2002; Kovalčiková, 2004). We use here the heterogeneity of pollen size as an indirect method to estimate the ploidy level in herbarium specimens.

The aim of the present paper is: (1) to find evidence about the recent hybrid origin of our plants, (2) to find a name, which can be applied for them, and (3) to provide a list of localities in the Ukrainian Carpathians.

Material and methods

Plants

Putative hybrid plants were collected by J. C. and R. Letz (1996), P. M. and V. Jurkovičová (1999) and J. Zahradníček (2005) in the Ukrainian Carpathians. Living plants from 2005 (Mt. Berlebashka) were transferred in the experimental garden of the Institute of Botany, Průhonice and used for chromosome counting. Herbarium specimens from following institutions were examined (acronyms according to Holmgren et al., 1990): BP, GLM, KRA, KRAM, PR, PRA, PRC, SAV, W.

Pollen was examined in herbarium plants from the original material of *H.* ×*grofae* (KRAM) and in plants collected by P.M. at Mt. Unharias'ka (the Svydovets' Mts) and Mt. Berlebashka (the Marmarosh Mts) (Fig. 1A–C). Furthermore, it was examined in herbarium plants of *Hieracium alpinum* and *H. umbellatum* from the Ukrainian Carpathians, as well as in experimental diploid hybrids between high mountain form of diploid *H. umbellatum* (mother plant) and diploid *H. alpinum* (pollen donor) (for details cf. Mráz & Paule, 2006; cross no. X9 ut *H. umbellatum* ×*H. alpinum*, Fig. 1D). The localities of the plants examined are summarized in Appendix 1.

Chromosome number, pollen grains

Chromosome counts were estimated in two cultivated plants originating from Mt. Berlebashka. Root tips of mature plants were used for karyological studies. The material was pretreated with a saturated solution of p-dichlorobenzene, fixed in a mixture of ethanol and acetic acid (3:1 v/v) and stored in 70% ethanol. The squash method and staining by lacto-propionic orceine were used (Dyer, 1963).

The pollen (its shape and size) was observed using light microscope. After acetolysis following Erdtman (1960) the pollen grains were observed using light microscope. Usually three to five flowers per plant in the stage before anthesis were used and at least 30 pollen grains per plant were measured (all values include echinae).

Results

Extensive search in public herbarium collections lead us to conclusion that our plants are identical with those described by E. Wołoszczak as *Hieracium* ×*grofae* Woł.

Hieracium ×***grofae*** Woł. Spraw. Komis. Fizjogr. 27: 142, 1892

Ind. loc. (Wołoszczak 1892: 143): "Galicia in regione Mughi montis Grofae Lomnicensis, Carpathorum orientalium 1600 m s. m.".

Lectotypus (hoc loco designatus): W dziedzinie kosodrzewa na Grofie, Karp. Wsch. okol. rz. Lomnicy [in dwarf-pine stands at Mt. Grofa, the Eastern Carpathians, near the river of Lomnica], ca 1550 m, 20. lip. [July] 1889, leg. Wołoszczak, KRAM no. 148408. (Fig. 1A).

Fig. 1. Herbarium specimens of diploid hybrid *Hieracium* ×*grofae*. **A.** Original voucher selected as a lectotype (KRAM 148408). **B.** Plants collected W of Mt. Unharias'ka. **C.** Plants collected on Mt. Berlebashka. **D.** Plants from experimental hybridization. Scale bar = 10 cm (on Fig. 1A).

Table 1. Main distinguishing characters of *Hieracium alpinum*, *H.* ×*grofae* and *H. umbellatum*. Delimitation of *H. alpinum* follows that in CHRTEK (1997). Thus, plants completely lacking simple eglandular hairs, occurring in some parts of the Ukrainian Carpathians, are treated separately [*H. augusti-bayeri* (ZLATNÍK) CHRTEK f.]. The data for *H. umbellatum* refer to plants from the Ukrainian Carpathians only, do not cover the whole variation range of the species.

Character	*H. alpinum*	*H.* ×*grofae*	*H. umbellatum*
Height of plants (cm)	6–24	15–30	10–80
Basal leaves	distinct rosette of basal leaves	usually withering in the flowering time	plants without basal leaves
No. of stem leaves	0–1(–2)	4–8, the lower sometimes withering in the flowering time	10–40
Shape of stem leaves	(if present) linear to linear-lanceolate	oblong-elliptical to lanceolate	lanceolate, elliptical-lanceolate, oblong-elliptical, elliptical
Indumentum of leaves	numerous (less often scattered) simple eglandular hairs, on the margins of leaves scattered minute yellowish glands	scattered, on the margins numerous simple eglandular hairs (on the upper site sometimes nearly glabrous around the midrib), on the margins of leaves scattered minute yellowish glands	leaves glabrous, sometimes with short simple eglandular hairs on the margins, without minute yellowish glands
No. of heads	1	1–4	10–40 (high mountain type 3–5 only)
Simple eglandular hairs at the apex of ligules	numerous	very few	none
Colour of styles and stigmas	purely yellow	olivaceous with black scales	yellow to olivaceous with black scales

Description

Phyllopodous or hypophyllopodous. Stem 15–30 cm high, simple (unbranched), usually slightly flexuous, usually purplish below, with scattered to numerous pale towards the top of stem pale but dark-based 1.5–2.5(–3.0) mm long simple eglandular hairs, scattered 0.2–0.4 mm long dark glandular hairs in the upper part and with numerous stellate hairs in the middle and upper parts. Leaves medium green, slightly glaucous below, with scattered, on the margins numerous, (0.8–)1.0–2.0(–2.6) mm long pale simple eglandular hairs (sometimes nearly glabrous around the midrib above), scattered 0.10–0.13 mm long yellowish glandular hairs mainly on the margins, and stellate hairs. Basal leaves 1–3, usually withering at the time of flowering, petiolate, oblanceolate to oblong-elliptical, 5–7 × 1.2–1.6 cm, rounded at apex, attenuate to a petiole or cuneate-based, entire with remote mucronate glands. Cauline leaves 4–8, gradually decreasing in size towards the top of stem, the lower cauline leaves (sometimes withering at the flowering time) ± petiolate, oblong-elliptical to lanceolate, rounded to obtuse-acute at apex, cuneate-based; the middle cauline leaves lanceolate to oblong-elliptical, 3.2–7.5 × 1.0–1.5 cm, obtuse-acute to acute at apex, abruptly narrowed to a sessile base, entire (with remote mucronate glands) to remotely denticulate with teeth to 1 mm long; upper cauline leaves lanceolate, linear-lanceolate to linear, acute at apex, narrowed (often abruptly) to a sessile base, entire or occasionally remotely denticulate. Heads solitary or 2–4 (acladium 1.6–1.8 cm long); peduncles blackish-green, with scattered to numerous, 1–2 mm long, shortly (1/5–1/4 of their length) dark-based simple eglandular hairs, scattered to numerous 0.2–0.5 mm long dark glandular hairs and numerous to dense stellate hairs. Involucres barrel-shaped, (10–)12–14 mm long; phyllaries linear-lanceolate, obtuse at apex, brownish to blackish-green, the inner with paler margins, the outer with numerous to 2.5 mm long pale but dark-based (1/4–1/3 of their length) simple eglandular hairs and numerous glandular hairs, the inner with scattered eglandular and glandular hairs. Ligules flat, yellow, with few, very short hairs at the apex, the outer ligules 14–16 mm long. Styles olivaceous with black scales. Achenes 3.1–3.4 mm long, brown, empty. Flowering from July to August.

According to our study (chromosome number, morphological studies – see Tab. 1) the hybrid formula should be *H. alpinum* × *H. umbellatum*, instead of *H. decipiens* × *H. umbellatum*.

Hieracium ×*grofae* can be easily distinguished from its parents. It is in general appearance more similar to *H. alpinum*, but clearly differs first of all in the leafy stems, usually 2–3(–4) heads and dark (olivaceous with black scales) styles (Tab. 1, Fig. 1A-D).

Chromosome number

$2n = 18$ (Ukrainian Eastern Carpathians, Marmarosh Mts, Mt. Berlebashka, coll. J. ZAHRADNÍČEK, 4 August 2005, counted by J.C., 2005, 2 plants).

Pollen size in H. ×*grofae, H. alpinum and H. umbellatum*

All measured samples including putative parental taxa

Table 2. Size of acetolyzed pollen grains (including echinae) of parental *Hieracium* taxa and their natural and experimental hybrids. For parental taxa, the pollen measurements were taken from two or more individual plants per taxon; for hybrids, each line represents just one plant. Origin, number of studied plants and codes are given in Appendix 1. N – number of pollen grains.

Taxon (and code or other characteristic)	*N*	Equatorial size $x \pm$ SD (μm)	cv (%)	Polar size $x \pm$ SD (μm)	cv (%)
alpinum	*312*	44.3 ± 2.2	4.9	42.2 ± 2.3	5.5
umbellatum (lower altitude morphotype)	*117*	37.4 ± 2.0	5.2	35.4 ± 2.2	6.2
umbellatum (high mountain morphotype)	*119*	39.6 ± 2.7	6.8	37.6 ± 2.7	6.7
×*grofae* (lectotype specimen KRAM 148408)	*30*	37.3 ± 1.8	4.8	34.5 ± 2.2	6.4
×*grofae* (Ber1)	*43*	36.8 ± 2.3	6.3	34.7 ± 2.3	6.6
×*grogae* (Ungar1)	*30*	36.1 ± 2.1	5.8	33.4 ± 2.3	6.9
×*grofae* (Ungar2)	*49*	36.6 ± 1.6	4.4	34.3 ± 1.6	4.7
×*grofae* (X9/35, 1 headed plant)	*35*	38.4 ± 2.3	6.0	36.1 ± 2.3	6.4
×*grofae* (X9/29, 5 headed plant)	*47*	34.0 ± 1.8	5.3	31.5 ± 2.1	6.6

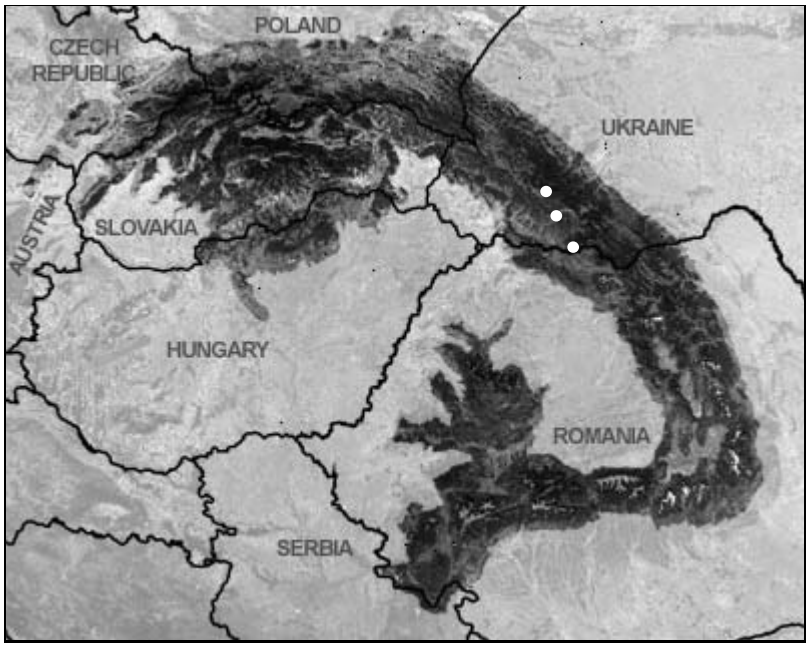

Fig. 2. Distribution of *Hieracium* ×*grofae*. The easternmost dot represents a group of localities in the Pip Ivan massif (Marmarosh Mts).

and putative natural hybrids and progeny from experimental hybridization produced homogeneously sized pollen with standard deviations and coefficients of variation lower then upper limit values for diploids – 3 μm or 7.5%, respectively (Tab. 2). This suggests that the hybrid plants found in Mt. Unharias'ka and lectotype specimens from the locus classicus are also diploids. *Hieracium alpinum* produced the largest pollen grains, while *H. umbellatum* and their hybrids *H.* ×*grofae* smaller ones. We found a variation in pollen size between lowland and mountain morphotypes of *H. umbellatum*. Similarly, two experimental hybrids analyzed show differences in pollen size.

Ecology

Subalpine secondary grasslands (less intensively grazed or abandoned pastures, usually more or less disturbed places) and rocky outcrops.

Distribution

Local and very rare in the Marmarosh Mts, the Svydovets' Mts and the Horhany Mts (all the Ukrainian Eastern Carpathians, Fig. 2), occurring in very small number of individuals on the localities. Its occurrence in adjacent part of Rumanian Eastern Carpathians, mainly in the Munţii Maramureşului, is highly probable.

Specimens studied:

Ukraine

Oblasť Ivano-Frankivs'ka: [Horhany Mts] Galicia in regione Mughi montis Grofae Lomnicensis,

Carpathorum orientalium 1600 m s. m. (20. VII. 1889 Wołoszczak, KRAM; Fig. 1A).
Oblasť Zakarpats'ka: [Svydovets' Mts] Svidovec, ca 2 km V od kóty Ungariaska (1711), na hrebeni, ca 1600 m (7. VIII. 1996 P. Mráz & V. Jurkovičová, herb. P. Mráz, det. P. Mráz 2003 ut *Hieracium fritzei* f. *marmarosense*, rev. P. Mráz ut *H. alpinum* × *H. hryniawiense*; Fig. 1B). – [Marmarosh Mts] Huculské Alpy, hřeben mezi Žerbánem a Pop Ivanem (VIII. 1938 s. coll., SAV). – Marmaroš, in graminosis et saxosis montis Pop Ivan prope vicum Trebušany, solo granitico, alt. 1650-1940 m s.m. (VIII. 1933 M. Deyl, PR, SAV). – Marmaros: Alpenmatten des Popp Ivan, Glimmerschiefer, 1800-1900 m (24. VIII. 1894 Weberbauer, SAV). – Marmaroš, Berlebaška Mt., skaly pod vrcholom, ca 1800 m (31. VII. 1996 R. Letz, herb. P. Mráz, det. P. Mráz 2004 ut *Hieracium alpinum* × *hryniawiense* (*conicum* agg.); Fig. 1C). – Ostkarpaten, 13 km SE Rachiv: Marmaroski Alpi, Berlebaška, W unterhalb des Gipfels, 1700 m, 47°57.2' N, 24°18.9' E, Gneisfelsen (31. VII. 1996, S. Bräutigam & J. Chrtek jun., GLM, PRA). – Marmarosh, Dilove: Mt. Berlebashka, W slope below the top, 10.5 km ENE of Dilove (plant cultivated at the experimental garden in Průhonice, leg. 20 September 2005, J. Chrtek; collection in the field 4 August 2005, J. Zahradníček).

Discussion

The chromosome number ($2n = 18$, diploid) supports our hypothesis of a recent hybrid origin of the plants from *H. alpinum* and *H. umbellatum*. All stabilized hybridogeneous species in *Hieracium* s.str. are polyploids (triploids and tetraploids, very rarely pentaploids). The studied plants produce homogeneously sized pollen and would most likely serve as pollen parents in backcrosses or in hybridization with another sexual *Hieracium* species (either the parent species or *Hieracium transsilvanicum* co-occurring in the area). Similarly, pollen production and quite high stainability of pollen grains (75 and 85%) was reported for experimental hybrids *H.* ×*grofae* (Mráz & Paule, 2006; cf. Tab. 2, cross no. X9). Only empty achenes were found in all examined plants of *H.* ×*grofae*; it is most probably completely seed sterile (examination of heads of the herbarium specimens), similar to *H.* ×*krasanii* Woł. (Mráz et al., 2005). Nearly complete seed sterility has also been documented in experimental hybrids between *H. alpinum* and *H. umbellatum* (Mráz & Paule, 2006). Moreover, the morphological comparison of natural hybrids with those from an experimental hybridization (Mráz, 2003; Mráz & Paule, 2006; cross no. X9 *H. umbellatum* × *H. alpinum*) revealed that they are identical.

The original plants of *H.* ×*grofae* collected by E. Wołoszczak are with high probability diploid as is evident from the pollen size homogeneity, an indirect indicator of diploidy, found in lectotype voucher. While particular ploidy levels in *Hieracium* s. str. do not differ significantly from each other in the mean pollen size, pollen from diploids strongly differs from that in polyploids with respect to its size homogeneity. In diploids, values of standard deviation and coefficient of variation do not exceed 3 μm or 7.5%, respectively. In contrast, triploid and tetraploid apomictic taxa (if they produce pollen at all) produce lower quantity of heterogeneously sized pollen grains (values of standard deviation and coefficient of variation always exceed 3 μm or 7.5%, respectively) (Mráz et al., 2002; Kovalčiková, 2004). In the genus *Taraxacum*, Sterk at al. (1982) found that diploids have standard deviation lower than 3 μm and polyploids higher than 3 μm. Considering the genus *Hieracium* as a whole, there is probably a correlation between the number and size of capitula and pollen size. Plants with small number of capitula per plant produced usually larger pollen than the plants with higher number of small capitula per plant. Although, this tendency was observed also in other analyzed diploid taxa (Kovalčiková, 2004), more detailed studies should be undertaken.

Wołoszczak (1892) proposed the parentage of *H.* ×*grofae* as *H. decipiens* × *H. umbellatum* var. *lactaris* Bertol. However, he was aware that this decision was rather arbitrary and he was not convinced that it was really correct. He has found the hybrids growing with *H. umbellatum* var. *lactaris*, *H. alpinum* and *H. decipiens* (from the taxonomical point of view, the plants determined by Wołoszczak as *H. decipiens* do not match *H. decipiens* Tausch and cannot be referred to it). While the parentage of *H. umbellatum* was clear, Wołoszczak (1892: 143) much doubted about the second parent (*H. alpinum* vs. *H. decipiens*).

According to our studies, plants from the Ukrainian Eastern Carpathians determined by E. Wołoszczak as *H. decipiens* (e.g. from Mt. Guretvyn, herb. W no. 15285) have no pollen grains and could hardly be involved in any natural hybridization. Unfortunately, we do not know the ploidy level of this taxon. However, morphologically nearly identical plants collected in 2003 by J.C. and P.M. at Mt. Pikuj (the Beskyds'ke vysokohir'ya Mts, the Ukrainian Carpathians) were found to be tetraploid (J.C. unpubl.). Thus, even if these plants had produced viable pollen grains, their hybrids with diploid plants (*H. umbellatum*) should have been triploid. *Hieracium alpinum* is much more likely to be a parent – it is diploid and sexual in the Ukrainian Eastern Carpathians (Chrtek, 1997; Mráz, 2001, 2003) and its capability to produce hybrids with other *Hieracium* species from different section has been proved experimentally (Mráz & Paule, 2006). Furthermore, *H. decipiens* sensu Woł. non Tausch has distinctly dentate leaves, while hybrid plants have entire to finely denticulate leaves (similarly to *H. alpinum* and *H. umbellatum*). Thus, we are sure that the hybrid formula should be *H. alpinum* × *H. umbellatum*.

Although many polyploid *Hieracium* species occu-

pying an intermediate position between two or more other species are undoubtedly results of past hybridization and introgression processes, recent spontaneous hybrids in *Hieracium* (s. str.) are most likely extremely rare. Besides *H.* ×*grofae*, only the hybrid between *H. alpinum* and *H. transsilvanicum* (*H.* ×*krasanii*) has been reported in the literature (Mráz et al., 2005). Two common features of the two hybrids deserve brief discussion:

(1) Both of them were discovered in the Ukrainian and/or Rumanian Eastern Carpathians. This part of the Carpathian arc harbours 3 diploid sexual hawkweed species, i.e. *Hieracium alpinum*, *H. transsilvanicum* and *H. umbellatum* (both lower altitude and high mountain morphotypes). Intermingled populations have never been observed mostly due to their different ecological demands. However, they are sometimes found in rather close vicinity allowing effective pollination (all hawkweeds are entomophilous) and hybrid formation.

(2) Both *H.* ×*grofae* and *H.* ×*krasanii* are very rare, only individual plants are found in the nature. This can be explained by only occasional effective pollination, due to distance between the parent plants, and/or by prezygotic fertilization barriers (competition between conspecific and heterospecific pollen/pollen tubes). Hybrid zones are situated on the secondary mountain grassland ("poloniny"), thus low germination rates due to an absence of suitable gaps and disturbed places can also play an important role. Low seedling vigour and competitive ability should also be suggested.

Hieracium ×*grofae* resembles taxa of the *Hieracium fritzei* group, but differs by having nearly glabrous ligule apices (ligules at apex with scattered to numerous short hairs in the *H. fritzei* group), obtuse phyllaries (acute in the *H. fritzei* group) and obtuse leaves (rather acute in the *H. fritzei* group). Plants of *H.* ×*grofae* are diploid and produce regularly-sized pollen grains, the *H. fritzei* group embraces tri- and tetraploid agamosperms with disturbed microsporogenesis (aborted or irregularly sized pollen). The total geographic range of the *H. fritzei* group includes the high Sudeten mountains (the Krkonoše Mts, Mt. Králický Sněžník), highest parts of the Western Carpathians, and Rumanian South Carpathians, but the occurrence in the Ukrainian Eastern Carpathians is unclear. Zahn (1930–1939) listed 5 localities from this area, namely "B. [Berg] Gorgan ilemski", "Grofa", "Alpe Sywula", "Pop Ivan" and "Czorna hora". The former 3 lie in the Horhany Mts; the latter localities ("Pop Ivan" and "Czorna hora") are situated in (or nearby) the area where we have discovered the hybrid plants and have never observed plants referring to the *H. fritzei* group. Chopyk (1977) reported *H. fritzei* from the Chornohora Mts, Prokudin (1987) gave only brief note "V Karpatach" [in the Carpathians]. Unfortunately, we have not found the respective herbarium voucher specimens and thus the occurrence of *H. fritzei* in the Marmarosh Mts (Mt. Pip Ivan) and the Chornohora Mts remains a moot point. However, based on our own observation (excursion in 1996) we are fairly certain that the two localities in the Zahn's monograph refer to *H.* ×*grofae* and that *H. fritzei* is absent from this area.

Hieracium umbellatum, one of the parent species of H. ×*grofae*, is well-known by an extreme range of variation mainly in the total height, number of stem leaves, leaf shape, and number and size of capitula (e.g. Turesson, 1922; Zahn 1930–1939; Lövkvist, 1962). Particular morphologically distinct types are often classified as varieties (Sennikov, 2003). All plants from the mountain grasslands in our area are characterized by distinctly broad cauline leaves. Moreover, the plants from the subalpine or even alpine belt of the Ukrainian Carpathians growing on exposed slopes of glacial cirques have only few (usually 3–5) large heads. This mountain morphotype is morphologically stable in cultivation and clearly distinct from lowland populations of *H. umbellatum*. In our previous papers we have used the names *H. conicum* (Chrtek, 1996) or *H. hryniawiense* (Mráz, 2003) for these diploid mountain populations. The former taxon was described from the Western Alps, for the latter name we have recently discovered an original herbarium material in KRAM. Neither of the names can be clearly applied to our mountain populations, and for this reason we treat them under the name *H. umbellatum* in this paper.

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Appendix 1

Origin of plant material used for pollen measurements

Localities are abbreviated and simplified; voucher specimens are deposited in the Herb. P. Mráz, except for lectotype specimen *H.* ×*grofae* (deposited in KRAM). Abbreviations: Sk – Slovakia, Uk – Ukraine.

H. alpinum (diploid cytotype; other plants from identical populations were counted karyologically, cf. Chrtek, 1997; Mráz, 2001, 2003)
Uk, Svydovets' Mts, Mt. Unharias'ka, coll. P. Mráz & V. Jurkovičová 1999 (1 plant)
Uk, Svydovets' Mts, Mt. Tatul, coll. P. Mráz & V. Jurkovičová 1999 (1 plant)
Uk, Svydovets' Mts, Mt. Blyznytsia, coll. P. Mráz & V. Jurkovičová 1999 (2 plants)
Uk, Chornohora Mts, Mt. Hoverla, coll. P. Mráz et al. 1996 (1 plant)

H.* ×*grofae – natural diploid hybrids
Uk, Horhany Mts, Mt. Grofa, coll. E. Wołoszczak 1889 (KRAM 148408)
Uk, Marmarosh Mts, Mt. Berlebashka, coll. R. Letz 1996 (1 plant, Ber1)
Uk, Svydovets' Mts, E of Mt. Unharias'ka, coll. P. Mráz & V. Jurkovičová 1999 (2 plants, Ungar1 and 2)

H.* ×*grofae – experimental diploid hybrids
X9/29 and X9/35 (for details on provenience of parental species, crossing scheme, chromosome numbers etc. see Mráz, 2003 and Mráz & Paule, 2006)

H. umbellatum – morphotype of low altitude (diploid cytotype; diploid chromosome number for plant for the first locality was published in Mráz, 2003):

Sk, Volovské vrchy Mts, village of Prakovce, coll. P. MRÁZ 1999 (1 plant, cult. no. 736).
Sk, Volovské vrchy Mts, town of Gelnica, coll. P. MRÁZ 1999 (1 plant)

H. umbellatum – high mountain morphotype (diploid cytotype; cf. CHRTEK, 1996 ut *H. conicum*; MRÁZ, 2003 ut *H. hryniawiense*)

Uk, Svydovets' Mts, Mt. Unharias'ka, coll. P. MRÁZ & V. JURKOVIČOVÁ 1999 (1 plant)
Uk, Svydovets' Mts, Mt. Heryshas'ka , coll. P. MRÁZ & V. JURKOVIČOVÁ 1999 (1 plant)

Príloha Q

Mráz P

Mentor effects in the genus *Hieracium* sstr (Compositae, Lactuceae)

Folia Geobotanica 38: 345–350, 2003

Folia Geobotanica 38: 345–350, 2003

MENTOR EFFECTS IN THE GENUS *HIERACIUM* S.STR. (*COMPOSITAE*, *LACTUCEAE*)

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Abstract: Self-incompatibility was demonstrated in the diploid taxa *Hieracium alpinum* and *H. umbellatum* using isolation experiments. However, self-incompatibility was broken down in diploid *H. alpinum* under the influence of foreign pollen from another species of this genus (mentor effects) during a series of crossing experiments. Interestingly, failure of the SI system was also recorded in diploid *H. alpinum* in an intergeneric cross with the representative of the closely related genus of *Pilosella*. This is the first record of autogamy in the genus *Hieracium* s.str. The possible evolutionary significance of this phenomenon is discussed.

Keywords: *Asteraceae*, Autogamy, Chromosome numbers, Hybridization, Self-incompatibility

INTRODUCTION

Self-incompatibility (SI) is the inability of a fertile hermaphrodite seed-plant to produce zygotes after self pollination (autogamy). It is an effective mechanism that encourages obligate outbreeding and enhances genetic diversity (e.g. RICHARDS 1997, CRUZ-GARCIA & MCCLURE 2001). However, there are several natural conditions under which an otherwise normally functional SI system may fail, such as delayed pollination, high temperature and mixture of self and heterospecific pollen (so-called mentor effects) (RICHARDS 1997). Mentor effects have been recorded from several genera of *Asteraceae* with sporophytic SI, e.g. *Helianthus* (DESROCHERS & RIESEBERG 1998), *Pilosella* (KRAHULCOVÁ et al. 1999), and *Taraxacum* (e.g. MENKEN et al. 1989, TAS & VAN DIJK 1999). Examples from other families include *Lotus corniculatus* (MIRI & BUBAR 1966), *Paspalum notatum* (BURTON & HANNA 1992) and other taxa cited by DE NETTANCOURT (2001) with references therein.

Although many species have been described in *Hieracium* s.str., we know far too little about the mechanisms of speciation that caused such immense diversity. Many experimental studies have been performed in order to clarify the evolutionary processes leading to the recent variation in the closely related genus *Pilosella*, often treated as subgenus *Pilosella* of *Hieracium* (for review see KRAHULCOVÁ et al. 2000). Sexual diploid species in the genus *Hieracium* s.str. are very rare; triploid and tetraploid taxa are much more common (MERXMÜLLER 1975, SCHUHWERK 1996, SCHUHWERK & LIPPERT 1998). For polyploids, the apomictic mode of reproduction (diplospory of the *Antennaria* type) was established by embryological experiments (e.g. BERGMAN 1941, SKAWIŃSKA 1963). Diploid taxa are considered as obligatory sexuals with regular mega- and microsporogenesis (GUSTAFSSON 1947). Until now, probably only one species at the diploid level (*H. alpinum*, from the

Ukrainian Eastern Carpathians) has been tested for self-incompatibility (CHRTEK 1997). Isolated capitula did not produce any viable seeds and the diploid *Hieracium alpinum* was considered as strictly self-incompatible under field conditions, requiring cross-pollination for seed production.

MATERIAL AND METHODS

In 2000 I started a large series of crosses between different species of the genus *Hieracium* s.str. of various ploidy levels occurring in the Carpathians (Slovakia, Ukraine and Romania). The plants used in hybridization experiments were grown and cross-pollinated in the lowland experimental garden under field conditions and also in a semi-open greenhouse (protection against rain) in the Botanical Garden of P.J. Šafárik University in Košice. Two main experiments were carried out: crosses (i) among diploid species, and (ii) between diploid (preferably as seed parents) and polyploid (tetraploid and triploid) species. All inflorescences of each parent involved in hybridization were isolated in nylon bags before anthesis (before opening of the first outer ligular flowers). At the stage of stigma receptivity, when the bifurcate stigmas protruded from the flowers, both parental capitula were rubbed together to enable transport of pollen to stigmas. Pollination was usually repeated 2–5 times per week for each pair of capitula. The pollinated capitula were isolated until the harvest of mature seeds. The progeny from cross-pollination was evaluated by means of morphology, because the parental species involved in the crosses could be easily distinguished by their morphological characters. For two diploid taxa (*H. alpinum* and *H. umbellatum*) an isolation experiment (by nylon bags) was done to confirm or exclude the presence of a self-incompatibility system.

For parental species the chromosome numbers were stated on mitotic metaphases prepared by method of MURÍN (1960). The ploidy level and chromosome number of each particular parent is given in Table 1 and in Appendix. From among the studied parental plants the diploid chromosome number was published previously only for *Pilosella lactucella* (ROTREKLOVÁ et al. 2002). Other chromosome numbers represent new counts.

RESULTS

Self-incompatibility

For one plant of diploid *Hieracium alpinum* (cultivation number 1018 / 1 isolated capitulum) and diploid *H. umbellatum* (cultivation number UMB12JP / 5 isolated capitula) self- -incompatibility was demonstrated. All seeds from the isolated capitula were small, white and completely empty, whereas some portion of full seeds was obtained from cross pollination.

Breakdown of SI system

Most of the F1 plants, as results of crossing experiments, were true hybrids (see Table 1). However, in some crosses clearly matroclinal plants arising from self-pollination were detected. This is the first record of autogamy in the genus *Hieracium* s.str.

In the crosses between diploid *H. alpinum* (mother seed plant, cultivation number 649) × *H. transsilvanicum* (diploid pollen donor, cultivation number 1064), altogether 4 mother-like

Table 1. Summarized results of hybridization experiments. Abbreviations used: ALP – *Hieracium alpinum* L., HRY – *H. hryniawiense* WOŁ., sect. *Alpina* – *H.* sp. (probably yet unnamed taxon of sect. *Alpina*), LAC – *Pilosella lactucella* (WALLR.) P.D. SELL et C. WEST, POJ – *H. pojoritense* WOŁ., UMB – *H. umbellatum* L., TRANS – *H. transsilvanicum* HEUFF., VAL s.l. – *H. valdepilosum* s.l. Ploidy level is given in parentheses after the cultivation number of each plant used in hybridization.

Parents		Progeny		
mother (seed) plant	father (pollen) plant	no. of total evaluated plants	no. of matroclinal plants	proportion of autogamously arisen progeny (%)
ALP (649, 2x)	TRANS (1064, 2x)	21	4	19
ALP (661, 2x)	LAC (763, 2x)	14	14	100
ALP (664, 2x)	sect. *Alpina* (827, 3x)	17	17	100
HRY (699, 2x)	ALP (639, 2x)	54	0	0
HRY (701, 2x)	VAL s.l. (374, 4x)	2	0	0
POJ (776, 2x)	VAL s.l. (374, 4x)	1	0	0
TRANS (1064, 2x)	ALP (649, 2x)	10	0	0
TRANS (1067, 2x)	UMB (736, 2x)	8	0	0
TRANS (1067, 2x)	POJ (776, 2x)	13	0	0

plants arising by autogamy (cultivation numbers X5/12, X5/13, X5/14, X5/21) and 17 true hybrids were detected. Interestingly, all hybrid plants flowered in the first year of cultivation, contrary to the selfed progeny. The reciprocal crosses between the same parental plants did not bring any autogamous progeny, but did produce true hybrids, all flowering in the first year of cultivation.

The ability of diploid *H. alpinum* to produce an F1 generation by selfing was also confirmed in the crosses between diploid *H. alpinum* (mother plant, cultivation number 664) and an unnamed triploid taxon from *Hieracium* sect. *Alpina* (pollen donor, cultivation number 827). Contrary to the above-mentioned case, all progeny (17 plants, arising from this experiment were evaluated as matroclinal plants. Three plants from selfing at the seedling stage were morphologically deviant: three true cotyledons on one seedling; very deeply cut lamina of one cotyledon in two seedlings. Normally, the leaf lamina of cotyledons in the genus *Hieracium* is entire.

Additionally to the crosses within the genus *Hieracium*, crosses between some representatives of *Hieracium* and *Pilosella* were undertaken. So far only the results from the crosses between diploid *Hieracium alpinum* (cultivation number 661) and diploid *Pilosella lactucella* (cultivation number 763) are available. All F1 mature progeny, 14 plants, obtained from one capitulum of *H. alpinum* (mother plant) have originated from autogamous events. It is noteworthy that 2 seedlings arising in this cross were complete albinos and they died after 3 weeks of cultivation. No plant from the F1 generation flowered in the first year of cultivation, as in previous experiments. No viable seeds were produced in the reciprocal cross combination of the same parental plants (*P. lactucella* as seed parent, *H. alpinum* as pollen donor). The lack of hybrids between the representatives of the closely related *Pilosella* and *Hieracium* underlines the differences between these two genera. No true hybrid between representatives of these genera (often treated as subgenera within large genus *Hieracium* s.l.) has been found in the field up to now.

DISCUSSION

Different proportions of offspring arising by selfing have been recorded in different SI incompatible taxonomic groups. High selfing rates were detected in diploid-triploid crosses in the genus *Taraxacum*. Almost 90% of the viable offspring were diploids with the same isozyme phenotype as mother plants (TAS & VAN DIJK 1999). High inbreeding in some crosses between diploid taxa of two taxonomically distant sections of *Taraxacum* was found by MENKEN et al. (1989). The total proportion of autogamously derived progeny, however, was lower in the genus *Pilosella*, varying between 6.2% (or 7.6% in those crosses in which mother-like offspring occurred) in sexual diploid *Pilosella lactucella* and 13% (or 25% in those crosses in which mother like offspring occurred) in sexual tetraploid *Pilosella officinarum* (KRAHULCOVÁ et al. 1999). GADELLA (1987, 1992) did not record any matroclinal offspring in his experimental hybridization in this genus. A lower proportion of matroclinal plants (5.1%) was obtained in interspecific crosses between strictly allogamous diploid species of *Helianthus* (DESROCHERS & RIESEBERG 1998). It seems that the selfing rate depends not only on the taxonomic position of the taxa used in crosses, but also on the particular combination of parental species or even individuals, and on environmental conditions (e.g. DESROCHERS & RIESEBERG 1998, KRAHULCOVÁ et al. 1999, MENKEN et al. 1989). Although the adaptive significance of the failure of the SI system is not clear, the breakdown of self-incompatibility could play an important role in reproductive isolation and hybrid speciation mainly in hybrid zones where mixed pollen loads are common. The breakdown of the SI system in *Hieracium* could be one of several causes, why natural primary hybrids in this genus are extremely rare. Until now, only one recent, truly hybrid plant of the genus is known from the field (MRÁZ et al. 2003).

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APPENDIX

List of the localities of taxa used in the crosses, or used in the tests for SI system (numbers of particular plants, accompanied by chromosome number, are given in brackets after each locality; * – counted by J. PAULE):

Hieracium alpinum L. – Ukraine, Svydovets' Mts.: Mt. Ungaryas'ka, N rocky slopes with *Alnus viridis*, 1705 m a.s.l., 48°18′ N, 24°06′ E, coll. P. MRÁZ and V. JURKOVIČOVÁ, 7 August 1999 (no. 639, 2n=18*). – Ukraine, Svydovets' Mts.: on the ridge between Mt. Stih and Mt. Blyznytsya, 1750–1850 m a.s.l., 48°14′ N, 24°14′ E, coll. P. MRÁZ and V. JURKOVIČOVÁ, 10 August 1999 (no. 649, 2n=18). – Ukraine, Svydovets' Mts.: Tatulska polonina ridge, saddle below Mt. Tatul, 1760 m a.s.l., 48°16′30″ N, 24°12′ E, coll. P. MRÁZ and V. JURKOVIČOVÁ, 10 August 1999 (nos. 661, 664, 2n=18). – Romania, Mţii Retezatului Mts.: on the ridge between Mt. Bârlea and Mt. Seşele Mari, 2300 m a.s.l., 45°20′ N, 22°22′ E, coll. P. MRÁZ, 8 July 2001 (1018, 2n=18).

Hieracium hryniawiense WOŁ. – Ukraine, Svydovets' Mts.: Gereshas'ka glacial cirque, below Mt. Dogyas'ka, rocky slopes, 1750 m a.s.l., 48°18′ N, 24°10′ E, coll. P. MRÁZ and V. JURKOVIČOVÁ, 7 August 1999 (no. 701, 2n=18).

Hieracium pojoritense WOŁ. – Romania, Mţii Bistriţei Mts.: Tulgeş, Mt. Piatra Runcului, calcareous rocks, 1150–1200 m a.s.l., coll. P. MRÁZ and V. JURKOVIČOVÁ, 17 July 2000 (no. 776, 2n=18).

Hieracium sect. *Alpina* – Romania, Mţii Bistriţei Mts.: Mt. Bogolini in the massive of Mt. Pietrosul Brostenilor, 1650–1700 m a.s.l., coll. P. MRÁZ, 20 July 2000 (no. 827, 2n=27)

Hieracium transsilvanicum HEUFF. – Romania, Mţii Rodnei Mts.: Mt. Pietrosul Mare, N slopes, spruce forest by the path Borşa – Staţia Meteo, 1300–1400 m a.s.l., 47°39′ N, 24°39′ E, coll. P. MRÁZ, 5 July 2001 (nos. 1064, 1067, 2n=18).

Hieracium umbellatum L. – Slovakia, Volovské vrchy Mts.: Prakovce, meadow below the ski lift, 390 m a.s.l., 48°48′45″ N, 20°54′33″ E, May 2000, coll. P. MRÁZ (no. 736, UMB 12JP – the plant from seed collected on this locality, 2n=18).

Hieracium valdepilosum s.l. – Slovakia, Veľká Fatra Mts., Malá Ramžiná valley, southern slope below the elevation 1497 m a.s.l., ca. 1 km WSW of Mt. Krížna (1574), 1340 m a.s.l., 48°52′34″ N, 19°04′02″ E, coll. P. MRÁZ, 12 July 1997 (no. 374, 2n=36).

Pilosella lactucella (WALLR.) P.D. SELL et C. WEST (syn. *Hieracium lactucela* WALLR.) – Slovakia, Volovské vrchy Mts.: Krivé sedlo saddle between Mt. Ramzová and Mt. Biele Skaly, 1110 m a.s.l., 48°43′50″ N, 20°38′08″ E, coll. P. MRÁZ and V. JURKOVIČOVÁ, 14 June 2000 (no. 763, 2n=18, originally published in ROTREKLOVÁ et al. 2002).

Príloha R

Mráz P, Tomčíková D

Experimental hybridization in the genus *Hieracium* s. str. – crosses between diploid *H. umbellatum* and triploid *H. sabaudum*

Thaiszia – Journal of Botany 14, Supplement 1: 15–16, 2004

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Experimental hybridization in the genus *Hieracium* s. str. – crosses between diploid *H. umbellatum* and triploid *H. sabaudum*

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Natural hybridization may have a variety of evolutionary consequences. The reticulate pattern of morphological variation in the genus *Hieracium* may result from large scale hybridization. However, at present we know far too little about the detailed processes, which led to such a complicated taxonomic structure within this genus. Moreover, there are very few studies on recent natural hybridization in *Hieracium* (MRÁZ et al. unpubl.).

Relevant data on possible hybrid origin, hybrid fitness, mode of reproduction, direction of gene flow, etc., may be obtained from controlled experimental hybridization. In 2000 we started with large series of crosses among diploid taxa and between diploid and polyploid taxa. The partial results from mainly diploid × diploid crosses were showed at the 7th *Hieracium* workshop (MRÁZ & PAULE 2003). Here, we present the preliminary data mainly from crosses between strictly allogamous diploid *Hieracium umbellatum* L. (mother plant) and apomictic triploid species *H. sabaudum* s. l. (pollen donor). These taxa are probably very closely related: they are very similar from morphological and phenological point of view, and they can be found in a similar range of biotopes, although no data of spontaneous recent hybrids were recorded. An indisputable advantage of both taxa in experimental hybridization is the presence of many flower heads per stem in both species, and thus several kinds of experiment can be carried out on one plant (control cross, open pollination, isolation, emasculation).

Four crosses using different parents of both taxa were carried out. One cross failed completely and no developed seeds were obtained. In 2 crosses, all progeny, altogether 10 plants, arose from self pollination (mentor effect). In the final cross all F1 offspring, 32 plants together, were identified as true hybrids. Morphologically, the plants surprisingly resembled *H. racemosum* s. l., with the leaves typically arranged just above the stem base. All hybrids counted so far

(14 plants) are diploid with a somatic chromosome number 2n = 18. This means that only reduced haploid pollen (n = 9) of triploid *H. sabaudum* s. l. contributed to the hybrid formation. Six studied hybrids did not produce normally developed seeds after isolation in nylon bags, and six studied hybrids produced (0–)7.3–15.7 % of good seeds after open pollination, much higher than we obtained from diploid × diploid crosses (0–5.4%; MRÁZ & PAULE 2003, PAULE 2003). The good seed set could be explained by the close relationship of the parental species (in diploid crosses we used morphologically very distant taxa), and by presence of many *H. umbellatum* plants surrounding of our hybrids. On the other hand, the percentage of good seed set in open pollination of diploid *H. umbellatum* was higher (48–84 %) than in hybrid plants. Partially seed-sterile hybrids produced a considerable amount of pollen of homogeneous size.

Additionally, we crossed the diploid *Hieracium umbellatum* (mother plant), with triploid *H. virgicaule* NÄGELI & PETER (*umbellatum* – *bupleuroides*) and diploid *Pilosella echioides* (LUMN.) F. W. SCHULTZ & SCH. BIP. (pollen donor). We obtained only maternal plants in both cases, 42 and 4 plants respectively. The mentor effect (induced autogamy) in otherwise strictly self-incompatible diploid *H. umbellatum* was found and this phenomenon may have a great influence in the low rate of recent natural hybridization within genus *Hieracium*.

Acknowledgement

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Príloha S

Mráz P, Paule J

Experimental hybridization in the genus *Hieracium* s.str. (Asteraceae): crosses between selected diploid taxa

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Experimental hybridization in the genus *Hieracium* s. str.: crosses between diploid taxa

Experimentálna hybridizácia v rode *Hieracium* s. str.: kríženie vybraných diploidných druhov

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Mráz P. & Paule J. (2006): Experimental hybridization in the genus *Hieracium* s. str.: crosses between diploid taxa. – Preslia 78: 1–26.

The few attempts to produce artificial hybrids in the genus *Hieracium* s. str. have usually failed due to the use of polyploid parental taxa reproducing via agamospermy. Presented here for the first time are data on artificial hybridization in *Hieracium* s. str. which may help in understanding the microevolutionary processes resulting in the great morphological and genetic diversity in this genus. Diploid, sexually reproducing species (*H. alpinum*, *H. pojoritense, H. transsilvanicum* and two stable morphological types of *H. umbellatum* – of a low altitude and a high mountain type) were used as parent plants in experimental crosses. In most cases true hybrids, with intermediate morphology, were obtained. All the hybrids tested were diploid and produced a high amount of stainable pollen (65–92%). Hybrid progeny resulting from one cross exhibited a large range of morphological variation due to the combination of alleles from unrelated parental species. The percentage of well-developed achenes per capitulum, in capitula with at least one well-developed achene, in hybrids, ranged from 1.9 to 12.5% after free or controlled pollination, with an average of 4–5% per capitulum. Similar results (1.9–12.1%) were obtained from triple-cross hybrids. However, most of the capitula of hybrid progeny (either F1 or triple) were completely sterile after free or controlled pollination. Sterility is probably caused by genome incompatibility of unrelated parental taxa belonging to different sections. In two crosses, where strictly allogamous diploid plants of *H. umbellatum* (both morphotypes) were used as mother plants and F1 hybrids as pollen donors, some matroclinal progeny were obtained. This is a further example of the previously reported mentor effect. Diploid hybrids may be involved as pollen donors in gene flow as they produce uniformly sized and viable pollen. They are probably substantially less important as seed parents.

K e y w o r d s : apomixis, *Compositae*, homoploid hybridization, hybrid fitness, *Lactuceae*, sterility

Introduction

Interspecific hybridization is considered to be important in plant speciation (Arnold 1992, Rieseberg 1997). Information on natural hybridization and its rate is crucial for understanding the evolution of species. However, one or more hybrids have been recorded only for ca 6–16% of plant genera (Ellstrand et al. 1996). Apart from the data on spontaneous hybridization in the field, experimental crosses have contributed much to the identification of microevolutionary processes like species compatibility, pre- and postzygotic isolation barriers, pollen competition, reproductive capacity of hybrid progeny, selection and the role of introgression (Nieto Feliner et al. 1996, Nieto Feliner 1997, Rieseberg & Carney

1998, Khalaf & Stace 2000, Lihová et al. 2000, Burke & Arnold 2001, Mooring 2002, Lexer et al. 2003, Jarolímová 2005).

The holarctic genus *Hieracium* L. s. str. is taxonomically one of the most complicated and at the same time most species-rich genera in the plant kingdom (see the number of entries in Index Kewensis, Royal Botanical Gardens Kew, 1993). The complexity of the genus is reflected in the reticulate pattern of morphological variation (Zahn 1921–1923), widespread polyploidy and agamospermic formation of seeds in polyploid taxa. Most of the karyologically studied taxa are tri- or tetraploids (x = 9, 2n = 3x = 27, 2n = 4x = 36, respectively) (see Schuhwerk 1996 and references therein). There are few chromosome numbers above the tetraploid level (Stace et al. 1995, Chrtek 1996, Pul'kina & Tupitsyna 2000) and aneuploids are extremely rare (for references see Schuhwerk 1996). Polyploidy is connected with gametophytic apomixis, the diplospory of *Antennaria*-type (Gustafsson 1946). So far, there is no evidence of facultative apomixis in the genus and therefore obligate apomixis is usually considered as the sole mode of reproduction in *Hieracium* polyploids. In contrast, there is a high number of polyploids, but sexually reproducing diploids (2n = 2x = 18) are rare and confined mostly to the southern part of the distributional range of the genus (mainly S Europe, for actual list of diploids see Chrtek et al. 2004). For some diploid taxa strict allogamy (self-incompatibility) was proven by isolation experiments (Turesson 1922, Chrtek 1997, Mráz 2003). However, in a series of different interspecific crosses, an induced autogamy in diploids (so called "mentor effect") was recorded (Mráz 2003).

It seems that interspecific hybridization along with introgression were the main evolutionary processes resulting in the great diversity in this genus. The following facts support a hybrid origin of polyploid *Hieracium* taxa: (i) Polyploids are morphological intermediates among themselves as well as among several diploid species (Zahn 1921–1923). (ii) Molecular studies have shown fixed heterozygosity in polyploid taxa (Stace et al. 1997, Mráz et al. 2001, Štorchová et al. 2002, Chrtek & Plačková 2005; high level of heterozygozity was found also in polyploids of the closely related genus *Pilosella*, Peckert et al. 2005). Generally, fixed heterozygosity is considered sound support for an allopolyploid origin (e.g. Asker & Jerling 1992, Brochmann et al. 2004). (iii) Abnormal microsporogenesis is recorded in several *Hieracium* polyploid taxa. Various numbers of uni-, bi-, tri- or tetravalents show uneven chromosome pairing due to chromosome heterology (Rosenberg 1927, Gentcheff & Gustafsson 1940, Aparicio 1994) resulting in the production of variable sized pollen or even full male sterility in polyploids. This feature may be as a good indicator of polyploidy in this genus (Mráz et al. 2002). (iv) Many plant hybrids of recent origin do not produce seed. From this point of view, apomixis may represent an elegant mechanism for "escaping from sterility" (Asker & Jerling 1992).

Nowadays interspecific hybridization in the genus *Hieracium* is probably highly restricted. There are a few records of recent natural hybrids between diploid taxa (Mráz et al. 2005; and some other not yet published records of Chrtek & Mráz, see Material and Methods). Similarly, interspecific hybridization among diploid taxa within closely related genera *Stenotheca* Monn. (Guppy 1978) and *Pilosella* Hill. (Turesson 1972) may occur, but the hybrids are seldom found in nature. On the other hand in the genus *Pilosella* natural hybridization between diploid and polyploid taxa and among polyploids is relatively common (reviewed in Krahulcová et al. 2000)

Considering that *Hieracium* s. str. belongs to the most species-rich genera in the world it is surprising that there are almost no data on experimental crosses in this genus. This is in strong contrast to *Pilosella*, where several tens of papers on this topic have been published so far (see Krahulcová et al. 2000 and references therein).

After his successful experiments with *Pisum* L., Johann Gregor Mendel was among the first scientists to produce artificial hybrids of *Hieracium* s.l. (Krahulcová et al. 2000). While in the case of the closely related genus *Pilosella* the crossing ability was high (Mendel 1870), Mendel did not obtain interpretable and publishable data from crossing *Hieracium* s. str. (see some short notes in his letters to Carl Nägeli in Results and discussion) (Mendel 1950). Mendel obtained few hybrids from crosses in which *H. umbellatum* L. was used as the pollen donor and an unnamed species, later classified as *H. racemosum* Willd., as well as from crosses between *H. umbellatum* and *H. vulgatum* Fr. (s.l.). Mendel also obtained progeny from experimental hybridization of *H. murorum* L. and *H. umbellatum*, but they did not look like hybrids and clearly resembled the maternal plant. Their origin was explained by self-fertilization (Mendel 1950: 2), although it was likely agamospermy, an unknown phenomenon at the time Mendel was doing his experiments. Similarly, all efforts of Ostenfeld to produce hybrids via artificial hybridization were unsuccessful. However, he noted that the offspring from unprotected plants of the sexual species *H. virgicaule* Nägeli et Peter were very heterogeneous in the terms of morphology (Ostenfeld 1921). Later, Zlatník (1938: 41) outlined the results of artificial reciprocal crosses between *H. alpinum* L. and *H. murorum*. He obtained matroclinal progeny that reproduced apomictically ("... apogamishe Bastarde ... sind matroklin ..."), but considered them to be hybrids.

In order to study microevolution within the genus *Hieracium*, the first author of the present paper, later on with help of his diploma students, experimentally hybridized selected diploid, and diploid and polyploid taxa. Among the interesting results was the first record of autogamy in crosses among diploid and between diploid and polyploid taxa (Mráz 2003).

In this paper, the results of crosses between several diploid taxa, focusing on the characterization of hybrid progeny by means of morphology, pollen quality, chromosome number and reproductive capacity, are presented.

Material and methods

Parental taxa used in crosses

Parent plants were collected during the years 1996–2001 at various sites in Slovakia, Romania and Ukraine (for exact localities see Appendix 1 in Mráz 2003). After transplantation, plants were cultivated and cross-pollinated in a lowland experimental garden under field conditions and in some cases in unheated semi-open greenhouse in the Botanical Garden of P. J. Šafárik University of Košice. The following taxa were used as mother and / or pollen donor plants: *Hieracium alpinum, H. pojoritense* Woł., *H. transsilvanicum* Heuff. and two morphologically distinct types of *H. umbellatum* (Figs 1–5; see Electronic Appendix 1 (http://www.ibot.cas.cz/preslia) for colour images of Figs 1–12). High mountain type of *H. umbellatum* from the Eastern Carpathians differs from the morphotype of low altitude having small number of capitula (usually 3–5; low altitude type usually 20–50),

distinctly bigger capitula than the type of low altitude, low number of stem leaves (up to 15; the most usually above 20 in the type of low altitude), broadly elliptic to ovate stem leaves (type of low altitude has lanceolate, oblong lanceolate to oblong elliptic stem leaves). Moreover, the plants of high mountain type are smaller (up to 30–40 cm) then *H. umbellatum* coming from the low altitude (usually above 50 cm). The differences between both types are stable also in cultivation (3 years of observations). For high mountain populations of *H. umbellatum* from the Ukrainian Carpathians two names have been recently used in the literature: *H. conicum* Arv.-Touv. (Chrtek 1996) and *H. hryniawiense* Woł. (Mráz 2003). The first taxon was originally described from the French Alps and probably does not reach the territory of the Carpathians (in a narrow sense). The latter one, described from the Ukraine, with high probability represents another taxon closely related *H. sabaudum* s.l. (based on study of scanned original material of *Hieracium hryniawiense* Woł. kindly sent to us by J. Chrtek jun.). In this respect, the use of both names for these high mountain populations seems to be incorrect. For these reasons we treated these high mountain populations within hypervariable *H. umbellatum*.

Each parent was examined karyologically and all proved to be diploids (Mráz 2003). The hybridization scheme is given in Table 1. All parent taxa were easily recognized by means of their morphology and all belong to different sections sensu Stace (1998). In addition to the difference in morphology each particular taxon differs in ecology and altitudinal preferences. The diploid cytotype of *H. alpinum* (*H*. sect. *Alpina*) is confined to the alpine and subalpine belts of the Eastern and Southern Carpathians (Chrtek 1997, Mráz & Szeląg 2004). The second species, *H. pojoritense* (*H*. sect. *Italica*), is a stenoendemic taxon of calcareous relict rocks of the montane and submontane belt in NE Romania (Ştefureac & Tăcină 1979). *Hieracium transsilvanicum* (*H*. sect. *Vulgata*) is a typical species of spruce and fir-beech forests in the Eastern and Southern Carpathians and Balkan Peninsula. While the high mountain populations of *H. umbellatum* usually grow on rocky slopes in glacial cirques or on subalpine meadows, a form of low altitude prefers abandoned meadows and forest margins in most parts of Eurasia (Zahn 1921–1923, both morphological types of *H. umbellatum* belong to the *H*. sect. *Hieracioides*). The above-mentioned taxa usually do not co-occur. However, they were rarely observed in close proximity at some localities (e.g. *H. alpinum* and high mountain type of *H. umbellatum*, *H. alpinum* and *H. transsilvanicum* or *H. pojoritense* and *H. transsilvanicum*; personal observations of the first author). In two cases, spontaneous hybridization in the field was observed between *H. alpinum* and *H. transsilvanicum* (Mráz et al. 2005 at two sites, J. Chrtek & P. Mráz, unpubl., at one site), and *H. alpinum* and high mountain *H. umbellatum* (J. Chrtek et al., in prep., at two localities).

Artificial hybridization

The inflorescences of plants used in the experiments were enclosed in nylon bags until anthesis to prevent cross-pollination. The crosses were made at the stage of stigma receptivity by softly rubbing the capitula together, to facilitate transfer of pollen to the stigmas, 1–2 times per day for 2–5 consecutive days, if possible. Apart from the primary crosses between the above-mentioned taxa, the progeny of these crosses (F1 hybrids) were subjected to further artificial (triple) hybridization with diploid plants, which flowered at the same time as the F1 hybrids.

Fig. 1. – Habitus of parental taxon *Hieracium alpinum* L., scale bar = 10 cm. See Electronic Appendix 1 for colour images of Figs 1–12.

Fig. 2. – Habitus of parental taxon *Hieracium pojoritense* Woł., scale bar = 10 cm.

Fig. 3. – Habitus of parental taxon *Hieracium transsilvanicum* Heuff., scale bar = 10 cm.

Fig. 4. – Habitus of parental taxon *Hieracium umbellatum* L. (high mountain morphotype). This plant (no. X22/3) arose from selfing in the cross no. X22 (see text), scale bar = 10 cm.

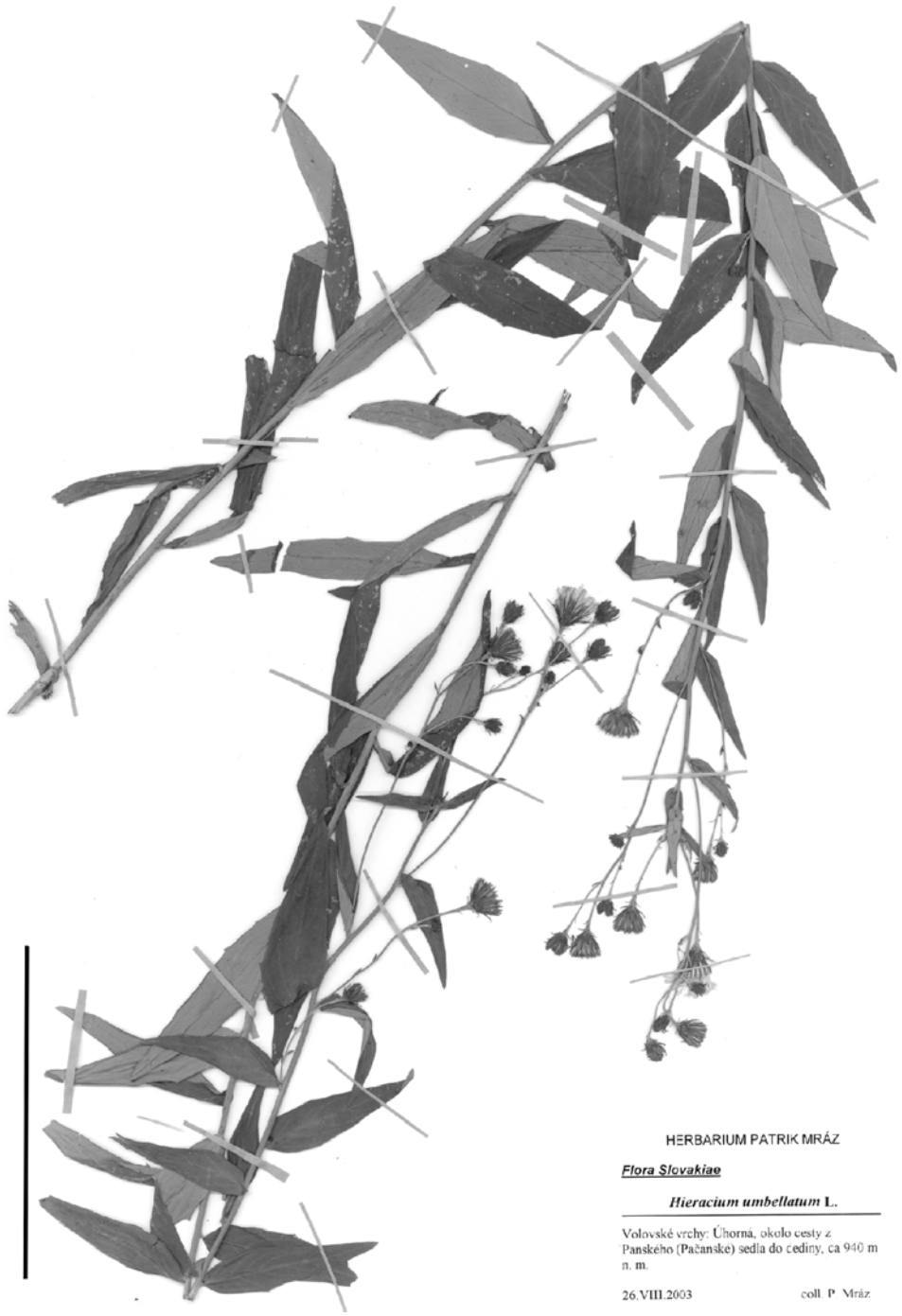

Fig. 5. – Habitus of parental taxon *Hieracium umbellatum* L. (morphotype of low altitude), scale bar = 10 cm.

Fig. 6. – Hybrid progeny from cross no. X1: *Hieracium transsilvanicum* × *H. umbellatum* (morphotype of low altitude), scale bar = 10 cm.

Table 1. – Experimental interspecific hybridization involving some diploid species of the genus *Hieracium* L. The first parent in a cross is always the mother plant, the second the pollen donor. Numbers below species names are the cultivation numbers of the parental taxa. (L) – morphotype of low altitude, (H) – high mountain morphotype, R – reciprocal cross.

Cross no.	Parents	Well-developed fruit (%)	Germination (%)	No. of F1 progeny		
				Evaluated	Hybrids	Selfs
	H. transsilvanicum × *H. umbellatum* (L)					
X1	1067 × 736	100	53	8	8	0
	H. transsilvanicum × *H. alpinum*					
X2	1064 × 649	68	32	10	10	0
X5	R	72	40	21	17	4
	H. umbellatum (H) × *H. alpinum*					
X9	699 × 639	100	72	54	54	0
	H. transsilvanicum × *H. pojoritense*					
X10	1067 × 776	100	42	13	13	0

Estimates of fruit quality

Full mature achenes were collected and classified as well-developed or poorly developed. While the well-developed fruits were stiff, plump, well-pigmented, black, brown or dark red, poorly developed achenes were narrow, flimsy and weakly pigmented or even white. Well-developed fruit may or may not contain an embryo; poorly developed fruit always lack embryos (confirmed by stereomicroscopy). The fruit-set was determined as the percentage of well-developed achenes among the fruit within a capitulum of those capitula with at least one well-developed fruit. If the capitulum did not produce any well-developed achenes, just the number of sterile capitula and the number of plants with no well-developed fruit were recorded.

Germination

After a 6–8 month cold stratification, the well-developed achenes were germinated in Petri dishes on wet filter paper. Germination was measured as the percentage of the well-developed achenes that germinated.

Evaluation of progeny resulting from artificial hybridization

The progeny from cross-pollination were evaluated by means of morphology (shape, size, number and position of the leaves on the stem; shape and size of phyllaries; indumentum of the leaves, stems, peduncles and phyllaries; style colours; stem branching pattern; number of capitula). Parental species involved in the crosses were easily distinguished by their morphological characters, which were also apparent in the progeny.

Estimate of pollen viability

Striking differences in the production of pollen by diploid and polyploid taxa of *Hieracium* s. str. have been recorded. The diploids produce an abundance of uniformly sized pollen, while polyploids produce either a little pollen of variable size or no pollen (Aparicio 1994, Mráz et al. 2002, Kovalčiková 2004). As F1 diploid hybrids produce uniformly sized pollen, we focused on estmating pollen viability. Male fertility of hybrid progeny was estimated by staining the pollen with acetocarmine in glycerol jelly (Marks 1954). In order to remove the pollen three unopened tubular flowers per capitulum were dissected with a razor blade in a drop of acetocarmine jelly. One-hundred pollen grains per individual were evaluated, and both viable (well-stained) and non-viable (unstained) grains were scored.

Chromosome number

The chromosomes were counted in mitotic metaphases in the root tips of the hybrid progeny cultivated in pots. For details of the method see Mráz in Chrtek et al. (2004). The number of karyologically analyzed plants are given in Tables 2 and 4.

Reproductive capacity of hybrid progeny

In order to determine the reproductive capacity, the number of well-developed fruit produced by F1 and triple hybrids from three types of experiments were scored: (i) F1 and triple hybrids were crossed with diploid *Hieracium* taxa, (ii) were reciprocally crossed or (iii) they were kept unisolated and exposed to free pollination. The source of pollen for free pollination, in addition to the hybrids and their parents, may have been from ca 200 plants of *Hieracium*, belonging to different taxa and ploidy levels, cultivated together with the hybrid progeny. Some capitula of F1 or triple hybrids were isolated during the whole flowering period to test the self-incompatibility system (SI).

Results and discussion

Hybridity

The proportion of well-developed fruit produced by the primary crosses between diploid taxa was relatively high. Mean fruit set per cross ranged between 68–100% with an average of 88% (Table 1). The mean germination of the achenes that originated from these crosses was 48%. Almost all F1 progenies exhibited clear hybrid origin. Only 4 plants (19%) in cross no. X5 arose via autogamy, the rest of the offspring were true hybrids. This is the first detected occurrence of autogamy in the otherwise self-incompatible genus *Hieracium* (Mráz 2003), although a total failure of the self-incompatibility system is recorded for several diploid – triploid crosses (see Mráz 2003, Mráz & Tomčíková 2004). From the above results it is obvious that hybridization at the diploid level is possible. The results of the very rare attempts at artificial hybridization in *Hieracium* s. str., which were unsuccessful or at least not clearly interpretable (cf. experiments of Mendel, Ostenfeld and Zlatník mentioned in Introduction), can now be acounted for. Mendel had no idea about

Fig. 7. – Hybrid progeny from cross no. X5: *Hieracium alpinum* × *H. transsilvanicum*, scale bar = 10 cm.

Fig. 8. – Hybrid progeny from cross no. X9: *Hieracium umbellatum* (high mountain morphotype) × *H. alpinum*, scale bar = 10 cm.

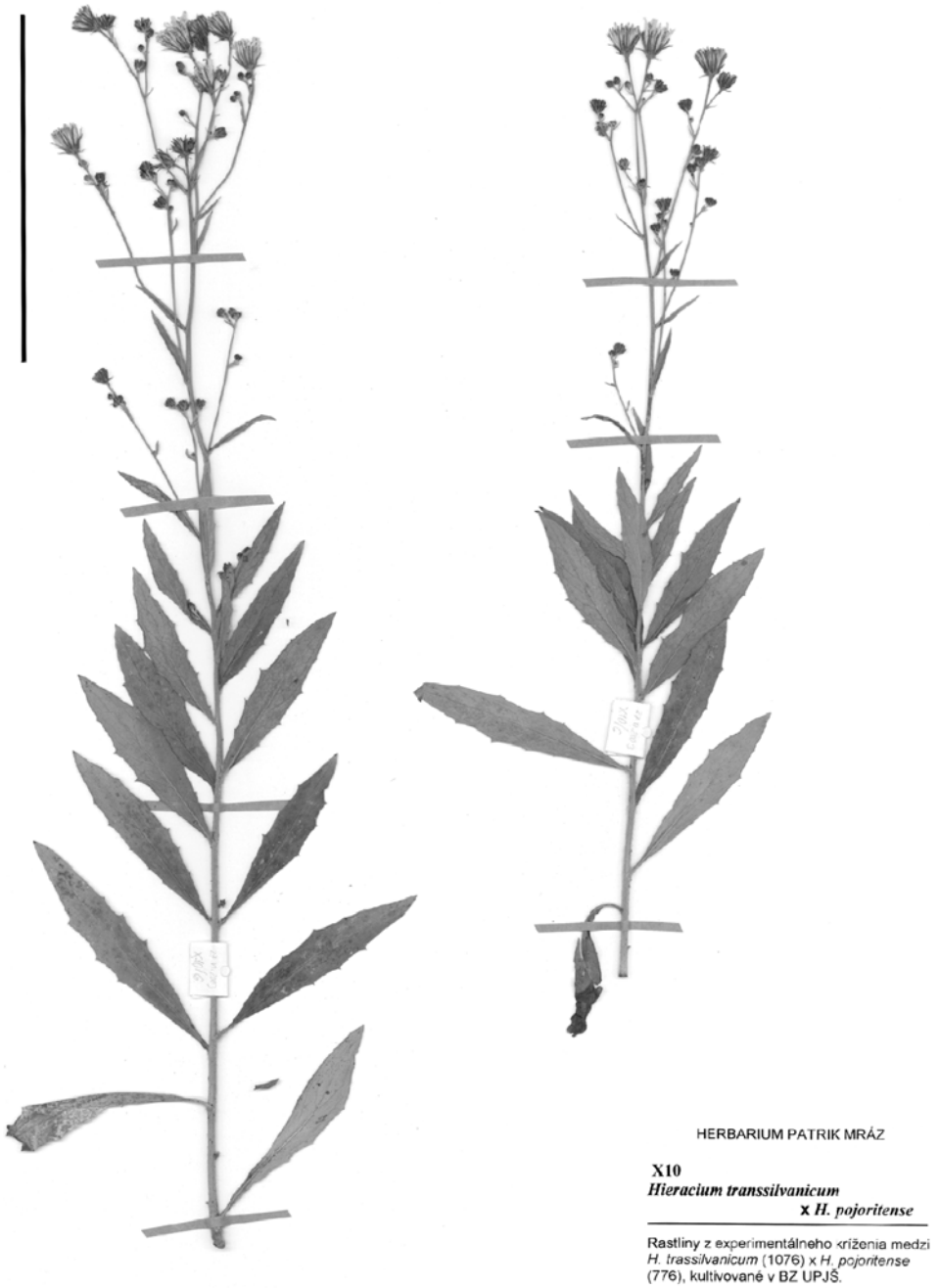

Fig. 9. – Hybrid progeny from cross no. X10: *Hieracium transsilvanicum* × *H. pojoritense*, scale bar = 10 cm.

Fig. 10. – Hybrid progeny from cross no. X10: *Hieracium transsilvanicum* × *H. pojoritense*, scale bar = 10 cm.

Fig. 11. – Triple hybrids from cross no. X21: (*Hieracium transsilvanicum* × *H. umbellatum* – morphotype of low altitude) × *H. umbellatum* – high mountain morphotype, scale bar = 10 cm.

Fig. 12. – Triple hybrids from cross no. X22: *H. umbellatum* – high mountain morphotype × (*Hieracium transsilvanicum* × *H. umbellatum* – morphotype of low altitude), scale bar = 10 cm.

the existence of apomixis in *Hieracium* the first embryological evidence for which was reported early in the 20th century (Rosenberg 1917). Most of the taxa used by Mendel were polyploids, which form seed apomictically. His notes in letters to C. Nägeli (Mendel 1950: 2, 21, 26): "..., I fear that in spite of all precautions, self-fertilization did occur"; "I have not yet succeeded in producing hybrids of Archieracia, ..."; "In the Archieracia it is very difficult to prevent self-fertlization"; "Thus far only two hybrids have been obtained", clearly show that almost all the progeny Mendel obtained were of apomictic origin. The important feature of this diplospory of *Antennaria* type is precocious embryony, where the unreduced egg cell develops into an embryo before the flowers open so that fertilization is impossible (Bergman 1941: 29). Thus, the possibility of hybridization, with *Hieracium* polyploids serving as mother plants, is highly limited. For this reason, Zlatník's record of obtaining apomictically reproducing hybrids from reciprocal crosses between *H. alpinum* and *H. murorum* (Zlatník 1938: 41), is rather surprising. Both taxa are usually triploid apomicts. On the other hand, Zlatník might have used a diploid cytotype of *H. alpinum* from the Eastern Carpathians in the Ukraine, obtained on one of his many botanical field trips there in the first half of 20th century. Unfortunately, he did not mention the exact provenance of the plant material used in his experiments.

Morphology of hybrid progeny

The F1 hybrids are usually intermediate in most morphological characters. However, in the reciprocal crosses (X2 and X5) between *Hieracium alpinum* and *H. transsilvanicum* and in the cross between high mountain type of *H. umbellatum* and *H. alpinum* (X9), the hybrids usually more closely resemble the mother plant than the pollen donor (mainly in the size and number of capitula, or in general habit/branching pattern in the latter case; Fig. 8). This is also the case of the rare recent natural hybrids between *H. transsilvanicum* and *H. alpinum* (Mráz et al. 2005). Although coming from two different crosses (X1: *H. transsilvanicum* × *H. umbellatum* – a type of low altitude and X10: *H. transsilvanicum* × *H. pojoritense*), the F1 hybrids are more or less similar to each other in general habit (Figs 6 and 9), number of stem leaves, position of stem leaves, and character of indumentum.

Hybrid progeny of one cross exhibit high levels of morphological variation (number and size of capitula, character of indumentum, shape and position of the stem leaves, branching pattern, etc.; Figs 6–12) due to the different allelic combinations of the unrelated parental taxa. The characteristic trait of the hybrids from the different crosses is a distinctive complex branching pattern, unusual in the parental taxa. Hybrids usually form numerous lateral branches often along the whole stem.

Interestingly, intermediacy in phenology was recorded in the hybrid plants from the cross (X1) between two phenologically different taxa *H. transsilvanicum* and *H. umbellatum*. While the former species usually flowers in cultivation from mid-May to the beginning of June, the latter blooms from mid-July to the mid-September. The hybrids start to bloom from the end of June.

Chromosome numbers

The diploid chromosome number (2n = 18) was recorded in all the hybrids (Table 2). This means that the homoploid artificial hybridization yielded hybrids of the same ploidy level

Table 2. – Characterization of F1 hybrid plants obtained from primary crosses (details given in Table 1.). Percentage of well-developed fruit as counted only for those capitula that produced at least one well developed achene. Abbreviations: Ncap – number of capitula, Nfr – total number of fruit, Npl – number of plants, Nwellfr – number of well-developed fruit, x – mean. Note: Only well-developed fruit was germinated. The percentage germination is scored as germinated fruit/well-developed fruit × 100.

Cross no.	Chromosome number		Stainable pollen			Well-developed fruits in free pollination (only successful pollination scored)			Unsuccessful free pollination	Germination (%)
	2n	Npl	range (%)	x (%)	Npl	range (%)	x (%)	Npl / Ncap / Nfr / Nwellfr	Npl/Ncap	
X1	18	4	80–87	85	3	3.2–5.4	4.2	3 / 5 / 184 / 8	4 / 24	50
X2	18	4	–	91	1	–	–	–	1 / 3	–
X5	18	7	–	92	1	1.9–12.5	5.5	1 / 3 / 148 / 8	1 / 3	12.5
X9	18	5	75–85	80	3	2.8–7.2	4.9	3 / 4 / 263 / 13	3 / 6	50
X10	18	8	65–85	77	3	3.0–6.1	4.5	3 / 8 / 243 / 11	11 / 115	33.3

Table 3. – Control crosses in which the F1 hybrids were the mother plants or pollen donors. The first parent in a cross is the mother plant, the second the father plant. Abbreviations: Ncap – number of capitula, Nfr – total number of fruit, Npl – number of plants, NT – not tested, Nwellfr – number of well-developed fruit, x – mean, s.n. – sine numero. Abbreviations of species names: ALP – *Hieracium alpinum*, POJ – *H. pojoritense*, TRANS – *H. transsilvanicum*, UML – *H. umbellatum,* morphotype of low altitude, UMH – *H. umbellatum,* high mountain morphotype. Note: Only well-developed fruit was germinated. The percentage germination is scored as germinated fruit/well-developed fruit × 100.

Cross no.	Parents	Well-developed fruits (only successful pollination scored)			Unsuccessful pollination	Germination	No. of triple progeny		
		range (%)	x (%)	Ncap / Nfr / Nwellfr	Ncap	(%)	Evaluated	Hybrids	Selfs
	(TRANS × UML) × UMH								
X21	X1/1 × 700	3.2–5.7	4.8	5 / 187 / 9	0	55.6	3	3	0
X22	R	13.8–59.4	37.9	4 / 259 / 98	0	60.2	16	7	9
	(TRANS × UML) × UMH								
s.n.	X1/7 × 700	–	3.7	1 / 27 / 1	0	NT	–	–	–
s.n.	R	27.0–28.9	27.9	2 / 75 / 21	0	NT			
	ALP × TRANS								
s.n.	X5/9 × X2/18	–	1.7	1 / 57 / 1	1	100	–	–	–
s.n.	R	–	–	–	4	–	–	–	–
	(TRANS × POJ) × UML								
s.n	X10/15 × 12JP	–	4.2	1 /24 / 1	6	100	–	–	–
X26	R	8.6–28.8	18.7	2 / 94 / 20	2	65	5	4	1

as the parental taxa, with the participation of both male and female reduced gametes. Rare recent hybrids between different diploid parental taxa are also diploid (Mráz et al. 2005, J. Chrtek et al., in prep.). Merxmüller (1975: 193) records an allegedly spontaneous diploid hybrid *H. leiocephalum* Bartl. ex Griseb. which arose from an interspecific cross between two unprotected diploid taxa, *H. porrifolium* L. and *H. umbellatum* L., cultivated in Munich botanical garden.

Pollen viability

The percentage of pollen of the hybrids that stained was in the range 65–92% (Table 2), which is quite similar to that of some diploid taxa and diploid natural hybrids (Mráz et al. 2005). High stainability and production of uniformly sized pollen (although not measured here) clearly indicate regular microsporogenesis in our F1 interspecific progeny. No abnormality in pollen size of different diploid *Hieracium* taxa is recorded (Kovalčiková 2004). Also no abnormalities in chromosome pairing during microsporogenesis are recorded in diploid interspecific hybrids within a closely related genus *Stenotheca* (syn. *Hieracium* subgen. *Chionoracium* Sch. Bip.), although some hybrids have a very low percentage of stainable pollen (Guppy 1978).

Reproductive capacity of artificial interspecific hybrids

The reproductive capacity of hybrid progeny was evaluated in terms of the percentage of well-developed fruit produced when flowers were isolated, freely pollinated and by controlled crosses. No well-developed achenes were produced when flowers of hybrid plants of crosses X1 (1 plant) and X5 (1 plant) were isolated.

The proportion of well developed fruit in capitula of freely pollinated plants, with at least one achene, was 1.9–12.5% in F1 hybrids. However, many of the capitula of several hybrids were completely sterile (Table 2). Although, some well-developed achenes were obtained from artificially produced hybrids, only a small percentage germinated. Moreover, some of the seedlings were abnormal morphologically, e.g. the seedling that resulted from the free pollination of hybrid plant no. X5/8 had an undeveloped radicula and died early. Abnormalities (lack of chlorophyll, unusually cut leaf lamina of cotyledons) were observed also in seedlings from primary crosses (Mráz 2003).

Five hybrid plants were crossed either reciprocally (X2 and X5) or with two different morphotypes of *H. umbellatum* (see Table 3), which were flowering at the same time as the hybrids. Only one well-developed achene (from 281 fruits in total) was obtained from the cross between two hybrid plants, which originated from the cross between the same parental species of *H. alpinum* and *H. transsilvanicum* (crosses no. X2 and X5), but in a different direction. Although in cultivation the plant reached the rosette leaf stage, it died before flowering. According to the shape and indumentum of the leaves this plant was clearly a hybrid, but it was not clear if this plant was matroclinal or arose via hybridization. Three plants of the natural hybrid *H.* ×*krasani* (*H. alpinum* × *H. transsilvanicum*) were fully sterile as mother plants when freely pollinated, or back- or reciprocal cross (Mráz et al. 2005).

In the three crosses where the hybrid plants served as the mother plants the percentage of well-developed achenes was very low (3.2–5.7%, see Table 3). On the other hand, in the reverse cross (diploid species as maternal plant, diploid hybrid as pollen donor) the pro-

Table 4. – Characterization of triple hybrid plants. Percentage of well-developed fruit as counted only for those capitula that produced at least one well developed achene. Abbreviations used: Ncap – number of capitula, Nfr – total number of fruit, Npl – number of plants, Nwellfr – number of well-developed fruit, x – mean.

Cross no.	Chromosome number		Well-developed fruits in free pollination (only successful pollination scored)			Unsuccessful free pollination
	2n	Nplants	range (%)	x (%)	Npl / Ncap / Nfr / Nwellfr	Npl / Ncap
X21	18	1	–	1.8	1 / 1 / 57 / 1	1 / 8
X22	18	6	1.9–4.0	2.6	1 / 3 / 201 / 5	1 / 2
X26	18	1	2.8–12.1	6.5	2 / 8 / 302 / 19	2 / 10

duction of well-developed fruits was significantly higher (8.6–59.4%). Interestingly, this reverse cross (crosses no. X22 and X26) resulted in some matroclinal offspring. This is further confirmation of previously reported induced autogamy (so-called mentor effect, Mráz 2003). Characterization of the triple hybrids that originated from the above mentioned control crosses is given in Table 4. The average percentage of well-developed achenes produced by triple hybrids, in capitula with at least one well-developed fruit, when freely or control pollinated was similar to that produced by the F1 plants (1.8–6.5%).

Sterility (or almost sterility) was observed in both experimental and natural *Hieracium* hybrids (cf. Mráz et al. 2005) and is probably caused by chromosomal and genic incompatibilities between parental taxa. Currently known diploids taxa are morphologically not related and belong to the diferent sections (Stace 1998). All crosses presented in this paper are in fact intersectional (see Material and Methods). Full sterility or low production of well-developed achenes were recorded also in triple hybrids, where the two parental taxa of the total of three were closely related (high mountain type and type of low altitude of *H. umbellatum*) (Table 3, cross X21).

Considering the genus *Pilosella*, there are little data on homoploid hybridization at the diploid level, but many results of crosses between diploid and polyploid taxa as well as among various polyploids (Krahulcová et al. 2000). Gadella (1992) found sexually reproducing interspecific diploid hybrids from crosses between two closely related diploids; *P. hoppeana* (Schult.) F. W. Schultz et Sch. Bip. and *P. peleteriana* (Mérat) F. W. Schultz, and two distinct species belonging to the different sections – *P. lactucella* (Wallr.) P. D. Sell et C. West and *P. hoppeana*. On the other hand, the hybrid *P. lactucella* × *P. peleteriana* (intersectional cross found in nature as a sterile diploid, cf. Turesson 1972) could not be resynthezid experimentaly (Gadella 1992).

Evolutionary considerations

Most of the recent *Hieracium* polyploid taxa probably arose via interspecific hybridization, although formation of meiotic trivalents in triploids may indicate an autopolyploid origin in some cases (Bergman 1935, Guppy 1978). It seems that recent hybridization is rare. Some factors (internal and external), which inhibit recent natural hybridization in this genus, were discussed by Mráz et al. (2005). Different diploid taxa are usually geographically and/or ecologically separated and moreover, some internal factors, like preference for conspecific pollen or an induced autogamy, may contribute to the rarity of natural interspecific hybridization in nature. In the present paper a mentor effect (an induced

autogamy) is confirmed, which may contribute to the low rate of hybridization, although most of the progeny were true interspecific hybrids. The rare occurrence of natural diploid hybrids may also be linked with their very low reproductive capacity via achenes. Further, if natural selection acts on germination and on seedlings, which was not considered here, the propagation of hybrids seems to be limited. Consequently, the participation of diploid hybrids in gene flow as pollen donors in nature is probably more frequent than their role as mother plants.

Only diploid × diploid natural hybrids have been recorded (Mráz et al. 2005, J. Chrtek et al., in prep.), although gene flow between pollen producing polyploids and diploids is likely as illustrated by experimental crosses (Paule 2004, Mráz & Tomčíková 2004 and unpubl. data). If we assume that the diploid ploidy level is the most primitive, the polyploids should evolve from the diploid state. Interspecific hybridization followed by an increase in ploidy level of the diploid hybrids could be a possible pathway of polyploid formation. Another possibility is the production of unreduced gametes (2n). Certain meiotic abnormalities may occasionally occur and result in a proportion of gametes with the somatic chromosome number (for role of unreduced gametes in polyploidization see e.g. Thompson & Lumaret 1992, Bretagnolle & Thompson 1995, Ramsey & Schemske 1998). Although this phenomenon is unknown in *Hieracium* diploids, it is recorded in diploid *Pilosella peleteriana* (syn. *Hieracium peleterianum* Mérat) (Gadella 1988).

See http://www.ibot.cas.cz/preslia for Electronic appendix 1.

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Súhrn

V práci sú po prvýkrát prezentované ucelenejšie výsledky experimentálnych krížení v rode *Hieracium* s. str. Doteraz publikované informácie o umelom krížení sú veľmi kusé a len ťažko interpretovateľné. Viaceré pokusy J. G. Mendela boli neúspešné z dôvodu použitia apomikticky sa rozmnožujúcich rodičovských rastlín, o čom však samotný Mendel pochopiteľne nevedel. Agamospermia v rode bola objavená až na začiatku 20. storočia. V pokusoch sme použili ako rodičovské rastliny nasledujúce diploidné, sexuálne sa rozmnožujúce druhy: *H. alpinum*, *H. pojoritense, H. transsilvanicum* a dva morfologicky odlišné typy *H. umbellatum* (vysokohorský typ a typ nižších polôh) patriace do odlišných sekcií. V drvivej väčšine prípadov sme získali hybridné potomstvo. Rastliny z jedného kríženia boli navzájom značne morfologicky premenlivé. Všetky karyologicky analyzované rastliny mali diploidný počet chromozómov (2n = 18) rovnako ako rodičovské rastliny. Hybridy sa vyznačovali značnou produkciou veľkostne homogénneho peľu a vysokým zastúpením životaschopných peľových zŕn (65–92 %) podobne ako rodičovské diploidné druhy. Percento dobre vyvinutých nažiek v úboroch, v ktorých bola prítomná aspoň jedna dobrá nažka bolo v rozmedzí 1.9–12.5 % (priemerne 4–5 %) na úbor po voľnom alebo kontrolovanom opelení. Podobné výsledky sme získali aj u trojnásobných (“triple”) hybridov. Avšak väčšina úborov hybridných rastlín (F1 aj trojnásobných hybridov) netvorila žiaden dobre vyvinutý plod. Medzidruhové hybridy tak možno považovať za takmer sterilné (ako materské rastliny). Nakoľko boli v pokusoch zahrnuté morfologicky veľmi odlišné rodičovské druhy patriace do rôznych sekcií, nekompatibilita na úrovni chromozómov, resp. celého genómu rodičovských druhov pravdepodobne spôsobuje značné poruchy v tvorbe zárodočných mieškov primárnych aj trojnásobných hybridov. V dvoch prípadoch recipročných krížení, kde materské rastliny boli *H. umbellatum* a darcovia peľu hybridy, sme zistili indukovanú autogamiu (tzv. mentor efekt). Diploidné medzidruhové hybridy sa v prírode vyskytujú len zriedkavo, čo je zrejme dané ekologickými, geografickými (diploidy sú zvyčajne alopatrické druhy

s rozdielnymi ekologickými nárokmi) ale aj vnútornými (pravdepodobne preferencia peľu iného jedinca toho istého druhu, indukovaná autogamia, strerilita hybridov na úrovni nažiek) faktormi. Pri zriedkavom výskyte sa však diploidné krížence môžu zapojiť do toku génov vďaka tvorbe dobre vyvinutého a životaschopného peľu, zriedkavejšie aj prostredníctvom nažiek ako materské rastliny.

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Morphological and allozyme diversity in the *Hieracium nigrescens* group (Compositae) in the Sudety Mountains and the Western Carpathians

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The overall pattern of morphological variation and genetic diversity (allozyme analysis) was studied in the *Hieracium nigrescens* group (*H. nigrescens s.l.*, *H. alpinum* ≥ *H. murorum*) in the Sudety Mountains and the Western Carpathians. A morphological analysis was performed on 180 plants from 12 populations belonging to six *a priori* distinguished taxa. Altogether, 25 characters were measured or scored. Morphometric (canonical discriminant analysis) data separated five taxa, evaluated here at the species rank: *H. chrysostyloides, H. decipiens, H. nigrescens* (all from the Sudety Mountains), *H. jarzabczynum,* and *H. vapenicanum* (the Western Carpathians). A distinct local population from Mount Babia hora (the Western Carpathians) comprised a further possible taxon, given the preliminary name '*H. babiagorense*'. Genetic diversity was studied in 17 populations of H. chrysostyloides, H. decipiens, *H. jarzabczynum*, *H. nigrescens*, *H. vapenicanum* and '*H. babiagorense*' using five enzyme systems. All *a priori* recognized species were proved to be genetically homogeneous, each consisting of one unique multilocus allozyme genotype, except '*H. babiagorense*' which shared the same genotype with *H. jarzabczynum*. For the first time, a chromosome number is reported for *H. vapenicanum* ($2n = 3x = 27$) and previously published numbers were confirmed for *H. chrysostyloides* ($2n = 5x = 45$), *H. decipiens* ($2n = 4x = 36$), *H. jarzabczynum* ($2n = 4x = 36$), *H. koprovanum* ($2n = 4x = 36$), and *H. nigrescens* ($2n = 4x = 36$). All species have been shown to be endemic to either the Sudety Mountains or the Western Carpathians. Except for the species studied, two further ones (*H. apiculatum, H. nivimontis*) are recognized in the area, giving a total of seven species from the *Hieracium nigrescens* group in the area studied. The morphologically slightly different local population from Mount Babia hora/Babia Góra ('*H. babiagorense*') requires further study. Two new combinations are proposed: *Hieracium jarzabczynum* (Pawł. & Zahn) Mráz & Chrtek f. and *Hieracium vapenicanum* (Lengyel & Zahn) Chrtek f. & Mráz. *Botanical Journal of the Linnean Society*, 2007, **153**, 287–300.

ADDITIONAL KEYWORDS: apomixis – Asteraceae – chromosome numbers – flow cytometry – polyploidy.

INTRODUCTION

The genus *Hieracium* L. in the narrow sense (*Hieracium* subgen. *Hieracium*; Sell, 1987) contains herbs distributed mainly in the temperate regions of the northern hemisphere (Zahn, 1921–23; Sell & West, 1976). It is well known as a genus with widespread

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agamospermy (seed apomixis), giving rise to a very large number of variants that have been described as species (narrow species concept) or subspecies (broad species concept). Agamospermy is closely associated with polyploidy; the great majority of taxa are either triploids ($2n = 27$) or tetraploids ($2n = 36$) (Schuhwerk, 1996). Sexuality is rare and is confined to a few diploid species (e.g. Merxmüller, 1975; Chrtek, Mráz & Sevéra, 2004).

Two kinds of species in the broad sense (= species groups) are traditionally distinguished: (1) basic species ('species principales', 'Hauptarten') with a unique set of morphological characters; (2) intermediate species ('species intermediae', 'Nebenarten', 'Zwischenarten') sharing morphologically intermediate position between two or more basic species (Nägeli & Peter, 1885; Zahn, 1921–23). They are supposed to be a result of past hybridization between two or more basic species.

The *Hieracium nigrescens* group (*H. nigrescens s.l.*), together with the *H. atratum* group, occupies an intermediate position between the *H. alpinum* and *H. murorum* groups (species in the broad sense). Worldwide Zahn (1921–23) recognized 154 subspecies within *H. nigrescens* (in the broad sense), primarily on the basis of differences in leaf shape and dentation, the indumentum of peduncles and phyllaries, the number of stem leaves and the style colour. Since then, many new taxa mostly at the species rank have been described from the British Isles (e.g. Pugsley, 1948; Sell, West & Tennant, 1995) and from north-west Russia (Üksip, 1960; Shlyakov, 1966, 1989). The total distribution area of the group includes Greenland, Iceland, the British Isles, and through the Scandinavian mountains extending to north-west Russia. In Central Europe the group has a discontinuous distribution at high-altitude locations: the Alps, the highest Sudety Mountains, the Carpathians, and an isolated population in the Harz Mountains (Germany) (cf. Hultén & Fries, 1986; map incomplete). Plants of this group are typically found in high mountain grasslands (usually open-canopy), rocky knolls and ledges in gullies and on cliffs.

There have been relatively few chromosome studies of the group. All previously examined plants were polyploid. Tetraploids seem to prevail, while triploids are less common; two pentaploid species are unique within the genus *Hieracium (s.s.)* (e.g. Chrtek, 1994; Stace *et al.*, 1995; Chrtek, 1996). Agamospermy has been reported for *H. decipientiforme* from the Ukrainian Carpathians (Chrtek, 1997) and is supposed to be a common mode of reproduction in the group. It is widely supposed that the group includes hybridogeneous taxa originated from past crosses between *H. alpinum* and *H. murorum* (or their hybrids). It is unknown whether the group is monophyletic or whether particular taxa of this group have originated repeatedly through polytopic hybridization events between different taxa of both putative parental groups.

Both the Sudety Mountains and the Western Carpathians belong to genetic and diversity centres of the *Hieracium nigrescens* group. In the Sudety Mountains, members of the group occur in the Krkonoše Mountains (the western Sudety), the Hrubý Jeseník Mountains and Mount Králický Sněžník (both the eastern Sudety). Results of long-term research (the first studies in the Krkonoše Mountains go back to second half of the 19th century) of *Hieracium* are summarized in Zahn's monographs (Zahn, 1921–23; Zahn, 1936–38). Zahn (1936–38) recognized five subpecies of *H. nigrescens* in the Krkonoše Mountains, and four in the eastern Sudety Mountains Later on, Zlatník (1938, 1939) recognized in the Krkonoše Mountains four more or less sympatrically occurring species (in the narrow sense) within the 'group IV'; delimitation of the group is nearly identical to that of the *Hieracium nigrescens* group. Diversity of high mountain *Hieracium* species in the eastern Sudety is lower; recent revision of the *H. nigrescens* group revealed two endemic taxa treated at the species level here (Chrtek, 1995). More recent taxonomic treatment of the *H. nigrescens* group in the Western Carpathians is still lacking. Zahn (1936–38) distinguished 11 subspecies within the highest Western Carpathian mountain ranges. Local diversity centres of *Hieracium* sect. *Alpina* are situated mostly in the Tatra Mountains. Considering the total distribution range, important studies (apart from Zahn's monographs) come from the British Isles (e.g. Pugsley, 1948; Sell *et al.*, 1995), Scandinavia (e.g. Elfstrand, 1893, 1894; Norrlin, 1912; Omang, 1928) and north-west Russia (e.g. Üksip, 1960; Shlyakov, 1966, 1989).

The *Hieracium nigrescens* group is considered here to consist of eight *a priori* recognized species in the Sudety Mountains and the Western Carpathians. They are as follows: *Hieracium apiculatum* Tausch, *H. decipiens* Tausch, *H. nigrescens* Tausch, *H. chrysostyloides* (Zahn) Chrtek f., *H. nivimontis* (Oborný & Zahn) Chrtek f. (all the Sudety Mountains), *H. jarzabczynum* (Pawł. & Zahn) Mráz & Chrtek f. (formerly placed by Zahn in *H. pietroszense* Degen & Zahn) and *H. vapenicanum* (Lengyel & Zahn) Chrtek f. & Mráz (both from the Western Carpathians). Morphologically distinct plants from Mount Babia hora/Babia Góra (the Oravské Beskydy Mountains, Western Carpathians) are preliminarily treated as a separate unit and named '*H. babiagorense*'. The *a priori* concept proposed here is based on the revision of herbarium specimens (including most of the original collections of previously described taxa) and our own field observations.

Two recognized species, namely *H. apiculatum* and *H. nivimontis* were excluded from our analyses. Both of them are very rare and any sampling might seriously influence their survival. *Hieracium koprovanum* (Rech. f. & Zahn) Mráz & Chrtek f., *ined*., previously treated by Zahn (1936–38) as a subspecies of *H. nigrescens*, is considered here to belong to either the *H. rohacsense* or *H. pietroszense* group (owing to stellate hairs on phyllaries). It was therefore excluded from our analyses, except for allozyme analysis (to show its relationship with the *H. nigrescens* group) and chromosome number counts (necessary for a proper interpretation of the allozyme banding patterns).

The aims of this paper are: (1) to examine the overall morphological variation of the *H. nigrescens* group in the study area, (2) to assess genetic variation and diversity using allozyme analysis, and (3) to determine chromosome numbers/ploidy levels, and mode of reproduction.

MATERIAL AND METHODS

Plants

Plants for morphological studies

Altogether, 12 population samples, of 15 plants each, were collected during 1995–2002. The populations were selected according to previous studies in order to: (1) include all *a priori* recognized taxa in the area, except for those very rare and extremely endangered (*H. apiculatum, H. nivimontis*), i.e. *H. chrysostyloides, H. decipiens, H. jarzabczynum, H. nigrescens, H. vapenicanum* and '*H. babiagorense*'; and (2) to cover the geographical range of the recognized taxa. A list of the population samples is given in Table 1. The number of populations collected per species ranged from one to three, and reflects the geographical distribution and population abundance. In most cases, only the aboveground part of the plant was carefully removed to allow the plant to survive at the locality. All morphological characters were measured (scored) on herbarium specimens. Voucher specimens are deposited in PRA (plants collected by JC) and in private herbarium of P. Mráz (plants collected by PM).

Plants for allozyme analysis, and karyological and breeding system studies

Living plants from the same localities as above (ten plants from each, except of *H. koprovanum*; see Table 1) were collected, transferred to the experimental garden of the Institute of Botany, Průhonice, and cultivated in pots under field conditions. Only leaves were collected in the field for the endangered species *H. chrysostyloides* and *H. nigrescens*. In contrast to the morphometric studies, *H. koprovanum* was also included in the allozyme and karyological (chromosome counting) analysis.

Ploidy level and number of chromosomes

Either the chromosome number or the ploidy level was estimated for all the cultivated plants used for the allozyme and breeding system studies (five plants per population, a total of 65 plants). Chromosome counts were made for at least two plants per population. Root tip cuttings of mature plants were used. The material was pretreated with a saturated solution of p-dichlorobenzene, fixed in a mixture of ethanol and acetic acid (3:1) and stored in 70% ethanol. The squash method with staining by lacto-propionic orceine followed Dyer (1963). Determination of ploidy level by flow cytometry followed Krahulcová, Papoušková & Krahulec (2004).

Breeding system

The mode of reproduction was tested in ten cultivated plants per species; if more than one population per species was collected, the plants studied originated proportionally from all such populations. The top portions of unopened flower buds containing anthers and stigma were sliced off with a razor. Heads were than bagged to prevent loss of achenes. Fully developed achenes were considered to be the result of agamospermous mode of reproduction. Differences in the percentage of fully developed (viable) achenes were assessed by one way ANOVA.

Morphological analysis

Altogether 25 quantitative characters (Table 2) were measured or scored on herbarium material from our own collections. Two datasets were used in the analyses: (1) a complete dataset that included all plants and all variables (matrix A), and (2) a reduced dataset that included plants attributed *a priori* to *H. chrysostyloides*, *H. decipiens* and *H. vapenicanum* and all variables (matrix B). Data analysis was performed in the following steps:

1. The normality of the distribution of all characters was tested using the Shapiro-Wilks test. Pearson and nonparametric Spearman correlation coefficients were calculated on the matrix of the whole material and on the individual matrices of taxa distinguished *a priori*.
2. Two canonical discriminant analyses (CaDA) were performed based on individual plants and populations as groups. The first one was based on the full dataset (matrix A) and the second one on the reduced dataset (matrix B).

Table 1. List of samples of the *Hieracium nigrescens* group studied. M, number of plants for morphometric analysis; A, number of plants for allozyme analysis, CR, number of plants for chromosome counting (C, counted by J Chrtek; M, counted by P Mráz, counts were originally published in Mráz, 2001); PL, determination of ploidy level by flow cytometry (by A. Krahulcová)

		No of individuals		
Population code	Origin and sampling data	M	A	CR/PL
H. jarzabczynum				
1/1, Jarz 1	Sk: Západné Tatry Mts., Zuberec: Roháčske plesá mountain lakes, 10.5 km south-east of village,1620 m a.s.l., 49°12′30″N, 19°44′08″E, 22.vii.1997 & 7.vii.2000, *J. Chrtek & M. Odvodyová.*	15	10	5/5 (C)
1/2, Jarz 2	Sk: Západné Tatry Mts., Zuberec: Mt. Lúčna, south-west slopes, 11.5 km east-south-east of village, 1400–1620 m a.s.l., 49°13′46″N, 19°45′40″E, 23.vii.1997 & 8.vii.2000, *J. Chrtek.*	15	10	4/6 (C)
1/3, Jarz 3	Sk: Západné Tatry Mts., Pribylina: Mt. Hrubý vrch, western slopes, 10.5 km north of village, 1820 m a.s.l., 49°11′54″N, 19°47′20″E, 25.vii.1998 & 27.vii.2001, *J. Chrtek & D. Vaňková.*	15	10	5/5 (C)
'H. babiagorense'				
2/1, Babiag 1	Sk: Oravské Beskydy Mts., Oravská Polhora: Mt. Babia hora, eastern slopes, 8 km north-west of village,1700 m a.s.l., 49°34′15″N, 19°31′40″E, 23.vii.1998, *J. Chrtek.*	15	10	3/7 (C)
H. decipiens				
3/1, Dec 1	Cz: Krkonoše Mts., Pec pod Sněžkou: Mt. Sněžka, western slopes, 5 km north of village, 1390 m a.s.l., 50°44′15″N, 15°43′45″E, 24.vi.2000, *J. Chrtek.*	15	10	8/2 (C)
3/2, Dec 2	Cz: Krkonoše Mts., Špindlerův Mlýn: Mt. Kotel, 5.5 km west-north-west of village, 1420 m a.s.l., 50°45′05″N, 15°31′45″E, 15.vii.2001, *J. Chrtek & M. Odvodyová.*	15	10	5/5 (C)
3/3, Dec 3	Cz: Krkonoše Mts., Špindlerův Mlýn: Mt. Vysoké kolo, south-east slopes, 6 km south-west of village, 1460 m a.s.l., 50°46′32″N, 15°33′20″E, 4.viii.2000, *J. Chrtek.*	15	10	5/5 (C)
H. chrysostyloides				
4/1, Chrys 1	Cz: Hrubý Jeseník Mts., Karlova Studánka: Petrovy kameny rocks, 5.5 km west of village, 1430 m a.s.l., 50°03′57″N, 17°14′40″E, 4.viii.2000, *J. Chrtek & D. Vaňková.*	15	10*	4/0 (C)
H. vapenicanum				
5/1, Vapen 1	Sk: Nízke Tatry Mts., Hel'pa: Mt. Vel'ká Vápenica, 5.5 km north-east of village, 1690 m a.s.l., 48°54′30″N, 19°59′00″E, 17.vii.2001, *J. Chrtek.*	15	10	6/4 (C)
5/2, Vapen 2	Sk: Západné Tatry Mts., Roháče, Zuberec: mountain ridge between Mt. Osobitá and Mt. Lúčna, 8.5–11 km east-south-east of the village, 1500–1550 m a.s.l., 49°15′12″N, 19°43′50″E–49°14′20″N, 19°45′30″E, 15.vii.1995, 8.vii.2000 & 28.vii.2001, *J. Chrtek.*	15	10	6/4 (C)
5/3, Vapen 3	Sk: Západné Tatry Mts., Roháče, Zuberec: Zábrat' saddle, 10.5 km east-south-east of village, 1650 m a.s.l., 49°13′15″N, 19°44′58″E, 7.vii.2000 & 27.vii.2001, *J. Chrtek.*	15	10	5/5 (C)
H. nigrescens				
6/1, Nigres 1	Cz: Krkonoše Mts., Pec pod Sněžkou: Mt. Sněžka, W slopes, 5 km north of village, 1390 m a.s.l., 50°44′15″N, 15°43′45″E, 17.vii.1997 & 24 June 2000, *J. Chrtek.*	15	10*	5/0 (C)
H. koprovanum				
7/1, Koprov 1	Sk: Západné Tatry Mts., Podbanské: Zadná Tichá dolina valley, 9 km north east of village, 1550 m a.s.l., 49°12′30″N, 19°59′10″E, 24.viii.1997, *P. Mráz (cult. nos. 491, 493, 494, 496).*	–	4	–

Table 1. *Continued*

Population code	Origin and sampling data	No of individuals M	A	CR/PL
7/2, Koprov 2	Sk: Vysoké Tatry Mts., Starý Smokovec: Vel'ká Studená dolina valley, 4.5 km north-north-west of village, 1550 m a.s.l., 49°10′32″N, 20°11′50″E, 13.viii.1996, *P. Mráz (cult. nos. 211, 213, 225).*	–	10 + 3	4/6 (C) 2/0 (M)
7/3, Koprov 3	Sk: Vysoké Tatry Mts., Štrbské Pleso: Važecká dolina valley (Zahandel), 1 km south-east of Mt. Kriváň, 5.5 km north-west of village, 1720 m a.s.l., 49°08′43″N, 20°00′29″E, 16.vii.1998, *P. Mráz & V. Jurkovičová (cult. nos. 517, 519).*	–	2	1/0 (M)
7/4, Koprov 4	Po: Tatry Wschodnie Mts., Łysa Polana: south-east slopes of Mt. Swistowa Czuba, 0.3 km south-east of Mt. Swistowa Czuba, 7.8 km south-west of village, 1840 m a.s.l., 49°12′50″N, 20°03′44″E, 23.vii.1997, *P. Mráz (cult. nos. 462, 464).*	–	2	2/0 (M)
7/5, Koprov 5	Sk: Nízke Tatry Mts., Mýto pod Ďumbierom: Demänovské sedlo saddle, 10 km north of village, 1750 m a.s.l., 48°56′25″N, 19°37′21″E, 15.vii.1996, *P Mráz (cult. no. 13).*	–	1	2/0 (M)

Cz, Czech Republic; Po, Poland; Sk, Slovakia. *see Material and methods.

3. principal component analysis (PCA; Sneath & Sokal, 1973; Krzanowski, 1990) based on a correlation matrix of characters and individual plants as OTUs (operational taxonomic units) was conducted (based on matrix A), to elucidate morphological homogeneity of the taxa recognized *a priori*.
4. Exploratory data analysis (EDA) was performed on the data matrices of the six recognized taxa. Within each group, the mean, standard deviation, and 5% and 95% percentiles were computed for each character.

The numerical analyses were computed using statistical packages SYN-TAX 2000 (Podani, 2001), SAS, v.8 (SAS Institute, 2000) and STATISTICA (StatSoft, 1998).

Allozyme analysis

Young leaves of cultivated plants were used. Plant material was ground in extraction buffer generally according to Kato (1987) with some modifications: 0.1 M Tris-HCl (pH 8.0), 70 mM mercaptoethanol, 26 mM sodium metabisulfite, 11 mM L-ascorbic acid, 4% (w/v) soluble PVP-40, pH-adjusted after the addition of the ascorbate. Crude homogenates were centrifuged for 10 min at 15 000 r.p.m. Clear supernatant was stored in a deep freeze at –75 °C. The PAGE was carried out using separating gel (8.16%) with the buffer 1.82 M Tris-HCl, pH 8.9; the stacking gel (4.0%) with the buffer (0.069 M Tris-HCl, pH 6.9); the electrode buffer was 0.02 M Tris, 0.24 M glycine, pH 8.3. The following enzymes were analysed: AAT (Aspartate aminotransferase, EC 2.6.1.1), LAP (Leucine aminopeptidase, EC 3.4.11.1), PGM (Phosphoglucomutase, EC 5.4.2.2), 6-PGDH (6-phosphogluconate dehydrogenase, EC 1.1.1.44), and SKD (Shikimate dehydrogenase, EC 1.1.1.25).

The staining procedures followed Vallejos (1983) to visualize 6-PGDH and SKD, and Wendel & Weeden (1989) for PGM and EST, with the following modifications: 6-PGDH (0.1 M tris-HCl, pH 8.4, 30 mg 6-phosphogluconic acid), SKD (0.1 M tris-HCl, pH 8.4), colorimetric EST (Na-phosphate buffer pH 6.45, 25 mg β-naphthyl phosphate, 50 mg Fast Blue BB), PGM (24 mg $MgCl_2$, 50 mg glucose-1-phosphate, 10 mg NADP). Visualization of LAP was performed using buffer 0.2 M tris-maleate pH 6. The gel was rinsed with the buffer and then incubated for 10 min in a solution of 30 mL of the buffer, 40 mg L-leucyl-β-naphthylamide, HCl (in 50% acetone) and 60 mg $MgCl_2$. Then 25 mg Fast Black K Salt in 30 mL of the buffer was added. The gel was incubated in the dark until bands appeared. Two staining solutions were prepared for AAT: A (20 mL 0.1 M tris-HCl, pH 8.4, 240 mg aspartic acid, 40 mg α-ketoglutaric acid) and B (20 mL 0.1 M tris-HCl, pH 8.4, 50 mg Fast Blue BB Salt, 50 mg Fast Violet B, 25 mg pyridoxal-5-phosphate). Solution A was prepared at least 15 min before application. The gel was rinsed in water and then in buffer tris-HCl pH 7. Solutions A and B were mixed and poured onto gel. The gel was incubated in the dark at 32 °C until bands appeared. It was then rinsed and fixed (1:1:3:5, glycerine, acetic acid, H_2O, methanol).

As a consequence of the complex 'nonsegregating' banding pattern in our agamospermous taxa, patterns were compared with those produced by closely related

Table 2. Variables used for morphometric analysis of *Hieracium nigrescens* group

Abbreviation	Description
PH	Plant height
NBL	Number of basal leaves
Longest basal leaf (L1), primordial leaves were excluded	
LL1	Length of L1
WL1	Width of L1
MWL1	Position of maximal width of L1 (distance between the widest part and the top)
TLL1	Length of longest tooth on L1
ATLL1	Mean length of three longest teeth on L1
MGL1	Total of teeth and mucronate glands on L1
HUL1	Number of simple eglandular hairs at 0.5 cm^2 of upper leaf surface (L1) (scored in the middle part of the leaf, central vein and margins are not included)
HLL1	Number of simple eglandular hairs at 0.5 cm^2 of lower leaf surface (L1) (scored in the middle part of the leaf, central vein and margins are not included)
Stem leaves	
NSL	Number of non bract-like stem leaves
BSL	Number of bract-like stem leaves
Lowest stem leaf (L2)	
LL2	Length of L2
WL2	Width of L2
TLL2	Length of longest tooth on L2
ATLL2	Mean length of three longest teeth on L2
MGL2	Total of teeth and mucronate glands on L2
Heads, akladium, peduncles	
NH	Number of heads
SEHP	Number of simple eglandular hairs (SEH) on 0.5 cm-long part of peduncle
GHP	Number of glandular hairs (GH) on 0.5 cm-long part of peduncle; microglands (gland usually shorter than 0.1 mm) were not scored
LSEH	Length of SEH on peduncle (mean of 10 consequently inserted SEH)
LGH	Length of GH on peduncle (mean of 10 consequently inserted GH)
LI	Length of involucrum
WB	Width of middle involucral bract (measured in the middle of their length, mean of 3 bracts)
LL	Length of outer ligules (mean of 3 ligules)

sexual *Hieracium alpinum* (Kirschner & Chrtek, unpubl. data). We used an approach indicating no increase in locus number for agamosperms; interpretation was accomplished by assigning bands within a prescribed 'zone' to a locus corresponding to 'zones' occupied by individual loci for sexual species. According to the migration distance, alleles at a particular locus were marked by lowercase letters (a,b…), corresponding to alleles identified in previous studies in *Hieracium* sect. *Alpina* (Mráz, Chrtek & Kirschner, 2001; Štorchová *et al*., 2002).

For each multilocus genotype (genotypes correspond to taxa delimited *a priori*) the average number of alleles per locus (A) and observed heterozygosity (H_O) were calculated. Nei's standard genetic distances between all pairs of multilocus genotypes were calculated using the program FREETREE (Pavlíček, Hrdá & Flégr, 1999). The distance matrix was subjected to principal coordinate analysis (PCoA), using the programs SYN-TAX 2000 (Podani, 2001) and STATISTICA (StatSoft, 1998). Furthermore, Euclidean distances between all pairs of genotypes were calculated. Based on this matrix, UPGMA clustering method was performed, using the STATISTICA (StatSoft, 1998) program.

RESULTS

Chromosomes and mode of reproduction

We report here for the first time the chromosome number for *H. vapenicanum* ($2n = 27$, 17 plants from three populations). In 13 other plants, the triploid ploidy level was determined by flow cytometry. Previously reported chromosome counts (Chrtek, 1994, 1996; Mráz, 2001) were confirmed for *H. decipiens* ($2n = 36$),

H. nigrescens ($2n = 36$), *H. chrysostyloides* ($2n = 45$), *H. koprovanum* ($2n = 36$) and *H. jarzabczynum* ($2n = 36$; the count for '*H. nigrescens s.l.*' in Mráz, 2001: 327 refers to this species as well). We have found no variation in chromosome number within a species.

The percentage of fully developed achenes ranges from 55.8 to 100 in *H. chrysostyloides*, 38.1–90.5 in *H. decipiens*, 66.3–97.1 in *H. jarzabczynum*, 62.5–90.5 in *H. nigrescens*, 39.1–93.8 in *H. vapenicanum* and 74.8–91.4 in '*H. babiagorense*' (Table 3). One-way ANOVA did not show statistically significant differences among species at the $P < 0.05$ level.

Table 3. Percentage of fully developed (black) achenes after emasculation in *Hieracium chrysostyloides*, *H. decipiens*, *H. jarzabczynum*, *H. nigrescens*, *H. vapenicanum* and '*H. babiagorense*'

Taxon	Mean ± SD	Min. – max.
H. chrysostyloides	83.14 ± 15.86	55.8–100
H. decipiens	77.60 ± 15.48	33.1–93.5
H. jarzabczynum	84.94 ± 10.38	66.3–97.1
H. nigrescens	82.96 ± 11.55	62.5–90.5
H. vapenicanum	77.28 ± 16.88	39.1–93.8
'*H. babiagorense*'	81.86 ± 6.20	74.8–91.4

MORPHOLOGICAL ANALYSES

Normality of the distribution of morphological characters and character correlations

All but two (PH and LL1) variables showed certain deviations from the normal distribution in the Shapiro-Wilks test. A very high correlation (exceeding the value 0.95) was found between characters TLL1 and ATLL1 (Spearman $R = 0.98$, Pearson coefficient = 0.98) and TLL2 and ATLL2 (Spearman $R = 0.99$, Pearson coefficient = 0.98). Therefore, characters TLL1 and TLL2 were removed from further analyses.

Discriminant analysis

In CaDA, based on individual plants and populations as groups performed on matrix A (the complete dataset), rather distinct separation of populations attributed *a priori* to *Hieracium jarzabczynum, H. nigrescens* and '*H. babiagorense*' was achieved (Fig. 1). The first canonical axis represents 55% of the variation among the groups, with the variables strongly contributing to the separation of plants along this axis (in descending order): WB, WL1, LL, HUL1, LSEH, GHP, BSL and MGL1. The second canonical axis accounts for 16% of variation among the groups. The variables highly correlated with this axis are: SEHP, LGH, LI, GHP and Ll. Finally, the third axis

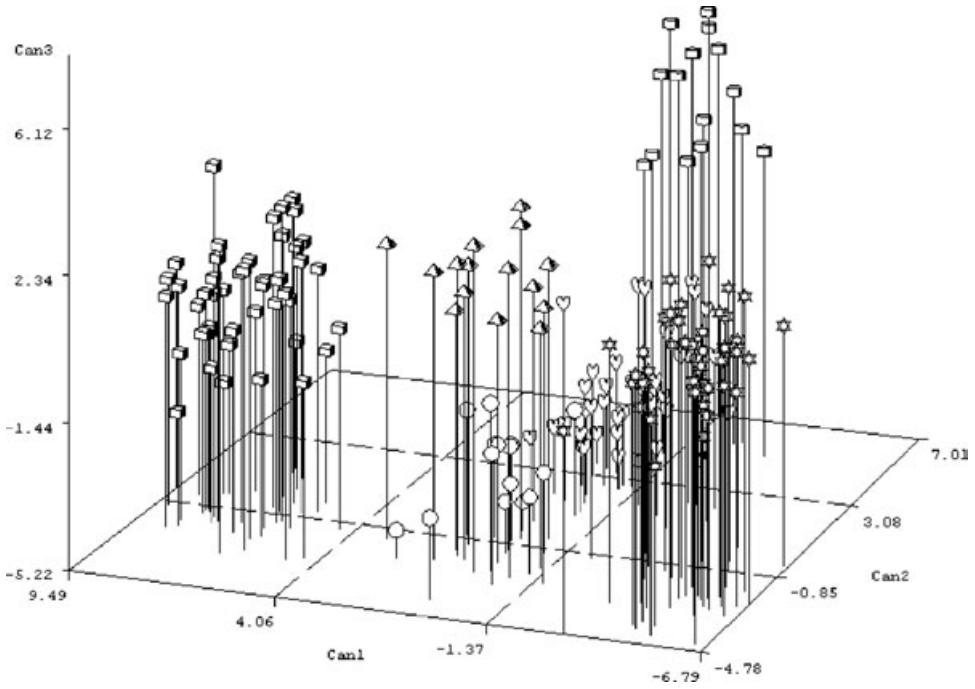

Figure 1. Canonical discriminant analysis based on 23 morphological characters of individuals of *Hiercium chrysostyloides* (C, $N = 15$), *H. decipiens* (V, $N = 45$), *H. jarzabczynum* (S, $N = 30$), *H. nigrescens* (□, $N = 15$), *H. vapenicanum* (✡, $N = 45$), and '*H. babiagorense*' (△, $N = 15$).

represents 12% of variation, the highly correlated variables with this axis are LSEH and LI (Table 4).

CaDA performed on matrix B (all populations attributed *a priori* to *Hieracium chrysostyloides, H. decipiens* and *H. vapenicanum*) showed a good separation of populations attributed to the particular species (Fig. 2). *Hieracium vapenicanum* is well separated from *Hieracium chrysostyloides* and *H. decipiens* along the first canonical axis (most strongly correlated with LSEH, LGH, WB, LI, LL, WL1 and BSL), which accounts for 61% of variation among the groups. The population of *H. chrysostyloides* is separated along the second axis (most strongly correlated with SEHP, MWL1 and NBL, accounting for 18% of variation among the groups) (Table 5).

Principal component analysis

Two groups corresponding to the *a priori* recognized Carpathian species *H. jarzabczynum* and *H. vapenicanum* were revealed. However, the remaining samples did not show any clear structure, due to within-population variation (not shown).

Exploratory data analysis

The results of the evaluation of the variation of all characters in six taxa recognized on the basis of the previous analyses are given in Table 6. The taxa cannot be distinguished clearly by any single character: the ranges of characters overlap at least among some of the taxa studied. However, a combination of the characters does allow separation. *Hieracium vapenicanum* can be separated from the other taxa (except for a very small overlap with *H. decipiens*) by the width of the involucral bracts, *Hieracium nigrescens* by a very low number of simple eglandular leaves on the peduncles (except for a very small overlap with *H. decipiens*). *Hieracium nigrescens* and *H. jarzybczynum* have deeply dentate basal leaves (TLL1) in comparison with the shallowly dentate ones in *H. chrysostyloides, H. decipiens* and '*H. babiagorense*'.

Allozyme analysis

Five enzyme systems with five allelically interpretable loci (*Skd*, *Lap*-1, *Pgm*-1, *6Pgdh*-2 and *Aat*-2) were observed; allelic interpretation of the remaining loci

Table 4. Total canonical structure (correlation of the characters with canonical axes) obtained in the canonical discriminant analysis of plants *H. chrysostyloides, H. decipiens, H. jarzabczynum, H. nigrescens, H. vapenicanum* and '*H. babiagorense*'. Groups defined as populations

Character	Axis 1	Axis 2	Axis 3
PH	0.313	0.284	–0.094
NBL	–0.125	0.323	0.270
LL1	0.153	0.177	–0.163
WL1	0.766	0.207	0.098
MWL1	0.205	0.016	–0.108
ATLL1	0.439	0.241	0.455
MGL1	–0.504	0.359	0.431
HUL1	–0.652	0.201	–0.027
HLL1	–0.162	–0.088	0.191
NSL	–0.222	–0.046	–0.060
BSL	–0.526	–0.314	0.149
LL2	–0.128	0.020	–0.101
WL2	0.294	0.045	0.123
ATLL2	0.163	0.296	0.413
MGL2	–0.224	0.476	0.109
NH	0.229	0.039	0.074
SEHP	0.325	–0.701	–0.138
GHP	–0.556	0.522	0.079
LSEH	0.651	0.191	–0.607
LGH	0.261	0.664	–0.466
LI	0.232	0.609	–0.536
WB	0.952	0.102	0.003
LL	0.701	0.500	–0.049

Table 5. Total canonical structure (correlation of the characters with canonical axes) obtained in the canonical discriminant analysis of plants *H. chrysostyloides, H. decipiens,* and *H. vapenicanum*. Groups defined as populations

Character	Axis 1	Axis 2	Axis 3
PH	0.378	0.218	0.223
NBL	–0.157	0.626	–0.382
LL1	0.356	–0.305	0.189
WL1	0.701	0.171	0.157
MWL1	0.275	–0.532	0.101
ATLL1	0.061	0.091	0.198
MGL1	–0.324	0.241	0.317
HUL1	–0.218	–0.155	0.009
HLL1	–0.272	–0.071	–0.170
NSL	–0.053	–0.047	0.472
BSL	–0.688	0.236	–0.088
LL2	0.113	–0.255	0.344
WL2	0.203	–0.192	0.374
ATLL2	0.128	–0.219	0.369
MGL2	0.273	0.103	0.541
NH	0.154	–0.098	0.511
SEHP	–0.286	–0.688	0.053
GHP	–0.005	0.493	–0.164
LSEH	0.866	–0.172	–0.101
LGH	0.850	0.252	0.106
LI	0.816	0.156	–0.007
WB	0.843	–0.322	–0.218
LL	0.810	–0.075	0.308

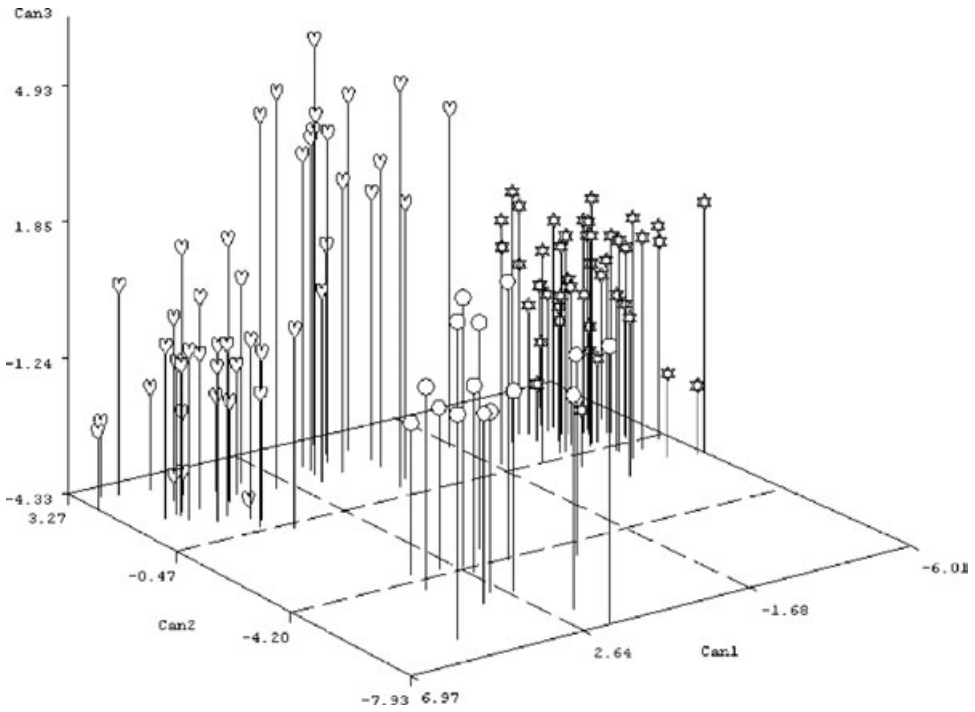

Figure 2. Canonical discriminant analysis based on 23 morphological characters of individuals of *Hieracium chrysostyloides* (♁, $N = 15$), *H. decipiens* (♈, $N = 45$), and *H. vapenicanum* (♎, $N = 45$).

was impossible due to either the complex banding pattern or insufficient staining. Among the interpreted loci, four were found to be polymorphic (*Skd,* *Lap*-1, *Pgm*-1, *6Pgdh*-2), while *Aat*-2 is monomorphic in all populations studied (Table 7). Six multilocus allozyme genotypes were detected corresponding to the taxa defined *a priori* (taxa of the *Hieracium nigrescens* group and *H. koprovanum*). Each taxon consists of a unique genotype, except '*H. babiagorense*' which shares the same genotype with *H. jarzabczynum*. Except for *H. decipiens*, each species has at least one unique allele: *H. nigrescens* (*Pgm*-1d), *H. chrysostyloides* (*Skd*-1d), *H. koprovanum* (*Lap*-1b, *Lap*-1d), *H. jarzabczynum* (*6Pgdh*-2b) and *H. vapenicanum* (*Lap*-1a). Assuming a geographical pattern of the variation, two alleles (*Skd*-1d, *Pgm*-1d) were only found in the Sudetic species, and four alleles (*Lap*-1a, *Lap*-1b, *Lap*-1d, *6Pgdh*-2b) in the Carpathian species. Measures of allelic variation (mean number of alleles per locus, and frequency of observed heterozygotes) are summarized in Table 8.

A dendrogram constructed from the corresponding Euclidean distances (Fig. 3) clearly separates *H. koprovanum*. In the remaining major cluster, two taxa from the Sudety Mountains (*H. decipiens* and *H. chrysostyloides*) are grouped together, and similarly also *H. vapenicanum* (the Carpathians) with *H. nigrescens* (the Sudety Mountains). The PCoA scatterplot based on Nei's standard distances (Fig. 4) shows a group formed by *H. nigrescens* and *H. vapenicanum*, separated along the first coordinate. *Hieracium jarzabczynum* is separated along the second coordinate; the remaining species are only loosely grouped.

DISCUSSION

CHROMOSOME NUMBERS, MODE OF REPRODUCTION

We confirmed the previously reported diversity of ploidy levels in the *H. nigrescens* group. A similar pattern of cytological diversity in the *H. nigrescens* group has been detected in the British Isles (Stace *et al*., 1995; further counts from the British Isles were published by Mills & Stace, 1974 and Morton, 1974). Both triploid and tetraploid counts were reported for *H. nigrescens* (agg.) from the North Ural (Komi Autonomous Republic, north-west Russia; Lavrenko, Serditov & Ulle, 1988; 1990) and from Iceland (Löve, 1970). Recently Schuhwerk & Lippert (1999) published $2n = 27$ for *H. nigrescens* ssp. *cochleare* (Huter) Zahn from the Bavarian Alps.

The percentage of fully developed achenes did not vary substantially among the species. Similar percentages have been reported by Štorchová *et al*. (2002) in several species of the *Hieracium fritzei* group. Empty

Table 6. Summary statistics for characters of '*Hieracium babiagorense*' (Babiag, *n* = 15), *H. chrysostyloides* (Chrys, *n* = 15), *H. decipiens* (Dec, *n* = 45), *H. jarzabczynum* (Jarz, *n* = 60), *H. nigrescens* (Nigres, *n* = 15), and *H. vapenicanum* (Vapen, *n* = 45). Upper line: mean ± SD, lower line 5% and 95% percentiles

	Taxon					
Character	Babiag	Chrys	Dec	Jarz	Nigres	Vapen
PH (cm)	17.80 ± 3.63 12–24	20.03 ± 3.52 16–29	23.38 ± 4.43 17–30	23.91 ± 4.21 18.5–32.5	22.70 ± 3.13 17.5–27.5	18.93 ± 3.34 12.5–24
NBL	4.73 ± 1.28 2–6	2.73 ± 1.22 1–5	5.93 ± 1.64 3–8	5.51 ± 2.01 3–9	7.80 ± 2.31 4–12	6.11 ± 1.58 4–9
LL1 (cm)	7.65 ± 2.18 5.3–11	10.88 ± 2.30 7.5–15	9.22 ± 1.95 6.5–12.0	9.13 ± 2.03 6–12.5	9.80 ± 1.99 5.8–13	7.75 ± 2.14 3.5–10.8
WL1 (cm)	1.35 ± 0.29 1–2	1.27 ± 0.21 0.9–1.6	1.48 ± 0.24 1–1.8	2.18 ± 0.47 1.6–3	1.75 ± 0.38 1.3–2.6	1.01 ± 0.20 0.7–1.3
MWL1 (cm)	2.39 ± 0.58 1.4–3.5	3.43 ± 1.04 2–5	2.38 ± 0.55 1.4–3.3	2.62 ± 0.62 1.7–3.9	2.66 ± 0.79 1.5–4.5	2.16 ± 0.61 1.2–3.3
TLL1 (mm)	2.69 ± 1.27 1–5.5	1.47 ± 1.35 0–5	1.97 ± 1.43 0–5	4.68 ± 2.36 1.7–9	5.57 ± 2.93 1.8–12	1.55 ± 0.97 0–3
ATLL1 (mm)	2.09 ± 0.84 0.9–3.8	1.21 ± 1.18 0–4.4	1.50 ± 1.07 0–3.5	3.44 ± 1.81 0.9–6.3	4.35 ± 1.93 1.2–8.3	1.24 ± 0.78 0–2.3
MGL1	8.73 ± 2.12 5–12	6.33 ± 3.52 0–13	8.09 ± 2.54 4–12	5.31 ± 1.95 3–9	13.80 ± 2.08 10–18	9.16 ± 2.54 5–13
HUL1	4.93 ± 3.39 0–13	16.73 ± 4.99 11–28	13.42 ± 5.04 6–22	3.07 ± 4 0–10	20.07 ± 7.46 10–34	16.84 ± 7.65 8–35
HLL1	16.60 ± 6.37 7–28	15.47 ± 4.69 9–24	13.09 ± 5.49 6–24	13.93 ± 5.98 5–23	17.33 ± 7.67 7–37	17.91 ± 8.73 6–32
NSL	1.13 ± 0.35 1–2	1.73 ± 0.70 1–3	1.64 ± 0.77 1–3	1.27 ± 0.62 0–2	1.33 ± 0.49 1–2	1.51 ± 0.66 0–2
BSL	2.40 ± 0.63 1–3	2.00 ± 0.66 0–3	2.82 ± 0.91 2–4	2.47 ± 0.87 1–4	2.73 ± 0.70 2–4	4.49 ± 1.10 3–6
LL2 (cm)	4.47 ± 1.86 1.5–8.3	7.06 ± 3.36 2–13.5	5.28 ± 2.70 2–11	4.08 ± 2.51 0–8.3	4.99 ± 2.43 2–10	4.46 ± 2.17 0–7.2
WL2 (mm)	7.13 ± 3.18 3–15	8.20 ± 3.85 1.5–14	6.59 ± 3.54 2–12	9.05 ± 5.82 0–21	8.13 ± 5.60 2–16	4.90 ± 2.39 0–9
TLL2 (mm)	1.29 ± 1.25 0–3.5	1.09 ± 1.57 0–5.5	0.58 ± 0.97 0–2.5	1.71 ± 2.39 0–6.5	3.95 ± 3.48 0–11	0.29 ± 0.77 0–2
ATLL2 (mm)	1 ± 1.04 0–3.3	0.93 ± 1.32 0–4.4	0.46 ± 0.76 0–2	1.18 ± 1.75 0–5	3.14 ± 2.78 0–8.2	0.19 ± 0.50 0–1.3
MGL2	3.33 ± 3.09 0–10	3.87 ± 3.52 0–11	5.00 ± 3.13 0–10	1.67 ± 1.61 0–4	7.80 ± 4.40 0–14	2.22 ± 2.52 0–7
NH	1 ± 0 1–1	1.33 ± 0.49 1–2	1.22 ± 0.56 1–3	1.44 ± 0.87 1–3	1.20 ± 0.41 1–2	1 1
SEHP	42.07 ± 11.17 19–56	54.07 ± 13.10 31–72	21.29 ± 9.43 9–37	46.13 ± 13.87 24–66	4.47 ± 3.52 0–12	36.67 ± 16.08 15–61
GHP	138.40 ± 21.61 103–171	125.07 ± 22.58 93–166	191.80 ± 49.85 117–271	112.18 ± 24.83 79–156	247.47 ± 49.58 183–365	182.67 ± 33.10 133–231
LSEH (mm)	1.32 ± 0.18 1.05–1.67	2.41 ± 0.50 1.65–3.69	2.26 ± 0.44 1.41–2.73	2.49 ± 0.38 1.89–3.09	1.55 ± 0.18 1.28–1.86	1.34 ± 0.22 1.01–1.78
LGH (mm)	0.38 ± 0.05 0.33–0.50	0.44 ± 0.07 0.32–0.57	0.54 ± 0.07 0.42–0.65	0.44 ± 0.05 0.37–0.54	0.47 ± 0.05 0.36–0.56	0.34 ± 0.03 0.3–0.39
LI (mm)	13.27 ± 0.96 12–15	14.63 ± 1.01 13–16	15.98 ± 1.59 13–18	14.36 ± 1.03 13–16	14.80 ± 0.68 14–16	12.69 ± 1.13 11–15
WB (mm)	1.19 ± 0.16 1–1.5	1.23 ± 0.09 1.1–1.4	1.09 ± 0.17 0.8–1.3	1.65 ± 0.13 1.4–1.8	1.20 ± 0.12 1–1.5	0.82 ± 0.08 0.7–0.9
LL (mm)	15.97 ± 0.93 15–18	16.55 ± 1.01 14–18	16.69 ± 1.59 14.7–19.3	18.63 ± 1.91 16.3–22	18.73 ± 1.55 16–20.7	12.19 ± 1.77 9.7–15.7

Table 7. Allelic frequencies for all scored loci for *Hieracium chrysostyloides*, *H. decipiens*, *H. jarzabczynum*, *H. koprovanum*, *H. nigrescens* and *H. vapenicanum*. Allelic frequencies for *'H. babiagorense'* are the same as for *H. jarzabczynum*

	Skd-1				*Lap*-1				*Pgm*-1			*Aat*-2		6-*Pgdh*-2		
Species	a	b	c	d	a	b	c	d	b	c	d	a	c	a	b	c
H. chrysostyloides	1	1	0	1	0	0	1	0	1	1	0	1	1	1	0	1
H. decipiens	1	0	0	0	0	0	1	0	1	1	0	1	1	1	0	1
H. jarzabczynum	1	0	1	0	0	0	1	0	1	1	0	1	1	0	1	1
H. koprovanum	1	1	1	0	0	1	1	1	1	1	0	1	1	1	0	1
H. nigrescens	1	0	1	0	0	0	1	0	1	0	1	1	1	1	0	1
H. vapenicanum	1	0	1	0	1	0	1	0	1	0	0	1	1	1	0	1

Table 8. Measures of allelic variation of *Hieracium chrysostyloides*, *H. decipiens*, *H. jarzabczynum*, *H. koprovanum*, *H. nigrescens*, *H. vapenicanum* and *'H. babiagorense'* (each species consists of unique multilocus allozyme genotype, *'H. babiagorense'* shares the same one with *H. jarzabczynum*). *N*, number of plants, *A*, mean number of alleles per locus, H_o, observed frequency of heterozygotes

	N	*A*	H_o
H. chrysostyloides	10	2.0	0.8
H. decipiens	30	1.6	0.6
H. jarzabczynum + *'H. babiagorense'*	40	1.8	0.8
H. koprovanum	22	2.2	1.0
H. nigrescens	10	1.8	0.8
H. vapenicanum	30	1.8	0.8

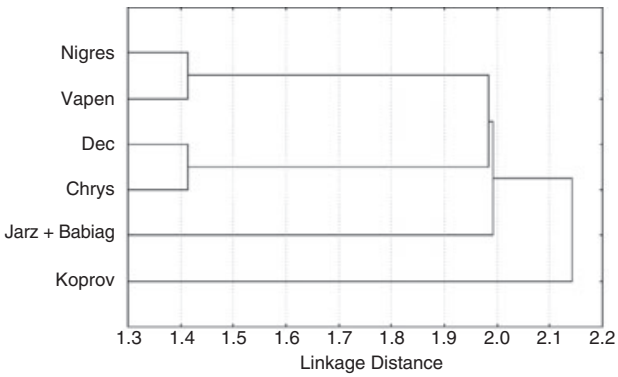

Figure 3. Cluster analysis, UPGMA method, of Euclidean distances between *Hieracium chrysostyloides* (Chrys), *H. decipiens* (Dec), *H. jarzabczynum* (Jarz), *H. koprovanum* (Koprov), *H. nigrescens* (Nigres), *H. vapenicanum* (Vapen) and *'H. babiagorense'* (Babiag) using four polymorphic allozyme loci (each species consists of one unique multilocus allozyme genotype).

achenes can be explained in several ways. Besides the possibility of meiotic development of the archesporial cell and a lack of fertilization, the empty achenes are much more likely explained by environmental conditions or the improper cutting of the upper part of the flowering buds.

GENETIC DIVERSITY

We have found no allozyme variation within species distinguished *a priori*: each consists of one unique allozyme genotype. '*Hieracium babiagorense*' shares the same allozyme genotype with *H. jarzabczynum*. However, we are aware that more sensitive methods/markers might reveal genetic differences. Thus, the morphologically distinguishable taxa (treated here at the species rank) represent single genotypes fixed through agamospermy, which became rather widespread or at least scattered in the Sudety Mountains and Western Carpathians. Except for *H. decipiens*, each species has at least one unique allele; two alleles were found to be unique to the Sudety Mountains and four to the Carpathians.

Genetic homogeneity seems to be a case of many *Hieracium* species (in the narow sense), mostly of those supposed to be of hybrid origin (Shi *et al*., 1996; Stace, Gornall & Shi, 1997; Gornall, 1999; Mráz *et al*., 2001; Štorchová *et al*., 2002). On the other hand, considerable genetic variation was found in nonhybrid 'basic' species *Hieracium alpinum* (triploid, agamospermous) in the Carpathians (Štorchová *et al*., 2002), the Krkonoše Mountains (the Sudety Mountains; Chrtek & Plačková, 2005), the Alps and the British Isles (Shi *et al*., 1996; Stace *et al*., 1997). Some level of genetic variation has also been found in several hybrid species of *Hieracium* sect. *Alpina* (Shi *et al*., 1996; Mráz *et al*., 2001; Štorchová *et al*., 2002) and in the progeny of three endemic *Hieracium* microspecies from Wales (Lledó & Rich, 2004).

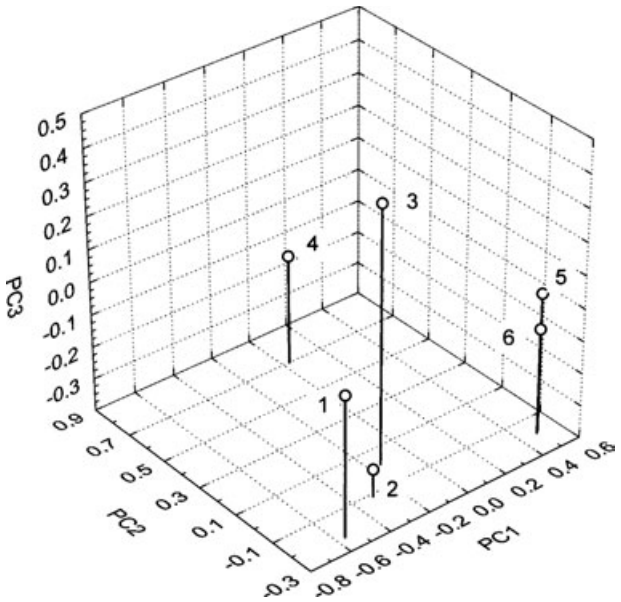

Figure 4. Principal coordinate analysis of allozyme data of *H. chrysostyloides* (3), *H. decipiens* (2), *H. jarzabczynum* and *'H. babiagorense'* (4, they share the same multilocus allozyme genotype), *H. koprovanum* (5), *H. nigrescens* (1) and *H. vapenicanum* (6). The first three coordinates explaining 43.42%, 29.65% and 12.50% of variation, respectively. Each species consists of one unique multilocus allozyme genotype, *'H. babiagorense'* shares the same one with *H. jarzabczynum*.

MORPHOLOGICAL ANALYSIS

Based on the results of CaDA computed on a complete dataset (matrix A), it is apparent that morphological differences between *Hieracium jarzabczynum, H. nigrescens* and '*H. babiagorense*' are sufficient to justify their separation into distinct taxa. *Hieracium vapenicanum* is also rather well separated along the first canonical axis. A second, more detailed, CaDA performed on a reduced dataset (matrix B, *H. chrysostyloides, H. decipiens, H. vapenicanum*) showed good separation of these taxa. Considering the geographical distribution of the target taxa, all taxa from the Western Carpathians (*H. jarzabczynum, H. vapenicanum* and '*H. babiagorense*') are well separated from each other. However, '*H. babiagorense*' fell rather close to a couple of Sudeten taxa, i.e. *H. chrysostyloides* and *H. decipiens*. *Hieracium nigrescens*, the third Sudeten taxon, seems to be well separated from both the Carpathian and remaining Sudetic taxa.

In the PCA of individual plants, the *a priori* recognized Carpathian taxa *H. jarzabczynum* and *H. vapenicanum* are rather separated from each other. The remaining samples belonging to *H. chrysostyloides, H. decipiens, H. nigrescens* and '*H. babiagorense*' did not show a clear structure. Partly separated along the second axis is *H. nigrescens*, which differs from *H. chrysostyloides, H. decipiens* and '*H. babiagorense*' first of all by the indumentum of the peduncles (only a few simple eglandular hairs in *H. nigrescens*, rather numerous in the remaining taxa; see Table 6), and the base of basal leaves (more or less truncate in *H. nigrescens*, cuneate in the remaining taxa). The lack of separation of some taxa in the PCA analysis reflects their overall similarity, while differences appear only in a few characters.

Apart from the characters used in the present morphometric studies, the colour of the styles and stigmas can be used for the reliable determination of some species. Yellow styles and stigmas were consistently observed in *H. chrysostyloides* and *H. vapenicanum*. In the rest of the species, brownish to dark styles and stigmas were observed (Chrtek, unpubl. data).

TAXONOMIC IMPLICATIONS

The combined morphological and allozyme study confirmed our preliminary concept of the six *a priori* recognized taxa treated here (except from '*H. babiagorense*', see below) at the species level. In the Sudety Mountains, the present study more or less confirmed the previously proposed taxonomic concepts (Zlatník, 1938; Zahn, 1936–38). In contrast, the taxonomic treatment of the group in the Western Carpathians deviates from previously proposed concepts (Zahn, 1936–38).

The Sudety Mountains. The three species treated in the present paper are recognized in this area, i.e. *Hieracium chrysostyloides, H. decipiens* and *H. nigrescens*. Two other species *H. apiculatum* and *H. nivimontis* have previously been recognized in this area (and are accepted by the present authors); thus, five species of the *Hieracium nigrescens* group are recognized in the Sudety Mountains.

The Western Carpathians. The two well-separated species, *Hieracium jarzabczynum* and *H. vapenicanum*, with rather overlapping geographical areas (the core of their distribution is in the Západné Tatry Mountains) can be recognized. A morphologically distinct population from Mount Babia hora/Babia Góra (named here '*H. babiagorense*') needs further study: we have refrained here from its formal taxonomic recognition.

All but one ('*H. babiagorense*') taxa clearly differ from each other in their multilocus allozyme genotypes (each consisting of one genotype/clone only). '*Hieracium babiagorense*' shares the same genotype with *H. jarzabczynum* and thus the nature of the morphological differences remains a puzzle that needs fur-

ther detailed examination. Based on the morphology, it is more or less grouped with the Sudeten taxa *Hieracium decipiens* and *H. chrysostyloides* (see above), which is also reflected in Zahn's monographs, where the plants from Mount Babia hora/Babia Góra refer to *H. nigrescens* ssp. *decipiens*. Geographically, Mount Babia hora/Babia Góra is situated between the highest parts of the Western Carpathians (where both *H. jarzabczynum* and *H. vapenicanum* occur) and the Sudety Mountains (with *H. decipiens, H. chrysostyloides* and *H. nigrescens*).

Differences in ploidy level support the separation of *H. chrysostyloides* ($2n = 45$) and *H. decipiens* ($2n = 36$). Generally, tetraploids are the most common ploidy level within the *Hieracium nigrescens* group. The chromosome number $2n = 3x = 27$ found in *H. vapenicanum* is rather rare in the group in Central Europe and contributes to the separation of this taxon.

Further differences were detected in pollen production and the size of pollen grains (J. Chrtek and P. Mráz, unpubl. data). The high production of pollen grains of a homogeneous size was found in *H. nigrescens* and *H. decipiens*, while rather small amounts of pollen of heterogeneous size were found in *H. chrysostyloides* and *H. jarzabczynum*; *H. vapenicanum* lacks any pollen.

Based on our results, two new combinations are proposed.

HIERACIUM JARZABCZYNUM (PAWŁ. & ZAHN) MRÁZ & CHRTEK F., **COMB. & STAT. NOV.**

≡*Hieracium pietroszense* ssp. *jarzabczynum* [*jarząbczynum*] Pawł. & Zahn in Zahn, Bul. Acad. Polon., Cl. Sci. Math. Nat., Sér. B, Sci. Nat. (Botanique) 1928: 210, 1929.

HIERACIUM VAPENICANUM (LENGYEL & ZAHN) CHRTEK F. & MRÁZ, **COMB. & STAT. NOV.**

≡*Hieracium nigrescens* ssp. *brachytrichellum* var. [γ] *vapenicanum* Lengyel & Zahn in Zahn, Magyar Bot. Lapok, 25 (1926): 369, 1927.

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