Habitat differentiation, hybridization and gene flow patterns in mixed populations of diploid and autotetraploid Dactylorhiza maculata s.l. (Orchidaceae)

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ORIGINAL PAPER

Habitat differentiation, hybridization and gene flow patterns in mixed populations of diploid and autotetraploid *Dactylorhiza maculata* s.l. (Orchidaceae)

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Abstract Detailed ecological, morphological and molecular analyses were performed in mixed populations of diploid and autotetraploid *Dactylorhiza maculata* s.l. in Scandinavia. Comparisons were made with pure populations of either diploid ssp. *fuchsii* or tetraploid ssp. *maculata*. It was shown that mixed populations are the result of secondary contact between ssp. *fuchsii* and ssp. *maculata*. No patterns of recent and local autopolyploidization were found. Morphology and nuclear DNA markers (internal transcribed spacers of nuclear ribosomal DNA) showed that diploids and tetraploids from mixed populations have similar levels of differentiation to diploids and tetraploids from pure populations. Vegetation analyses, as well as analyses of environmental variables, revealed that diploid and tetraploid individuals in mixed populations are ecologically well differentiated on a microhabitat level. Diploids and tetraploids in pure populations have wider ecological amplitudes than they do in mixed populations. Triploid hybrids grew in intermediate microhabitats between diploids and tetraploids in the mixed populations. Plastid DNA markers indicated that both diploids and tetraploids may act as the maternal parent. Based on morphology and nuclear markers triploids are more similar to tetraploids than to diploids. There were indications of introgressive gene flow between ploidy levels. Plastid markers indicated that gene flow from diploid to tetraploid level is most common, but nuclear markers suggested that gene flow in opposite direction also may occur. Similar patterns of differentiation and gene flow appeared in localities that represented contrasting biogeographic regions. Disturbance and topography may explain why hybridization was slightly more common and the differentiation patterns somewhat less clear in the Scandinavian mountains than in the coastal lowland.

Keywords Autopolyploidy · *Dactylorhiza* · Hybrid zone · Introgression · ITS · Morphometry · Niche differentiation · Plastid DNA · Vegetation analysis

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Introduction

Polyploid speciation has long been recognized as an important process in plant evolution (Müntzing 1936; Stebbins 1950; Grant 1981). Recent genomic studies have made it clear that angiosperms possess genomes with considerable gene redundancy, indicating that "most (if not all) plants have undergone one or more episodes of polyploidization" (Soltis et al. 2003). Many taxa are ancient polyploids that have become diploidized due to gene silencing and other genomic processes (e.g. Lynch and Conery 2000), and furthermore have no close relatives at lower ploidy levels. Functional polyploids in the traditional sense refer to polyploids that are integral parts of polyploid complexes that consist of closely related taxa at various ploidy levels (cf. Grant 1981; Soltis et al. 2003). Polyploidization is probably the most common mechanism of sympatric speciation (Otto and Whitton 2000), and it is widely accepted that a single taxon may arise several times by independent polyploidization events, via both allopolyploidization and autopolyploidization (Soltis and Soltis 1993, 1999; Soltis et al. 2003).

Chromosome doubling is an instantaneous mode of speciation that produces effective postzygotic barriers between new polyploids and their diploid progenitors (Ramsey and Schemske 1998). On the other hand, new polyploid plants are subjected to competitive constraints because they are also exposed to pollen from diploid plants, which results in triploid offspring that have low fitness (minority cytotype disadvantage; Levin 1975). Adaptive strategies are needed for establishment and persistence of new polyploids. Such strategies include apomixis (Hedrén et al. 2000), self-pollination (Levin 1975), pollinator shift (Segraves and Thompson 1999) and habitat differentiation (Felber-Girard et al. 1996). However, even though the literature on polyploidy is comprehensive there are relatively few studies that focus on the ecological aspects of polyploidization (Soltis et al. 2003).

Contact zones between diploid and polyploid cytotypes might be designated as primary or secondary hybrid zones (cf. Hewitt 1988; Arnold 1997). A primary hybrid zone would then be the consequence of recent and local (in situ) polyploidization, whereas a secondary hybrid zone would be the consequence of past polyploidization, followed by habitat differentiation and random migration and finally contact between the diploid and polyploid cytotypes. The differentiation patterns would consequently be less distinct in a primary hybrid zone than in a secondary hybrid zone. Holocene range expansion may have brought divergent lineages of diploid and polyploid cytotypes into contact, which implies that many hybrid zones in previously glaciated areas should be the result of secondary contact (cf. Stebbins 1984; Hewitt 1988).

The autopolyploid complex *Dactylorhiza maculata* (L) Soó s.l. (the spotted-orchids) consists of diploid and tetraploid cytotypes with overlapping distributions in Eurasia (Averyanov 1990; Hedrén 1996). This polyploid complex is morphologically very variable and at least 30 species, subspecies and varieties have been described (Soó 1960; Delforge 1995). In Scandinavia it is represented by the diploid *Dactylorhiza maculata* ssp. *fuchsii* (Druce) Hyl. (Common Spotted-orchid) and tetraploid ssp. *maculata* (Heath Spotted-orchid). In a previous study that contains populations of both subspecies from throughout all of Scandinavia, it was shown that pure populations of either diploids or tetraploids were well differentiated with respect to morphology and nuclear DNA markers, whereas plastid DNA markers revealed patterns of past introgressive gene flow from the diploid to tetraploid level, and between separate lineages of tetraploids (D. Ståhlberg and M. Hedrén, in preparation). It also indicated that diploids generally were associated with semi-open woodlands on base-rich soils, whereas tetraploids were associated with a wide range of open natural and semi-natural grassland habitats. In the present paper I focus on mixed

populations of diploids and tetraploids. I test four hypotheses: (1) that mixed populations in Scandinavia represent secondary contact zones between divergent lineages of diploids and tetraploids, i.e. that autotetraploids in mixed populations are not the result of recent polyploidization in situ; (2) that diploids and tetraploids in mixed populations are ecologically differentiated on a microhabitat level; (3) that gene flow between cytotypes is limited; (4) that similar patterns of differentiation between diploids and tetraploids appear in localities that represent contrasting biogeographic regions.

Materials and methods

Sampling

Field work was carried out in Sjösa nature reserve (58°45′N, 17°07′E) in the coastal lowland of central Sweden in the beginning of July 2004 and in Hamra nature reserve (62°34′N, 12°15′E) in the central part of the Scandinavian mountain ridge in the middle of July 2003 (Fig. 1). The lowland locality is a flat fen on acid bed-rock. Postglacial deposits of limestone and shell gravel have created opportunities for a diverse flora. The central area is open and scattered stands of spruce, birch and ash grow at the edges. The fen is managed by grazing. The mountain locality is a calcareous sloping fen slightly below the tree limit. The fen is open to semi-open with scattered stands of mountain birch and willow. The open area is sparsely managed by mowing.

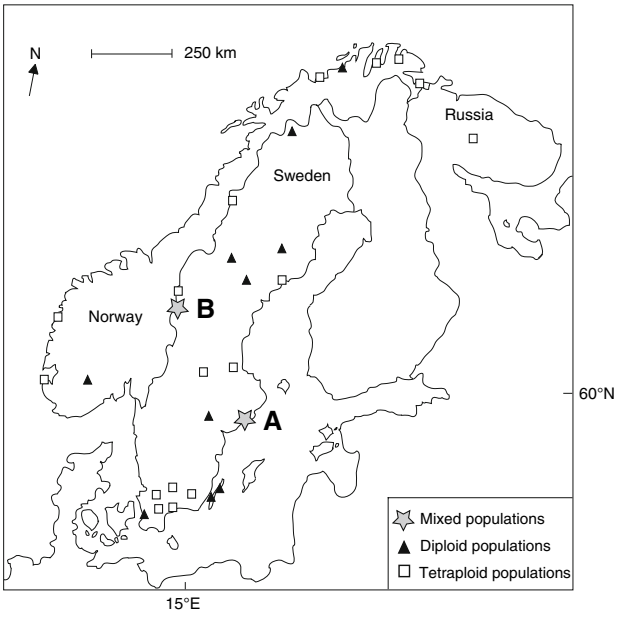


Fig. 1 Localities for the two mixed populations of diploid and tetraploid *Dactylorhiza maculata* s.l., and localities for cytologically homogeneous reference populations of either diploid ssp. *fuchsii* or tetraploid ssp. *maculata* (cf. D. Ståhlberg and M. Hedrén, in preparation). All reference populations were included in the analysis of plastid DNA haplotypes. Five tetraploid populations from northernmost Norway and Russia were excluded in the analysis of ITS. *A* = Sjösa (the lowland locality); *B* = Hamra (the mountain locality)

In each locality, about 50 individuals (45 in the lowland locality and 50 in the mountain locality) of *Dactylorhiza maculata* s.l. were sampled within an area of 100 m × 100 m. To avoid any clonality all sampled plants were separated from each other by a distance of at least 1 m (cf. Batygina et al. 2003). Three classes of plants were selected based on diagnostic features in field floras treating the Nordic countries (Hylander 1966; Krok and Almquist 1994; Mossberg and Stenberg 2003; Lid and Lid 2005): morphologically typical specimens of diploid *Dactylorhiza maculata* ssp. *fuchsii*, morphologically typical specimens of tetraploid *Dactylorhiza maculata* ssp. *maculata* and morphologically intermediate specimens, i.e. hypothetical triploid hybrids. The number of plants from each class was similar. The field classification of individuals was then adjusted according to cytological data (see next section).

Ploidy determination

The relative ploidy level of each individual was assessed by flow cytometry. The analyses were performed by Gerard Geenen, Plant Cytometry Services (Schijndel, The Netherlands). Fresh leaf material was chopped in an ice-cold buffer (modified from Arumuganathan and Earle 1991) containing the DNA binding fluorochrome 4′,6-Diamidino-2-phenylindole. The mixture was passed through a 40 μm nylon filter and run through a flow cytometer (PAS II Partec GMbH). The flourescence was measured by a photomultiplier and converted into voltage pulses processed electronically to yield peak signals. The flourescence is expected to vary proportionally to the DNA content (e.g. Husband and Schemske 1998).

Tetraploid *Dactylorhiza maculata* ssp. *maculata* from a cytologically homogeneous reference population was used as an external calibration standard.

The flow cytometry revealed that 25 individuals from the lowland locality were diploids, 17 individuals were tetraploids and three individuals were triploids. In the mountain locality there were 18 diploids, 25 tetraploids and seven triploids.

Ecology

The niche of each sampled individual was characterized by data of presence and cover of associated plant species (herbaceous plants, dwarf shrubs and saplings) in a 40 cm × 40 cm quadrat centred on the target *Dactylorhiza* individual. The cover of each associated species was estimated according to the Hult-Sernander–Du Rietz scale (Du Rietz 1921; 1 = >0–6%; 2 = 6–12%; 3 = 12–25%; 4 = 25–50%; 5 = >50%). Absent species were given a zero-value for cover. Eight environmental variables were recorded in each quadrat in addition to the vegetation data: (1) shading (percentage cover of trees and bushes); (2) exposed soil (percentage cover); (3) litter (percentage cover of dead non-decomposed plant material); (4) *Sphagnum* (percentage cover); (5) mosses other than *Sphagnum* (percentage cover); (6) pH on the top 10 cm of soil immediately adjacent to the target plant; (7) moisture, on a scale 1–4 (1 = dry through to 4 = wet); (8) grass sward density immediately adjacent to the target plant, on a scale 1–4 (1 = absent through to 4 = very dense and coherent; cf. Barthram 1986; Ekstam and Forshed 1996). Percentage cover, where appropriate, was assessed according to the Hult-Sernander–Du Rietz scale. Some variables are obviously interacting or overlapping. The variables 4–5 were chosen as rough predictors of the physical texture of the bottom layer rather than as indirect predictors of chemical features.

Morphology

A total of 35 quantitative and qualitative characters were measured on all individuals (Table 1). Both floral and vegetative characters were taken into account. The methodology was slightly modified from 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 colour on the lower part of the labellum was matched to the nearest colour block on a colour chart of the Natural Color System (Anonymous 2004). According to this system, a visually perceived colour can be characterized by three attributes: hue (the relative position between two of four elementary chromatic colours in a circle, in this case red and blue), blackness (the relative position between white and black

Table 1 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 × A1/(A2 (A3)
5. Labellum, maximum width (mm)
6. Labellum colour, hue[b] (arbitrary values ranging from 0 to 100)
7. Labellum colour, blackness[b] (arbitrary values ranging from 0 to 100)
8. Labellum colour, 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)

Table 1 continued

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

[b] See text

for a certain hue) and chromaticness (the degree of resemblance to a full chromatic colour of the same hue).

Molecular methods

Fresh flowers from all 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.

From the nuclear genome, the internal transcribed spacer (ITS) region of ribosomal DNA (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 2). 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 may differ among individuals (cf. Shipunov et al. 2004; Pillon et al. 2007; D. Ståhlberg and M. Hedrén, in preparation).

Ten polymorphic plastid DNA loci (seven microsatellite loci and three loci with indel variation) were amplified with a set of *Dactylorhiza* specific primers (Table 2; cf. M. Hedrén et al., in preparation). Size variants (alleles) were scored and combined into multilocus genotypes (hereafter referred to as haplotypes).

All nuclear and plastid 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 2) for 1 min, 72°C for 1 min 30 s, with a final step of 72°C for10 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

Table 2 List of primers used in the study. Initially, a larger number of 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	

Table 2 continued

Nr.	Locus, type of variation	Specific primers	Sequence 5′→3′	Ann. temp.
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

amplified simultaneously in the same PCR by multiplexing. The PCR product from each reaction was mixed with appropriate size standards to enable exact size determination of the amplified fragments. Dye labelled fragments were separated on an ALF Express II DNA analyzer (GE Healthcare), and the size of the fragments was determined using ALFwin Fragment Analyser 1.03.01 software (GE Healthcare).

Data analysis

Ecology

Ecological differentiation among quadrats was summarized by canonical correspondence analysis (CCA) and by detrended correspondence analysis (DCA). The CCA included both vegetation data and data from environmental variables, whereas only vegetation data was included in the DCA. The Shannon diversity index (Shannon and Weaver 1949) was calculated for each quadrat and the number of associated species was counted (species richness). Comparisons of quadrats containing diploids with those containing tetraploids were performed for all environmental variables using a Mann–Whitney *U*-test. Similarly, the quadrats were also compared for cover of all associated species, Shannon diversity index and species richness using a Mann–Whitney *U*-test. Statistical comparisons with triploids were not performed because only ten triploids were recorded. Separate analyses were performed for the lowland locality and for the mountain locality.

Morphology

Morphological differentiation was analysed using a principal component analysis (PCA). Since the combined character labellum shape index (A4; Table 1) depends on three other characters, one of these (A3) was excluded from the analysis to avoid redundancy. Diploids and tetraploids were compared for all characters using a Mann–Whitney *U*-test. Separate analyses were performed for the lowland locality and for the mountain locality.

Nuclear DNA markers

Based on ITS allele frequencies of individual plants, mean genetic distances (chord distances; Cavalli-Sforza and Edwards 1967) within and between diploid, triploid and tetraploid subpopulations in both the lowland locality and the mountain locality were calculated. All subpopulations from the lowland locality and the mountain locality were compared with each other and with diploid ssp. *fuchsii* and tetraploid ssp. *maculata* from previously investigated pure populations (D. Ståhlberg and M. Hedrén, in preparation); 83 individuals of ssp. *fuchsii* from ten pure populations and 108 individuals of ssp. *maculata* from 12 pure populations were included (Fig. 1). The reference populations for both ssp. *fuchsii* and ssp. *maculata* were from throughout all of Scandinavia, but genetically and morphologically aberrant tetraploid populations from the northernmost area (= var. *kolaënsis*) were excluded from the analysis.

Plastid DNA markers

All alleles at the different plastid DNA loci were treated as ordered characters based on the assumption that mutations primarily follow a stepwise mutation model (Ohta and Kimura

1973; cf. Cozzolino et al. 2003). Phenetic relationships among haplotypes were summarized by non-metric multidimensional scaling (NMDS). Haplotypes detected in this study were analysed together with haplotypes previously detected in pure populations of either diploid ssp. *fuchsii* (84 individuals from ten populations) or tetraploid ssp. *maculata* (152 individuals from 17 populations) from all parts of Scandinavia (Fig. 1; D. Ståhlberg and M. Hedrén, in preparation).

Separation between diploids and tetraploids

Discriminant analyses based on vegetation data, morphology and ITS allele frequencies, respectively, were performed to evaluate how well individual plants could be classified into either diploid *Dactylorhiza maculata* ssp. *fuchsii* or tetraploid *Dactylorhiza maculata* ssp. *maculata*. For each data set all specimens were plotted along an axis that maximized the differences between diploids and tetraploids. Triploid specimens were classified a posteriori. The axes resulting from the different data sets were then put together into a 3D scatter plot. Separate analyses were performed for the lowland locality and the mountain locality.

Multivariate analyses were performed in PAST version 1.44 (Hammer et al. 2001) whereas SPSS version 11.5 (SPSS, Chicago, IL, USA) was used for univariate analyses.

Results

Ecology

The eigenvalues for the first axis were of same order of magnitude in all ordination analyses (0.51–0.68; see Figs. 2, 3).

Based on vegetation data and environmental variables, diploids and tetraploids from the lowland locality, as well as from the mountain locality, were well differentiated along the first axis in the CCA (Fig. 2). Triploid hybrids were placed towards the centre of the first axis, but in the CCA of the mountain locality they were closer to the tetraploids than to the diploids. The relative impacts of the environmental variables to the differentiation patterns are illustrated as vectors. Statistical data for environmental variables are given in Table 3. In both localities, diploid plants were significantly associated with high levels of shading, whereas tetraploid plants were associated with open areas. Moreover, tetraploids in both localities were associated with dense grass sward whereas diploids were associated with loosened grass sward and exposed soil. Diploids were generally associated with soils with higher pH than tetraploids were, whereas tetraploids were more associated with *Sphagnum* than diploids were. The influence of moisture appeared to differ between the lowland locality and the mountain locality. In the lowland locality, quadrats with diploids got higher scores than quadrats with tetraploids, whereas the opposite was found in the mountain locality. Cover of mosses other than *Sphagnum* appeared to be a poor habitat predictor, probably because ecologically very different species were treated collectively.

The DCA, which only included vegetation data, also showed a clear pattern of differentiation between diploids and tetraploids (Fig. 3). Again triploids were placed towards the centre of the first axis, but closer towards tetraploids than towards diploids in the DCA of the mountain locality. A total of 51 herbaceous plant species were found in the lowland locality (35 species were associated with quadrats with diploids and 37 species were

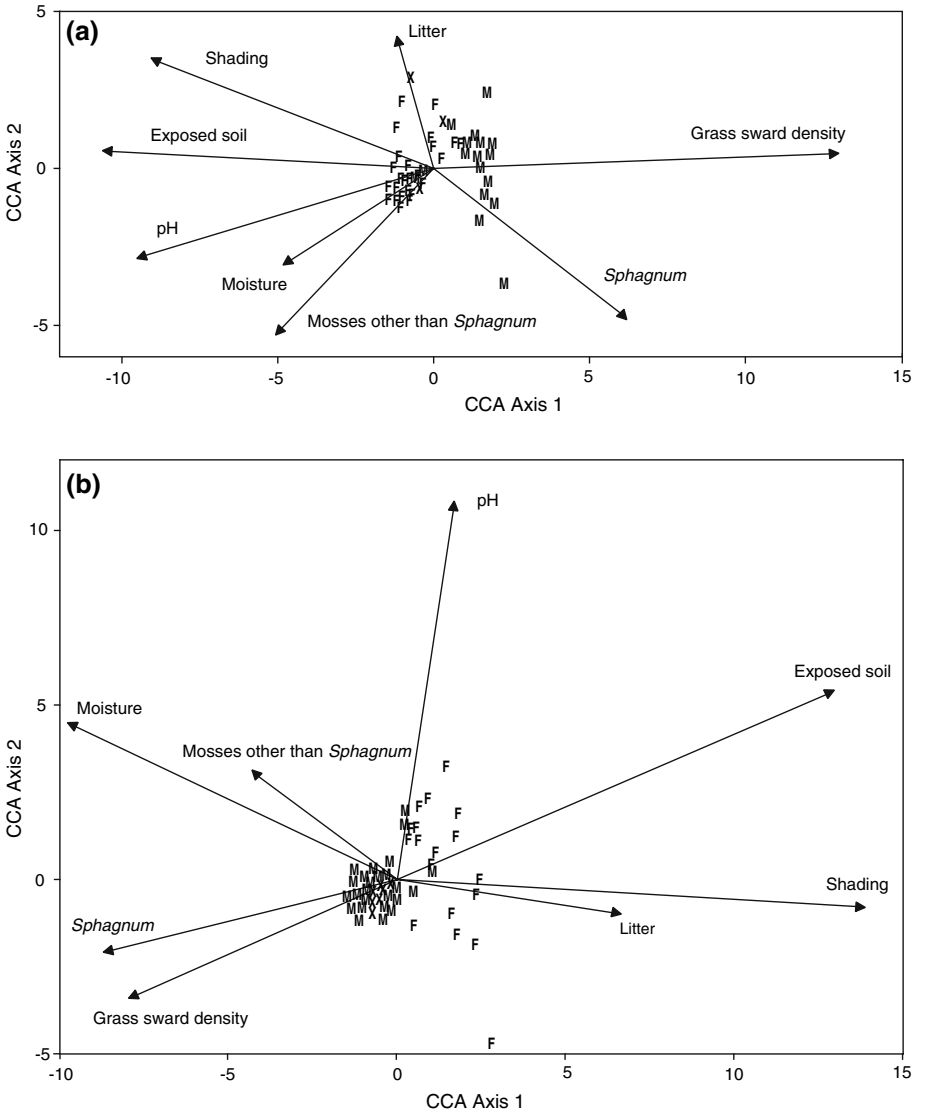
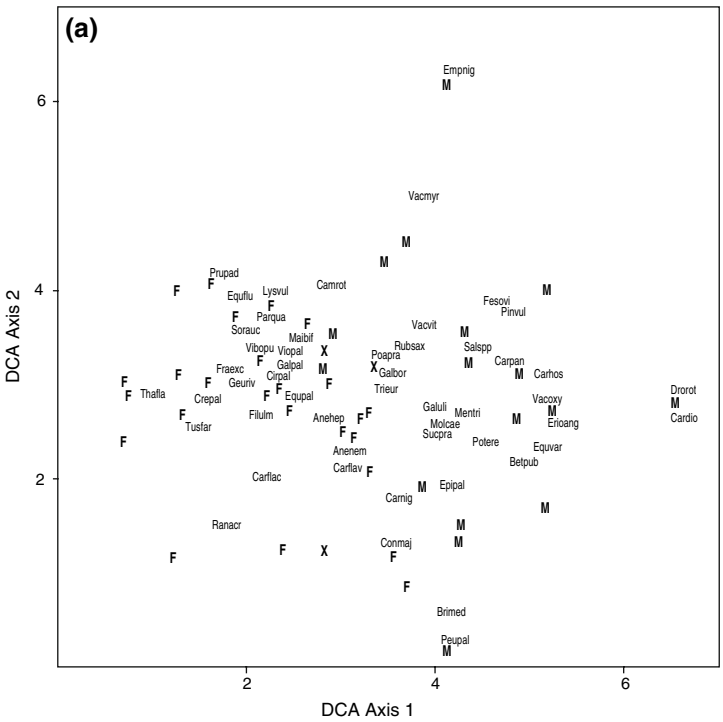


Fig. 2 Canonical correspondence analysis based on vegetation data and environmental variables. The positions of associated species are not shown. See text for explanation of environmental variables. F = quadrats with diploid *Dactylorhiza maculata* ssp. *fuchsii*; X = quadrats with triploid hybrids; M = quadrats with tetraploid ssp. *maculata*. (**a**) The lowland locality. The eigenvalues of axis 1 and axis 2 are 0.54 and 0.19, respectively. (**b**) The mountain locality. The eigenvalues of axis 1 and axis 2 are 0.51 and 0.31, respectively

associated with quadrats with tetraploids) and 81 species were found in the mountain locality (51 associated with diploids vs. 61 associated with tetraploids). The positions of associated species in relation to diploid, triploid and tetraploid samples of *Dactylorhiza* are indicated in the ordination plots. In the lowland locality six species were significantly associated with quadrats with diploids and 14 species were significantly associated with


(a)
DCA Axis 2
DCA Axis 1
2
4
6
Empnig
Vacmyr
Prupad
Equflu
Lysvul
Camrot
Parqua
Sorauc
Maibif
Vacvit
Fesovi
Pinvul
Salspp
Rubsax
Vibopu
Viopal
Poapra
Galpal
Carpan
Carhos
Fraexc
Cirpal
Galbor
Geuriv
Trieur
Thafla
Crepal
Equpal
Vacoxy
Drorot
Cardio
Galuli
Mentri
Filulm
Anehep
Molcae
Sucpra
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Carflav
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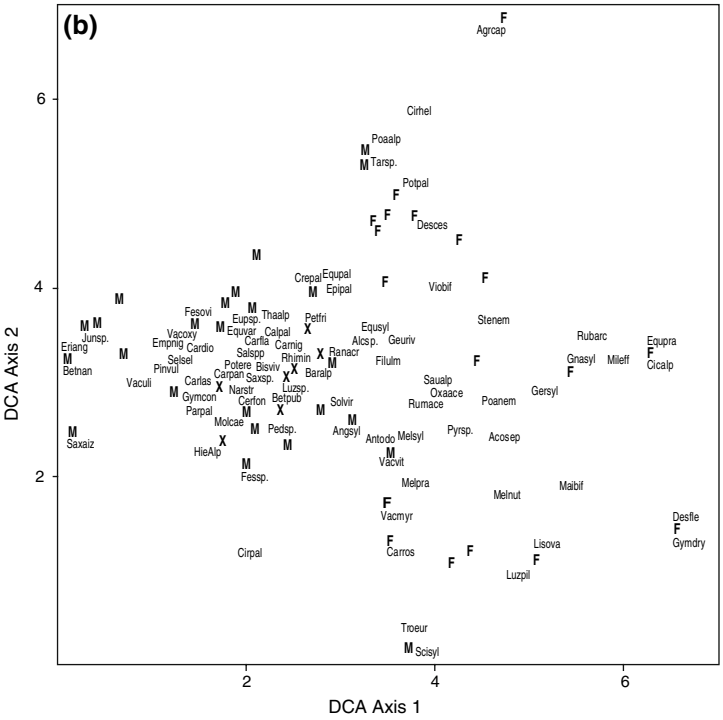
Epipal
Carnig
Ranacr
Conmaj
Brimed
Peupal


(b)
DCA Axis 2
DCA Axis 1
2
4
6
Agrcap
Cirhel
Poaalp
Tarsp.
Potpal
Desces
Crepal
Equpal
Epipal
Viobif
Stenem
Fesovi
Eupsp.
Thaalp
Petfri
Equsyl
Alcsp.
Geuriv
Rubarc
Equpra
Junsp.
Vacoxy
Equvar
Calpal
Carfla
Carnig
Eriang
Empnig
Cardio
Salspp
Ranacr
Rhimin
Filulm
Gnasyl
Mileff
Cicalp
Betnan
Selsel
Pinvul
Potere
Bisviv
Baralp
Saxsp.
Carpan
Vaculi
Carlas
Narstr
Luzsp.
Saualp
Oxaace
Gersyl
Gymcon
Cerfon
Betpub
Rumace
Poanem
Parpal
Solvir
Molcae
Pedsp.
Angsyl
Pyrsp.
Saxaiz
Antodo
Melsyl
Acosep
HieAlp
Vacvit
Fessp.
Melpra
Maibif
Melnut
Vacmyr
Desfle
Gymdry
Cirpal
Carros
Lisova
Luzpil
Troeur
Scisyl

◀**Fig. 3** Detrended correspondence analysis of vegetation data. Species abbreviations are explained in Appendix 1. F = quadrats with diploid *Dactylorhiza maculata* ssp. *fuchsii*; X = quadrats with triploid hybrids; M = quadrats with tetraploid ssp. *maculata*. (**a**) The lowland locality. The eigenvalues of axis 1 and axis 2 are 0.68 and 0.31, respectively. (**b**) The mountain locality. The eigenvalues of axis 1 and axis 2 are 0.63 and 0.39, respectively

quadrats with tetraploids; corresponding numbers for the mountain locality were 9 vs. 17. Statistical data for all associated species are summarized in Appendix 1. The Shannon diversity index and the species richness were highest for quadrats with triploid hybrids in both populations, and in both populations the Shannon diversity index and the species richness were higher in quadrats with tetraploids than in quadrats with diploids (Table 4).

Morphology

In both localities, diploids and tetraploids were separated along the first two axes in the PCA (Figs. 4, 5), but there was more overlap in the mountain population. In the lowland locality 31 and 8% of the total variation was described by the first two axes. Corresponding values for the mountain locality were 18 and 13%. Triploid hybrids from both localities were placed closer to tetraploids than to diploids in the ordination plots. The contributions of different characters to the ordination patterns are illustrated as vectors. Statistical data for all characters are summarized in Appendix 2. Diploids generally had higher values for diagnostic characters such as leaf width (B27) and labellum shape index (A4) compared to tetraploids (cf. Delforge 1995). Leaf width had a strong impact on the differentiation patterns irrespective of locality, whereas labellum shape only differed significantly between diploids and tetraploids in the lowland locality. In the mountain locality anthocyanin pigmentation (A16 and A19) contributed clearly to the differentiation patterns (high values for tetraploids), whereas in the lowland locality anthocyanin pigmentation rather separated between plants within the diploid and tetraploid subpopulations. In both the lowland population and the highland population diploid plants generally had higher scores for vegetative characters, for example plant height (B20), compared to tetraploids.

Nuclear DNA markers

In both the lowland locality and the mountain locality three different ITS alleles were scored. The same alleles were found in both localities. The relationships among individuals with respect to the frequencies of these three alleles are illustrated by triangular diagrams (Fig. 6). The alleles are numbered according to Pillon et al. 2007. Each allele is comprised of two fragments from the primer combinations i and ii (Table 2): 75, 72 bp (Allele I); 75, 80 bp (Allele IIIb) and 70, 80 bp (Allele V). In both localities, diploids were essentially characterized by absence of Allele I and high frequencies of Allele V, whereas tetraploids were characterized by having Allele I (generally in high frequencies), and by low frequencies of Allele V. Allele IIIb contributed less to the differentiation patterns, although it generally appeared in higher frequencies in diploids than in tetraploids. In both localities, triploids were more similar to tetraploids than to diploids. Diploids and tetraploids were more differentiated in the lowland locality than in the mountain locality. One diploid specimen from the mountain locality differed from all other diploids by having Allele I and very low frequency of Allele V.

Table 3 Population mean values of environmental variables; standard deviation is given for pH

Variable	Diploids ($N = 25$)	Triploid hybrids ($N = 3$)	Tetraploids ($N = 17$)	P-value
(a) The lowland locality				
Shading, cover of trees and bushes on a scale 0–5[a]	2.16	2.67	0.76	**<0.001**
Exposed soil, cover on a scale 0–5[a]	2.16	2.00	1.35	**<0.001**
Litter, cover on a scale 0–5[a]	2.92	3.00	2.71	0.282
Sphagnum, cover on a scale 0–5[a]	0.00	0.00	0.29	0.083
Mosses else than *Sphagnum,* cover on a scale 0–5[a]	3.68	3.00	3.24	0.112
pH	5.54 ± 0.30	5.50 ± 0.10	5.32 ± 0.33	**<0.05**
Moisture, on a scale 1–4[b]	2.48	2.33	2.18	0.102
Grass sward, density on a scale 1–4[b]	2.12	2.33	2.88	**<0.001**
(b) The mountain locality				
Variable	Diploids ($N = 18$)	Triploid hybrids ($N = 7$)	Tetraploids ($N = 25$)	P-value
Shading, cover of trees and bushes on a scale 0–5[a]	1.83	0.00	0.28	**<0.001**
Exposed soil, cover on a scale 0–5[a]	2.56	0.43	0.80	**<0.001**
Litter, cover on a scale 0–5[a]	3.44	3.00	2.96	0.061
Sphagnum, cover on a scale 0–5[a]	0.00	1.86	1.80	**<0.001**
Mosses else than *Sphagnum,* cover on a scale 0–5[a]	1.00	1.14	1.20	0.337
pH	5.80 ± 0.64	5.33 ± 0.26	5.62 ± 0.41	0.068
Moisture, on a scale 1–4[b]	1.83	2.57	2.74	**<0.01**
Grass sward, density on a scale 1–4[b]	2.06	2.43	2.56	**<0.01**

The P-values represent comparisons between diploids and tetraploids (Mann–Whitney U-test). Significant P-values are given in bold

[a] Hult-Sernander-du Rietz scale; Du Rietz 1921

[b] See text

Table 4 Population mean values (±SD) of species diversity

Index	Diploids ($N = 25$)	Triploid hybrids ($N = 3$)	Tetraploids ($N = 17$)	*P*-value
(a) *The lowland locality*				
Shannon diversity index[a]	1.80 ± 0.30	2.14 ± 0.42	2.01 ± 0.19	**<0.05**
Species richness (number of associated species)	7.12 ± 2.11	9.33 ± 3.51	8.29 ± 1.53	0.072
(b) *The mountain locality*				
Index	Diploids	Triploid hybrids	Tetraploids	*P*-value
	($N = 18$)	($N = 7$)	($N = 25$)	
Shannon diversity index[a]	2.07 ± 0.33	2.62 ± 0.15	2.38 ± 0.21	**<0.001**
Species richness (number of associated species)	9.11 ± 2.74	15.00 ± 2.16	11.88 ± 2.15	**<0.01**

The *P*-values represent comparisons between diploids and tetraploids (Mann–Whitney *U*-test). Significant *P*-values are given in bold

[a] Shannon and Weaver 1949

Mean genetic distances based on chord distances (Cavalli-Sforza and Edwards 1967) within and among diploid, triploid and tetraploid subpopulations, and cytologically homogeneous reference populations of diploid ssp. *fuchsii* and tetraploid ssp. *maculata* are presented in Table 5. The mean genetic distance between diploids and tetraploids from the lowland locality was similar to the mean genetic distance between diploids and tetraploids from cytologically homogeneous populations. In the mountain locality the mean genetic distance between diploids and tetraploids was slightly lower. The mean genetic distances within subpopulations of diploids and tetraploids were slightly lower or markedly lower (lowland tetraploids) compared to the mean genetic distances within pure populations of either diploids or tetraploids. In both the lowland locality and the mountain locality, the mean genetic distance between triploids and tetraploids was smaller than the mean genetic distance between triploids and diploids.

Plastid DNA markers

A total of 29 fragment size variants at ten loci were scored and combined to give 26 haplotypes (Appendix 3). The haplotypes were numbered according to a larger dataset that encompasses *Dactylorhiza* from all over Europe (M. Hedrén et al., in preparation). Eight haplotypes were detected in the lowland locality and 19 haplotypes were detected in the mountain locality. The NMDS (Fig. 7) revealed that the haplotypes could be sorted into two discrete groups (referred to as Groups I and II; D. Ståhlberg and M. Hedrén, in preparation) together with haplotypes from other Scandinavian populations of diploid and tetraploid *Dactylorhiza maculata* s.l. Most haplotypes within each group are very similar. The distribution of specific Group I and Group II haplotypes with respect to ploidy level and locality is summarized in Fig. 8. In the lowland locality, as well as in the mountain locality, all diploids had Group I haplotypes. Only one Group I haplotype (H59) was detected in the lowland locality whereas six different Group I haplotypes, including H59, were found among diploids in the mountain locality. In the lowland locality 88% of the tetraploids had Group II haplotypes (seven different). The remaining specimens had the

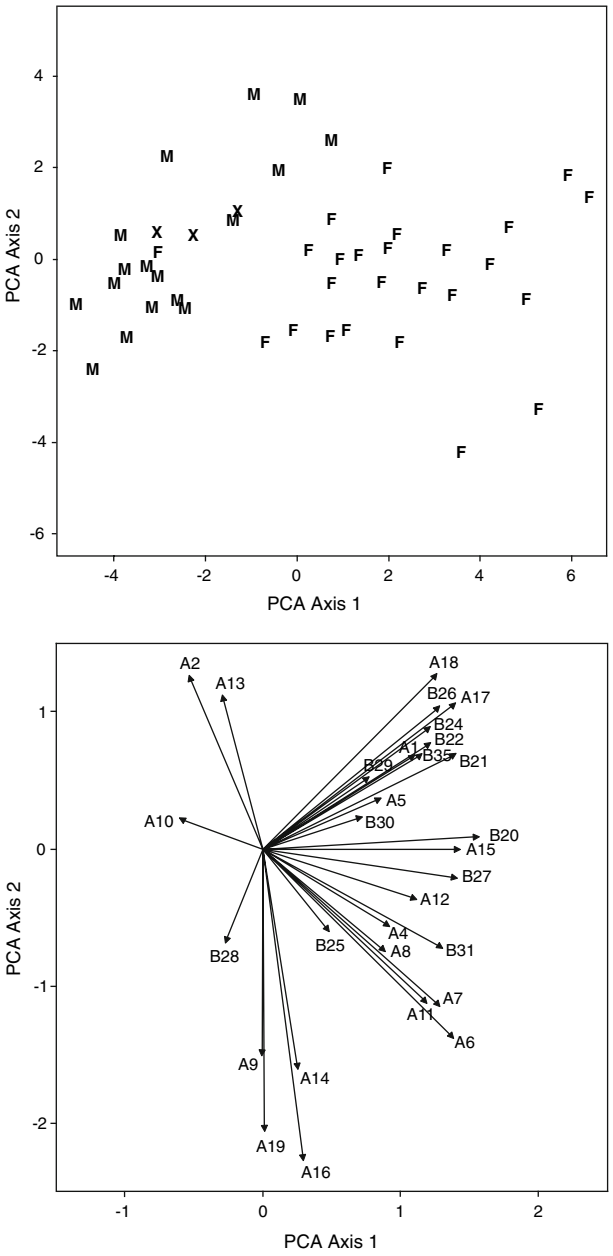


Fig. 4 Principal component analysis of morphological data from the lowland locality. The relative impacts of different characters are illustrated as vectors. The first two axes accounted for 31 and 8% of the total variation. F = diploids (*Dactylorhiza maculata* ssp. *fuchsii*); X = triploid hybrids; M = tetraploids (ssp. *maculata*). The characters are numbered according to Table 1. Characters with a low impact are not shown

Group I haplotype H59. In the mountain locality 56% of the tetraploids had Group II haplotypes (six different). Some of the remaining tetraploids had unique Group I haplotypes (H80, H81 and H83) that differed by several steps from the common Group I haplotypes that were found in diploids and other tetraploids from the mountain locality. All triploids from the lowland locality had Group I haplotypes, whereas both Group I and Group II haplotypes where detected in triploids from the mountain locality. All haplotypes

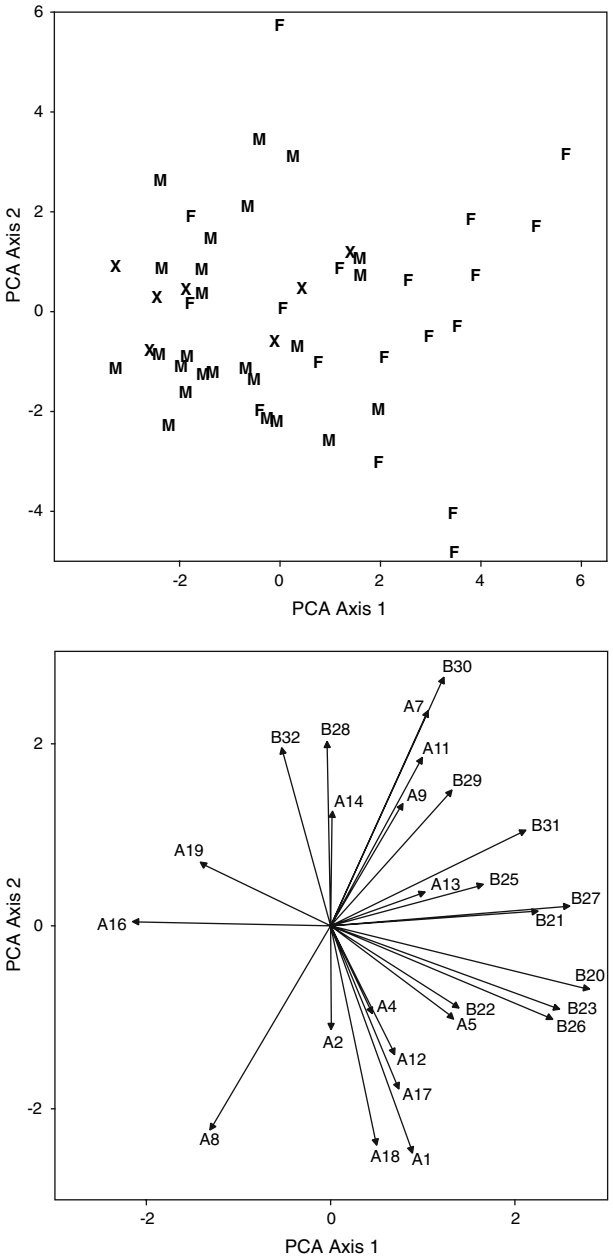


Fig. 5 Principal component analysis of morphological data from the mountain locality. The relative impacts of different characters are illustrated as vectors. The first two axes accounted for 16 and 13% of the total variation. F = diploids (*Dactylorhiza maculata* ssp. *fuchsii*); X = triploid hybrids; M = tetraploids (ssp. *maculata*). The characters are numbered according to Table 1. Characters with a low impact are not shown

that were detected in triploids were identical or very similar to the haplotypes that were detected in diploids and/or tetraploids from the same locality.

Separation between diploids and tetraploids

The discriminant analyses resulted in distinct groups of diploids and tetraploids in both localities (Fig. 9). Analyses based on vegetation data, morphology and ITS allele

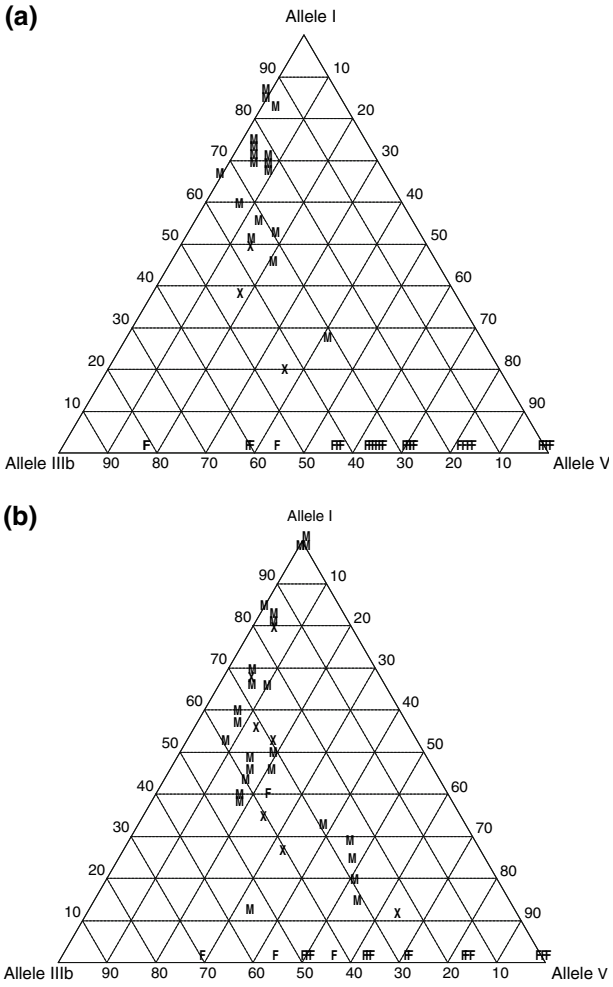


Fig. 6 Relative abundances of ITS alleles (%). The alleles are numbered according to Pillon et al. (2007). Each allele is comprised of two fragments from the primer combinations i and ii (Table 2); 75, 72 bp (Allele I); 75, 80 bp (Allele IIIa); 70, 80 bp (Allele V). F = diploids (*Dactylorhiza maculata* ssp. *fuchsii*); X = triploid hybrids; M = tetraploids (ssp. *maculata*). (**a**) The lowland locality. (**b**) The mountain locality

frequencies, respectively, gave similar patterns. In the lowland locality, all data sets resulted in 100% correct classification. Triploids were more similar to tetraploids than to diploids. In the mountain locality 98% of the plants were correctly classified with respect to vegetation data (one diploid was classified as a tetraploid) and 98% were correctly classified with respect to morphology (one tetraploid was classified as a diploid), whereas ITS allele frequencies resulted in 88% correct classification (one diploid was classified as a tetraploid and four tetraploids were classified as diploids). Triploids were more similar to diploids based on vegetation data and morphology, whereas they were more similar to tetraploids based on ITS allele frequencies.

Table 5 Mean genetic distances (chord distances; adapted from Cavalli-Sforza and Edwards 1967) within and between diploid, triploid and tetraploid subpopulations

	2x			3x hybrids		4x		
	Pure	Lowland	Mountain	Lowland	Mountain	Pure	Lowland	Mountain
2x Pure	0.50							
2x Lowland	0.45	0.37						
2x Mountain	0.48	0.40	0.43					
3x Lowland	0.83	0.84	0.80	0.27				
3x Mountain	0.94	0.93	0.90	0.49	0.50			
4x Pure	1.19	1.19	1.16	0.71	0.63	0.67		
4x Lowland	1.15	1.16	1.12	0.54	0.47	0.47	0.27	
4x Mountain	1.02	1.01	0.98	0.52	0.53	0.59	0.45	0.53

The mixed populations from the lowland locality and the mountain locality are compared with pure populations consisting of either diploids (ten populations) or tetraploids (12 populations) from throughout all Scandinavia

Discussion

Secondary contact between divergent lineages of diploids and tetraploids

As for many hybrid zones in previously glaciated areas (Hewitt 1988), it can be concluded that the mixed populations from the lowland locality, as well as from the mountain locality, are the results of secondary contact between divergent lineages. Previous studies on pure populations of either diploid *Dactylorhiza maculata* ssp. *fuchsii* or tetraploid *Dactylorhiza maculata* ssp. *maculata* in Scandinavia have clearly indicated that pure populations of autotetraploids have originated from diploid ancestors that differ from present-day ssp. *fuchsii* (D. Ståhlberg and M. Hedrén, in preparation). The two taxa are separated by qualitative and quantitative differences in nuclear DNA, by absolute DNA amount (the autotetraploid genome is less than twice the genome of present-day diploids), by phylogeography based on plastid DNA haplotypes, by morphology and by ecological preferences. These findings are corroborated by other studies on Scandinavian *Dactylorhiza* (Heslop-Harrison 1951; Reinhard 1985; Hedrén 1996; Hedrén et al. 2001; Aagaard et al. 2005). The clear patterns of differentiation that were observed in the present study are in agreement with the results from these previous studies on Scandinavian *Dactylorhiza*, and give no support for the alternative hypothesis of recent formation and establishment of autotetraploids from present-day diploids in heterogeneous environments in Scandinavia (Fig. 9).

In both the lowland locality and the mountain locality, diploids were characterized by the absence of the ITS allele I and high frequencies of Allele V, whereas tetraploids were characterized by having Allele I and by low frequencies or lack of Allele V (Fig. 6). The same alleles separate between diploid ssp. *fuchsii* and tetraploid ssp. *maculata* from pure populations in Scandinavia (Ståhlberg and Hedrén, in preparation). Similar patterns have been reported from western Continental Europe by Pillon et al. (2007).

The occurrence of Group II haplotypes in tetraploids from both the lowland locality and the mountain locality (Fig. 8) can not be explained by recent autopolyploidization from local diploids. In central and northern Scandinavia (D. Ståhlberg and M. Hedrén, in

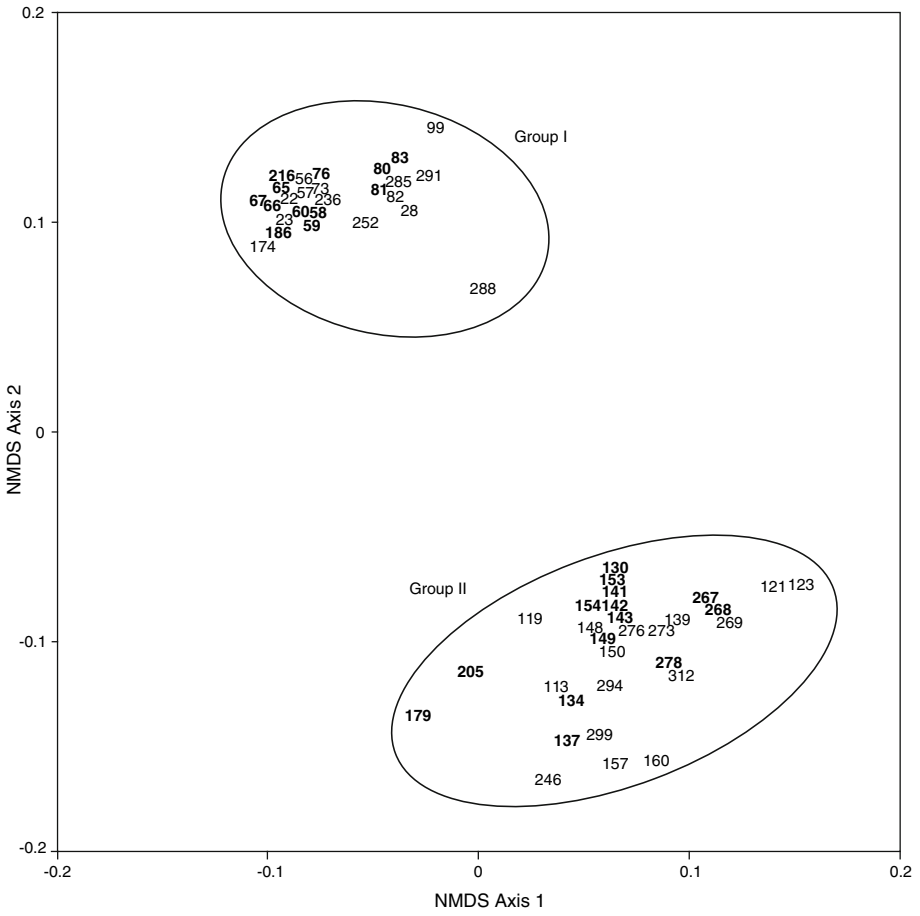


Fig. 7 Non-metric multidimensional scaling of plastid haplotypes. Stress = 0.14. Haplotypes from the two mixed populations of the present study were analysed together with haplotypes from pure populations consisting of either diploid *Dactylorhiza maculata* ssp. *fuchsii* (ten populations) or tetraploid ssp. *maculata* (17 populations) from throughout all of Scandinavia (D. Ståhlberg and M. Hedrén, in preparation). The numbering is based on a larger data set that encompasses *Dactylorhiza* from all over Europe (M. Hedrén et al., in preparation). *Numbers in bold* represent haplotypes from the mixed populations

preparation), as well as in northern Russia (Shipunov et al. 2004), populations of diploid ssp. *fuchsii* are invariably characterized by having Group I plastid haplotypes. All diploids from the two central Scandinavian populations in the present study had Group I haplotypes (Fig. 8). The distribution of Group I and Group II haplotypes in populations of tetraploid ssp. *maculata* is more complicated. In northernmost Europe, Group I haplotypes are prevalent (Shipunov et al. 2004; D. Ståhlberg and M. Hedrén, in preparation), whereas Group II haplotypes are prevalent in southern Scandinavia, as well as in western and southwestern Europe (Hedrén 2003; Devos et al. 2003, 2006; Pillon et al. 2007). The two localities in the present study are located in areas where Group I and Group II haplotypes often occur together in pure populations of ssp. *maculata* (D. Ståhlberg and M. Hedrén, in preparation). Therefore, the occurrence of Group I haplotypes in tetraploids in both the lowland locality and the mountain locality does not need to be attributed to recent introgressive gene flow

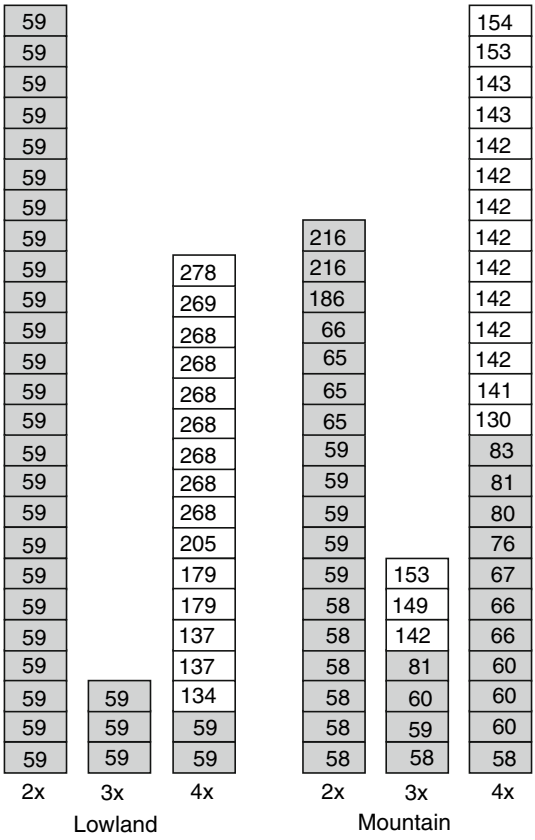


Fig. 8 Distribution of plastid haplotypes with respect to ploidy level and locality. *Each box* represents one individual. Group I and Group II haplotypes according to Fig. 7 are *grey* and *white*, respectively. The numbering is based on a larger data set that encompasses *Dactylorhiza* from all over Europe (M. Hedrén et al., in preparation)

from diploids. Moreover, most of the Group I haplotypes in the mountain population were not identical in diploids and tetraploids. Particularly, three aberrant Group I haplotypes (H80, H81 and H83; Fig. 7) were restricted to tetraploids.

Diploids and tetraploids from the mixed populations in this study had similar levels of differentiation to diploid ssp. *fuchsii* and tetraploid ssp. *maculata* from pure populations in Scandinavia, based on the same 35 morphological characters (cf. D. Ståhlberg and M. Hedrén, in preparation).

In contrast to a scenario of secondary contact between well-defined lineages of diploid ssp. *fuchsii* and tetraploid ssp. *maculata* as outlined here, primary hybrid zones may exist elsewhere in Europe, particularly in Central Europe where diploid and tetraploid *Dactylorhiza maculata* s.l. often are morphologically indistinguishable, and sometimes occur in mixed populations together with triploids (Groll 1965; Vöth and Greilhuber 1980). These hybrid zones may be derived from local glacial refugia. Recent studies have suggested that areas of sheltered topography in Central Europe may have provided refugia for woodland species and other temperate plant and animal species (Stewart and Lister 2001; Pinceel et al. 2005; Ursenbacher et al. 2006). Furthermore it has been clearly indicated that several alpine-subarctic plant species probably survived the glaciations at the periphery of the Alps (Schönswetter et al. 2003, 2005; Tribsch and Schönswetter 2003). According to the present-day distribution of *Dactylorhiza maculata* s.l. (Averyanov 1990), it has a wide ecological amplitude and can tolerate severe climatic conditions at high altitudes and latitudes.

Niche differentiation between diploids and tetraploids on a microhabitat level

Diploids and tetraploids from the two mixed populations were ecologically well differentiated (Figs. 2, 3). The area of each study site was restricted to 100 m × 100 m. The observed patterns of separation thus reflect niche differentiation on a microhabitat level. Similar fine-scale patterns of niche differentiation in hybrid zones between diploids and autotetraploids have previously been described in only a few studies (e.g. Lumaret et al. 1987 on *Dactylis glomerata*; Felber-Girard et al. 1996 on *Anthoxanthum alpinum*). On a landscape level there are some more studies (e.g. Rothera and Davy 1986 on *Deschampsia caespitosa*; Hardy et al. 2000 on *Centaurea jacea*; Johnson et al. 2003 on *Galax urceolata*), but in general there is a shortage of ecological approaches within the polyploidy literature (Soltis et al. 2003).

Analyses based on only vegetation data gave similar, or even more distinct, results than analyses that also incorporated environmental variables (Fig. 3). By integrating and summarizing interacting abiotic and biotic components, plant community composition may accordingly be regarded as a good predictor of the realized niche of species in natural populations (cf. Prentice and Cramer 1990). However, reciprocal transplant experiments should ideally be carried out in the mixed *Dactylorhiza* populations to confirm if the two cytotypes are adapted to different niches (cf. Buggs and Pannell 2007).

The degree of shading was the individual parameter that best explained the differentiation patterns, with high scores for diploids and low scores for tetraploids (Fig. 2; Table 3). Cover of exposed soil and litter, which obviously are dependent on high cover of trees and bushes, were positively correlated with shading, whereas density of grass sward was negatively correlated with shading. Consequently, many forest herbs were associated with the microhabitat of diploid ssp. *fuchsii* (e.g. *Anemone hepatica* and *Paris quadrifolia* in the lowland population, and *Melica nutans* and *Poa nemoralis* in the mountain locality; Fig. 3; Appendix 1) (cf. Ellenberg indicator values; Ellenberg et al. 1991). Also, many tall herbs and saplings that indirectly benefit from shading were associated with the habitat of ssp. *fuchsii* (e.g. *Filipendula ulmaria* and *Fraxinus excelsior* in the lowland locality, and *Geranium sylvaticum* and *Aconitum septentrionale* in the mountain locality). In contrast, many light-demanding grassland species were clearly associated with the microhabitat of tetraploid ssp. *maculata* (e.g. *Carex hostiana* and *Festuca ovina* in the lowland locality, and *Bartsia alpina* and *Nardus stricta* in the mountain locality). The Shannon diversity index and the species richness were higher in quadrats of tetraploids than in quadrats of diploids (Table 4). It could be explained by the association of tetraploids with grassland patches with short and dense vegetation. These patches were characterized by many weak competitors rather than by few dominant species.

Together with shading, pH is often considered to be the most discriminating environmental variable for separating between habitats of diploid ssp. *fuchsii* and tetraploid ssp. *maculata* (Heslop-Harrison 1951; Averyanov 1990). This study confirmed that diploids are generally associated with higher pH values than tetraploids, even though the patterns were not unambiguous (Fig. 2; Table 3). Cover of *Sphagnum* was negatively correlated with pH, indicating that acidophilous *Sphagnum* species were abundant in patches with low pH. Characteristically, tetraploids grew on oligotrophic, somewhat elevated and moderately damp tussocks with relatively dense grass sward intermingled with *Sphagnum*. Hydrophilous fen species were found between the tussocks. Such habitat heterogeneity was often detected within the 40 cm × 40 cm sample squares, particularly in the mountain locality. The fine-scale complexity was further illustrated by the fact that hydrophilous, open fen species commonly associated with high pH, such as *Epipactis palustris* in the lowland

locality and *Equisetum variegatum* in the mountain locality (cf. Ellenberg et al. 1991), were also significantly associated with quadrats with tetraploid ssp. *maculata* (Fig. 3; Appendix 1). Diploids were typically found on base-rich soils towards the edges of the open fen areas.

The ecological constraints for both diploid ssp. *fuchsii* and tetraploid ssp. *maculata* seem to be strengthened in Scandinavian mixed populations due to inter-ploidy competition. In pure populations consisting of either diploids or tetraploids, the ecological amplitudes of both taxa are wider; diploids are not obligately restricted to shaded environments and tetraploids are growing on both base-poor and base-rich soils (D. Ståhlberg and M. Hedrén, in preparation; D. Ståhlberg, unpublished data).

Triploid hybrids

Even though one third of the plant samples were chosen because of their morphological intermediacy between diploid ssp. *fuchsii* and tetraploid ssp. *maculata*, only about 10% of the samples were confirmed to be triploid F1 hybrids. Groll (1965) found a markedly higher number of triploids (about 25%) in a putatively primary hybrid zone between morphologically indistinguishable diploid and tetraploid cytotypes of *Dactylorhiza maculata* s.l. in the Eastern Alps.

I found that triploids in general were more similar to tetraploids than to diploids, both in morphology and molecular ITS data (Figs. 4, 5, 6). This similarity is logical since a tetraploid parent contributes two haploid genomes to the hybrid, whereas a diploid parent only contributes one haploid genome. In the discriminant analyses based on vegetation data and morphology, the posteriori classification of triploids from the mountain locality resulted in closer similarity with diploids (Fig. 9b), but this could be an artefact since the analyses were biased towards those characters that best separated between diploids and tetraploids.

When it comes to ecology, the triploids were placed in a relatively intermediate position between diploids and tetraploids in the ordination plots, particularly when based solely on vegetation data, even though the triploids still were slightly more similar to tetraploids than to diploids (Figs. 2, 3). The number of associated species was higher in quadrats with triploids than in quadrats with diploids or tetraploids. It may be assumed that hybrids are to some extent growing along a habitat gradient where species from the habitat of diploids, as well as from the habitat of tetraploids, are coming together. However, the intermediate location of triploids is not necessarily due to intermediate ecological adaptation, but could be simple due to the location where cross-fertilization took place between diploids and tetraploids.

The plastid DNA haplotypes, which are generally maternally inherited (Corriveau and Coleman 1988; Cafasso et al. 2005), indicated that both diploid ssp. *fuchsii* and tetraploid ssp. *maculata* can act as maternal parent in the formation of triploid hybrids. Group II haplotypes were dominating among tetraploids (particularly in the lowland locality), but where absent in diploids (Fig. 8). In the triploid hybrids both Group I and Group II haplotypes were detected and a slight predominance of Group I haplotypes was observed.

Introgressive gene flow between ploidy levels

The clear differentiation between diploids and tetraploids suggest that introgressive gene flow between ploidy levels is limited (Fig. 9). Only a few individuals were misclassified in the discriminant analyses. Based on morphology, less than 3% were misclassified. A

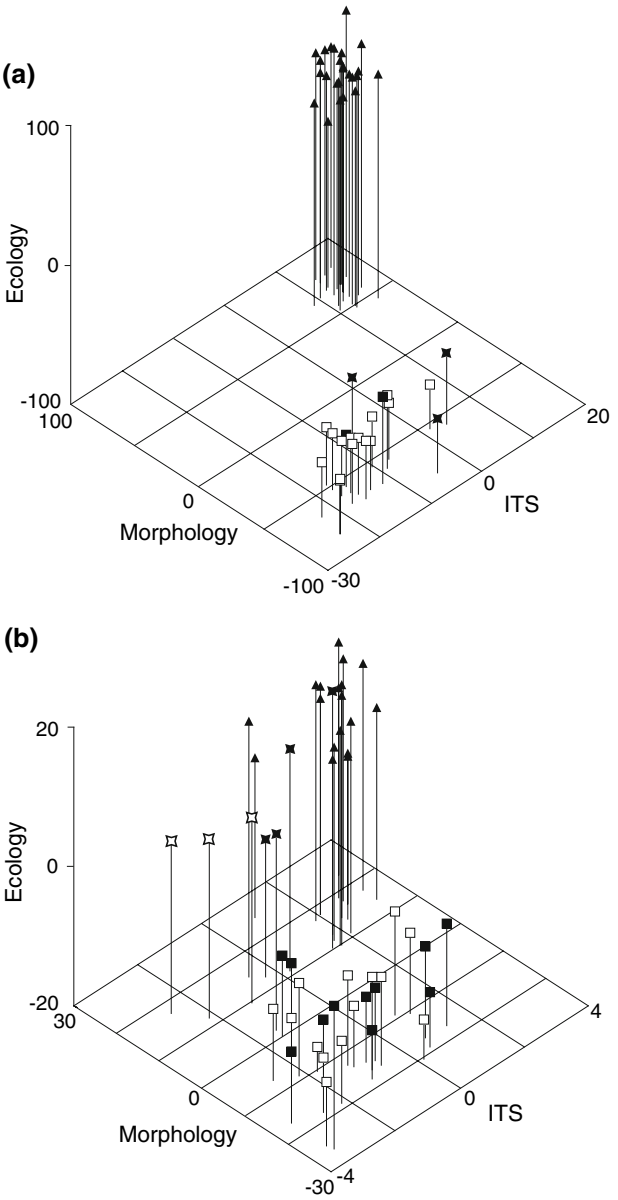


Fig. 9 Discriminant analysis based on vegetation data, morphology and ITS allele frequency. *Triangles* indicate diploids, *crosses* indicate triploid hybrids, *squares* indicate tetraploids. Samples with Group I haplotypes are shown in *black* and samples with Group II haplotypes are shown in *white* (cf. Fig. 7). (**a**) The lowland locality. (**b**) The mountain locality

similar percentage was acquired when diploids and tetraploids from cytologically homogeneous populations were analysed (D. Ståhlberg and M. Hedrén, in preparation). The ordinations based on morphology resulted also in relatively good separations of diploids and tetraploids, although there was some overlap, especially in the mountain

locality. Irrespective of whether mixed or pure populations are considered, it should be observed that the morphological variation between ssp. *fuchsii* and ssp. *maculata* is clinal, and within each taxon there can be substantial variation both within and between populations.

The ITS data from the mountain locality gave indications that some gene flow between ploidy levels is occurring. In contrast to the patterns found at the lowland locality, where the genetic distance between diploid ssp. *fuchsii* and tetraploid ssp. *maculata* was similar to the genetic distance between ssp. *fuchsii* and ssp. *maculata* from pure populations, the genetic distance between the two cytotypes was lower in the mountain locality (Table 5). Moreover, about 10% of the specimens (diploids and tetraploids) from the mountain locality were misclassified in the discriminant analysis based on ITS data, compared to 0% misclassification of specimens from the lowland locality. Patterns of introgression in the mountain locality may also be inferred from the triangular diagram of ITS allele frequencies (Fig. 6b). One diploid displayed a relatively high frequency of Allele I (38%) and a low frequency of Allele V (23%), whereas four tetraploids had low frequencies of Allele I (13–27%) and markedly high frequencies of Allele V (45–54%). Populations of diploid ssp. *fuchsii* in Scandinavia are characterized by absence of Allele I and high frequencies of Allele V (>40%), whereas populations of tetraploid ssp. *maculata* typically have higher frequencies of Allele I (>30%) and lower frequencies of Allele V (<40%) (D. Ståhlberg and M. Hedrén, in preparation).

Gene flow from the diploid to the tetraploid level should be more common than gene flow in the opposite direction (Grant 1981), but the ITS data indicated that some gene flow also can happen in the opposite direction. This assumption is supported by Devos et al. (2005) for *Dactylorhiza maculata* s.l. in western Europe. However, I did not find any Group II plastid haplotypes in the diploid subpopulations, which would have been expected if gene flow from the tetraploid to the diploid level was common, since Group II haplotypes were dominating among tetraploids.

Differences between localities from contrasting biogeographic regions

Similar patterns of differentiation between diploids and tetraploids were observed in the two mixed populations, despite the fact that the lowland locality and the mountain locality were very different from a biogeographic point of view, as indicated by the floristic compositions. The mountain locality was, for example, characterized by a large number of boreal-alpine elements, and less than 20% of the species were found in both localities. However, the differentiation between diploids and tetraploids was more distinct in the lowland population than in the mountain locality. Based on studies on *Iris*, Arnold (1997) points out that the significance of introgressive gene flow in hybrid zones may be directly correlated to the frequency of F1 hybrids. In the lowland locality, very few triploid hybrids were detected and introgressive gene flow appeared to be of limited importance. The frequency of triploid hybrids was somewhat higher in the mountain locality, and a more obvious pattern of introgressive gene flow was observed.

Hybridization is often associated with disturbance (Anderson 1948; Arnold 1997). In mixed populations of *Dactylorhiza* on industrial waste sites, Shaw (1998) observed markedly higher frequencies of hybrids than is typical for mixed populations on less disturbed habitats. The higher levels of hybridization and introgression in the mountain locality compared to the lowland locality may likewise be attributed to disturbance. The mountain locality—a sloping fen in the Scandinavian mountain ridge, subjected to harsh

winters, avalanches, spring floods and landslides, should clearly be a more dynamic and heterogeneous environment than the flat lowland locality.

Conclusions

(1) Mixed populations of diploid and autotetraploid *Dactylorhiza maculata* s.l. in Scandinavia represent secondary contact zones between diploid ssp. *fuchsii* and tetraploid ssp. *maculata*. No patterns of recent and local autopolyploidization were detected. Diploids and tetraploids from mixed populations are not less differentiated than diploids and tetraploids from cytologically homogeneous populations. (2) Diploids and tetraploids are spatially separated on a microhabitat level in mixed populations. Both cytotypes appear to have wider ecological amplitudes in pure populations. The ecological constraints may thus be strengthened in mixed populations, which should contribute to the maintenance of hybrid zones. (3) Relatively few triploid hybrids were found. Most of them grew in intermediate habitats between diploids and tetraploids. Both diploid ssp. *fuchsii* and tetraploid ssp. *maculata* may act as maternal parent. Some patterns of introgressive gene flow between ploidy levels appeared. Plastid markers indicated that gene flow from diploid to tetraploid level is most common, but nuclear markers suggested that gene flow in opposite direction may also occur. (4) Comparable patterns of differentiation and gene flow appeared in localities that represent contrasting biogeographic regions. Disturbance and topography may explain why hybridization was more common and the differentiation patterns less clear in the Scandinavian mountains than in the coastal lowland.

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Appendices

Appendix 1 Mean cover of species associated to quadrates with diploid, triploid or tetraploid *Dactylorhiza maculata* s.l. A 0-5 graded Hult-Sernander-Du Rietz scale (Du Rietz 1921) was used

Abbreviation	Taxon	Diploids ($N = 25$)	Triploid hybrids ($N = 3$)	Tetraploids ($N = 17$)	P
(a) The lowland locality					
Anehep	*Anemone hepatica*	0.12	0.00	0.00	0.238
Anenem	*Anemone nemorosa*	0.40	0.67	0.06	**<0.05**
Betpub	*Betula pubescens*	0.04	0.00	0.71	**<0.01**
Brimed	*Briza media*	0.04	0.00	0.18	0.329
Camrot	*Campanula rotundifolia*	0.04	0.00	0.06	0.781
Cardio	*Carex dioica*	0.00	0.00	0.06	0.225
Carflac	*Carex flacca*	0.20	0.67	0.00	0.052
Carflav	*Carex flava*	0.08	0.00	0.00	0.238
Carhos	*Carex hostiana*	0.00	0.00	0.18	**<0.05**
Carnig	*Carex nigra*	0.40	0.33	0.35	0.829

Appendix 1 continued

Abbreviation	Taxon	Diploids ($N = 25$)	Triploid hybrids ($N = 3$)	Tetraploids ($N = 17$)	P
Carpan	*Carex panicea*	0.00	0.00	0.76	**<0.001**
Cirpal	*Cirsium palustre*	0.56	1.00	0.12	**<0.01**
Conmaj	*Convallaria majalis*	0.08	0.67	0.12	0.781
Crepal	*Crepis paludosa*	0.08	0.00	0.00	0.238
Drorot	*Drosera rotundifolia*	0.00	0.00	0.06	0.225
Empnig	*Empetrum nigrum*	0.00	0.00	0.18	0.225
Epipal	*Epipactis palustris*	0.00	0.00	0.18	<0.05
Equflu	*Equisetum fluviatile*	0.08	0.00	0.00	0.238
Equpal	*Equisetum palustre*	3.00	2.00	0.65	**<0.001**
Equvar	*Equisetum variegatum*	0.00	0.00	0.18	**<0.05**
Erioang	*Eriophorum angustifolium*	0.00	0.00	0.06	0.225
Fesovi	*Festuca ovina*	0.00	0.33	0.24	**<0.05**
Filulm	*Filipendula ulmaria*	1.56	0.67	0.00	**<0.001**
Fraexc	*Fraxinus excelsior*	0.88	1.33	0.12	**<0.01**
Galbor	*Galium boreale*	0.04	0.33	0.06	0.781
Galpal	*Galium palustre*	0.04	0.00	0.00	0.410
Galuli	*Galium uliginosum*	0.24	0.67	0.65	**<0.05**
Geuriv	*Geum rivale*	0.68	0.67	0.00	**<0.001**
Lysvul	*Lysimachia vulgaris*	0.08	0.00	0.00	0.238
Maibif	*Maianthemum bifolium*	0.68	1.33	0.35	0.179
Mentri	*Menyanthes trifoliata*	0.60	0.00	2.18	**<0.001**
Molcae	*Molinia caerulea*	0.00	0.67	0.18	**<0.05**
Parqua	*Paris quadrifolia*	0.08	0.00	0.06	0.796
Peupal	*Peucedanum palustre*	0.00	0.00	0.12	0.225
Pinvul	*Pinguicula vulgaris*	0.00	0.00	0.12	0.083
Poapra	*Poa pratensis*	0.04	0.00	0.06	0.781
Potere	*Potentilla erecta*	0.28	0.33	1.59	**<0.001**
Prupad	*Prunus padus*	0.04	0.00	0.00	0.410
Ranacr	*Ranunculus acris*	0.16	0.00	0.00	0.143
Rubsax	*Rubus saxatilis*	0.64	1.33	1.47	**<0.01**
Salspp	*Salix* spp.	0.00	0.00	0.12	0.225
Sorauc	*Sorbus aucuparia*	0.04	0.00	0.00	0.410
Sucpra	*Succisa pratensis*	0.08	0.33	0.53	**<0.05**
Thafla	*Thalictrum flavum*	0.12	0.00	0.00	0.238
Trieur	*Trientalis europaea*	0.04	0.00	0.06	0.781
Tusfar	*Tussilago farfara*	0.04	0.00	0.00	0.410
Vacmyr	*Vaccinium myrtillus*	0.00	0.00	0.18	**<0.05**
Vacoxy	*Vaccinium oxycoccos*	0.00	0.00	0.47	**<0.001**
Vacvit	*Vaccinium vitis-idaea*	0.00	0.33	0.12	0.083
Vibopu	*Viburnum opulus*	0.52	0.00	0.12	0.158
Viopal	*Viola palustris*	0.20	0.00	0.06	0.205

Appendix 1 continued

Abbreviation	Taxon	Diploids ($N = 18$)	Triploid hybrids ($N = 7$)	Tetraploids ($N = 25$)	P
(b) The mountain locality					
Acosep	*Aconitum septentrionale*	0.33	0.00	0.00	<**0.05**
Agrcap	*Agrostis capillaris*	0.17	0.00	0.00	0.239
Alcsp.	*Alchemilla* sp.	0.83	1.29	0.76	0.440
Angsyl	*Angelica sylvestris*	0.17	0.43	0.08	0.784
Antodo	*Anthoxanthum odoratum*	0.39	0.57	0.20	0.936
Baralp	*Bartsia alpina*	0.00	0.29	0.12	<**0.05**
Betnan	*Betula nana*	0.00	0.00	0.08	1.000
Betpub	*Betula pubescens*	0.00	0.43	0.20	<**0.05**
Bisviv	*Bistorta vivipara*	0.17	0.43	0.48	<**0.05**
Calpal	*Caltha palustris*	0.00	0.29	0.08	0.078
Cardio	*Carex dioica*	0.00	0.29	0.48	<**0.01**
Carfla	*Carex flava*	0.06	0.71	0.56	<**0.01**
Carlas	*Carex lasiocarpa*	0.00	0.43	0.08	0.078
Carnig	*Carex nigra*	0.00	1.14	0.68	<**0.01**
Carpan	*Carex panicea*	0.00	0.57	0.16	<**0.05**
Carros	*Carex rostrata*	0.06	0.00	0.00	0.239
Cerfon	*Cerastium fontanum*	0.00	0.00	0.04	0.396
Cicalp	*Cicerbita alpina*	0.11	0.00	0.00	0.239
Cirhel	*Cirsium helenioides*	0.06	0.00	0.08	0.759
Cirpal	*Cirsium palustre*	0.00	0.00	0.08	0.396
Crepal	*Crepis paludosa*	0.61	0.43	0.68	0.518
Desces	*Deschampsia cespitosa*	1.22	0.00	0.12	<**0.01**
Desfle	*Deschampsia flexuosa*	0.22	0.00	0.00	0.239
Empnig	*Empetrum nigrum*	0.00	0.57	0.52	<**0.05**
Epipal	*Epilobium palustre*	0.06	0.14	0.16	0.298
Equpal	*Equisetum palustre*	0.22	0.00	0.28	0.602
Equpra	*Equisetum pratense*	0.06	0.00	0.00	0.239
Equsyl	*Equisetum sylvaticum*	0.17	0.43	0.00	0.667
Equvar	*Equisetum variegatum*	0.06	0.57	0.52	<**0.01**
Eriang	*Eriophorum angustifolium*	0.00	0.00	0.04	1.000
Eupsp.	*Euphrasia* sp.	0.06	0.57	0.60	<**0.01**
Fesovi	*Festuca ovina*	0.00	0.00	0.08	1.000
Fessp.	*Festuca* sp.	0.00	0.00	0.04	0.396
Filulm	*Filipendula ulmaria*	0.28	0.29	0.16	0.432
Gersyl	*Geranium sylvaticum*	1.28	0.00	0.00	<**0.001**
Geuriv	*Geum rivale*	0.67	0.57	0.00	<**0.01**
Gnasyl	*Gnaphalium sylvaticum*	0.06	0.00	0.00	0.239
Gymcon	*Gymnadenia conopsea*	0.00	0.14	0.04	0.396
Gymdry	*Gymnocarpium dryopteris*	0.22	0.00	0.00	0.239

Appendix 1 continued

Abbreviation	Taxon	Diploids ($N = 18$)	Triploid hybrids ($N = 7$)	Tetraploids ($N = 25$)	P
HieAlp	*Hieracium* sect. *Alpina*	0.00	0.00	0.12	0.225
Junsp.	*Juncus* sp.	0.00	0.00	0.04	0.396
Lisova	*Listera ovata*	0.11	0.00	0.00	0.239
Luzpil	*Luzula pilosa*	0.22	0.00	0.04	0.069
Luzsp.	*Luzula* sp.	0.06	0.14	0.16	0.478
Maibif	*Maianthemum bifolium*	0.22	0.00	0.00	**<0.05**
Melnut	*Melampyrum pratense*	0.17	0.00	0.00	**<0.05**
Melpra	*Melampyrum sylvaticum*	0.17	0.00	0.16	0.954
Melsyl	*Melica nutans*	0.06	0.14	0.08	0.478
Mileff	*Milium efusum*	0.11	0.00	0.00	0.092
Molcae	*Molinia caerulea*	0.00	1.00	0.44	**<0.05**
Narstr	*Nardus stricta*	0.11	0.86	0.68	**<0.05**
Oxaace	*Oxalis acetosella*	0.11	0.00	0.00	0.092
Parpal	*Parnassia palustris*	0.00	0.14	0.12	0.225
Pedsp.	*Pedicularis* sp.	0.00	0.14	0.04	0.225
Petfri	*Petasites frigidus*	0.06	0.14	0.04	0.759
Pinvul	*Pinguicula vulgaris*	0.00	0.14	0.12	0.132
Poaalp	*Poa alpina*	0.00	0.00	0.04	0.396
Poanem	*Poa nemoralis*	0.33	0.00	0.00	**<0.05**
Potere	*Potentilla erecta*	0.39	1.14	1.44	**<0.01**
Potpal	*Potentilla palustris*	0.06	0.00	0.00	0.239
Pyrsp.	*Pyrola* sp.	0.28	0.00	0.12	0.087
Ranacr	*Ranunculus acris*	0.22	0.57	0.48	0.052
Rhimin	*Rhinanthus minor*	0.00	0.43	0.12	**<0.05**
Rubarc	*Rubus arcticus*	0.28	0.00	0.00	**<0.05**
Rumace	*Rumex acetosa*	0.06	0.00	0.04	0.813
Salspp	*Salix* spp.	0.00	0.71	0.36	**<0.01**
Saualp	*Saussurea alpina*	0.28	0.00	0.12	0.205
Saxaiz	*Saxifraga aizoides*	0.00	0.00	0.04	1.000
Saxsp.	Saxifraga sp.	0.00	0.14	0.08	0.132
Scisyl	*Scirpus sylvaticus*	0.00	0.00	0.04	0.396
Selsel	*Selaginella selaginoides*	0.00	0.29	0.24	**<0.05**
Solvir	*Solidago virgaurea*	0.89	0.86	0.64	0.818
Stenem	*Stellaria nemorum*	0.11	0.00	0.00	0.092
Tarsp.	*Taraxacum* sp.	0.11	0.00	0.08	0.732
Thaalp	*Thalictrum alpinum*	0.22	0.71	0.92	**<0.001**
Troeur	*Trollius europaeus*	0.06	0.00	0.08	0.840
Vacmyr	*Vaccinium uliginosum*	0.44	0.29	0.12	0.603
Vacoxy	*Vaccinium myrtillus*	0.00	0.14	0.16	0.225
Vaculi	*Vaccinium oxycoccos*	0.00	0.00	0.16	0.225

Appendix 1 continued

Abbreviation	Taxon	Diploids ($N = 18$)	Triploid hybrids ($N = 7$)	Tetraploids ($N = 25$)	P
Vacvit	*Vaccinium vitis-idaea*	0.39	0.29	0.28	0.633
Viobif	*Viola biflora*	0.56	0.00	0.08	**<0.01**

The P-values represent comparisons between diploids and tetraploids (Mann–Whitney U-test). Significant P-values are given in bold

Appendix 2 Population means of morphological characters (see Table 1); standard deviations (where appropriate) in italics

Character	Diploids ($N = 25$)		Triploid hybrids ($N = 3$)		Tetraploids ($N = 17$)		P
(a) The lowland locality							
A1	8.63	*0.88*	7.27	*0.65*	7.34	*1.03*	**<0.001**
A2	4.62	*0.71*	4.77	*0.31*	5.39	*0.75*	**<0.01**
A3	5.91	*1.14*	6.13	*1.00*	6.27	*0.85*	0.396
A4	1.69	*0.37*	1.34	*0.12*	1.27	*0.19*	**<0.001**
A5	10.15	*1.49*	9.40	*0.95*	9.48	*1.00*	0.093
A6	9.40	*3.00*	5.00	*0.00*	5.00	*0.00*	**<0.001**
A7	42.40	*6.63*	18.33	*12.58*	17.35	*12.51*	<0.001
A8	39.20	*7.02*	33.33	*5.77*	35.29	*5.14*	0.073
A9	2.68		2.33		2.41		0.433
A10	2.48		3.00		2.82		**<0.05**
A11	2.52		1.67		1.47		**<0.001**
A12	8.85	*0.92*	7.53	*0.67*	7.75	*1.44*	**<0.05**
A13	3.36		3.33		3.53		0.282
A14	1.04		1.00		0.94		0.155
A15	10.12	*1.64*	8.50	*1.21*	8.62	*1.19*	**<0.01**
A16	1.08		0.33		0.76		0.085
A17	17.26	*2.82*	13.70	*1.08*	12.61	*3.48*	**<0.001**
A18	12.29	*1.97*	9.90	*0.70*	9.81	*2.52*	**<0.01**
A19	1.24		1.00		1.18		0.725
B20	49.88	*10.35*	35.67	*4.04*	31.24	*8.13*	**<0.001**
B21	6.64	*1.81*	5.17	*0.76*	5.09	*1.38*	**<0.01**
B22	24.28	*7.80*	22.67	*4.04*	19.24	*4.70*	**<0.05**
B23	3.36	*1.25*	2.53	*0.58*	3.39	*4.07*	**<0.05**
B24	4.08		3.67		3.41		**<0.05**
B25	1.64		1.00		1.41		0.203
B26	12.78	*2.61*	9.50	*0.50*	10.06	*2.96*	**<0.05**
B27	2.74	*0.88*	1.40	*0.17*	1.29	*0.39*	**<0.001**
B28	1.04		1.00		1.18		0.144
B29	1.80		1.67		1.41		**<0.05**
B30	1.92		1.67		1.65		**<0.05**
B31	2.56		2.00		1.94		**<0.001**

Appendix 2 continued

Character	Diploids ($N = 25$)		Triploid hybrids ($N = 3$)		Tetraploids ($N = 17$)		P
B32	3.44		4.00		3.71		0.192
B33	2.96		3.00		2.94		0.781
B34	3.12		3.33		3.00		0.274
B35	2.58		1.83		1.85		**<0.001**
Character	Diploids ($N = 18$)		Triploid hybrids ($N = 7$)		Tetraploids ($N = 25$)		P
(b) The mountain locality							
A1	8.08	*1.55*	8.03	*1.05*	7.65	*0.80*	0.402
A2	5.62	*0.97*	6.60	*0.75*	5.86	*0.71*	0.182
A3	6.88	*1.12*	7.11	*0.67*	7.04	*0.78*	0.321
A4	1.30	*0.19*	1.17	*0.10*	1.20	*0.16*	0.065
A5	9.72	*1.43*	9.37	*0.92*	9.88	*1.10*	0.921
A6	5.00	*0.00*	5.00	*0.00*	5.00	*0.00*	1.000
A7	23.33	*12.72*	20.00	*8.16*	14.40	*9.05*	**<0.05**
A8	32.78	*4.61*	32.86	*4.88*	37.20	*4.58*	**<0.01**
A9	1.94		1.86		2.48		**<0.05**
A10	1.33		1.57		1.92		**<0.01**
A11	1.83		1.43		1.84		0.989
A12	6.15	*1.32*	6.26	*0.61*	6.19	*0.99*	0.498
A13	3.44		3.43		3.12	*0.33*	**<0.05**
A14	0.72		1.00		1.00	*0.41*	**<0.05**
A15	8.35	*2.22*	9.39	*0.95*	8.73	*1.54*	0.853
A16	0.17		0.71		0.52		**<0.05**
A17	13.62	*1.84*	12.03	*1.72*	13.93	*2.69*	0.692
A18	8.92	*1.45*	8.40	*1.35*	9.43	*1.42*	0.135
A19	0.83		1.57		1.40		**<0.01**
B20	35.22	*6.53*	22.64	*6.00*	22.32	*5.62*	**<0.001**
B21	5.36	*1.55*	3.50	*1.04*	4.08	*0.61*	**<0.001**
B22	17.00	*5.78*	13.29	*3.68*	13.72	*3.51*	0.063
B23	2.97	*0.96*	2.30	*0.75*	2.41	*0.60*	0.094
B24	3.00		3.14		3.12		0.813
B25	1.72		1.43		1.52		0.249
B26	11.11	*2.61*	7.86	*2.66*	8.14	*1.77*	**<0.001**
B27	2.15	*0.59*	1.40	*0.37*	1.42	*0.41*	**<0.001**
B28	1.22		1.29		1.20		0.861
B29	1.17		1.29		1.08		0.387
B30	1.50		1.29		1.32		0.488
B31	2.50		2.00		2.08		**<0.01**
B32	2.94		4.86		4.60		**<0.01**
B33	3.00		3.14		3.08		0.488
B34	3.17		3.00		3.04		0.192
B35	2.00		1.50		1.50		**<0.01**

The P-values represent comparisons between diploids and tetraploids (Mann-Whitney U-test). Significant P-values are given in bold

Appendix 3 Characterization of plastid haplotypes identified in the present study by means of the primer pairs described in Table 2

Nr.	Group	Locus										*N*
		1	6	6B	8	9	10b	11b	17	18	19	
58	I	185	222	470	75	196	144	84	132	93	149	8
59	I	185	222	470	75	196	145	84	132	93	149	36
60	I	185	222	470	75	196	146	84	132	93	149	4
65	I	186	222	470	75	196	144	84	132	93	149	3
66	I	186	222	470	75	196	145	84	132	93	149	3
67	I	186	222	470	75	196	146	84	132	93	149	1
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	1
81	I	185	281	560	75	196	145	84	132	93	149	2
83	I	186	281	560	75	196	144	84	132	93	149	1
130	II	185	281	560	73	185	147	85	136	93	149	1
134	II	185	275	610	73	177	146	85	136	97	149	1
137	II	186	275	610	73	177	147	85	136	97	149	2
141	II	185	281	560	73	177	145	85	136	97	149	1
142	II	185	281	560	73	177	146	85	136	97	149	9
143	II	185	281	560	73	177	147	85	136	97	149	2
149	II	186	281	560	73	177	146	85	136	97	149	1
153	II	185	281	560	73	185	146	85	136	97	149	2
154	II	186	281	560	73	185	146	85	136	97	149	1
179	II	185	222	470	73	177	148	87	136	93	149	2
186	I	185	222	470	75	177	145	84	132	93	149	1
205	II	186	222	470	73	177	146	85	136	97	149	1
216	I	184	222	470	75	196	144	84	132	93	149	2
267	II	185	281	560	73	177	148	87	136	93	149	1
268	II	185	281	560	73	177	149	87	136	93	149	7
278	II	185	281	560	73	177	148	85	136	103	149	1

The numbering of the haplotypes is based on a larger data set that encompasses *Dactylorhiza* from all over Europe (M. Hedrén et al. in preparation). Group refers to major haplotype groups according to Fig. 7. *N* is number of individuals found with a particular haplotype. Assessed fragment lengths are given in bp

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