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Polyploid evolution in the European orchid genus *Nigritella*: evidence from DNA fingerprinting

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Masters thesis, May 1999
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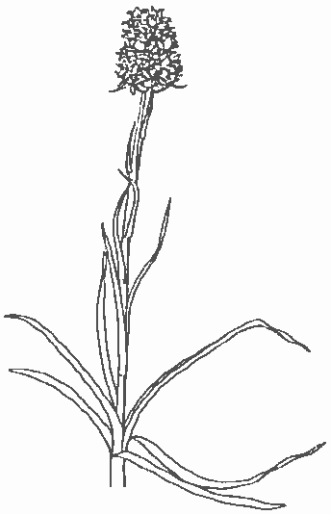

ABSTRACT

The genus *Nigritella* (Orchidaceae-Orchidinae) is a complex of species at four different ploidy levels. Fourteen taxa at species or subspecies level have been distinguished. The triploid *N. nigra* ssp. *nigra* is endemic to Scandinavia, whereas the remaining taxa are confined to different mountain regions in Central and Southern Europe. The polyploids are of hybrid origin and have arisen from taxa at lower ploidy levels by allopolyploidization. By using a recently developed PCR based DNA fingerprinting technique (AFLP), I have investigated the evolutionary history of the genus. I have also considered the genetic relationship between *Nigritella* and the closely related genus *Gymnadenia*.

Tetraploid members of *Nigritella* are supposed to be the result of hybridization between diploids and triploids. The latter may have contributed unreduced gametes. Three different ancient triploid taxa would explain the origin of all present-day tetraploids. *Nigritella nigra* ssp. *nigra* and the other – today probably extinct – triploids may have survived the last glacial maximum in different Central and Southern European refugia. They may then have mixed with populations of diploids, thereby giving rise to new, tetraploid taxa.

All the polyploids displayed several DNA bands that were rare or absent in the extant diploids. It is therefore reasonable to assume that ancestral diploids, somewhat different from present-day taxa, have been involved – at least in the formation of the intermediate triploid forms.

The diploid samples only partly clustered in accordance with morphologically defined entities. Plants with the same geographic origin tended to cluster together, regardless of taxonomic belonging.

The polyploids are mainly apomicts, but in spite of this fact, certain taxa varied in a complex way. This pattern could partly be explained by recurrent polyploidization, by which a given taxon may have several independent origins. The number of origins was estimated for different taxa, and varied between one and seven.

It is not necessary to amalgamate the genera *Nigritella* and *Gymnadenia*. They were well separated from each other. Bigeneric hybrids clustered in between, and the origin of *Gymnigritella runei* was confirmed: this rare Scandinavian tetraploid is most likely the result of hybridization between *N. nigra* ssp. *nigra* and *G. conopsea*.

INTRODUCTION

Polyploidy

Polyploidy, the occurrence of more than two chromosome sets per nucleus, is central to our understanding of evolution and biodiversity. Since the discovery of the phenomenon one century ago, it has proved to be a very common feature among plants (reviewed in Briggs and Walters 1997). It is well-known that ferns have high chromosome numbers. Among angiosperms, it is estimated that 30–70% of all species may be of polyploid origin. Conservative estimates only include functional polyploids, i.e. species that have close relatives at a lower ploidy level (Stebbins 1971). In contrast, Grant (1981) and Masterson (1994) also considered ancient polyploids. Grant assumed that all species with chromosome numbers in excess of n = 13 (~ 50%) have had a history of polyploidy, whereas Masterson argued that n = 7 to 9 is the primitive haploid chromosome number. Ancient polyploids are generally considered as functional diploids. Given sufficient time, polyploid genomes may become diploidized. Redundant gene copies may be silenced, or evolve into new functions (Soltis and Soltis 1993, Leitch and Bennett 1997).

Polyploidy calls attention to a mode of speciation that is distinct from the traditional Darwinian concept. Instead of speciation by means of slow, gradual evolution within divergent lineages, new species are formed abruptly by hybridization (Grant 1981). This type of evolution causes a reticulate pattern in which divergent lineages are linked together. Allopolyploid derivatives are formed by combination of taxa at lower ploidy levels. Polyploidization permits normal meiosis and reproduction of the hybrids, and prevents backcrossing with parental taxa. It is thus a way of reproductive isolation (Grant 1981). The combination of characteristics from different species may enable the new allopolyploid species to colonize habitats inaccessible to the parental species (Thompson and Lumaret 1992). Brochmann and Elven (1992) found an intriguing pattern of niche separation among Arctic *Draba* at different ploidy levels. In several taxonomic groups, reticulate speciation has generated complexes of polyploid taxa. Of particular interest for the present study, is the finding that endemic taxa in previously glaciated areas to a great extent belong to polyploid complexes (Brochmann et al. 1992a, 1992b and 1996, Borgen 1997).

In general, fusion of unreduced gametes seems to be of supreme importance as a mechanism generating polyploids, even if somatic chromosome doubling is a possibility as well (Briggs and Walters 1997). It is reasonable to suppose that there is a certain low frequency of 2n gametes in a diploid population (Grant 1981). Studies on diploid *Dactylis glomerata*, for example, have shown that on average 1% of the pollen grains produced are unreduced (Maceira et al. 1992). The corresponding value for 2n eggs is about 0.5% (De Haan et al. 1992). Regarding orchids, already Hagerup (1947) noticed the occurrence of unreduced gametes in some European species. The probability for random fusion of one unreduced gamete with one normal, haploid gamete should be significantly higher than fusion of two unreduced gametes. Recurrent formation of triploids may thus be a characteristic element in diploid populations, even if the frequency is extremely low. In a study of different cultivars of diploid *Hordeum vulgare*, Sandfaer (1970) estimated the frequency of triploid seedlings to be less than 3%. It is reasonable to assume that tri-

ploids act as evolutionary links between diploid and tetraploid taxa. It has been demonstrated that the tetraploid species*Galeopsis t tetrahit* probably could be derived from the diploid species *G. pubescens* and *G. speciosa*, via an intermediate triploid form (Münzing 1960).

During the last decades, molecular techniques have provided a lot of new insights into polyploid evolution (Thompson and Lumaret 1992, Soltis and Soltis 1993). The evolutionary potential of a polyploid complex is greater than has been assumed previously. Polyploid taxa are not genetically isolated and uniform. Variation can be introduced in different ways. There is a number of studies showing that polyploid taxa usually have several independent origins (references in Soltis and Soltis 1993). It has further been indicated that gene flow may act between taxa in a polyploid complex, even between different ploidy levels (Brochmann et al. 1992c).

Nigritella

Taxa within *Nigritella* (Orchidaceae-Orchidinae) constitute a polyploid complex. According to Teppner and Klein (1998), there are at least fourteen taxa at species or subspecies level: five diploids, one triploid, seven tetraploids, and one pentaploid (Table 1). All taxa are restricted to European mountain regions (Fig. 1). Hybridization with diploid members of the closely related genus *Gymnadenia* is common. Like most orchids in the European

Table 1. Taxa of *Nigritella*, *Gymnadenia* and hybrids. Taxonomic delimitations within *Nigritella* correspond to Teppner and Klein (1998), but *Nigritella* is treated as a genus separated from *Gymnadenia* in accordance with the results of the present investigation. *Gymnigritella runei* is a tetraploid apomict and is considered as a separate species, in contrast to other hybrids which are temporary and sterile. * = Taxa not included in the present study.

Diploids	Nigritella	Polyploids
N. carpatica (Zapalowicz) Teppner, Klein & Zagulskij*		N. nigra (L) Rchb. f.
N. corneliana (Beauverd) Götz & Reinhard		ssp. nigra (3x)
N. gabasiana Teppner & Klein		ssp. austriaca Teppner & Klein (4x)
N. lithopolitanica Ravnik		ssp. iberica Teppner & Klein (4x)
N. rhellicani Teppner & Klein		N. miniata (Cranz) Janchen (4x)
		N. widderi Teppner & Klein (4x)
		N. buschmanniae Teppner & Ster (5x)*
		N. dolomitensis Teppner & Klein (4x)*
		N. stiriaca (K. Rechinger) Teppner & Klein (4x)
		N. archiducis-joannis Teppner & Klein (4x)

Gymnadenia
G. conopsea (L.) R.Brown
G. odoratissima (L.) L.C.M. Richard

Hybrids	
N. x wettsteiniana (Abel) Ascher & Gräbn*	(N. rhellicani x N. miniata)
(x)Gymnigritella runei Teppner & Klein	(N. nigra ssp. nigra x G. conopsea)
xG. suaveolens (Villars) E.G. Camus	(N. rhellicani x G. conopsea)
xG. truongae (Demares)*	(N. corneliana x G. conopsea)
xG. turnowskyi (W.Foelsche)*	(N. lithopolitanica x G. conopsea)
xG. godferyana (Wettst.) K.Richter*	(N. miniata x G. conopsea)
xG. heufleri (A.Kern) E.G. Camus	(N. rhellicani x G. odoratissima)
xG. albelii (Hayek) Ascher & Gräbn*	(N. miniata x G. odoratissima)

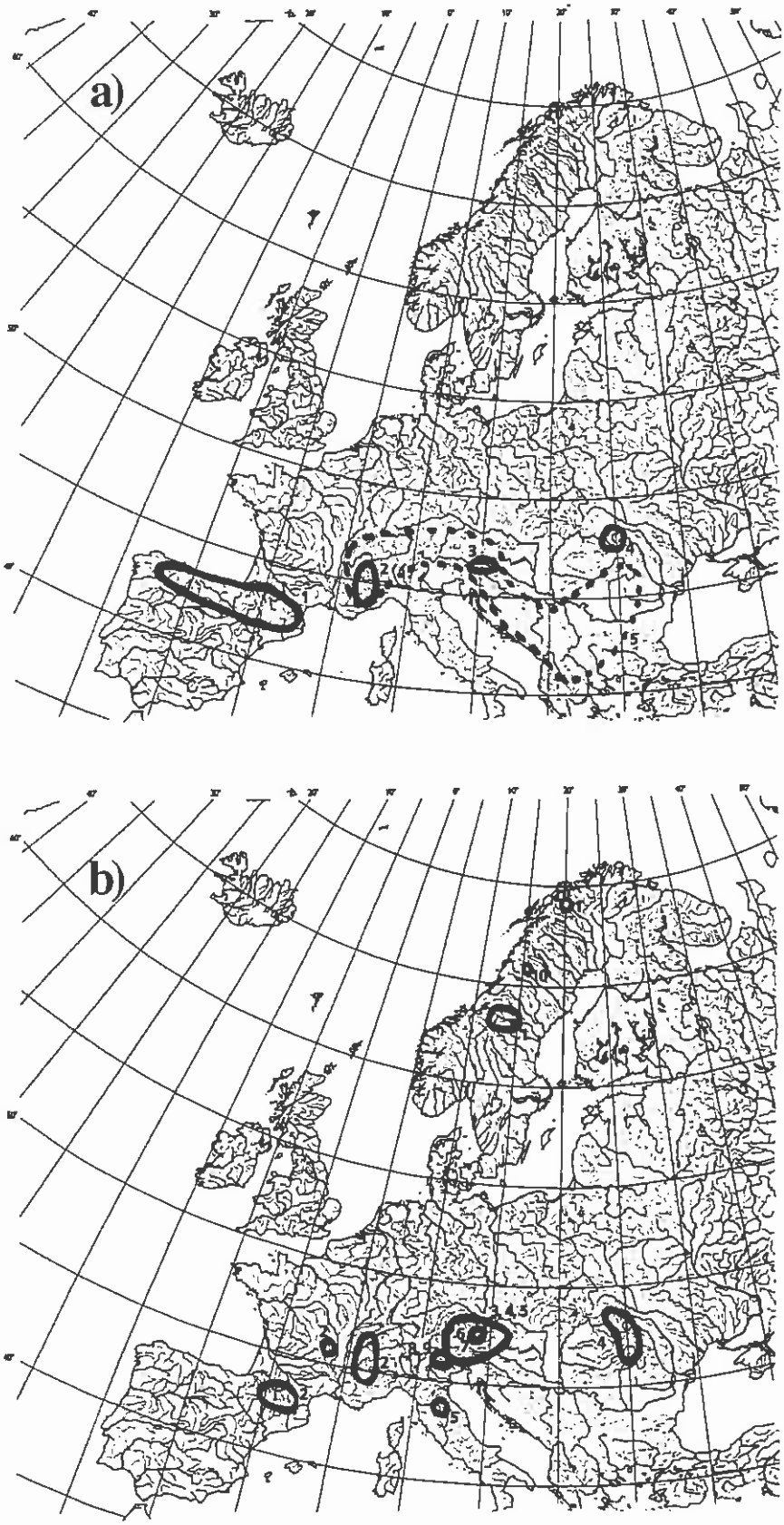

Fig. 1. Distribution of *Nigritella* and *Gymnigritella runei*.
a) Diploid taxa: 1 = *N. gabasiana*, 2 = *N. corneliana*, 3 = *N. lithopolitanica*, 4 = *N. carpatica*, 5 = *N. rhellicani*.
b) Polyploid taxa: 1 = *N. nigra* ssp. *nigra*, 2 = *N. nigra* ssp. *iberica*, 3 = *N. nigra* ssp. *austriaca*, 4 = *N. miniata*, 5 = *N. widderi*, 6 = *N. archiducis-joannis*, 7 = *N. stiriaca*, 8 = *N. buschmanniae*, 9 = *N. dolomitensis*, 10 = *Gymnigritella runei*.

flora, both *Nigritella* and *Gymnadenia* have a diploid chromosome number of 2n = 40, indicating a history of ancient polyploidization.

A polyploid complex is often associated with vexing taxonomic problems. *Nigritella* is no exception. The close relationship with *Gymnadenia*, and the occurrence of hybrids are further reasons for debate. Until the end of the last century only one species, *N. nigra*, was recognized. This single species was treated in different ways and it was transferred between at least five genera (Foelsche 1993). Since the separation of the genus *Nigritella* during the last century, several taxonomists have focused on the intrageneric variation, and new species have continuously been distinguished. The discovery of cytological discrepancy among *Nigritella* from different geographic areas, has been challenging (Afzelius 1928 and 1932). During the last decades, several more detailed investigations have been performed on taxonomic delimitations within the genus (Teppner and Klein 1985a,b, 1990, 1993, 1998, Teppner and Ster 1996, Teppner et al. 1994, Teppner 1996, Rossi et al. 1987, Klein and Drescher 1996). Also, recent phylogenetic studies based on DNA sequencing (the ITS-region of nuclear rDNA), have suggested that *Nigritella* should be included in *Gymnadenia* (Pridgeon et al. 1997 and Bateman et al. 1997).

Due to irregular meiotic chromosome pairing, polyploidy often results in a reduced ability to reproduce sexually (Grant 1981). Polyploid *Nigritella* are apomicts. Seeds are formed by agamospermy, a feature not demonstrated in any other European orchid genus (Delforge 1995). However, some pollen formation is normal, and crossing experiments have suggested that polyploids occasionally may reproduce sexually (Teppner 1996, Deutsch 1998). In agamospermous *Nigritella*, development of seeds from particular embryo cells in nucellus (adventitious embryony), begins already in the plant´s bud stage (Teppner 1996). This strategy is obviously adventageous for alpine plants occasionally exposed to extreme weather conditions and scarcity of pollinators. Intergeneric hybrids with *Gymnadenia* are usually temporary and sterile, but interestingly, a tetraploid apomict (*Gymnigritella runei*) is found in Scandinavia (Teppner and Klein 1989, Rune 1993).

Even if only one example of a triploid *Nigritella* individual in an otherwise diploid population has been reported hitherto (Teppner et al. 1994), it has been assumed that formation of triploids may constitute intermediate stages in the evolution of tetraploid *Nigritella* taxa (Teppner 1996, Hedrén et al. in manuscript). Once formed, a triploid stage must not necessarily be temporary, but may well be as stable and prolonged as the tetraploid stage, if reproducing apomictically. The Scandinavian *Nigritella* (*N. nigra* ssp. *nigra*) is an illuminating example. Even if triploids usually have to rely on vegetative propagation, a low percentage of viable gametes may be formed. Triploid pollen are probably regulary formed in *N. nigra* ssp. *nigra*. Fusion with normal reduced egg cells through backcrossing, or crossing with other diploid taxa, could then give rise to new tetraploid taxa. Allozyme data (Hedrén et al. in manuscript) have clearly indicated that *N. nigra* ssp. *nigra* has contributed to the formation of the tetraploids *N. nigra* ssp. *austriaca* and *N. nigra* ssp. *iberica*, and if another two triploid ancestors are hypothesized, the origin of remaining tetraploid *Nigritella* taxa could be explained.

In the present study, I have considered the new insights concerning polyploid evolution as presented above. By means of molecular tools, my intension has been to bring light on the evolutionary patterns within *Nigritella*. Recently, Hedrén et al. (in manuscript) studied *Nigritella* by means of allozymes, and

a model of evolutionary pathways within the genus was proposed. The idea of Delforge (1995) that polyploid *Nigritella* might be autopolyploids could be rejected – the constituent genomes in the polyploids were obviously derived from divergent taxa. The allozyme data also indicated that polyploid taxa have arisen recurrently. However, the genetic variation provided by allozymes was limited and the analysis had low resolution. Therefore, I wanted to test if a DNA fingerprinting method, amplified fragment length polymorphism (AFLP), could provide further information. Recently, this technique was used successfully in a study of polyploid evolution in the Eurasian orchid genus *Dactylorhiza* (Hedrén, Fay, and Chase in manuscript).

Aims of this study

I addressed the following specific questions:

1) Evolutionary pathways between diploids and polyploids: How are polyploid taxa related to the diploids? Is it necessary to invoke hypothetical diploid ancestors to explain the genomic constitutions of the polyploids? Do the AFLP data give support to the suggestion of Hedrén et al. (in manuscript) that triploids represent intermediate stages between diploids and tetraploids?

2) Multiple origins of polyploid taxa: Have polyploid taxa arisen repeatedly? How common is polyploidization?

3) The history of *Nigritella*: How old is the polyploid complex? What influence have geography and distribution patterns had on the origin of new taxa?

4) Taxonomic implications: How should taxa within the *Nigritella* complex be treated? Is it necessary to transfer *Nigritella* to *Gymnadenia*?

5) Finally, I will examine the origin of *Gymnigritella runei*, since its bigeneric origin recently has been questioned (Ericsson 1997).

MATERIAL AND METHODS

The species

Relevant taxa of *Nigritella*, *Gymnadenia* and hybrids are compiled in Table 1.

Nigritella is characterized by long, digitate stolons, linear leaves and small and vanilla scented flowers with a short, thick spur and a non-resupinate ovary. Flower colour vary from pale pink to dark reddish brown. Of the diploids, *N. rhellicani*, *N. gabasiana* and *N. carpatica* have brownish flowers, whereas *N. lithopolitanica* and *N. corneliana* have pinkish flowers. Of the polyploids, the flowers of the three subspecies of *N. nigra* are brownish. *Nigritella miniata*, *N. dolomitensis*, and *N. buschmanniae* have red flowers. The flowers of *N. archiducis-joannis*, *N. stiriaca*, and *N. widderi* are bright-coloured. Apart from flower colours, different species and subspecies are distinguished by more or less subtle floral characters (Delforge 1995, Klein and Kerschbaumsteiner 1996). Sometimes cytological data is needed for certain determination (Teppner and Klein 1990). The closely related genus *Gymnadenia* is distinguished by a long, thin spur and a resupinate ovary. Hybrids between the genera have intermediate characters (Delforge 1995).

The triploid *N. nigra* ssp. *nigra* is endemic to Scandinavia. The remaining taxa of *Nigritella* are confined to different mountain regions of Central and Southern Europe (Fig. 1). Widespread taxa as well as taxa with restricted distribution areas are found both among diploids and polyploids. *Gymnadenia* is spread all over the Eurasian continent, and is not restricted to alpine habitats. It contains more variable taxa than *Nigritella*. A dozen species have been described, two of them (*G. conopsea* and *G. odoratissima*) almost completely overlapping the distribution range of *Nigritella*. *Gymnigritella runei* is restricted to Scandinavia, whereas a number of different temporary, bigeneric hybrids are found in the European mainland.

There are no major differences among the taxa of *Nigritella* concerning preference of habitat. They are all found on calcareous, dry grasslands (Delforge 1995). Yet, it could be noted that *N. nigra* ssp. *nigra* preferably grow on subalpine meadows dependent on grazing and cutting. Due to changed land use, a lot of suitable meadows have disappeared during this century (Stenar 1947, Björkbäck et al. 1976). Today, *N. nigra* ssp. *nigra* is endangered, and is the object of intensive conservation efforts (Björkbäck and Lundqvist 1982). The Central and Southern European taxa are to a higher degree also found on natural, alpine grasslands. *Gymnadenia* has a wider ecological amplitude, which covers that of *Nigritella*.

Sampling

Most of the plant material was collected during the summer of 1998. I tried to get samples representing as much as possible of the whole distribution range of *Nigritella*. Collegues in different parts of Europe were of great help. When selecting samples, the data on allozyme variation in Hedrén et al. (in manuscript) were guiding. The aim was to get representatives from all allozyme multilocus genotypes detected in that study. More samples were collected from diploid taxa, as they were more variable, and because it was supposed that polyploids had evolved from them. A few samples deviated somewhat from described taxa and were collected for that reason. Some samples of *Gymnadenia* and hy-

Table 2. Origin of the plant material studied. 'Code' refers to code numbers used in Figs. 3-4. Allozyme multilocus genotypes according to Hedrén et al. (in manuscript) are noted for certain samples.

Code	Taxon	Country	Province	Locality	Comments	Date	Collector
a-j087	N. archiducis-joannis	Austria	Styria	Totes Gebirge, Tauplitzalm, Lawinenstein, 1960m	multil. genotype F	6 July 1998	D. Ståhlberg & E. Klein
a-j141	N. archiducis-joannis	Austria	Styria	Totes Gebirge, Tauplitzalm, Lawinenstein, 1960m	multil. genotype F	7 July 1998	K. Redl
aus049	N. nigra ssp. austriaca	Austria	Styria	Hochschwab, Aflenzer-Bürgeralm, 1450m		5 July1998	D. Ståhlberg & E. Klein
aus152	N. nigra ssp. austriaca	Austria	Upper Austria	Schafberg 1660m		30 June 1998	K. Redl
aus153	N. nigra ssp. austriaca	Austria	Eastern Tyrol	Hinteregger-Alm, Matrei, 1520m	really aus?	6 July 1998	K. Redl
aus154	N. nigra ssp. austriaca	Germany	Bavaria	Chiemgauer Berge, Feichtenalm, 1485m		28 June 1998	K. Redl
con005	G. conopsea	Sweden	Medelpad	Ånge, Granboda		6 July 1998	M. Hedrén
con111	G. conopsea	Austria	Carinthia	Heiligenbach-Alm, 1930m		10 July 1998	D. Ståhlberg & E. Klein
con112	G. conopsea	Austria	Carinthia	Heiligenbach-Alm, 1930m		10 July 1998	D. Ståhlberg & E. Klein
con115	G. conopsea	Austria	Carinthia	Heiligenbach-Alm, 1930m		10 July 1998	D. Ståhlberg & E. Klein
con165	G. conopsea	Switzerland	Uri	Furkapass		23 July 1998	W. Foelsche
con166	G. conopsea	France	Savoie	Roselend		23 July 1998	W. Foelsche
con194	G. conopsea	Sweden	Gotland	Jacobsberg		11 Aug. 1998	M. Hedrén
con200	G. conopsea	Sweden	Gotland	Lärbro, Hoburgsmyr		8 Aug. 1998	M. Hedrén
con201	G. conopsea	Sweden	Gotland	Lärbro, Hoburgsmyr		8 Aug. 1998	M. Hedrén
cor169	N. corneliana	France	Isère	Massif de la Chartreuse, Col de l´Alpe, 1790m		28 June 1998	D. Gerbaud
cor170	N. corneliana	France	Isère	Massif de la Chartreuse, Col de l´Alpe, 1790m		28 June 1998	D. Gerbaud
cor175	N. corneliana	France	Savoie	Croix de Fer		5 July 1998	D. Gerbaud
cor176	N. corneliana	France	Savoie	Croix de Fer		5 July 1998	D. Gerbaud
cor179	N. corneliana	France	Savoie	Mont Cenis		5 July 1998	D. Gerbaud
cor180	N. corneliana	France	Savoie	Mont Cenis		5 July 1998	D. Gerbaud
gab102	N. gabasiana	Spain	Huesca	Formigal		17 June 1998	M. Lewin
gab109	N. gabasiana	Spain	Huesca	Formigal		17 June 1998	M. Lewin
gab185	N. gabasiana	France	Pyrénées Orientales	Porté Puymorens, 2000m		11 July 1998	M. Lewin
gab186	N. gabasiana	France	Pyrénées Orientales	Porté Puymorens, 2000m		11 July 1998	M. Lewin
heu025	x G-n. heufleri	Austria	Carinthia	Heiligenbach-Alm, 1930m		10 July 1998	D. Ståhlberg & E. Klein
ibe039	N. nigra ssp. iberica	France	Cantal	Massif Central, Puy de Prat de Bouc, 1520m	extr. of leaves	29 June 1995	E. Klein
ibe167	N. nigra ssp. iberica	France	Isère	Mont de Lans		14 June 1998	D. Gerbaud
ibe168	N. nigra ssp. iberica	France	Savoie	Croix de Nivolet		21 June 1998	D. Gerbaud
ibe182	N. nigra ssp. iberica	France	Pyrénées-Orientales	Porté Puymorens		11 July 1998	M. Lewin
ibe188	N. nigra ssp. iberica	France	Pyrénées-Orientales	Fontrabiouse (Val de Galle)		13 July 1998	M. Lewin
lit083	N. lithopolitanica	Austria	Carinthia	Petzen, 2050m		9 July 1998	D. Ståhlberg & E. Klein
lit084	N. lithopolitanica	Austria	Carinthia	Petzen, 2050m		9 July 1998	D. Ståhlberg & E. Klein
lit086	N. lithopolitanica	Austria	Carinthia	Petzen, 2050m		9 July 1998	D. Ståhlberg & E. Klein
min068	N. miniata	Austria	Styria	Teichalm, 1450m	multil. genotype H	30 June 1998	E. Klein
min071	N. miniata	Austria	Styria	Teichalm,1450m	multil. genotype H	30 June 1998	E. Klein
min072	N. miniata	Austria	Styria	Hochschwab, Aflenzer-Bürgeralm, 1450m	multil. genotype E	5 July 1998	D. Ståhlberg & E. Klein
min080	N. miniata	Austria	Styria	Schöckel, 1400m	E; "widderi-colour"	28 June 1998	E. Klein
min135	N. miniata	Germany	Bavaria	Chiemgauer Berge, Brandlberg-Sattel, 1410m		28 June 1998	K. Redl
min136	N. miniata	Austria	Eastern Tyrol	Hinteregger-Alm, Matrei, 1520m		25 June 1998	K. Redl
min138	N. miniata	Austria	Styria	Ardninger-Alm, Stubenschlag, 1240m		22 June 1998	K. Redl

Table 2. (continued)

Code	Taxon	Country	Province	Locality	Comments	Date	Collector
min140	N. miniata	Austria	Styria	Totes Gebirge, Tauplitzalm, Lawinenstein, 1960m	multil. genotype E	7 July 1998	K. Redl
nig003	N. nigra ssp. nigra	Sweden	Härjedalen	Klinken		9 July 1998	M. Hedrén
nig007	N. nigra ssp. nigra	Sweden	Jämtland	Oviken		8 July 1998	M. Hedrén
odo126	G. odoratissima	Austria	Carinthia	Heiligenbach-Alm, 1930m		10 July 1998	D. Ståhlberg & E. Klein
odo195	G. odoratissima	Sweden	Gotland	Lojsthöjd		5 Aug. 1998	M. Hedrén
rhe015	N. rhellicani	Austria	Carinthia	Heiligenbach-Alm, 1930m		10 July 1998	D. Ståhlberg & E. Klein
rhe016	N. rhellicani	Austria	Carinthia	Heiligenbach-Alm, 1930m		10 July 1998	D. Ståhlberg & E. Klein
rhe017	N. rhellicani	Austria	Carinthia	Heiligenbach-Alm, 1930m		10 July 1998	D. Ståhlberg & E. Klein
rhe089	N. rhellicani	Austria	Carinthia	Dobratsch, 1800m		9 July 1998	D. Ståhlberg & E. Klein
rhe092	N. rhellicani	Austria	Styria	Altes Almhaus, 1800m		7 July 1998	D. Ståhlberg & E. Klein
rhe097	N. rhellicani	Austria	Styria	Altes Almhaus, 1800m		7 July 1998	D. Ståhlberg & E. Klein
rhe106	N. rhellicani	Austria	Styria	Gleinalpe, Sattelhaus, 1410m		7 July 1998	D. Ståhlberg & E. Klein
rhe107	N. rhellicani	Austria	Carinthia	Dobratsch, 1800m		9 July 1998	D. Ståhlberg & E. Klein
rhe142	N. rhellicani	Austria	Eastern Tirol	Kals, Lucknerhütte, 2090m		6 July 1998	K. Redl
rhe144	N. rhellicani	Austria	Carinthia	Nockalm, Grünleitenrock, 1900m		12 July 1998	K. Redl
rhe146	N. rhellicani	Austria	Carinthia	Astental,Rudnigalm, 2000		13 June 1998	K. Redl
rhe148	N. rhellicani	Austria	Salzburg	Trattberg bei Kuchl, 1560m		7 July 1998	K. Redl
rhe156	N. rhellicani	Switzerland	Uri	Oberalppass, 1800m		23 July 1998	W. Foelsche
rhe157	N. rhellicani	Switzerland	Uri	Furkapass Est, 1880m		23 July 1998	W. Foelsche
rhe158	N. rhellicani	Switzerland	Bern	Grimselpass, 2100m		24 July 1998	W. Foelsche
rhe159	N. rhellicani	France	Savoie	Col du Petit St Bernard, 2188m		28 July 1998	W. Foelsche
rhe160	N. rhellicani	Italy	Aosta	Grosser St. Bernard		29 July 1998	W. Foelsche
rhe161	N. rhellicani	France	Savoie	Val Thorens	late flowering	26 July 1998	W. Foelsche
rhe163	N. rhellicani	France	Savoie	Col du Petit St. Bernard, Chanovisia	late flowering	28 July 1998	W. Foelsche
rhe164	N. rhellicani	Italy	Aosta	Colle del Nivolet, Gran Paradiso	late flowering	29 July 1998	W. Foelsche
rhe171	N. rhellicani	France	Isère	Dauphine´Alpen, Chaine de Belledone, le Collet d'Allevard, 1800m		1 July 1998	D. Gerbaud
rhe173	N. rhellicani	France	Savoie	Croix de Fer		5 July 1998	D. Gerbaud
rhe177	N. rhellicani	France	Savoie	Mont Cenis, 2100m	late flowering	5 July 1998	D. Gerbaud
rhe178	N. rhellicani	France	Savoie	Mont Cenis, 2100m	late flowering	5 July 1998	D. Gerbaud
rhe181	N. rhellicani	Yugoslavia	Montenegro	Durmitor, Zabljak, 1500m	extr. of leaves	1 July 1988	K-A Olsson
run012	G-n. runei	Sweden	Åsele Lappmark	Ransaren, 600m		4 Aug. 1997	S. Ericsson
sti081	N. stiriaca	Austria	Styria	Teichalm, 1450m	multil. genotype E	20 June 1998	E. Klein
sti131	N. stiriaca	Austria	Salzburg	Schafberg, 1640m	multil. genotype E	30 June 1998	K. Redl
sua030	x G-n. suaveolens	Austria	Carinthia	Heiligenbach-Alm, 1930m		10 July 1998	D. Ståhlberg & E. Klein
sua124	x G-n. suaveolens	Austria	Carinthia	Heiligenbach-Alm, 1930m		10 July 1998	D. Ståhlberg & E. Klein
wid041	N. widderi	Austria	Styria	Teichalm, 1450m	multil. genotype G	20 June 1998	E. Klein
wid044	N. widderi	Austria	Styria	Hochschwab, Aflenzer-Bürgeralm, 1450m	multil. genotype G	5 July 1998	D. Ståhlberg & E. Klein
wid047	N. widderi	Austria	Styria	Hochschwab, Aflenzer-Bürgeralm, 1450m	multil. genotype F	5 July 1998	D. Ståhlberg & E. Klein
wid132	N. widderi	Austria	Upper Austria	Totes Gebirge, Warschenk-gruppe, Halssattel, 1645m		25 June 1998	K. Redl
wid133	N. widderi	Austria	Eastern Tyrol	Hinteregger-Alm, Matrei, 1220m		24 June 1998	K. Redl

brids between *Nigritella* and *Gymnadenia* were also collected.

Only fresh plant material was taken. Flowers were preferred, because they have a thinner cuticule than vegetative tissue, thereby making dessication of material and preservation of DNA more efficient. The material was dried in bags with silica gel (cf. Chase and Hills 1991). In a few cases, material was taken from plants that had been transplanted to gardens.

A complete sample list is presented in Table 2. Deviating samples are indicated, as well as allozyme multilocus genotypes for certain samples. Taxonomic delimitations correspond to Teppner and Klein (1998), except that *Nigritella* is treated as a genus separated from *Gymnadenia*, in accordance with results from the present study and data presented by Hedrén et al (in manuscript). Instructive field floras have been Klein and Kerschbaumsteiner (1996) and Delforge (1995).

DNA analysis

AFLP is a fairly recently developed DNA fingerprinting technique (Vos et al. 1996). Its use is steadily increasing, but up to now, the number of studies reported is limited. A high percentage deals with genetic relationships within crops and other cultivars (Becker et al. 1995, Sharma et al. 1996, Hill et al. 1996, Paul et al. 1997). A few population genetic studies on endangered species are present (Travis et al. 1996, Winfield et al. 1998, Quamruz-Zaman et al. 1998). Also, more ecological problems have been addressed (Beismann et al. 1997, Arens et al. 1998). AFLP has proved to be most appropriate for studies on the species or population level (Hedrén, Fay, and Chase in manuscript). The technique is based on the polymerase chain reaction (PCR). From a total digest of DNA, restriction fragments are selectively amplified. It is a very efficient and reliable technique. Scoring of bands can be highly automized if an automatic sequencer is used (Quamruz-Zaman et al. 1998; Hedrén, Fay, and Chase in manuscript). In the investigation of Hedrén, Fay, and Chase (in manuscript) on Dactylorhiza, an average of ca 70 bands were scored per reaction. Only minimal amounts of starting material is required (a few mg dw), a desirable feature if dealing with rare plants. Unlike randomly amplified polymorphic DNA (RAPD), it has a very high reproducibility. One disadvantage of AFLP compared to allozymes, is the uncertain genetic background of the generated fragments. If no breeding schemes are performed, the DNA markers may be regarded as dominant and the identity of homozygotes and heterozygotes cannot be established.

Isolation of DNA

A modified version of the Jodrell Laboratory (Royal Botanic Gardens, Kew) protocoll for small amounts of orchid material was followed (cf. Qamaruz-Zaman et. al (1998) and Doyle and Doyle (1987)).

10 - 50 mg dw (i.e. 1-5 flowers) were ground to a fine powder, and 1000 µl of a preheated (65°C) master mix of CTAB/2-mercaptoethanol was added. CTAB (cetyltrimethylammonium bromide) is a cationic detergent which solubilizes membranes and forms a complex with DNA. Mercaptoethanol inhibits oxidization processes, which otherwise can cause damage to DNA (Weising et al. 1995). The homogenized material was transferred to an extraction tube together with another 500 µl of the master mix, used for rinsing. After incubation at 65°C for 15 minutes, 1500 µl wet chloroform – an organic

solvent – was added. The tubes were then continuously shaken for one hour, and spun at 10 000 rpm for 10 minutes. A dark-coloured aqueous phase (upper phase) was separated from an yellowish organic phase. The DNA-containing upper phase (about 500 µl) was removed to a 1.5 ml Eppendorf tube.

The Qiaquick purifying kit (Qiagen Inc., Chatsworth, California), which includes trade marked chemicals, columns and collection tubes, was used to purify DNA. The columns contain a micro filter of silica, which selectively captures DNA. 625 µl buffer PB and 125 µl DNA extract were transferred to a column. The column was put in a collection tube, and the sample was spun at 10 000 rpm for another minute. The flow through was discarded and the step was repeated once again. The column was washed by adding 750 µl buffer PE (containing ethanol), followed by centrifugation (10 000 rpm for 1 minute). Buffer PE efficiently removes CTAB from DNA. The centrifugation was repeated until all buffer PE had passed through the silica filter. Finally, the column was placed in a clean 1.5 ml Eppendorf tube. The DNA was eluted from the silica filter by adding 30 µl buffer EB. After 1 minute, the sample was spun at 10 000 rpm for 1 minute. The elution step was repeated, to get less concentrated DNA. Accordingly, a DNA extract of 60 µl was finally received. Samples were stored at 80°C until required for analysis.

The quality of all DNA samples was checked. 2 µl was taken from each sample and run on a 0.7% agarose gel. To a buffer solution of 50 ml TBE (Tris-Borate-EDTA), 8 µl ethidium bromide (EtBr) was added. EtBr binds to DNA and is flourescent in UV-light. DNA of good quality was indicated by the presence of a single distinct band. Concentration of DNA was quantified by a fluorometer (Hoefer DyNA Quant200). The concentrations varied between 30 and 200 ng/ml, with an average of about 150 ng/ml. Samples having a concentration below 90 ng/ml were incubated at 70°C for a few hours in order to get more concentrated DNA.

AFLP

The general AFLP™ Plant Mapping Protocol for Regular Plant Genomes, described by the Perkin-Elmer Corporation (PE Applied Biosystems Inc., Foster City, California), was followed. This protocol may thus be consulted for exact information and details concerning chemicals and reaction conditions. Four major steps are distinguished:

1. *Restriction-ligation.* Two different restriction endonucleases, EcoR1 and Mse1 were used. The sequences recognized by these enzymes are dispersed throughout the entire genome, and thousands of fragments can be generated. Mse1 is a frequent 4-base cutter yielding fragments that are in optimal size for both PCR and gel separation, whereas EcoR1 is a relatively rare 6-base cutter. More than 90% of the fragments have Mse1 sequences on both ends. For technical reasons, however, only fragments having one Mse1 end and one EcoR1 end will be properly amplified in the following steps. Accordingly, the use of one rare cutter reduces the overall complexity of fragments. After the restriction, specific adaptors were ligated to the ends of the DNA fragments. The adaptors are oligonucleotides consisting of a core sequence and an enzyme-specific sequence.

2. *Preselective amplification.* Preselective primers were added to the reaction tubes. These primers are composed of three parts: a core sequence and an enzyme-specific sequence fitting the adaptors, and a selective extension of one base. Only fragments that have an adaptor on each end are exponentially amplified during the PCR, which effectively

eliminates irrelevant bands. The preselective amplification results in a 16-fold reduction in the number of amplified fragments. After this step, an agarose gel with EtBr was run to ensure that the amplification successfully had occurred. A smear of several thousand fragments of 100-1500 bp should then appear.

3. *Selective amplification.* A further reduction in number of fragments was necessary. Thus, primers with three (1+2) selective bases were added. Only that subset of fragments having matching nucleotides at all three positions will be amplified, resulting in a further, 256-fold, reduction in the number of fragments. The EcoR1-based primers were labeled with fluorescent dye to permit detection. About 50-200 fragments should be detectable after this step. Three combinations of selective bases were used in this study (codes in brackets): ACC+CAC (Y10), ACG+CAG (G11), and ACT+CTT (B16). The triplet combinations correspond to EcoR1-based and Mse1-based sequences, respectively. The number and strength of bands generated may vary greatly between combinations. Previous studies on the same species or on closely related genera may be a guidance when selecting combinations, but this procedure is still somewhat arbitrary.

4. *Data analysis.* The fragments were separated by using an automized sequencer (ABI Prism 377). The samples were applied on lanes on a gel and each fragment migrated past a fluorescence detector. Fluorescent signals from the fragments were transformed and collected by ABI Genescan software. Every fragment was ranked according to size, or more correctly, migration speed. The migration speed depends primarily on length, but also on sequence. Fragments that were equal in length but differed in sequence could thereby be separated. An internal size standard applied to each lane enabled exact calibration among individuals.

The aquired fragment data was further analyzed in Genotyper Version 1.1. The band patterns were visualized as electropherograms (Fig. 2). Only fragments in the range 50–500 bp were considered by this program, and bands weaker than a recommended fixed threshold value were discarded. The information was extracted as a binary matrix showing presence/absence of bands. The matrix was carefully compared to the electropherogram. Certain corrections and completions of the matrix had to be done. Sometimes bands were obviously present but not automatically scored. That was the case in several weak reactions, but also when bands were too close in the electropherogram. Bands that were nearly equal in size especially had to be checked between samples, to separate between homologous and non-homologous fragments.

Statistics

The presence/absence data were subjected to phenetic analyses, i.e. methods that simply consider similarities between samples. Data from different primer combinations were treated separately, as well as combined into one matrix. Similarities between all possible pairs of samples were estimated using the Jaccard coefficient (Jaccard 1908). Negative matches are not taken into consideration by the Jaccard index. The resulting similarity matrices were used for UPGMA cluster analysis and for principal coordinates analysis (PCO).

The UPGMA cluster analysis ("unweighted pair-group method using arithmetic averages") is a simple and widespread method for constructing phenetic trees. An algorithm is used that in a hierarchial manner sequentially groups similar units together (Li 1997).

The PCO analysis is another way to or-

ganize multivariate data. Instead of providing a tree of hierarchial classification, similarity data are summarized and visualized in a low dimensional ordination space (Gauch 1982). The first PCO axis is rotated in such a way that it accounts for a maximum of the variation among samples. The second axis is perpendicular to the first one and accounts for as much as possible of the remaining variation, and so on. Usually two or three axis will be enough. In the aquired ordination space, similar samples will be closer to each other than dissimilar ones. PCO analyses were performed on the total data set (*Nigritella* + *Gymnadenia* + hybrids), and on three different subsets that only included samples of *Nigritella*: 1) all samples of *Nigritella*, 2) diploids, 3) polyploids.

Correlation between matrices aquired from the different primer combinations was tested by Mantel tests (Mantel 1967). Matrix correlation was tested both with and without *Gymnadenia* and the hybrids included in the matrices. All phenetic analysis were performed in NTSYS-pc 1.80 (Rohlf 1994).

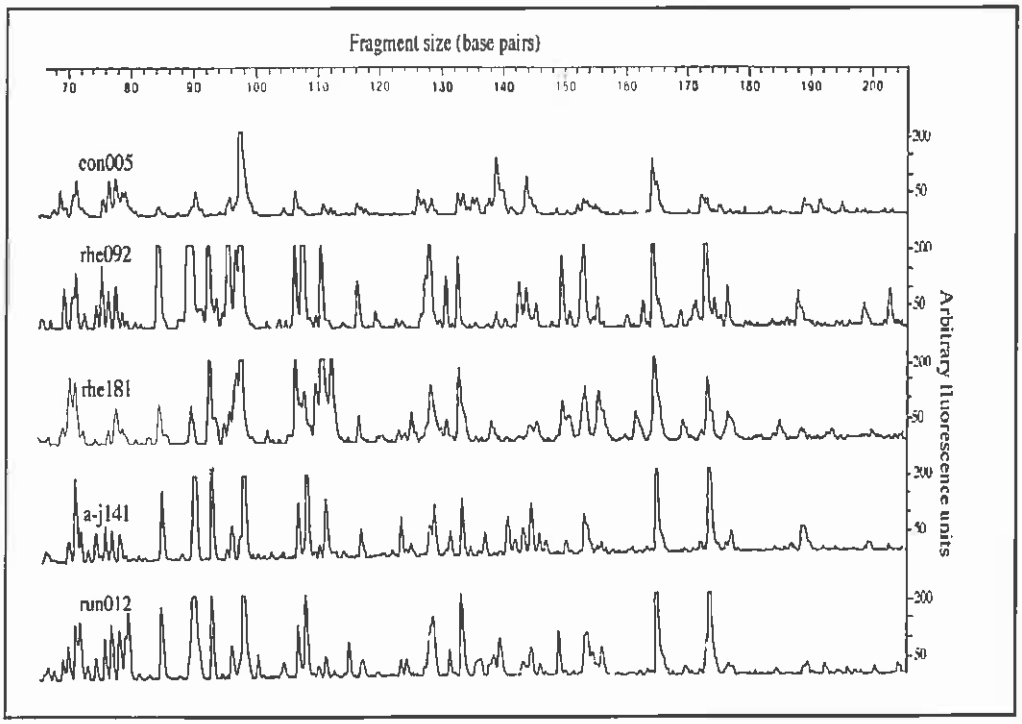

Fig. 2. Representative band patterns as visualized in electropherograms. The sample codes are explained in Table 2.

RESULTS

AFLP data

The number and strength of bands generated by the AFLP technique varied greatly between primer combinations. G11 was the best combination and generated in total 56 recognizable bands in the size range 70–200 bp. In the same range, Y10 rendered only 17 usable bands, whereas B16 had to be rejected because of too few bands. The percentage of polymorphic bands generated by G11 and Y10 were 77% and 76%, respectively. The G11 and Y10 matrices were significantly correlated when all taxa were included in the Mantel test ($p < 0.0001$), but the matrix correlation was not significant when *Gymnadenia* and *Nigritella* x *Gymnadenia* hybrids were excluded ($p = 0.25$). Bands from diploid samples of *Nigritella* were in general stronger than corresponding bands from polyploids. On average 39 ± 1 bands were scored in diploids and 33 ± 1 in polyploids. The corresponding values for *Gymnadenia* and hybrids were 31 ± 1 and 39 ± 5, respectively. The mean number of bands scored in different taxa are listed in Table 3.

Table 3. The mean number of DNA bands scored in different taxa.

Taxon	n	Mean (± s.e.)
G. conopsea	9	31.6 ± 1.5
G. odoratissima	2	27.5 ± 4.5
N. rhellicani	25	38.8 ± 1.2
N. corneliana	6	44.8 ± 1.3
N. lithopolitanica	3	38.7 ± 2.6
N. gabasiana	4	27.8 ± 2.8
G-n. runei	1	49.0 —
x*G-n. suaveolens*	2	39.5 ± 6.5
x*G-n. heufleri*	1	26.0 —
N. nigra ssp. *nigra*	2	31.0 ± 11.0
N. nigra ssp. *austriaca*	4	32.8 ± 3.5
N. nigra ssp. *iberica*	5	36.6 ± 3.2
N. stiriaca	2	30.5 ± 7.5
N. widderi	5	33.0 ± 2.4
N. miniata	8	31.5 ± 2.3
N. archiducis-joannis	2	41.0 ± 1.0

Nigritella/Gymnadenia

The taxa of *Nigritella* and *Gymnadenia* were well separated from each other on the first axis in the PCO ordination (Fig. 3a). Hybrids (run012, sua030, sua124, heu025) clustered in between, albeit somewhat closer to *Nigritella*. The first three PCO axes accounted for 25.1, 12.9, and 8.6% of the total variation, respectively. All taxa of *Nigritella* were more homogenous (mean similarity coefficient for comparisons of samples within taxa ranged between 79 and 100%) than *G. conopsea* and *G. odoratissima* (71 and 77%, respectively) (Table 4).

The general pattern revealed by the PCO ordination also appeared in the UPGMA phenogram (Fig. 4). Of the two most basal branches, both species of *Gymnadenia* were included in one, whereas hybrids and taxa belonging to *Nigritella* were included in the other one. Within the first branch, *Gymnadenia* was separated into three subgroups. One subgroup consisted of *G. odoratissima* only. The other two both consisted of *G. conopsea*, and partly reflected geographic origin of samples (cf. Table 2): all samples from the island of Gotland (con194, con 200, con201) clustered together with one sample from the Western Alps (con166), whereas the only sample from Northern Scandinavia (con005) clustered together with samples from different Central European populations. The hybrids and all *Nigritella* taxa formed two distinct subgroups within the latter branch.

Pairwise comparisons between the hybrids and *Nigritella* (Table 4) resulted in higher mean similarity coefficients (on average 66%) than comparisons with *Gymnadenia* (57%). *Gymnigritella runei*,the tetraploid hybrid, was most similar to *N. nigra* s.lat.

Table 4. Mean Jaccard similarity coefficients (per cent) within and between the taxa investigated. The species codes are explained in Table 2; * = Only one sample available.

	con	odo	rhe	cor	lit	gab	run	sua	heu	nig	aus	ibe	sti	wid	min	a-j
con	71															
odo	64	77														
rhe	42	49	79													
cor	40	47	80	85												
lit	44	53	77	80	81											
gab	46	52	69	68	76	94										
run	58	54	65	63	64	62	*									
sua	57	58	68	66	69	61	77	79								
heu	55	59	61	62	70	62	70	74	*							
nig	37	47	74	74	81	74	69	65	72	100						
aus	41	52	77	77	83	77	71	67	74	97	96					
ibe	44	53	76	78	85	81	70	69	71	91	90	97				
sti	41	53	73	75	80	69	58	62	72	80	79	78	88			
wid	44	53	71	73	79	71	58	64	75	76	77	78	82	89		
min	42	55	71	72	79	69	57	62	73	80	78	78	92	84	91	
a-j	45	53	77	79	80	71	68	69	68	79	81	80	82	80	79	91

(70%) and to *G. conopsea* from Northern Scandinavia/Central Europe (65%). *Gymnigritella runei* and *Nigritella nigra* s.lat. shared a few DNA markers that were absent or rare in other taxa. Samples of *Nigritella* and *Gymnadenia* from Heiligenbach-Alm, where hybrids frequently were found together with the parental species, were not more similar to each other than samples of *Nigritella* and *Gymnadenia* from other populations.

Diploids

Apart from *N. gabasiana*, diploid taxa of *Nigritella* were variable and did not constitute any discrete groups in the PCO ordination for diploids (Fig. 3c). The first three PCO axes accounted for 21.1, 10.3, and 9.3% of the total variation, respectively.

Samples of *N. rhellicani* were scattered all over the *Nigritella* branch in the UPGMA phenogram (Fig. 4). A number of *N. rhellicani* individuals from the Western Alps formed a group with *N. corneliana*, a western species. This geographic pattern was more obvious in the UPGMA phenogram than in the PCO ordination. In Table 2, a small group of N. *rhellicani* samples were characterized as phenologically deviating (rhe161, rhe163, rhe164, rhe177, rhe178). Genetically, these samples were not more similar to each other than to any other samples of *N. rhellicani*. *Nigritella lithopolitanica*, the diploid with the smallest distribution area, was not homogenous, but appeared in several different subgroups. All individuals of *N. gabasiana*, the westernmost diploid, clustered very close to each other. Furthermore, *N. gabasiana* was the only diploid that distinctly deviated from other diploid species, from which it was separated on the first PCO axis.

The mean similarity coefficient for comparisons of samples within diploid species varied between 79 and 94% (Table 4), the lowest value corresponding to *N. rhellicani* and the highest to *N. gabasiana*. Sixteen individual DNA bands were restricted to the diploids. Of those, seven were confined to *N. rhellicani*. No other species had any unique markers. The bands restricted to the diploids generally appeared in low frequencies.

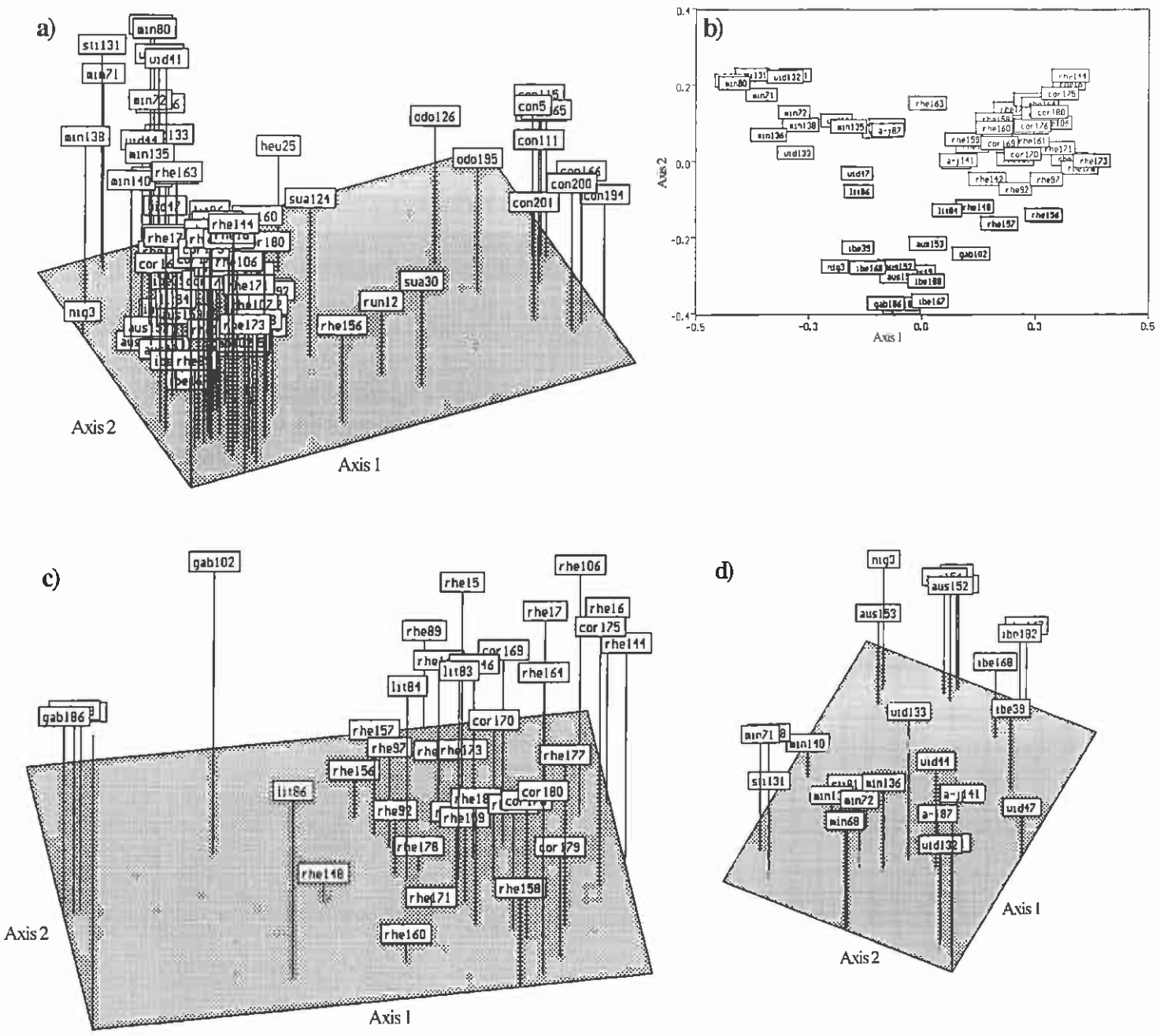

Fig. 3. Two- or three-dimensional principal coordinate (PCO) plots illustrating the differentiation among taxa of *Nigritella*, *Gymnadenia*, and their hybrids. Number of dimensions have been chosen to give the best visual description of the variation pattern. Sample codes are explained in Table 2.
a) All samples included. The axes account for 25.1, 12.9, and 8.6% of the total variation, respectively.
b) Only samples of *Nigritella* included (20.6 and 14.4%).
c) *Nigritella* – only diploids included (21.1, 10.3, and 9.3%).
d) *Nigritella* – only polyploids included (41.9, 16.4, and 12.1%).

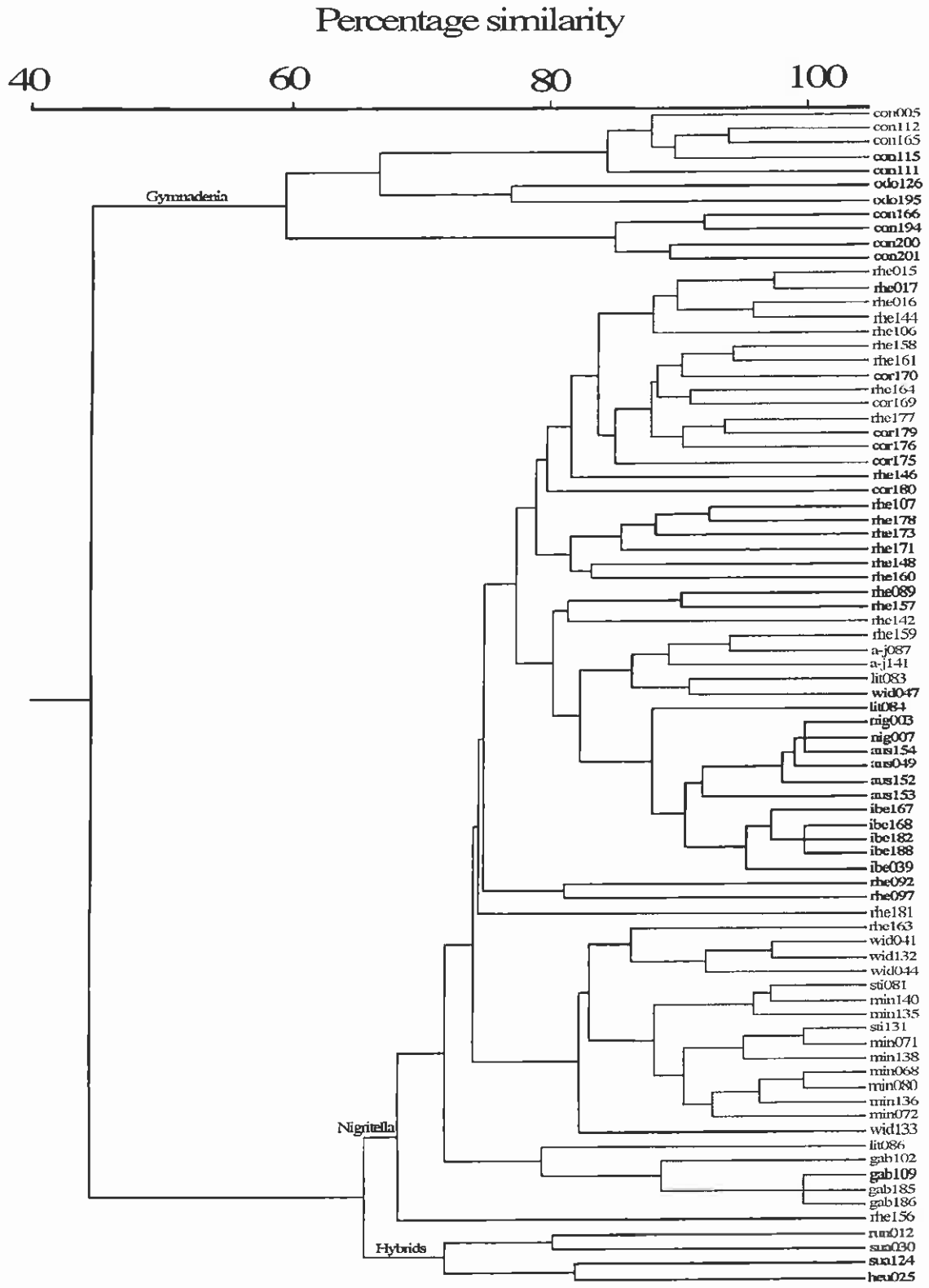

Fig. 4. UPGMA phenogram based on the Jaccard coefficient of similarity, showing relationships within *Nigritella/Gymnadenia*. The data set includes 56 polymorphic bands from 81 samples. Data from two different AFLP reactions are combined. Sample codes are explained in Table 2.

Polyploids

Polyploid taxa were less variable than diploid taxa. The mean similarity coefficient for comparisons of samples within taxa varied between 88 and 100% (Table 4). A few individual DNA bands were restricted to the polyploids, but also the frequency of several particular bands was considerable higher in polyploid taxa compared to the diploids. Comparisons between different polyploid taxa generally resulted in higher similarity coefficients (range 76–100%) than comparisons with diploids (range 69–85%). In the PCO ordination for all *Nigritella* taxa, polyploids were separated from all diploids but *N. gabasiana* on the second axis (Fig. 3b). The first three axes accounted for 20.6, 14.4, and 10.0% of the total variation, respectively.

Two main clusters appeared in the PCO ordination for polyploids (Fig. 3d). The first axis clearly separated *N. nigra* s.lat. from the remaining polyploids. The samples of *N. nigra* s.lat. clustered fairly well in accordance with the three subspecies. The variation among the remaining polyploids appeared to be more complex. The first three axes accounted for 41.9, 16.4, and 12.1% of the total varation, respectively.

Most of the bands that were found in the triploid *N. nigra* ssp. *nigra*, also appeared in the tetraploid subspecies, whereas the tetraploids contained bands not found in the triploid. A few of these bands were restricted to either *N. nigra* ssp. *austriaca* or *N. nigra* ssp. *iberica*. All three subspecies of *N. nigra* differed from each other on the second PCO axis (Fig. 3d). Samples of *N. nigra* ssp. *austrica* constituted an intermediate position, partly overlapping *N. nigra* ssp. *nigra*, but clearly distinguished from *N. nigra* ssp. *iberica*. In the PCO ordination where all samples of *Nigritella* were included (Fig. 3b), all three subspecies clustered together with the diploid *N. gabasiana* on the second axes. When individual DNA bands were considered, it was further revealed that the most western subspecies, *N. nigra* ssp. *iberica*, shared a rare DNA band with the diploid *N. gabasiana*.

In the PCO ordination for polyploids (Fig. 3d), *N.widderi* and *N. archiducis-joannis* clustered together and were separated from *N. miniata* and *N. stiriaca* along the second axis. Samples of *N. widderi* were indistinguishable from samples of *N. archiducis-joannis*, and the same was true for *N. miniata* and *N. stiriaca*. Samples within the *N. widderi/N. archiducis-joannis* group partly clustered in accordance with different allozyme multilocus genotypes (cf. Hedrén et al. in manuscript). Such pattern could not be detected in the *N. miniata/N. stiriaca* group. Allozyme multilocus genotypes for certain samples are indicated in Table 2. One sample of *N. miniata* (min080) had the same flower colour as *N. widderi*. However, genetically, it belonged to the *N. miniata/N. stiriaca* group.

DISCUSSION

The high number of DNA markers generated appeared to be very informative when combined with allozyme data (provided by Hedrén et al. in manuscript). An outline of possible evolutionary pathways within the *Nigritella* complex is presented in Fig. 5.

Nigritella/Gymnadenia

The genetic differentiation within *Nigritella/Gymnadenia* as a whole was less extensive than could be expected from the taxonomic delimitations. Still, fairly distinct groups appeared in the PCO ordination, as well as in the UPGMA phenogram (Figs. 3a and 4). Taxa belonging to *Nigritella* were clearly separated from the two species of *Gymnadenia*, and all the hybrids clustered somewhere in between. The recently proposed hypothesis that *Gymnigritella runei* might be of pure *Nigritella* origin (Ericsson 1997) can therefore be rejected. In the Mantel test, the clear separation of *Nigritella*, *Gymnadenia*, and the hybrids, resulted in a significant correlation between the G11 and Y10 matrices, in spite of the fact that Y10 only generated 17 characters (compared with 56 characters from G11).

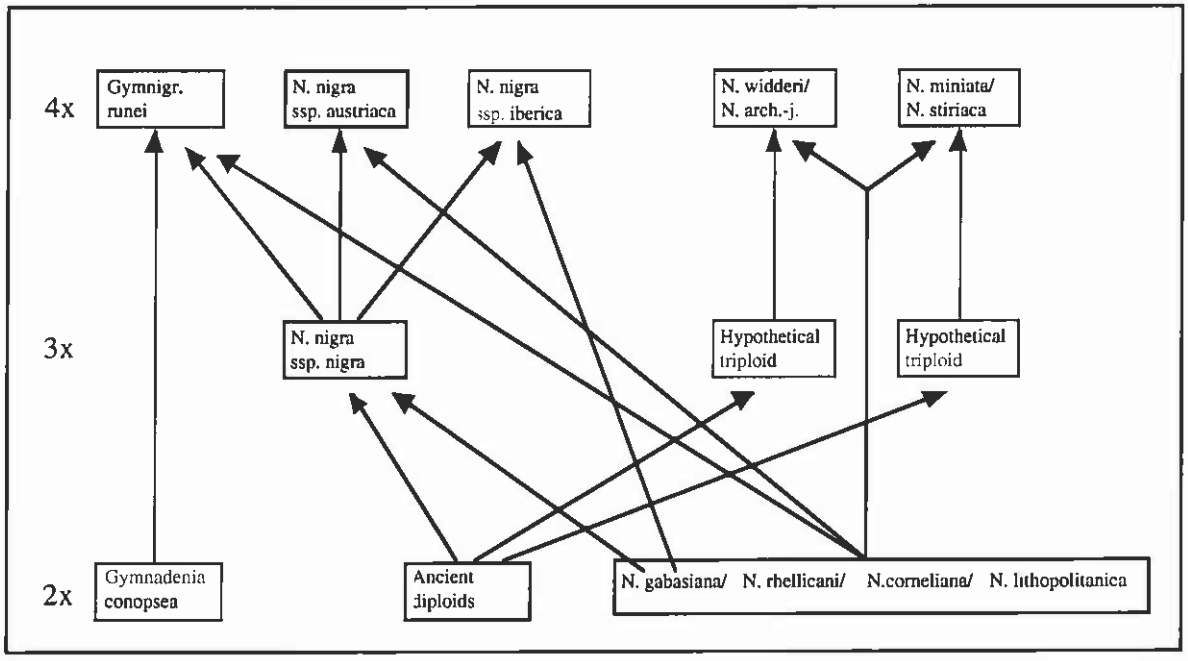

Fig. 5. Possible evolutionary pathways between diploids and polyploids. The number of hypothetical steps has been minimized. Tetraploids are thought to have evolved from diploids via intermediate, triploid forms. The triploids could be derived from hypothetical diploid ancestors, and may be the result of fusion of unreduced (2x) and normal, haploid (x) gametes. Unreduced (3x) pollen may then have fused with haploid egg cells, thereby giving rise to new, tetraploid taxa. In the last step, extant diploids may have been involved. All extant diploid species are treated as one collective unit, since they appeared to be very similar. Within this unit, *N. gabasiana* is however treated somewhat differently; AFLP data indicate that ancestors similar to this taxon may have contributed to the formation of *N. nigra* s.lat. in general, and *N. nigra* ssp. *iberica* in particular. *Nigritella widderi* + *N. archiducis-joannis*, and *N. miniata* + *N. stiriaca* are treated as two collective groups. Due to recurrent polyploidization, the phylogeny of these taxa appeared to be very complex.

The two species of *Gymnadenia* proved to be more variable than the *Nigritella* taxa (Table 4), a pattern revealed also from allozyme studies (Scacchi and De Angelis 1989, Hedrén et al. in manuscript). For example, the difference between *G. conopsea* from Gotland and Northern Scandinavia was of same order of magnitude as the differences between different species of *Nigritella*. The low level of variation within *Nigritella* is probably the reason why the hybrids clustered closer to *Nigritella* than to *Gymnadenia*: the *Nigritella* samples included in this study are likely to be more similar to the true parental individuals that gave rise to the hybrids, than are the *Gymnadenia* samples.

Concerning *Gymnigritella runei* , individual DNA markers indicated that this tetraploid hybrid partly could be derived from the triploid *N. nigra* ssp. *nigra*.

Ancient diploids

The band pattern of polyploid *Nigritella* reflected some kind of additivity of different diploid genomes, but the origin of the polyploids could not simply be explained by adding bands from different diploid taxa. In the PCO ordination for all *Nigritella* samples, the polyploids were fairly well separated from the diploids (Fig. 3b). When considering that the polyploids in general generated a lower number of DNA bands than the diploids (Table 3), it could be argued that this displacement is an artefact due to the AFLP technique: the polyploids with their combined genomes should contain a higher number of fragments than the diploids, especially if they are the result of hybridization between divergent parental taxa. Competition between fragments during the PCR process may have resulted in several fragments only becoming poorly amplified, and accordingly not scored. However, even if certain bands are missing, inspection of the electropherograms reveals that very clear presence/absence differences do exist: all polyploid taxa display a high frequency of bands that are rare or absent in the present-day diploids.

Typical "polyploid" bands may correspond to alleles that once were common among diploids, but that have subsequently decreased in frequency. Consequently, the polyploids may, at least partly, be derived from diploids different from modern taxa. The possibility that the polyploid genomes to any larger extent have evolved after the hybridization events, can be excluded because of the predominantly apomictic mode of reproduction.

Hypothetical diploid ancestors may have constituted taxa morphologically more or less divergent from extant diploid species; it is not possible to know how patterns of genetic variation are correlated to phenotypic characters. Actually, deviating diploids may still exist, and it cannot be ruled out that ancient alleles are hidden in the isolated populations of *N. carpatica* from the Eastern Carpathians (not included in this study).

Could it then be stated that diploid taxa similar to those included in this study, have not contributed to the formation of the polyploids? *Nigritella nigra* ssp. *nigra*, and other, hypothetical, triploids should probably be derived from ancient diploids. Tetraploid taxa, on the other hand, may well be the result of hybridization between triploids with ancient genomes, and diploids with genomes similar to those of present-day diploids.

Present-day diploids

In the PCO ordinations as well as in the UPGMA phenogram, only samples of *N. gabasiana* constituted a distinct group

(Figs. 3c and 4). The remaining taxa were not distinct from each other, and the variation pattern only partly corresponded to morphologically defined entities. Hedrén et al. (in manuscript) estimated that less than 10% of the genetic variation could be explained by differences between taxa, whereas about 80% were due to variation within populations.

To some extent, samples from same geographic area were grouped together. In the UPGMA phenogram, samples of the brown-coloured *N. rhellicani* from the Western Alps tended to form a group with *N. corneliana*, a western, bright-coloured species. *Nigritella rhellicani*, the most widespread species, may represent the oldest diploid, whereas the other diploids appear to have arisen as isolated derivatives of *N. rhellicani*, given their relative small AFLP variation (Table 4) and their restricted distribution areas (Fig. 1a). Samples of *N. rhellicani* were spread all over the *Nigritella* branch in the UPGMA phenogram, and most of the unique DNA bands were found in samples of *N. rhellicani*. Notably, several of these unique bands were confined to rhe181, a sample from Montenegro, which represents the southernmost limit of the distribution area. *Nigritella lithopolitanica*, in contrast, has a very restricted distribution, composed only of a few isolated populations in the Southeastern Alps. Yet, individuals of this bright-coloured species were genetically indistinguishable from *N. rhellicani*. Samples of *N. gabasiana*, the most western species, clustered very close to each other, and were well separated from other diploids. This pattern could partly be explained by the comparatively low number of bands scored (Table 3). However, also from allozyme data it was concluded that *N. gabasiana* was the least variable species, even if the separation from other diploids was less striking (Hedrén et al. in manuscript). Morphologically, *N. gabasiana* is very similar to *N. rhellicani* (Delforge 1995).

Most likely, all populations of diploids have previously been linked together by gene flow, either by pollen or seed dispersal. The present morphological differentiation between taxa should have evolved since then. The separation of *N. gabasiana* may be due to its isolation in the Pyrenées and the Cantabrian Mts., far away from other diploids.

All present-day diploids may be treated as one variable ancestor species when reconstructing evolutionary pathways in the *Nigritella* complex (Fig. 5). Still, within this unit it might be justifiable to treat *N. gabasiana* somewhat differently. The PCO ordination in which all *Nigritella* samples were included (Fig. 3b), suggests that a taxon similar to *N. gabasiana* has contributed to the formation of *N. nigra* s.lat. Furthermore, the western-most subspecies, *N. nigra* ssp. *iberica*, shared a rare DNA marker with *N. gabasiana*, indicating a close relationship and a possible origin of *N. nigra* ssp. *iberica* in the present area of distribution (cf. Fig. 5).

Polyploids

In accordance with their apomictic mode of reproduction, polyploid taxa were less variable than the diploids. Two main groups appeared in the PCO ordination (Fig. 3d): the three brown-coloured subspecies of *N. nigra* were separated from the remaining, red- or bright-coloured, taxa.

Nigritella nigra ssp. *austrica* and *N. nigra* ssp. *iberica* could most likely be derived from triploid individuals very closely related to the extant triploid *N. nigra* ssp. *nigra*. DNA bands found in *N. nigra* ssp. *nigra* generally also appeared in the tetraploids, whereas the tetraploids contained bands that were not found in the triploid. One indistinct band confined to *N. nigra* ssp. *nigra* may be an artefact due to incorrect band interpretation, but could

also be an indication that the two samples of *N. nigra* ssp. *nigra* included in this study may differ somewhat from the individuals that gave rise to the tetraploids. Certain bands also suggest that *N. nigra* ssp. *austriaca* and *N. nigra* ssp. *iberica* have separate origins, since a few bands were restricted to only one of the subspecies.

The allozyme data suggested that *N. widderi* and *N. archiducis-joannis* could be derived from one hypothetical triploid, whereas *N. miniata* and *N. stiriaca* could be derived from another one. Indeed, the subgroups consisting of the same pairs of taxa also appeared in the PCO ordination based on AFLP data, thus giving support to the hypothesis. Samples of *N. widderi* formed a complex with *N. archiducis-joannis*, and *N. miniata* formed a complex with *N. stiriaca*, suggesting that the taxonomic delimitations do not reflect phylogenetic patterns. The allozyme data showed that some individuals of *N. widderi* shared a multilocus genotype with *N. archiducis-joannis*, whereas some individuals of *N. miniata* shared a multilocus genotype with *N. stiriaca*. The AFLP data corresponded only partly to these multilocus genotypes, indicating that taxa within both groups probably are even more inter-linked than suggested from allozymes. If morphological characters are considered, this pattern may seem surprising, especially regarding *N. miniata* with its typically red flowers.

Recurrent polyploidization and gene flow

A polyploid taxon often has several independent origins. Actually, a polyploid taxon may comprise several races, each originating from different progenitor species (Brochmann et al. 1992b). From recent molecular analyses it has even been suggested that multiple origins are the rule rather than the exception (Soltis and Soltis 1993).

Presence of different fixed multilocus genotypes has been interpreted as an indication of recurrent polyploidization (Brochmann et al. 1992a,b). This is particularly valid for apomictic species where the genomes of all individuals are more or less uniform. Hedrén et al. (in manuscript) found two different multilocus genotypes in *N. widderi* and *N. miniata*, respectively, but allozymes are conservative gene markers, and the number of origins of different polyploid taxa may therefore be underestimated. What could then be said about recurrent polyploidization from the AFLP data?

Since the polyploids are apomicts, every event of polyploidization should result in a new, uniform taxon. Because of the heterogeneity within present polyploid taxa (Fig. 3d; Table 4), it could be imagined that numerous occasions of polyploidization have taken place. After excluding a few weak and uncertain bands, in order to get a conservative estimate, I estimated the number of origins by counting the AFLP genotypes in each taxon. Both the tetraploid subspecies of *N. nigra* probably only have a single origin each, whereas *N. widderi/N. archiducis-joannis* and *N. miniata/N. stiriaca* may have as many as seven and five separate origins, respectively. The pattern revealed from the AFLP analysis may however give a somewhat exaggerated impression of the variation. The presence/absence matrices are not free from errors, since they partly are a result of manual interpretation. On the other hand, errors should be evenly spread all over the samples. It seems therefore unlikely that the comparatively high variation within the *N. widderi/N. archiducis-joannis* and *N. miniata/N. stiriaca* complexes should be the result of incorrect interpretations. A significant fraction of the variation should indeed re-

flect true genetic diversity, and polyploidization is probably more frequent than indicated by allozymes.

In addition to recurrent polyploidization, variation may also have been introduced by occasional events of gene flow between polyploids. Apomictic polyploids may be more heterogeneous than expected. Individuals within an apomictic taxon do not necessarily have identical genotypes. Asker (1979) meant, as a general statement, that there are no obligate apomicts. Exchange of genes may take place either directly between polyploids, or via back-crossing with taxa at lower ploidy levels. This has been indicated in other polyploid complexes (Briggs and Walters 1997, Brochmann et al. 1992c) and may also apply to *N. widderi*, *N. archiducis-joannis*, *N. miniata*, and *N. stiriaca*, all of which have more or less overlapping distributions in the Eastern Alps (Fig. 1b). It has been shown that polyploid *Nigritella* produce fertile pollen, and crossing experiments have suggested that a limited number of functional egg cells regularly may develop (Teppner 1996). Moreover, the origin of the pentaploid *N. buschmanniae* (see below), and the occurrence of hybridization between *N. rhellicani* and *N. miniata*, and between *N. miniata* and *Gymnadenia* spp. (Table 1), provide indirect evidence that functional gametes may be produced by the tetraploids. Yet, it should be stressed that gene flow cannot be a common phenomenon. There is no indication from allozyme data that recombination of polyploid genomes has taken place, nor have any natural triploid hybrids resulting from hybridization between diploids and tetraploids been found (Deutsch 1998).

If polyploid taxa of *Nigritella* are not obligate apomicts, but sometimes reproduce sexually, recurrent polyploidization and occasional events of gene flow between taxa should be of significant evolutionary importance. Variation is introduced to various taxa, thereby enhancing the evolutionary potential. In general, polyploid complexes are dynamic genetic systems, and should not be designated as evolutionary dead ends, as opposed to Wagner (1970).

History of Nigritella

Stebbins (1984) argued that polyploidization and reticulate speciation largely has been forced by the Pleistocene glaciations. Advance and retreat of ice sheets has brought about changes in distribution of plants, and contact between previously isolated and divergent populations may have resulted in hybridization and formation of new polyploid taxa.

Allozyme studies and AFLP data have suggested that some tetraploid species of *Dactylorhiza* probably arose during the end of the last glaciation, 15000–10000 BP (Hedrén 1996a,b; Hedrén, Fay, and Chase in manuscript). A similar age could be imagined for tetraploid taxa of *Nigritella*, whereas the triploid *N. nigra* ssp. *nigra* may be older. Together with other, hypothetical triploids, it may have arisen from ancient diploids during an interstadial episode of the last glaciation.

A scenario that would account for the present-day distribution and the relationships between *Nigritella* taxa, may be described as follows: *N. nigra* ssp. *nigra* and two other – today probably extinct – triploid taxa survived the last glacial maximum in different Central and Southern European refugia. (Other authors have suggested that *N. nigra* ssp. *nigra* survived the Weichselian ice age in Scandinavian coastal refugia (Holmboe 1936, Gjærevoll 1992), but this seems most unlikely.) When the ice sheet started to retreat, the plants colonized new areas. The triploids met populations of diploids closely related to extant taxa, and new, tetraploid taxa arose. Accord-

ing to the PCO ordination (Fig. 3b), it could be assumed that *N. nigra* ssp. *nigra* had a distribution that overlapped the distribution of a predecessor to the western diploid *N. gabasiana*. In this area of contact, *N. nigra* ssp. *austriaca* and *N. nigra* ssp. *iberica* then arose – the former towards the eastern limit and the latter towards the western limit. *Nigritella widderi/N. archiducis-joannis* and *N. miniata/ N.stiriaca* have no typical "western" DNA markers, and their hypothetical triploid ancestors had probably more eastern distributions. During Holocene, *N. nigra* ssp. *nigra* as well as the hypothesized triploids, seem to have become extinct from the European mainland. *Nigritella nigra* ssp. *nigra* managed however to settle in Scandinavia.

A comparatively early origin of the polyploids and the presence of isolated populations, do not necessarily imply that the evolutionary potential has been lost or that the distribution areas have been fixed. The pentaploid *N. buschmanniae* (not included in the present study) presumably has a recent origin as a hybrid between *N. widderi* and a present-day diploid (Teppner and Ster 1996, Hedrén et al. in manuscript), and there are indications that *Nigritella* may have a considerable ability to disperse. Monitoring of *N. nigra* ssp. *nigra* in Jämtland, for example, has repeatedly shown that colonization of new localities has taken place (Björkbäck and Lundqvist 1982). The present disjunct distribution of *N. nigra* ssp. *nigra* in Scandinavia (Fig. 1b) may reflect events of dispersal during historical time. Rune (1993) proposed that *N. nigra* ssp. *nigra* expanded northwards during a period of warm and dry climate about 1000 BP. A few centuries later, a climatic deterioration brought about a regression, leaving a very isolated population at Mt. Balgesoaivve, Troms, 200 km north of the present-day main distribution (Engelskjøn and Skifte 1984, Sætra 1987). During the expansion phase, *N. nigra* ssp. *nigra* may have hybridized with *G. conopsea*, giving rise to the tetraploid *Gymnigritella runei*. A high degree of similarity between *Gymnigritella runei* and *G. conopsea* from northern Sweden indicates that *Gymnigritella runei* has arisen in that area and that it is thus not a postglacial immigrant. Its origin is probably the result of one single, unusually successful coincidence. Attempts to artificially cross *N. nigra* ssp. *nigra* with *G. conopsea* have not been successful (Malmgren 1992).

Taxonomy

Orchids are spectacular. No other plant group has been met by such a broad popular interest. This is obviously reflected in the taxonomy. There is a tendency to assign taxa a higher rank than they would have got if they had belonged to less popular plant groups. However, a sound taxonomy should be consistent, and different plant groups treated in a similar way.

Given the genetic patterns revealed in the present study, it could be questioned whether it is correct to treat diploid taxa of *Nigritella* as separate species. The taxonomic delimitations are based on a few morphological characters, and there is a relatively weak association between morphology and DNA characters. This condition became especially obvious when morphologically deviating samples were considered. Morphological characters, e.g. flower colour, may be controlled by few genes, and simple mutations may have a high ability to establish in *Nigritella* populations. In addition, different diploid taxa are probably interfertile – something that could be tested experimentally.

The complex relationships within the species-pairs *N. widderi/N. archiducis-joannis* and *N. miniata/N. stiriaca* demonstrate the taxonomic difficulties that may arise in poly-

ploid complexes due to recurrent polyploidization and gene flow among ploidy levels (cf. Brochmann et al. 1992c). Taxa within each pair are obviously linked to each other in a highly elaborate way. If the current species concept is used, the groups may be polyphyletic or paraphyletic in the sense that they include several polyploid races, each originating from a separate polyploidization event. If distinct units of genetically and morphologically similar individuals could be distinguished, taxa within each species pair should be split into several new apomictic microspecies. However, the lack of useful morphological characters in practise excludes such a solution. If genetically coherent taxa are attempted, it may be better to use a wider species concept. For example, in conservation biology it is important to consider units that correspond to genetically distinct entities. Three groups of polyploids may accordingly be considered in *Nigritella*: *N.widderi/N. archiducis-joannis*, *N. miniata/N. stiriaca* and *N. nigra* s.lat. Subunits within the first two groups cannot be distinguished, whereas lower units within *N. nigra* s.lat. still may be considered.

This study has confirmed that *Nigritella* and *Gymnadenia* are closely related. However, it is not necessary to transfer *Nigritella* to *Gymnadenia*: samples belonging to the two genera are clearly separated from each other. Furthermore, sampling at Heiligenbach-Alm, where hybrids grew together with *Nigritella* and *Gymnadenia*, gave no indications of gene flow between the genera. The finding in Pridgeon et al. (1997) that *Nigritella* together with *G. conopsea* var. *borealis* form a sister group to *G. conopsea* var. *conopsea* is remarkable, but needs to be confirmed with extended studies including additional taxa and data from other parts of the genome. It is not certain that the nrDNA ITS region studied by Pridgeon et al. (1997) reflects the relationship of the entire nuclear genome.

This study has mainly focused on *Nigritella*. A future challenge would be to perform a more inclusive investigation on *Gymnadenia* as well. It could be assumed that the high variation in *Gymnadenia* partly is a result of polyploid evolution, as both diploids and tetraploids are found in this genus.

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Appendix 1. Distribution and frequency of DNA bands, expressed as percentage of samples within a taxon displaying a particular band. Band 1–56 refer to primer combination g11, and band 57–73 refer to y10; ? = Indistinct bands.

Band / Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
con	100	100	100	100	100	100	100	78	100	0	44	38	100	100	29	0	11	100	44	44	56	56	0	100	0
odo	100	100	100	100	100	100	100	0	100	100	0	50	50	100	0	0	0	100	0	0	100	50	0	100	0
rhe	22	56	100	100	96	100	100	4	100	100	0	100	40	8	96	96	100	100	4	0	100	64	42	100	4
cor	0	33	100	100	100	100	100	33	100	100	33	100	33	0	100	100	100	100	0	0	100	67	100	100	0
lit	0	67	100	100	100	100	100	0	100	100	33	100	67	0	100	100	100	100	0	0	100	100	100	100	0
gab	0	75	100	100	100	100	100	0	100	0	100	0	0	0	100	100	100	100	0	0	100	0	0	100	0
run	100	100	100	100	100	100	100	100	100	0	0	100	100	0	100	0	100	100	0	0	100	100	0	100	0
sua	50	100	100	100	100	100	100	50	100	100	0	100	100	0	100	100	100	100	50	0	100	50	50	100	0
heu	0	0	100	100	?	100	100	0	100	?	0	100	0	0	100	?	?	100	0	0	100	100	100	100	0
nig	0	0	100	100	100	100	100	0	100	100	0	100	0	0	100	100	100	100	0	0	100	100	100	100	0
aus	0	0	100	100	100	100	100	0	100	67	0	100	0	0	100	100	100	100	0	0	100	75	100	100	0
ibe	0	100	100	100	100	100	100	0	100	80	100	100	0	0	100	100	100	100	0	0	100	100	100	100	0
sti	0	50	100	100	100	100	100	0	100	100	0	100	0	0	100	100	100	100	0	0	100	100	100	100	0
wid	0	60	100	100	100	100	100	0	100	100	20	100	0	0	100	100	100	100	0	0	100	100	100	100	0
min	0	62	100	86	100	100	100	0	100	100	0	100	0	0	100	100	100	100	0	0	100	100	100	100	0
a-j	0	100	100	100	100	100	100	0	100	50	0	100	0	0	100	50	100	100	0	0	100	100	100	100	0

Band / Taxon	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
con	0	0	100	0	0	0	0	100	33	100	100	0	78	67	22	78	56	22	0	0	11	100	100	56	56
odo	0	0	100	0	0	0	0	100	0	100	100	0	0	0	0	100	50	0	0	50	0	100	100	100	0
rhe	8	0	100	100	28	33	4	100	96	100	0	72	4	4	74	52	4	96	79	100	39	100	100	8	46
cor	50	0	100	100	50	50	0	100	100	100	0	100	0	0	83	100	0	100	75	100	67	100	100	17	83
lit	0	0	100	100	33	50	0	100	100	100	0	33	0	0	33	100	0	67	0	100	0	100	100	0	0
gab	0	0	100	100	25	0	0	100	100	100	0	25	0	0	0	100	0	0	0	100	0	100	100	0	0
run	0	100	100	100	0	0	0	100	100	100	100	0	100	100	0	100	100	100	100	100	0	100	100	0	0
sua	0	0	100	100	0	0	0	100	100	100	100	0	100	100	100	100	0	100	50	100	0	100	100	100	0
heu	0	0	100	?	0	0	0	100	100	100	?	0	100	100	0	100	100	?	0	0	0	100	100	100	0
nig	0	100	100	100	0	0	0	100	100	100	0	0	0	0	0	100	0	100	0	100	0	100	100	0	0
aus	0	100	100	100	0	0	0	100	100	100	0	0	0	0	0	100	0	100	0	100	0	100	100	0	0
ibe	0	80	100	100	0	0	0	100	100	100	0	0	0	0	0	100	0	100	0	100	0	100	100	0	0
sti	0	0	100	0	0	100	0	100	100	100	0	100	0	0	0	100	50	50	0	100	0	100	100	0	0
wid	20	0	100	100	0	100	0	100	100	100	0	40	0	0	80	100	80	0	0	0	0	100	100	0	0
min	0	0	100	0	0	100	0	100	100	100	0	88	0	0	0	100	50	25	0	100	0	100	100	0	0
a-j	0	0	100	100	0	100	0	100	100	100	0	100	0	0	100	100	0	100	0	100	0	100	100	0	0

Band / Taxon	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	n
con	56	0	0	11	0	0	100	100	0	100	83	100	0	0	100	100	0	0	0	33	0	17	0	9
odo	100	0	0	0	50	0	100	100	0	100	0	100	0	100	100	100	0	0	0	0	0	0	0	2
rhe	100	8	100	0	0	100	20	100	5	0	0	100	35	100	100	95	95	5	15	95	80	5	100	25
cor	100	0	100	0	0	100	0	100	17	17	0	100	0	100	100	100	100	0	50	100	100	0	100	6
lit	100	0	100	0	0	100	0	100	33	0	0	100	0	100	100	100	67	0	67	100	67	0	100	3
gab	100	0	100	0	0	100	0	100	0	0	0	100	0	?	100	100	100	0	0	100	100	?	?	4
run	100	0	100	0	0	100	100	100	0	100	100	100	0	100	100	100	100	0	0	100	100	100	100	1
sua	100	0	100	50	0	0	100	100	0	100	100	100	0	100	100	100	100	0	0	100	100	?	?	2
heu	100	0	100	100	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1
nig	100	0	100	0	0	100	100	100	0	0	100	100	0	100	100	100	100	0	0	100	100	100	100	2
aus	100	0	100	0	0	100	100	100	0	0	0	100	50	100	100	100	100	0	0	100	50	50	100	4
ibe	100	0	100	0	0	100	0	100	0	0	0	100	0	100	100	100	100	0	0	100	100	100	100	5
sti	100	0	100	0	0	?	0	100	0	0	0	100	0	100	100	100	100	0	0	100	0	0	100	2
wid	100	20	100	0	0	80	0	100	0	0	0	100	0	100	100	100	0	100	0	0	0	0	0	5
min	100	0	100	0	0	100	0	100	0	0	0	100	0	100	100	100	40	60	0	40	0	0	40	8
a-j	100	0	100	0	0	100	100	100	0	100	0	100	0	100	100	100	100	0	0	50	50	0	100	2

Appendix 2. Presence/absence of DNA bands in individual samples. Band 1–56 refer to primer combination g11, and band 57–73 refer to y10; ? = Indistinct bands.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
con005	1	1	1	1	1	1	1	1	1	0	0	0	1	1	?	0	1	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0
con111	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0
con112	1	1	1	1	1	1	1	1	?	0	0	0	1	1	1	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0
con115	1	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0
con165	1	1	1	1	1	1	1	1	1	0	0	0	1	1	?	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0
con166	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	1	0
con194	?	1	1	1	1	1	1	1	?	0	1	?	1	1	0	0	0	1	1	1	0	0	0	?	0	0	0	?	0	0	0	0	1	1	1	1	0
con200	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0
con201	1	1	1	1	1	1	1	0	1	0	1	1	1	?	0	0	0	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	1	0
odo126	1	1	1	1	1	1	1	0	1	1	0	0	0	?	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0
odo195	?	1	1	1	1	1	1	0	1	1	0	1	1	1	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	0
rhe015	?	0	1	1	1	1	1	0	1	1	?	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	?	0	1	?	1	0	1
rhe016	1	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1	0	1
rhe017	?	0	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	0	1	1	1	0	1
rhe089	?	0	?	?	0	?	?	0	1	?	0	1	0	0	1	?	1	1	0	0	1	1	0	1	0	0	0	1	1	0	0	0	1	?	1	0	0
rhe107	?	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	0	1
rhe106	1	0	1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1	0	0
rhe142	0	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	1	1	1	0	0
rhe144	1	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1	0	1
rhe146	?	0	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	1	1	1	0	1	1	1	0	1
rhe148	?	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	0	0
rhe156	1	1	1	1	1	?	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	0	0
rhe157	0	1	1	1	1	?	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0	0
rhe158	0	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	1	1	1	0	1
rhe159	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	1	1	1	0	1
rhe160	0	1	1	1	1	1	1	0	1	?	0	1	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	?	0	0	0	1	1	1	0	1
rhe177	?	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	1	0	1	1	1	1	0	1	1	1	0	1
rhe178	0	1	1	1	1	1	1	0	1	?	0	1	1	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	0	1
rhe181	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	1	0	1	1	?	1	1	0	0	1	1	0	0	1	1	1	1	0	1
rhe171	0	1	1	1	1	?	1	0	1	?	0	1	0	0	1	1	1	1	0	0	1	0	0	1	0	1	0	1	?	0	0	0	1	1	1	0	1
rhe092	0	1	1	1	1	1	1	0	1	?	0	1	0	1	1	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	0	0	1	1	1	0	0
rhe097	0	1	1	1	1	1	1	0	1	?	0	1	0	1	1	1	1	1	0	0	1	0	0	1	0	0	0	1	1	1	0	0	1	1	1	0	1
rhe173	0	0	?	1	1	?	?	0	1	1	?	1	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	0	1
rhe161	0	1	1	1	1	1	1	0	1	?	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	1	1	1	0	1
rhe163	0	0	1	1	1	1	1	0	1	?	0	1	0	0	1	1	1	1	0	0	1	1	0	1	0	0	0	1	1	0	1	0	1	1	1	0	1
rhe164	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	0	1	1	0	1	0	0	0	1	1	1	1	0	1	1	1	0	1
cor169	0	0	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	1	1	1	0	1
cor170	0	0	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	1	1	1	0	1
cor175	0	0	?	1	1	1	1	1	?	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1	0	1
cor176	0	0	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	0	1	1	0	1	0	1	1	0	?	0	1	1	1	0	1
cor179	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	1	0	1	1	0	?	0	1	1	1	0	1
cor180	0	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1	0	1
lit083	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1	0	0
lit084	0	0	1	1	1	1	1	0	1	?	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	?	0	1	1	1	0	0
lit086	0	1	1	1	?	1	1	0	1	?	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	1	1	1	0	1
gab102	0	0	1	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	1	1	0	0	1	1	1	0	1
gab109	0	1	1	1	1	1	1	0	?	0	1	0	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	0	0
gab185	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	0	0
gab186	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	0	0
run012	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	0	1	1	0	0	1	1	0	1	0	0	1	1	1	0	0	0	1	1	1	1	0
sua030	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	?	1	1	0	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	1	0
sua124	0	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	0	?	?	0	0	0	1	1	1	1	0
heu025	0	0	1	1	?	1	1	0	1	?	0	1	0	0	1	?	?	1	0	0	1	1	1	1	0	0	0	1	?	0	0	0	1	1	1	?	0
nig003	0	0	?	?	?	?	?	0	1	?	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	1	?	?	0	0	0	1	1	1	0	0
nig007	0	?	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0	0	0	1	1	1	0	0
aus049	0	?	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0	0	0	1	1	1	0	0
aus152	0	0	1	1	1	?	1	0	1	0	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	?	1	1	1	0	0	0	1	1	1	0	0
aus153	0	0	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	0	?	1	0	?	?	1	?	0	0	0	1	1	1	0	0
aus154	0	0	1	1	1	?	?	0	1	?	0	1	0	0	1	1	1	1	0	0	1	1	?	1	0	0	1	1	1	0	0	0	1	1	1	0	0
ibe167	0	1	1	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	0	0	0	1	1	1	0	0
ibe168	0	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	?	0	0	0	1	1	1	0	0
ibe182	0	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	?	1	1	1	0	0	0	1	1	1	0	0
ibe188	0	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	?	1	1	1	0	0	0	1	1	1	0	0
ibe039	0	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	0	0	1	1	1	0	0
sti081	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	1	0	1	1	1	0	1
sti131	0	0	?	?	1	?	1	0	1	1	0	?	0	0	1	1	1	?	0	0	1	1	1	1	0	0	0	1	0	0	1	0	1	1	1	0	1
wid041	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	1	0	1	1	0	1	0	1	1	1	0	1
wid044	0	0	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	0	1	1	1	0	0
wid047	0	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	0	1	1	1	0	0
wid132	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	0	1	1	1	0	1
wid133	0	0	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	?	0	1	1	1	0	0
min138	0	0	?	?	?	?	?	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	?	0	1	1	1	0	1
min068	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	?	0	0	1	0	1	1	1	0	1
min071	0	0	?	0	?	?	?	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	?	0	1	1	1	0	1
min072	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	1	0	1	1	1	0	1
min080	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	1	0	1	1	1	0	1
min135	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	1	0	1	1	1	0	1
min136	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	1	0	1	1	1	0	0
min140	0	0	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	1	0	1	1	1	0	1
a-j087	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	0	1	1	1	0	1
a-j141	0	1	1	1	1	1	1	0	1	0	0	1	0	0	1	0	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	0	1	1	1	0	1

Appendix 2. (continued)

	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73
con005	1	1	0	1	1	0	0	0	0	1	1	0	1	1	0	0	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0
con111	1	1	0	1	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	1	0	1	0
con112	1	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	1	0	0	0
con115	1	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	1	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0
con165	1	1	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0
con166	1	0	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0
con194	1	1	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
con200	0	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
con201	0	0	0	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
odo126	0	0	0	1	1	0	0	1	0	1	1	1	0	1	0	0	0	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	0	0	0	0
odo195	0	0	0	1	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
rhe015	0	0	?	0	0	0	1	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	1	1	1	1	1	0	0	1	1	0	1
rhe016	0	0	1	0	0	1	1	1	0	1	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	1	1	1	1	1	0	0	1	1	0	1
rhe017	0	0	1	0	0	1	1	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	1	1	1	1	1	0	0	1	1	0	1
rhe089	0	0	?	0	0	1	1	1	0	1	1	0	0	1	0	1	0	0	1	0	?	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1
rhe107	0	0	1	0	0	1	1	1	0	1	1	0	0	1	0	1	0	0	1	1	1	0	0	0	1	0	1	1	1	1	0	0	1	1	0	?
rhe106	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	1	1	1	1	1	0	0	1	1	0	1
rhe142	0	0	0	0	0	1	1	1	1	1	1	0	0	1	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
rhe144	0	0	1	1	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	1	1	1	1	1	0	0	1	1	0	1
rhe146	0	0	0	1	0	1	1	1	1	1	1	0	0	1	0	1	0	0	?	0	1	0	0	0	1	0	1	1	1	1	0	0	1	0	0	1
rhe148	0	0	1	1	0	1	0	?	0	1	1	0	0	1	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
rhe156	0	0	0	0	0	?	0	1	0	1	1	0	1	1	0	1	0	0	?	1	1	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1
rhe157	?	0	0	1	0	1	1	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1
rhe158	0	0	1	1	0	1	0	1	1	1	1	0	1	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
rhe159	0	0	1	1	0	1	1	1	0	1	1	0	0	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
rhe160	0	0	1	1	0	1	1	1	1	1	1	0	1	1	0	1	0	0	?	0	1	0	0	0	1	0	1	1	1	0	0	0	0	0	0	?
rhe177	0	0	1	1	0	1	1	1	?	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	1	1	1	0	1
rhe178	0	0	1	0	0	1	1	1	?	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	1	1	1	0	1
rhe181	1	0	0	1	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	1	1	1	1	1
rhe171	0	0	1	0	0	1	1	1	0	1	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	1	1	0	0	1
rhe092	0	1	1	1	0	1	1	1	0	1	1	0	1	1	1	1	0	0	1	0	1	0	0	0	1	1	1	1	0	1	0	0	1	0	0	1
rhe097	0	0	1	1	0	1	1	1	0	1	1	0	0	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	0	0	1	1	0	1
rhe173	0	0	1	0	0	1	1	1	0	1	1	1	1	1	0	1	0	0	1	1	?	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1
rhe161	0	0	1	1	0	1	?	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1
rhe163	0	0	1	0	1	1	0	?	0	1	1	0	0	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
rhe164	0	0	1	1	0	1	0	1	1	1	1	0	?	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1
cor169	0	0	1	1	0	1	?	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1
cor170	0	0	1	1	0	1	?	1	0	1	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1
cor175	0	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1
cor176	0	0	1	1	0	1	0	1	1	1	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	1	1	1	0	1
cor179	0	0	1	1	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	1	1	1	0	1
cor180	0	0	0	1	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	0	1	1	1	0	1	0	1	1	1	1	0	1	1	1	0	1
lit083	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	0	1	1	0	0	1	0	1	1	1	1	0	0	1	1	0	1
lit084	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	1	1	1	0	1
lit086	0	0	0	1	0	0	0	1	0	1	1	0	0	1	0	?	0	0	?	0	1	0	0	0	1	0	1	1	1	0	0	1	?	0	0	?
gab102	0	0	0	1	0	0	0	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	?	1	1	1	0	0	1	1	1	1
gab109	0	0	0	1	0	0	0	1	0	1	1	0	0	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
gab185	0	0	0	1	0	0	0	1	0	1	1	0	0	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
gab186	0	0	0	1	0	0	0	?	0	1	1	0	0	1	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
run012	1	1	0	1	1	1	1	1	0	1	1	0	0	1	0	1	0	0	1	1	1	0	1	1	1	0	1	1	1	1	0	0	1	1	1	1
sua030	1	1	1	?	0	?	0	1	0	1	1	1	0	1	0	1	0	0	0	1	1	0	1	1	1	0	1	1	1	1	0	0	1	1	1	1
sua124	1	1	1	1	0	1	1	?	0	1	1	?	0	1	?	1	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
heu025	1	1	0	1	1	?	0	0	0	1	1	1	0	1	0	1	1	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
nig003	0	0	0	?	0	?	0	1	0	1	1	0	0	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
nig007	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	1	1	0	0	1	1	0	1	1	1	1	0	0	1	1	1	1
aus049	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	1	1	0	0	0	1	0	1	1	1	1	0	0	1	1	1	1
aus152	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
aus153	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	1	1	0	0	0	1	1	1	1	1	1	0	0	1	0	0	1
aus154	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
ibe167	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	1	1	1	1
ibe168	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
ibe182	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	1	1	1	1
ibe188	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	1	1	1	1
ibe039	0	0	0	?	0	?	0	?	0	1	1	0	0	1	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
sti081	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	?	0	1	0	0	0	1	0	1	1	1	1	0	0	1	0	0	1
sti131	0	0	0	1	1	0	0	?	0	1	1	0	0	1	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
wid041	0	0	1	1	1	0	0	0	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	0	1	0	0	0	0	0
wid044	0	0	1	1	1	0	0	?	0	1	1	0	0	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
wid047	0	0	1	1	0	0	0	?	0	1	1	0	0	1	0	1	0	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
wid132	0	0	1	1	1	0	0	0	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	0	1	0	0	0	0	0
wid133	0	0	0	1	1	0	0	0	0	1	1	0	0	?	1	1	0	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
min138	0	0	0	1	0	0	0	1	0	1	1	0	0	1	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
min068	0	0	0	1	1	0	0	?	0	1	1	0	0	1	0	1	0	0	?	0	1	0	0	0	1	0	1	1	1	0	1	0	0	0	0	0
min071	0	0	0	1	1	0	0	?	0	1	1	0	0	1	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
min072	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	?	0	1	0	0	0	1	0	1	1	1	0	1	0	0	0	0	0
min080	0	0	0	1	1	0	0	?	0	1	1	0	0	1	0	1	0	0	?	0	1	0	0	0	1	0	1	1	1	0	1	0	0	0	0	0
min135	0	0	0	1	0	0	0	1	0	1	1	0	0	1	0	1	0	0	?	0	1	0	0	0	1	0	1	1	1	1	0	0	1	0	0	1
min136	0	0	0	1	1	0	0	?	0	1	1	0	0	1	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
min140	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	1	0	0	1
a-j087	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	1	1	0	1	0	1	0	?	1	1	?	0	0	0	0	0	1
a-j141	0	0	1	1	0	1	0	1	0	1	1	0	0	1	0	1	0	0	1	1	1	0	1	0	1	0	1	1	1	1	0	0	1	1	0	1