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Evolution of Polyploids in the European Orchid Genus *Nigritella*: Evidence from Allozyme Data

By

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With 5 Figures

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Summary

HEDRÉN M., KLEIN E. & TEPPNER H. 2000. Evolution of polyploids in the European orchid genus *Nigritella*: Evidence from allozyme data. – Phyton (Horn, Austria) 40 (2): 239–275, with 5 figures. – English with German summary.

The orchid genus *Nigritella* constitutes a polyploid complex which is widespread in mountain regions in Europe. Diploid members of the genus have sexual reproduction, whereas polyploid members are characterised by agamospermy. We used allozyme data to estimate levels of variation at different hierarchical levels and to describe the evolution of polyploids. – The variation patterns at allozyme loci agree with the mode of reproduction. Thus, populations of diploid species are variable, whereas populations of polyploid species contain one or two multilocus genotypes. The two tetraploids *N. widderi* and *N. miniata* contained two different multilocus genotypes each, indicating either multiple origins, or else sexual recombination or mutation at the tetraploid level. – The two tetraploids *N. nigra* subsp. *austriaca* and *N. nigra* subsp. *iberica* are closely related to the triploid *N. nigra* subsp. *nigra*, and they may have evolved by hybridization of this triploid and a diploid species. – In agreement with previous data, allozyme data confirm that the tetraploid apomict *Gymnigritella runei* is formed by fusion of an unreduced gamete from *N. nigra* subsp. *nigra* with a normal, haploid gamete from *Gymnadenia conopsea*. – The multilocus

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genotype found in *Nigritella archiducis-joannis* was identical to one multilocus genotype found in *N. widderi*, indicating that they may have evolved from a similar set of parental taxa. The pentaploid *N. buschmanniae* may be derived by hybridization of *N. widderi* with a sexual diploid species. – The multilocus genotype found in *N. stiriaca* was identical to one of the multilocus genotypes found in *N. miniata*, indicating a close relationship of these taxa as well. – The polyploid species investigated appear to combine divergent genomes and are likely to be derived by allopolyploidization. They all contain alleles that are rare or absent from present-day diploids, indicating that the polyploid taxa are derived from extinct ancestors and that they may have evolved at least before the last glaciation. – A comparison with two species of *Gymnadenia*, *G. conopsea* and *G. odoratissima*, revealed that *Gymnadenia* and *Nigritella* are more divergent from each other than species within each genus, which agrees with the view that the genera are sister groups.

Zusammenfassung

HEDRÉN M., KLEIN E. & TEPPNER H. 2000. Evolution von Polyploiden in der europäischen Orchideen-Gattung *Nigritella:* Ergebnisse aufgrund von Allozym-Daten. – Phyton (Horn, Austria) 40 (2): 239–275, 5 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Orchideen-Gattung *Nigritella* bildet einen, in den Gebirgen Europas weit verbreiteten Polyploidkomplex. Diploide Arten haben sexuelle Fortpflanzung, während polyploide Arten Agamospermie aufweisen. Wir nutzten Allozym-Daten um das Ausmaß der Variabilität auf verschiedenen hierarchischen Niveaus abzuschätzen und die Evolution der Polyploiden zu deuten. – Die Variabilitätsmuster an Allozymloci stimmen mit den Fortpflanzungsmodi überein. Folglich sind diploide Populationen variabel, während polyploide Arten ein oder zwei Multilocus-Genotypen enthalten. Die beiden Tetraploiden *N. widderi* und *N. miniata* enthalten jede zwei verschiedene Multilocus-Genotypen, was entweder multiplen Ursprung oder auch sexuelle Rekombination oder Mutation auf tetraploiden Niveau anzeigt. – Die zwei Tetraploiden *N. nigra* subsp. *austriaca* und *N. nigra* subsp. *iberica* sind mit der triploiden *N. nigra* subsp. *nigra* nahe verwandt und dürften durch Hybridisierung dieser Triploiden mit einer diploiden Art entstanden sein. – Übereinstimmend mit früheren Ergebnissen bestätigen die Allozym-Daten den Ursprung von *Gymnigritella runei* aus der Kombination eines unreduzierten Gameten von *N. nigra* subsp. *nigra* und eines normalen haploiden von *Gymnadenia conopsea*. – Der Multilocus-Genotyp von *Nigritella archiducis-joannis* ist mit einem der in *N. widderi* gefundenen identisch, was für die Evolution aus einem ähnlichen Satz von Eltern-Taxa spricht. Die pentaploide *N. buschmanniae* dürfte aus Hybridisierung von *N. widderi* mit einer sexuellen Diploiden entstanden sein. – Der Multilocus-Genotyp von *N. stiriaca* ist identisch mit einem der in *N. miniata* gefundenen, was ebenfalls eine enge Verwandtschaft dieser Taxa anzeigt. – Die polyploiden Taxa kombinieren verschiedene Genome und sind daher offensichtlich Allopolyploide. Sie alle enthalten Allele, die bei den heutigen Diploiden selten sind oder fehlen; die polyploiden Taxa entstanden daher wohl aus ausgestorbenen Diploiden und zumindest vor der letzten Eiszeit. – Ein Vergleich mit den zwei *Gymnadenia*-Arten *G. conopsea* und *G. odoratissima* zeigt, daß *Gymnadenia* und *Nigritella* voneinander stärker verschieden sind, als die

Arten jeder dieser beiden Gattungen untereinander; die beiden Gattungen können daher als Schwestergruppen angesehen werden.

Introduction

Polyploidy is an important feature of higher plants. It has been estimated that about one third of the number of plant species are functional polyploids as they have close relatives within the same genus at lower ploidy levels (STEBBINS 1980). An even higher number, around 47 % must have had a history of polyploidy, if plants with a base number of more than 13 are considered as polyploids (GRANT 1981). Among European terrestrial orchids in *Orchdoideae-Orchidinae*, the diploid numbers 2n=36 to 2n=42 prevail (PRIDGEON & al. 1997), which indicate that the whole group may have had a history of polyploidization. However, plants with these chromosome numbers in, e.g. *Orchis* s.l. (e.g., SCACCHI & al. 1990, CORRIAS & al. 1991, ROSSI & al. 1992, ARDUINO & al. 1995), *Gymnadenia* (SCACCHI & DE ANGELIS 1989), and *Dactylorhiza* (ROSSI & al. 1995, HEDRÉN 1996) are apparently functional diploids and do not have any higher numbers of isozyme loci than is expected in diploid plants (cf. WEEDEN & WENDEL 1989). In addition to these functional diploids, some genera contain polyploids that appear to be the result of more recent polyploidization events: *Gymnadenia* (LÖVE & LÖVE 1975), *Ophrys* and *Orchis* (BIANCO & al. 1991), *Dactylorhiza* (VERMEULEN 1947, HESLOP-HARRISON 1953), and *Nigritella* (TEPPNER 1996), the two latter genera with a large proportion of the described species being polyploids.

One of us (MH) has previously studied polyploid evolution in *Dactylorhiza* and by using allozymes as genetic markers, it has been possible to describe the general patterns of species relationships and polyploid evolution in that genus. In this paper, we explore the extent to which allozymes could be used to reveal details of polyploid evolution in *Nigritella*. Whereas diploid species of *Nigritella* are sexual, polyploid members of the genus all reproduce asexually by agamospermy (TEPPNER 1996; TEPPNER & KLEIN 1985a, 1985b, 1990, 1993, 1998; TEPPNER & STER 1996; TEPPNER & al. 1994). Although agamospermy is normally associated with polyploidy, in *Orchidoideae-Orchidinae*, agamospermy has only been demonstrated in *Nigritella*. Polyploids in other genera appear all to reproduce sexually, and in *Dactylorhiza* they are apparently outcrossing to a large degree (HEDRÉN, unpublished data).

Allozymes have several desired properties which makes them useful in studies of polyploid evolution. (i) They evolve relatively slowly, and different taxa in a polyploid complex could be expected to retain some alleles present in ancestral taxa. (ii) they are expressed as codominant markers and hybrid derivatives express alleles from each of the parental genomes, (iii) the numbers of copies of various alleles expressed at a locus could

often be estimated from band intensities seen on the electrophoresis gels, which enable us to estimate relative contribution of the various putative parental genomes, and (iv) if variation patterns at several loci are compared, restructuring of the polyploid genome as a whole may be studied.

Nigritella is closely related to *Gymnadenia*, and the genus *Nigritella* has variously been treated as part of *Gymnadenia* or as an independent genus. A study of nrDNA ITS sequences (PRIDGEON & al. 1997) indicated that *Nigritella* may be embedded in *Gymnadenia*, for which reason several recent authors have treated members of *Nigritella* in *Gymnadenia* (e.g., BATEMAN & al. 1997, ERICSSON 1998, TEPPNER & KLEIN 1998, DELFORGE 1998). We investigate whether allozymes are useful in describing the relationship between the two genera. As a part of this study, we use allozyme data to describe the degree of separation between populations of diploid species in *Nigritella* and *Gymnadenia*. Furthermore, we also describe patterns of hybridization in mixed populations of *Gymnadenia conopsea* and *Nigritella rhellicani*, and *G. odoratissima* and *N. rhellicani*, respectively.

The genus *Nigritella* is a genus of about twelve species confined to the mountain regions of Europe (TEPPNER 1996, TEPPNER & KLEIN 1998). Diploids with 2n=40: *Nigritella carpatica* (ZAPAL.) TEPPNER, KLEIN & ZAGULSKIJ is considered as a relic species confined to the Eastern Carpathians (TEPPNER & al. 1994). The most widely distributed diploid is *N. rhellicani* TEPPNER & KLEIN, which is distributed in the entire Alp region extending into the Apennines, Romania, and N. Greece (TEPPNER & KLEIN 1990). *Nigritella gabasiana* TEPPNER & KLEIN is confined to the Pyrenées and the Cantabrian Mts. (TEPPNER & KLEIN 1993). *Nigritella corneliana* (BEAUV.) GÖLZ & REINHARD is found in the south-western Alps and *N. lithopolitanica* RAVNIK in the south-eastern Alps in Austria and Slovenia. The polyploids are: *Nigritella nigra* (L.) RCHB. fil. subsp. *nigra*, a triploid with 2n=3x=60, endemic to the Scandinavian mountain range from Sør-Trøndelag and Härjedalen to Troms (HULTÉN 1971, TEPPNER & KLEIN 1990). *Nigritella nigra* subsp. *austriaca* TEPPNER & KLEIN and subsp. *iberica* TEPPNER & KLEIN are tetraploids with 2n=4x=80. The first of these is distributed in the eastern Alps (TEPPNER & KLEIN 1990) and the second in the Massif Central, in the Pyrenées, in the westernmost Alps and the Jura (TEPPNER & KLEIN 1993, 1998, KLEIN & DRESCHER 1996). The remaining tetraploids are *N. widderi* TEPPNER & KLEIN in the eastern Alps (TEPPNER & KLEIN 1985b), *N. archiducis-joannis* TEPPNER & KLEIN with a small area of distribution in Steiermark (Salzkammergut, Eisenerzer Alpen), eastern Alps (TEPPNER & KLEIN 1985a), *N. stiriaca* (K. RECH.) TEPPNER & KLEIN likewise in the Salzkammergut and the Grazer Bergland, in the eastern Alps (TEPPNER & KLEIN 1985a), *N. miniata* (CRANTZ) JANCH. with a relatively wide area of distribution from the central Alps and eastwards up to

the Carpathians (TEPPNER & KLEIN 1985a), and the recently described *N. dolomitensis**) (as *Gymnadenia)* from the South Tyrol in Italy (TEPPNER & KLEIN 1998). *Nigritella buschmanniae* TEPPNER & STER, a pentaploid with 2n=5x=100, is also confined to South Tyrol (TEPPNER & STER 1996). *Gymnigritella runei* TEPPNER & KLEIN is known from four localities in southern Lapland, Sweden (RUNE 1993). It is a tetraploid, apomictic species which has arisen as the hybrid between *Nigritella nigra* subsp. *nigra* and *Gymnadenia conopsea* (TEPPNER & KLEIN 1989, HEDRÉN 1999).

In this study we included material of all taxa except for *N. carpathica* and *N. dolomitensis*. We also investigated material of the recently described *N. cenisia* FOELSCHE & GERBAUD (FOELSCHE & al. 1998, 1999) which is based on diploid plants from Mt Cenis in Savoie, France. However, for reasons given in the results section (3.2.), we included these plants in *N. rhellicani* from the same locality in our analysis.

Finally, we included some populations of *Gymnadenia conopsea* and *G. odoratissima* for comparison. Both diploids with 2n=40 and tetraploids with 2n=80 are known from the genus (LÖVE & LÖVE 1975). According to variation patterns at allozyme loci, and known chromosome counts for Swedish populations (Berndt NYBERG, unpublished data), our investigated material of *Gymnadenia* was all diploid.

In the present study we use the systematic treatment of TEPPNER & KLEIN 1998, but we recognise the genus *Nigritella* for reasons given in the discussion (4.3.).

2. Materials and Methods

2.1. The plants

Locality data for the material studied is given as Table 1. About 800 plants were studied.

2.2. Enzyme Electrophoresis

Parts of leaves were detached from living specimens in the field and shipped to the laboratory as soon as possible. Some additional material was taken from plants cultivated by the second and the third authors (cf. Table 1). The leaves were then kept refrigerated at 4° C until analysis. For each individual, about 1 cm^2 of leaf area was ground with a small amount of washed sea sand in 80 µl of a Tris-HCl grinding buffer (SOLTIS & al. 1983) modified by replacing β-mercaptoethanol by dithiothreitol (LÖNN & PRENTICE 1990). Extracts were absorbed onto chromatography paper wicks and proteins were separated on 8–9 % horizontal starch gels at ca. 10 Vcm^{-1}. A Lithium-borate/Tris-citrate buffer system (ASHTON & BRADEN 1964), modified according to LÖNN & PRENTICE 1990 was used to separate allozymes of Aspartate aminotransferase

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*) *Nigritella dolomitensis* (TEPPNER & KLEIN) HEDRÉN, KLEIN & TEPPNER, comb. nova. – Basionym: *Gymnadenia dolomitensis* TEPPNER & KLEIN, Phyton (Horn, Austria) 38 (1): 223–224 (1998).

Table 1

Material investigated for allozyme variation in *Nigritella, Gymnigritella* and *Gymnadenia*. Diploid populations have been given code numbers which are also used in Figs. 1 & 2 and Table 2. Abbreviations: a–j = *Nigritella archiducis-joannis*, aus = *N. nigra* subsp. *austriaca*, bus = *N. buschmanniae*, con = *Gymnadenia conopsea*, cor = *N. corneliana*, gab = *N. gabasiana*, ibe = *N. nigra* subsp. *iberica*, min = *N. miniata*, lit = *N. lithopolitanica*, nig = *N. nigra* subsp. *nigra*, odo = *Gymnadenia odoratissima*, rhe = *N. rhellicani*, run = *Gymnigritella runei*, sti = *N. stiriaca*, wid = *N. widderi*

Taxon	Country	Province	Locality	Date	Collector
gab (28)	Spain	Cantabria	Burgos, Puerto de la Lunada, 1350 m	5 July 1997	M. Lewin
ibe	France	Pyrénées-Orientales	Pic del Moros, 3 km W Font-Romeu, 2000 m	12 July 1997	M. Lewin
ibe	France	Pyrénées-Orientales	Costa Guillem, 1 km NNE Eyne, 1605m	12 July 1997	M. Lewin
gab (29)	France	Pyrénées-Orientales	Prats-de-Molló, 1 km WNW du Col d'Ares, Vallée du Tech, 1530 m	16 July 1997	M. Lewin
gab (30)	France	Pyrénées-Orientales	8 km NW Formigueres, 1900 m	29 July 1997	M. Lewin
ibe	France	Haute Loire	Massif Central, Mont Mézenc, 1480 m	27 June 1995	E. Klein & A. Drescher, cult. garden E. Klein
ibe	France	Haute Loire	Massif Central, Chaudeyrolles, 1340 m	27 June 1995	E. Klein & A. Drescher, cult. garden E. Klein
ibe	France	Puy-de-Dôme	Massif Central, Monts du Cézallier, 1410 m	28 June 1995	E. Klein & A. Drescher, cult. garden E. Klein
rhe	France	Hautes Alpes	Lautaret, 2050 m	6 July 1997	O. Gerbaud
cor (27)	France	Isère	Col de l'Alpe, 1790 m, Massif de la Chartreuse	16 June 1997	O. Gerbaud
ibe	France	Isère	Col de l'Alpe, 1790 m, Massif de la Chartreuse	16 June 1997	O. Gerbaud
rhe (01)	France	Isère	Col de l'Alpe, 1790 m, Massif de la Chartreuse	16 June 1997	O. Gerbaud
rhe (10)	France	Isère	Dauphiné Alpen, Chaine de Belledonne, le Collet d'Allevard, 1800 m	15 July 1997	O. Gerbaud
rhe (18)	France	Savoie	Mont Cenis, 2100 m	25 July 1997	W. Foelsche
rhe (19)	France	Savoie	Coll Iseran, ca. 2700 m	26 July 1997	W. Foelsche
rhe (14)	France	Ain	Grand Colombier, 1500 m	20 July 1997	O. Gerbaud
rhe (09)	Switzerland	Graubünden	Avers, 1 km W–WNW Juf, 2100 m	15 July 1997	H. Reinhard
rhe (12)	Switzerland	Graubünden	Avers, 3 km SE Cresta, 1980–2000 m	16 July 1997	H. Reinhard
rhe (13)	Switzerland	Graubünden	Schatzalp near Davos, 1900–2000 m	16 July 1997	H. Reinhard
aus	Austria	Eastern Tyrol	Hinteregg, Matrei, 1450 m	30 June 1997	K. Redl
rhe (02)	Austria	Salzburg	Postalm, ENE of the Strobler Hütte, 1250–1270 m	29 Jun 1997	K. Redl
min	Austria	Salzburg	W Hofpürgelhütte, Dachsteingruppe, 1640–1660 m	4 July 1997	K. Redl
rhe (08)	Austria	Salzburg	Trattberg, 1600–1680 m	5 July 1997	K. Redl
sti	Austria	Salzburg	Schafberg, 1600–1620 m	1 July 1997	K. Redl
aus	Austria	Salzburg	Schafberg, 1730–1750 m	1 July 1997	K. Redl
rhe (07)	Austria	Carinthia	Dobratsch, 1760 m	12 July 1997	G. Deutsch
lit (24)	Austria	Carinthia	Hochobir, 1700–1950 m	6 July 1997	K. Redl
lit (26)	Austria	Carinthia	Petzen, 1850–1950 m	16 July 1997	K. Redl
con (34)	Austria	Carinthia	Heiligenbachalm, 1930 m	15 July 1997	E. Klein
odo (35)	Austria	Carinthia	Heiligenbachalm, 1930 m	15 July 1997	E. Klein
rhe (16)	Austria	Carinthia	Heiligenbachalm, 1930 m	15 July 1997	E. Klein
rhe (15)	Austria	Carinthia	Astental, 2000–2200 m	17 Jul 1997	K. Redl
sti	Austria	Styria	Teichalm, 1450 m	20 June 1997	E. Klein
aus	Austria	Styria	Teichalm, 1450 m	20 June 1997	E. Klein
min	Austria	Styria	Teichalm, 1450 m	20 June 1997	E. Klein
min	Austria	Styria	Schöckel, 1400 m	21 June 1997	E. Klein
wid	Austria	Styria	Schöckel, 1400 m	21 June 1997	E. Klein
aus	Austria	Styria	Hochschwab, Trenchtling, 1820 m	25 June 1997	T. & H.E. Schmid
wid	Austria	Styria	Hochschwab, Trenchtling, 1770 m	25 June 1997	T. & H.E. Schmid
min	Austria	Styria	Hochschwab, Trenchtling, 1800 m	25 June 1997	T. & H.E. Schmid
min	Austria	Styria	Hochschwab, Aflenzer Bürgeralm, 1680–1710 m	1 July 1997	E. Klein
wid	Austria	Styria	Hochschwab, Aflenzer Bürgeralm, 1680–1710 m	1 July 1997	E. Klein
aus	Austria	Styria	Hochschwab, Aflenzer Bürgeralm, 1680–1710 m	1 July 1997	E. Klein
rhe (03)	Austria	Styria	Stubalpe, Wölkerkogel, 1650 m	26 June 1997	E. Klein
rhe (06)	Austria	Styria	Stubalpe, Brandkogel, 1600 m	26 June 1997	E. Klein
rhe (04)	Austria	Styria	Gleinalpe, Sattelhaus, 1390 m	27 June 1997	E. Klein
rhe (05)	Austria	Styria	Pleschaitz, Wölzer Tauern, 1500 m	27 June 1997	E. Klein
min	Austria	Styria	Totes Gebirge, Tauplitzalm, 1680–1955m	9 July 1997	K. Redl
rhe	Austria	Styria	Totes Gebirge, Tauplitzalm, 1630 m	9 July 1997	K. Redl
a-j	Austria	Styria	Totes Gebirge, Tauplitzalm, Traweng, 1970 m	17 July 1997	K. Redl
aus	Austria	Styria	Koralpe, Lavanttaler Alpen, ca. 2000 m	17 July 1997	E. Klein
rhe (23)	Austria	Styria	Pusterwald/Wölzer Tauern, Kamm vom Schiesseck zum Predigstuhl, 1860–2070 m	30 Jul 1997	K. Redl
rhe (11)	Austria	Styria	Liesing, Rottenmanner Tauern, Schwarz Kogel, 1990 m	4 July 1997	K. Redl
aus	Austria	Styria	Schneeberggruppe, Hohe Veitsch, 1750 m	26 June 1997	G. Deutsch

Table 1. Cont.

Taxon	Country	Province	Locality	Date	Collector
min	Austria	Styria	Schneeberggruppe, Hohe Veitsch, 1800 m	26 June 1997	G. Deutsch
wid	Austria	Styria	Schneeberggruppe, Hohe Veitsch, 1750 m	26 June 1997	G. Deutsch
min	Austria	Styria	Bosruck, Südseite, unweit Eder-Hütte, 1385 m	28 Jun 1997	K. Redl
min	Austria	Styria	Ardninger Alm, Stubenschlag, 1130 m	26 Jun 1997	K. Redl
wid	Austria	Styria	Gesäuse, Kreuzkogel, 1700–1850 m	30 Jun 1997	K. Redl
min	Austria	Styria	Gesäuse, Tamischbachturm, 1960 m	13 Jul 1997	K. Redl
wid	Austria	Styria	Gesäuse, Tamischbachturm, 1850 m	13 Jul 1997	K. Redl
min	Austria	Lower Austria	Schneeberg, ca. 1900 m	16 July 1997	E. Klein
rhe (22)	Italy	Piemonte	Grand St. Bernhard, ca. 2700 m	27 Jul 1997	W. Foelsche
rhe (21)	Italy	Lombardia	Passo di Croce Domine, 1890 m	24 Jul 1997	W. Foelsche
bus	Italy	Trentino-Alto Adige	Brenta, Le Crosette, 2500 m	23 Jul 1997	H. Teppner & T. Ster, cult. in Bot. Gard. Graz
rhe (20)	Italy	Trentino-Alto Adige	Brenta, Le Crosette, 2500 m	23 Jul 1997	H. Teppner & T. Ster, cult. in Bot. Gard. Graz
rhe (17)	Italy	Trentino-Alto Adige	Hochalm, W Pragser Wildsee, 2190 m	27 Jul 1997	J. Claessens
lit (25)	Slovenia	Savinjske Alpe	Velika Planina, 1660 m	9 Jul 1997	W. Foelsche
con (31)	Sweden	Gotland	Lärbro, Hoburgsmyr	23 Jun 1997	M. Hedrén
nig	Sweden	Härjedalen	Klinken	19 Jul 1997	M. Hedrén & S. Hansson
run	Sweden	Åsele Lappmark	Ransaren, 600 m	21 Jul 1997	M. Hedrén & S. Hansson
con (33)	Sweden	Åsele Lappmark	Ransaren, 600 m	21 Jul 1997	M. Hedrén & S. Hansson
run	Sweden	Lycksele Lappmark	Artfjällen, Rödingsnäset, 900 m	22 Jul 1997	M. Hedrén & S. Hansson
con (32)	Sweden	Lycksele Lappmark	Artfjällen, Rödingsnäset, 900 m	22 Jul 1997	M. Hedrén & S. Hansson

(AAT, E.C. 2.6.1.1), Diaphorase (DIA, E.C. 1.6.99.-), Phosphoglucoisomerase (PGI, E.C. 5.3.1.9), Phosphoglucomutase (PGM, E.C. 5.4.2.2), and Triose-phosphate isomerase (TPI, E.C. 5.3.1.1). A Tris-citrate/Histidine-EDTA buffer system at pH 7.0, modified from KING & DANCIK 1983, was used to separate allozymes of Isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), Malate dehydrogenase (MDH, E.C. 1.1.1.37), Phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44), and Shikimate dehydrogenase (SKD, E.C. 1.1.1.25). This system used an electrode buffer of 0.125 M Tris adjusted to pH 7.0 with 1M citric acid and a gel buffer containing 10 mM L-histidine, 0.28 mM EDTA (tetrasodium salt), and 20 mM Tris adjusted to pH 7.0 with 1M hydrochloric acid. The gel buffer was prepared as a stock solution of 5 × concentration. Staining recipes followed WENDEL & WEEDEN 1989 with only minor modifications. Loci were numbered sequentially beginning with the most rapidly migrating enzyme, and alleles were annotated by alphabetic letters beginning with the most rapidly migrating allozyme at each locus.

2.3. Data Analysis

2.3.1. Variation within populations of diploid taxa

All genetic data were compiled in a data table containing genotype data for each individual (not shown). This table could be obtained from the first author upon request. Each population was tested for significant deviations from Hardy-Weinberg equilibria at each locus by means of X^2-tests.

2.3.2. Differentiation among Populations of Diploid Taxa

Mean allele frequencies at each locus in 35 diploid populations from which a fair number of plants were examined was compiled as Table 2. The differentiation between pairs of populations were estimated by Rogers' genetic distance (ROGERS 1972), and the subsequent cluster analysis and ordinations were based on the resulting triangular matrix of distance data.

The cluster analysis was performed according to the UPGMA algorithm (SNEATH & Sokal 1973). The correlation between the original matrix of Rogers' genetic distances and the distances between populations in the UPGMA phenogram is described as the cophenetic correlation coefficient (SNEATH & SOKAL 1973).

Patterns of allelic differentiation between population samples were also summarised by nonmetric multidimensional scaling (MDS) (KRUSKAL 1964a; 1964b). The MDS analyses were carried out on the triangular matrix of Rogers' genetic distances, and was repeated ten times to avoid problems with local minima. The run with the lowest stress value (lowest amount of ranking order distortion between genetic distances in the original matrix of Rogers' genetic distances and distances in the final MDS solution) was chosen for interpretation. The MDS solution was rotated in a principal component analysis (PCA) in order to maximise the variance along the horizontal axis.

2.3.3. Evolution of Polyploids

Based on previous knowledge on chromosome numbers and on band intensities seen on the gels, the numbers of alleles at the various loci in the polyploid samples were estimated. These samples were then grouped according to taxon, site, and multilocus genotype as Table 3. This table was used to estimate the (minimum) number of origins of each polyploid.

A summary of the genetic variation in the diploid taxa, and the multilocus genotypes found in the polyploid taxa is presented as Table 4. This table was used to construct a hypothetical scheme for the polyploid evolution in *Nigritella*. A number of assumptions were made: (i) The construction of the scheme was primarily based on observed combinations of alleles in taxa at lower ploidy levels, and we tried to invoke as few hypothetical ancestral taxa as possible. Furthermore, in choosing between alternative scenarios, we selected the one that invoked the lowest number of hybridization and polyploidization events. (ii) Each allozyme allele is assumed to have a unique origin. Alleles present in both polyploids and diploids are thus believed to have been transferred when the polyploidization took place. Alleles unique to the polyploids may indicate that they have arisen from diploid ancestors that were different from extant ones, or that new alleles have arisen by somatic mutations in the polyploids. (iii) Polyploids are assumed to have evolved by unreduced gametes. If several tetraploids could be traced back to the same triploid, this would require a lower amount of change in ploidy levels than if tetraploids arose directly from the diploids.

It should be observed that our hypothesis on the relationships between diploid and polyploid members of *Nigritella* is based on available data. We were unable to obtain material of the eastern diploid *N. carpatica* and it is possible that data from this species would have given further detail to the hypothesis presented by us.

2.3.4. Hybridization at the Diploid Level

We described hybridization between *Gymnadenia conopsea* and *Nigritella rhellicani*, and *G. odoratissima* and *N. rhellicani* in two separate analyses. Each analysis included representatives of the two parental taxa and their hybrid. The differentiation between any pair of samples was estimated with Rogers' genetic

distance and then the complete triangular matrix of distances was summarised by means of MDS as described above.

2.3.5. Genetic Diversity

Genetic diversity statistics was calculated separately for the dataset consisting of *Nigritella rhellicani* only, and the dataset consisting of all diploid populations of *Nigritella*. The partitioning of genetic diversity was estimated by NEI's (1973) diversity statistic H, adjusted for sample size according to PRENTICE & WHITE 1988, i.e. by multiplying with a correction term $2n/(2n-1)$, where n is the number of individuals sampled.

For *N. rhellicani*, the total genetic diversity for each locus is given by H_T and the mean within-site diversity by $\overline{H}_S$. The proportion of the total diversity that is due to variation among sites is given by G_{ST} $((H_T - \overline{H}_S)/H_T)$. The mean values for G_{ST} over loci were calculated both as an arithmetic mean, and according to CHAKRABORTHY & al. 1982 in which G_{ST} values are weighted by H_T at each locus, i.e., a rough measure of the locus' information content.

For the dataset consisting of all diploid populations of *Nigritella*, the total genetic diversity at each locus is given by H_{Genus}, the mean within-species diversity by $\overline{H}_{Species}$, and the mean within-site diversity by $\overline{H}_{Site}$. The proportion of the total diversity that is due to variation among species is given by $G_{SpecGen}$ $((H_{Genus} - \overline{H}_{Species})/\overline{H}_{Genus})$, and the proportion of the total diversity that is due to variation among sites within species by $G_{SiteSpec}$ $((\overline{H}_{Species} - \overline{H}_{Site})/H_{Genus})$. Mean values were calculated as for *N. rhellicani*.

Allele frequencies, X^2-tests, and genetic diversity statistics were calculated in MULE 1.70 (WHITE 1995). Rogers' genetic distances, UPGMA cluster analysis, and MDS ordinations were performed in NTSYS-pc 1.80 (ROHLF 1994).

3. Results

3.1. Allozyme Data

Seven of the nine enzyme systems were possible to interpret. The exceptions were AAT at which bands were too close to each other to permit interpretation and at which variation patterns from at least two loci appeared to be overlapping, and DIA, the staining of which often became too faint. At the other seven systems, ten variable loci were scored. MDH contained three variable and interpretable loci, TPI two, and the remaining systems one locus each.

IDH. We interpreted one strong zone of activity as variation at one locus. At least one further zone was seen, but could not be reliably interpreted.

MDH. At least four putative loci were present at MDH, of which the three anodal ones were interpreted. At the most anodal locus, homodimeric enzymes gave rise to triplet bands, but the interpretation was nevertheless straightforward. The second locus gave rise to moderately strong, somewhat diffuse, but still interpretable bands. The third locus gave rise to

relatively faint, but distinct bands. There was some variation at the forth locus as well, but the various bands were poorly separated.

PGD. At least two zones of activity were seen at PGD. A strong zone was interpreted as one locus, whereas a more anodal, weak zone of activity, was identical to the bands interpreted as IDH.

PGI. One strong, anodal zone of activity was interpreted as one locus. A more cathodal, weaker zone was also seen, but the variation pattern was not clear enough to be interpreted.

PGM. Two zones of activity were seen, the stronger, more anodal of which was interpreted as one locus.

SKD. One moderately strong zone of activity was interpreted as one locus at SKD. In addition to this moderately strong zone, some additional, more faint zones of activity were seen on the gels. The most anodal zone was identical to the strongest IDH locus. The interpretation of Skd was sometimes uncertain because of several, partially overlapping loci.

TPI. The variation seen at TPI was interpreted as being caused by two loci, both of which were variable in *Nigritella*.

Some individuals produced faint bands at some loci, for which reason the complete data table contain missing values. In the polyploid species, individuals with missing data were sorted into the same multilocus genotypes as the individuals with complete data, based on the observed data.

3.2. Distribution of Alleles Found in Diploid Taxa

In almost no comparison between diploid taxa were the species fixed for different alleles at any locus. The only exception was Skd at which *Gymnadenia conopsea* was characterised by alleles d and f, whereas the single population investigated of *Nigritella corneliana* contained the alleles b, c and e (Table 2). Still, these species were linked by allelic combinations at Skd found in other species. Also, most species were similar to each other in allele frequencies with the same dominating allele at most loci. However, the two species of *Gymnadenia* differed quite considerably from each other at Mdh-3 and Skd. These species also differed from the *Nigritella* species at Mdh-2. Within *Nigritella*, most populations of *N. rhellicani* and *N. lithopolitanica* differed from those of *N. corneliana* and *N. gabasiana* at Pgm.

Some species contained unique alleles, mostly at low frequencies. Thus *N. rhellicani* contained unique alleles at Idh, Pgm, and Tpi-1, *N. lithopolitanica* at Pgd, *N. corneliana* at Pgm, *N. gabasiana* at Idh, *Gymnadenia conopsea* at Mdh-1, Mdh-2, and Pgi, and *G. odoratissima* at Pgi and Tpi-1.

The material of *Nigritella* from Mt. Cenis was morphologically variable and contained plants with inflorescences of the normal size of *N. rhellicani* as well as plants with unusually large inflorescences

(= *N. cenisia* FOELSCHE & GERBAUD; FOELSCHE & al. 1998). However, although the material was variable at several loci (Table 2), there was no genetic differentiation corresponding to the morphological variation and we treated all the plants from the locality as one population sample.

The number of alleles per polymorphic locus, A_p, was 1.6, 1.9, 1.7, 1.4, 2.5, and 2.1 in *Nigritella rhellicani*, *N. lithopolitanica*, *N. corneliana*, *N. gabasiana*, *Gymnadenia conopsea*, and *G. odoratissima*, respectively. These numbers mainly reflect the numbers of plants analysed of each species. At the population level, the mean number of alleles per polymorphic locus for the above-mentioned species was 1.48, 1.57, 1.7, 1.17, 1.9, and 2.1. Thus, populations of the *Gymnadenia* species were more variable than populations of the *Nigritella* species.

3.3. Levels of Heterozygosity in Populations of Diploid Species

Chi-square tests for variable loci within populations show that in most populations the variation patterns do not deviate significantly from those expected under random mating (data not shown). The few deviations that were found were mostly confined to single loci. Most of these deviations were due to a slight deficiency of heterozygotes and may be due to inbreeding, or else to random effects. However, in two populations of *N. rhellicani*, St. Bernhard and Brenta, the majority of loci had variation patterns deviating from those expected under random mating. These deviations consisted in both deficiency and excess of heterozygotes, and a closer inspection of these populations revealed that a few multilocus genotypes dominated.

3.4. Polyploid Species

The polyploid species of *Nigritella* contained three alleles that were not present in the diploid species. *Nigritella widderi*, *N. archiducis-joannis*, and *N. buschmanniae* contained Skd^a, and *Gymnigritella runei*, *Nigritella nigra* subspp. *nigra*, *austriaca*, and *iberica* contained $Tpi\text{-}1^c$. Some plants of *Nigritella widderi* contained $Mdh\text{-}1^a$.

3.5. Differentiation between Populations of Diploid Taxa

All MDS ordinations gave a stress value of 0.25056. The resulting plot from one of these ordinations is given as Fig. 1. Two major clusters are formed, one containing populations of *Gymnadenia* s.str. and the other containing populations of *Nigritella*. Within the *Gymnadenia* cluster, the single population of *G. odoratissima* is somewhat separated from the remaining populations representing *G. conopsea*. Within the *Nigritella* cluster, populations representing *N. rhellicani* are spread out, whereas populations of the remaining diploids are concentrated to the lower portion of the *Nigritella* cluster. However, *N. lithopolitanica*, *N. corneliana*

and *N. gabasiana* are still somewhat differentiated from each other. The same general pattern is seen in the UPGMA phenogram (Fig. 2). The cophenetic correlation coefficient in this analysis was 0.91179. In both analyses, most western populations, plus *N. lithopolitanica* group together, whereas most eastern populations of *N. rhellicani* form another subgroup. Some populations of *N. rhellicani* form outliers. As there was no locus fixed for alternative alleles in the two major subgroups, the differentiation was apparently due to the combined patterns of variation at several loci (Table 2).

Table 2

Allele frequencies found in populations of diploid, sexual taxa of *Nigritella* and *Gymnadenia*. For population codes, see Table 1.

Population	Locus/alleles																					
	idh			mdh1			mdh2			mdh3			pgd				pgi					
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	d	a	b	c	d	e	f
Nigritella rhellicani																						
1	1.00			.97	.03			1.00		1.00				1.00					1.00			
2	.98	.02		.81	.19		.10	.90		.84	.16		.04	.96					1.00			
3	.71	.29		.68	.32		.15	.85		.95	.05			1.00					1.00			
4	.85	.15		.95	.05		.05	.95		.95	.05			1.00					1.00			
5	.72	.28		.78	.22		.08	.92		1.00				1.00					1.00			
6	.78	.22		.80	.20		.07	.93		.93	.07			1.00					1.00			
7	.92	.08		.83	.17			1.00		.83	.17			1.00					1.00			
8	1.00			.60	.40		.15	.85		.95	.05			1.00					1.00			
9	.88	.12		.81	.19		.08	.92		.91	.09			1.00					1.00			
10	1.00			.93	.07			1.00		.93	.07			1.00					1.00			
11	.92	.08		.33	.67		.50	.50		1.00				1.00					1.00			
12	1.00			.79	.21			1.00		.82	.18			1.00					1.00			
13	.95	.05		.91	.09		.14	.86		.85	.15			1.00					1.00			
14	1.00			.62	.38		–	–	–	–	–	–		1.00					1.00			
15	.97	.03		.59	.41		.03	.97		.03	.69	.28	.09	.91					1.00			
16	.89	.11		.81	.19		.06	.94		.91	.09		.03	.97					1.00			
17	1.00			.62	.38		.12	.88		1.00				1.00					1.00			
18	.95	.05		.85	.15			1.00		1.00				1.00					1.00			
19	1.00			1.00				1.00		1.00				1.00					1.00			
20	1.00			.64	.36		.29	.71		.29	.71			1.00					.64	.36		
21	.60	.40		.95	.05		.50	.50		.56	.44			1.00					1.00			
22	1.00			.81	.19		.44	.56		.81	.19			1.00					1.00			
23	.39	.61		.80	.20		.02	.98		.98	.02			1.00					1.00			
mean	*.91*	*.09*		*.78*	*.22*		*.13*	*.87*		+	*.87*	*.13*	*.01*	*.99*					*.99*	*.01*		
N. lithopolitanica																						
24	1.00			.95	.05		.05	.95		.94	.06			.85	.15				1.00			
25	1.00			.90	.10			1.00		1.00				1.00					1.00			
26	1.00			1.00				1.00		.83	.17			.91		.09			1.00			
mean	*1.00*			*.95*	*.05*		*.02*	*.98*		*.92*	*.08*			*.92*	*.05*	*.03*			*1.00*			
N. corneliana																						
27	1.00			.90	.10		.06	.94		1.00				1.00					1.00			
N. gabasiana																						
28	.93	.07		1.00				1.00		1.00				.93	.07				1.00			
29	.88	.12		1.00				1.00		1.00				1.00					1.00			
30	1.00			1.00				1.00		1.00				1.00					1.00			
mean	*.94*	*.06*		*1.00*				*1.00*		*1.00*				*.98*	*.02*				*1.00*			
Gymnadenia conopsea																						
31	1.00			.17	.33	.50		.75	.25	.83	.17			1.00				.17	.50	.25	.08	
32	1.00				.53	.47	.13	.87		1.00				.97	.03			.23	.60	.17		
33	1.00				.42	.58	.12	.88		1.00			.04	.92	.04		.04	.17	.71	.08		
34	1.00				.81	.19		.88	.12	–	–	–		1.00				.19	.62	.06	.13	
mean	*1.00*			*.04*	*.52*	*.44*	*.06*	*.85*	*.09*	*.94*	*.06*		*.01*	*.97*	*.02*		*.01*	*.19*	*.61*	*.14*	*.05*	
G. odoratissima																						
35	1.00			.88	.12		.94	.06		.08	.92			1.00					.44	.38	.12	.06

3.6. Genetic Diversity

The genetic diversity statistics for all diploid populations of *Nigritella* is summarised as Table 5. Only a small proportion of the total genetic variation observed, at most 6.6%, could be explained by differences between taxa. The differentiation between populations within taxa was higher, 16.1%. Thus, the remaining proportion of the genetic diversity, 77.3%, was due to variation within populations, either as differences between individuals, or as individual heterozygosity. In *N. rhellicani* (Table 6) 19.2% of the genetic diversity was due to differentiation between populations, and 80.8% due to variation within populations.

Table 2. Cont.

Population	Locus/alleles	pgm						skd						tpi1					tpi2			N
		a	b	c	d	e	f	a	b	c	d	e	f	a	b	c	d	e	a	b	c	
Nigritella rhellicani																						308
1			.73	.27						.50		.46	.04		.03		.97		.03	.94	.03	16
2			.21	.79						.04		.96					1.00			1.00		26
3			.10	.88	.02					.32		.68					1.00			1.00		31
4			.05	.95						.15		.85					1.00			1.00		10
5			.14	.83		.03				.06		.94					1.00			1.00		18
6			.04	.96						.24		.76					1.00			1.00		23
7			.08	.92								1.00					1.00			1.00		6
8			.40	.60						.10		.90					1.00			1.00		10
9			.19	.81						.27		.73					1.00			1.00		13
10			.86	.14						.40		.60					1.00		.07	.93		7
11			.25	.75									1.00				1.00			1.00		6
12			.11	.89					.04			.96					1.00			1.00		14
13			.09	.91						.19		.81					1.00			1.00		15
14			.38	.62				–	–	–	–	–	–				1.00			1.00		8
15			.25	.75								1.00					1.00			1.00		16
16			.14	.86								1.00					1.00			1.00		18
17			.50	.50								1.00					1.00			1.00		4
18			.55	.45						.50		.50					1.00		.15	.85		10
19			1.00						.50	.50							1.00			1.00		4
20			.50	.50								1.00					1.00			1.00		7
21			.05	.95						.05		.95					1.00			1.00		10
22			.19	.81						.56		.19	.25				1.00			1.00		8
23			.04	.96						.05		.95					1.00			1.00		28
mean			*.29*	*.71*	+	+			*.02*	*.17*		*.76*	*.05*		+		*1.00*		*.01*	*.99*	+	*13.4*
N. lithopolitanica																						31
24			.30	.70					.22	.17		.44					1.00			1.00		10
25			.30	.70					.10	.50		.30	.10				1.00			1.00		5
26			.38	.62					.03	.59		.38					1.00			1.00		16
mean			*.33*	*.67*					*.12*	*.42*		*.37*	*.09*				*1.00*			*1.00*		*10.3*
N. corneliana																						16
27			.72	.25			.03		.35	.45		.20					1.00			.97	.03	16
N. gabasiana																						15
28		1.00										1.00					1.00			1.00		7
29		1.00								.33		.50	.17				1.00			1.00		4
30		1.00										1.00					1.00			1.00		4
mean		*1.00*								*.11*		*.83*	*.06*				*1.00*			*1.00*		*5*
Gymnadenia conopsea																						41
31		.08	.50	.42							.91		.09				.92	.08		1.00		6
32			.97	.03							1.00						.90	.10		1.00		15
33			.71	.29							1.00						.94	.04		1.00		12
34			.94	.06							1.00						.88	.12		1.00		8
mean		*.02*	*.78*	*.20*							*.98*		*.02*				*.92*	*.08*		*1.00*		*10.2*
G. odoratissima																						8
35		.13	.56	.31							.09	.91		.06			.50	.44		1.00		8

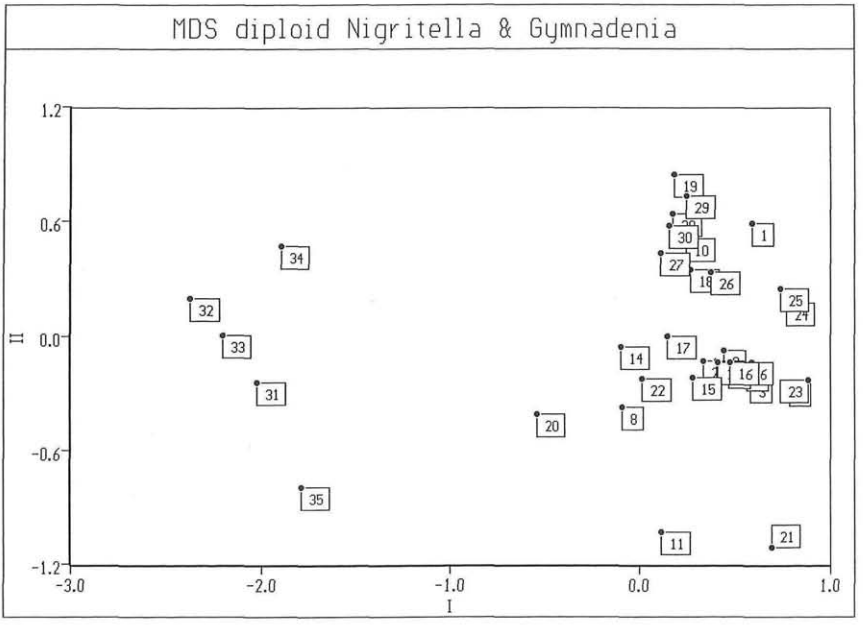

Fig. 1. MDS ordination of all diploid populations investigated of *Nigritella* and *Gymnadenia*. Stress = 0.25056. The resulting MDS solution was rotated in a PCA in order to maximize variation along the horizontal axis.

3.7. Multilocus Genotype Variation in the Polyploid Taxa

We found a small number of multilocus genotypes in each of the polyploid taxa, which is in accordance with their apomictic mode of reproduction. In Table 3, we have compared all polyploid populations and taxa for multilocus genotype variation and each multilocus genotype is indicated by a capital letter. *Nigritella widderi* and *N. miniata* comprised two multilocus genotypes each, whereas the other polyploid taxa were each characterised by a single multilocus genotype. In *N. widderi*, four populations contained multilocus genotype F, two populations contained multilocus genotype G, and one population, Aflenzer Bürgeralm, contained both multilocus genotypes. In *N. miniata*, nine populations contained multilocus genotype E, one population contained multilocus genotype H, and one population, Teichalm, contained both multilocus genotypes.

The single multilocus genotype found in *N. archiducis-joannis* was identical with multilocus genotype F found in *N. widderi* and the single multilocus genotype found in *N. stiriaca* was identical with the multilocus genotype E found in *N. miniata*.

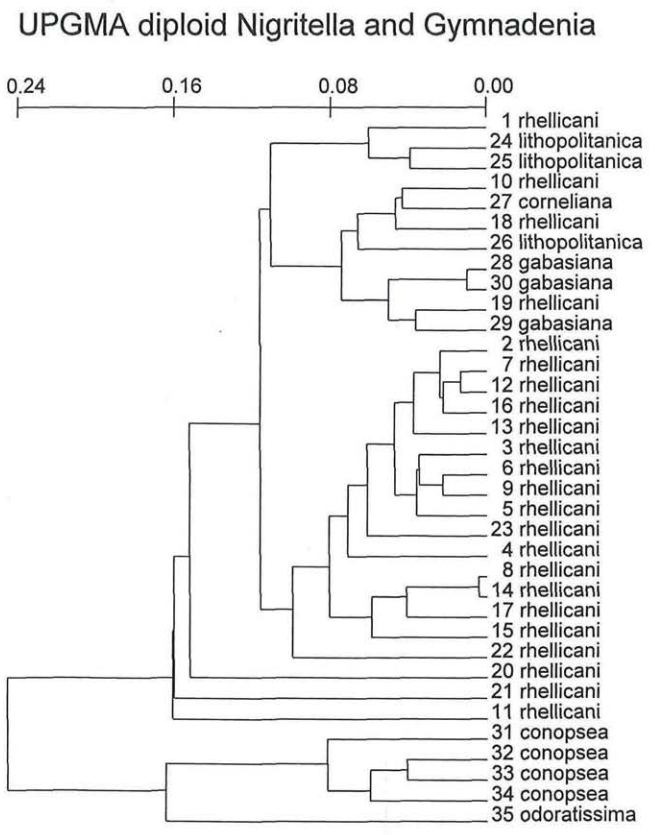

Fig. 2. UPGMA phenogram summarizing genetic distances among diploid populations of *Nigritella* (1–30) and *Gymnadenia* (31–35). The cophenetic correlation coefficient was 0.91179.

The tetraploid members of *Nigritella* could be sorted pairwise into three groups, within which the multilocus genotypes differ by a single dose difference, and between which there are 4–7 differences (either as presence/absence of a certain allele, or as a relative difference of alleles present in both multilocus genotypes). Thus, multilocus genotypes C and D (Fig. 3), comprising *N. nigra* subsp. *iberica* and *N. nigra* subsp. *austriaca*, respectively, differ in relative numbers of the Pgm alleles b and c (Table 3); multilocus genotypes G & F, comprising *N. widderi* and *N. archiducis-joannis* differ by the presence of one copy of the allele $Mdh1^a$ in genotype G that is absent from genotype F; finally, multilocus genotypes E & H, comprising *N. miniata* and *N. stiriaca* differ by the presence of one copy of the allele Pgm^b in genotype E that is absent from genotype H. The tetra-

ploid *Gymnigritella runei* differs by 3–8 differences from the other tetraploid multilocus genotypes, but is most similar to the group of tetraploid *Nigritella nigra*.

Table 3

Multilocus genotypes found at each locality from which polyploids were studied. Dashes indicate that no individual from the particular locality could be studied for a certain locus. In some additional cases, some individuals from a locality did not give results for all studied loci, but they were amalgamated with the remaining individuals if they agreed in other loci. The banding pattern at Skd in *N. buschmanniae* was difficult to estimate as exact doses. x gives the ploidy level, and ML the multilocus genotype as denoted in Tables 3 & 4 and in Fig. 3. The names of taxa are abbreviated as in Table 1.

Taxon	x	ML	Locality	N	idh	mdh1	mdh2	mdh3	pgd	pgi	pgm	skd	tpi1	tpi2
			Alleles		a b c	a b c	a b c	a b c	a b c d	a b c d e f	a b c d e f	a b c d e f	a b c d e	a b c
run	4	A	Rödingsnäset	16	4	4	1 3	1 3	2 2	4	2 2	1 1 2	1 3	4
run	4	A	Ransaren	14	4	4	1 3	1 3	2 2	4	2 2	1 1 2	1 3	4
nig	3	B	Klinken	16	3	3	3	3	1 2	3	1 2	1 2	1 2	3
ibe	4	C	Font Romeu	7	4	4	4	4	2 2	4	2 2	1 3	1 3	4
ibe	4	C	Eyne	8	4	4	4	4	2 2	4	2 2	1 3	1 3	4
ibe	4	C	Mt Mézenc	1	–	4	4	4	2 2	4	2 2	1 3	1 3	4
ibe	4	C	Chaudeyrolles	1	–	4	4	4	2 2	4	2 2	1 3	1 3	4
ibe	4	C	Mts de Cézallier	2	–	4	4	4	2 2	4	2 2	1 3	1 3	4
ibe	4	C	Col de l'Alpe	9	–	4	4	4	2 2	4	2 2	1 3	1 3	4
aus	4	D	Hinteregg	6	4	4	4	4	2 2	4	1 3	1 3	1 3	4
aus	4	D	Schafberg	13	4	4	4	4	2 2	4	1 3	1 3	1 3	4
aus	4	D	Teichalm	10	4	4	4	4	2 2	4	1 3	1 3	1 3	4
aus	4	D	Trenchtling	4	4	4	4	4	2 2	4	1 3	1 3	1 3	4
aus	4	D	Aflenzer Bürgeralm	15	4	4	4	4	2 2	4	1 3	1 3	1 3	4
aus	4	D	Koralpe	11	4	4	4	4	2 2	4	1 3	1 3	1 3	4
aus	4	D	Hohe Veitsch	5	4	4	4	4	2 2	4	1 3	1 3	1 3	4
sti	4	E	Schafberg	13	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
sti	4	E	Teichalm	15	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
wid-1	4	F	Trenchtling	12	4	4	4	4	4	4	3 1	1 1 2	4	4
wid-1	4	F	Aflenzer Bürgeralm	8	4	4	4	4	4	4	3 1	1 1 2	4	4
wid-1	4	F	Hohe Veitsch	8	4	4	4	4	4	4	3 1	1 1 2	4	4
wid-1	4	F	Kreuzkogel	12	4	4	4	4	4	4	3 1	1 1 2	4	4
wid-1	4	F	Tamischbachturm	5	4	4	4	4	4	4	3 1	1 1 2	4	4
wid-2	4	G	Teichalm	6	4	1 3	4	4	4	4	3 1	1 1 2	4	4
wid-2	4	G	Schöckel	1	4	1 3	4	4	4	4	3 1	1 1 2	4	4
wid-2	4	G	Aflenzer Bürgeralm	12	4	1 3	4	4	4	4	3 1	1 1 2	4	4
min-1	4	E	W. Hofpürgelhütte	6	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
min-1	4	E	Teichalm	2	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
min-1	4	E	Schöckel	10	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
min-1	4	E	Trenchtling	15	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
min-1	4	E	Aflenzer Bürgeralm	26	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
min-1	4	E	Tauplitzalm	9	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
min-1	4	E	Hohe Veitsch	2	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
min-1	4	E	Bosruck	16	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
min-1	4	E	Tamischbachturm	6	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
min-1	4	E	Schneeberg	8	4	3 1	4	3 1	3 1	4	1 3	1 3	4	4
min-2	4	H	Teichalm	20	4	3 1	4	3 1	3 1	4	4	1 3	4	4
min-2	4	H	Ardninger Alm	5	4	3 1	4	3 1	3 1	4	4	1 3	4	4
a–j	4	F	Traweng	6	4	4	4	4	4	4	3 1	1 1 2	4	4
bus	5	I	Brenta	12	5	5	5	4 1	5	5	3 2	*1 1 3*	5	5

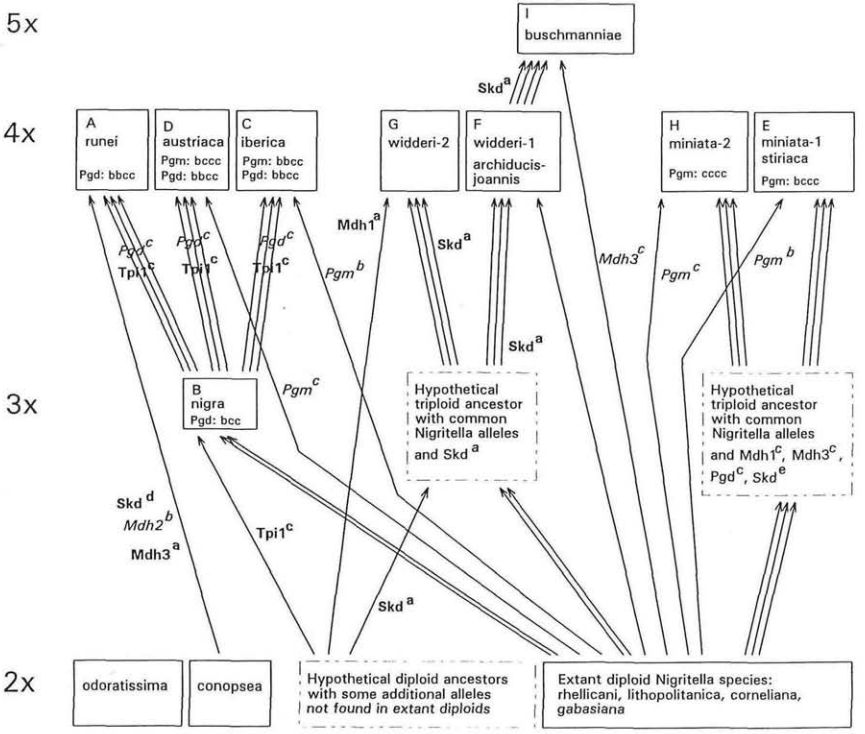

Fig. 3. A hypothetical scheme for the phylogeny of polyploid members of *Nigritella* and *Gymnigritella*. Each multilocus genotype is enclosed by a separate box which may in some cases contain material of different taxa. The numbers of arrows between boxes give the number of haploid chromosome sets thought to have been transferred. Transfer of alleles unique to certain polyploids, or groups of polyploids are put in bold face, whereas alleles supporting certain pathways by their frequency distribution are put in italics; see text for further details.

Two hypothetical triploids are inserted in the scheme in an attempt to minimize the number of polyploidization events. The scheme is likely to underestimate the number of origins of certain multilocus genotypes. Likewise, in the scheme, as much as possible of the allozyme composition of polyploids is explained by alleles found in extant diploid members of *Nigritella*, which may give an overestimate of the contribution of extant diploids to the polyploids.

Alternative pathways involving autotetraploid intermediates may be considered for some of the tetraploid taxa. See text for further discussion.

3.8. Hybridization between *Nigritella rhellicani* and *Gymnadenia conopsea*

The pattern of hybridization between *Nigritella rhellicani* and *Gymnadenia conopsea* was described from one site, Heiligenbachalm (Table 1). In the resulting plot of an MDS ordination on genetic distances (Fig. 4),

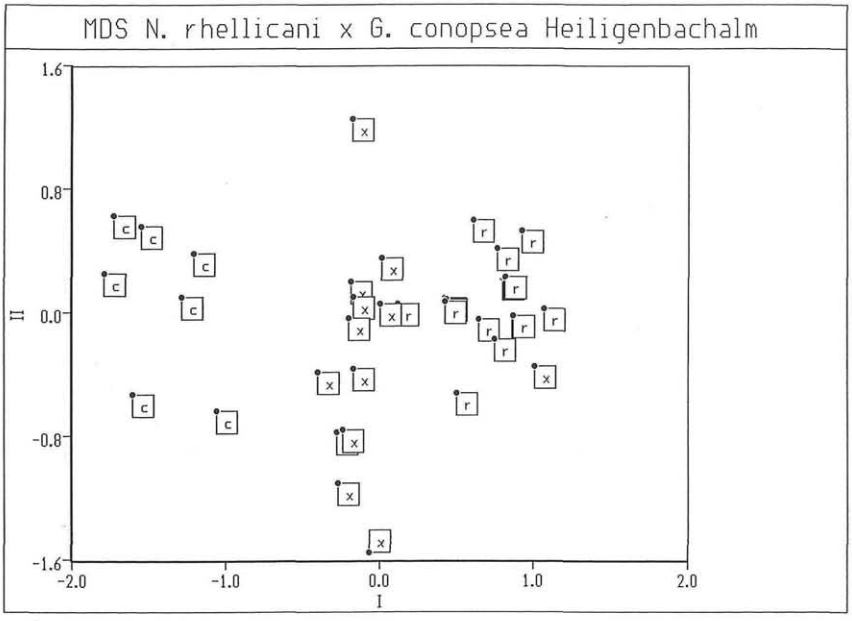

Fig. 4. MDS ordination on *Gymnadenia conopsea*, *Nigritella rhellicani* and their hybrid on material from Heiligenbachalm. Stress = 0.27124. The resulting MDS solution was rotated in a PCA in order to maximize variation along the horizontal axis.

most individuals classified as hybrids appear at an intermediate position between the two parental groups. Much of the separation of the parental groups was due to allele frequency differences. The only locus at which the parental taxa had different alleles was Skd. Most hybrids were heterozygous for alleles from the different parental genomes at this locus, but a few hybrid specimens had alleles from only one of the parental genomes, which may indicate some degree of back-crossing with the parental taxa. However, since Skd produced faint bands in many individuals, the interpretation of these hybrid specimens was somewhat uncertain.

3.9. Hybrids between *Nigritella rhellicani* and *Gymnadenia odoratissima*

Hybridization between *Nigritella rhellicani* and *Gymnadenia odoratissima* was also described from Heiligenbachalm. In the MDS ordination on genetic distances (Fig. 5), the hybrids all appeared at intermediate positions between the parental groups, but they were not well separated from any of the parents. All differences between the parental taxa consist of allele frequency differences, and accordingly, a clear separation of the groups of parental taxa and the hybrids is not to be expected even though all hybrids may be primary hybrids.

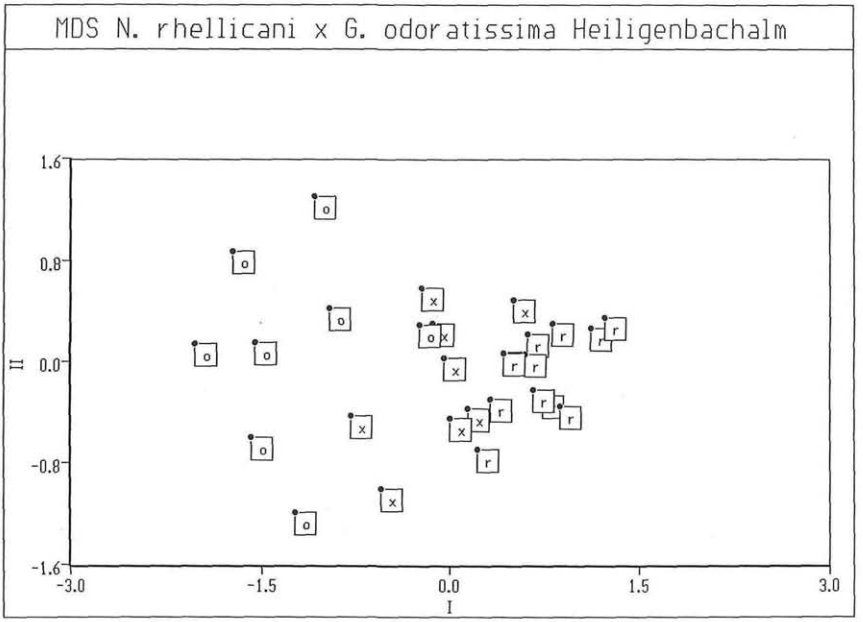

Fig. 5. MDS ordination on *Gymnadenia odoratissima*, *Nigritella rhellicani* and their hybrid on material from Heiligenbachalm. Stress = 0.28315. The resulting MDS solution was rotated in a PCA in order to maximize variation along the horizontal axis.

4. Discussion

4.1. Breeding systems

The variation patterns seen at allozyme loci agree with previous observations based on embryological studies, viz. that diploid members of *Nigritella* are sexual, whereas polyploid members reproduce apomictically (TEPPNER 1996; TEPPNER & KLEIN 1985a, 1985b, 1990, 1993, 1998; TEPPNER & STER 1996; TEPPNER & al. 1994). Furthermore, allozyme data reveal that the diploid taxa are outcrossing to a large degree. However, the finding that the St. Bernhard and Brenta populations of *N. rhellicani* are dominated by a few multilocus genotypes may imply that some diploids are able to produce seed by agamospermy under certain conditions. The mentioned populations are among the ones growing at the highest altitudes (Table 1). Still, it is possible that other causes are responsible for the dominance of a low number of multilocus genotypes in these populations, for example asexual propagation by vegetative dispersal, or strongly unequal reproductive success between individuals in the population and between years. These observations indicate that further embryological studies are needed in diploid populations from high altitudes.

In insect-pollinated plants apomixis could be seen "... as a mechanism to avoid pollination problems due to harsh climate conditions with uncertain availability of pollinators" (ASKER & JERLING 1992: 207). Selfing would also permit plants to reproduce in situations which pollinators are scarce, but this mechanism does not occur in *Nigritella*. See also ASKER & JERLING 1992: 207, 228 for a discussion of apomixis in relation to pollination and altitude. However, it is uncertain whether there is any clear correlation between altitude and ploidy level in *Nigritella*. In the Alps, e.g. in the Hohe Tauern, the diploid *N. rhellicani* ascends to higher altitudes than the polyploids *N. nigra* subsp. *austriaca* and *N. miniata* (TEPPNER unpubl.). With respect to independence of pollination, apomicts would not be advantageous if they are pseudogamous, that is if stimulus of pollination is

Table 4

Comparison of allele frequencies found in diploid, sexual taxa of *Nigritella* and *Gymnadenia*, and multilocus genotypes found in polyploid, apomictic taxa of *Nigritella* and *Gymnigritella*. For sexual taxa in which several localities were sampled, and in which loci were variable, the given values were calculated as the arithmetic mean values of the frequencies found at these localities. For such taxa, the minimum and the maximum frequencies are also given. For polyploid taxa the numbers of copies of each allele is given. In cases where the numbers of copies were difficult to estimate from the band intensities found on the gels, the copy numbers are given in italics. + denote the presence of an allele in a frequency $\ll 0.01$.

		idh			mdh1			mdh2			mdh3			pgd				pgi					
		a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	d	a	b	c	d	e	f
rhe		.91		.09		.78	.22		.13	.87	+	.87	.13	.01	.99					.99	.01		
max		*1.00*		*.61*		*1.00*	*.41*		*.50*	*1.00*	*.03*	*1.00*	*.71*	*.09*	*1.00*					*1.00*	*.36*		
min		*.39*		*.00*		*.33*	*.00*		*.00*	*.50*	*.00*	*.29*	*.00*	*.00*	*.91*					*.64*	*.00*		
lit		1.00				.95	.05		.02	.98		.92	.08		.92	.05	.03			1.00			
max						*1.00*	*.10*		*.05*	*1.00*		*1.00*	*.17*		*1.00*	*.15*	*.09*						
min						*.90*	*.00*		*.00*	*.95*		*.83*	*.00*		*.85*	*.00*	*.00*						
cor		1.00				.90	.10		.06	.94		1.00			1.00					1.00			
gab		.94	.06			1.00				1.00		1.00			.98	.02				1.00			
max		*1.00*	*.12*												*1.00*	*.07*							
min		*.88*	*.00*												*.93*	*.00*							
con		1.00			.04	.52	.44	.06	.85	.09	.94	.06		.01	.97	.02		.01	.19	.61	.14	.05	
max					*.17*	*.81*	*.58*	*.13*	*.88*	*.25*	*1.00*	*.17*		*.04*	*1.00*	*.04*		*.04*	*.23*	*.71*	*.25*	*.13*	
min					*.00*	*.33*	*.19*	*.00*	*.75*	*.00*	*.83*	*.00*		*.00*	*.92*	*.00*		*.00*	*.17*	*.50*	*.06*	*.00*	
odo		1.00				.88	.12		.94	.06	.08	.92			1.00					.44	.38	.12	.06
run	(A)	4				4			1	3	1	3			2	2				4			
nig	(B)	3				3				3		3			1	2				3			
ibe	(C)	4				4				4		4			2	2				4			
aus	(D)	4				4				4		4			2	2				4			
sti	(E)	4				3	1			4		3	1		3	1				4			
wid-1	(F)	4				4				4		4			4					4			
wid-2	(G)	4			1	3				4		4			4					4			
min-1	(E)	4				3	1			4		3	1		3	1				4			
min-2	(H)	4				3	1			4		3	1		3	1				4			
a-j	(F)	4				4				4		4			4					4			
bus	(I)	5				5				5		4	1		5					5			

necessary for seed development. In *Nigritella* the initiation of embryogenic cells (embryocytes) is clearly autonomous because it begins as early as in closed flower buds (TEPPNER 1996: 329).

Embryological observations indicate that sexual reproduction may sometimes occur in polyploid *Nigritella* species (TEPPNER 1996). It might be argued that the different multilocus genotypes found in *N. miniata* and in *N. widderi* would be a result of rare sexual recombination at the tetraploid level. However, given the numbers of loci with the same unbalanced heterozygous genotype in these cases, it seems more likely that multiple origins are responsible for the variation seen. Sexual recombination would affect the entire genome, and we would have found variation at other heterozygous loci as well.

4.2. Differentiation of Diploid *Nigritella* Species

We found relatively little total genetic diversity in diploid members of *Nigritella*. The mean genetic diversity at the genus level at ten variable loci was 0.178 (H_{Genus} in Table 5). A small proportion of this diversity, 6.6% was due to differentiation among species. This degree of differentiation among species was apparently considerably lower than in some Mediterranean species of *Orchis* (SCACCHI & al 1990), in which Nei's genetic

Table 4. Cont.

	pgm						skd						tpi1					tpi2				
	a	b	c	d	e	f	a	b	c	d	e	f	a	b	c	d	e	a	b	c	x	N
rhe		.29	.71	+	+			.02	.17		.76	.05		+		1.00		.01	.99	+	2	323
max		*1.00*	*1.00*	*.02*	*.03*			*.50*	*.56*		*1.00*	*1.00*		*.03*		*1.00*		*.15*	*1.00*	*.03*		
min		*.00*	*.00*	*.00*	*.00*			*.00*	*.00*		*.00*	*.00*		*.00*		*.97*		*.00*	*.85*	*.00*		
lit		.33	.67					.12	.42		.37	.09				1.00			1.00		2	33
max		*.38*	*.70*					*.22*	*.59*		*.44*	*.17*										
min		*.30*	*.62*					*.10*	*.17*		*.30*	*.00*										
cor		.72	.25			.03		.35	.45		.20					1.00			.97	.03	2	16
gab		1.00							.11		.83	.06				1.00			1.00		2	15
max									*.33*		*1.00*	*.17*										
min									*.00*		*.50*	*.00*										
con	.02	.78	.20						.98			.02				.92	.08		1.00		2	41
max	*.08*	*.97*	*.42*						*1.00*			*.09*				*.96*	*.12*					
min	*.00*	*.50*	*.03*						*.91*			*.00*				*.88*	*.04*					
odo	.13	.56	.31						.09	.91			.06			.50	.44		1.00		2	8
run		2	2						1	1	2				1	3			4		4	30
nig		1	2						1		2				1	2			3		3	16
ibe		2	2						1		3				1	3			4		4	28
aus		1	3						1		3				1	3			4		4	69
sti		1	3						1		3					4			4		4	28
wid-1		3	1				1		1		2					4			4		4	45
wid-2		3	1				1		1		2					4			4		4	19
min-1		1	3						1		3					4			4		4	100
min-2			4						1		3					4			4		4	25
a-j		3	1				1		1		2					4			4		4	6
bus		3	2				*1*		*1*		3					5			5		5	12

Table 5

The distribution of allozyme diversity in the studied diploid species of *Nigritella*. H_{Genus}, $\overline{H}_{Species}$, and $\overline{H}_{Site}$ describe the total genetic diversity, the mean genetic diversity in each species, and the mean genetic diversity in each locality, respectively. Separate calculations are made for each locus. The proportion of the total genetic diversity that is due to differentiation among species is given as $G_{SpecGen}$, and the proportion of the total genetic diversity that is due to differentiation among localities within species is given as $G_{SiteSpec}$. The mean proportions of genetic diversity over loci are given as the arithmetic mean, and as a weighted mean, weighted by the total genetic diversity at each locus.

locus	H_{Genus}	SE	$\overline{H}_{Species}$	$G_{SpecGen}$	$\overline{H}_{Site}$	$G_{SiteSpec}$
idh	0.2324	0.01861	0.2264	0.0257	0.1748	0.2220
mdh1	0.3081	0.01804	0.2997	0.0272	0.2839	0.0513
mdh2	0.1684	0.01818	0.1666	0.0107	0.1464	0.1200
mdh3	0.1810	0.01831	0.1794	0.0086	0.1525	0.1486
pgd	0.0349	0.00948	0.0342	0.0198	0.0332	0.0287
pgi	0.0134	0.00596	0.0134	0.0011	0.0094	0.2985
pgm	0.4135	0.01448	0.3487	0.1569	0.2756	0.1768
skd	0.4065	0.01993	0.3728	0.0828	0.2793	0.2300
tpi1	0.0027	0.00270	0.0027	–0.0000	0.0027	0.0000
tpi2	0.0188	0.00703	0.0188	–0.0004	0.0180	0.0426
mean	0.1780			0.0332		0.1318
weighted mean				0.0657		0.1612

identity, I, ranged between 0.934 and 0.995 in comparisons of different populations within species, and between 0 and 0.767 in comparisons between species. However, *Orchis* s.l. was shown to be a paraphyletic group in PRIDGEON & al. 1997 and should be split into three clearly genetically differentiated genera (BATEMAN & al. 1997). Still, genetic identities are also quite low in species comparisons within these generic segregates. Compared to the genus *Dactylorhiza*, diploid members of *Nigritella* are less separated than the diploid *D. fuchsii* and *D. incarnata* s.l., but are more clearly separated than the various segregates of *D. incarnata* s.l. (HEDRÉN 1996).

The relatively small proportion of the total genetic diversity in diploid members of *Nigritella* due to differentiation among species is also shown by the incomplete separation of taxa in the MDS ordination of Rogers' genetic distances (Fig. 1). The allozyme data thus indicate that the present-day diploid members of *Nigritella* are relatively closely related, and it is likely that other, more divergent diploids also contributed to the formation of polyploids (see below).

A clear pattern of differentiation was correlated to the geographic origin of the populations (Figs. 1 & 2). Thus, most western populations, including those from the western Alps grouped together, whereas most

eastern populations of *N. rhellicani*, including those from Switzerland, formed another cluster. The eastern *N. lithopolitanica* grouped with the western populations, and some populations of *N. rhellicani* deviated from both major groups. A close relationship of *N. lithopolitanica* and *N. corneliana* was suggested by TEPPNER & KLEIN 1985a, and is thus in agreement with allozyme data. However, the variation pattern in *N. rhellicani* and the genetic similarity of western populations of this species with other western diploids, indicate that the taxonomy of the western diploids may need to be revised after extended studies.

In *N. rhellicani*, which is the single diploid species that was investigated for a fair number of population samples, the mean over variable loci of total genetic diversity was 0.17 (H_T in Table 6). This estimate is comparable to species studied of *Orchis* (SCACCHI & al. 1990, CORRIAS & al. 1991, ROSSI & al. 1992, ARDUINO & al. 1995, 1996), in which H_T ranged between 0.077 in *O. morio* to 0.338 in *O. mascula* (SCACCHI & al. 1990). However, the proportion of genetic diversity due to differentiation among populations, G_{ST} (Table 6) was 0.19 in *N. rhellicani*, which was higher than in most species of *Orchis*, in which G_{ST} was generally less than 0.10. Exceptions were found in *O. palustris* and in *O. laxiflora* (ARDUINO & al. 1996), which were both investigated from a large geographic area. A study of *Gymnadenia conopsea* (SCACCHI & DE ANGELIS 1989) also revealed a high value of population differentiation, which, however, was interpreted as

Table 6

The distribution of allozyme diversity in *Nigritella rhellicani*. H_T and $\overline{H}_S$ describe the total genetic diversity, and the mean genetic diversity at the studied localities, respectively. Separate calculations are made for each locus. The proportion of the total genetic diversity that is due to differentiation among localities is given as G_{ST}. The mean proportions of genetic diversity over loci are given as the arithmetic mean and as a weighted mean, weighted by the total genetic diversity at each locus.

locus	H_T	SE	$\overline{H}_S$	G_{ST}
idh	0.2635	0.02020	0.2018	0.2341
mdh1	0.3418	0.01886	0.3229	0.0553
mdh2	0.1897	0.02030	0.1659	0.1254
mdh3	0.1965	0.02041	0.1647	0.1618
pgd	0.0194	0.00782	0.0188	0.0309
pgi	0.0161	0.00714	0.0112	0.3032
pgm	0.3505	0.01887	0.2615	0.2540
skd	0.3384	0.02229	0.2333	0.3106
tpi1	0.0032	0.00325	0.0032	0.0000
tpi2	0.0193	0.00781	0.0184	0.0516
mean	0.1738			0.1527
weighted mean				0.1918

differentiation into two ecological races. Chi-square tests or other comparisons of levels of expected and observed heterozygosity reveal that the taxa discussed above are all outbreeding to a large degree (papers cited above), and this was also true for the diploid *Nigritella* species analysed in this study. Accordingly, the relatively high differentiation of populations of *N. rhellicani* appears not to be related to breeding system, but rather to geographic isolation of the populations and differentiation into geographic races.

4.3. Generic Delimitation

A close relationship of *Gymnadenia* and *Nigritella* has been recognised for long. REICHENBACH 1856 included *Nigritella nigra* in *Gymnadenia* and he has been followed, among recent authors, by LØJTNANT 1977, SUNDERMANN 1980, and BATEMAN & al. 1997. However, most 20th century authors have regarded the genera different enough to motivate separation, e.g., SCHLECHTER 1919, KELLER & SCHLECHTER 1928, MOORE 1980.

Another argument to unite these genera was presented by PRIDGEON & al. 1997, who studied phylogenetic relationships and generic delimitation in European *Orchidaceae-Orchidinae* based on ITS sequences. They found that *Nigritella nigra* clustered with *Gymnadenia conopsea* subsp. *borealis* and that these taxa in turn formed a sister group to *G. conopsea* subsp. *conopsea*. Because of this pattern, *Nigritella nigra* was treated as *Gymnadenia* by BATEMAN & al. 1997.

If members of *Nigritella* were more closely related to some members of *Gymnadenia conopsea* than members of *Gymnadenia* with each other, we would expect that these relationships were reflected also in the pattern revealed by allozyme data. In the MDS ordination (Fig. 1), we would have seen samples of *Gymnadenia* being spread out over the plot, whereas populations of *Nigritella* would have been concentrated to one smaller area. In the UPGMA phenogram (Fig. 2), populations of *Gymnadenia* would join at a relatively basal level, whereas the *Nigritella* samples would join one of the *Gymnadenia* branches. However, we do not find these patterns. Although we included *Gymnadenia conopsea* from Austria as well as from Sweden, these populations fall relatively close to each other and to *G. odoratissima*, and all members of *Gymnadenia* are relatively differentiated from the *Nigritella* samples. Our data indicate that *Gymnadenia* and *Nigritella* may indeed be sister groups, and that it is not necessary to unite the genera in order to reflect the phylogenetic pattern. We regard the morphological differences between genera large enough to treat *Nigritella* as a separate genus. However, the findings by PRIDGEON & al. 1997 are thought-provocative and it should be of interest to perform extended studies on the relationship between the genera. In order to get more precise information from allozyme data, it would be necessary to char-

acterise other populations of *Gymnadenia* over the entire distribution area. It would also be necessary to examine the allozyme composition of a relevant outgroup and to perform strict cladistic analyses of these data.

4.4. Evolution of Polyploids

All subspecies of *Nigritella nigra* have one copy each of the allele $Tpi1^c$, which was absent from the diploid taxa, and two copies each of the allele Pgd^c, which was rare in the diploids. Accordingly, it could be assumed that at least one of the diploid ancestors to the triploid *N. nigra* subsp. *nigra* is lacking among the studied diploids. The multilocus genotypes of the tetraploid *N. nigra* subsp. *austriaca* and *N. nigra* subsp. *iberica* could be explained by an addition of a set of alleles common in the extant diploid *Nigritella* species to the multilocus genotype found in *N. nigra* subsp. *nigra*. This observation fits the hypothesis that triploid taxa have contributed to the formation of tetraploid taxa by unreduced gametes. The two tetraploid subspecies differ by a dose difference at Pgm where the eastern subsp. *austriaca* contains an extra copy of the allele Pgm^c as compared to subsp. *nigra*, and the western subsp. *iberica* contains an extra copy of Pgm^b. As a matter of fact, Pgm^c is the most common allele in the eastern diploids *N. rhellicani* and *N. lithopolitanica*, whereas Pgm^b appears to be the common allele in the western diploids *N. corneliana* and *N. gabasiana*. The two tetraploid subspecies of *N. nigra* have therefore probably originated separetely somewhere within their present distribution areas.

The multilocus genotype of *Gymnigritella runei* could be explained by an addition of the *N. nigra* subsp. *nigra* genome to a set of alleles common in *Gymnadenia conopsea*. *Gymnadenia conopsea* contained three alleles that were rare or absent in the *Nigritella* species: Skd^d, $Mdh3^a$, and $Mdh2^b$ (Table 2). These observations fit the hypothesis presented by TEPPNER & KLEIN 1989, based on morphological, karylogical, and embryological data, that *Gymnigritella runei* was formed by fertilization of a normal haploid egg cell in *Gymnadenia conopsea* with an unreduced gamete from pollen of *Nigritella nigra* subsp. *nigra*.

The remaining tetraploids may also have arisen from triploid ancestors contributing unreduced gametes, but no such triploid candidates have been identified. We still regard origins from triploid ancestors as probable, but in addition we also consider the possibility that these tetraploids have been formed by hybridization between different autotetraploid lineages, such as in, e.g., *Dactylis* (LUMARET & BARRIENTOS 1988). The tetraploid multilocus genotypes F & G (Table 4), both containing *N. widderi*, and one containing *N. archiducis-joannis*, differ by the presence of the unique allele $Mdh1^a$ in genotype G. Both genotypes were characterised by one dose of the allele Skd^a, which was also present in the pentaploid

N. buschmanniae, but which was absent from all other investigated material. A plausible explanation to this pattern is that both genotypes originated from a triploid ancestor with one copy of Skd^a, which contributed an unreduced gamete, and a diploid ancestor, which contributed a normal reduced gamete, and which at least in the formation of genotype G, must have been different from the studied diploids.

Similarly, genotypes H & E (Table 4), both containing *N. miniata*, and one containing *N. stiriaca*, differ by the presence of one copy of the allele Pgm^b in genotype E, whereas genotype H contains Pgm^c alleles only. At each of three other loci both genotypes had in common one copy of an allele that was rare in the diploids. It seems likely also in this case that the two tetraploid multilocus genotypes have their origins in a fusion of an unreduced gamete from a triploid ancestor containing the rare alleles with a normal reduced gamete from a diploid similar to the studied diploids containing common *Nigritella* alleles. As already commented upon, the allele Pgm^b is predominant in the western diploids, but it does occur in the eastern diploids as well.

The multilocus genotype observed in the pentaploid *N. buschmanniae* could be explained by an addition of the tetraploid multilocus genotype F to a set of alleles common in present-day diploids. This observation fits the view presented by TEPPNER & STER 1996, who regarded the species as being related to *N. widderi*. In addition, *N. buschmanniae* contained the allele $Mdh3^c$, which occurred infrequently in the diploids *N. rhellicani* and *N. lithopolitanica*.

Banding patterns found in tetraploid members of *Nigritella* exclude the possibility that they have evolved through somatic doubling. If so, the tetraploids would not have contained unbalanced genotypes at any allozyme locus.

An origin of the tetraploid genotypes by hybridization of autotetraploid lineages should also be considered. In populations of diploid, sexual species the presence of unreduced gones or gametes respectively at a very low scale is common (e.g. TEPPNER 1974: 69, 70, 1991: 275, 277, 1996: 324, ASKER & JERLING 1992: 69), and unreduced gametes may be produced in high frequencies by certain individuals in populations of non-hybrid plants (cf. MACEIRA & al 1992, ORTIZ & al. 1992; FINCH & BENNETT 1979, RHOADES & DEMPSEY 1966, IWANAGA & PELOQUIN 1982, HARLAN & DE WET 1975), or by primary hybrids between different species (DE WET 1980). In the former case usually autopolyploids will be formed, in the latter case allopolyploids. Tetraploids may be formed in a single step by fusion of two unreduced gametes, or more commonly by stepwise increase of chromosome number via an intermediate triploid stage (HARLAN & DE WET 1975, DE WET 1980, RAMSEY & SCHEMSKE 1998): Fertilization of an unreduced gamete by a haploid one may give rise to triploid plants, and in a second

step tetraploid plants may be formed. In *Nigritella*, such an autotriploid has been observed in *N. carpathica* (TEPPNER & al. 1994: 180–181).

In order to produce allotetraploid offspring, the autotetraploids must have normal meiosis and at least partial sexuality. This assumption requires that apomictic seed production is associated with hybridity rather than with polyploidy in the genus. Given the amount of heterozygous loci observed in extant apomictic tetraploids, these sexual autotetraploids should have been genetically variable. If the different multilocus genotypes in *N. widderi* or in *N. miniata* were the result of repeated hybridization events, we would thus expect that these multilocus genotypes differed by more than one allele copy, which is not in agreement with our data.

However, an origin of present-day tetraploids by hybridization between autotetraploid lineages would still be possible if it is assumed that the two multilocus genotypes in each of *N. widderi* and *N. miniata* were not the results of repeated hybridization events, but the results of rare somatic mutations within each apomictic lineage. Several authors emphasise that such somatic mutations are probably more important than previously thought in contributing to genetic variation in apomictic groups (ELLSTRAND & ROOSE 1987, ASKER & JERLING 1992, KASHIN & KUPRIJANOV 1994, BIRKY 1996). Most mutations result in loss of function of existing genes rather than gain of new functional genes. Thus, in *N. widderi* genotype F could be derived from genotype G by loss of $Mdh1^a$ function, and in *N. miniata* genotype H could be derived from genotype E by loss of Pgm^b function. It follows that *N. archiducis-joannis* would have been derived from *N. widderi* after the mutation occurred, but that *N. stiriaca* would have been split off from the *N. miniata* lineage before the mutation.

To summarise, it is clear that an origin of tetraploid apomicts in *Nigritella* by hybridization of autotetraploid lineages is feasible. However, this scenario is rather complex and requires a fairly high number of events and hypothetical conditions. An origin from triploid intermediates is more simple (Fig. 3), and should therefore be considered as more likely (cf. RAMSEY & SCHEMSKE 1998). However, we also emphasise that it is extremely difficult to estimate the likelihood of single steps in polyploid evolution, and that likelihood estimates for each group of polyploids must be regarded as largely independent of corresponding estimates for other such groups.

The species relationships proposed in Fig. 3 are exclusively based on the variation patterns we have seen at allozyme loci. Still, we found comparatively little allozyme differentiation within *Nigritella* as a whole, and it is possible that the true species relationships may be more complex than indicated. For instance, it has been sufficient to invoke just two hypothetical triploid ancestors to account for the various multilocus genotypes found in *Nigritella widderi*, *N. archiducis-joannis*, *N. miniata*, and

N. stiriaca. Given the apparent differences in flower morphology of *N. widderi* and *N. archiducis-joannis*, it is possible that they are derived from different triploid ancestors, which have had the same allozyme composition (in the loci studied by us). Still, *N. widderi* and *N. archiducis-joannis* are similar to each other in other morphological traits (TEPPNER & KLEIN 1985) and they have narrow and partly sympatric distribution areas in the eastern Alps. Similarly, the multilocus genotype found in *N. stiriaca* was identical to one of the two multilocus genotypes found in the widespread *N. miniata*. Although *N. stiriaca* is distinct from *N. miniata* in its flower colour and relatively broad floral parts (TEPPNER & KLEIN 1985), they are still similar to each other in many characters and an origin from a common triploid ancestor does not seem improbable.

The occurrence of more than one multilocus genotype in *N. widderi* and in *N. miniata* may be taken as evidence for multiple origins. Multiple origins of polyploid taxa seem to be common and have been well documented in, e.g., *Draba* (BROCHMANN & al. 1992a, 1992b), *Tragopogon* (ROOSE & GOTTLIEB 1976, SOLTIS & SOLTIS 1989, SOLTIS & SOLTIS 1991, SOLTIS & al. 1995, SCHIERENBECK & al. 1992, COOK & al. 1998), *Heuchera* (WOLF & al. 1990, SEGRAVES & al. 1999), *Anthoxanthum odoratum* (TEPPNER 1970, 1998) and *Dactylis* (LUMARET & BARRIENTOS 1990). Furthermore, considering the low degree of allozyme differentiation in *Nigritella*, it is possible that the number of independent origins of tetraploid *Nigritella* is underestimated, and that some independent origins have given rise to identical multilocus genotypes. In *Tragopogon*, DNA-based studies have revealed that most allotetraploid populations have arisen independently of each from their diploid ancestors, despite of the fact that several of these populations were indistinguishable at common allozyme loci (COOK & al. 1998).

4.5. Age of the *Nigritella* species

Several species of *Nigritella* are restricted to classical relict areas in the Alp region. These areas are known to contain many plants that probably have survived the last glaciation in close vicinity of their present locations. It is assumed that species of *Nigritella* with this type of distribution also have survived the last glaciation in situ.

The distribution of rare alleles suggest that at least the triploid intermediates which are supposed to have given rise to the species at higher ploidy levels should be quite old. Allozyme data alone give no clear indication to the timing of further polyploidization events. However, distribution data combined with the tentative evolutionary pathways given in Fig. 3, provide some further insights. *Gymnigritella runei*, *Nigritella nigra* subsp. *austriaca* and *N. nigra* subsp. *iberica* may have evolved from *N. nigra* subsp. *nigra* by addition of normal, reduced gametes from present-day diploids. Based on estimated distribution areas of *G. conopsea*

and *N. nigra* subsp. *nigra* during historical times, a recent origin of *G. runei* was proposed by RUNE 1993, which is thus in concordance with our allozyme data.

The Central European *N. nigra* subsp. *austriaca* and *N. nigra* subsp. *iberica* are today isolated from the Scandinavian *N. nigra* subsp. *nigra*. In addition, *N. nigra* subsp. *nigra* is also isolated from any of the diploid species. No triploids have been found in Central European material of *N. nigra* despite of extensive chromosome counts (TEPPNER & KLEIN 1990, 1993). A possible explanation for the present distribution patterns is that the triploid survived the last glaciation in Central European refugia, followed the retreating ice towards the north, and became extinct from the more southern area (cf. TEPPNER & KLEIN 1990: 23–24). Alternatively, the triploid has been isolated from the Central European members of *Nigritella* for a longer period of time and survived the Weichselian glaciation close to the Northern European ice sheet. It is probable, therefore, that the different subspecies of *N. nigra* date back at least to the Postglacial, possibly even much longer given the wide present-day distribution areas of tetraploid *N. nigra* in Central and Western Europe.

Nigritella widderi and *N. archiducis-joannis* (multilocus genotypes F & G) may also be old taxa, which agrees with the relictual character of their present-day distribution areas. These tetraploids contain the alleles Skd^a and $Mdh1^a$, which have not been found in the diploids, and no triploid possible ancestor containing these alleles is known. The genetic composition of *N. buschmanniae* could be explained by an addition of multilocus genotype F to a haploid set of chromosomes containing alleles that are common in present-day diploids, and it could accordingly be of relatively recent origin. TEPPNER & STER 1996 regarded *N. buschmanniae* as morphologically close to *N. widderi* and this observation is in agreement with our data.

Nigritella miniata and *N. stiriaca* (multilocus genotypes E & H) could also be comparatively old. We explain the close similarity of these multilocus genotypes by that they evolved from a common triploid ancestor. No such triploid has been found. Furthermore, this triploid must have contained a number of alleles that are rare in present-day diploids and it is likely that it evolved from diploids with different allele frequencies from those that have been investigated here.

The extant diploid members of *Nigritella* have relatively low levels of genetic variation and are poorly separated from each other in allozymes. This pattern indicate that they may be relatively young and that they have separated from each other relatively recently. If the group of tetraploids were old, more variation in the group would be expected, and if the low levels of variation within single taxa was a result of bottleneck effects related to Pleistocene glaciations, it would be expected that different diploid

species were fixed for different alleles at several loci. However, the present-day distribution areas of the diploids, except for *N. rhellicani* which is more widespread, are also restricted to relict areas, just as in the polyploids. We cannot explain this discrepancy, but it is seems that although the diploids may have evolved before the last ice age, they are nevertheless younger than some of the extant polyploids.

4.6. Autopolyploidy versus Allopolyploidy

DELFORGE 1995 speculated that *Nigritella miniata* (as *N. rubra*) is an autotetraploid derived from *N. rhellicani*. Furthermore, in DELFORGE 1998 it was suggested that *Nigritella nigra* subsp. *nigra* and *N. nigra* subsp. *austriaca* were derived independently from *N. rhellicani* as autopolyploids. Similarly, ERICSSON 1998 proposed that *Gymnigritella runei* should have evolved by autopolyploidy from unknown diploid ancestors of *Nigritella*.

However, from karyological data it could be concluded that it is highly unlikely that any of the tetraploids mentioned is an autotetraploid (TEPPNER & KLEIN 1998: 221). The allozyme patterns agree with this view, and rather indicate that the polyploids are derived by hybridization between genetically divergent genomes.

Autopolyploids are characterised by having multiple sets of homologous chromosomes in their genomes (STEBBINS 1980). In sexual taxa, any chromosome may pair with any of its homologous counterparts during meiosis, and at variable loci, any combination of alleles may appear in the offspring. In contrast, allopolyploids are composed of more or less divergent genomes between which recombination is rare. If the constituent genomes have different alleles at a locus, the offspring would normally be characterised by fixed heterozygosity, and this heterozygosity would be transferred to subsequent generations as well.

Because polyploid members of *Nigritella* set seed asexually, the offspring would normally be identical to the parent, and the genome composition of the polyploid would not be possible to reveal from variation patterns seen at allozyme loci. If inheritance patterns are used to differentiate between the two types of polyploids, then it would be in a way meaningless to discuss whether an apomict is an allo- or an autopolyploid. However, the question has bearing on the origin of polyploid species.

Several lines of evidence suggest that polyploid *Nigritella* species include divergent genomes and that they have not originated by autopolyploidization. The genome composition of the tetraploid members of *Nigritella* could most parsimoniously be explained by invoking triploid forms that have contributed unreduced gametes. These triploids should have been quite different from the diploids that contributed the reduced gamete to the formation of the tetraploid.

The triploid that gave rise to multilocus genotypes E and H (characterising *N. miniata* and *N. stiriaca*) must have contained a number of alleles that are rare in present-day diploids, but these alleles are present in equal doses in the two tetraploid genotypes. If these tetraploids evolved by autotetraploidization, it is also possible that they would have been formed by intermediate triploid stages, but, if so, we would expect that the alleles that are present in one copy each in the tetraploids and that are rare in the present-day diploids, would have been common in the diploids, and we would have seen varying numbers of these alleles in the tetraploid derivatives. The same reasoning holds for the multilocus genotypes G and F (characterising *widderi* and *archiducis-joannis*), but there are fewer heterozygous loci to consider in this case.

It seems very likely that a plant nearly or fully identical to the triploid *N. nigra* subsp. *nigra* contributed to the two tetraploid subspecies of *N. nigra*. However, it is unlikely that *N. nigra* subsp. *nigra* is directly derived from *N. rhellicani* (as supposed in DELFORGE 1998), considering the allele $Tpi1^c$, which we did not find in the latter, despite a large material investigated.

As already indicated, we do not consider *Gymnigritella runei* as an autotetraploid because it combines perfectly the full genome of *N. nigra* subsp. *nigra* and a haploid genome of *Gymnadenia conopsea*, and these taxa are obviously quite divergent (HEDRÉN 1999).

Most gametophytic apomicts are polyploids that have arisen by hybridization between sexual parents (ASKER & JERLING 1992), and they could accordingly be described as allopolyploid in origin. Few examples of groups with adventitious embryony have been studied, but it seems that apomictic members of *Nigritella* also agree with this pattern.

4.7. Inbreeding and Apomixis in Polyploid Species

The evolutionary potentials of polyploid apomicts and polyploid inbreeders are often regarded as being similar, due to uniparental reproduction and low variation in the offspring. However, there are further similarities, but also fundamental differences between these two categories of polyploids that could be examplified by polyploid members of *Nigritella*.

(i) As in most polyploids, polyploid members of *Nigritella* are the results of hybridization between genetically divergent genomes, allopolyploids. Such polyploids are likely to be different from any parental taxon in their habitat requirements, and their establishment would be dependent on whether they are preadapted to available habitats in which they are not experiencing competition from their parents (cf. DE WET 1980, THOMPSON & LUMARET 1992). Because of their uniparental reproduction, inbreeders, as

well as apomictic polyploids would be able to establish new populations from single diaspores.

(ii) Considering that transfer of genes from diploid to polyploid level is restricted at any polyploidization event, polyploids have often been seen as dead ends with little evolutionary potential (WAGNER 1970). However, recent studies using molecular markers have shown that polyploid taxa generally are derived by multiple polyploidization events, and that the amount of genetic variation within polyploid taxa is relatively high, often comparable to that of their diploid ancestors (SOLTIS & SOLTIS 1993). For example, our data indicate that there may be at least two independent origins in each of *N. widderi* and *N. miniata*. The high degree of genetic variation at the polyploid level has been taken as argument that polyploids are capable of adaptation to changed conditions of various kinds and that they would not be evolutionary dead ends in this respect (e.g., COOK & al. 1998). However, the argument does only apply to polyploids that are sexual and outcrossing to some degree. In inbreeding and apomictic polyploids, the species is a collection of independent evolutionary lineages rather than a single evolutionary unit. In the long run some of these lineages may be successful, but the majority are likely to go extinct, just as commented by WAGNER 1970.

(iii) Polyploid inbreeders retain high degrees of heterozygosity as fixed heterozygosity, if the constituent genomes are divergent enough to prevent recombination of the different parental genomes (allopolyploidy). In contrast, polyploid apomicts retain heterozygosity because of lack of recombination (ASKER & JERLING 1992, RICHARDS 1997), in which case the degree of differentiation of the parental genomes does not matter.

(iv) An inbreeding allopolyploid must (at least in the case of allotetraploidy) contain a balanced genome, and at heterozygous loci there must be equal expression of alleles from the different parental genomes. Polyploid apomicts may have unbalanced genomes as examplified in *Nigritella*. This enables the existence of taxa at odd ploidy levels, such as the triploid *N. nigra* subsp. *nigra* or the pentaploid *N. buschmanniae*, in which the entire genomes may be unbalanced in the case they were derived by allopolyploidization. Unbalanced genomes may also be found in tetraploids, e.g., in *Gymnigritella runei*, containing the entire triploid genome of *Nigritella nigra* subsp. *nigra* and the haploid genome of *Gymnadenia conopsea*. Both HANSSON 1992 and ERICSSON 1998 have commented that *Gymnigritella* is morphologically remarkably similar to a typical member of *Nigritella*, thus suggesting that the quantitative composition of the genome is reflected in the phenotype. *Nigritella nigra* subsp. *nigra* appears in itself to be an allopolyploid, and it is even possible that *Gymnigritella* contain three different genomes. Finally, apomicts may retain unbalanced heterozygosity at individual loci, which is seen in most tetraploid multi-

locus genotypes in *Nigritella*. In conclusion, apomictic reproduction may widen the possibilities for variation within a genus at polyploid levels, allowing for establishment in niches not available to sexual relatives.

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