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Published in:
Plant Systematics and Evolution

DOI:
10.1007/s00606-008-0035-x

2008

Link to publication

Citation for published version (APA):
Ståhlberg, D., & Hedrén, M. (2008). Systematics and phylogeography of the Dactylorhiza maculata complex (Orchidaceae) in Scandinavia: insights from cytological, morphological and molecular data. *Plant Systematics and Evolution*, *273*(1-2), 107-132. https://doi.org/10.1007/s00606-008-0035-x

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Plant Syst Evol (2008) 273:107–132
DOI 10.1007/s00606-008-0035-x

ORIGINAL ARTICLE

Systematics and phylogeography of the *Dactylorhiza maculata* complex (Orchidaceae) in Scandinavia: insights from cytological, morphological and molecular data

David Ståhlberg · Mikael Hedrén

Received: 13 August 2007 / Accepted: 21 February 2008 / Published online: 7 May 2008
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Abstract Flow cytometry, morphometry and molecular markers [plastid DNA and internal transcribed spacers (ITS) of nuclear ribosomal DNA] were used to determine taxonomic and phylogeographic patterns in *Dactylorhiza maculata* s.l. from Scandinavia. A total of 238 individuals from 27 populations from throughout all of Scandinavia, including the adjacent Kola Peninsula of Russia, were analyzed. Diploid *D. maculata* ssp. *fuchsii* and autotetraploid *D. maculata* ssp. *maculata* are morphologically differentiated. Fragment size variants from 10 plastid DNA loci (seven microsatellite loci and three loci with indel variation) were combined to give 43 haplotypes. Three major groups of haplotypes were found. Group I haplotypes were prevalent in the north and the northeast, whereas Group II haplotypes were prevalent in the south and the southwest. Group III was represented by only a single haplotype and appeared to be the result of introgression from *D. incarnata* s.l. Group I and Group II haplotypes did not correspond with cytologically and morphologically defined *D. maculata* ssp. *fuchsii* or *D. maculata* ssp. *maculata*. Past introgressive gene flow rather than recent hybridization is envisaged. Intermediate Group I haplotypes between Group II and the rest of Group I were detected in a zone of contact in central Sweden, which may suggest plastid DNA recombination. The six ITS alleles scored showed strong positive correlation with taxonomy. All data sets obtained for ssp. *maculata* were significantly correlated with geography. Three different autotetraploid lineages are hypothesized. One lineage may represent postglacial immigration from the south and the other two lineages may represent eastern immigration routes. Morphology and ITS data suggested that subarctic populations of ssp. *maculata* should be recognized as var. *kolaënsis*.

Keywords *Dactylorhiza* · Flow cytometry · Introgression · ITS · Morphometry · Plastid DNA · Polyploidy

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Introduction

Dactylorhiza Necker ex Nevski is one of the most taxonomically investigated genera in the orchid family (cf. Pedersen 1998; Tyteca 2001). The genus is widespread (Eurasia, Northern Africa, Alaska), and consists of a confusing variety of forms that are difficult to sort into discrete taxa. Consequently, the number of species varies greatly, as reported by different authors, from 12 (Klinge 1898) to 75 (Averyanov 1990) species being recognized. Part of the taxonomic complexity could be explained by the frequent interpretation of aberrant populations and specimens as separate taxa (Bateman and Denholm 2003; Pillon and Chase 2007). However, more important explanations may be innate factors such as phenotypic plasticity, or that many taxa are young and have not yet acquired good distinguishing characters. Molecular data have shown that most species of *Dactylorhiza* form a polyploid complex that has undergone extensive reticulate evolution (summarized by Hedrén 2002). The majority of taxa are allotetraploids that have evolved repeatedly by hybridization among three broadly defined parental lineages: the *D. euxina* (Nevski) Czerep. lineage (restricted to Asia Minor and the Caucasus), the *D. incarnata* (L.) Soó s.l.

lineage and the *D. maculata* (L.) Soó s.l. lineage (Hedrén 1996, 2001a; Hedrén et al. 2001). Several critical taxa in Europe [i.e. taxa belonging to the *D. majalis* (Rchb.) P.F. Hunt & Summerh. s.l. aggregate] can be derived from *D. incarnata* s.l. and *D. maculata* s.l., among which the *D. maculata* s.l. lineage is invariably the maternal lineage, and is also the most genetically variable one (Hedrén 2003; Devos et al. 2003, 2006; Shipunov et al. 2004; Pillon et al. 2007). A better understanding of this variation, particularly in relation to morphology and geography, would thus provide deeper insights into the origin of allotetraploid taxa. This is an urgent issue also for conservation reasons, since many allotetraploid taxa in the *D. incarnata/maculata* polyploid complex are rapidly declining due to habitat loss (e.g. Janečková et al. 2006).

At least 30 taxa at the species, subspecies or variety level have been recognized in the *D. maculata* s.l. lineage (Soó 1960; Delforge 1995). Most authors recognize two cytologically defined subgroups. Within these, a minimum of three diploid taxa [*D. maculata* ssp. *fuchsii* (Druce) Hyl., *D. maculata* ssp. *saccifera* (Brongn.) Diklic. and *D. foliosa* (Sol. ex Lowe) Soó] and one tetraploid taxon (*D. maculata* ssp. *maculata*) are distinguished. The diploid number $2n = 40$ is widely accepted (Averyanov 1990; Dressler 1993; Baumann et al. 2006), although there is a single report of $2n = 20$ (Stepanov 1994). *Dactylorhiza maculata* ssp. *fuchsii* and ssp. *maculata* have overlapping distributions in northwestern Eurasia (Averyanov 1990), ssp. *saccifera* replaces ssp. *fuchsii* in southeastern Europe, Asia Minor and the Caucasus (M. Hedrén and D. Ståhlberg, personal observation), whereas *D. foliosa* is endemic to Madeira (Averyanov 1990).

Dactylorhiza maculata ssp. *maculata* is functionally an autotetraploid with four homologous chromosome sets that recombine freely during meiosis, as shown by allozymes (Hedrén 1996). The origin of ssp. *maculata* is debated. Jagiełło and Lankosz-Mróz (1986–1987) observed smaller dimensions of chromosomes in tetraploids compared to diploids, and sequences of both internal and external transcribed spacers of nuclear ribosomal DNA (ITS and ETS nrDNA, respectively; Pridgeon et al. 1997; Bateman et al. 2003; Devos et al. 2005), as well as amplified fragment length polymorphisms (AFLPs; Hedrén et al. 2001), have revealed differences between ssp. *maculata* and present-day diploids. Plastid DNA markers have indicated a slight dominance of certain haplotypes in diploids and other in tetraploids (Hedrén 2003; Devos et al. 2003, 2006; Pillon et al. 2007). Devos et al. (2005) noted similarities in ITS and ETS between *D. maculata* ssp. *maculata* and *D. foliosa* and suggested an autotetraploid origin from a *D. foliosa*-like ancestor. However, the sampling efforts have almost invariably been biased towards certain biogeographic regions, and particularly towards areas where diploid and tetraploid taxa are morphologically distinct, viz. western Continental Europe (cf. morphometric studies performed by Dufrêne et al. 1991; Tyteca and Gathoye 2004) and the British Isles (Heslop-Harrison 1951; Bateman and Denholm 1989), but also the southern part of Scandinavia (Heslop-Harrison 1951; Reinhard 1985). In contrast, there is little molecular data from, for example, Central Europe where diploid and tetraploid cytotypes often are indistinguishable (Groll 1965; Vaucher 1966; Scharfenberg 1977; Vöth 1978; Vöth and Greilhuber 1980; Jagiełło 1986–1987; Jagiełło and Lankosz-Mróz 1986–1987; Reinhardt 1988; Gölz and Reinhard 1997; Bertolini et al. 2000). Combined plastid DNA and ITS data have been obtained from morphologically difficult populations in northwestern Russia and a complex relationship between diploid *D. maculata* ssp. *fuchsii* and tetraploid *D. maculata* ssp. *maculata* has been revealed (Shipunov et al. 2004, 2005; Shipunov and Bateman 2005). No molecular data have been obtained from the northern part of Scandinavia. Morphologically conspicuous tetraploids have sometimes been interpreted as independent autotetraploid lineages (e.g. the form "*sudetica*" in Central Europe; Devillers and Devillers-Terschuren 2000). In polyploid groups other than *Dactylorhiza*, recurrent polyploidization (including autopolyploidization) is a common phenomenon (Ramsey and Schemske 1998; Soltis and Soltis 1999; Soltis et al. 2003). In fact, there are few examples of well-studied polyploid taxa for which only a single origin appears likely. Accordingly, it could be hypothesized that *D. maculata* ssp. *maculata* has arisen several times by autopolyploidization.

Classification in Scandinavia

Most field floras treating the Nordic countries only recognize diploid *D. maculata* ssp. *fuchsii* and tetraploid *D. maculata* ssp. *maculata*, but both taxa are considered to be morphologically variable and their taxonomic rank varies with reports by various authors (Krok and Almquist 1994; Mossberg and Stenberg 2003; Lid and Lid 2005). *Dactylorhiza maculata* ssp. *maculata* occupies a wide range of open, natural and semi-natural habitats: tussocks and margins in bog–fen complexes, pastures, mountain meadows and coastal moorland. *Dactylorhiza maculata* ssp. *fuchsii* typically grows in more shaded habitats in soils with higher pH. Compared with ssp. *maculata*, ssp. *fuchsii* characteristically has a pronounced three-lobed labellum (Fig. 1) and the lowest leaf is broad and obtuse. However, both taxa are very variable and overlap in supposedly diagnostic features. Mixed populations occur in heterogeneous environments. A few cytological investigations have been performed in Scandinavia (Löve and Löve 1944; Afzelius 1958; Heslop-Harrison 1951; Knaben and Engelskjön 1967; Aagaard et al. 2005), but there are no reports

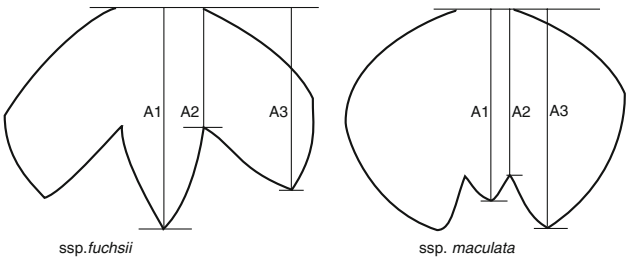

Fig. 1 Labellum shape of *Dactylorhiza maculata* ssp. *fuchsii* and *D. maculata* ssp. *maculata*. Labellum shape index (Heslop-Harrison 1948) is defined as $I = 2 \times A1/(A2 + A3)$

of triploid hybrids, as has been found elsewhere in Europe (Groll 1965; Averyanov 1977; Vöth and Greilhuber 1980; Gathoye and Tyteca 1989).

In addition to *D. maculata* ssp. *maculata* and *D. maculata* ssp. *fuchsii*, a varying number of taxa have been discussed in the literature. Most of these taxa are defined on the basis of ecology/biogeography and there is no consensus regarding morphological delimitations and taxonomic ranks (Table 1).

Diploid populations, often consisting of dwarfed plants with strong anthocyanin pigmentation, from subarctic areas have occasionally been assigned to *D. psychrophila* (Schltr.) Aver. (Heslop-Harrison 1951; Averyanov 1990; Delforge 1995). Other authors have recognized tetraploid populations of "*psychrophila*" from mountain areas in Central Europe (von Wegener 1966; Vöth and Greilhuber 1980).

Dactylorhiza maculata ssp. *maculata* var. *kolaënsis* (Montell) Soó [=*D. maculata* ssp. *montellii* (Verm.) Soó] was originally applied to tetraploid populations from northernmost Scandinavia and the Kola Peninsula (Montell 1947; Vermeulen 1947; Väre 2004). Recent authors have also assigned populations of ssp. *maculata* from this area to var. *kolaënsis* (Breiner and Breiner 2001). Moreover, some authors have included populations from similar habitats along the Scandinavian mountain range within var. *kolaënsis*, noting morphological similarities such as intense pigmentation and few flowers (Hylander 1966; Nilsson 1991; Delforge 1995; Baumann et al. 2006). Heslop-Harrison (1951) and later Sahlin (1960, 1980) argued that a western, oceanic taxon with morphological and ecological similarities with British tetraploids should be distinguished as *D. maculata* ssp. *ericetorum* (E.F. Linton) P.F. Hunt & Summerh. Tetraploid populations on coastal moorlands, particularly in southwestern Norway, should then typically be referred to as "*ericetorum*" (cf. Skrede 2001). *Dactylorhiza maculata* ssp. *elodes* (Griseb.) Soó has been applied to tetraploid populations consisting of plants with very slender habit, associated with boreal *Sphagnum* bogs, in contrast to ssp. *maculata* s.str. that has been applied to populations of robust plants from pastures and other open lowland habitats, mainly in the nemoral zone

Table 1 Treatment of lower taxa of *Dactylorhiza maculata* s.l. in European Floras and checklists (cf. Bateman and Denholm 2003). Hylander (1966) and Karlsson (1997) have only considered the Nordic countries

Taxon	Ploidy level	Vermeulen (1947)	Soó (1960)	Hylander (1966)	Senghas (1968)	Nelson (1976)	Sundermann (1980)	Soó (1980)	Buttler (1986)	Averyanov (1990)	Delforge (1995)	Karlsson (1997)	Baumann et al. (2006)
deflexa	4× (?)	-	-	-	-	-	-	-	x	-	kol	-	-
elodes	4×	x	x	-	x	eri	-	x	x	x	x	-	mac
ericetorum	4×	?	-	-	elo	x	-	-	x	x	x	?	mac
fuchsii	2× (4×[a])	x	x	x	x	x	x	x	mey	x	x	x	x
hebridensis	2×	?	-	-	/	/	?	-	-	x	-	-	/
kolaënsis	4×	mon	x	x	mon	-	-	-	mon	x	x	mon	mon
maculata	4×	x	x	x	x	eri	x	x	x	x	x	x	x
meyeri	2×	?	x	-	-	fuc	-	-	x	x	?	-	-
montellii	4×	x	kol	kol	x	-	-	-	x	kol	kol	x	x
psychrophila	2× (4×)	x	x	-	x	-	-	x	sud	x	x	-	x
sudetica	4×	?	x	kol	elo	-	-	-	x	x	-	-	/

[a] Populations from Central Europe

x = considered to occur in Scandinavia; / = not considered Scandinavian; - = not mentioned or accepted; ? = authors' intensions unclear

Taxon considered synonymous with *elo* elodes, *eri* ericetorum, *fuc* fuchsii, *kol* kolaënsis, *mac* maculata, *mey* meyeri, *mon* montellii, *sud* sudetica

(Vermeulen 1947; Delforge 1995). In Continental Europe and the British Isles, the relationships among "*elodes*", "*ericetorum*" and ssp. *maculata* s.s. have long been debated (Stephenson and Stephenson 1921; Godfery 1921; Vermeulen 1947; Van Straaten et al. 1988; Bateman and Denholm 2003).

In addition, some authors (Table 1) have recognized from northern Europe *D. maculata* ssp. *meyeri* (Rchb.f.) Tournay, *D. hebridensis* (Wilmott) Aver., *D. maculata* ssp. *sudetica* (Rchb.f.) Vöth and *D. maculata* ssp. *deflexa* Landwehr. The relationship between "*meyeri*" and ssp. *fuchsii* s.str. is unclear and the names have furthermore been used synonymously (e.g. Hagerup 1944); "*hebridensis*" has been treated as a subarctic element, in parallel with "*psychrophila*", but traditionally "*hebridensis*" is regarded as a heavily pigmented diploid taxon restricted to the British Isles (cf. Bateman and Denholm 1989); "*sudetica*" has been treated as a disjunct tetraploid subarctic-alpine taxon, but the relationship between populations of "*sudetica*" from Northern and Central Europe is unclear; "*deflexa*" has been referred to as a Scandinavian taxon morphologically intermediate between ssp. *fuchsii* and ssp. *maculata* s.str. Analysis of morphometric data obtained from Russian herbarium specimens has demonstrated the general indistinctiveness of these taxa (I. Blinova, Polar-Alpine Botanical Garden-Institute, Kirovsk, unpublished data).

Aims of the present study

In this study, we combine different data sets (cytology, morphology, plastid DNA markers and nuclear DNA markers) to gain an overview of the variation in *D. maculata* s.l. in Scandinavia. We address the question of whether it is possible to clearly distinguish between ssp. *fuchsii* and ssp. *maculata*, and we examine the potential existence of taxa at the lower levels outlined above. Particular attention is paid to geographic trends. By avoiding cytologically heterogeneous populations with putatively ongoing hybridization between ssp. *fuchsii* and ssp. *maculata*, we will be able to focus on patterns of historical gene flow. The possibility of multiple immigration routes and the hypothesis of multiple autotetraploid lineages are considered.

Materials and methods

Sampling

Field work was carried out between 2003 and 2005 in 27 populations of *D. maculata* s.l. (Table 2, Fig. 2). Between 5 and 20 individuals were randomly sampled in each population (yielding a total of 238 individuals). The populations were chosen to cover as much as possible of the biogeographic variation in Scandinavia. One population from the adjacent Kola Peninsula of Russia was included to improve the representation of taxonomically controversial northern populations. All populations were categorized as either diploid *D. maculata* ssp. *fuchsii* or as tetraploid *D. maculata* ssp. *maculata*, in accordance with the field Floras treating the Nordic countries (Hylander 1966; Krok and Almquist 1994; Mossberg and Stenberg 2003; Lid and Lid 2005). Only morphologically homogeneous populations were analyzed. Diploid populations were further classified into two ecologically/biogeographically defined types and tetraploid populations were classified into five such types (Table 2). The types may correspond to taxa referred to in the literature (Table 1). This approach was chosen as an unbiased way of analyzing taxonomically loosely defined entities.

Ploidy determination

The ploidy level of 40 individuals from 12 populations was confirmed by flow cytometry. In 19 individuals from three populations absolute DNA content was quantified in order to evaluate the accuracy (cf. Aagaard et al. 2005). The populations for the analyses were chosen as a geographically representative subset of all the populations (Table 3).

Leaves were mailed to Gerard Geenen, Plant Cytometry Services (Schijndel, The Netherlands). In order to isolate nuclei from plant cells, fresh leaf material was chopped in an ice-cold DNA buffer, using a method modified from Arumuganathan and Earle (1991). The buffer contained a DNA binding fluorochrome; 4′-6-diamidino-2-phenylindole (DAPI) was used in analyses of relative ploidy level, whereas propidium iodide (PI) was used in the analyses of absolute DNA content (DAPI binds specifically to adenine and thymine). In the latter procedure, RNase was added to the buffer. The mixture was passed through a 40-μm nylon filter and run through a flow cytometer (PAS II Partec GMbH). The fluorescence was measured by a photomultiplier and converted into voltage pulses processed electronically to yield peak signals. The florescence is expected to vary proportionally with DNA content (e.g. Husband and Schemske 1998). *Ilex crenata* 'Fastigiata' of known DNA content (2C = 2.16 pg; Gerard Geenen, unpublished data) was used as an internal standard in the analyses of absolute DNA content. Initially *Lactuca sativa* var. *capitata* (2C = 6.10 pg) was used, but the fluorescence peak of this standard was partly superimposed on those of diploid *D. maculata* ssp. *fuchsii.*

Morphology

A total of 35 quantitative and qualitative characters were measured on all 238 individuals (Table 4). Both floral and vegetative characters were taken into account. The

Table 2 Origin and classification of material

Population	Taxon	Location	Latitude	Longitude	Habitat	Ecological/ biogeographic classification	Sample size
A	*fuchsii*	Sweden, Skåne, Måryd	55°41′N	13°23′E	Mixed broadleaved forest	Nemoral/boreal type[a]	8
B	*maculata*	Sweden, Skåne, Mosslunda	55°59′N	14°04′E	Pasture	Nemoral type[b]	20
C	*maculata*	Sweden, Skåne, Gyetorp	56°01′N	14°27′E	Pasture	Nemoral type	9
D	*maculata*	Sweden, Skåne, Åraslöv	56°06′N	13°58′E	Eutrophic fen/meadow	Nemoral type	6
E	*maculata*	Sweden, Skåne, Kylen	56°27′N	13°57′E	Pasture	Nemoral type	5
F	*maculata*	Sweden, Blekinge, Målatorp	56°15′N	15°32′E	Pasture	Nemoral type	5
G	*fuchsii*	Sweden, Öland, Torp	56°36′N	16°37′E	Mixed broadleaved forest	Nemoral/boreal type	10
H	*fuchsii*	Sweden, Öland, Kåtorp	56°38′N	16°32′E	Mixed broadleaved forest	Nemoral/boreal type	6
I	*fuchsii*	Sweden, Östergötland, Ombergsliden	58°22′N	14°41′E	Mixed forest	Nemoral/boreal type	10
J	*maculata*	Sweden, Västmanland, Högfors	59°59′N	14°58′E	*Sphagnum* bog	Boreal type[c]	5
K	*maculata*	Sweden, Uppland, Långbromossen	60°02′N	18°21′E	*Sphagnum* bog	Boreal type	10
L	*maculata*	Sweden, Härjedalen, Klinken	62°43′N	12°18′E	Semi-natural meadow	Mountain type[d]	10
M	*fuchsii*	Sweden, Medelpad, Granboda	62°34′N	15°42′E	Spruce forest	Nemoral/boreal type	5
N	*maculata*	Sweden, Ångermanland, Villmyran	62°59′N	18°31′E	*Sphagnum* bog	Boreal type	10
O	*fuchsii*	Sweden, Jämtland, Vackermyran	63°28′N	15°20′E	Fen with spruce	Nemoral/boreal type	10
P	*fuchsii*	Sweden, Lycksele lappmark, Toskfors	64°51′N	17°57′E	Spruce forest	Nemoral/boreal type	10
Q	*maculata*	Sweden, Lycksele lappmark, Västansjö	65°45′N	15°05′E	Sloping fen	Mountain type	10
R	*fuchsii*	Sweden, Torne lappmark, Abisko	68°20′N	18°47′E	Fen with mountain birch	Diploid subarctic type [e]	10
S	*maculata*	Norway, Rogaland, Tungeneset	59°02′N	05°35′E	Coastal moorland	Oceanic type[f]	10
T	*fuchsii*	Norway, Telemark, Dalen	59°26′N	08°00′E	Roadside with tall herbs	Nemoral/boreal type	10
U	*maculata*	Norway, Hordaland, Rebnor	60°47′N	04°52′E	Coastal moorland	Oceanic type	10
V	*maculata*	Norway, Troms, Jøkelfjordeidet	70°04′N	22°03′E	Subarctic moorland	Tetraploid subarctic type[d]	10
W	*fuchsii*	Norway, Finnmark, Talvik	70°03′N	22°57′E	Mountain birch forest	Diploid subarctic type	5
X	*maculata*	Norway, Finnmark, Tårnvika	70°27′N	26°50′E	Subarctic moorland	Tetraploid subarctic type	10
Y	*maculata*	Norway, Finnmark, Smalfjord	70°25′N	28°06′E	Semi-natural meadow	Tetraploid subarctic type	5
Z	*maculata*	Norway, Finnmark, Kirkenes	69°42′N	30°01′E	Subarctic moorland	Tetraploid subarctic type	10
Ø	*maculata*	Russia, Kola Peninsula, Apatity	67°34′N	33°23′E	Mesotrophic fen	Tetraploid subarctic type	9

The ecological/biogeographic classifications may correspond to taxa referred to in the literature (see "Introduction" and Table 1); [a] *fuchsii* s.s; [b] *maculata* s.s; [c] *elodes*; [d] *kolaënsis*; [e] *psychrophila*; [f] *ericetorum*

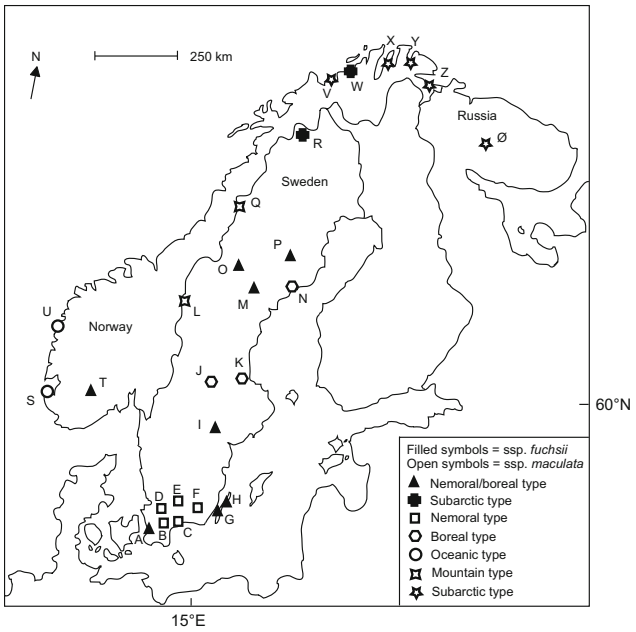

Fig. 2 Localities of the 27 sampled populations of *D. maculata* s.l.

Table 3 Flow cytometry; inferred ploidy level and absolute DNA content (mean ± SD)

Population	Taxon	Inferred ploidy level	Absolute DNA content (2C, pg)	CV (%)	*N*
A	*fuchsii*	2×			8
B	*maculata*	4×			7
C	*maculata*	4×	12.96 ± 0.241	8.0	4
C	*incarnata*[a] × *maculata*	3×	10.49	10.0	1
D	*maculata*	4×			9
D	*maculata*	6×			1
I	*fuchsii*	2×	6.97 ± 0.134	9.0	10
K	*maculata*	4×			10
O	*fuchsii*	2×			3
P	*fuchsii*	2×			2
R	*fuchsii*	2×	6.88 ± 0.168	13.5	4
V	*maculata*	4×			2
W	*fuchsii*	2×			2
X	*maculata*	4×			3

CV refers to mean coefficient of variation and *N* is number of investigated individuals

[a] *Dactylorhiza incarnata* s.str., 2C = 8.11 pg (D. Ståhlberg, unpublished data)

methodology was slightly modified from that of Bateman and Denholm (1985). All measurements were performed in situ. Floral characters were measured on one fully expanded flower from the base of the inflorescence. The color on the lower part of the labellum was matched to the nearest color block on a color chart of the Natural Colour System (Anonymous 2004). According to this system, a visually perceived color can be characterized by three attributes: hue (the relative position between two of four elementary chromatic colors in a circle, in this case red and blue), blackness (the relative position between white and black for a certain hue) and chromaticness (the degree of resemblance to a full chromatic color of the same hue).

Molecular methods

Fresh flowers from all 238 individuals were dried in silica gel (Chase and Hills 1991). Total genomic DNA was extracted by the CTAB method (Doyle and Doyle 1990). DNA was further cleaned with QIAquick columns (QIAGEN Ltd); following the manufacturer's protocol.

Ten polymorphic plastid DNA loci (seven microsatellite loci and three loci with indel variation) were amplified with a set of *Dactylorhiza*-specific primers (Table 5; cf. Ståhlberg 2007). Size variants (alleles) were scored and combined into multilocus genotypes (hereafter referred to as haplotypes).

From the nuclear genome, the ITS region (including the ITS1 spacer, the 5.8S rDNA gene and the ITS2 spacer) was analysed. Two pairs of *Dactylorhiza*-specific primers were used to amplify short length-variable fragments from different loci (Table 5). The amplified fragments were combined and interpreted as alleles, and the frequency of each allele was assessed—the repetitive nature of the ITS region means that the frequency of each allele can differ among individuals (cf. Shipunov et al. 2004; Pillon et al. 2007).

All plastid and nuclear fragments were amplified by an initial round of denaturing at 94°C for 2 min, followed by 40 cycles of 94°C for 1 min, 50–58°C (depending on primer pairs; Table 5) for 1 min, 72°C for 1 min 30 s, with a final step of 72°C for 10 min. PCR reactions were performed in a reaction volume of ca. 5 µl containing 3.9 µl ddH_2O, 0.5 µl 10× reaction buffer (100 mM Tris–HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.1 µl dNTPs (10 mM of each nucleotide), 0.0625 µl Cy5-labelled primer (1.5 pmol/µl), 0.025 µl unlabelled complementary primer (25 pmol/µl), 0.023 µl AmpliTaq Gold polymerase (5 U/µl; Applied Biosystems), and 0.4 µl template DNA (14 ng/µl). Some fragments were amplified simultaneously in the same PCR by multiplexing. The PCR product from each reaction was mixed with appropriate size standards to enable the exact size determination of the amplified fragments. Dye-labelled fragments were separated on an ALF Express II DNA analyser (GE Healthcare), and the size of the fragments was determined using ALFwin Fragment Analyser 1.03.01 software (GE Healthcare).

Table 4 List of morphological characters (modified after Bateman and Denholm 1985)

A. Floral characters

1. Labellum, length from base of spur entrance to apex of central lobe (mm)
2. Labellum, length from base of spur entrance to base of sinus separating central and right lateral lobe (mm)
3. Labellum, length from base of spur entrance to apex of right lateral lobe (mm)
4. Labellum shape index,[a] $I = 2 \times A1/(A2 + A3)$
5. Labellum, maximum width (mm)
6. Labellum color, hue[b] (arbitrary values ranging from 0 to 100)
7. Labellum color, blackness[b] (arbitrary values ranging from 0 to 100)
8. Labellum color, chromaticness[b] (arbitrary values ranging from 0 to 100)
9. Labellum markings, type of markings on a scale 0–5 (0 = no markings; 1 = spots; 2 = spots and dashes; 3 = dashes and loops; 4 = loops; 5 = ±solid blotch)
10. Labellum markings, distribution on a scale 0–3 (0 = no markings through to 3 = extensive coverage)
11. Labellum markings, contrast on a scale 0–3 (0 = no markings; 1 = pale; 2 = well-defined; 3 = bold)
12. Spur, length (mm)
13. Spur, curvature on a scale 1–5 (1 = strongly recurved through to 5 = strongly decurved)
14. Perianth markings, contrast on a scale 0–2 (0 = no markings; 1 = pale; 2 = bold)
15. Ovary, length (mm)
16. Anthocyanin pigmentation immediately below inflorescence on a scale 0–2 (0 = none; 1 = diffuse; 2 = heavy)
17. Bract, length at the base of the inflorescence (mm)
18. Bract, length halfway up the inflorescence (mm)
19. Bracts, anthocyanin pigmentation on a scale 0–2 (0 = none; 1 = diffuse; 2 = heavy).

B. Vegetative and general characters

20. Plant height (cm)
21. Inflorescence length (cm)
22. Number of flowers
23. Stem diameter immediately above lowermost sheathing leaf (mm)
24. Number of sheathing leaves
25. Number of non-sheathing leaves
26. Length of longest sheathing leaf (cm)
27. Maximum width of widest sheathing leaf (cm)
28. Relative positions of longest and widest sheathing leaves along the stem on a scale 1–3 (1 = longest above widest; 2 = longest is widest; 3 = longest below widest).
29. Shape of uppermost sheathing leaf (maximum width/length) on a scale 1–4 (1 = 0–10%; 2 = 10–25%; 3 = 25–50%; 4 ≥ 50%)
30. Shape of longest sheathing leaf (see 29)
31. Shape of lowest sheathing leaf (see 29)
32. Sheathing leaf markings, mean cover on a scale 0–5 (0 = no markings; 1 ≥ 0–6%; 2 = 6–12%; 3 = 12–25%; 4 = 25–50%; 5 ≥ 50%)
33. Sheathing leaf markings, distribution on a scale 1–5 (1 = slightly concentrated towards base through to 5 = extremely concentrated towards apex)
34. Sheathing leaf markings, mean shape on a scale 1–5 (1 = strongly longitudinally elongated through to 5 = strongly transversely elongated)
35. Sheathing leaf markings, mean diameter on a scale 1–5 (1 = ca. 1 mm; 2 = ca. 1.5 mm; 3 = ca. 2.5 mm; 4 = ca. 4 mm; 5 = ca. 6 mm)

[a] Heslop-Harrison (1948) (cf. Fig. 1)

[b] See text

Data analysis

Morphology

Morphological differentiation among populations was summarized using canonical variates analysis (CVA). In CVA the axes of variation are chosen to maximize the separation among predefined groups relative to the variation within groups. Populations were chosen as groups since they could be objectively defined. In the first analysis, all populations were included in order to highlight the overall relationship between *D. maculata* ssp. *fuchsii* and *D. maculata* ssp. *maculata*. Separate analyses were then performed on populations of either ssp. *fuchsii* or ssp. *maculata*. Characters B33-B35 (Table 4) were excluded from the analyses to avoid bias caused by series of blank

Table 5 List of primers used in the study. Initially, many loci were screened

Nr.	Locus, type of variation	Specific primers	Sequence 5′ → 3′	Ann. temp.
1	*trn*T–*trn*L intergenic spacer, polyA[a]	Cy5trnL5	CGA AAT CGG TAG ACG CTA CGC	57
		trnLR5	CGT TAG AAC AGC TTC CAT TG	
6	*psb*C–*trn*S pseudospacer, indel[b]	Cy5trnS2	AGA GTT TCA GGT CCT ACC TA	54.4
		psbC2	GTG TTC CTA ACT GCC CAC TT	
6B	*psb*C–*trn*S pseudospacer, indel[b]	Cy5trnS1	GGT TCG AAT CCC TCT CTC TC	54.4
		trnS2f	TAG GTA GGA CCT GAA ACT CT	
8	*rps*19–*psb*A intergenic spacer, polyT	Cy5HK7F	CAC CTA GAC ACT TAT CAT TC	54
		HK8R	CCG ATT TCT CCA AAT TTT CG	
9	*rps*19–*psb*A intergenic spacer, indel	Cy5HK9R	CTA GCT TCT GTG GAA GTT CC	54
		HK8F	CGA AAA TTT GGA GAA ATC GG	
10b	*psb*A–*trn*K exon 1 interg. spacer, polyA-TA-T	Cy5trnK1A	CCG ACT AGT TCC GGG TTC GA	56
		HK10F	GAA AGG CTT GTT ATT TCA CAG	
11b	*rpl*16 intron, polyA	Cy5F71	GCT ATG CTT AGT GTG TGA CTC GTT G	53
		F71R2	AGT TTA TAG TGG GGT CAG CC	
17	*trn*S–*trn*G interg. spacer, poly[T_nA(C,G)]	Cy5trnSf	GCC GCT TTA GTC CAC TCA GC	54
		trnSGr1	GGA TAA ATC CGT TTC GAA TC	
18	*trn*S–*trn*G intergenic spacer, polyTA	Cy5trnSGf2	CCT AAT TCT TAG AAA GAA TAT GAG	54
		trnSGr2	GAA TAG ATA TAG AAT CTT ACT C	
19	*trn*S–*trn*G intergenic spacer, polyT[c]	Cy5trnSGf3	GAG TAA TAG TGT TCT AAT AAG AG	58
		trnSGr3	CAG ACG CAG TCA AGA TAG CA	
i	ITS, indel[c]	Cy5ITS.d.fuc	ATT GAA TCG CTC CAT AAG AC	52
		ITS.d.fuc	ACC GCA TGA CGG GCC ATT CT	
ii	ITS, indel[c]	Cy5ITS.d.mac	TGT GCC AAG GTA AAT ATG CA	52
		ITS.d.mac	TAG GAG CAA ACA ACT CCA CA	

[a] Soliva and Widmer (1999)

[b] Hedrén (2003)

[c] Pillon et al. (2007)

values reflecting the absence of a single feature (i.e. leaf markings). Since the combined character labellum shape index (A4; Fig. 1) depends on three other characters, one of these (A3) was excluded from the analyses to avoid duplication. The relative contributions of each character to the canonical axes were inferred from raw canonical coefficients. To estimate the influence of environmentally dependent characters to the ordination patterns, all vegetative characters were excluded in a separate analysis. Discriminant analysis using SAS version 9.1 (SAS Institute 1990) was performed to evaluate how well individual plants could be classified into the predefined groups a posteriori. Discriminant analyses were performed using both populations and cytotypes as predefined groups.

Plastid DNA

All alleles for the different loci were treated as ordered characters assuming that mutations primarily follow a stepwise mutation model (Ohta and Kimura 1973; cf. Cozzolino et al. 2003), and squared distances between pairs of haplotypes were calculated according to Slatkin (1995) using Arlequin version 3.01 (Excoffier et al. 2005). A minimum spanning network was constructed to illustrate the minimum number of differences among haplotypes. Phenetic relationships among haplotypes were summarized by non-metric multidimensional scaling (NMDS) and phenetic relationships among populations were summarized by principal coordinates analysis (PCO) using PAST version 1.44 (Hammer et al. 2001). For the PCO analysis, the mean distances among haplotypes in every population pair were first calculated in order to take into account not only the proportion of different haplotypes among populations, but also the degree of differentiation among haplotypes.

Nuclear DNA

Mean ITS allele frequencies were calculated for each population. A PCO based on chord distances (Cavalli-Sforza and Edwards 1967) was performed in PAST to

describe the differentiation patterns between pairs of populations.

Comparisons among data sets

Pairwise correlations between distance matrices obtained for each data set (see above) were investigated by Mantel tests using Pearson's correlation coefficient (*r*) in NTSYSpc version 2.2 (Rohlf 2005). Each data set was also correlated to geographic distances. Separate tests were performed on populations of ssp. *fuchsii* and ssp. *maculata*, respectively.

Results

Cytology

The results from the flow cytometry are reported in Table 3. The analyzed populations were very uniform with respect to ploidy level. Exceptions were one hexaploid sample observed in a tetraploid population (D) from southern Sweden and one triploid sample observed in another tetraploid population (C) in the same region. Based on absolute DNA content it could be concluded that the latter was a hybrid between *D. maculata* ssp. *maculata* and *D. incarnata* s.str., which also occurred in the colony.

Morphology

Population mean and SD for morphological characters are listed in Appendix 1. In all ordinations, between 25 and 30% of the total variation was described by the first axis, whereas the second axis accounted for between 17 and 26% of the total variation (Figs. 3, 4, 5).

In the CVA of all populations (Fig. 3), populations of diploid *D. maculata* ssp. *fuchsii* were placed on the upper right, whereas populations of tetraploid *D. maculata* ssp. *maculata* were placed on the lower left, but as indicated from 95% confindence limits, there was an overlap. The variation within populations of both ssp. *fuchsii* and ssp. *maculata* was considerable, but the total variation was larger within ssp. *fuchsii* than in ssp. *maculata*. The relative contributions of different characters to the first two axes are illustrated as vectors. Labellum shape index (A4) and width of the widest sheathing leaf (B27) were the most discriminating characters (high values for diploid populations). No apparent differences in the ordination plot appeared when vegetative characters were excluded from the analysis. Leaf width was substituted by the leaf shape characters as especially important discriminating features (more slender leaves in ssp. *maculata*). A posteriori classification (discriminant analysis) of individual

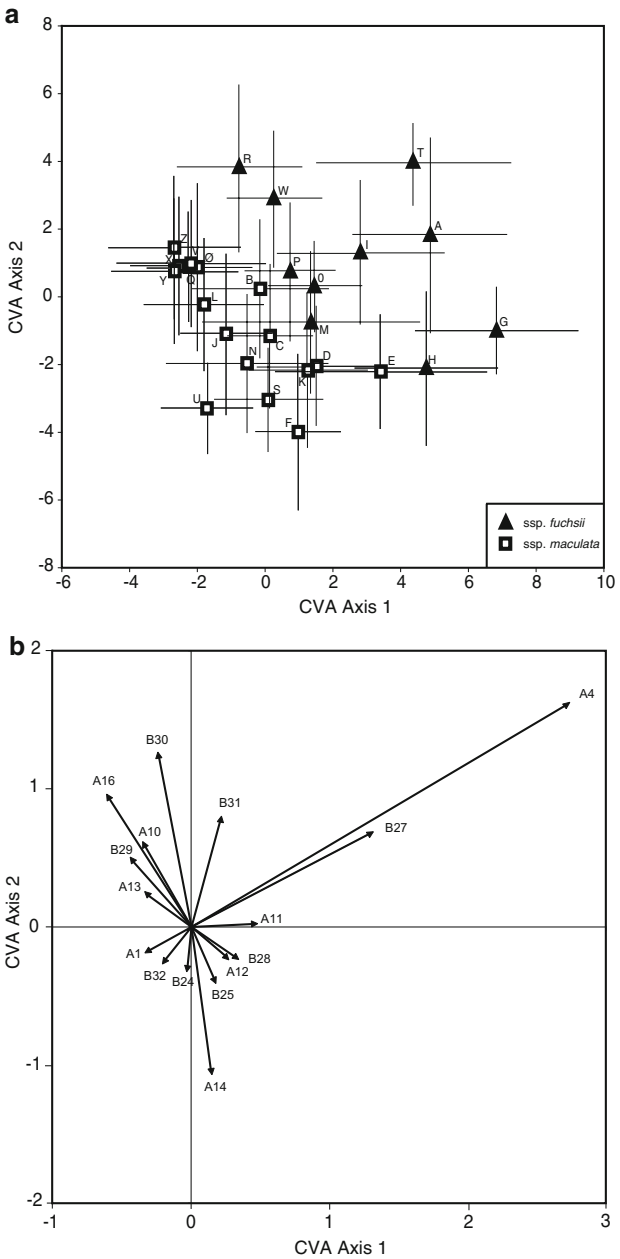

Fig. 3 Canonical variates analysis (*CVA*) of morphological data, when all populations are included; 25 and 17% of the total variation was described by the first two axes; 95% confidence limits for population means are indicated. The relative contributions of different characters (numbered according to Table 4) are shown as *vectors*. Characters with low discriminating impact are omitted

plants into populations resulted in misclassification of six specimens (3%). Five of these specimens belonged to ssp. *maculata* populations and were reclassified into other ssp. *maculata* populations. One ssp. *fuchsii* specimen was reclassified into another diploid population. A posteriori classification into cytotypes resulted in misclassification of 18 specimens (8%); 13 of these belonged to ssp. *maculata* and 5 to ssp. *fuchsii*.

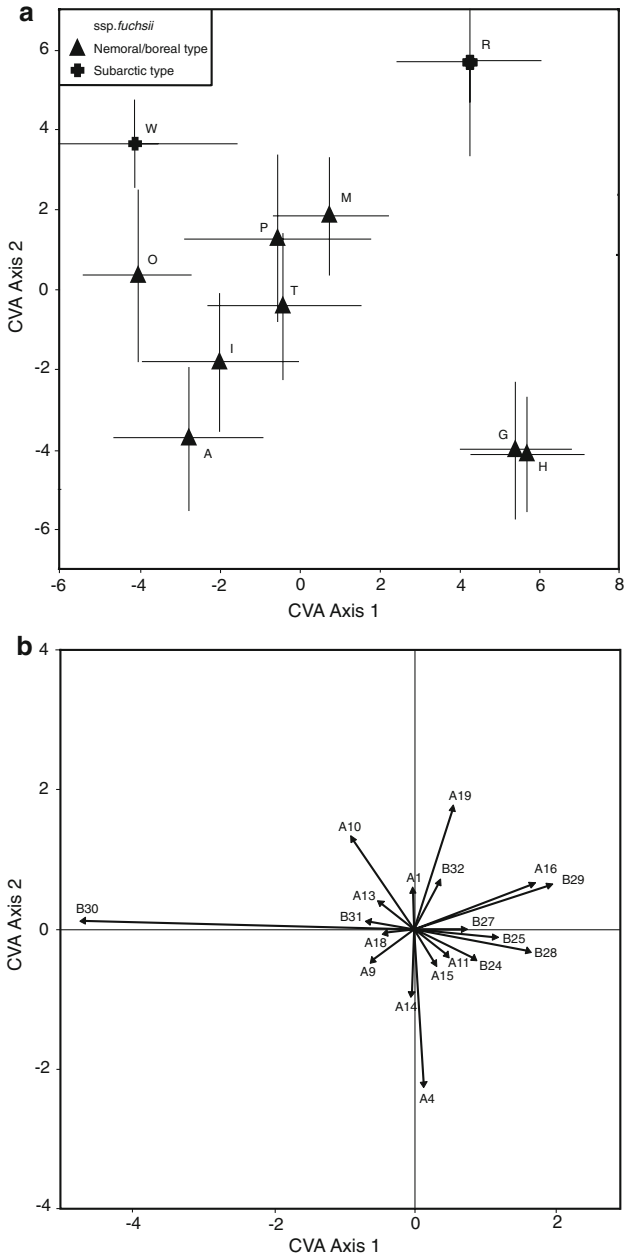

Fig. 4 **a** Canonical variates analysis of morphological data from populations of diploid *D. maculata* ssp. *fuchsii*; 30 and 26% of the total variation was described by the first two axes; 95% confidence limits for population means are indicated. **b** The relative contributions of different characters (numbered according to Table 4) are shown as *vectors*. Characters with low discriminating impact are omitted

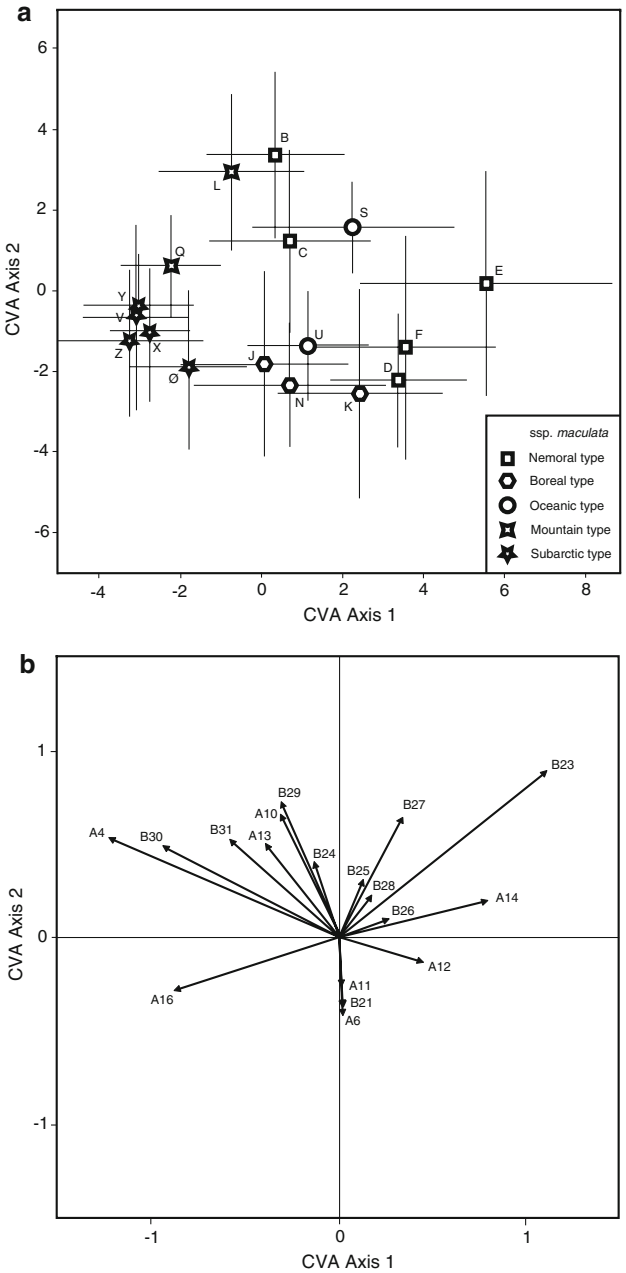

Fig. 5 **a** Canonical variates analysis of morphological data from populations of tetraploid *D. maculata* ssp. *maculata*; 25 and 21% of the total variation was described by the first two axes; 95% confidence limits for population means are indicated. **b** The relative contributions of different characters (numbered according to Table 4) are shown as *vectors*. Characters with low discriminating impact are omitted

In the CVA restricted to populations of ssp. *fuchsii* (Fig. 4) no groups of systematic relevance were revealed. The subarctic populations (R and W) were distant from each other on the first axis, but on the second axis they had similar values and were slightly separated from all other populations. Two populations (G and H) from the Baltic island of Öland were separated from the rest and grouped together, which should be ascribed to geographic proximity. The shape of the longest sheathing leaf (B30) and labellum shape index (A4) contributed most to the separation of populations of ssp. *fuchsii*; values of the latter character decreased northwards.

In the CVA using only populations of ssp. *maculata* (Fig. 5), subarctic and mountain populations were placed to the left. Populations associated with boreal *Sphagnum* bogs were placed in the lower centre. The oceanic

populations (S and U) were placed far apart. The nemoral lowland populations were very variable and showed no tendency to group together. Stem diameter (B23), which should be environmentally dependent, was the most discriminating character, with low values for populations from harsh northern habitats. However, three characters—labellum shape index (A4), shape of the longest sheathing leaf (B30) and anthocyanin pigmentation (A16)—had almost similar discriminating loadings to stem width, but operated in different directions. Anthocyanin pigmentation had a strong positive influence on the position of subarctic populations.

Plastid DNA markers

A total of 48 fragment size variants at 10 loci were scored and combined to give 43 haplotypes (Appendix 2). The haplotypes were numbered according to a larger data set that encompasses *Dactylorhiza* from all over Europe (M. Hedrén, S. Nordström and D. Ståhlberg, unpublished data). The distribution of individuals among haplotypes was highly skewed. The three most common haplotypes (H59, H143 and H58) were found in more than 40% of the individuals, whereas 20 haplotypes were found in just one individual. The number of haplotypes found in ssp. *maculata* was much higher than the number of haplotypes found in ssp. *fuchsii* (38 vs. 11). Similarly, 27 haplotypes were private to ssp. *maculata*, whereas only 5 haplotypes were private to ssp. *fuchsii*. No haplotypes unified any of the biogeographically defined groups. The distribution of haplotypes among populations is listed in Table 6.

Three distinct groups of haplotypes (Groups I, II and III) appeared in the NMDS (Fig. 6; stress = 0.14). Groups I and II both contained 21 haplotypes. Group I haplotypes were most common in ssp. *fuchsii* and Group II in ssp. *maculata*, but both groups were still represented in each taxon. Group III was very distant from Groups I and II (Fig. 7) and consisted of one single haplotype (H166) that appeared to be a typical *D. incarnata* s.l. haplotype (M. Hedrén, S. Nordström and D. Ståhlberg, unpublished data). It was

Table 6 Distribution of haplotypes among populations

Population	Taxon	Haplotype			*N*
		Group I	Group II	Group III	
A	*fuchsii*		149		8
B	*maculata*		123, 143, 148, 157, 160, 269, 299		20
C	*maculata*		121, 246, 268	166	9
D	*maculata*		123, 150, 317		6
E	*maculata*		113, 149, 150, 312		5
F	*maculata*		119, 143, 148, 294		5
G	*fuchsii*	23, 59, 76	149		10
H	*fuchsii*	59, *81*	148		6
I	*fuchsii*	59, 60, 236			10
J	*maculata*	59, *99*			5
K	*maculata*	22, 57, 60, *80*, *285*			10
L	*maculata*	*28, 288*	142, 143		10
M	*fuchsii*	59			5
N	*maculata*	57, 59, 60, *82*			10
O	*fuchsii*	59, 60			10
P	*fuchsii*	58			10
Q	*maculata*	23, 60	143		10
R	*fuchsii*	58, 59, 174, *252*			10
S	*maculata*		143		10
T	*fuchsii*	57, 58, 59			10
U	*maculata*	*285*	142, 143, 139, 273		10
V	*maculata*	23, 58, 59, 73			10
W	*fuchsii*	23, 58			5
X	*maculata*	23, 58, 59, 73			10
Y	*maculata*	59			5
Z	*maculata*	56, 59, 60			10
Ø	*maculata*	23, 58, 59, 65, *291*			9

The numbering of haplotypes is based on a larger data set that encompasses *Dactylorhiza* from all over Europe (M. Hedrén, S. Nordström and D. Ståhlberg, unpublished data). Group refers to major haplotype groups according to Fig. 6; intermediate Group I haplotypes are shown in italics. *N* sample size

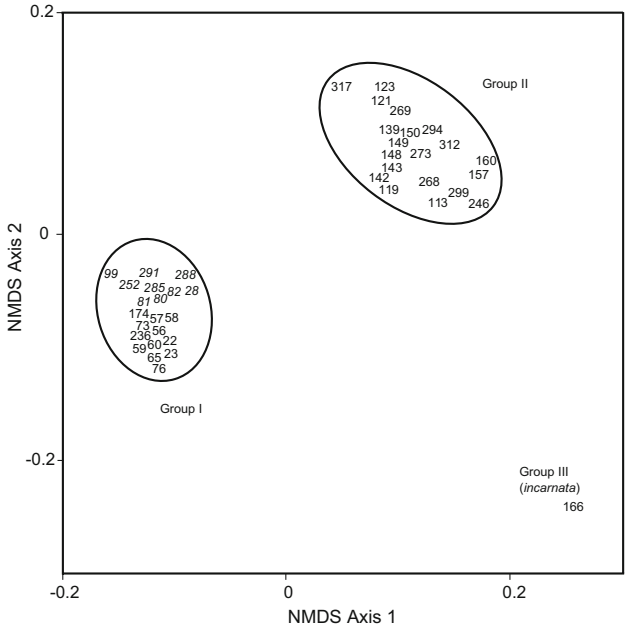

Fig. 6 Non-metric multidimensional scaling of the 43 plastid haplotypes. The numbering is based on a larger data set that encompasses *Dactylorhiza* from all over Europe (M. Hedrén, S. Nordström and D. Ståhlberg, unpublished data). Stress = 0.14. *Numbers in italics* correspond to intermediate Group I haplotypes according to the minimum spanning network (Fig. 7)

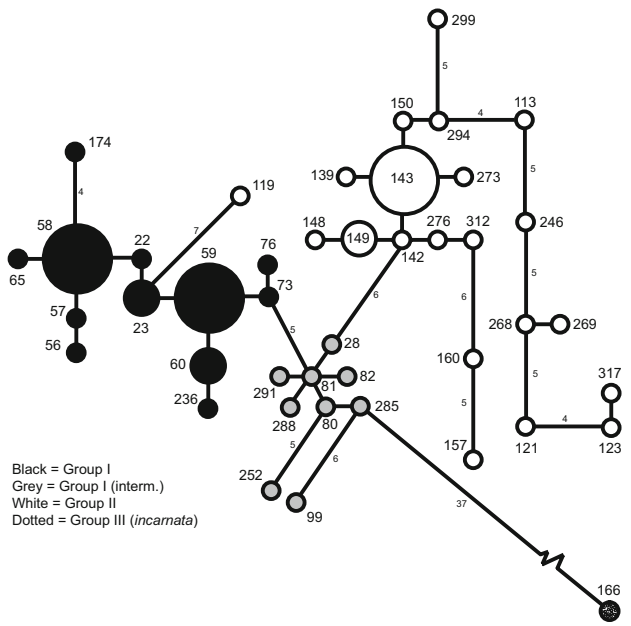

Fig. 7 Minimum spanning network of the 43 plastid haplotypes. The network is based on squared Euclidean distances. The numbering is based on a larger data set that encompasses *Dactylorhiza* from all over Europe (M. Hedrén, S. Nordström and D. Ståhlberg, unpublished data). Connection lengths above one unit are indicated. There are alternative links between H58 and H59, H73 and H236, H149 and H150, and H113 and H299. *Small circles* haplotypes detected in 1–10 individuals; *medium circles* haplotypes detected in 11–24 individuals; *large circles* haplotypes detected in 25–48 individuals

detected in a South Swedish individual of ssp. *maculata* in population C. The NMDS plot indicated that Group II was more heterogeneous than Group I, and this observation was supported by the minimum spanning network (Fig. 7), in which the total number of steps among haplotypes was largest in Group II. One Group II haplotype (H119) differed from other Group II haplotypes by two or more steps on one locus (Nr 6; Appendix 2), but was otherwise similar to other Group II haplotypes. In the minimum spanning network, a cluster of Group I haplotypes had an intermediate position between Group II and the rest of Group I.

The geographic distribution of Group I and Group II haplotypes is shown in Fig. 8. The frequency of Group I haplotypes increased towards the north and the northeast in populations of both ssp. *fuchsii* and ssp. *maculata*, whereas Group II haplotypes were predominant in southern and western populations, particularly in populations of ssp. *maculata*. Group I haplotypes of intermediate character were most common in Central Sweden.

In the PCO of populations (Fig. 9; the first two axes accounted for 78 and 8% of the total variation), no absolute differentiation between ssp. *fuchsii* and ssp. *maculata*, or among any biogeographically defined groups, appeared. Nine of ten populations of ssp. *fuchsii* formed a coherent group together with mainly northern and northeastern populations of ssp. *maculata*. Group I haplotypes dominated in all these populations. Subarctic populations of ssp. *maculata* were separated from mountain populations of ssp. *maculata*. The remaining populations, dominated by Group II haplotypes, were widely scattered in the ordination plot. Three nemoral lowland populations of ssp. *maculata* were placed together. Population K (ssp. *maculata*) from Central Sweden had a marked frequency of Group I haplotypes of intermediate character and so received a solitary position in the plot.

Nuclear DNA markers

Six different ITS alleles were scored [Table 7; numbered according to Pillon et al. (2007)]. All six alleles were present in ssp. *maculata*, whereas only three alleles were present in ssp. *fuchsii*. In individual plants of ssp. *maculata* up to four different alleles were scored. Only two individuals of ssp. *fuchsii* displayed more than two different alleles. Allele frequencies for each population are listed in Table 7. Allele I was present in all populations of ssp. *maculata*. In subarctic ssp. *maculata* populations the frequency of this allele varied between 9 and 16%. In all other ssp. *maculata* populations the frequency was considerably higher (30–87%). Allele I was almost completely absent from ssp. *fuchsii*; it was detected in only two populations, and then only in low proportions (<3%). Allele V generally occurred in higher frequencies in populations of ssp. *fuchsii*

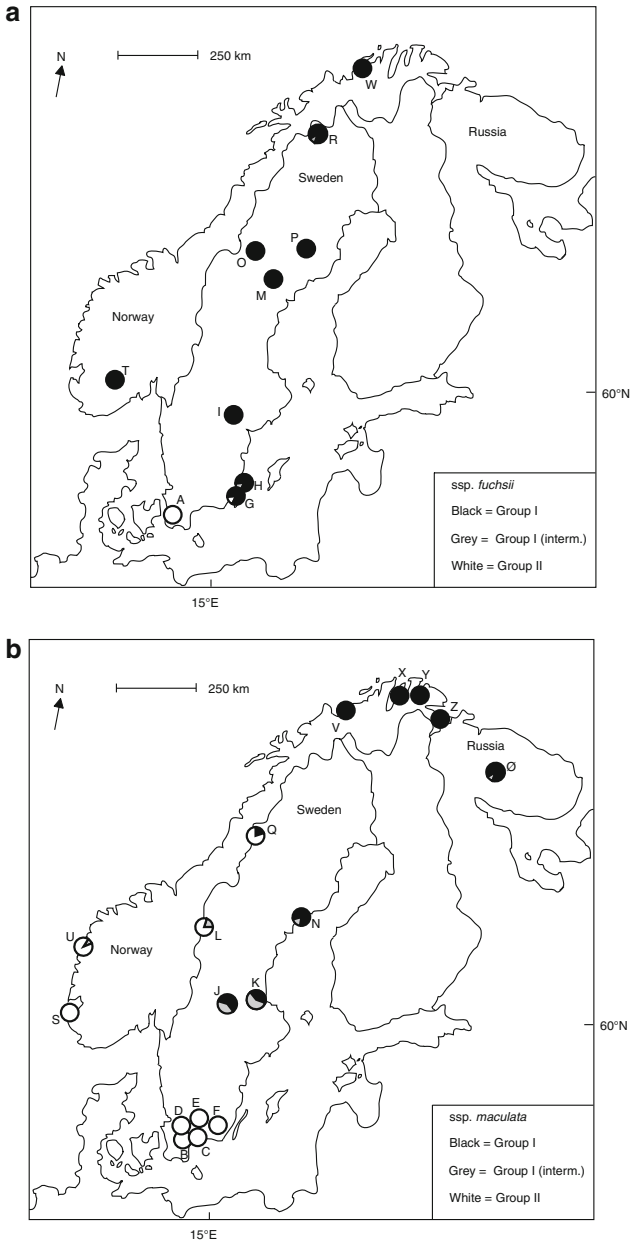

Fig. 8 Geographic distribution of Group I and Group II plastid haplotypes (see Figs. 6, 7) for **a** diploid *D. maculata* ssp. *fuchsii* and **b** tetraploid ssp. *maculata*. A single Group III (*incarnata*) haplotype was detected in one ssp. *maculata* individual in population C (not indicated on the map)

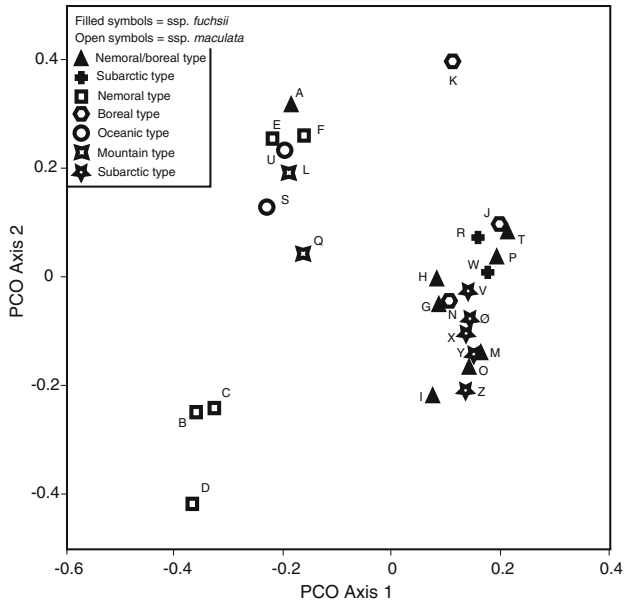

Fig. 9 Principal coordinates analysis of plastid haplotype data from all populations; 78 and 8% of the total variation was described by the first two axes

(38–91%) than in populations of ssp. *maculata* (1–46%). The frequency of this allele increased towards the north and the northeast in populations of ssp. *maculata*. Allele X is characteristic of *D. incarnata* s.l. (Pillon et al. 2007). It was scored in the same ssp. *maculata* individual from population C that displayed the private *D. incarnata* s.l. haplotype H166. In the PCO (Fig. 10; the first two axes accounted for 66 and 12% of the total variation), ssp. *fuchsii* was separated from ssp. *maculata* on the first axis. Subarctic populations of ssp. *maculata* occupied intermediate positions. No other groupings of taxonomic or phylogeographic relevance were evident.

Comparisons among data sets

The morphological and geographic distance matrices were significantly correlated for populations of ssp. *fuchsii*, as well as for populations of ssp. *maculata* ($r = 0.507$ and 0.396, respectively; $P < 0.01$). Plastid haplotype differentiation and geographic distances were significantly correlated only for ssp. *maculata* ($r = 0.556$; $P < 0.001$). The same was true for differentiation in ITS when compared to geographic distances ($r = 0.637$; $P < 0.001$), morphological differentiation versus haplotype differentiation ($r = 0.401$; $P < 0.001$), morphological differentiation versus ITS differentiation ($r = 0.637$; $P < 0.001$) and haplotype differentiation versus ITS differentiation ($r = 0.454$; $P < 0.01$).

Discussion

Cytotype differentiation

Flow cytometry invariably confirmed the field classifications of the populations into either diploid *D. maculata* ssp. *fuchsii* or tetraploid *D. maculata* ssp. *maculata*. The detection of a hexaploid plant in a tetraploid population

Table 7 Mean ITS allele frequencies (%)

Population	Taxon	Allele I 75, 72 bp	Allele IIIa 75, 80 bp	Allele IIIb 75, 77 bp	Allele IV 75, 78 bp	Allele V 70, 80 bp	Allele X 73, 80 bp	*N*
A	*fuchsii*	0.0	0.0	40.9	0.0	59.1	0.0	8
B	*maculata*	67.9	0.0	19.8	9.2	3.1	0.0	20
C	*maculata*	66.4	10.2	16.7	0.0	2.4	3.5	9
D	*maculata*	74.4	10.6	12.6	0.0	2.4	0.0	6
E	*maculata*	68.1	7.5	3.1	0.0	1.3	0.0	5
F	*maculata*	86.9	0.0	9.5	0.0	3.7	0.0	5
G	*fuchsii*	2.2	0.0	51.0	0.0	46.8	0.0	10
H	*fuchsii*	0.0	0.0	58.3	0.0	41.7	0.0	6
I	*fuchsii*	0.0	0.0	52.5	0.0	37.5	0.0	10
J	*maculata*	29.6	0.0	46.5	0.0	23.9	0.0	5
K	*maculata*	76.3	0.0	0.0	0.0	23.7	0.0	10
L	*maculata*	63.4	0.0	28.0	0.0	8.6	0.0	10
M	*fuchsii*	0.0	0.0	18.9	0.0	81.0	0.0	5
N	*maculata*	66.9	0.0	0.0	0.0	33.1	0.0	10
O	*fuchsii*	0.0	0.0	11.1	0.0	88.9	0.0	10
P	*fuchsii*	0.3	0.0	9.1	0.0	90.6	0.0	10
Q	*maculata*	37.4	0.0	43.6	0.0	19.1	0.0	10
R	*fuchsii*	0.0	0.0	49.2	0.0	50.8	0.0	10
S	*maculata*	84.4	0.0	0.0	0.0	15.6	0.0	10
T	*fuchsii*	0.0	0.0	14.8	0.0	85.2	0.0	10
U	*maculata*	65.9	0.0	30.1	0.0	4.0	0.0	10
V	*maculata*[a]	12.0	0.0	32.4	0.0	45.6	0.0	10
W	*fuchsii*	0.0	0.0	45.8	0.0	54.2	0.0	5
X	*maculata*[a]	14.2	0.0	45.7	0.0	30.1	0.0	10
Y	*maculata*[a]	9.3	0.0	50.5	10.0	30.1	0.0	5
Z	*maculata*[a]	14.5	0.0	44.3	0.0	41.1	0.0	10
Ø	*maculata*[a]	16.0	0.0	50.4	0.0	33.6	0.0	9

The alleles are numbered according to Pillon et al. (in press). Each allele is comprised of two fragments (from the primer combinations i and ii, respectively; Table 5). *N* sample size

[a] Subarctic populations of var. *kolaënsis*

could be explained by incidental formation of unreduced gametes (cf. Averyanov 1979). Our values of absolute DNA content are comparable to the estimates of Aagaard et al. (2005), but our estimates systematically became somewhat higher, which could be due to the use of different internal standards.

In contrast to several observations made in Central Europe (e.g. Scharfenberg 1977; Vöth 1978), our morphometric analyses resulted in a reasonably good correlation on an individual plant level between cytotype and morphology. Thus, phenetic division of specimens into either diploid *D. maculata* ssp. *fuchsii* or tetraploid *D. maculata* ssp. *maculata* is feasible (Fig. 3). This conclusion accords with previous data obtained from some Swedish localities by Heslop-Harrison (1951). However, the connection is more ambiguous than has been reported from the British Isles (e.g. Heslop-Harrison 1951) and from western Continental Europe (e.g. Tyteca and Gathoye 2004). In accordance with general belief (Delforge 1995; Baumann et al. 2006), labellum shape and width (shape) of the widest leaf are the most effective characters at distinguishing ssp. *fuchsii* from ssp. *maculata*. The values of these characters in Scandinavian populations are comparable to data obtained from elsewhere in Europe (Table 8).

To achieve a more satisfying determination of Scandinavian ssp. *fuchsii* and ssp. *maculata*, habitat features should also be taken into account. Even if habitat characterization was not a major objective of this study, it was observed that populations of ssp. *maculata* were associated with open grasslands or moorlands, whereas populations of ssp. *fuchsii* were to a large extent associated with semi-open woodlands on fertile soils. Averyanov (1990) argued that diploid and tetraploid populations in Russia can be separated by ecology, although they sometimes approach each other in morphology. On the other hand, complete lack of correspondence between cytotype and ecology has been reported from areas in Central Europe (Jagiełło and Lankosz-Mróz 1986–1987; Reinhardt 1988). A detailed study of ecological differentiation in cytologically mixed populations of *D. maculata* s.l. in Scandinavia is presented in a separate paper (Ståhlberg and Hedrén 2007).

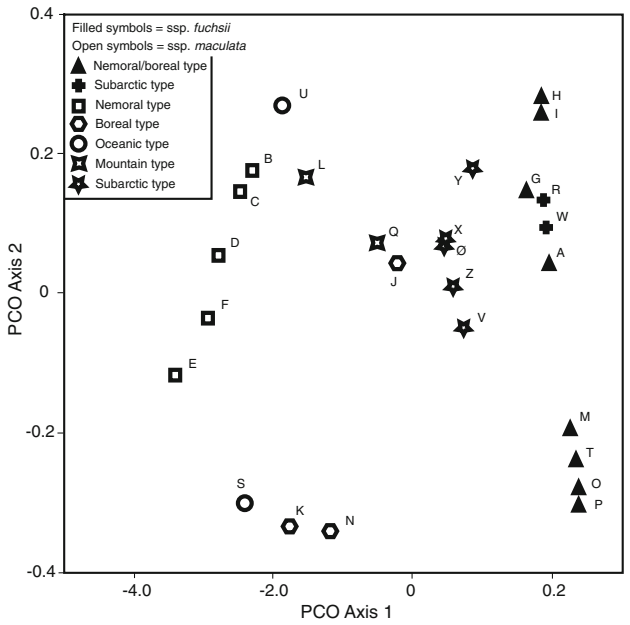

Fig. 10 Principal coordinate analysis of ITS data from all populations; 66 and 12% of the total variation was described by the first two axes

Plastid DNA variation

We found three major groups of plastid haplotypes (Fig. 6), with a slight dominance of Group I haplotypes in ssp. *fuchsii* and Group II haplotypes in ssp. *maculata*. Only one haplotype belonged to Group III. It was detected in a ssp. *maculata* sample, but appeared to be a typical *D. incarnata* s.l. haplotype (M. Hedrén, S. Nordström and D. Ståhlberg, unpublished data; discussed below). Similar overall patterns have previously been found in other studies of the *D. incarnata/maculata* polyploid complex from elsewhere in Europe (Hedrén 2003; Devos et al. 2003, 2006; Shipunov et al. 2004; Pillon et al. 2007). However, these studies rely on a smaller number of plastid loci, and the pattern of long distances between haplotypes from different groups relative to the short distance within groups is less evident. We found that the level of variation differed strongly among loci (Appendix 2). Some loci are more likely to be subjected to backmutations and parallel mutations than others. The combined use of many loci should therefore improve the strength of the interpretation.

Table 8 Comparison of diagnostic characters among studies. Both taxa are considered in a broad sense

Character	Area	Taxon	
		ssp. *fuchsii*	ssp. *maculata*
Labellum shape index (Fig. 1)	Scandinavia[a]	1.3–1.7	1.1–1.4
	Western Continental Europe[b]	1.4–1.5	1.1–1.2
	British Isles[c]	>1.2	<1.2
Widest leaf (mm)	Scandinavia[a]	1.6–3.0	0.6–2.3
	Western Continental Europe[b]	1.8–3.8	1.4–2.5
	British Isles[c]	>1.5	<1.5

[a] This study

[b] Tyteca and Gathoye (2004)

[c] Bateman and Denholm (2003)

Patterns of introgressive gene flow

The observed genetic structure in populations of ssp. *fuchsii* and ssp. *maculata* could be explained by three temporal aspects of gene flow.

(1) *Autopolyploidization* (i.e. formation of tetraploid *D. maculata* ssp. *maculata* from diploid *D. maculata* ssp. *fuchsii*) Studies using AFLPs (Hedrén et al. 2001) and, to a lesser extent, allozyme markers (Hedrén 1996), have indicated qualitative differences between ssp. *fuchsii* and ssp. *maculata* in Scandinavia. We found that the DNA content of the tetraploids was not double that of the diploids, which would have been expected if the tetraploids had originated recently from local diploids (Table 3). Morphological and ecological differences also point at a considerable period of separate history. We therefore conclude that ssp. *maculata* in Scandinavia has not evolved in situ from present-day populations of ssp. *fuchsii*. Our ITS data support this view (Fig. 10, Table 7). Allele I was essentially private to ssp. *maculata*. It was detected in almost all individuals of ssp. *maculata* and found in all populations, but was only occasionally detected in ssp. *fuchsii*. Allele I most likely was present in the diploid ancestor(s) that gave rise to ssp. *maculata*. A complementary pattern is shown by Allele V, which generally occurred in higher frequencies in populations of ssp. *fuchsii* than in populations of ssp. *maculata*. Pillon et al. (2007) found the same alleles to be taxonomically discriminating between these two taxa in other parts of Europe.

Studies of other genera [e.g. Van Dijk and Bakx-Schotman (1997) on *Plantago media*; Segraves et al. (1999) on *Heuchera grossulariifolia*] have shown that autopolyploid taxa can arise recurrently. In general, recurrent autopolyploidization is thought to be a common phenomenon (Ramsey and Schemske 1998). The fact that populations of ssp. *maculata* were fixed for either Group I or Group II plastid haplotypes throughout large parts of its Scandinavian distribution area (Fig. 8b) may indicate a past pattern of recurrent formation of different autotetraploid ssp. *maculata* lineages, from diploid predecessors containing Group I haplotypes as well as from predecessors containing Group II haplotypes. The split of haplotypes into Group I and Group II may then be ancient and pre-date the origin of the autotetraploids. Analyses of sequence

data, including comparisons with outgroups, have indicated that Group I haplotypes have probably been derived from Group II haplotypes (Hedrén 2003).

(2) *Past gene flow* Since all populations in this study were cytologically homogeneous (i.e. either diploid or tetraploid) the mixed pattern of Group I and Group II haplotypes observed in populations of both ssp. *fuchsii* and ssp. *maculata* should be due to past gene flow. Such a scenario is further supported by the contrasting distributions of Group I and Group II haplotypes (Fig. 8). Glaciations and climatic shifts during the Pleistocene may have repeatedly brought various tetraploid and diploid lineages together, forming hybrid zones in disturbed, mosaic or intermediate habitats. Holocene migration routes may have further promoted introgressive gene flow and plastid capture by putting different lineages into contact (cf. Petit et al. 2003). Dispersal and divergence in accordance with ecological demands may have subsequently resulted in the observed mixture of Group I and Group II haplotypes in taxonomically homogeneous populations of either ssp. *fuchsii* or ssp. *maculata*. Populations containing multiple haplotype groups seem to be more common in ssp. *maculata* than in ssp. *fuchsii*. This could be explained by the skewed distribution of haplotype groups between taxa, and to the predominant directions of gene transfer; that is between different lineages on the same ploidy level, and from diploid to tetraploid level (Grant 1981).

In a context of past gene flow and remixing of plastid haplotypes between taxa, it is notable that our ITS data, as well as previous nuclear data (Hedrén 1996; Hedrén et al. 2001) distinguish between ssp. *fuchsii* and ssp. *maculata* in Scandinavia. The biparental inheritance of the nuclear genome, in contrast to the uniparental inheritance of the plastid genome (maternally inherited; Corriveau and Coleman 1988), can result in incongruence. Genetic drift can have contrasting effects (Levy and Neal 1999). Similarly, nuclear alleles introduced to a population by introgression may not affect the frequency of alleles that are already prevalent, whereas plastid capture can result in a marked spread of novel plastid haplotypes (Rieseberg and Soltis 1991). Allele I can thus be present in all populations of ssp. *maculata* even if there have been periods of introgressive gene flow from ssp. *fuchsii*. Nevertheless, there was a negative correlation between the frequency of Allele I and Allele V in populations of ssp. *maculata* (Table 7), which could reflect past gene flow from ssp. *fuchsii* to ssp. *maculata*.

The almost complete absence of Allele I from populations of ssp. *fuchsii* is consistent with the theory that gene flow from the tetraploid to the diploid level should be rare (Stebbins 1971). Nonetheless, gene flow from ssp. *maculata* to ssp. *fuchsii* cannot be excluded (Devos et al. 2005), and the fact that low frequencies of Allele I were actually found in two populations (G and P), indicates that introgressive gene flow may have occurred. A complementary explanation to the low frequencies of Allele I is concerted evolution (Hillis and Dixon 1991). Although the ITS region is present in thousands of copies within a genome, intra-genomic diversity is generally low since differing copies are sorted out (Baldwin et al. 1995). In populations influenced by past or recent hybridization, the direction of gene conversion may be strongly biased, leading to a gradual loss of certain alleles (Franzke and Mummenhoff 1999). This could be the fate for the Allele I in introgressed populations of ssp. *fuchsii*. Previous studies in the *D. incarnata/maculata* polyploid complex, in which allotetraploid derivatives were shown to often be fixed for ITS alleles from either parental genome, have also invoked concerted evolution to explain the observed patterns (Pridgeon et al. 1997; Bateman et al. 2003, Devos et al. 2005; Pillon et al. 2007).

(3) *Recent gene flow* Pillon et al. (2007) argued that the degree of loss of one allele due to concerted evolution could be used to infer the relative age of hybridogenous lineages. Here, a high frequency of Allele I was scored in one individual of ssp. *fuchsii* in one population (G). This could be an occasional case of comparably recent gene flow from populations of ssp. *maculata* that occur in the same region. However, even if concerted evolution is a widely recognized phenomenon, the exact mechanisms involved are poorly understood, and considerable differences have been observed among hybridogenous lineages. Gene conversion may be completed in less than 100 generations (e.g. *Cardamine*; Franzke and Mummenhoff 1999) or it may be practically zero for thousands of years (e.g. *Paeonia*; Sang et al. 1995).

The occurrence of a typical *D. incarnata* s.l. plastid haplotype in one morphometrically well-defined *D. maculata* ssp. *maculata* individual (population C) suggests introgressive gene flow. The same individual was characterized by ITS allele X, which is characteristic of *D. incarnata* s.l. (Pillon et al. 2007), and the discovery of a triploid F1 hybrid in the same population (Table 3) further supports the picture of present-day gene flow between *D. maculata* ssp. *maculata* and *D. incarnata* s.l. in this population. These findings illustrate the potential for allotetraploid speciation, but when it comes to explaining the overall variation patterns within *D. maculata* s.l. in Scandinavia, gene flow from *D. incarnata* s.l. appears to be a negligible factor.

Geographic patterns

Differentiation in morphology, in plastid haplotypes and in the ITS region were each significantly correlated with the geographic distances between populations of ssp. *maculata*. The strong morphological similarity among populations

from the northernmost area (Fig. 5) substantially strengthened the correlation between morphology and geography. Figure 8 indicates a southwestern–northeastern haplotype gradient. Group I haplotypes prevail east of the Scandinavian mountain ridge, in northernmost Norway and on the Kola Peninsula in northeast, whereas Group II haplotypes prevail in the southern part of Sweden and in western Norway. Populations that mix Group I and Group II haplotypes are located in intermediate areas. Group I haplotypes of intermediate character between Group I and Group II are also most common in these areas. The low and high frequencies, respectively, of ITS Alleles I and V in subarctic populations of ssp. *maculata* contribute strongly to the geographic structuring (Table 7).

The populations of ssp. *maculata* from the northernmost area are of decisive importance in generating the strong positive correlations between various data sets and geography. The concordant differences between these populations and more southerly populations should also explain why there are significant correlations between other pairs of data sets for ssp. *maculata*; for example, between plastid haplotypes and ITS. It can be assumed that ssp. *maculata* has immigrated into Scandinavia independently from the northeast and the south. The occurrence of populations with mixed haplotype groups in central Sweden fits with such a hypothesis. Similar immigration patterns have been proposed for *Silene dioica* (Malm and Prentice 2005) and *Viola rupestris* (Nordal and Jonsell 1998), and the disjunct distribution of other species, such as *Dianthus superbus* and *Oxytropis campestris* (Hultén and Fries 1986), clearly indicates that other elements in the Scandinavian flora have immigrated from both the northeast and the south. The scenario of two independent migration routes for ssp. *maculata* is supported by the distributions of the Group I and Group II haplotypes outside Scandinavia. Group I haplotypes predominate in eastern Europe, and in northern Russia Group II haplotypes appear to be entirely absent (Shipunov et al. 2004). This pattern is evident in both ssp. *maculata* and ssp. *fuchsii*. In western Europe, Group II haplotypes appear dominant, even though this trend is much more pronounced for ssp. *maculata* than for ssp. *fuchsii* (Hedrén 2003; Devos et al. 2003, 2006; Pillon et al. 2007). Given this picture, Scandinavian populations of ssp. *maculata* may well represent at least two contrasting autotetraploid lineages, a northeastern Group I lineage and a southern Group II lineage.

Furthermore, when the ITS data are taken into account, it is conceivable that the northeastern migration pathway represents two separate lineages. Compared with all other populations of ssp. *maculata*, the populations from northernmost Norway and from the Kola Peninsula have conspicuously low frequencies of Allele I and high frequencies of Allele V, thus showing a marked similarity to ssp. *fuchsii*. These populations may represent a comparably young lineage that could be derived from a diploid ancestor similar to present-day ssp. *fuchsii*. Hedrén et al. (2001) did not include material from the subarctic area in their AFLP study; an extended study incorporating material from this area might prove conclusive. Two explanations of the fact that Allele I was not completely absent from this putative lineage (like in present-day ssp. *fuchsii* in Scandinavia) can be envisaged. First, the hypothesized diploid ancestor could have represented an intermediate state between ancient diploids (which should have contained high frequencies of Allele I, as discussed above) and present-day diploids. Second, the presence of Allele I could be explained by lateral gene flow from the other northeastern lineage. Shipunov et al. (2004) noted close similarity between ssp. *fuchsii* and ssp. *maculata* in northern Russia and proposed that northern ssp. *maculata* might be recent allotetraploids formed from ssp. *fuchsii* and ssp. *maculata*. However, we did not find the patterns of fixation of certain allele frequencies within the populations that would have been expected if different genomes had been combined. Autotetraploidy (possibly combined with lateral gene flow) therefore remains a more plausible explanation.

Analysis of ssp. *maculata* from outside Scandinavia for ITS and plastid DNA data (Ståhlberg 2007) revealed that populations from the Ural Mountains are similar to ssp. *maculata* from northernmost Norway and the Kola Peninsula, whereas populations from the St. Petersburg area and Estonia are similar to the other eastern lineage.

Morphological differentiation was also significantly correlated with geography in ssp. *fuchsii*. However, unlike the uniform variation patterns in ssp. *maculata*, molecular data were not correlated with geography in ssp. *fuchsii*. The morphological differentiation may be a response to variation in environmental conditions, such as climate, which in turn are likely to be correlated with geography. The labellum shape index of ssp. *fuchsii* decreased towards north, converging towards ssp. *maculata*. Since labellum shape is a crucial diagnostic feature, this observation supports the previous assertions that it is more difficult to distinguish ssp. *fuchsii* from ssp. *maculata* in northern areas (e.g. Shipunov et al. 2004). However, the relevance of this trend should not be overemphasized. A complementary explanation for the morphological differentiation may be due to ontogeny (cf. Bateman and Denholm 1989).

Group II haplotypes were restricted to southernmost Sweden in ssp. *fuchsii*. Variation in ITS frequencies showed no geographic trend. Based on the ecology and distribution of ssp. *fuchsii* outside Scandinavia, we conclude that this taxon is most likely to have immigrated to Scandinavia from the south. *Dactylorhiza maculata* ssp.

fuchsii is absent from or rare in the northern part of Finland (Hultén 1971; D. Ståhlberg, personal observation), a fact consistent with this hypothesis. The dominance of Group I haplotypes in Scandinavian ssp. *fuchsii* reflects the general dominance of Group I haplotypes in ssp. *fuchsii* in Continental Europe (e.g. Pillon et al. 2007).

Recognition of the various lineages of *D. maculata* s.l. has implications for understanding the origin of allotetraploid taxa in the *D. incarnata/maculata* polyploid complex (see Introduction). Combined with data from allotetraploids (Hedrén 2003; Pillon et al. 2007), this study reveals that the maternal lineage of Scandinavian *D. majalis* ssp. *sphagnicola* is similar to the southern lineage of *D. maculata* ssp. *maculata*, whereas the maternal lineage of most populations of *D. majalis* ssp. *traunsteineri* and ssp. *lapponica* is more closely related to the *D. maculata* ssp. *fuchsii* lineage.

Indications of plastid DNA recombination

It is interesting that Group I haplotypes of intermediate character between Group I and Group II (Fig. 7) are well-represented in central Sweden, which could therefore be characterized as a contact zone between Group I and Group II haplotypes (Fig. 8b). An interpretation in terms of plastid DNA recombination is logical, but this is a controversial idea that currently receives little support in the literature (but see Marshall et al. 2001; Wolfe and Randle 2004). However, there is an increasing amount of data that suggest recombination in mitochondrial DNA (mtDNA) (e.g. Städler and Delph 2002; Tsaousis et al. 2005). Jaramillo-Correa and Bousquet (2005) found intermediate mitochondrial haplotypes in a contact zone between the common haplotypes of *Picea mariana* and *P. rubens*, which was interpreted as patterns of recombination promoted by heteroplasmy caused by occasional leakage of paternal mtDNA.

One individual with an intermediate Group I haplotype was detected in a northern ssp. *fuchsii* population (R). Similarly, one individual with an intermediate Group I haplotype was detected in a northern ssp. *maculata* population (Ø). These cases are most likely examples of local mutations.

Taxonomic inferences

In an attempt to bring order to the inconsistent and unstable *Dactylorhiza* taxonomy, Pedersen (1998) proposed a taxonomic ranking where *species* should correspond to the biological species concept in a botanically focused way (Mayr 1940; Jonsell 1984), *subspecies* to the ecological species concept (Van Valen 1976) and *varieties* to the phenetic species concept (Sneath 1976). All units should have some evolutionary relevance. *Forms* should not be formally named to avoid nomenclatural ambiguity. Hedrén (2001b) and Pillon et al. (2006) emphasized the significance of delimiting phylogenetically defined entities for proper conservation strategies. Pillon et al. (2007) further stressed the importance of ecological distinctness as a key to understand the evolutionary patterns in a polyploid complex. Since it is not practically possible to distinguish between diploid and tetraploid cytotypes in the field in every part of Eurasia, particularly in the Central European mountain regions, it is preferable to apply a wide species concept which includes both ssp. *fuchsii* and ssp. *maculata*. It would probably be most appropriate to treat them as subspecies, in accordance with Pedersen's (1998) suggestions. Hybridization is not prohibited by post-zygotic barriers; there is morphological overlap, the genome compositions are basically similar, and there is a high probability of recurrent autopolyploidization and gene flow between taxa.

The morphological variation among putative autotetraploid lineages is clinal, but ssp. *maculata* from the subarctic area may represent a comparably coherent entity, also in relation to populations of ssp. *maculata* from similar habitats in the Scandinavian mountain range. In terms of conservation it could therefore be useful to separate populations from the subarctic area as var. *kolaënsis*. According to this study, plants of var. *kolaënsis* characteristically have thin stems (1.2–1.8 mm; cf. Table 4, Appendix 1), high levels of anthocyanin pigmentation and few flowers (10–15). It is not possible to reliably distinguish any other groups of ssp. *maculata* in Scandinavia. The notion of Heslop-Harrison (1951) and Sahlin (1960, 1980) that tetraploid populations might be separated in a western oceanic taxon and a Continental eastern taxon is not supported. Similarly, there is no support for a discrete subarctic diploid taxon (Vermeulen 1947; Heslop-Harrison 1951; Delforge 1995), even if the population from Abisko (R) was relatively distinct. The specimens in this population had a *maculata*-like labellum and many individuals were dark purple relative to *D. maculata* s.l. in general.

Taken together, we conclude that the morphological variation within both ssp. *fuchsii* and ssp. *maculata* is continuous in Scandinavia.

Conclusions

When morphology and habitat features are considered together, it is possible to distinguish between diploid and tetraploid populations of *D. maculata* s.l. in Scandinavia. The cytotypes are more difficult to separate in other parts of the distribution area. As diploids and tetraploids are kept apart by ecological, prezygotic barriers rather than by postzygotic barriers, it is appropriate to treat them as

subspecies (ssp. *fuchsii* and ssp. *maculata*, respectively) *sensu* Pedersen (1998). Plastid haplotype data indicate that ssp. *maculata* probably has immigrated to Scandinavia from both the south and the northeast. Intermediate haplotypes in a zone of contact between southern and northeastern haplotypes are consistent with plastid DNA recombination, a controversial process. The southern pathway may represent a single autotetraploid lineage, whereas ITS data suggest that the northeastern pathway may represent two different autotetraploid lineages. One of these lineages consists of morphologically, relatively coherent subarctic populations that loosely could be referred to as var. *kolaënsis*. There is no support for any other taxa in Scandinavia. *Dactylorhiza maculata* ssp. *fuchsii* has probably immigrated from the south. The genetic structure within populations of both ssp. *fuchsii* and ssp. *maculata* reflects past introgressive gene flow rather than recent hybridization events.

Acknowledgments We thank friends and colleagues for providing information about sample localities. We are especially grateful to Ilona Blinova for practical help on the Kola Peninsula, Ingela Ståhlberg for assistance in the field, Lina Steinke for assistance in the lab and Louise Hathaway for correcting and improving the language. We also thank Richard M. Bateman and one anonymous reviewer for valuable comments. Financial support was given by Anna och Svante Murbecks minnesfond and Lunds botaniska förening to DS, and The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning, FORMAS (grant 2002–0102) to MH.

Appendix 1

See Table 9.

Table 9 Population means of morphological characters (see Table 4)

Population	Taxon	*N*	Character										
			A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
A	*fuchsii*	8	8.95	5.13	6.45	1.56	10.84	9.38	40.00	41.25	2.63	2.88	1.75
			1.18	*0.78*	*0.88*	*0.24*	*1.02*	*3.20*	*11.95*	*8.35*			
B	*maculata*	20	7.90	5.79	6.80	1.26	9.51	5.50	29.50	31.50	2.95	2.95	2.10
			0.71	*0.81*	*0.57*	*0.11*	*1.09*	*1.54*	*9.45*	*7.45*			
C	*maculata*	9	8.55	5.89	6.91	1.33	9.41	8.00	31.00	41.00	2.33	2.11	2.22
			1.62	*0.86*	*0.68*	*0.18*	*0.71*	*3.50*	*13.70*	*8.76*			
D	*maculata*	6	9.16	6.52	7.98	1.28	10.28	6.00	26.00	36.00	3.00	2.20	2.20
			1.67	*1.33*	*1.51*	*0.19*	*1.87*	*2.24*	*15.17*	*5.48*			
E	*maculata*	5	8.76	6.92	7.04	1.26	9.78	7.00	32.00	36.00	2.40	2.40	2.20
			0.89	*0.98*	*0.88*	*0.11*	*0.75*	*2.74*	*14.83*	*8.94*			
F	*maculata*	5	8.84	6.76	7.24	1.26	10.92	6.00	18.00	40.00	2.40	2.20	2.60
			1.56	*0.47*	*0.81*	*0.14*	*1.05*	*2.24*	*13.04*	*7.07*			
G	*fuchsii*	10	8.69	4.99	6.19	1.59	9.94	9.00	44.00	39.00	2.80	2.00	2.20
			1.03	*0.80*	*0.91*	*0.32*	*0.78*	*3.16*	*9.66*	*7.38*			
H	*fuchsii*	6	8.38	4.45	5.72	1.65	9.63	7.50	27.50	40.00	2.50	1.50	2.17
			0.78	*0.53*	*0.53*	*0.14*	*1.44*	*4.18*	*17.25*	*8.94*			
I	*fuchsii*	10	8.08	5.44	6.40	1.37	10.06	6.00	27.00	36.00	3.10	2.50	2.50
			1.13	*0.94*	*0.82*	*0.19*	*1.50*	*2.11*	*11.60*	*8.43*			
J	*maculata*	5	7.90	6.14	6.84	1.22	9.56	5.00	12.00	38.00	2.40	2.20	1.60
			0.33	*0.36*	*0.91*	*0.11*	*1.42*	*0.00*	*4.47*	*4.47*			
K	*maculata*	10	7.49	4.77	5.85	1.42	9.48	5.00	11.00	37.00	2.10	2.60	1.90
			0.95	*0.31*	*0.92*	*0.22*	*1.52*	*0.00*	*6.58*	*4.83*			
L	*maculata*	10	7.71	6.61	7.21	1.14	9.59	5.00	14.00	36.00	1.70	3.00	2.00
			0.86	*1.32*	*1.20*	*0.18*	*0.83*	*0.00*	*5.16*	*5.16*			
M	*fuchsii*	5	9.16	6.30	7.42	1.36	10.98	5.00	16.00	34.00	3.20	3.00	2.20
			0.69	*1.20*	*1.36*	*0.16*	*1.65*	*0.00*	*5.48*	*5.48*			
N	*maculata*	10	8.28	6.51	7.10	1.22	9.33	5.00	9.50	39.00	2.20	2.20	1.70
			0.73	*0.60*	*0.68*	*0.13*	*0.86*	*0.00*	*4.38*	*3.16*			
O	*fuchsii*	10	7.84	5.65	6.70	1.27	9.76	5.00	21.00	31.00	2.80	2.50	1.90
			0.53	*0.59*	*0.62*	*0.11*	*1.03*	*0.00*	*5.68*	*3.16*			

Table 9 continued

Population	Taxon	*N*	Character										
			A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
P	*fuchsii*	10	9.10	5.40	6.51	1.53	10.14	5.00	22.00	32.00	2.70	2.80	2.40
			0.94	*0.27*	*0.67*	*0.15*	*1.30*	*0.00*	*7.89*	*4.22*			
Q	*maculata*	10	6.82	5.03	5.60	1.30	8.43	5.00	9.00	38.00	1.40	2.70	1.80
			1.12	*0.84*	*0.90*	*0.24*	*1.14*	*0.00*	*6.15*	*4.22*			
R	*fuchsii*	10	8.07	6.09	6.69	1.27	10.27	17.50	53.00	49.00	2.60	2.90	2.50
			0.99	*0.92*	*0.67*	*0.12*	*0.89*	*7.17*	*6.75*	*3.16*			
S	*maculata*	10	7.87	6.14	7.13	1.19	9.09	5.50	27.50	32.00	2.90	3.00	2.80
			0.58	*0.66*	*0.31*	*0.09*	*2.84*	*1.58*	*10.34*	*4.22*			
T	*fuchsii*	10	7.95	4.74	5.90	1.51	10.26	10.00	47.00	39.00	2.90	2.80	2.90
			0.63	*0.59*	*0.90*	*0.19*	*0.91*	*0.00*	*4.83*	*3.16*			
U	*maculata*	10	6.63	5.61	6.63	1.08	8.28	5.00	16.00	35.00	3.20	3.00	2.10
			0.83	*0.68*	*0.83*	*0.05*	*0.92*	*0.00*	*6.99*	*5.27*			
V	*maculata* [a]	10	7.59	5.48	6.25	1.31	8.72	5.00	13.00	37.00	3.00	2.80	2.10
			0.61	*0.92*	*0.68*	*0.18*	*1.28*	*0.00*	*4.83*	*4.83*			
W	*fuchsii*	5	8.98	6.00	7.34	1.36	9.78	5.00	24.00	30.00	3.00	3.00	2.00
			0.91	*0.84*	*1.28*	*0.14*	*1.37*	*0.00*	*5.48*	*0.00*			
X	*maculata*[a]	10	7.94	6.12	6.84	1.23	9.50	6.00	29.00	33.00	2.90	2.90	2.40
			0.90	*0.68*	*0.86*	*0.11*	*1.24*	*2.11*	*11.97*	*4.83*			
Y	*maculata* [a]	5	7.28	6.10	5.98	1.21	8.84	7.00	34.00	34.00	3.00	3.00	2.00
			0.73	*1.10*	*0.84*	*0.16*	*1.10*	*2.74*	*11.40*	*5.48*			
Z	*maculata* [a]	10	7.49	5.13	5.70	1.40	8.43	7.50	27.00	35.00	3.00	3.00	2.10
			0.47	*0.53*	*0.84*	*0.15*	*0.99*	*4.86*	*17.03*	*5.27*			
Ø	*maculata* [a]	9	7.78	5.93	6.72	1.24	9.99	5.00	15.56	33.33	2.78	3.00	2.00
			0.78	*0.79*	*0.83*	*0.18*	*1.00*	*0.00*	*6.82*	*5.00*			

Population	Taxon	Character										
		A12	A13	A14	A15	A16	A17	A18	A19	B20	B21	B22	B23
A	*fuchsii*	7.90	3.38	0.75	10.79	0.00	17.14	11.48	0.00	45.31	6.00	24.25	3.24
		0.97			*1.11*		*3.13*	*1.18*		*8.92*	*1.71*	*6.36*	*0.67*
B	*maculata*	7.49	3.95	1.10	8.94	0.80	14.45	11.40	1.15	23.57	3.91	23.40	3.08
		0.85			*1.03*		*3.12*	*2.32*		*6.08*	*1.16*	*8.07*	*0.73*
C	*maculata*	8.22	3.80	1.00	9.09	1.00	14.68	9.96	1.60	23.30	4.32	22.80	2.58
		0.76			*1.47*		*3.04*	*1.90*		*5.78*	*1.33*	*9.38*	*0.52*
D	*maculata*	8.82	4.40	0.80	10.04	0.80	13.72	9.72	1.20	40.40	6.70	22.00	3.20
		1.76			*1.40*		*3.21*	*2.20*		*10.01*	*2.33*	*7.58*	*1.05*
E	*maculata*	8.20	4.00	1.00	10.72	0.00	17.22	11.54	1.00	47.60	6.10	29.20	4.36
		1.56			*1.24*		*1.39*	*1.26*		*6.73*	*0.89*	*6.83*	*1.08*
F	*maculata*	8.48	3.80	1.60	9.70	0.40	13.98	9.90	1.20	32.00	5.00	20.40	3.00
		0.54			*1.04*		*1.68*	*2.65*		*5.57*	*1.58*	*5.08*	*0.72*
G	*fuchsii*	7.14	2.90	1.00	10.19	0.38	16.73	10.40	0.89	48.40	6.05	37.33	4.88
		0.87			*1.33*		*4.15*	*1.89*		*11.97*	*1.77*	*11.88*	*1.38*
H	*fuchsii*	7.23	3.17	1.00	9.52	0.17	13.45	8.40	0.67	34.50	4.33	22.33	8.77
		1.11			*1.46*		*2.85*	*1.20*		*11.76*	*1.03*	*6.06*	*13.83*
I	*fuchsii*	7.22	3.30	1.00	9.87	0.10	15.69	11.82	0.40	42.45	6.70	25.00	2.98
		1.83			*1.90*		*4.96*	*3.67*		*12.24*	*1.65*	*7.73*	*1.27*
J	*maculata*	6.34	3.40	0.80	9.84	1.20	12.76	10.30	2.00	33.50	5.50	18.20	2.74
		0.50			*1.08*		*1.70*	*1.12*		*2.55*	*0.61*	*3.19*	*0.35*

Table 9 continued

Population	Taxon	Character											
		A12	A13	A14	A15	A16	A17	A18	A19	B20	B21	B22	B23
K	*maculata*	7.50	3.20	0.90	9.20	0.20	12.96	9.95	0.30	38.10	4.95	19.00	2.37
		1.56			*1.45*		*1.64*	*1.48*		*8.65*	*1.86*	*6.13*	*0.60*
L	*maculata*	5.36	3.20	1.00	7.88	0.00	17.90	12.23	0.40	23.75	3.50	16.40	2.78
		1.05			*0.91*		*2.47*	*1.48*		*5.62*	*0.91*	*4.97*	*0.38*
M	*fuchsii*	7.00	4.00	1.00	10.04	0.40	16.06	10.44	1.00	48.20	7.40	30.40	3.58
		1.63			*0.65*		*1.89*	*1.79*		*13.08*	*2.58*	*8.23*	*0.90*
N	*maculata*	7.19	3.70	0.90	9.28	0.30	14.31	10.40	0.30	31.35	4.75	14.40	1.74
		1.49			*1.28*		*3.19*	*1.85*		*5.10*	*1.14*	*4.22*	*0.35*
O	*fuchsii*	6.25	3.90	1.00	8.83	0.00	12.15	9.04	1.00	44.20	5.15	18.20	2.36
		1.10			*1.04*		*1.50*	*1.23*		*4.87*	*0.94*	*2.39*	*0.44*
P	*fuchsii*	6.99	3.70	1.10	9.75	0.90	13.68	10.11	1.10	34.40	5.00	17.80	2.08
		0.90			*0.95*		*2.90*	*1.48*		*9.01*	*1.18*	*6.16*	*0.23*
Q	*maculata*	4.37	3.20	0.80	6.80	0.60	12.19	8.95	0.80	24.25	3.25	17.70	2.21
		0.76			*1.27*		*1.45*	*1.00*		*3.61*	*0.59*	*2.63*	*0.43*
R	*fuchsii*	6.23	3.20	0.80	8.79	1.50	12.69	9.52	1.90	27.55	4.30	18.90	2.46
		0.92			*0.79*		*2.48*	*1.12*		*6.45*	*1.60*	*6.51*	*0.48*
S	*maculata*	6.53	3.70	1.50	8.07	0.50	12.51	9.29	0.80	22.20	3.20	24.20	2.60
		0.92			*1.56*		*1.66*	*1.57*		*5.06*	*0.95*	*12.08*	*0.89*
T	*fuchsii*	8.00	3.80	1.00	9.52	0.80	17.35	11.46	1.00	47.20	6.65	32.00	3.48
		0.84			*0.98*		*3.66*	*1.93*		*6.11*	*2.12*	*15.67*	*0.80*
U	*maculata*	6.23	3.20	1.00	7.20	0.60	10.39	7.85	1.10	23.65	3.00	16.30	1.84
		0.67			*0.99*		*1.68*	*1.45*		*2.21*	*0.85*	*5.44*	*0.16*
V	*maculata*[a]	6.04	4.00	1.00	7.75	1.50	9.33	7.32	1.80	18.25	3.20	13.00	1.38
		0.65			*0.87*		*1.38*	*1.16*		*4.64*	*0.98*	*4.24*	*0.43*
W	*fuchsii*	6.04	3.80	1.00	9.84	0.60	16.80	11.00	1.20	38.60	6.20	30.00	2.76
		1.07			*1.85*		*2.53*	*0.98*		*7.02*	*1.75*	*8.80*	*0.70*
X	*maculata*[a]	6.47	3.90	1.10	8.27	1.90	10.72	7.38	2.00	23.00	3.75	13.50	1.38
		0.74			*1.41*		*1.70*	*0.76*		*3.47*	*0.68*	*3.06*	*0.26*
Y	*maculata*[a]	5.82	3.80	1.00	8.00	2.00	10.58	8.76	2.00	18.80	3.80	13.80	1.30
		0.94			*1.40*		*1.72*	*1.58*		*1.89*	*0.76*	*3.77*	*0.07*
Z	*maculata*[a]	5.96	4.00	1.00	8.30	2.00	11.06	7.88	2.00	20.10	3.30	10.70	1.16
		0.83			*0.83*		*1.55*	*1.10*		*2.58*	*0.92*	*4.08*	*0.38*
Ø	*maculata*[a]	6.92	3.33	1.00	8.99	1.44	11.57	8.47	1.89	28.17	4.72	14.89	1.84
		1.07			*0.73*		*1.44*	*1.05*		*6.56*	*0.91*	*6.41*	*0.44*

Population	Taxon	Character											
		B24	B25	B26	B27	B28	B29	B30	B31	B32	B33	B34	B35
A	*fuchsii*	4.13	1.88	13.50	2.88	1.50	1.00	2.00	2.50	2.50	2.25	2.88	1.31
				2.52	*0.72*								
B	*maculata*	3.75	2.45	10.00	1.75	1.25	1.70	1.90	2.05	3.00	3.16	3.05	2.03
				2.03	*0.45*								
C	*maculata*	3.20	2.60	9.54	1.47	1.10	1.40	1.40	1.90	3.30	2.80	3.10	1.90
				2.24	*0.25*								
D	*maculata*	3.20	2.20	12.70	1.20	1.20	1.00	1.00	1.40	3.40	3.00	3.00	1.60
				1.82	*0.21*								
E	*maculata*	3.60	2.60	14.80	2.30	1.20	1.00	1.00	1.40	4.20	3.00	3.00	1.90
				1.92	*0.99*								

Table 9 continued

Population	Taxon	Character											
		B24	B25	B26	B27	B28	B29	B30	B31	B32	B33	B34	B35
F	*maculata*	3.20	2.40	10.40	1.44	1.40	1.00	1.00	1.20	3.80	3.00	3.00	1.50
				2.33	*0.31*								
G	*fuchsii*	5.00	2.70	16.92	2.97	1.00	1.00	1.10	2.30	2.50	3.00	3.00	2.63
				3.75	*0.89*								
H	*fuchsii*	4.00	2.83	10.83	2.33	1.00	1.00	1.00	2.33	3.17	3.00	3.00	2.00
				0.68	*0.27*								
I	*fuchsii*	4.10	1.50	11.78	2.39	1.00	2.00	2.00	2.50	3.20	3.00	3.50	2.35
				3.47	*0.51*								
J	*maculata*	4.00	2.20	8.50	1.22	1.00	1.20	1.40	2.20	4.80	3.00	3.00	1.40
				1.27	*0.26*								
K	*maculata*	3.60	1.50	12.30	1.16	1.00	1.10	1.10	1.50	3.10	3.00	3.10	1.70
				2.62	*0.28*								
L	*maculata*	3.30	1.60	8.32	1.89	1.40	1.90	1.90	2.20	4.20	3.00	3.10	1.55
				1.32	*0.28*								
M	*fuchsii*	4.40	2.00	11.90	1.92	1.00	1.20	1.20	2.20	4.40	2.80	3.40	2.10
				2.16	*0.41*								
N	*maculata*	2.80	1.10	10.65	0.96	1.20	1.10	1.20	1.70	2.10	2.00	2.10	0.85
				2.89	*0.24*								
O	*fuchsii*	3.50	1.80	9.10	1.61	1.10	1.10	2.00	2.20	2.70	2.40	2.80	1.05
				1.17	*0.17*								
P	*fuchsii*	3.80	1.10	9.65	1.56	1.40	1.30	1.80	2.60	4.00	3.00	3.00	1.45
				1.47	*0.30*								
Q	*maculata*	3.30	1.50	6.59	1.22	1.10	2.00	2.00	2.10	2.40	3.25	3.63	1.31
				0.93	*0.25*								
R	*fuchsii*	3.50	1.30	6.37	1.68	1.30	2.10	2.10	2.80	5.00	3.00	3.70	2.60
				1.94	*0.40*								
S	*maculata*	3.80	1.90	12.70	1.09	1.40	1.00	1.00	1.40	3.10	3.00	3.10	1.70
				2.57	*0.49*								
T	*fuchsii*	3.90	1.90	12.15	2.92	1.00	2.00	2.00	3.10	2.90	3.00	3.40	2.10
				2.93	*1.06*								
U	*maculata*	3.20	2.10	8.75	0.58	1.10	1.00	1.00	1.10	4.40	3.00	3.00	2.35
				0.89	*0.16*								
V	*maculata*[a]	2.90	1.50	4.47	0.95	1.30	1.30	1.90	2.10	1.50	1.50	1.20	0.45
				0.93	*0.25*								
W	*fuchsii*	4.00	1.00	9.32	2.46	1.00	1.80	2.40	3.00	4.00	3.00	3.80	1.90
				1.04	*0.33*								
X	*maculata*[a]	2.80	1.40	5.10	0.82	1.10	1.50	1.80	2.20	5.00	3.00	3.20	1.50
				1.02	*0.23*								
Y	*maculata*[a]	3.40	1.20	5.04	0.86	1.20	1.00	1.40	2.20	4.80	3.00	3.00	1.30
				1.87	*0.26*								
Z	*maculata*[a]	2.80	0.90	5.35	0.82	1.20	1.40	1.80	1.90	4.80	3.00	3.00	1.45
				1.43	*0.20*								
Ø	*maculata*[a]	2.67	1.22	5.69	1.13	1.11	1.78	1.78	2.33	4.67	3.00	3.33	1.56
				1.15	*0.26*								

SD (where appropriate) in italics. *N* sample size

[a] Subarctic populations of var. *kolaënsis*

Appendix 2

See Table 10.

Table 10 Characterization of plastid haplotypes identified in the present study by means of the primer pairs described in Table 5

Nr.	group	Locus										
		1	6	6B	8	9	10b	11b	17	18	19	*N*
22	I	185	222	470	74	196	144	84	132	93	149	1
23	I	185	222	470	74	196	145	84	132	93	149	12
28	I	185	281	560	74	196	145	84	132	93	149	1
56	I	185	222	470	75	196	142	84	132	93	149	1
57	I	185	222	470	75	196	143	84	132	93	149	10
58	I	185	222	470	75	196	144	84	132	93	149	25
59	I	185	222	470	75	196	145	84	132	93	149	48
60	I	185	222	470	75	196	146	84	132	93	149	12
65	I	186	222	470	75	196	144	84	132	93	149	1
73	I	185	228	470	75	196	145	84	132	93	149	6
76	I	186	228	470	75	196	145	84	132	93	149	1
80	I	185	281	560	75	196	144	84	132	93	149	2
81	I	185	281	560	75	196	145	84	132	93	149	1
82	I	185	281	560	75	196	146	84	132	93	149	3
99	I	185	281	560	76	202	141	84	132	93	149	2
113	II	185	275	610	73	177	147	85	136	93	149	1
119	II	185	222	560	73	177	146	85	136	97	149	1
121	II	185	281	560	73	177	149	85	136	93	146	2
123	II	185	281	560	73	177	153	85	136	93	146	8
139	II	185	281	560	73	177	147	85	136	97	148	1
142	II	185	281	560	73	177	146	85	136	97	149	2
143	II	185	281	560	73	177	147	85	136	97	149	32
148	II	186	281	560	73	177	145	85	136	97	149	5
149	II	186	281	560	73	177	146	85	136	97	149	11
150	II	186	281	560	73	177	147	85	136	97	149	3
157	II	185	275	610	73	177	149	85	136	103	149	2
160	II	185	323	610	73	177	148	85	136	103	149	3
166	III	184	367	610	73	171	138	85	130	93	138	1
174	I	185	222	470	75	171	144	84	132	93	149	6
236	I	185	228	470	75	196	146	84	132	93	149	7
246	II	185	275	610	73	177	149	87	136	93	149	1
252	I	185	281	470	75	171	144	84	132	93	149	1
268	II	185	281	560	73	177	149	87	136	93	149	6
269	II	185	281	560	73	177	150	87	136	93	149	6
273	II	184	281	560	73	177	147	85	136	97	149	1
276	II	185	281	560	73	177	146	85	136	101	149	1
285	I	185	281	560	75	196	143	84	132	93	149	6
288	I	185	281	560	75	196	145	84	136	93	149	1
291	I	185	281	560	75	202	145	84	132	93	149	1
294	II	186	281	580	73	177	147	85	136	97	149	1
299	II	185	323	610	73	177	148	85	136	93	149	1
312	II	185	281	560	73	177	147	85	136	107	149	1
317	II	185	281	560	73	177	153	84	136	93	146	1

The numbering of the haplotypes is based on a larger data set that encompasses *Dactylorhiza* from all over Europe (M. Hedrén, S. Nordström and D. Ståhlberg, unpublished data). Group refers to major haplotype groups according to Fig. 6; intermediate Group I haplotypes are in italics. *N* number of individuals found with a particular haplotype. Assessed fragment lengths are given in bp

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