Evolution of *Veronica* (*Plantaginaceae*) on the Balkan Peninsula

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Abstract. With more than 6500 species of native seed plants on the Balkans and almost a third of them endemic, the Balkan Peninsula is known to be a place for diversification and formation of new species and an important refugium during the Ice Ages. One plant group, which exemplifies this well, is the genus *Veronica* (*Plantaginaceae*, formerly *Scrophulariaceae*). Four groups from this genus (*V.* subg. *Stenocarpon*; *V.* subg. *Chamaedrys*; *V.* subg. *Pseudolysimachium*, *V. alpina*-complex) display putative tertiary relict species, speciation within Pleistocene refugia and Pleistocene or Holocene speciation by hybridization and polyploidization on the Balkan Peninsula. I here review earlier published results for these groups and present new data. DNA sequence analyses from the nuclear ribosomal DNA (ITS) and plastid genome (*trnLF* region) were examined so as to shed more light on the relationship of the species from the Balkans. In addition, AFLP fingerprints were used to study *V.* subg. *Pseudolysimachium*, which exhibits limited DNA sequence divergence. Results support the distinctiveness of taxa from the Balkans as a divergent group of plants on the intra- and interspecific level. Limited resolution and support of the results further demonstrate the need for another marker system to continue the study of evolution of these plants of the Balkan Peninsula.

Introduction

The plant-life on the Balkan Peninsula is richer than any comparable area in Europe. There are more than 6500 species of native seed plants in the Balkans (Turrill 1929; Horvat & al. 1974: 72–78; Polunin 1980: 22). Several factors are responsible for this diversity. Apart from human impact, the combination of varied topography and habitats at the crossroads of several major floras (Central European, Mediterranean, Anatolian, and Pontic), which had found favorable refugia there to survive the Ice Age, is exceptional. Almost one-third of the species is endemic to the Balkans (Turrill 1929; Horvat & al. 1974: 72–78).

The Balkan Peninsula is known as an Ice Age refugium for many important plant species (Willis 1996; Taberlet & al. 1998). The climate during the maximum glaciation did not allow continuous forest vegetation but was rather a steppe with scattered trees and isolated pockets of forest (Frenzel 1968). Steppe species included mainly species from Asia and led to a major influx of Asian taxa into the European flora (Frenzel 1968; Ozenda 1988). In addition to being a source for the recolonisation of Europe (Taberlet & al. 1998; Hewitt 2000; Schmitt & Seitz 2002), the Balkan Peninsula was a place for further diversification and speciation, given its richness in endemic species.

The Pleistocene climate shifts have led to cycles of isolation of the plants in refugia and to their subsequent spread in more favorable times for a more continuous distribution. Whereas the more temperate elements were restricted to refugia in glacial times, the

cold-adapted plants had likely a more or less continuous distribution during these times and were restricted to the refugia during the interglacials. Many alpine plants might have originated in the Pleistocene in restricted alpine regions and had diversified owing to genetic drift (Zhang & al. 2001). Those taxa that were restricted in the Pleistocene to small refugia and afterwards expanded often did so into areas inhabited by plants from other refugia, which provided ample opportunity for hybridization and formation of new species (Hewitt 2000).

Veronica is a genus of the *Plantaginaceae* sensu APG (1998) and Albach & al. (2005), to which have been transferred most of the well-known genera of *Scrophulariaceae* from the Northern Hemisphere floras. *Veronica* includes about 500 species (Albach & al. 2004a). It is distributed over much of the Northern Hemisphere and beyond and is ecologically highly diverse, with species living in aquatic to dry steppe habitats, from sea level to high alpine regions. This diversity may explain the interest *Veronica* has evoked for a long time. Recently, molecular techniques and phylogenetic analyses have been applied to *Veronica* and related genera (Wagstaff & Garnock-Jones 1998; Albach & Chase 2001, Albach & al. 2004b, c). These studies have helped revolutionise our ideas about the evolution of the genus and have led to a new phylogenetic intrageneric classification of *Veronica* (Albach & al. 2004a). Combined with the vast amount of information from other aspects of their biology, we have now a much better understanding of how major groups in *Veronica* are delimited, related, and evolved. In the present article I summarise what we currently know about the evolution of the species of *Veronica* native to the Balkan Peninsula. Apart from a review of earlier publications, I present new sequence data on the species from *V.* subg. *Stenocarpon* and *V.* subg. *Chamaedrys* and AFLP-fingerprint data on *V.* subg. *Pseudolysimachium*.

Materials and Methods

DNA Extraction, Amplification, Sequencing

The total genomic DNA was extracted from herbarium material and silica gel-dried samples according to the 2x CTAB procedure of Doyle and Doyle (1987) and then washed twice with 70 % ethanol or using NucleoSpin plant DNA extraction kits (Macherey-Nagel; Düren, Germany) following the manufacturer's specifications. Nine sequences (seven for the ITS-region, two for the *trnLF*-region) are used here for the first time.

The *trnLF* region was amplified with primers **c** and **f** of Taberlet & al. (1991) and includes the *trnL* intron, 3′ *trnL*-exon and the *trnL-trnF* spacer. ITS sequences were amplified and sequenced using the primers 17SE (Sun & al. 1994) and ITS4 (White & al. 1991) and include ITS1, 5.8S rDNA and ITS2. PCR products were either separated on 1 % TBE-agarose gels; fragments corresponding to the expected size were excised and cleaned using the QIAquick™ PCR purification and gel extraction kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocols or cleaned using the Macherey-Nagel PCR purification kit (Macherey-Nagel; Düren, Germany) following manufacturer's specifications. Sequencing reactions (10 µl) were carried out using one µl of the BigDye Terminator Cycle Sequencing mix (Applied Biosystems Inc.). Both strands were sequenced on a Prism 377 automated sequencer (Applied Biosystems Inc.). Sequences were assembled and edited using Sequence Navigator™ (Applied Biosystems Inc.) or Sequencher™4.1 (Gene Codes Corp., Ann Arbor, MI, USA). Assembled sequences were manually aligned prior to analysis. Origin, voucher information and GenBank accession numbers for all sequences used in this study are given in Table 1.

AFLP Fingerprints

AFLP profiles were generated following the established procedures (Vos & al. 1995) and according to the PE Applied Biosystems (1996) protocol with only minor modifications (Tremetsberger & al. 2003). Genomic DNA (approx. 500 ng) was digested with *Mse*I (New England BioLabs) and *Eco*RI (Promega) and ligated (T4 DNA-Ligase; Promega) to double-stranded adapters in a thermal cycler for 2hr at 37°C. Preselective amplification (5 µL reactions) was performed using primer pairs with a single selective nucleotide, *Mse*I-C and *Eco*RI-A. Selective amplifications were performed with the primer combinations *Mse*I-CAT/*Eco*RI-ACT, *Mse*I-CTC/*Eco*RI-AAG, and *Mse*I-CAT/*Eco*RI-ACC. Selective amplification products were run in a 5 % denaturing polyacrylamide gel with an internal size standard (GeneScan®-500 [ROX], PE Applied Biosystems) on an automated DNA se-

quencer (ABI 377). Polyacrylamide gels run on automated sequencers increase the resolution and decrease the probability of scoring fragments of similar size as homologues. Fragments from the polyacrylamide gel were analyzed using the ABI Prism Gene-Scan® 2.1 Analysis Software (PE Applied Biosystems) and Genographer 1.1 (Benham 1998). Peaks (i.e. fragments) were scored manually as present (1) or absent (0) in a readable region of bands from 50 to 500 base pairs in length and used to construct a presence/absence data matrix with individual plants in rows and bands in columns. Origin and vouchers for all specimen used in the AFLP analysis are given in Table 2.

Data Analysis – sequences

Data matrices were analysed separately and combined using maximum parsimony and maximum likelihood criteria in PAUP*4.0b10 (Swofford 2002). Potentially parsimony informative gap characters were coded as present/absent. Heuristic parsimony searches were conducted using 10 replicates, starting from random trees with tree bisection reconnection (TBR) branch swapping and MulTrees in effect and no tree limit. Parsimony bootstrap analyses involved 1000 replicates with the same search parameters as above but simple taxon addition. Maximum

Table 1. Species name, origin, voucher information and GenBank accession number of accessions (ITS/*trnLF*).

Outgroups

Veronica jacquinii Baumg. – cult. BG Bonn (Albach 70, WU – AF313000/AF513341); *V. scutellata* L. – Waldviertel, Austria (Dobes 7026, WU – AF509805/AF486393); *V. serpyllifolia* L. – Bonn, Germany (Albach 64, WU – AF313017/AF486400); *V. triloba* Opiz – Aphrodisias, Turkey (Albach 242, WU – AF509803/AF486366); *V. triphyllos* L. – Aphrodisias, Greece (Albach 244, WU – AF509795/AF486396);

Data set of *V.* subg. *Stenocarpon* (Boriss.) M.M. Mart. Ort., Albach, & M.A. Fisch

Paederotella pontica (Rupr.) Kem.-Nath. – Georgia (Sachokia, 01.9.1951, TBS – AF515214/AF486382); *Veronica ciliata* Fisch. – Qinghai, China (Miehe & al., 98-33313, GOET – AF515215/AF486385); *V. densiflora* Ledeb. – Altai (Staudinger s.n., SALA – AY741521/AY776282); *V. erinoides* Boiss. & Sprun. – Gioua Mts, Greece (Hagemann, Scholz & Schmitz 461, SALA – AY741523/n.a.); *V. fruticans* Jacq. – Scotland, UK (Viv Halcro VH030, K – AY144462/n.a.); *V. fruticulosa* L. – cult. BG Bonn (Albach 71, WU – AF313004/AF486383); *V. lanosa* Royle ex Benth. – Pakistan (Schickhoff 1377, GOET – AY540868/AY486442); *V. lanuginosa* Benth. – 13 km east of Mt Everest, Nepal (Dickore 6482, GOET – AF509793/AF486386); *V. macrostemon* Bunge ex Ledeb. – Altai (Staudinger AL23-18, SALA – AY741522/AY486441); *V. mampodrensis* Losa & P. Monts. – Velilla de Río Carrión , Spain (Martínez Ortega 713, SALA – DQ227331/ DQ227337); *V. mexicana* S. Watson – Mesa el Campanero, Sonora, Mexico (Fishbein 2586, TEX – DQ227332/n.a.); *V. monticola* Trautv. – Georgia (Ivanisvili 26.07.1983, WU – DQ227333 & DQ227334/ n.a.); *V. nummularia* Gouan – Tosses, pr. La Molina, subida al pico pico Niu d'Aliga, Spain (Martínez Ortega 718, SALA – DQ227335/n.a.); *V. saturejoides* Vis. subsp. *kelleri* (Degen & Urum.) P. Monts. – Mt Pirin, Bulgaria (Albach 558, WU – AY144461/AY486450); *V. thessalica* Benth. – Olymbos, Greece (Raus & Rogl 5072, SALA – AF509792/AF513343).

Data set of *V.* subgenus *Chamaedrys* (W.D.J. Koch) M.M. Mart. Ort., Albach & M.A. Fisch.

Veronica arvensis L. – Stromberg, Germany (Albach 147, WU – AF313002/AF486380); *V. arvensis* – Richmond Park, UK (M. Sheahan 9, K – DQ227328/n.a.); *V. chamaedryoides* Bory & Chaub. – Peloponnes, Greece (Albach 393, WU – AF673611/AY673631); *V. chamaedrys* subsp. *chamaedrys* L. – Homefield Wood, UK (M. Fay 149, K – DQ227329/n.a.); *V. chamaedrys* subsp. *chamaedrys* – cult. RBG Kew, ex Norway (Albach 121, K – AF313003/ AF486377); *V. chamaedrys* subsp. *micans* M.A. Fisch. – Austria (Schönswetter 2567, WU – AY673616/AY673632); *V. krumovii* (Peev) Peev – Eastern Rhodopes, Bulgaria (Albach 484, WU – AY673612/AY673633); *V. laxa* Benth. – Pakistan (Dickoree 13042, GOET – AY673613/AF486378); *V. magna* M.A. Fisch. – Orbetia, Georgia (Albach 360, WU – AY6736152/AY673634); *V. micrantha* Hoffmanns. & Link – Portugal (Martínez Ortega 1754, SALA – DQ227330/ DQ227336); *V. verna* L. – Bad Kreuznach, Germany (Albach 149, WU – AF509789/ AF486379); *V. vindobonensis* (M.A. Fisch.) M A. Fisch. – cult. BG Wien (M.A. Fischer s.n., WU – AY673614/AF510426).

Table 2. Samples (with vouchers) used in the AFLP analysis of *V.* subg. *Pseudolysimachium*.

Veronica bachofenii Heuff. – cult. Bot. Gard. Wien, ex Bot. Gart. Halle (Albach 978, MJG); *V. barrelieri* Schott ex Roem. & Schult. – Bulgaria (Vladimirov s.n., WU); Croatia (Schneeweiss & Schönswetter, WU); Croatia (5 individuals, Martínez Ortega 908, SALA); *V. crassifolia* Wierzb. ex Heuff. – Demogled-Herkulesbach, Romania (Köster s.n., WU); *V. daurica* Steven – cult. Bot. Gard. Jena (Albach s.n., WU); *V. incana* L. – cult. Bot. Gard. Bonn (Albach 155, WU); *V. longifolia* L. – cult. Bot. Gard. Bonn (Albach 66, WU); Kühkopf, Germany (Fay et al. s.n., K); Frey´s Is., UK (Sheahan 48, K); Altai (Tribsch 31.7.2002, WU); *V. orchidea* Crantz – Wien-Salmansdorf, Austria (Fischer 21.7.2000, WU); Montana, Bulgaria (Albach 540, WU); White Carpathians, Czech Republic (Köster s.n., WU); Klausenburg, Romania (Köster s.n., WU); *V. porphyriana* Pavlov – Altai (Tribsch 2002, WU); *V. schmidtiana* Regel – Kushiro-mashi, Hokkaido, Japan (Umezawa 20130, WU); *V. spicata* L. – Stanner Rock, UK (5 samples, Jones s.n., K); Avon Gorge, UK (2 samples, M. Fay s.n., K); Uppsala, Sweden(Thulin 10035, UPS); Neusiedler See, Austria (Krefft s.n., WU); Brey, Germany (Albach 224, WU); Thorenburg canyon (Koester s.n., WU); cult. RBG Kew, ex Hainburg, Austria (Kew 1970-759 – Chase s.n., K); cult. Bot. Gard. Bonn (Albach 65, WU); ex Knauber, Bonn (Albach 214, WU); "Nana Alba" cult. RBG Kew (Kew 1979-6053 – Chase s.n., K); „Sarabande" cult. RBG Kew (Kew 1973-21579 – Chase s.n., K); Ukraine (2 samples, WU); *V. spuria* L. – Burgenland, Austria (Fischer 04.06.2000, WU).

likelihood analyses were conducted using a model of sequence evolution as inferred by Modeltest 3.06 (Posada & Crandall 1998) using the Akaike Information Criterion, three to six replicates of random taxon addition starting from random trees with TBR branch swapping, MulTrees in effect and no tree limit. Likelihood bootstrap analyses for the combined dataset involved 500 replicates with the same search parameters as above but simple taxon addition. For analyses of *V.* subg. *Stenocarpon*, sequences of *V. scutellata, V. serpyllifolia, V. triloba, V. triphyllos, V. jacquinii,* and *V. chamaedrys* were designated as outgroups. For analyses of *V.* subg. *Chamaedrys*, the same taxa were designated as outgroups except for replacing *V. chamaedrys* with *V. thessalica*. To evaluate different phylogenetic hypotheses in *V.* subg. *Stenocarpon*, the likelihood-based Shimodaira-Hasegawa test (with the same model used in the maximum likelihood analysis and RELL optimization) and parsimony-based Templeton test were used as implemented in PAUP4.0b (Swofford 2002) with the combined data set.
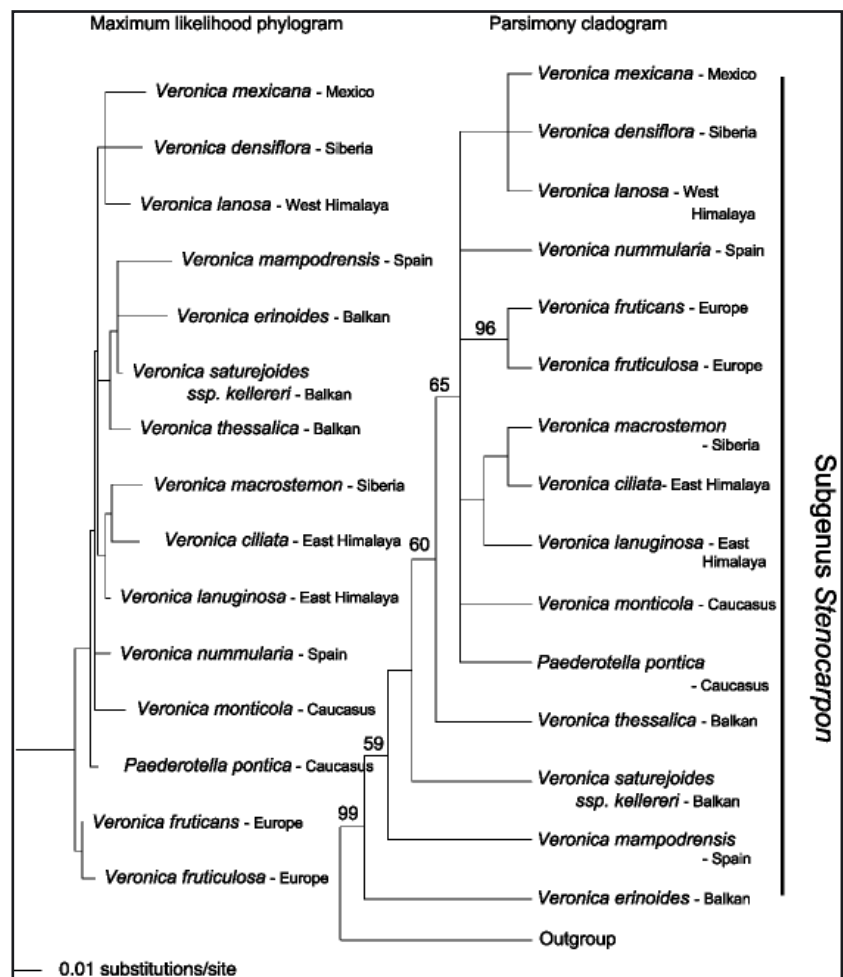
Data Analysis – AFLP

AFLP fingerprints were examined using neighbour-joining and principal coordinate analysis (PCoA). Neighbour-joining (Saitou & Nei 1987) and neighbour-joining bootstrap analyses using the Nei-Li-distances have been conducted in PAUP4.0b10 (Swofford 2002). Principal coordinate analysis (PCoA) was conducted using Dice genetic distances between all pairs of accessions as implemented in R4.0 (Casgrain & Legendre 2001) and the first two axes projected in two two-dimensional graphs.

Results

Veronica subg. *Stenocarpon*

The ITS-dataset included 21 taxa with 745 aligned characters, 91 of them potentially parsimony-informative and no indel character, whereas the *trnLF*-dataset with 17 taxa included 998 aligned characters, 43 of them potentially parsimony informative with one indel character. The combined dataset included 17 taxa and 1743 characters with 135 of them potentially parsimony-informative. Modeltest (Posada & Crandall 1998) chose the GTR+Γ-model (Γ=0.18) for the ITS data set, the TVM+Γ-model model (Γ=0.74) for the *trnLF* dataset and the GTR+I+Γ-model (Γ=0.72) for the combined analysis as the optimal model. The 14 most parsimonious trees of the ITS-analysis required 365 steps (Fig. 1; CI=0.72; RI=0.59), with the 1755 most parsimonious tree from the *trnLF*-analysis requiring 239 steps (not shown; CI=0.90; RI=0.69). The most likely tree of the maximum likelihood analysis of the ITS-dataset (Fig. 1) required six more steps under parsimony, whereas that from the analy-

Fig. 1. Results from the analysis of the ITS-dataset. Left, optimal tree from the maximum likelihood analysis. Right, strict consensus of 14 most parsimonious trees. Numbers above the branches indicate bootstrap support.

sis of the *trnLF*-dataset being one of the most parsimonious trees (ITS ML vs. MP: -ln = 2858.12 vs. 2860.88-2862.06; *trnLF*: -ln = 2792.57). The combined dataset had a single optimal parsimony tree requiring 593 steps (Fig. 2; CI = 0.79; RI = 0.72; -ln = 5583.40) and the best tree chosen by the likelihood analysis required 594 steps under parsimony (-ln = 5579.49).

Veronica subg. *Stenocarpon* is a well supported group by both the ITS-analysis and the combined analysis (Figs 1, 2; >98 % BP). Support for internal relationships within *V.* subg. *Stenocarpon* is low, with internal branches being short (Fig. 1). The combined analysis (Fig. 2) reveals a clade of Asian species with two Eastern Himalayan and the two Siberian species, forming each well supported (>75 % bootstrap percentage (BP)) sister-groups. Support for other internal groups is lacking. For example, *V. densiflora* groups in the results of the ITS-analysis (Fig. 1) with the Western Himalayan *V. lanosa* and the Mexican species, *V. mexicana*, for which a *trnLF*-sequences is still missing and which therefore was not included in the *trnLF*- and combined analysis, but the relationship in the ITS-analysis is not supported by the bootstrap analysis. Results from the parsimony analysis of the *trnLF* dataset are largely unresolved and even some trees in which the subgenus is not monophyletic are equally parsimonious (result not shown). The only clade retrieved in this analysis is the sister-group relationship of *V. ciliata* and *V. lanuginosa*. The tree resulting from the maximum likelihood analysis of the *trnLF*-dataset is identical with the maximum likelihood analysis of the combined dataset (Fig. 2) and is therefore also not shown. Surprisingly, the rooting inferred by parsimony and maximum likelihood analyses differs dramatically. Whereas parsimony analyses from both ITS and the combined dataset (Figs 1, 2) reveal a grade of Balkan-Iberian species (*V. thessalica*, *V. saturejoides*, *V. mampodrensis*) as sisters to the rest of the subgenus, all maximum likelihood analyses (Figs 1, 2) support a sister-group relationship of *V. fruticulosa* (plus *V. fruticans* in the ITS-analysis) to the rest of the subgenus. All Central Asian-Himalayan species form a clade with the exception of *V. lanosa*. Shimodaira-Hasegawa tests and Templeton tests were insignificant (p = 0.29 and p = 0.80, respectively). Increasing outgroup sampling did not lead to different results (Albach, unpubl.).
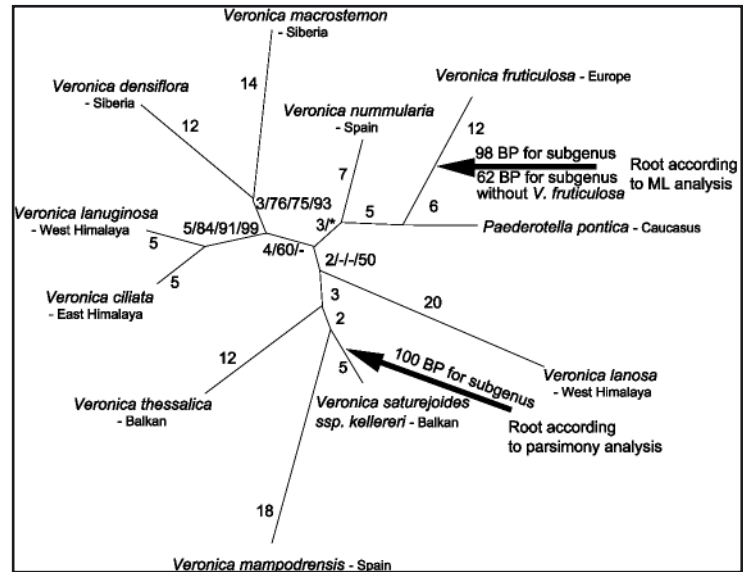

Fig. 2. Unrooted phylogram from the maximum likelihood analysis of the combined dataset.
Numbers along the branches indicate parsimony branch lengths/ parsimony bootstrap percentage/ maximum likelihood bootstrap percentage/ parsimony bootstrap percentage from the analysis of the *trnL-F*-dataset only. Asterisk indicates branch not present in the most parsimonious tree. **ML** – Maximum likelihood; **BP** – bootstrap percentage.

Veronica subg. *Chamaedrys*

The ITS-dataset included 18 taxa with 743 aligned characters, 111 of them potentially parsimony-informative with four indel characters, whereas the *trnLF*-dataset included 16 taxa with 988 aligned characters, 48 of them potentially parsimony informative with two indel characters and the combined dataset with 16 taxa had 1731 characters and 151 of them potentially parsimony-informative. Modeltest (Posada & Crandall 1998) chose the GTR + I + Γ-model (Γ = 0.38) for the ITS data set, the GTR + Γ-model (Γ = 0.45) for the *trnLF* dataset and the TIM + I + Γ-model (Γ = 0.63) for the combined data set as the optimal model. The ten most parsimonious trees of the ITS-analysis required 367 steps (Fig. 3A; CI = 0.73; RI = 0.73), with the four most parsimonious tree from the *trnLF*-analysis requiring 223 steps (Fig. 3B; CI = 0.89; RI = 0.81). The most likely trees (Fig. 3A, B) were just one step longer under parsimony in both cases (ITS ML vs. MP: -ln = 2834.53 vs. 2835.07-2837.93; *trnLF*: ML vs. MP: -ln = 2618.47 vs. 2619.47). The combined dataset had a single optimal parsimony tree requiring 593 steps (CI = 0.79; RI = 0.72; -ln = 5583.40) and the best tree chosen by the likelihood analysis required 594 steps under parsimony (Fig. 3C; -ln = 5579.49).

All analyses support four groups in *V.* subg. *Chamaedrys*: *V. arvensis*, *V. verna*, the Asian perennial species, and the European perennials. Support for *V. arvensis* as sister to the rest of the subgenus is high (99 BP in combined analysis). The branching order of the other three groups varies with all three possibilities realized in at least one analysis. Monophyly of the perennials is, however, only shown in the parsimony analysis of the ITS dataset (Fig. 3A), with most analyses supporting the sister-group relationship of *V. verna* and the perennial Europeans (Fig. 3B, C; 77 BP in maximum likelihood analysis of combined dataset). Within the European perennials, the Balkan endemics, *V. chamaedryoides* and *V. krumovii*, are consistently occupying first branches but support for relationships within this group is low.

Veronica subg. *Pseudolysimachium*

The AFLP dataset consists of 42 taxa and 732 bands scored with 73 of them constant. The number of AFLP fragments per taxon ranged between 97 and 159 bands. Ploidy level is not known for the individuals used in this study but there was a tendency for taxa known to be diploid ($2n = 34$) to have fewer bands (e.g. *V. porphyriana*, which had the lowest number of bands) than tetraploid ($2n = 68$) taxa (*V. spicata*, which is mostly tetraploid had the highest number of bands). Intraspecific variation was large in *V. spicata* ($n = 104–159$). Intraspecific variation in *V. barrelieri* was significant with respect to geographical origin with the Bulgarian sample having markedly fewer bands than the Croatian samples (109 vs. on average 140). No species-specific fragments were found for species for which individuals from more than one population was analyzed.
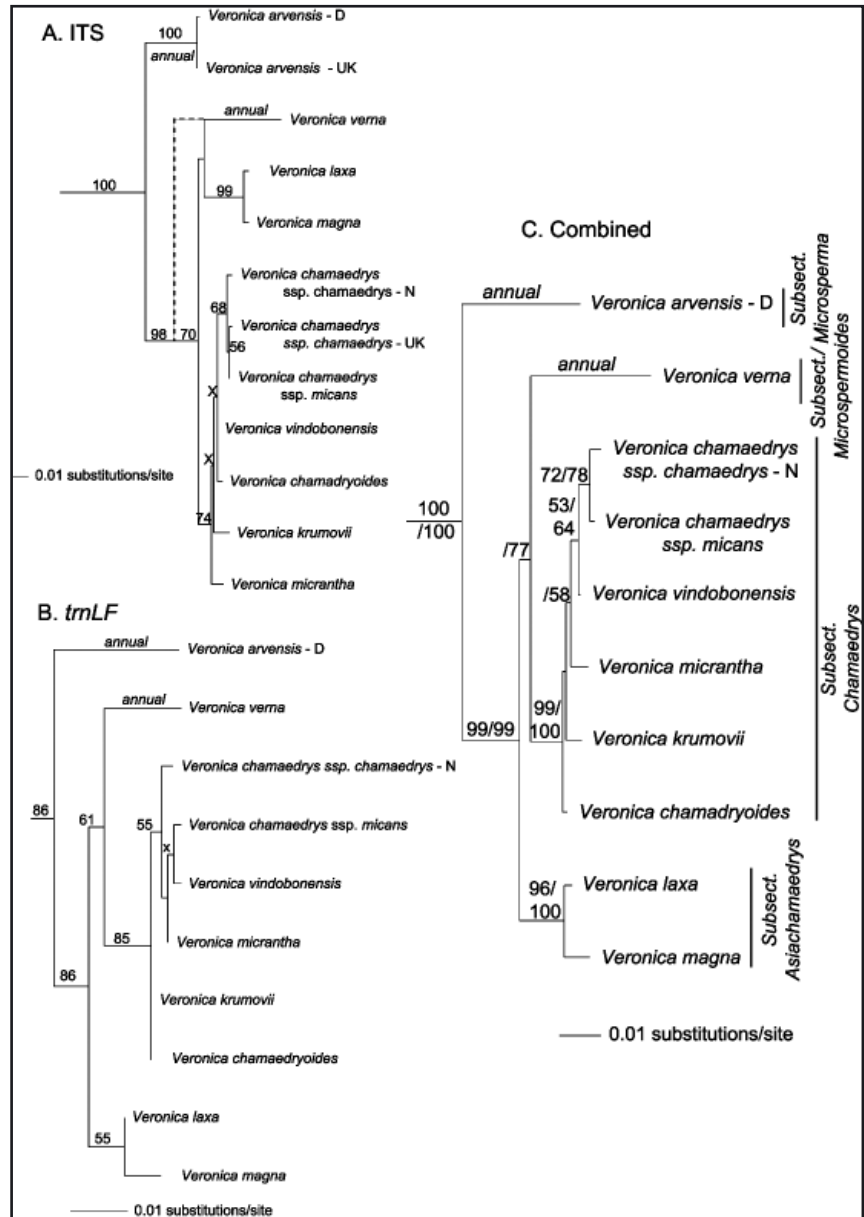
Fig. 3. Phylograms derived from the maximum likelihood analyses of *V.* subg. *Chamaedrys*. Numbers above the branches indicate bootstrap support.
D, German accession; **N**, Norwegian accession; **UK**, English accessions; **X**, branch not present in most parsimonious tree; **A**, optimal tree for the ITS-dataset. Dashed line indicates branch present in most parsimonious tree; **B**, optimal tree for the *trnL-F*-dataset; **C**, optimal tree for the combined dataset.

Neighbour-joining analysis and Principal Coordinate Analysis (PCoA) did not cluster samples into species specific groups but show similar results. Hybridization and polyploidy are common in *V.* subg. *Pseudolysimachium* (see below). Relationships based on hybridization are difficult to show on a phylogenetic tree. Therefore, only the results from the PCoA are shown (Fig. 4). The x-axis explains 17.0 %; the y-axis explains 9.0 % of the variation. Both are low values and further emphasize the complexity of the results.
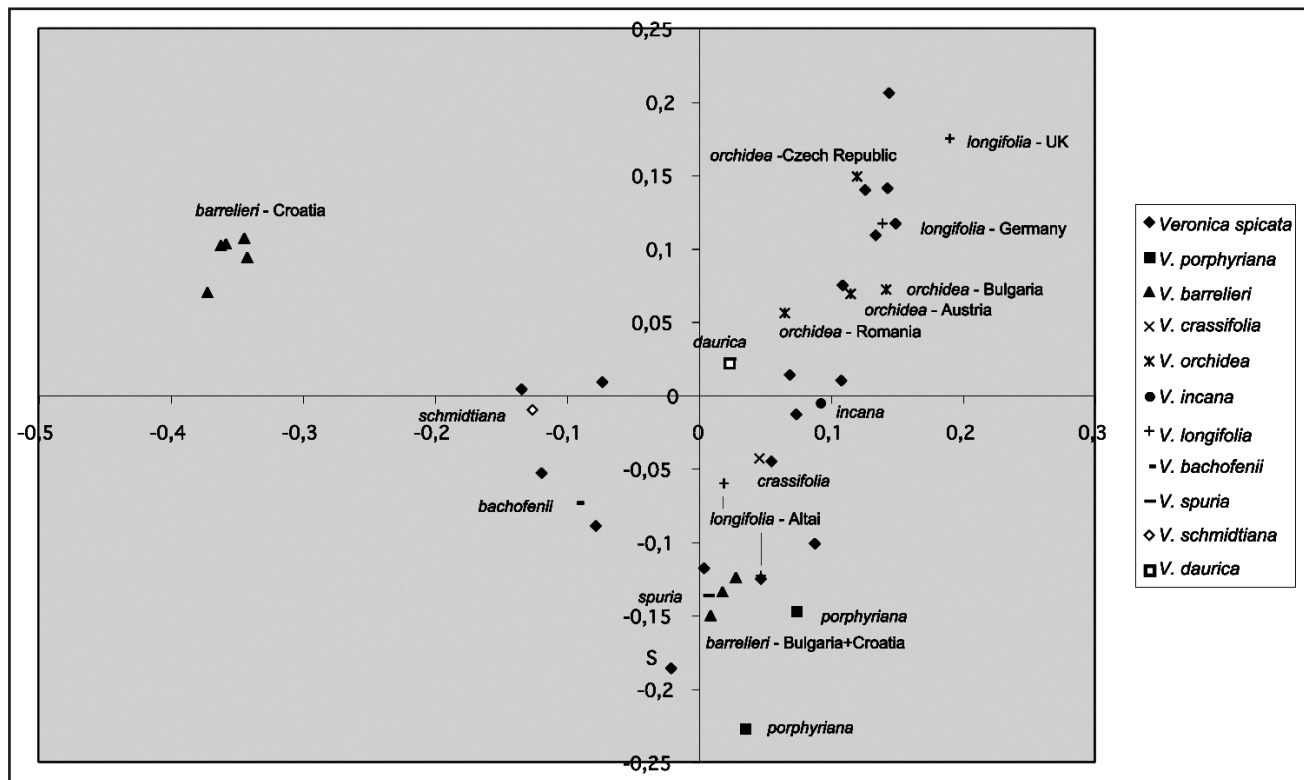
Fig. 4. First two axes of the principal coordinate analysis of *V.* subg. *Pseudolysimachium*.

Discussion

Veronica in Europe

Based on detailed morphological analyses (e.g., Fischer 1972, 1973b, 1975a, b, 1978, 1991; Trávníček 1998, 2000; Martínez-Ortega & Rico 2001; Albach & Fischer 2003), we currently have a well-founded estimate of species diversity of *Veronica*, especially in Europe. Molecular analyses have been contributing to this (Martínez-Ortega & al. 2004; Albach & al. in press; Albach, submitted). On the base of these analyses our estimate that about 80 species of *Veronica*, representing 10 of 13 subgenera recognized by Albach & al. (2004a), are found in Europe. About 40 species found in nine different subgenera are endemic to Europe. On the Balkan Peninsula, we can find about 60 species of *Veronica*, with 14 of them endemic (*V. chamaedryoides, V. contandriopouli, V. crinita, V. erinoides, V. glauca, V. krumovii, V. oetaea, V. orbelica, V. orbiculata, V. rhodopea, V. sartoriana, V. saturejoides, V. thessalica, V. turrilliana*). Some of these species have been investigated in the present study using DNA sequence and AFLP data. Furthermore, I discuss results from earlier analyses shedding more

light on the evolution of *Veronica* on the Balkan Peninsula.

Veronica subg. *Stenocarpon* – relics of alpine regions

Veronica subg. *Stenocarpon* is probably the most surprising group revealed by molecular phylogenetic analysis. The morphological diversity and especially its biogeographical distribution pattern make it a fascinating group to study. *Veronica* subg. *Stenocarpon* includes approximately 25 species, most from Central Asia but also seven species from Europe plus the Mexican endemic representative of the genus, *V. mexicana*. This Mexican-Himalayan disjunction appearing in the analysis of ITS-sequences (Fig. 1), however without bootstrap support, merits further study. Among the European species most are endemic in the Balkans, often known only from one or very few localities, such as *V. saturejoides* subsp. *kelleri* (Mt Pirin), *V. saturejoides* subsp. *munellensis* (Mt Mnela), and *V. thessalica* (Mt Gjaliqa e Lumes; Mt Olymbos; Mt Jakupica; Sar Planina; Mt Koritnik). *Veronica erinoides* is known from only five localities (Giona, Vardousia, Lidorikiou Ori, Parnassos,

Killini). *Veronica thessalica* and *V. erinoides* were thought to be synonyms (e.g., Stroh 1942) but even before the results presented here (Fig. 1) a detailed morphological analysis had shown that one is having terminal and the other only lateral inflorescences (Fischer 1969), a character formerly used for dividing sections in *Veronica*. Molecular analyses on the genus level (Albach & Chase 2001; Albach & al. 2004c) has shown that the character is labile and not useful for delimiting natural groups in *Veronica*.

So far, all species for which chromosome numbers have been counted are diploid, although apart from the seven European species only one Asian species has been investigated (Albach & al. submitted). Sequence analysis using parsimony (Figs 1, 2) revealed that the Balkan endemics *V. saturejoides*, *V. thessalica* and *V. erinoides*, together with *V. mampodrensis* from the Iberian Peninsula constitute a paraphyletic grade of consecutive sisters to the Asian species of the subgenus, plus *V. mexicana* and three more European species (*V. fruticans*, *V. fruticulosa*, *V. nummularia*). *Veronica mampodrensis* has been included here for the first time in a phylogenetic analysis. Its position in *V.* subg. *Stenocarpon* is in general agreement with morphology, although Fischer & Fischer (1981) suggested a closer relationship with *V. fruticans* and *V. fruticulosa*. Based on their restricted distribution areas (in comparison with other species of the subgenus), diploid ploidy level and their position in parsimony analyses, the Balkan endemic species could be considered to be relictual species, in accord with the idea of the Balkan Peninsula as a refugium for plant groups since the Tertiary (Horvat & al. 1974; Willis 1996; Taberlet & al. 1998). However, support for the relationships is low and, more important, maximum likelihood analyses differ with regard to the topology of *V.* subg. *Stenocarpon* in *V. fruticulosa* being a sister to the rest of the subgenus (Figs 1, 2). The reason for this difference is solely a difference in rooting the tree, because unrooted phylogenies from both kinds of analyses are identical (Fig. 2). I evaluated several reasons for the difference in rooting. For example, addition of other outgroup taxa to the dataset does not change the results (results not shown). Rather the lack of sufficient informative characters leads to spurious differences in the results. Thus, the most parsimonious tree is not rejected as significantly worse by the likelihood-based Shimodaira-Hasegawa test, and the most likely tree is not rejected as significantly worse by the par-

simony-based Templeton test. This lack of sufficient informative characters currently renders it impossible to resolve the mysteries of the biogeographical and morphological evolution in this clade. Faster evolving DNA regions will be necessary for future analyses of the evolution of the group.

Furthermore, no calibration point for molecular dating is available for *Veronica*, and extensive rate heterogeneity in the genus (Albach & Müller in prep.) renders the common substitution rate unusable. Therefore, I am currently unable to substantiate the claim that the first branching event in *V.* subg. *Stenocarpon* represents a Tertiary event. Biogeographically, the situation resembles *Ramonda* (*Gesneriaceae*), which is likewise considered a Tertiary relict and found in the Pyrenees and the Balkan Peninsula (Meyer 1970), although, in contrast to *Veronica, Gesneriaceae* are of tropical origin. Work is in progress studying phylogeographical patterns within the Balkan endemics *V. saturejoides, V. thessalica, V. erinoides*, the Iberian endemics *V. nummularia* and *V. mampodrensis* and the more widespread *V. fruticans*, so as to investigate the hypothesis that the endemic species are relictual species that had failed to disperse out of their glacial refugia, whereas *V. fruticans* had managed to spread from a glacial distribution area into and close to the Alps, north towards to Scandinavia. Additional sequence data will be helpful to estimate the genetic variation within the Balkan endemic species in order to differentiate between the hypotheses of recent bottlenecks or long persistence in refugial areas, so as to explain the limited distribution of these taxa and to help protect these rare alpine species.

Veronica subg. *Chamaedrys* – Diploid taxa marking hypothesized forest refugia

Veronica subg. *Chamaedrys* consists of four different groups: *V. arvensis* (incl. *V. sartoriana* = *V.* subsect. *Microsperma* (Römpp) Stroh), *V. verna* (most likely together with *V. dillenii* and *V. brevistyla*; = *V. subsect. Microspermoides* Albach[1]), *V. laxa* and *V. magna* (= *V.*

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[1] *Veronica* subsectio *Microspermoides* Albach, subsect. nov. – Typus: *V. verna* L. Spec. Pl. 1: 14, 1753; lectotypus (ab E. Fischer 1997 designatus): LINN 26.63. Annua aut raro biennis, −30 cm, erecta, simplex. Folium sessile, pinnati- vel palmatipartitum vel −lobatum. Inflorescentia terminalis, multiflora, glandulosa. Pedicellus brevior quam calyx. Capsulae obcordatae, numquam glandulosae. Semina plana. Numerus basalium chromosomatium x = 9. Planta inutilis in Europa et Asia diffusa.

subsect. Asiachamaedrys Albach[2]), and the European perennial species (*V. Chamaedrys*, *V. vindobonensis*, *V. chamaedryoides*, *V. krumovii*, and *V. orbelica*; = *V. subsect. Multiflorae* Benth.). Surprisingly, the perennial species do not form a monophyletic group, except for the parsimony analysis of the ITS dataset (Fig. 3A). Thus, a likely scenario is that first a formerly widespread Eurasian perennial taxon split into a Mediterranean and an Asian taxon, and *V. verna* (and relatives) evolved from the Mediterranean group. *Veronica verna* is nowadays widespread across Eurasia and nothing is known about its place of origin to confirm this hypothesis.

Veronica subsect. *Multiflorae* includes prominent species of forest vegetation, mainly along the forest edges, in open places and clearings. Originally considered one species, the group consists of five species and three additional intraspecific taxa. Whereas *V. chamaedrys* subsp. *chamaedrys* is a tetraploid plant, all others are diploid. They differ not only in their ploidy level but also in the distribution range, with the tetraploid widespread throughout Europe and the only one that has reached North Europe. The diploids occur in South, especially South and Central Europe, and are mostly confined to small areas that have been assumed as Pleistocene refugia. *Veronica chamaedryoides* is endemic to Greece, where it is fairly widespread, and found in open forests, meadows and rocky slopes up to 1900 m. Greece is commonly suggested as a Pleistocene refugium for many submediterranean taxa (e.g. Huntley & Birks 1983; Tzedakis & al. 2002). *Veronica krumovii* is endemic to Bulgaria. It is morphologically separated but its ecology and exact distribution has not been studied adequately (Mirek & Fischer 1986). Although currently not restricted to the western Black Sea Coast, a suggested Pleistocene refugium by Huntley & Birks (1983), it could have expanded from there to its current distribution area. *Veronica orbelica* is another Bulgarian endemic. It is found in Southwest Bulgaria and is morphologically close to *V. vindobonensis* (Mirek & Fischer 1986). It grows in drier habitats and may have originated in the drier interior part of Bulgaria. Unfortunately, no authenticated

material of *V. orbelica* was available for this study because much of the material seen in nature in Bulgaria was intermediate between *V. orbelica* and *V. vindobonensis*. Clear delimitation of these two species will be an important task for the future.

Veronica vindobonensis is the most widespread diploid taxon occurring from Northwestern Anatolia to South Germany. It is probably adapted to the driest types of forests within the group. Its wide distribution argues either for rapid spread from a refugium (possibly furthest to the north in Hungary, according to Willis & al. 2000) after the last glaciation, or a more widespread distribution even in the Pleistocene. It sometimes reaches subalpine habitats in Bulgaria (Albach pers. obs.), which might contend for the latter. *Veronica chamaedrys* subsp. *micans* is mostly known from the Austrian and German northern calcareous Alps and is the only taxon that can be considered truly subalpine. Its distribution is especially intriguing, because it is exactly confined to the hypothesized Pleistocene refugia in the northeastern Alps (Tribsch & Schönswetter 2003). Another diploid taxon is found in South Austria and is informally called *V. chamaedrys* „carintho-stiriaca" (Fischer 1973a, pers. comm.). It is similar to *V. chamaedrys* subsp. *micans* occurring on drier habitats but is morphologically distinct (Fischer 1973b) and may have survived in a forest refugium in the southeastern Alps (Bennett & al. 1991). Another diploid population can be found further south in Dalmatia but has been not studied intensively (Fischer unpubl.). The tetraploid *V. chamaedrys* has the widest distribution of all taxa. It occurs throughout Europe, with the exception of the Mediterranean islands and the Arctic. In Northern Greece it barely overlaps with *V. chamaedryoides* (Fischer 1991).

Results from sequence analysis suggest that the southern diploid taxa of the group, *V. krumovii* and *V. chamaedryoides*, were not involved in the origin of tetraploid *V. chamaedrys* subsp. *chamaedrys* but were isolated in their Pleistocene refugia. Unfortunately, molecular dating is not possible in *Veronica* and therefore the age of divergence is not inferable. Several scenarios regarding the origin of tetraploid *V. chamaedrys* are possible on the base of sequence data. *Veronica chamaedrys* subsp. *micans* is very likely a parent of it, based on the high similarity of their ITS-sequences (Fig. 3A.). The other parent could be an unsampled diploid taxon, possibly the "stiriaca-carinthiaca"-type or the "dalmatica"-type, based on the fact that none of

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[2] *Veronica* subsectio *Asiachamaedrys* Albach, subsect. nov. – Typus: *V. laxa* Benth. Scroph. Ind.: 35, 1835; typus: K n.v. Perennis. 35–90 cm, erecta. Caulis ubique pubescens. Folia brevipetiolata, ovata, grosse dentate, plus quam 25 cm. Inflorescentia lateralis, plus quam 10 cm longa, multiflora. Calyx longior quam pedicellus, plus quam 4 mm. Corolla caerulea. Capsula obcordata, brevior quam calyx, base cuneata, glabra.

the sampled diploid taxa had the tetraploids plastid-sequence (Fig. 3B), although an autopolyploid event cannot be excluded. Another possibility to be considered is the polytopic origin, supported by morphological variation in the tetraploid cytotype (Fischer 1973a). More intensive sampling will be needed to reveal the parents of tetraploid *V. chamaedrys*. So far, we can be only reasonably certain that the tetraploid *V. chamaedrys* sampled in our analysis had ancestors that lived close to the ice-sheet during the Pleistocene. Such a scenario would support Stebbins' (1984) secondary-contact hypothesis, in which polyploids arise at the margin of distribution. Thus, diploid taxa survived in their Pleistocene refugia and, after the ice retreated, spread out into the formerly glaciated area and encountered other diploid survivors, with which they formed polyploid hybrids. Such kinds of neo-polyploids are known to surpass their ancestors in invasion potential, especially in habitats that have become available after the retreat of the Pleistocene ice sheets (Ehrendorfer 1965, 1980). The involvement of the subalpine *V. chamaedrys* subsp. *micans* in the origin of *V. chamaedrys* rather than a true forest species, such as *V. krumovii*, further supports such a scenario. Consequently, the Balkan endemic species of *V.* subsect. *Multiflorae*, in the sense applied here, represent relicts from Pleistocene refugia, which did not contribute to the recolonization of Central and North Europe – a situation resembling that of many animals, such as the brown bears (Taberlet & Bouvet 1994), grasshoppers (Cooper & al. 1995), shrews and bank voles (Bilton & al. 1998).

Veronica subg. *Pseudolysimachium* – speciation by hybridization

Veronica subg. *Pseudolysimachium* is one of the most beautiful but also systematically complex groups of *Veronica*. It is well differentiated from other species of *Veronica* by its dense inflorescence, different flower morphology, pollen, and embryology, as well as a different chromosome base number. The group contains about forty species but is notorious for its hybridization, intraspecific ploidy level changes, and phenotypic variability leading to vast amounts of synonyms and subspecies or varieties. Crossing experiments by Härle (1932) have shown the wide cross-compatibility within a ploidy-level in the group. In recent times, studies by Fischer (1974), Fischer & Bedalov (1988), Fischer

& Peev (1995), Albach & Fischer (2003) and Trávníček (1997, 1998, 2000) have helped greatly delimit morphologically distinct units in Europe. However, AFLP fingerprints failed to give support to those taxa studied here.

Within the European members of *V.* subg. *Pseudolysimachium*, we find about 11 diploid and 6–7 tetraploid taxa. Only two of the tetraploid taxa are exclusively tetraploid, *V. crassifolia* and *V. spicata* subsp. *fischeri*. Among the other tetraploids we find the most common European species, *V. spicata* and *V. longifolia* (= *V. maritima* sensu Trávníček 2000). On the base of crossing experiments Graze (1933) assumed widespread hybridization in nature, which Fischer (1974) doubted on the basis of extensive herbarium revision. DNA sequences from the ITS- and *trnLF*-regions were unable to distinguish species because of the low variability (Albach & al. 2005), so we depend on a faster evolving molecular marker. AFLP were chosen here, because they have been successful in detecting variation among closely related species and within species in other groups of *Veronica*, such as *V. tenuifolia* (Martínez-Ortega & al. 2004), *V. alpina* (Albach & al. in press) and *V. cymbalaria* (Albach submitted). AFLP have been useful in detecting patterns of hybridization in an earlier study of *Veronica* (*V. hederifolia* and *V. cymbalaria*; Albach submitted) and other plant taxa (e.g., Hedrén & al. 2001; Gobert & al. 2002) and thus were thought to be a reliable and cost-effective way to give some initial support for hypotheses of hybridization and its importance on the Balkan Peninsula. Hybrids have commonly been analyzed together with their parents (e.g., Hedrén & al. 2001; Hodkinson & al. 2002; Guo & al. 2005) but their effect on the analysis has never been investigated in great detail. Results from the analysis here show large-scale incongruence with currently accepted species delimitation (Fig. 4) and no fixed species-specific fragment was found. Several reasons that do not mutually exclude each other may explain this incongruence. Hybridization may be more prevalent than discernible by morphological inspection (Fischer 1974). Polyploidy increases homoplasy in cases in which either different species share one parent or one species is of polytopic origin with genetically different parentage (Albach submitted). Furthermore, *V.* subg. *Pseudolysimachium* is already of polyploid origin itself, which may increase the chances for homoplasy.

Hybridization and polyploidy may be very common in *V.* subg. *Pseudolysimachium* for historical reasons. Pleistocene glaciations had considerable impact on the vegetation of Europe. During glaciations, the climate in Europe was more continental, favoring steppe vegetation in larger parts of Europe (Mai 1995). This may have led to invasions of steppe species, such as *V.* subg. *Pseudolysimachium*, from West Siberia into Eastern Europe. Trávnícek (1998) hypothesized such an invasion and a subsequent retreat in more humid Holocene times for *V. incana*. Unfortunately, such steppe species have seldom been investigated phylogeographically in much detail (see Franzke & al. 2004). Climatological oscillations during the Ice Ages may have led to repeated advances of steppe species and repeated chances for hybridization. Another remarkable fact that may indicate a high chance for hybridization in this group is the prevalence of diploid cytotypes in marginal areas and relictual areas (Trávnícek 1998, 2000, pers. comm.), whereas tetraploids predominate in the central range of the species. Similar situations in other plant groups led to the hypothesis that tetraploid forms replaced diploid forms gradually during expansion after the Pleistocene (Ehrendorfer 1962, 1965). This situation seems on first sight in contrast to the general notion that polyploids evolve at the margins of distribution but supports Stebbins' (1984) secondary-contact hypothesis. Following this hypothesis, diploid taxa survived in their Pleistocene refugia and after the Ice Age spread and encountered other diploid survivors, with which they formed polyploid hybrids. Such kinds of neopolyploids are known to surpass their ancestors in invasion potential, e.g. in the habitats that have become available after the retreat of Pleistocene ice sheets (Ehrendorfer 1965, 1980). Consequently, a phylogeographic study of *V.* subg. *Pseudolysimachium* should retrieve a patchy distribution pattern of the Pleistocene refugia in Europe for the diploids and a pattern mirroring the post-Pleistocene migration for the tetraploid cytotypes, which blurs the genealogical history of the group. Unfortunately, it was not possible in this study to ascertain the ploidy level of the analyzed individuals. Subsequent studies will be need to confirm this factor.

Despite these fallacies, some results raise doubts about the earlier taxonomic conclusions. *Veronica barrelieri* is known to have diploid and tetraploid populations and is found here in two widely divergent groups (Fig. 4). It is also morphologically difficult to differen-tiate and sometimes considered part of a broad *V. spicata* (Elenevsky 1971; Walters & Webb 1972) but differentiated by us in five subspecies (Albach & Fischer 2003). Therefore the species needs closer inspection. *Veronica porphyriana* is also occasionally included in a broader *V. spicata* (Elenevsky 1971) but is clearly differentiated here from the European samples (Fig. 4). Its status as a distinct species therefore seems to be valid. *Veronica longifolia* is also found in two widely separated groups: the European samples in one group and the Asian sample from the Altai in another (Fig. 4). This would support Trávnícek´s (2000) hypothesis of two species being hidden in *V. longifolia*, which he termed *V. maritima* for the European populations and *V. longifolia* for the Asian samples. It should be borne in mind that the situation may be more complex, because only one population from Asia was analyzed and no geographically intermediate sample was included. Furthermore, it is not clear whether *V. longifolia* in Asia, where diploids are frequent (Albach & al. submitted), represent diploid progenitors of their mostly tetraploid European relatives, or just relatively unrelated diploid relatives that were not involved in the origin of the tetraploids.

Future investigations in *V.* subg. *Pseudolysimachium* will be need to take the lessons from this study into consideration. 1.) DNA sequence analyses are superior to anonymous markers, because hybridization renders homology assessment of markers more difficult. However, even DNA sequence studies will be problematic in the presence of hybridization and multiple markers will be necessary to infer a species tree. 2.) Ploidy level should be controlled in all samples. 3.) Morphological characters may be homoplastic due to character shuffling by hybridization. 4.) Ancestral genotypes are likely to be found in Asian members of the group, which is in line with the inferred Asian origin of the subgenus (Albach & al. 2005). The analysis of additional diploid taxa from East Europe and West Siberia will be crucial to understand the origin of the European taxa.

Veronica subsect. *Alpina* – relict populations on the Balkan Peninsula

Veronica alpina and *V. bellidioides* are closely related species of *V.* subg. *Veronica* (Albach & al. in press). They are both species of subalpine meadows but differ ecologically. This ecological difference is responsi-

ble for differences related to climate change as shown by simulation studies (Guisan & Theurillat 2000) and different phylogeographic histories (Albach & al. in press). Phylogeographic analyses of AFLP fingerprint data revealed that *V. alpina* shows much higher intraspecific genetic diversity than *V. bellidioides* (Albach & al. in press). Populations of *V. bellidioides* from the Balkan Peninsula (Bulgaria) form a monophyletic sister group to the populations from the Alps and the Pyrenees (Albach & al. in press) representing a long historic divergence. In the absence of molecular dating, we are unable to estimate the divergence time, but given the absence of plastid DNA variation (Albach & al. in press), a Pleistocene origin is likely. In contrast, populations of *V. alpina* from the Balkan Peninsula (Bulgaria) are not well separated from other populations of this species in Europe. Interestingly, however, there does not seem to be a close connection with populations from the Carpathians (Albach & al. in press). Unfortunately, populations from other localities of both species on the Balkan Peninsula could not be sampled. Populations of both *V. alpina* and *V. bellidioides* may nevertheless be Pleistocene relicts of similar age that were unable to migrate north after the Ice Ages, similar to the case in *V.* subg. *Chamaedrys* (see above). However, the cases of *V. alpina* and *V. bellidioides* differ in that extinctions and genetic bottlenecks in surviving populations of *V. bellidioides* led to a clear bifurcation in the phylogenetic tree.

Conclusions

The chosen examples highlight the different responses of plants to the Pleistocene climate changes. Plant species probably had very different histories on the Balkan Peninsula. They may have become relict taxa of probable Tertiary origin such as species from *V.* subg. *Stenocarpon* that differentiated into morphologically clearly distinguishable species. Other groups may have become separate taxa in the Pleistocene on the verge of speciation by genetic drift, such as taxa in *V.* subg. *Chamaedrys*. Or they may actively speciate by hybridization, such as taxa in *V.* subg. *Pseudolysimachium*. Despite these differences, the Balkan Peninsula represents an important center of genetic diversity for all investigated groups of *Veronica*. Other species and species complexes of *Veronica* on the Balkan Peninsula, such as *V.* sect. *Pentasepalae*, are currently

under investigation (Martínez Ortega, pers. comm.). Undoubtedly, they will further highlight the importance of the Balkan Peninsula for the survival and diversification of plant taxa in the Pleistocene.

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