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Patterns of chloroplast diversity among western European *Dactylorhiza* species (Orchidaceae)

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Abstract. In Europe, the genus *Dactylorhiza* comprises a bewildering variety of forms that are difficult to sort into discrete species. Most *Dactylorhiza* species are diploid or tetraploid and contrasting hypotheses have been proposed to explain the complex variation within this group. Using PCR-RFLP analysis in eight putative species, we could identify four highly differentiated chloroplast DNA lineages. The first lineage (clade A) included the unique haplotype found in *D. sambucina*. Clade B grouped four haplotypes belonging mostly to *D. incarnata*. Clades C and D included 27 haplotypes detected in diploid *D. fuchsii* and in all tetraploid species investigated. Eighty percent of the chloroplast variation were consistent with currently accepted species boundaries. The imperfect agreement between chloroplast variation and species boundaries may be ascribed to incomplete lineage sorting and/or reticulation. Our cpDNA results provide strong evidence that the allotetrapolyploids have been formed through asymmetric hybridization with a member of the *D. fuchsii*/*maculata* group as the maternal parent.

Key words: Orchidaceae, *Dactylorhiza*, PCR-RFLP, chloroplast DNA, neighbour-joining, haplotype diversity, species complex, polyploids.

The number of *Dactylorhiza* species varies strongly among authors (for review, see Pedersen 1998 and Tyteca 2001), who recognize from 12 (Klinge 1899) to 75 (Averyanov 1990) species overall or, for Europe only, from six (Sundermann 1980) to 49 species (Delforge 1994). Moreover, there is a current trend to recognize morphologically and geographically deviating groups of populations of *Dactylorhiza* as different species. In Scandinavia, for example, Hylander (1966) listed five species in the *D. incarnata*/*maculata* complex, whereas Delforge (2001) recognized 17 species.

Most *Dactylorhiza* species are diploid ($2n = 40$) or tetraploid ($2n = 80$) (Heslop-Harrison 1954, Soó 1980, Pridgeon et al. 2001) and the European species can be sorted into six groups on the basis of morphological and cytological data: (1) the *D. sambucina* group, comprising diploid and triploid species, (2) the *D. incarnata* group, comprising diploid species and sometimes called the ''diploid Marsh-Orchids'', (3) the *D. majalis* group, or the ''tetraploid Marsh-Orchids'', comprising only tetraploid species, (4) the *D. maculata*

group, or the "spotted Marsh-Orchids", comprising both diploid and tetraploid species, (5) the *D. iberica* group, comprising one diploid species, and (6) the *D. aristata* group, comprising also one diploid species (e.g. Heslop-Harrison 1954, 1956; Bateman and Denholm 1983, 1985, 1989; Tyteca and Gathoye 1989). Recent molecular work on subtribe Orchidinae suggested that *Coeloglossum viride* should be included in *Dactylorhiza* (Bateman et al. 1997).

The taxonomic complexity of this genus can be explained by the fact that (1) many species are young and have not yet acquired good discriminating morphological characters (Hedrén 2002), (2) most members of the genus hybridize frequently (Soó 1980, Stace 1991) or (3) phenotypic plasticity or age can modify more or less strongly the morphology of the different species (Bateman and Denholm 1983, Andersson 1996). Moreover, combined evidence from cytological, morphological and molecular studies suggested that the evolution in *Dactylorhiza*, tentatively associated with the last glaciation in the early Holocene, was highly reticulated because of hybridization between diploid species, between tetraploid species, between diploid and tetraploid species, or polyploidization following hybridization between diploid species (Averyanov 1990; Hedrén 1996a, 2002).

Tetraploid plants are often classified into two categories: autotetraploids, which include structurally similar parental genomes, and allotetraploids, which combine divergent parental genomes (Grant 1981). Based on cytological or morphological data, an auto- or allopolyploid origin has been suggested for various tetraploid *Dactylorhiza* species (Hagerup 1938, 1944; Vermeulen 1947; Heslop-Harrison 1953a, b; Nelson 1976, Gathoye and Tyteca 1989, Dufrêne et al. 1991). Allozyme data allowed a nearly complete distinction between the two diploids, *D. fuchsii* s.l. (referred to as "genome FF") and *D. incarnata* s.l. (referred to as "genome II"), which constitute the two principal parental lineages in the species complex (Hedrén 1996a). Among the descendants from these two parental lineages, *Dactylorhiza maculata* was considered to be autotetraploid (genome FFFF), whereas e.g. *D. majalis*, *D. praetermissa*, *D. sphagnicola* and *D. elata* appeared to be allotetraploids (genome FFII) (Hedrén 1996a, b, 2001b). The genetic distinction found by Hedrén (1996a) is consistent with the *Dactylorhiza incarnata*, the *D. maculata* and the *D. majalis* groups defined above as they contain only species with the genome composition II, FF/FFFF and FFII, respectively. The *Dactylorhiza sambucina*, *D. aristata* and *D. iberica* groups are not generally thought to be involved in the formation of the allotetraploid species although Bateman et al. (1997) suggested that *D. sambucina* might be an alternative parent to at least some of them. Hedrén (2001a) and Bateman and Denholm (2003) also suggested that *D. saccifera*, a diploid species from the *D. maculata* group, might be implicated in the formation of these allotetraploid species.

The uniparental inheritance of the chloroplast genome in most plant species (Birky 1995), its clonal mode of evolution and its slow rate of evolutionary change (Wolfe et al. 1987) have made the chloroplast an invaluable tool in plant evolutionary biology. If chloroplast DNA (cpDNA) is maternally inherited in *Dactylorhiza,* as in other Orchidaceae (Harris and Ingram 1991) and most angiosperms (Birky 1995), chloroplast markers could be of interest for tracing the maternal origin of taxa that have been formed by hybridization (e.g. Clegg et al. 1993, Comes and Abbott 2001). Allozymes and/or AFLP markers have already been used to reveal details of polyploid evolution in *Dactylorhiza* from Scandinavia (Hedrén 1996a, b, c; Hedrén et al. 2001) and Asia Minor (Hedrén 2001a). However, *Dactylorhiza* populations from large parts of western Europe have never been studied with nuclear nor chloroplast markers. The study of these populations, situated in one of the diversity centers of the genus, is nevertheless essential to better understand the evolution of the genus *Dactylorhiza*.

The present study was aimed at (1) developing cpDNA markers to describe the chloroplast genome of various *Dactylorhiza* species native to western Europe, (2) estimating the cpDNA diversity within and among the species examined, and (3) testing the utility of these cpDNA markers to reveal the maternal origin of the different polyploid species investigated.

Materials and methods

Investigated species and sampling. The species examined in this study all occur in western Europe (Bournérias et al. 1998, Delforge 2001). Three diploid species (2n = 40), *Dactylorhiza incarnata* (L.) Soó, *D. fuchsii* (Druce) Soó, and *D. sambucina* (L.) Soó, and five tetraploid species (2n = 80), *D. majalis* (Reichenbach) P.F. Hunt & Summerhayes, *D. maculata* (L.) Soó, *D. elata* (Poiret) Soó, *D. praetermissa* (Druce) Soó and *D. sphagnicola* (Höppner) Averyanov, were sampled for this study. A total of 262 specimens were sampled in 26 populations from western Europe (Table 1). All collected leaf material was snap-frozen in liquid nitrogen, if available, or dried with silica gel for subsequent DNA extraction.

Chloroplast DNA analysis (PCR-RFLP). Total DNA was extracted from approximately 50 mg of leaf tissue using a CTAB procedure slightly modified from Doyle and Doyle (1990). Using this procedure, DNA was efficiently isolated from both frozen and silica gel dried material. An initial screening for polymorphism was made in a subset of ten individuals using eleven pairs of universal primers described in Demesure et al. (1995) and Dumolin-Lapègue et al. (1997): *psb*C – *trn*S, *trn*H – *trn*K1, *trn*K1 – *trn*K2, *trn*Q – *trn*R, *trn*C – *trn*D, *trn*S – *trn*fM, *psa*A – *trn*S, *trn*V2 – *rbc*L, *trn*D – *trn*T, *trn*S – *trn*T and *trn*T – *trn*F. Amplicons were digested by three 4bp-cutter restriction enzymes (*Hin*fI, *Alu*I and *Rsa*I) and restriction products were separated on both 2% agarose and 19:1 polyacrylamide gels. Based on this initial screening, five amplicon/enzyme combinations yielding reliably scorable band patterns were used to characterize the cpDNA of all sampled individuals (Table 2). Chloroplast DNA was amplified in 25 μL reactions containing 5 μL DNA solution, 2.5 μL AmpliTaq buffer, 2.5 mM $MgCl_2$, 200 μg mL^{-1} BSA, 200 μM of each dNTP, 0.2 μM of each primer and 0.625 U TaqDNA polymerase (Roche). PCR reactions were performed using one denaturing step of 5 min at 95 °C, 30 amplification cycles of 45 s at 92 °C, 45 s at 48 to 62 °C (depending on the pair of primers used; see Table 2) and 2 to 4 min at 72 °C (depending on the pair of primers used; see Table 2), followed by one elongation step of 10 min at 72 °C. PCR reactions were carried out in a Perkin Elmer thermocycler (model 2400 or 9700). PCR products (10 μL) were digested at 37 °C for 4 hours with two units of restriction enzyme and its appropriate buffer in a total reaction volume of 20 μL. Digestion products were separated overnight by electrophoresis on polyacrylamide gels using Tris-borate EDTA (0.5 X) buffer. Polyacrylamide gels were prepared with different concentrations of 19:1 and 29:1 acrylamide-bisacrylamide solutions depending on the size of the polymorphic restriction fragments to be resolved (Table 2). Restriction products of fragment VL were separated on both 19:1 10% and 29:1 9% polyacrylamide gels in order to maximize the resolution of two polymorphic fragments having very different sizes. Gels were stained with ethidium bromide and photographed under UV light with the Gel Doc 2000 Image system (Bio-Rad). The data matrices are available from the first author on request.

Data analysis. The molecular data were scored as multistate characters: each polymorphic fragment was considered as a character and the different sizes of each polymorphic fragment were scored as different states. The length variants were noted as states 1 to 5; state 9 was used for restriction site mutations. Distances between each pair of haplotypes was defined and calculated as the number of characters differing in state. Based on the matrix of distances between haplotypes and haplotype frequencies, an analysis of molecular variance (AMOVA; Excoffier et al. 1992) was applied using Arlequin 2.00, in order to partition haplotypic diversity into within- and between-species components. A neighbour-joining tree of the haplotypes was obtained with the Neighbor procedure of PAUP 4.0 (Swofford 2001). Total genetic diversity (h_T), average genetic intrapopulation diversity (h_S), and the level of gene differentiation among populations (G_{ST}) and their standard errors were estimated for each investigated species using the Haplodiv software (Pons and Petit 1995). Haplotypic richness was computed for a uniform sample size of 14 individuals, which is the minimum

sample size for one of the eight species investigated (*D. sambucina*) (El Mousadik and Petit 1996).

Results

CpDNA polymorphism and genetic diversity. In the initial screening of a subset of ten individuals, no polymorphism was detected in the *psb*C–*trn*S, *trn*K1–*trn*K2, *trn*S–*trn*fM and *trn*S–*trn*T amplicons with any of the three restriction enzymes. All other amplicons displayed polymorphism with at least one of the three endonucleases used. However, the polymorphisms detected in the *trn*D–*trn*T and *trn*T–*trn*F amplicons, as well as in one restriction fragment of the AS amplicon, were not used further. Indeed, these polymorphisms could not be resolved satisfactorily on the polyacrylamide gels used, owing to the very small differences in size of the polymorphic restriction fragments.

The survey of the 262 individuals with the remaining five amplicon/enzyme combinations revealed 16 polymorphic fragments, which together displayed 34 polymorphisms and allowed the identification of 32 haplotypes (Table 3). Among the 34 polymorphisms resolved, six resulted from restriction site gains or losses, while the others seemed to be due to insertions or deletions (indels). The haplotype composition of the eight species examined is given in Table 4. Nineteen percent of the haplotypes were shared between two or more species. *Dactylorhiza sambucina* and *D. elata* were the only species that did not share any haplotype with other species. Five haplotypes (H04, H05, H20, H22 and H30) represented 53% of the total sample. Haplotype H22 alone represented 23% of the sample and was by far the most frequent over the studied area. Haplotypes H06, H08, H13, H14, H15, H16, H18, H23, H24, H25, H27 and H31 were extremely rare, each being observed only once. AMOVA analysis revealed that variation among species accounted for 80% of the total haplotype variation, while 20% of the total variation was found within species. For diploid species only, 85.5% of the haplotype diversity was found among species while for tetraploid species, 67.2% of the haplotype diversity was explained by taxonomic distinction.

For the species sampled in at least three sites, the total chloroplast diversity was high except for *D. sphagnicola* ($h_T = 0.03$; Table 5). The chloroplast diversity for *D. majalis* was mainly distributed within populations ($G_{ST} = 0.31$, $h_S = 0.62$; Table 5), whereas the chloroplast diversity for *D. elata* and *D. maculata* was mainly distributed among populations ($G_{ST} = 0.59$, $h_S = 0.27$ and $G_{ST} = 0.52$, $h_S = 0.47$, respectively). Haplotype richness was high for *D. majalis* and *D. maculata* (5.6 and 6.1, respectively), but very low for *D. sambucina* and *D. sphagnicola* (0.0 and 0.6, respectively; Table 5).

Haplotype lineages. In the neighbour-joining tree (Fig. 1), four main lineages (clades A–D) were distinguished and supported by modest and robust bootstrap values of 100%, 100%, 66% and 74 %, respectively. Clades were roughly species-specific, except for clades C and D, which were heterogeneous, but together formed a group also specific to some species. However, five individuals (two diploids and three tetraploids) appeared in a clade typical of other species. Clade A included the unique haplotype found in *D. sambucina* (H28). This haplotype was very distinct from those found in all the other taxa. It differed by nine mutations from the closest haplotype, and clade A was therefore quite distant from the other clades. Clade B grouped four haplotypes (H29–H32) and displayed little variation, since all haplotypes differed from their nearest neighbour by only one or two mutations. The four haplotypes of this clade were mostly found in *D. incarnata*. However, four individuals not belonging to *D. incarnata* were grouped in this clade. Clades C and D included 27 haplotypes (H01–H27). Together, clade C and D were specific to diploid *D. fuchsii* and all the tetraploid species investigated. Clade C included 14 haplotypes belonging mostly to *D. fuchsii*, *D. maculata*, *D. majalis* and *D. praetermissa*. Three of those haplotypes (H02, H04 and H05) were shared between

Table 1. Origin of the *Dactylorhiza* material studied. Abbreviations: inc *Dactylorhiza incarnata*, fuc *D. fuchsii*, mac *D. maculata*, ela *D. elata*, maj *D. majalis* s.str., sph *D. sphagnicola*, pra *D. praetermissa*, sam *D. sambucina*

Site code	Sample sites (Country, Muncipality, Local place)	Longitude	Latitude	Taxon							
				inc	fuc	mac	ela	maj	sph	pra	sam
A	Belgium, Houyet, Hérock	05° 03′ 12″ E	50° 10′ 33″ N					10			
B	Belgium, Braine-le-château, Housta	04° 16′ 15″ E	50° 39′ 45″ N					9			
C	Belgium, Arlon, Lagland	05° 43′ 00″ E	49° 39′ 47″ N			4			18		
D	Belgium, Rochefort, Tienne St Inal	05° 11′ 30″ E	50° 07′ 55″ N		4						
E	Belgium, Harinsart, Platte de souc les Monts	05° 32′ 07″ E	49° 41′ 40″ N	10				6			
F	Belgium, Virton, Latour	05° 34′ 52″ E	49° 33′ 07″ N	10							
G	Belgium, Anhée, Sossoye	04° 47′ 45″ E	50° 17′ 34″ N		1						
H	Belgium, Rochefort, Ri d' Hôwisse	05° 15′ 30″ E	50° 06′ 38″ N					9			
I	Belgium, Ferriéres, Wésomont	05° 40′ 30″ E	50° 22′ 25″ N			10		6			
J	Belgium, St Hubert, Plaine-Haie	05° 25′ 12″ E	50° 01′ 27″ N						16		
K	Belgium, St Hubert, Fange de la Borne	05° 24′ 38″ E	50° 02′ 38″ N						9		
L	France, Hargnies, Haut butés	04° 46′ 00″ E	49° 56′ 35″ N						3		
M	France, Plounévez-Lochirst, Keremma	04° 15′ 00″ W	48° 37′ 59″ N							8	
N	France, Crozon, Kerséguénou	04° 31′ 59″ W	48° 15′ 59″ N	14	6						
O	France, Lannéanou	03° 40′ 00″ W	48° 29′ 00″ N			6					
P	France, Guissény, Guissény marsh	04° 24′ 00″ W	48° 37′ 50″ N			15				8	
Q	France, Camprieu, Le Devois	03° 06′ 31″ E	43° 47′ 00″ N				7				
R	France, Lunas, Col de I'homme mort	03° 09′ 23″ E	43° 46′ 54″ N				3				
S	France, St Maurice/Sorgues, Mas des Combes	03° 04′ 17″ E	43° 52′ 41″ N		2		1				
T	France, Fondamente, Montpaon	03° 07′ 17″ E	43° 51′ 43″ N		2		7				
U	France, St Rome de Tarn	02° 53′ 49″ E	44° 03′ 15″ N				4				
V	Italy, Ne, Biscia marsh	09° 29′ 30″ E	44° 23′ 00″ N	12	12						
W	Spain, Courel-Samos, Pedrafita	07° 13′ 00″W	42° 39′ 50″ N								6
X	Spain, Courel, Seoane	07° 06′ 50″W	42° 37′ 50″ N								8
Y	Portugal, Beira litoral, Nossa Senhora de Vagos	08° 41′ 42″W	40° 33′ 48″ N				10				
Z	Portugal, Trás-os-Montes, Soutelo	07° 01′ 50″W	41° 50′ 18″ N				6				
	total			46	27	35	38	40	46	16	14

Table 2. Pairs of universal chloroplast primers used in this study and electrophoresis conditions

Primer 1	Primer 2	Abbr.	annealing temp (°C)	elongation (min)	restriction endonuclease	Polyacrylamide solution
[1]*trn*H	*trn*K1	HK	6.2	2	*Alu* I	19:1 8%
[1]*trn*C	*trn*D	CD	57.5	4	*Hin*f I	29:1 8%
[1]*psa*A	*trn*S	AS	62	4	*Hin*f I	19:1 8%
[1]*trn*V	*rbc*L	VL	58	2.5	*Hin*f I	19:1 10%
						29:1 9%
[2]*trn*Q	*trn*R	QR	62	4	*Hin*f I	19:1 7%

[1]Demesure et al. 1995; [2]Dumolin-Lapègue et al. 1997.

D. fuchsii and one or two of the tetraploid species, *D. majalis* and *D. maculata*, depending on the haplotype. One *D. incarnata* individual out of 45 was grouped in this clade instead of in clade B and displayed haplotype H05. All other haplotypes from this clade were species-specific. Clade D included 13 haplotypes belonging to *D. fuchsii* and *D. maculata* as in clade C, but also to *D. elata* and almost all *D. sphagnicola* individuals. Only one individual of *D. sphagnicola* out of 46 was found in clade C. Haplotype H22 was shared between *D. fuchsii*, *D. maculata* and almost all *D. sphagnicola* individuals. All other haplotypes from this clade were species-specific.

Discussion

cpDNA diversity. The amount of variation detected in this study (32 haplotypes) demonstrates the effectiveness of the PCR-RFLP methodology in detecting polymorphism in the chloroplast genome of *Dactylorhiza* species. Most of the observed polymorphism seemed to be due to small insertions or deletions (indels) of between 5 and 20 bp. We only detected six restriction site mutations, partly because of the higher frequency of indels as compared with point mutations in the chloroplast genome (Clegg et al. 1994) and partly because of the experimental approach used, which is more likely to detect indels than point mutations (Raspé et al. 2000). Indeed the only point mutations that may be detected are those that lead to the appearance or the disappearance of a restriction site.

As in other studies on cpDNA haplotype diversity in a complex of species (e.g. Golden and Bain 2000, Comes and Abbott 2001) the level of intraspecific cpDNA diversity was high (Table 5). *Dactylorhiza sphagnicola* and *D. sambucina* were the only species characterized by low diversity. Even if the total diversity was similar in most species, the haplotype richness (R) was quite different. The presence of a large number of rare haplotypes in the two tetraploid species (*D. majalis* and *D. maculata*) explain the discrepancy between the estimators of diversity, h_T and R. In our study, contrary to most other cpDNA studies, the cpDNA diversity was less distributed among populations than within populations, except for *D. elata* and *D. maculata* (Table 5; for review see Forcioli 1995).

Studies on cpDNA haplotype diversity in other species complexes (Golden and Bain 2000, Comes and Abbott 2001) have shown a lack of differentiation among the majority of the species in terms of cpDNA haplotypes, due to either incomplete lineage sorting or reticulation. In contrast to these studies, our results revealed a clear haplotypic distinction between species. Indeed, a large proportion (80%) of the haplotype diversity found in our samples can be explained by the taxonomic distinctions made. If we look at the five polyploid species only, 67.2% of the haplotype diversity can be explained by species distinction. This lower value was due to a higher number of shared haplotypes between these polyploid species and because these five species occurred in the same clades and displayed genetically close

Table 3. Description of the 32 haplotypes found in the genus *Dactylorhiza*

Haptotypes	Polymorphic fragments															
	HK5	CD2	CD3	CD5	CD6	AS4	AS5	AS6	AS10	VL7	VL9	QR5	QR6	QR7	QR8	QR11
H01	1	1	1	3	2	1	2	2	3	2	9	1	2	2	3	3
H02	1	1	1	3	2	1	2	2	3	3	9	1	2	2	3	3
H03	1	1	1	3	2	1	2	2	3	3	9	9	2	2	3	3
H04	2	1	1	3	2	1	2	2	3	2	9	1	2	2	3	3
H05	2	1	1	3	2	1	2	2	3	3	9	1	2	2	3	3
H06	2	1	2	2	2	1	2	2	3	3	9	1	2	2	3	3
H07	2	1	2	3	2	1	2	2	3	3	9	1	2	2	3	3
H08	3	1	1	3	1	1	2	2	3	1	9	1	2	2	3	3
H09	3	1	1	3	1	1	2	2	3	2	9	1	2	2	3	3
H10	3	1	1	3	1	1	2	2	3	3	9	1	2	2	3	3
H11	3	1	1	3	2	1	2	2	3	2	9	1	2	2	3	3
H12	3	1	1	3	2	1	2	2	3	3	9	1	2	2	3	3
H13	4	1	1	1	2	1	2	2	2	2	1	1	2	1	3	2
H14	4	1	1	1	2	1	2	2	2	3	1	1	2	1	3	2
H15	4	1	1	2	2	1	2	2	2	2	1	1	2	1	3	3
H16	4	1	1	2	2	1	2	2	2	3	1	1	2	1	3	2
H17	4	1	1	2	2	1	2	2	2	3	1	1	2	1	3	3
H18	4	1	1	3	2	1	2	2	2	2	9	1	2	1	3	2
H19	4	1	2	2	2	1	2	2	2	3	1	1	2	1	2	3
H20	4	1	2	3	2	1	2	2	2	2	1	1	2	1	1	2
H21	4	1	2	3	2	1	2	2	2	2	1	1	2	1	2	2
H22	4	1	2	3	2	1	2	2	2	2	1	1	2	1	2	3
H23	4	1	2	3	2	1	2	2	2	2	9	1	2	1	2	3
H24	9	1	1	2	2	1	2	2	3	2	9	1	2	2	3	3
H25	9	1	1	3	2	1	2	2	3	2	9	1	2	2	3	3
H26	9	1	2	3	2	1	2	2	2	2	1	1	2	1	1	2
H27	9	1	2	3	2	1	2	2	2	2	1	1	2	1	2	3
H28	9	2	3	2	2	3	1	1	1	3	9	1	3	4	3	4
H29	9	9	4	2	9	2	2	9	4	2	9	9	1	3	3	1
H30	9	9	4	2	9	2	2	9	5	1	9	9	1	3	3	1
H31	9	9	4	2	9	2	2	9	5	1	9	9	2	3	3	1
H32	9	9	4	2	9	2	2	9	5	2	9	9	1	3	3	1

Table 4. Haplotype composition of the eight sampled species. The characters in brackets correspond to the different sites where the haplotypes were found. The site codes are those given in Table 1. The last line gives the total number of haplotypes detected in each taxa. inc *Dactylorhiza incarnata*, fuc *D. fuchsii*, mac *D. maculata,* maj *D. majalis,* sph *D. sphagnicola,* ela *D. elata,* pra *D. praetermissa,* sam *D. sambucina*

Haplotypes	inc	fuc	mac	maj	sph	ela	pra	sam
H01				7 (A, I)				
H02		1 (V)		3 (A, E)				
H03		2 (V)						
H04		7 (G, N, S, T)	3 (I)	7 (B, H)	1 (J)			
H05	1 (N)	12 (N, V)	1 (C)	6 (H, E)				
H06			1 (C)					
H07			2 (C)					
H08							1 (M)	
H09							3 (M. P)	
H10							10 (M, P)	
H11				8 (A, I, H, B)				
H12				6 (A, B)				
H13			1 (I)					
H14			1 (I)					
H15					1 (J)			
H16			1 (I)					
H17			4 (I)					
H18						1 (Y)		
H19			6 (O)					
H20						18 (T, R, U, S, Q)		
H21						15 (Z,Y)		
H22		4 (D)	13 (P)		44 (C, J, K, L)			
H23			1 (P)					
H24				1 (E)				
H25				1 (E)				
H26						4 (R, U, Q)		
H27			1 (P)					
H28								14 (W, X)
H29	12 (V)							
H30	19 (N, E, F)						2 (P)	
H31				1 (E)				
H32	14 (N, E, F)	1 (N)						
	4	6	12	9	3	4	4	1

Table 5. Estimation of the total genetic diversity (h_T), the average genetic intrapopulation diversity (h_S), the level of gene differentiation among populations (G_{ST}) and the haplotype richness (R) for the eight species investigated. Standard errors are given in brackets. h_T, h_S and G_{ST} were not computed for *D. sambucina*, *D. praetermissa* because of the low number of populations. inc *Dactylorhiza incarnata*, fuc *D. fuchsii*, sam *D. sambucina,* mac *D. maculata,* maj *D. majalis,* ela *D. elata*, pra *D. praetermissa,* sph *D. sphagnicola*

Taxa	inc	fuc	sam	mac	maj	ela	pra	sph
Number of population	4	4	2	4	5	6	2	4
Mean number of individuals per population	11.25	6.25	7.5	8.75	7.6	6.17	8	11.5
h_T	0.758 (0.003)	0.850 (0.002)	–	1.000 (0.007)	0.910 (0.003)	0.670 (0.003)	–	0.03 (0.001)
h_S	0.417 (0.019)	0.259 (0.023)	–	0.472 (0.042)	0.620 (0.025)	0.270 (0.015)	–	0.03 (0.001)
G_{ST}	0.449 (0.100)	0.480 (0.050)	–	0.527 (0.043)	0.310 (0.033)	0.590 (0.047)	–	0
Haplotype richness	2.296	3.772	0	6.096	5.576	2.224	2.867	0.609

haplotypes. The taxonomic distinction between *D. incarnata*, *D. fuchsii* and *D. sambucina* explained 85.5% of the total cpDNA diversity found among them.

Relationship among species. Each one of the three diploid species was found in a different clade of the neighbour-joining tree, which suggests a long period of independent evolution with limited gene flow between them. However, cytoplasmic capture (i.e. the presence of the cytoplasmic genome of one species in the nuclear background of a related species; Rieseberg and Soltis 1991) seems to have occurred between *D. fuchsii* and *D. incarnata,* as shown by the presence of a *D. fuchsii* haplotype in one individual of *D. incarnata* and vice versa. Cytoplasmic capture may only occur throught hybridization and backcrosses between these two diploid species. A clear genetic distinction between these three diploid species was also found by Hedrén (1996a) on the basis of allozyme data and more recently by the use of AFLP markers (Hedrén et al. 2001) and/or ITS sequencing (Bateman et al. 1997, 2003).

As pointed out by several authors, *D. maculata* and *D. fuchsii* are, in some geographic regions, morphologically ill-defined and not always distinguishable (Heslop-Harrison 1951, Hylander 1966, Nelson 1976, Tyteca and Gathoye 1989). However, the use of molecular markers such as allozyme, ITS sequencing and AFLP fingerprints revealed that *D. maculata* is clearly separated from *D. fuchsii*, although with a close correspondence between their genomes usually explained by the autopolyploid origin of *D. maculata* (Hedrén 1996a, Bateman et al. 1997, Hedrén et al. 2001, Bateman and Denholm 2003). Our cpDNA data also indicated that *D. fuchsii* and *D. maculata* are quite distinct. However, some overlap between the haplotypes of these two species remained, since the most common *D. fuchsii* haplotypes were also found in *D. maculata* and vice versa. Although chloroplast DNA markers do not allow us to distinguish between species with an allo- or an autopolyploid origin, our data confirmed the existence of a close relationship between the polyploid *D. maculata* and the diploid *D. fuchsii*. Based on these results, we can assume that *D. maculata* has evolved from *D. fuchsii* either by autopolyploidization or by allopolyploidization with *D. fuchsii* as a maternal parent. The observation of three shared haplotypes and the fact that these two species were present in two distinct haplotype lineages (clades C and D) suggests that *D. maculata* has evolved from *D. fuchsii* more than once. Even if the actual extent of multiple origins for most

plant polyploids is unknown, it seems that most taxonomically recognized polyploid species are of multiple origin (Soltis and Soltis 1993, 1995, 1999). However, transfer of haplotypes from *D. fuchsii* to *D. maculata* by hybridization and introgression (reticulation) could be an other explanation of the existence of shared haplotypes between these two species.

Allotetraploid species constitute the most problematic group in the *Dactylorhiza* genus. All allotetraploids in Europe are thought to have evolved from more or less the same pair of parents, which is part of the reason why they are often so difficult to distinguish from each other. If these allotetraploids originated as hybrids between members of the *D. incarnata* and the *D. fuchsii* lineages including *D. maculata*, as pointed out by Hedrén (1996a, b), we might expect them to show haplotypes from both *D. fuchsii* and *D. incarnata* lineages and to cluster in both *D. incarnata* and *D. fuchsii* clades (clade B and clades C plus D, respectively). As expected, shared haplotypes between diploids and allotetraploids were observed. But, contrary to our expectations, almost all allopolyploid individuals examined (172 out of 175) were grouped in clades C and D with haplotypes of *D. fuchsii* and *D. maculata*. In the context of an allopolyploid origin for these tetraploid species, our data indicate an asymmetrical hybridization with a member of *D. fuchsii*/*maculata* group as a maternal parent. Some authors suggested that *D. sambucina* might be involved in the formation of some allotetraploid species (Bateman et al. 1997, 2003). Our results indicate that *D. sambucina* was not the

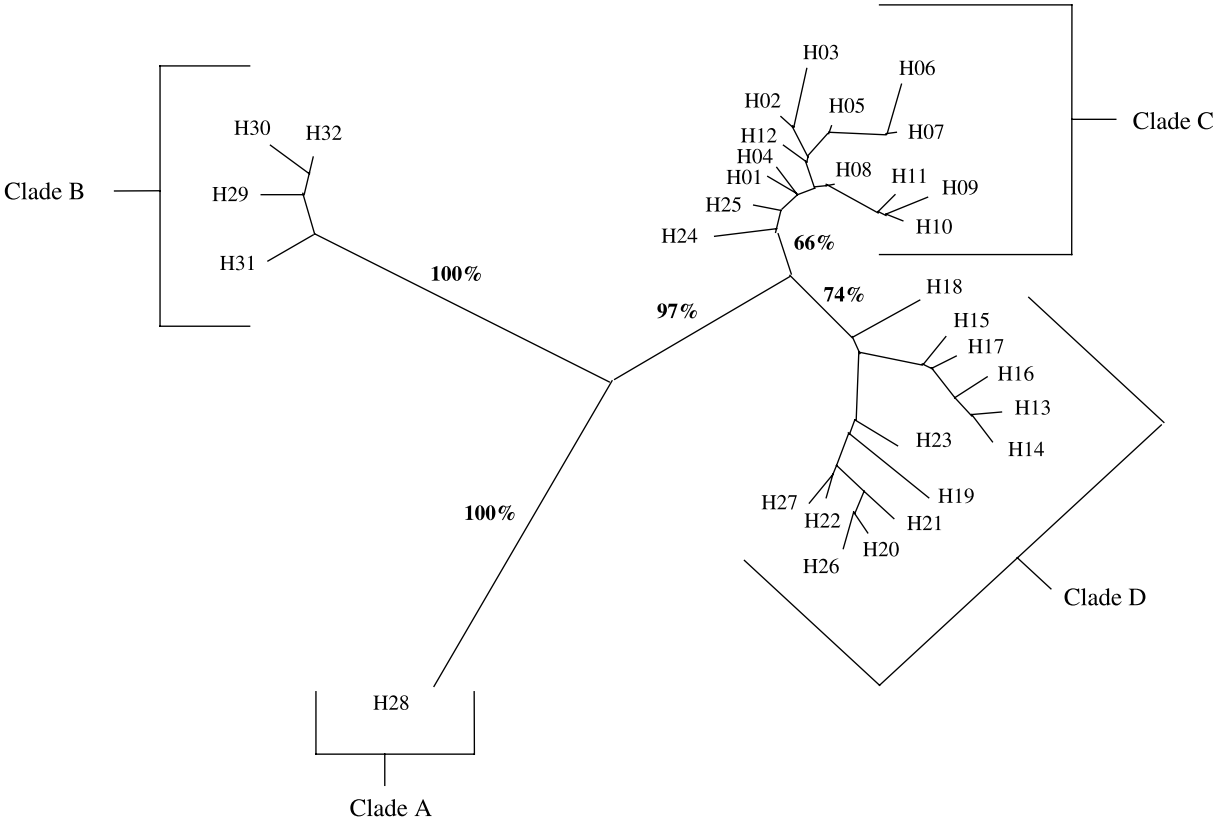

Fig. 1. Phenetic relations between haplotypes observed in the genus *Dactylorhiza* as assessed by neighbour-joining. Branch lengths are proportional to the distances between haplotypes. Bootstrap values of the main clades are in boldface

maternal parent of the allotetraploids examined although we cannot exclude the possibility that this species was involved in the formation of these allopolyploids as the paternal parent instead of *D. incarnata*. As in the case of *D. fuchsii* and *D. maculata*, transfer of haplotypes by reticulation from a member of the *D. fuchsii*/*maculata* group to an allopolyploid species could be an other explanation of the existence of shared haplotypes between these species.

The major conclusions from this survey were: (1) the high level of variation found in the chloroplast genome of the *Dactylorhiza* genus, (2) the large differentiation among the majority of species in terms of cpDNA haplotypes, (3) the large divergence among some haplotypes into four main lineages, and (4) the fact that all the allopolyploid species investigated have been formed by asymmetric hybridization with representatives of the *D. fuchsii*/*maculata* group as maternal parents.

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