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Evolutionary history of the *Dactylorhiza maculata* polyploid complex (Orchidaceae)

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Taxonomic complexity may be associated with migration history and polyploidy. We used plastid and nuclear DNA markers to investigate the evolutionary history of the systematically challenging *Dactylorhiza maculata* polyploid complex. A total of 1833 individuals from 298 populations from throughout Europe were analysed. We found that gene flow was limited between the two major taxa, diploid ssp. *fuchsii* (including ssp. *saccifera*) and tetraploid ssp. *maculata*. A minimum of three autotetraploid lineages were discerned: (1) southern/western ssp. *maculata*; (2) northern/eastern ssp. *maculata*; and (3) Central European ssp. *fuchsii*. The two ssp. *maculata* lineages, which probably pre-date the last glaciation, form a contact zone with high genetic diversity in central Scandinavia. Intermediate plastid haplotypes in the contact zone hint at recombination. Central Europe may have been a source area for the postglacial migration for the southern/western lineage of ssp. *maculata*, as well as for ssp. *fuchsii*. The northern/eastern lineage of ssp. *maculata* may have survived the LGM in central Russia west of the Urals. The tetraploid lineage of ssp. *fuchsii* is indistinguishable from diploid ssp. *fuchsii*, and is probably of postglacial origin. The Mediterranean region and the Caucasus have not contributed to the northward migration of either ssp. *fuchsii* or ssp. *maculata*.

ADDITIONAL KEYWORDS: genetic variation – glacial refugia – hybrid zone – ITS – phylogeography – plastid DNA – polyploid evolution – recombination – systematics.

INTRODUCTION

Quaternary climatic changes have had a profound impact on speciation, structuring of genetic diversity, and the shaping of the present-day distributions of plant and animal taxa (Vuilleumier, 1971; Hewitt, 1996, 2000, 2004; Avise, 2000). In Europe, the repeated cycles of glacials and interglacials during the Pleistocene (*c.* 2 Ma until 10 000 years BP) caused massive fluctuations in the distributions of taxa. Fragmentation and isolation of populations during the long-lasting glacials and expansion during the shorter interglacials resulted in marked differences among regions in intraspecific diversity. Oscillations of population sizes, bottlenecks, founder events and other population historical events associated with climatic shifts have further contributed to differentiation among regional population groups. As a combined effect of range shifts and population differentiation, divergent lineages have occasionally formed contact zones, leading to reticulate speciation via hybridization and polyploidization (Grant, 1981; Stebbins, 1984; Hewitt, 1988, 2001).

Analyses of macrofossil and pollen data, together with organellar markers, have shown that populations of many temperate species in the European flora and fauna survived the Last Glacial Maximum (LGM; *c.* 22 000–18 000 years BP) in various southern refugia in the Mediterranean region and the Caspian/Caucasian region (Huntley & Birks, 1983; Petit, Kremer & Wagner, 1993; Demesure, Comps & Petit, 1996; Hewitt, 2004). Similarly, patterns of postglacial migration have been reconstructed for many temperate species, and a general picture of high intraspecific genetic diversity in refugial areas in the south, and

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low diversity in previously glaciated areas in the north, has been established (Ferris, King & Hewitt, 1999). However, this picture may be simplistic (cf. Widmer & Lexer, 2001). Increasing evidence suggests that the southern refugia for temperate species were supplemented by more northern refugia during the LGM (reviewed by Stewart & Lister, 2001), which clearly would have resulted in more complex patterns of intraspecific genetic diversity.

Increased intraspecific genetic diversity also occurs in contact zones where divergent populations from separate refugia meet (Petit *et al*., 2003). Such zones of secondary contact have been demonstrated for both plants and animals in Central Europe (Petit *et al*., 2003). Polyploidization appears to be common in this region (Stebbins, 1984). Several studies have indicated that central–northern Scandinavia may be another area of secondary contact between divergent populations immigrating from the north-east and the south (Jaarola & Tegelström, 1995; Fredga, 1996; Nyberg Berglund & Westerbergh, 2001). In a recent study, we found that contrasting lineages of the widespread Eurasian orchid *Dactylorhiza maculata* (L.) Soó *s.l.* may form such a contact zone in central Scandinavia (Ståhlberg & Hedrén, 2008).

The *Dactylorhiza maculata* complex

Dactylorhiza maculata s.l. is a morphologically and genetically variable and intriguing polyploid complex that consists of diploid ($2n = 40$) and tetraploid ($2n = 80$) cytotypes (Averyanov, 1990; Hedrén, 1996; Hedrén, Fay & Chase, 2001; Tyteca, 2001; Bateman & Denholm, 2003; Shipunov *et al*., 2004; Devos *et al*., 2005; Ståhlberg & Hedrén, 2008). At least 30 taxa at various taxonomic levels have been described (Soó, 1960; Delforge, 1995), but most contemporary authors distinguish between three or four morphologically and cytologically defined taxa: (1) *D. maculata* ssp. *fuchsii* (Druce) Hyl., a predominantly diploid taxon that typically grows in semi-open woodlands on fertile soils throughout most of north-western Eurasia (absent from or rare in southern and south-eastern Europe); (2) *D. maculata* ssp. *saccifera* (Brongn.) Diklic, a diploid taxon that gradually replaces ssp. *fuchsii* on the Apennine Peninsula and in south-eastern Europe; (3) *D. maculata* ssp. *maculata*, a tetraploid taxon that characteristically is found in more open habitats such as grasslands, coastal moorlands and boreal–subarctic peatlands in western and northern Eurasia (absent in south-eastern Europe); (4) *Dactylorhiza foliosa* (Sol. ex Lowe) Soó, a geographically isolated Madeiran diploid. Tetraploid populations of *D. maculata* ssp. *fuchsii* are common in Central Europe, whereas ssp. *maculata* is rare or is absent from the same region (Ståhlberg, 2007; Fig. 1). Morphologically intermediate triploid hybrids between ssp. *fuchsii* and ssp. *maculata* have been reported from various parts of Eurasia (e.g. Averyanov, 1977; Gathoye & Tyteca, 1989).

Dactylorhiza maculata s.l. is morphologically and genetically distinct from other taxa in the genus (e.g. Pedersen, 1998), but many allopolyploid taxa are derived from hybridization between *D. maculata s.l.* and other distinct *Dactylorhiza* lineages (Hedrén, 2001, 2002, 2003; Devos *et al*., 2003, 2006; Shipunov *et al*., 2004; Pillon *et al*., 2007).

Allozyme studies have shown that tetraploid populations of *D. maculata s.l.* have originated by autopolyploidization (Hedrén, 1996). In most polyploid complexes recurrent polyploidization (including autopolyploidization) is a common phenomenon (Soltis, Soltis & Tate, 2003). A large number of regionally focused studies based on plastid DNA and/or nuclear ribosomal DNA (nrDNA) markers (e.g. Devos *et al*., 2003, 2005, 2006; Hedrén, 2003; Shipunov *et al*., 2004; Pillon *et al*., 2007; Hedrén, Nordström & Ståhlberg, 2008), as well as on morphometry and/or cytometry (e.g. Heslop-Harrison, 1951; Vöth, 1978; Reinhard, 1985; Jagiełło, 1986–1987; Bateman & Denholm, 1989; Dufrêne, Gathoye & Tyteca, 1991; Tyteca & Gathoye, 2004; Ståhlberg & Hedrén, 2008), suggest together that tetraploid populations of *D. maculata s.l.* include at least three separate autotetraploid lineages: (1) *D. maculata* ssp. *maculata* from southern and western Europe; (2) *D. maculata* ssp. *maculata* from northern and eastern Europe; and (3) *D. maculata* ssp. *fuchsii* from the mountain areas of Central Europe. According to morphological differences between ssp. *maculata* and present-day diploids, the first two lineages may be relatively ancient. Moreover, there are differences in chromosome size (Jagiełło & Lankosz-Mróz, 1986–1987) and in banding patterns of amplified fragment length polymorphism (AFLP; Hedrén *et al*., 2001) between ssp. *maculata* and present-day diploids. In contrast, the third lineage may be relatively young because diploid and tetraploid populations of ssp. *fuchsii* are morphologically indistinguishable (Groll, 1965; Vaucher, 1966; Scharfenberg, 1977; Vöth, 1978; Vöth & Greilhuber, 1980; Jagiełło, 1986–1987; Jagiełło & Lankosz-Mróz, 1986–1987; Reinhardt, 1988; Gölz & Reinhard, 1997; Bertolini, Del Prete & Garbari, 2000).

Aims of the present study

The association between taxonomic complexity, Quaternary migration and polyploid evolution makes the foundation for this study. Using both plastid and nuclear DNA markers we: (1) analyse relationships within and among major taxa in the *D. maculata* complex, and assess the influence of introgressive gene flow; (2) test the hypothesis that tetraploid

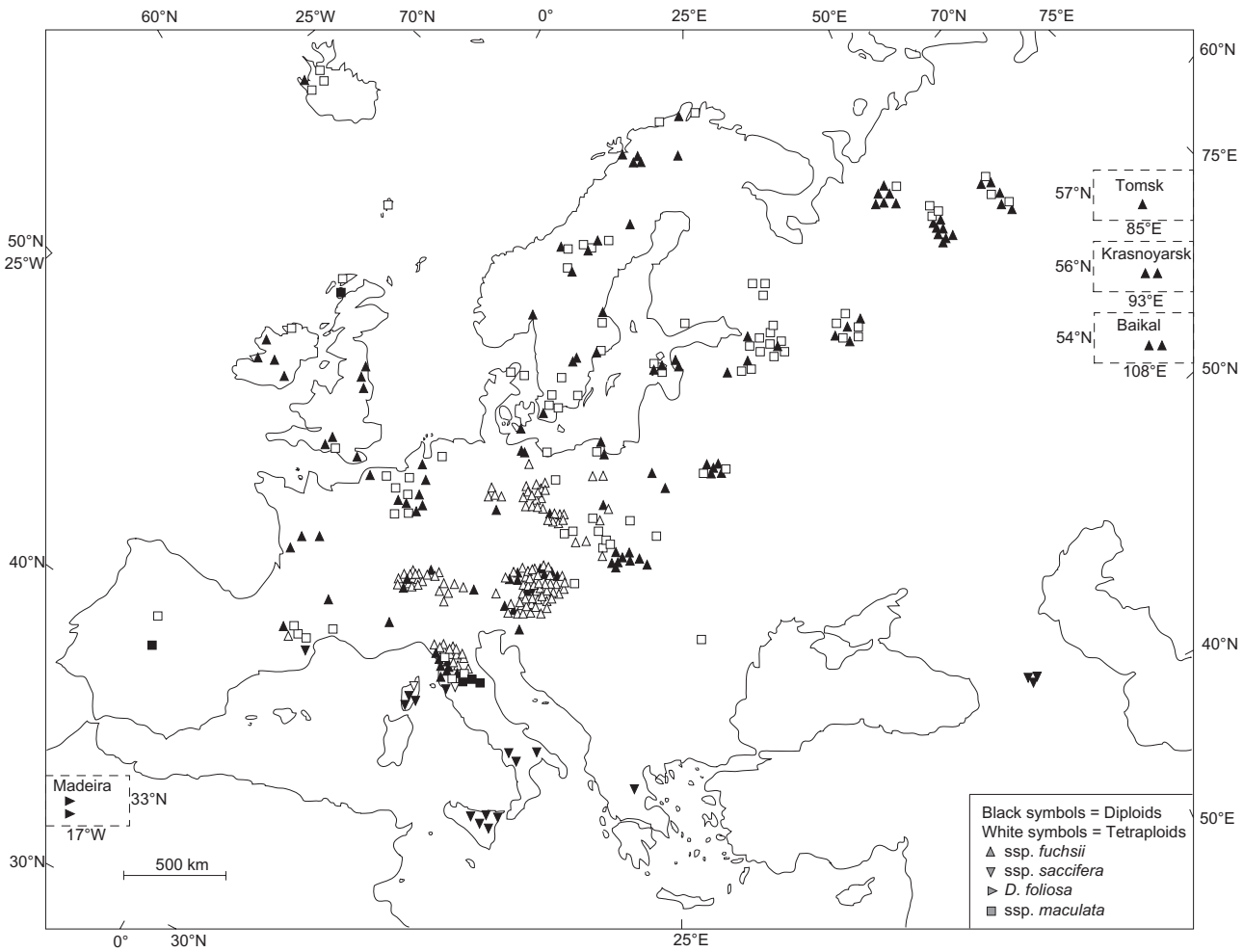

Figure 1. Distribution of diploid and tetraploid populations of *Dactylorhiza maculata s.l.* based on literature data (Ståhlberg, 2007).

lineages of *D. maculata s.l.* have arisen at least three times, as outlined above; (3) address the issue of whether glacial refugia in areas other than southern Europe have contributed significantly to the present-day distribution of genetic diversity in northern Europe; (4) investigate to what extent high genetic diversity is associated with secondary contact between divergent lineages. In this respect, we particularly scrutinize the role of a putative contact zone in central Scandinavia.

MATERIAL AND METHODS

SAMPLING

A total of 1833 individuals from 298 populations of *D. maculata s.l.* were sampled from throughout Europe (Fig. 2; Appendix S1). From each individual between five and ten fresh flowers or parts of fresh leaves were collected and dried in silica gel. The sampling effort was particularly directed towards Fennoscandia. The populations were taxonomically classified as described in the Introduction. However, as ssp. *fuchsii* and ssp. *saccifera* probably grade into each other, ssp. *saccifera* is included in ssp. *fuchsii* in the following discussion, unless explicitly stated otherwise.

MOLECULAR METHODS

All individuals were investigated with respect to plastid DNA variation, whereas a taxonomic and geographic representative subset of 820 individuals from 118 populations was investigated for nrDNA variation.

Ten polymorphic plastid DNA loci (seven microsatellite loci and three loci with indel variation) were amplified with a set of *Dactylorhiza*-specific primers (Table 1; cf. Hedrén *et al.*, 2008). Size variants (alleles) were scored and combined into multilocus genotypes (hereafter referred to as haplotypes).

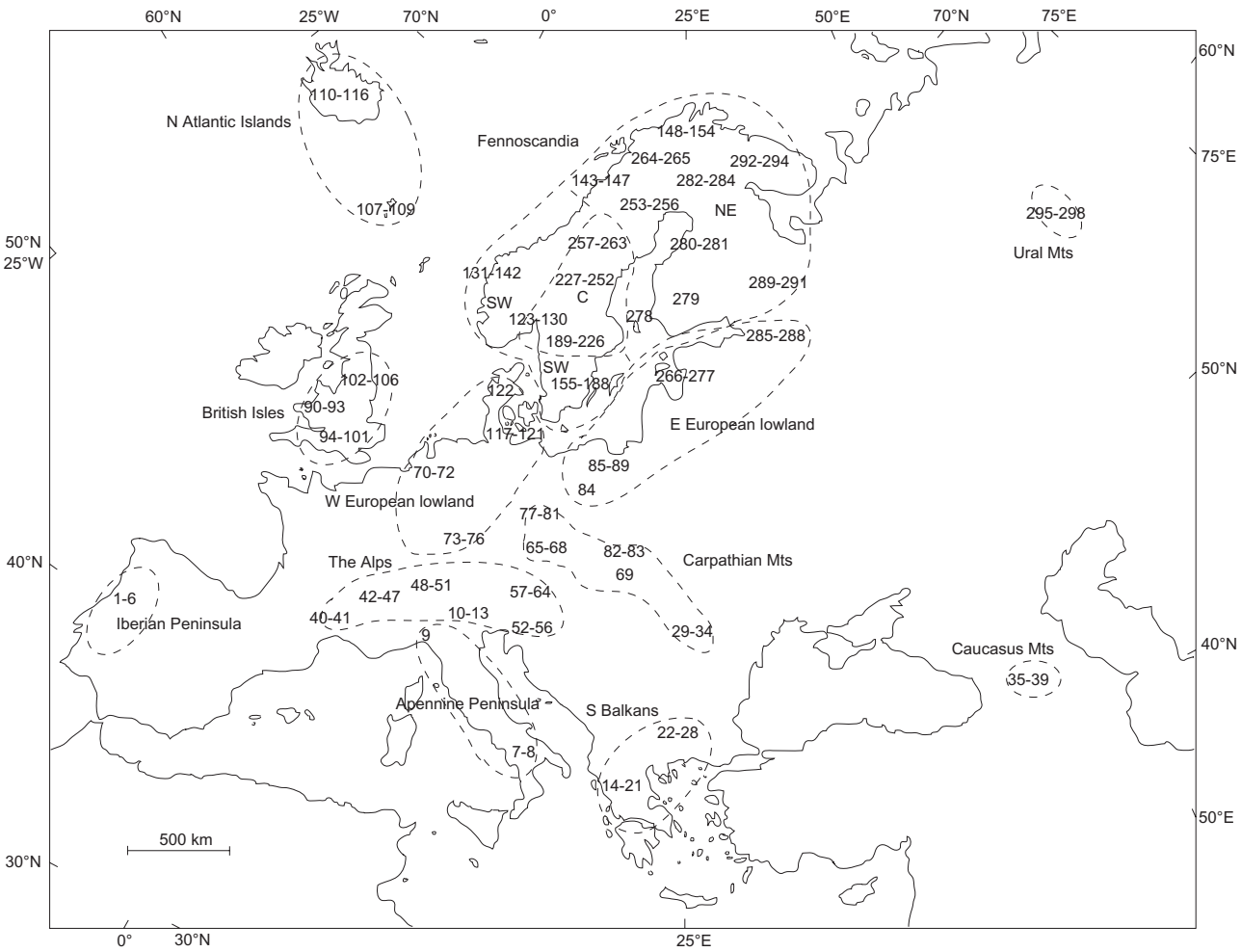

Figure 2. Localities for the 298 sampled populations of *Dactylorhiza maculata s.l.* (cf. Appendix S1). Regional population groups are delineated. Mountain areas adjacent to the Alps and the Carpathians are included in these regions. Fennoscandia is further divided into three subregions: south-western Scandinavia (SW), central Scandinavia (C), and north-eastern Fennoscandia (NE).

From the nuclear genome, portions of rDNA were analysed for allelic variation. rDNA genes occur in large numbers of copies, and individual plants may be heterozygous and contain different proportions of the constituent alleles. Following Shipunov *et al*. (2004) and Pillon *et al*. (2007), two pairs of *Dactylorhiza*-specific primers (Table 1) were used to amplify short (70 and 80 bp) length-variable fragments located in two different parts of the internal transcribed spacer (ITS) region (including the ITS1 spacer, the 5.8S rDNA gene and the ITS2 spacer). The fragments were combined and interpreted as alleles. The relative frequency of each allele was assessed from the relative peak areas of the fragments, as visualized in electropherograms on the automated sequencer.

Data analysis

Plastid markers: Parallel analyses (where appropriate) were performed based on unordered and ordered alleles, respectively, in order to consider both the infinite alleles model (IAM; Kimura & Crow, 1964) and the stepwise mutation model (SMM; Ohta & Kimura, 1973). Whereas the IAM is conservative (an allele is allowed to mutate into any other allele), the SMM makes assumptions about the actual mutation process that may occur at microsatellite loci (i.e. stepwise gain or loss of single repeat units) (cf. Lowe, Harris & Ashton, 2004). Using PAST v1.44 (Hammer, Harper & Ryan, 2001), relationships between haplotypes were summarized by principal coordinates analysis (PCO) based on Euclidean distances between

Table 1. List of primers used in the study

No.	Locus, type of variation	Specific primers	Sequence 5′ → 3′	Annealing temperature (°C)
1	*trn*T – *trn*L intergenic spacer, polyA[1]	Cy5trnL5 trnLR5	CGAAATCGGTAGACGCTACGC CGTTAGAACAGCTTCCATTG	57
6	*psb*C – *trn*S pseudospacer, indel[2]	Cy5trnS2 psbC2	AGAGTTTCAGGTCCTACCTA GTGTTCCTAACTGCCCACTT	54.4
6B	*psb*C – *trn*S pseudospacer, indel[2]	Cy5trnS1 trnS2f	GGTTCGAATCCCTCTCTCTC TAGGTAGGACCTGAAACTCT	54.4
8	*rps*19 – *psb*A intergenic spacer, polyT	Cy5HK7F HK8R	CACCTAGACACTTATCATTC CCGATTTCTCCAAATTTTCG	54
9	*rps*19 – *psb*A intergenic spacer, indel	Cy5HK9R HK8F	CTAGCTTCTGTGGAAGTTCC CGAAAATTTGGAGAAATCGG	54
10b	*psb*A – *trn*K exon 1 interg. spacer, polyA-TA-T	Cy5trnK1A HK10F	CCGACTAGTTCCGGGTTCGA GAAAGGCTTGTTATTTCACAG	56
11b	*rpl*16 intron, polyA	Cy5F71 F71R2	GCTATGCTTAGTGTGTGACTCGTTG AGTTTATAGTGGGGTCAGCC	53
17	*trn*S – *trn*G interg. spacer, poly[T_nA(C,G)]	Cy5trnSf trnSGr1	GCCGCTTTAGTCCACTCAGC GGATAAATCCGTTTCGAATC	54
18	*trn*S – *trn*G intergenic spacer, polyTA	Cy5trnSGf2 trnSGr2	CCTAATTCTTAGAAAGAATATGAG GAATAGATATAGAATCTTACTC	54
19	*trn*S – *trn*G intergenic spacer, polyT[3]	Cy5trnSGf3 trnSGr3	GAGTAATAGTGTTCTAATAAGAG CAGACGCAGTCAAGATAGCA	58
i	ITS, indel[3]	Cy5ITS.d.fuc ITS.d.fuc	ATTGAATCGCTCCATAAGAC ACCGCATGACGGGCCATTCT	52
ii	ITS, indel[3]	Cy5ITS.d.mac ITS.d.mac	TGTGCCAAGGTAAATATGCA TAGGAGCAAACAACTCCACA	52

Initially, many loci were screened.
[1]Soliva & Widmer, 1999; [2]Hedrén, 2003; [3]Pillon *et al*., 2007.

pairs of haplotypes. Minimum spanning networks (MSNs) were further constructed using ARLEQUIN v3.01 (Excoffier, Laval & Schneider, 2005). Relationships among populations with respect to haplotype composition were visualized by means of PCO. Separate analyses were performed for *D. maculata s.l.*, ssp. *maculata*, and ssp. *fuchsii*: only populations consisting of five or more individuals were included. For these analyses mean distances between individuals in every population pair were first calculated using ARLEQUIN, in order to take into account not only the proportion of different haplotypes between populations, but also the degree of differentiation between haplotypes. Potential recombination between haplotypes was analyzed by means of the four-gamete test (Hudson & Kaplan, 1985), which is based on pairwise combinations of alleles from different loci.

Nuclear markers: Mean ITS allele frequencies were calculated for each population. Relationships among populations were visualized by means of principal component analysis (PCA) using PAST.

Geographic analysis: Associations between geographic and genetic distances (both plastid and nuclear data) were investigated by Mantel tests using NTSYSpc v2.2 (Rohlf, 2005). Here, differentiation between populations in ITS was described by Cavalli-Sforza chord distances (Cavalli-Sforza & Edwards, 1967). Separate analyses were performed for ssp. *maculata* and ssp. *fuchsii*.

To facilitate the large-scale phylogeographic analyses, all populations were grouped into regions (Fig. 2). The regional divisions were based on major plains, Islands, and mountain ranges in Europe. Fennoscandia was further divided into three subregions based on preliminary results (see the Introduction; Fig. 2; Ståhlberg & Hedrén, 2008).

Using ARLEQUIN, and based on both plastid and nuclear markers, analysis of molecular variance (AMOVA) was performed to describe the partitioning of genetic diversity among regions, among populations within regions, and within populations (Φ statistics). Total between-population diversity (Φ_{ST}) was calculated by excluding the regional level from the

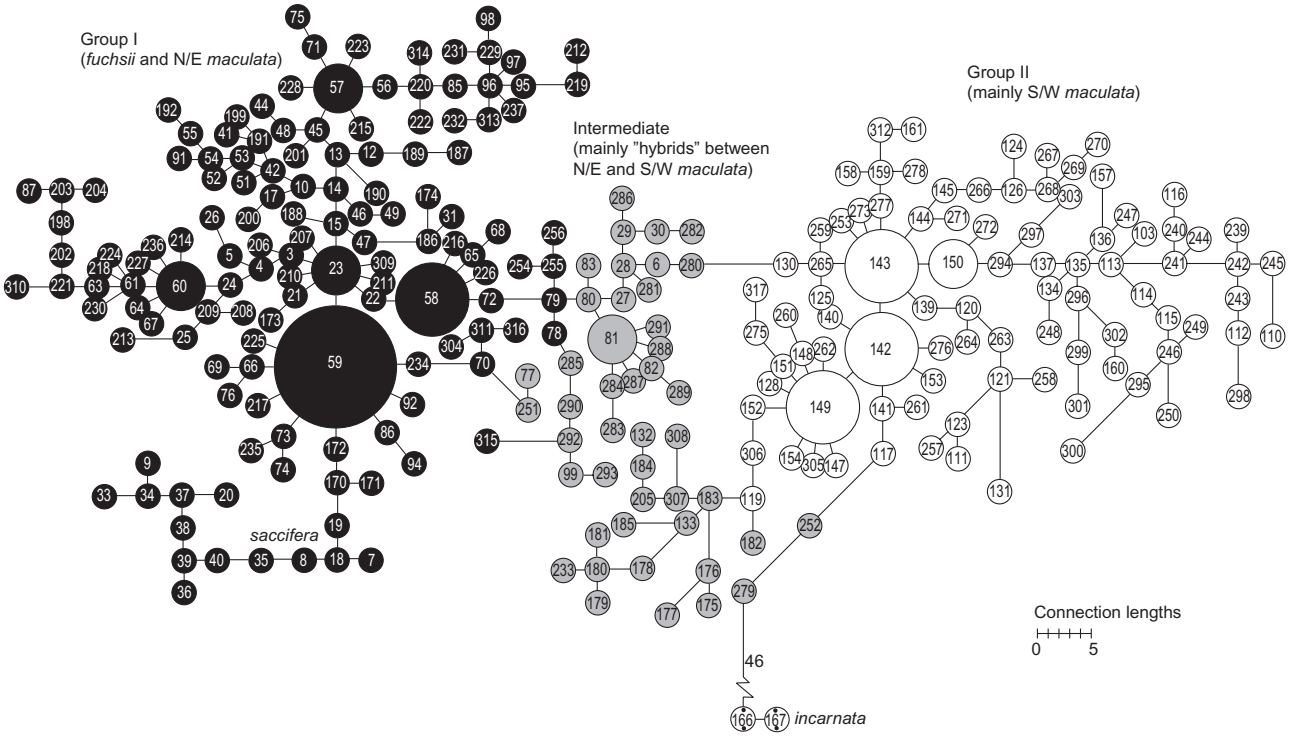

Figure 3. Minimum spanning network of the 274 plastid haplotypes (Appendix S2). The network is based on squared Euclidean distances. Alternative one-step links (not shown) connect many haplotypes within the three major groups. The sizes of the circles are proportional to the haplotype frequencies. The numbering of haplotypes is based on a larger data set that encompasses other taxa of *Dactylorhiza*.

analyses. Separate analyses were performed for ssp. *maculata* and ssp. *fuchsii*.

For plastid data, between-population diversity was calculated separately for all regions, and for the three Fennoscandian subregions, in order to unravel local differentiation patterns. Haplotype richness and frequency of private haplotypes were assessed for each region and subregion. Haplotype richness was expressed as the number of different haplotypes per 100 individuals. Frequency of private haplotypes was expressed as the proportion of individuals with region-specific haplotypes. Gene diversity and average gene diversity over loci (H and π, respectively, as implemented in ARLEQUIN) were calculated for regions and subregions by pooling populations. Gene diversity was based solely on haplotype frequencies, whereas average gene diversity over loci considers both haplotype frequencies and the degree of differentiation between haplotypes (cf. PCO of populations above). Populations from the three Fennoscandian subregions were compared by standard ANOVA using SPSS v11.5 (SPSS, Chicago, IL, USA). Separate diversity computations were performed for ssp. *maculata* and ssp. *fuchsii*.

RESULTS

Relationships among plastid DNA haplotypes

A total of 274 haplotypes were defined based on 61 fragment size variants (alleles) at ten loci (Appendix S2). The number of alleles per locus varied between three (loci 1 and 17) and 14 (locus 10b) (cf. Table 1). The ten most common haplotypes were geographically widespread, and were found in 54% of the individuals, whereas 138 haplotypes were just found in one individual. Two haplotypes (H166 and H167) that Hedrén *et al*. (2008) identified as typical for *Dactylorhiza incarnata*, that is, another *Dactylorhiza* lineage, were found in six individuals. We interpret their occurrence as the result of occasional introgressive gene flow.

Two major groups ('group I' and 'group II' *sensu* Hedrén *et al*., 2008) and one intermediate group of haplotypes appeared in the MSN and PCO (Figs S1, 3). Groups I and II consisted of 136 and 92 haplotypes, respectively, whereas 44 haplotypes were intermediate (Appendix S2). The same patterns appeared irrespective of whether the alleles were treated as unordered or ordered characters. As the degree of variation, and thus the probability of homoplasic

mutations, differed greatly among loci, we performed separate analyses, where we gradually excluded the most variable loci. However, this procedure did not affect the major structure.

The structuring of haplotypes was largely given by loci with indel variation (6, 6B, 9), and by three microsatellite loci (8, 11b, 17): each locus had one category of alleles typical for group I, and another category of alleles typical for group II (Table 1; Appendix S2). No unique alleles were found in the intermediate haplotypes. Most of them had two alleles from one category and four alleles from the other category, which explains why one fraction of the intermediate haplotypes was closer to group I, whereas another fraction was closer to group II. Haplotypes with only one 'incorrect' allele were placed in group I or group II. By applying the four-gametes test we detected a minimum of 15 possible recombination events.

Taxonomic patterns

The distribution of haplotypes among taxa and among populations is listed in Appendix S1, and is graphically visualized in Figure 4. Comparable numbers of haplotypes were found in ssp. *maculata* and ssp. *fuchsii* (157 versus 152). Similarly, 110 haplotypes were private to ssp. *maculata*, compared with 109 for ssp. *fuchsii*. However, most individuals of both ssp. *maculata* and ssp. *fuchsii* had haplotypes that were common to both taxa (62 versus 73%). Group-I haplotypes were slightly more common in ssp. *fuchsii* than in ssp. *maculata*. Group-II haplotypes were much more common in ssp. *maculata* than in ssp. *fuchsii*. Intermediate haplotypes were also more common in ssp. *maculata* than in ssp. *fuchsii*.

The structuring of populations was largely given by the proportion of group-I and -II haplotypes in the populations (Fig. 5). In the PCO of populations from both taxa, the proportion of group-I haplotypes increased to the left, and group II to the right (Fig. 5a). Most of the populations of ssp. *fuchsii* were placed in a very dense cluster to the left. A considerable fraction of the ssp. *maculata* populations were also placed in this cluster. Another major fraction of the ssp. *maculata* populations formed a dense group to the right. When ssp. *saccifera* was considered separately from ssp. *fuchsii*, it was observed that most of the ssp. *saccifera* population were placed somewhat above the dense *fuchsii* cluster. Of 45 haplotypes found in ssp. *saccifera*, 41 were private, but related to common group-I haplotypes (Fig. 3; Appendix S1). Common group-I haplotypes (also encountered in ssp. *maculata* and ssp. *fuchsii*) were found in 19% of the individuals of ssp. *saccifera*. Diploid and tetraploid populations of ssp. *fuchsii* were indistinguishable.

Six different ITS alleles were identified and numbered according to Pillon *et al*. (2007). Mean ITS allele frequencies were calculated for each population (Fig. 6). In the PCA that encompassed all populations, the first axis separated populations of ssp. *maculata* and ssp. *fuchsii* (Fig. 7). Populations of ssp. *fuchsii* mainly clustered to the left. One group of ssp. *maculata* formed a cluster to the right, whereas other populations were dispersed towards the centre. The second axis separated populations of ssp. *saccifera* from ssp. *fuchsii*. Allele I was detected in practically all populations of ssp. *maculata* (98%). By contrast, it was largely absent in ssp. *fuchsii* (detected in 17% of the populations). Allele IIIb was detected in 98% of the populations of ssp. *fuchsii*, mostly in high frequencies, especially in certain populations of ssp. *saccifera*. It was found in 75% of the populations of ssp. *maculata*, but here the frequencies differed much more among populations. Allele IV was detected in minute frequencies in just a few populations of both taxa. Allele V was rare in ssp. *saccifera* but was otherwise present in 95% of the ssp. *fuchsii* populations, mostly in high frequencies. It was furthermore found in 84% of the ssp. *maculata* populations, but here again the frequencies differed much more among populations. Allele VI was private to ssp. *saccifera* and was detected in 83% of the populations. Allele X was detected in minute frequencies in a few populations of both ssp. *maculata* and ssp. *fuchsii*. Previous studies have identified it as a typical *incarnata* allele (Pillon *et al*., 2007; Ståhlberg & Hedrén, 2008). We interpret its occurrence as the result of occasional introgressive gene flow (cf. haplotypes above).

Geographic patterns

Genetic distances based on both plastid and nuclear markers were correlated significantly with geographic distances for populations of ssp. *maculata* ($r = 0.17$ and 0.49, respectively; $P < 0.001$ for both). For populations of ssp. *fuchsii*, there was no correlation between differentiation in plastid markers and geographic distances ($r = 0.004$; $P = 0.47$), but there was a correlation between ITS differentiation and geographic distances ($r = 0.32$; $P < 0.001$).

For plastid data, the hierarchical AMOVA showed a stronger regional differentiation among populations for ssp. *maculata* than for ssp. *fuchsii* (Table 2). For ITS data, the regional differentiation was slightly stronger for ssp. *fuchsii* than for ssp. *maculata*. For both taxa, the proportion of between-population diversity was higher for plastid data than for nuclear data.

Distinct geographic patterns were highlighted in the PCO of plastid data when only populations of ssp. *maculata* were considered (Fig. 5b; cf. Fig. 4a). Populations from north-eastern Fennoscandia and the

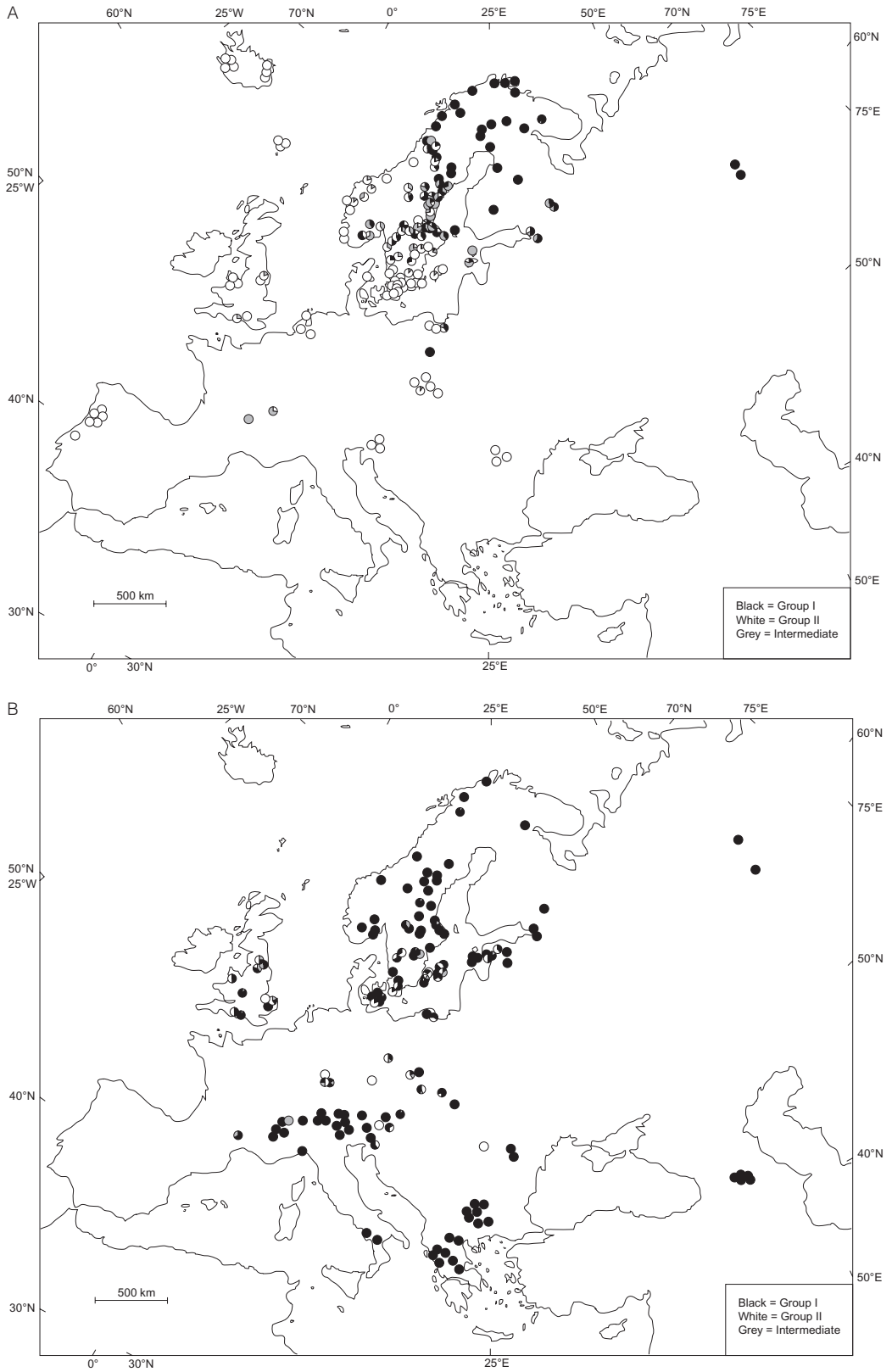

Figure 4. Geographic distribution of group-I, –II, and intermediate plastid haplotypes (cf. Fig. 3). A, populations of *Dactylorhiza maculata* ssp. *maculata*. B, populations of ssp. *fuchsii*.

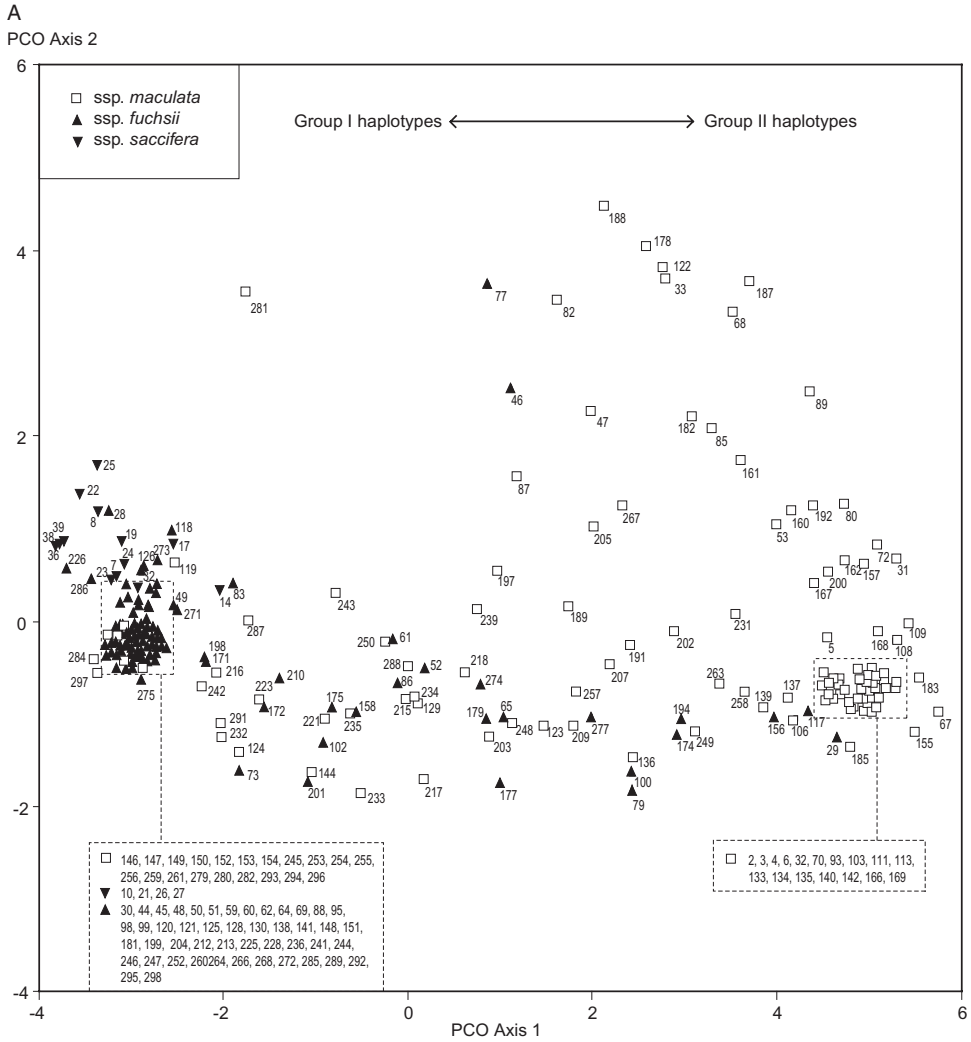

Figure 5. Principal coordinates analysis of plastid data. Population numbers refer to Appendix S1. A, all populations of *Dactylorhiza maculata s.l.* The first two axes account for 60 and 7% of the total variation, respectively. B, populations of ssp. *maculata*. Only populations consisting of five or more individuals are included. The first two axes account for 56 and 10% of the total variation, respectively. C, populations of ssp. *fuchsii*. Only populations consisting of five or more individuals are included. Numbers in boldface/italics refer to populations of ssp. *saccifera*. The first two axes account for 39 and 10% of the total variation, respectively.

Urals ('the northern/eastern lineage', characterized by group-I haplotypes) clustered to the left in the ordination plot. Populations from south-western Scandinavia, the North Atlantic Islands, the British Isles, and western and central Continental Europe, including the Eastern Carpathians ('the southern/western lineage', characterized by group-II haplotypes) clustered to the right. Most populations in the intermediate area of the ordination plot were from central Scandinavia, characterized by a mixed composition of group-I and -II haplotypes, or by high frequencies of intermediate haplotypes. Similar but less distinct geographic patterns were inferred from the nuclear markers (Fig. 6a). The frequency of alleles IIIb and V increase towards the north and the north-east, whereas the frequencies of allele I increase towards the south and the west. Populations from Atlantic Europe are almost entirely fixed for allele I.

Geographic structure was largely unresolved for ssp. *fuchsii*. A vast majority of the populations from the entire distribution range consist exclusively of individuals with group-I plastid haplotypes (Fig. 4b). In the PCO of plastid data for ssp. *fuchsii* (Fig. 5c), these populations were placed to the right in the ordination plot. Populations from the Caucasus were placed in the upper right corner, and populations from the southern

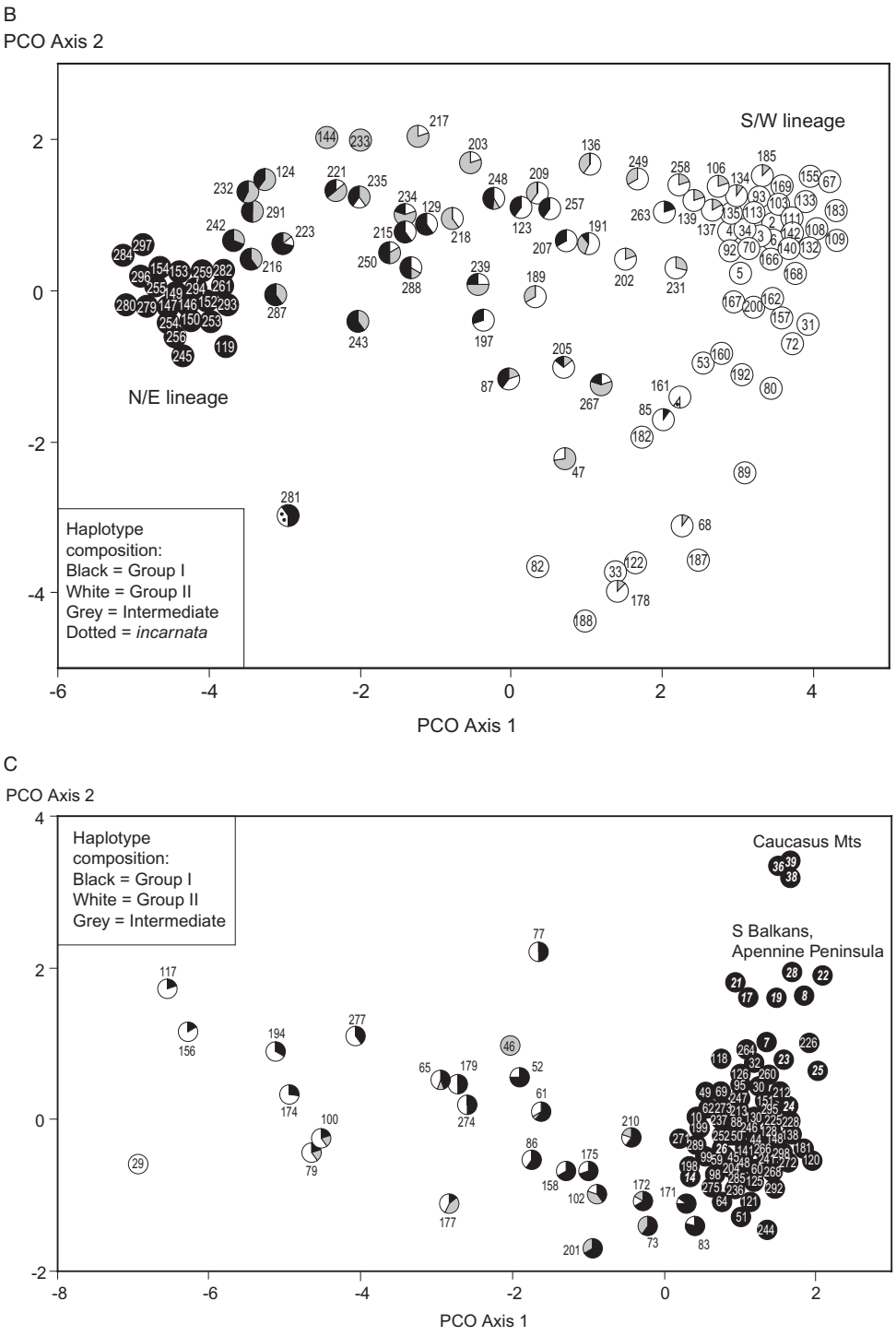

Figure 5. *Continued*

Balkans and the Apennine Peninsula were placed at intermediate positions along the second axis. The proportion of group-II haplotypes increased gradually in populations to the left along the first axis. The map (Fig. 4b) shows that a low number of populations with a mixed composition of group-I and -II haplotypes occur in most geographic regions, but not in the southern, northern, and eastern extremes of the distribution. A few populations that consist solely of group-II haplotypes, as well as a few populations with

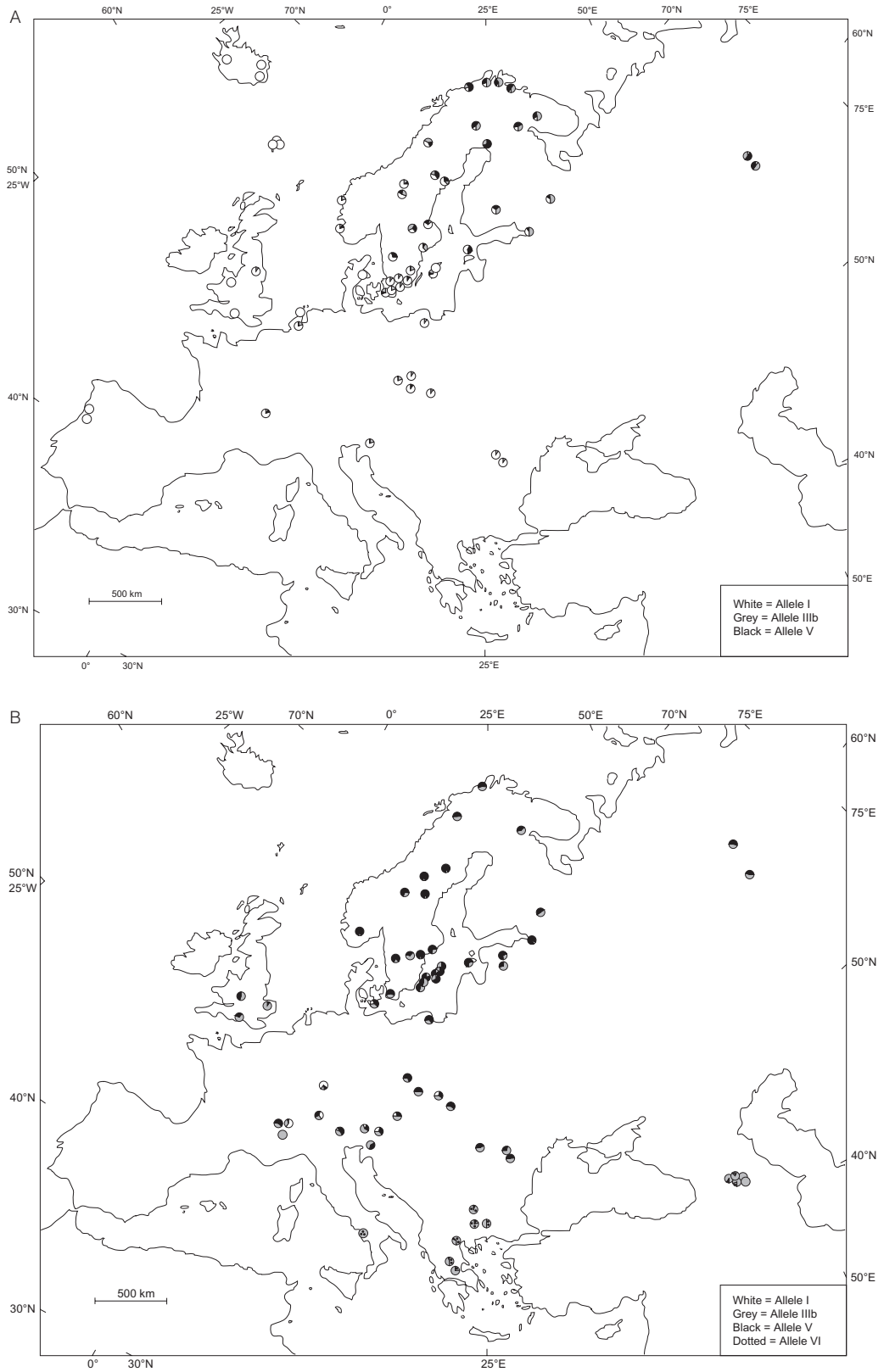

Figure 6. Geographic distribution of internal transcribed spacer (ITS) alleles. A, populations of *Dactylorhiza maculata* ssp. *maculata*; B, populations of ssp. *fuchsii*.

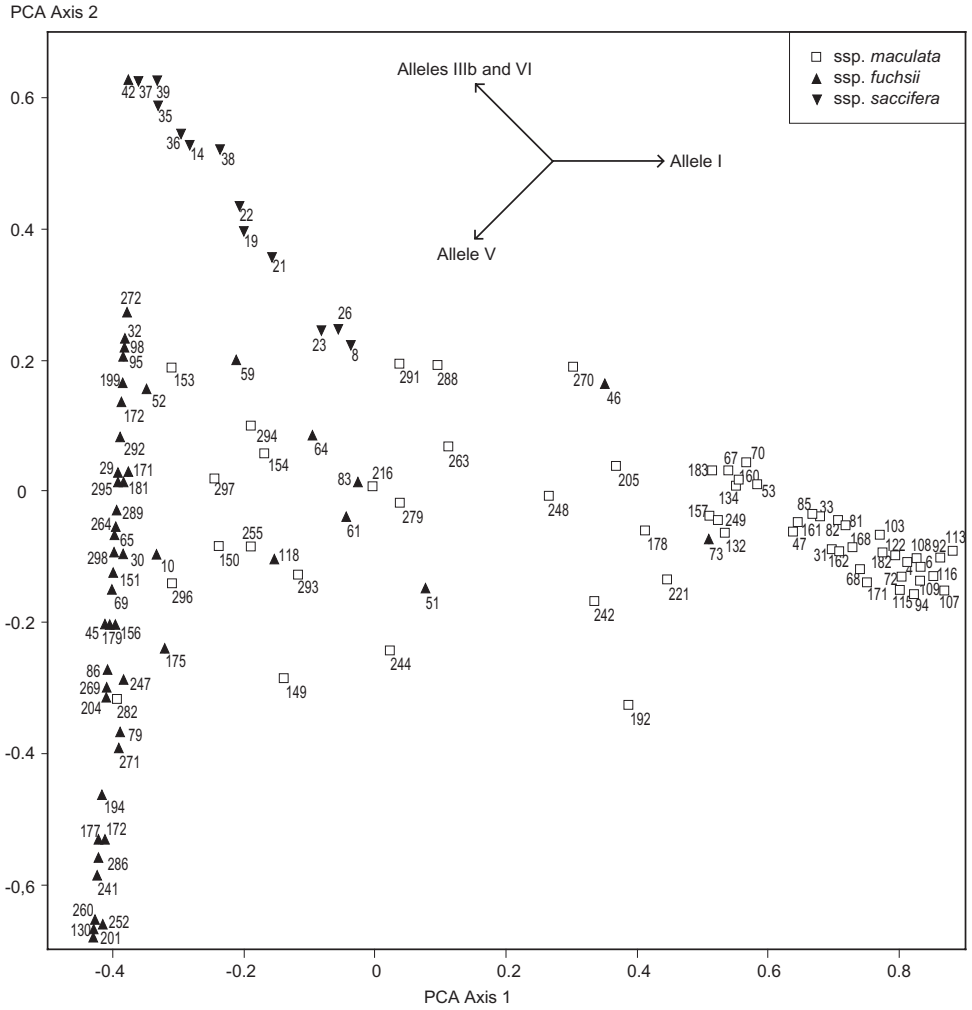

Figure 7. Principal components analysis based on internal transcribed spacer (ITS) allele frequencies. Population numbers refer to Appendix S1. The first two axes account for 68 and 22% of the total variation, respectively.

high frequencies of intermediate haplotypes, occur in the central part of the distribution. For nuclear markers, the observed Mantel test correlation between ITS differentiation and geographic distances was largely the result of differences between the southern/south-eastern extremes and other areas (Fig. 6b). Populations from the southern Balkans were characterized by alleles IIIb and VI, in similar frequencies, and populations from the Caucasus were almost fixed for allele IIIb. In other regions, most populations were characterized by comparably even frequencies of alleles IIIb and V. Allele I was restricted to a few populations in Central Europe.

Genetic diversity

In Table 3 (plastid data only), haplotype richness, frequency of private haplotypes, gene diversity, average gene diversity over loci, and between-population diversity are compiled for each region and subregion (cf. Fig. 2). Separate tables are given for ssp. *maculata* and ssp. *fuchsii*. The distribution of average gene diversity over loci for populations consisting of five or more individuals is shown on maps in Figure S2.

The regional distribution of haplotypes was comparable with the total distribution of haplotypes, that is, most regions were characterized by a few common haplotypes and a large number of rare haplotypes. This pattern was observed for both ssp. *maculata* and ssp. *fuchsii*, and was reflected by the values of haplotype richness and gene diversity. With some clear exceptions, similar values were obtained for all regions. Haplotype richness, which is sensitive to sample size, fluctuated more than gene diversity however.

Table 2. Analysis of molecular variance

Source of variation	Variation (%)	
	Plastid data	ITS data
Dactylorhiza maculata ssp. *maculata*		
Among regions	16.5	18.2
Among populations within regions	45.1	17.7
Within populations	38.4	64.1
Φ_{ST}	58.0	32.2
Dactylorhiza maculata ssp. *fuchsii*		
Among regions	4.9	25.6
Among populations within regions	45.0	13.5
Within populations	50.1	60.9
Φ_{ST}	49.8	36.3

The total between-population diversity (Φ_{ST}) was calculated by excluding the regional level from the analyses. All P values were < 0.001.

For ssp. *maculata*, particularly high values of haplotype richness were obtained for the Alps and the eastern European lowland. These regions were also characterized by high frequencies of private haplotypes. It should, however, be observed that the sample size for the Alps was low. In contrast to the Alps and the eastern European lowland, highly modest values of haplotype richness and frequency of private alleles were obtained for the North Atlantic Islands. Low frequencies of private haplotypes were also associated with the British Isles, the western European lowland, and the Urals. The measure of between-population diversity is sensitive to sampling strategy, which evidently makes comparisons among the regions difficult. Nevertheless, differentiation among British populations appeared to be exceptionally low compared with other regions. The highest values of both gene diversity and average gene diversity over loci were associated with the eastern European lowland and Fennoscandia. Populations from central Scandinavia were significantly more diverse than populations from south-western Scandinavia and north-eastern Fennoscandia (average gene diversity over loci: $F = 31.15$; $P < 0.001$; Fig. S2a). The structuring of diversity in Fennoscandia was also reflected by the between-population diversity. The differentiation among populations within subregions, and especially among populations from central Scandinavia, was markedly lower than the differentiation among populations from the region as a whole.

For ssp. *fuchsii*, somewhat lower values of haplotype richness were obtained for Fennoscandia and the British Isles, compared with Continental Europe. Frequencies of private haplotypes were by far highest in the southern Balkans and the Caucasus, where a majority of the individuals were characterized by regional-specific haplotypes. In contrast, the frequency of private haplotypes was almost negligible in the British Isles. High values of average gene diversity over loci were primarily found in the centre of the distribution range of ssp. *fuchsii*, where both group-I and -II haplotypes occurred (Fig. S2b). Among the regions in which group-I haplotypes were solely found, the southern Balkans was much more diverse than north-eastern Fennoscandia and the Urals. Differentiation among populations of ssp. *fuchsii* from the southern Balkans also appeared to be striking. The Caucasus was characterized by a remarkably low diversity. When populations from the three Fennoscandian subregions were compared, it was observed that the average gene diversity over loci decreased towards the north ($F = 7.66$; $P < 0.01$).

DISCUSSION

Intraspecific differentiation and introgressive gene flow

Our results reveal important aspects of glacial survival and postglacial expansion of *D. maculata s.l.* Both plastid and nuclear markers convincingly demonstrate that ssp. *maculata* consists of two distinct lineages: a southern/western lineage and a northern/eastern lineage (Figs 4a, 6a). To some extent, the two lineages are also morphologically distinct (e.g. with respect to pigmentation, number of flowers, and stem width; Ståhlberg & Hedrén, 2008). In contrast, populations of ssp. *fuchsii* form a coherent group, even though there are some differences in molecular markers between populations from the southern/south-eastern extremes and populations from other areas (Figs 4b, 6b).

This large-scale view agrees with previous regionally focused studies. One group of inter-related plastid haplotypes occurs in ssp. *maculata* from western Continental Europe, the British Isles, and southern Scandinavia (Devos *et al*., 2003, 2006; Hedrén, 2003; Pillon *et al*., 2007; Hedrén *et al*., 2008; Ståhlberg & Hedrén, 2008), as well as south-western Russia (Shipunov *et al*., 2004), whereas another group of inter-related haplotypes has been found in northern Scandinavia and north-western Russia (Shipunov *et al*., 2004; Ståhlberg & Hedrén, 2008). In all regions, the same authors have found group-I haplotypes in ssp. *fuchsii*. Regional studies based on nrDNA have revealed similar results (Shipunov *et al*., 2004; Devos *et al*., 2005; Pillon *et al*., 2007; Ståhlberg & Hedrén, 2008).

Hybrids between ssp. *fuchsii* and ssp. *maculata* have been reported from various parts of Europe (e.g.

Table 3. Regional distribution of haplotype richness, frequency of private haplotypes, gene diversity (H), average gene diversity over loci (π), and between-population diversity (Φ_{ST})

	N_{ind}	N_{pop}	Haplotype richness	Frequency of private haplotypes	H	π	Φ_{ST}
Dactylorhiza maculata ssp. *maculata*							
Iberian Peninsula	28	6	28.6	17.9	0.83	0.16	0.379***
The Alps	21	5	71.4	57.1	0.92	0.38	0.5481***
Carpathian Mts	64	8	25.0	29.7	0.88	0.37	0.640***
W European lowland	24	4	29.2	4.2	0.81	0.32	0.719***
E European lowland	41	8	65.9	46.3	0.97	0.56	0.488***
British Isles	30	7	30.0	3.3	0.81	0.16	0.038^{ns}
N Atlantic Islands	47	9	8.5	2.1	0.61	0.07	0.378***
Fennoscandia	669	100	18.5	23.3	0.96	0.50	0.540***
SW Scandinavia	196	31	19.4	24.0	0.92	0.33	0.372***
C Scandinavia	319	50	26.3	23.2	0.97	0.49	0.269***
NE Fennoscandia	158	25	12.7	10.1	0.82	0.15	0.450***
Ural Mts	10	2	20.0	0.0	0.53	0.11	0.750^{ns}
Dactylorhiza maculata ssp. *fuchsii*							
Apennine Peninsula	12	3	41.7	16.7	0.83	0.22	0.495*
S Balkans	110	15	38.2	80.0	0.95	0.33	0.653***
Caucasus Mts	30	5	6.7	100.0	0.07	0.01	0.245^{ns}
The Alps	112	24	29.5	25.7	0.88	0.32	0.461***
Carpathian Mts	61	10	39.3	23.0	0.92	0.44	0.480***
W European lowland	43	8	37.2	18.6	0.88	0.49	0.58****
E European lowland	78	13	32.1	15.4	0.78	0.30	0.178**
British Isles	46	10	28.3	2.2	0.86	0.35	0.364***
Fennoscandia	370	50	15.4	21.4	0.86	0.34	0.502***
SW Scandinavia	107	15	20.6	9.4	0.81	0.47	0.417***
C Scandinavia	225	29	19.1	18.7	0.85	0.26	0.502***
NE Fennoscandia	37	8	24.3	18.9	0.84	0.15	0.382***
Ural Mts	10	2	50.0	50.0	0.76	0.16	0.906**

Haplotype richness is expressed as the number of different haplotypes per 100 individuals. The frequency of private haplotypes is expressed as a proportion (%) of individuals with region-specific haplotypes. The three Fennoscandian subregions are given in italics.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

Averyanov, 1977; Gathoye & Tyteca, 1989). At least a limited introgressive gene flow may thus occur (cf. Devos *et al*., 2005). However, detailed studies of mixed populations (Ståhlberg & Hedrén, 2009) have suggested that introgression is probably less common in the *D. maculata* complex than has often been assumed (e.g. by Delforge, 1995). In particular, gene flow from tetraploid ssp. *maculata* to diploid ssp. *fuchsii* appears to be rare (Ståhlberg & Hedrén, 2009). This is in accordance with the general view that gene flow from tetraploid to diploid level is a rare process (Stebbins, 1971). In contrast, reproductive barriers between taxa at the same ploidy level are predicted to be lower (Grant, 1981). That introgression between ssp. *fuchsii* and ssp. *maculata* is indeed limited in both directions is strongly supported by the fact that group-I plastid haplotypes are completely absent from populations of ssp. *maculata* from southern/western Europe (Fig. 4a), despite the fact that ssp. *maculata* and ssp. *fuchsii* sometimes occur in sympatry. If gene flow from diploid ssp. *fuchsii* to tetraploid ssp. *maculata* is limited, then gene flow in the opposite direction should be negligible (cf. Stebbins, 1971). Theoretically, the occurrence of group-II haplotypes in some diploid populations of ssp. *fuchsii* in southern/western Europe could be interpreted as a consequence of introgression, but we consider an ancient phylogeographic pattern as a more probable explanation, because group-II is probably older than group I, and may formerly have been prevalent in diploid populations of *D. maculata s.l.* (discussed below; cf. Hedrén *et al*., 2008). However, a local influence of introgression (past or present) may be envisaged for Central Europe, where most populations

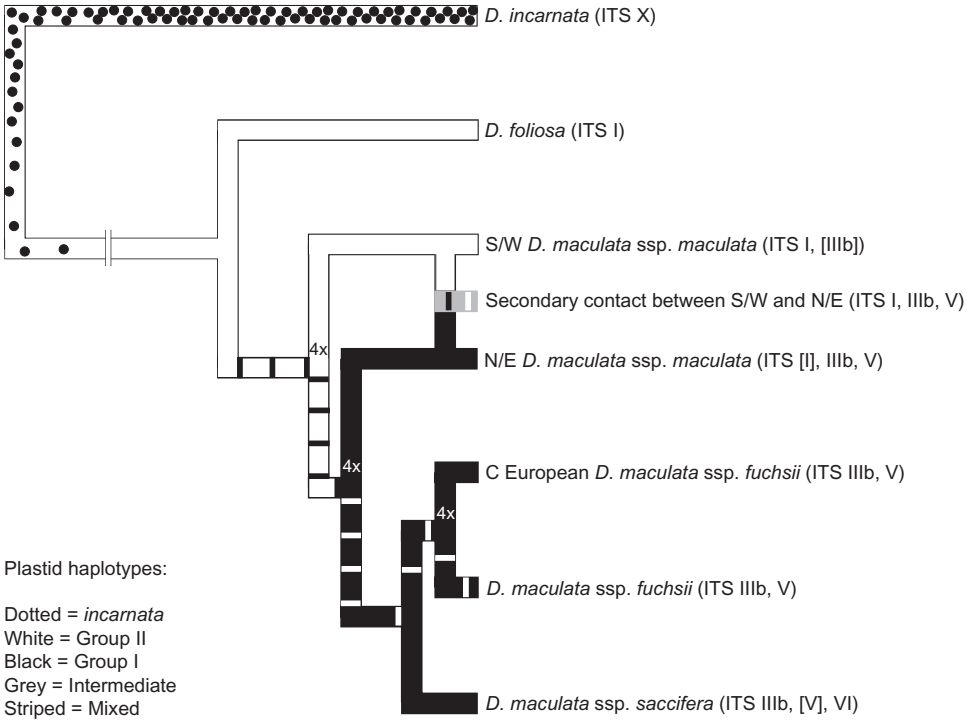

Figure 8. Evolutionary history of the *Dactylorhiza maculata* complex as indicated by plastid and nuclear ribosomal DNA (nrDNA) markers. Autopolyploidization events are marked '4×'. Note the changes in relative frequencies of group-II and -I haplotypes. Internal transcribed spacer (ITS) alleles that occur in low frequencies are placed between square brackets.

of ssp. *fuchsii* consist of tetraploid plants. For example, a relatively high incidence of the ssp. *maculata*-specific ITS allele I in populations of ssp. *fuchsii* from Central Europe is indicative of local introgression (Fig. 6b). Outside Central Europe, allele I is practically absent from ssp. *fuchsii*.

Polyploid evolution and phylogeny of the *Dactylorhiza maculata* complex

A hypothetical model of the evolutionary history of the *D. maculata* complex is given in Figure 8. If the group of *incarnata* plastid haplotypes is regarded as the out-group, it must be concluded that group I is derived from group II (Fig. 3; cf. Hedrén *et al*., 2008). Given the present distribution of group-I and -II haplotypes (Fig. 4), it can further be concluded that the differentiation between groups I and II must be ancient, and should pre-date the last glaciations. The occurrence of group-II haplotypes in some populations of present-day diploids may represent a relictual state. That the differentiation between groups I and II must be ancient is also dictated by the fact that group-II haplotypes have become completely replaced by group-I haplotypes in diploids in the Southern Balkans, and eastwards towards the Caucasus. In this area, group-II haplotypes are, however, preserved in some allotetraploid taxa (Hedrén *et al*., 2007; see the Introduction).

Interestingly, group-II haplotypes have been identified in the Madeiran diploid *D. foliosa* (Devos *et al*., 2006), which based on morphology is relatively close to *D. maculata* ssp. *maculata* (e.g. Sundermann, 1980). Data obtained from both nrDNA markers (Bateman *et al*., 2003; Devos *et al*., 2005; Pillon *et al*., 2007) and AFLP (Hedrén *et al*., 2001) have also indicated that *D. foliosa* is related to *D. maculata* ssp. *maculata*. It may thus be assumed that the southern/western lineage of ssp. *maculata* has arisen from ancestors common to this lineage and to *D. foliosa*, and that *D. foliosa* is of relictual character.

The northern/eastern lineage of ssp. *maculata*, which is characterized by group-I haplotypes, has probably arisen from diploid ancestors common to this lineage and to present-day ssp. *fuchsii*. Northern/eastern ssp. *maculata* is relatively close to ssp. *fuchsii* in nuclear markers (Fig. 6). Averyanov (1990) and Shipunov *et al*. (2004, 2005) have pointed to the morphological resemblance between ssp. *maculata* and ssp. *fuchsii* from northern Russia. Morphological differences between southern/western and northern/eastern populations of ssp. *maculata* have been

indicated in several studies. In Scandinavia, northern populations are, for example, relatively distinct from southern populations (Ståhlberg & Hedrén, 2008). In western Continental Europe, the British Isles, and southern Scandinavia, ssp. *maculata* is clearly distinct from ssp. *fuchsii* (e.g. Heslop-Harrison, 1951; Bateman & Denholm, 1989; Dufrêne *et al*., 1991; Tyteca & Gathoye, 2004; Ståhlberg & Hedrén, 2008).

Populations belonging to ssp. *maculata* are found in two different genetically defined lineages, and in each of these ssp. *maculata* is connected to a diploid taxon that is more-or-less contrasting in morphology. This pattern indicates restricted morphological evolution in the tetraploid lineages, which may thus have preserved some characters that have been modified in the diploids. The southern/western lineage of ssp. *maculata* should be older than the northern/eastern lineage, but both lineages are most likely to have arisen before the Holocene. The evolutionary potential for populations occupying contact zones between the two lineages remains an open question.

Based on molecular markers, tetraploid populations of ssp. *fuchsii* in Central Europe are indistinguishable from diploid populations of ssp. *fuchsii*. Previous studies have also shown that they are morphologically indistinguishable (e.g. Scharfenberg, 1977; Vöth, 1978). The tetraploid populations may accordingly represent an autotetraploid lineage that has arisen relatively recently. An origin during the Holocene accords with the present distributions of diploid and tetraploid populations: tetraploid populations of ssp. *fuchsii* are rare outside Central Europe (Ståhlberg, 2007; Fig. 1). Isolated tetraploid populations in other areas may reflect independent polyploidization events, and indicate that there is a potential for new lineages to arise and become established in the *D. maculata* complex.

Glacial refugia and postglacial recolonization

The separation of ssp. *maculata* in two distinct lineages indicates postglacial recolonization from two separate refugial areas. However, the phylogeographic signal within each lineage is weak. Both lineages are dominated by a few widespread plastid haplotypes, and by a large number of rare and geographically restricted haplotypes. The most common group-II haplotype (H143) is distributed from Portugal in the south to the Scandinavian mountain range in the north, and from Iceland in the west to Romania in the east. Similarly, the most common group-I haplotype (H59) is distributed from northern Norway in the west to the Urals in the east. In addition, many rare haplotypes differ from the common haplotypes by only one mutational step, and may have evolved locally and relatively recently (Fig. 3).

The weak phylogeographic signal within the two ssp. *maculata* lineages may reflect efficient seed dispersal and a propensity for long-distance gene flow, as suggested for other plant taxa with similar patterns of haplotype distribution (e.g. *Betula*; Palmé *et al*., 2003). Orchids have the smallest seeds among flowering plants (Dressler, 1993), permitting long-distance dispersal by wind. Evidence for long-distance seed dispersal in orchids has been provided by a case-study of *Calypso bulbosa* (Alexandersson & Ågren, 2000). Based on neutral markers, we found that the between-population diversity is moderate within ssp. *maculata* (Table 2a). Low between-population diversity is often a consequence of efficient dispersal, and low values are therefore generally reported for wind-dispersed, widespread and outcrossing taxa, such as *Dactylorhiza* (reviewed by Hamrick & Godt, 1989, 1996; Nybom & Bartish, 2000).

The Iberian Peninsula could potentially have served as a glacial refugium for the southern/western lineage of ssp. *maculata*. The Pyrenees do not appear to be a strong dispersal barrier for ssp. *maculata*, as the frequency of private haplotypes is low in the Iberian Peninsula as compared with, for example, the Caucasus for ssp. *fuchsii* (Table 3b; cf. Petit *et al*., 2003). However, we obtained relatively low values of haplotype richness and gene diversity. This limited diversity hints at a history of small population size and consequent loss of variation through genetic drift (cf. Petit *et al*., 2003). The Iberian Peninsula is therefore unlikely as a source for northward recolonization of ssp. *maculata*. According to the present-day distribution of genetic diversity, diffuse areas in more easterly parts of Europe have probably been more important for glacial survival of the southern/western lineage of ssp. *maculata*. The gene diversity is low across the western European lowland, and we found diversity to be even lower on the British Isles and the North Atlantic Islands, suggesting a history of recurrent founder events. As a comparison, southern Scandinavia, which is located entirely within the range of the southern/western lineage of ssp. *maculata*, exhibits much higher gene diversity. A low level of genetic diversity in westernmost Europe is also indicated by nuclear markers. Compared with more eastern areas, populations in westernmost Europe are almost entirely fixed for a single ITS allele.

The present distribution of ssp. *maculata* (Hultén & Fries, 1986; Averyanov, 1990) shows that the taxon has a wide ecological amplitude, and can tolerate severe climatic conditions at high latitudes and high altitudes. This fact, together with a presumed high

dispersal potential, suggests that ssp. *maculata* was widespread during the LGM. According to palaeobotanical data, suitable habitats were probably present on the European subarctic steppe-tundra (and perhaps even on the arctic tundra), during the LGM, although the westernmost regions were probably too arid (cf. Huntley & Birks, 1983; Adams, 1997; Adams & Faure, 1997). Molecular data have further indicated that several key species for temperate ecosystems survived close to the Fennoscandian ice sheet (Rendell & Ennos, 2002; Palmé *et al*., 2003; Alsos *et al*., 2005). In the east, extensive areas with low precipitation, rather than topographic barriers, may have kept the southern/western lineage of ssp. *maculata* separate and distinct from the northern/eastern lineage.

During the LGM, the northern/eastern lineage may have had a wide distribution in central Russia, between the Fennoscandian ice sheet and the Urals. Allozyme data have shown that *Picea abies* recolonized Fennoscandia from a glacial refugium with a centre close to the present-day area of Moscow (Lagercrantz & Ryman, 1990). The existence of a temperate tree species indicates that suitable habitats for many other organisms should have been present as well, and that the region may have been a common source area for westward migration.

Populations of ssp. *fuchsii* are almost exclusively characterized by group-I plastid haplotypes. As for ssp. *maculata*, few haplotypes are common and widespread, whereas a large number of haplotypes are rare and geographically restricted. With the exception of the Caucasus, the most common haplotype (H59) is found all over the distribution range of ssp. *fuchsii*. Nevertheless, the distribution of genetic diversity suggests that northern Europe has been colonized from the south to the north: there is no support for a north-eastern recolonization route for ssp. *fuchsii*. For Scandinavia, we observed that the average gene diversity over loci gradually decreases from the south to the north in accordance with a stepping-stone model of gene dispersal (Kimura, 1953). Group-II haplotypes sometimes occurred in the central area of the distribution range of ssp. *fuchsii*, but we did not find any particular source areas of group-II haplotypes. The mixture of groups-I and -II haplotypes in ssp. *fuchsii* is probably an ancient pattern, and is not primarily the consequence of population admixture during the Holocene (discussed above).

The southern Balkans and the Caucasus are characterized by high frequencies of region-specific haplotypes. We found that the frequency of private haplotypes is lower in the Apennine Peninsula. However, populations in the Apennine Peninsula have haplotypes in common with populations in the southern Balkans, indicating gene flow between the regions during the last glacials–interglacials. In general, populations from the southern and south-eastern extremes are distinct from more northern populations. A similar pattern is given by ITS markers. Southern refugia have not contributed to the present-day distribution of diversity in northern Europe. In a more extensive Quaternary time perspective that includes several glacial–interglacial cycles, southern refugia should, however, be considered as important areas for diversification and preservation of diversity. In this respect, the Southern Balkans seems to be particularly important (cf. Devey *et al*., 2009). Here, we found a high level of diversity within populations, but also a high level of differentiation among populations (Table 3b). In contrast, the Caucasus was by far much less diverse than expected, given its status as a global biodiversity hotspot (Myers *et al*., 2000; cf. Vellend & Geber, 2005; Pillon *et al*., 2006). Distinct plastid haplotypes and a conspicuously low diversity indicates a history of strong isolation and severe population bottlenecks. Such a scenario is further supported by the observation that populations from the Caucasus to a high extent are fixed for a single ITS allele.

In contrast with ssp. *maculata*, ssp. *fuchsii* is sensitive to intensive sun exposure and is generally confined to semi-open woodlands (e.g. Heslop-Harrison, 1951; Ståhlberg & Hedrén, 2009). It is therefore likely that ssp. *fuchsii* was absent from the vast tundra plains during the LGM. Fossil records, along with molecular data, have shown that areas of sheltered topography in mountainous parts of Central Europe may have provided suitable stable microclimates for many thermophilous organisms, including deciduous trees, during the LGM (Litynska-Zajac, 1995; Stewart & Lister, 2001; Willis & van Andel, 2004; Magri *et al*., 2006; Sommer & Nadachowski, 2006; Ursenbacher *et al*., 2006). Consequently, it is reasonable to assume that northern European populations of ssp. *fuchsii* originated from various source populations in Central Europe. Consistently high values of haplotype richness, gene diversity, and average gene diversity over loci were obtained from the Carpathians, emphasizing that this region has a particularly important gene pool (Table 3b).

High diversity in contact zones between divergent lineages

The present study confirms previous indications of a Scandinavian contact zone between divergent immigrant lineages (cf. Ståhlberg & Hedrén, 2008). Both plastid and nrDNA markers conclusively show that the northern/eastern and the southern/western lineages of ssp. *maculata* meet in central Scandinavia (Figs 4a, 6a). The main route of immigration for the

northern/eastern lineage is via northern Finland, but Figure 4a suggests that some immigration has also taken place via the Åland Archipelago in the Baltic Sea. A second contact zone involving the same two lineages seems to occur in the eastern European lowland, between Poland and Lake Ladoga.

For Scandinavia, two different immigration routes, from the north-east and the south, have previously been inferred by molecular markers for brown bear (*Ursus arctos*; Taberlet & Bouvet, 1994), field vole (*Microtus agrestis*, Jaarola & Tegelström, 1995), common shrew (*Sorex araneus*, Fredga, 1996), and some vascular plants (*Festuca ovina*, Bengtsson, Weibull & Ghatnekar, 1995; *Viola rupestris*, Nordal & Jonsell, 1998; *Silene dioica*, Malm & Prentice, 2005; *Arabidopsis thaliana*, Jakobsson *et al*., 2007). Some studies have especially stressed the possibility of separate immigration routes for boreal-arctic taxa (e.g. *Cerastium alpinum*, Nyberg Berglund & Westerbergh, 2001; *Vaccinium uliginosum*, Alsos *et al*., 2005; *Dryas octopetala*, Skrede *et al*., 2006). A disjunct distribution of other taxa (e.g. *Dianthus superbus*, *Oxytropis campestris*, and *Tephroseris integrifolia*, Hultén & Fries, 1986) suggests that many elements in the Scandinavian flora and fauna may have immigrated from both the north-east and the south. Contact zones between divergent immigrant lineages thus appear to be common in Scandinavia, with profound consequences for the structuring of genetic diversity.

We found that the contact zone between the northern/eastern and southern/western lineages of ssp. *maculata* has an extensive distribution in central Scandinavia. The centre is located in the provinces of Hälsingland, Medelpad, and Ångermanland, along the Bothnian Sea (Fig. 4a). Jaarola & Tegelström (1995) and Fredga (1996) localized hybrid zones for field vole and common shrew to the same Bothnian region. Nyberg Berglund & Westerbergh (2001) also suggested that north-eastern and southern lineages of *Cerastium alpinum* form a contact zone in this area. Such a pattern of coinciding contact zones could be explained by the deglaciation history of the Weichselian ice sheet. The centre of the ice sheet during the LGM was located in the Ångermanland area, and the deglaciation of southern Ångermanland took place only *c.* 9300 years BP (*c.* 10 500 cal. yrs. BP), when southern and north-eastern Fennoscandia was already ice-free (Berglund, 2004). Many species of plants and animals may thus have accumulated in the bordering areas left by the retreating ice. When the ice had finally melted away, the Bothnian region may have become quickly colonized from both the north and the south, which should explain the coincidence of contact zones.

For plastid data, we observed that genetic diversity is markedly higher in the contact zone in central Scandinavia than in adjacent areas to the north and the south. This is reflected by all measures of genetic diversity (Table 3a). The average gene diversity over loci, which considers divergence between haplotypes, reveals that populations in central Scandinavia, together with populations from the putative contact zone in the eastern European lowland, are more diverse than any other European populations of ssp. *maculata* (Fig. S2a). A comparable observation of high diversity in a contact zone in northern Europe was made by Skrede *et al*. (2006) for the mountain avens (*Dryas octopetala*).

Indications of plastid DNA recombination

Intermediate plastid haplotypes between northern/eastern group-I haplotypes and southern/western group-II haplotypes are conspicuously common in the contact zone in central Scandinavia (Fig. 4a). A quarter of the individuals of ssp. *maculata* in the area have intermediate haplotypes. Most of the 30 intermediate haplotypes identified in the area were private. It means that almost a half of the private haplotypes in central Scandinavia belong to the intermediate group. We also observed a high frequency of intermediate haplotypes in the putative contact zone in the eastern European lowland. These remarkable results suggest that recombination takes place in the plastid genome.

It is widely accepted that the plastid genome in plants is uniparentally inherited, which should preclude recombination. In angiosperms, maternal inheritance appears to be prevalent (Corriveau & Coleman, 1988). For orchids, maternal inheritance has been observed in a dozen species belonging to tropical genera (Corriveau & Coleman, 1988), and in a few species belonging to temperate genera (*Cypripedium acaule*, Corriveau & Coleman, 1988; *Anacamptis palustris*, Cafasso, Widmer & Cozzolino, 2005). On the other hand, variation in inheritance patterns has been documented in many plants. Smith (1989) noticed that biparental inheritance occurs, at least occasionally, in nearly a third of the angiosperms he surveyed. Recent studies have confirmed this figure (e.g. Frey, Frey & Forcioli, 2005), and Hansen *et al*. (2007) argued that heteroplasmy, the condition of cells having more than one organellar haplotype, may occur on a limited scale in most groups of angiosperms. So far, few suspected cases of plastid DNA recombination have been reported in gymnosperms (viz. *Pinus contorta*, Marshall, Newton & Ritland, 2001; *Cycas taitungensis*, Huang *et al*., 2001). These observations should be compared with an increasing body of evidence for mitochondrial genome (mtDNA) recombination (e.g. Bergthorsson *et al*., 2003; Barr, Neiman & Taylor, 2005; Tsaousis

et al., 2005). Recombination of mtDNA in plants is facilitated by an active DNA uptake system (Koulintchenko, Konstantinov & Dietrich, 2003), but a similar system has not been identified in the plastid genome (Richardson & Palmer, 2007). Plastid DNA markers are standard tools for population genetic and phylogenetic analyses. It is obvious that recombination can be problematic for phylogenetic inferences, especially at the species level and in plant groups where hybridization is common.

CONCLUSIONS

(1) Gene flow is limited between the two major taxa: diploid ssp. *fuchsii* (including ssp. *saccifera*) and tetraploid ssp. *maculata*. Populations of ssp. *fuchsii* form a coherent group, even though there are some differences between populations from the southern/southeastern extremes and populations from other areas. Populations of ssp. *maculata* form two distinct lineages: a southern/western and a northern/eastern. (2) There are at least three autotetraploid lineages: southern/western and northern/eastern ssp. *maculata*, which probably pre-date the last glaciation, and Central European ssp. *fuchsii*, which is probably of postglacial origin – diploid and tetraploid populations of ssp. *fuchsii* are indistinguishable. (3) Vast areas of Central Europe and central Russia between the Fennoscandian ice sheet and the Urals may have been source areas for the postglacial migration of the two lineages of ssp. *maculata*, respectively. Areas of sheltered topography in Central Europe may have provided suitable habitats for the more thermophilous ssp. *fuchsii* during the LGM. The Mediterranean region and the Caucasus have not contributed to the northward migration of either ssp. *maculata* or ssp. *fuchsii*. (4) The southern/western and the northern/eastern lineages of ssp. *maculata* meet in central Scandinavia and in the Baltic states. A high frequency of intermediate plastid haplotypes in the contact zones hints at recombination. Measures of genetic diversity reach higher values in the contact zones than in other areas.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Principal coordinates analysis of plastid haplotypes identified in this study.
Figure S2. Intrapopulation diversity measured as average gene diversity over loci (based on plastid data).
Appendix S1. Sampling localities and distribution of plastid haplotypes among populations.
Appendix S2. Characterization of plastid haplotypes identified in the present study by means of the primer pairs described in Table 1.

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