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The evolution of *Dactylorhiza* (Orchidaceae) allotetraploid complex: Insights from nrDNA sequences and cpDNA PCR-RFLP data

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Abstract

Sequence data from a portion of the external transcribed spacer (ETS) and from the internal transcribed spacers (ITS1 and ITS2) of 18S–26S nuclear ribosomal DNA were used together with chloroplast DNA PCR-RFLP data to unravel patterns of allotetraploid speciation within the Western European *Dactylorhiza* polyploid complex. A maximum likelihood tree based on combined ETS and ITS sequences suggests that the Western European *Dactylorhiza* allotetraploids have evolved by hybridization between four main diploid lineages. Cloned sequences and the topology of the ITS plus ETS tree indicate that the allotetraploid species *D. elata*, *D. brennensis*, and *D. sphagnicola* have originated from the autotetraploid *D. maculata* together with the diploid *D. incarnata*, while *D. majalis*, *D. traunsteineri*, and *D. angustata* seem to have evolved by hybridization between the *D. fuchsii s.str* and *D. incarnata* lineages. Finally, the diploid *D. saccifera* lineage seems to have been involved together with the *D. incarnata* lineage in the formation of the allotetraploid *D. praetermissa*. The observed congruence between the chloroplast tree and the ITS/ETS tree suggests a directional evolution of the nrDNA after polyploidization in favor of the maternal genome. Considered together with morphological, biogeographical, and ecological evidence, the molecular analysis leads us to recognize four species within the investigated allotetraploid complex, namely *D. majalis*, *D. praetermissa*, *D. elata*, and *D. sphagnicola*.

Keywords: Orchidaceae; *Dactylorhiza*; ITS; ETS; Polyploid complex; Allopolyploids; Concerted evolution; Recombination; cpDNA; Hybridization

1. Introduction

Sequences of both internal and external transcribed spacers (ITS and ETS) of nuclear ribosomal DNA (nrDNA) have been widely used to infer phylogenetic relationships in plants (e.g., Baldwin and Markos, 1998; Baldwin et al., 1995; Kelch and Baldwin, 2003) and to unravel reticulate evolution in a wide range of polyploid complexes (Campbell et al., 1997; Gaut et al., 2000; Hughes et al., 2002; Sang et al., 1995). However, nrDNA sequences can fail to reflect biparental lineages of homoploid and allopolyploid hybrids due to their particular mode of evolution. Plant nrDNA is indeed subjected to concerted evolution (reviewed by Alvarez and Wendel, 2003; Hershkovitz et al., 1999), a process by which differing intragenomic nrDNA copies are homogenized. In polyploids of hybrid origin, concerted evolution, which acts through gene conversion and recombination, can lead to the homogenization of the parental nrDNA types such that direct evidence of hybrid parentage is lost. In such cases, evidence of hybridization can often be found by comparing phylogenies from differently inherited markers such as nrDNA vs. cpDNA markers (Wendel and Doyle, 1998). A second possible outcome of the reunion of divergent ribotypes by hybridization is that those divergent ribotypes are maintained in the hybrid genome but undergo various degrees of recombination leading to chimeric nrDNA sequences (Baldwin et al., 1995; Van Houten et al., 1993; Wendel et al., 1995). Finally, the

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divergent copies present in a genome may be maintained, evolving independently without recombination (Aguilar and Feliner, 2003; Widmer and Baltisberger, 1999). In this case, concerted evolution is disrupted and nrDNA sequence data may provide unambiguous information on both the maternal and paternal progenitor lineages, documenting polyploidization events and reticulate evolution (Campbell et al., 1997; Gaut et al., 2000; Hughes et al., 2002; Sang et al., 1995). The intra-individual nrDNA polymorphism thus reflects both the history of organismal hybridization events and to some extent, the failure of concerted evolution, which together lead to the maintenance or the recombination of divergent ribotypes. The factors that might influence the degree and the direction to which concerted evolution homogenizes sequences in a genome are still poorly known (Raymond et al., 2002). A long generation time or vegetative reproduction are thought to be factors that may decrease rates of nrDNA homogenization in plants (Baldwin et al., 1995; Sang et al., 1995).

Processes other than hybridization need to be considered when interpreting polymorphic sequence patterns of nrDNA. Incomplete lineage sorting and divergence among paralogous loci (Buckler et al., 1997) could yield polymorphic, fixed sequence patterns similar to those resulting from hybridization (Bailey et al., 2003; Doyle, 1992; Wendel and Doyle, 1998). Determining the causes of intragenomic polymorphic nrDNA sequence patterns is a complex matter but it can be aided by other lines of evidence, such as chromosome numbers, interfertility data, allozymes, or sequence data from unlinked gene regions such as cpDNA (Doyle, 1992; Sang and Zhong, 2000; Wendel and Doyle, 1998).

Many orchid genera tend to be subdivided into larger numbers of species than genera in most other plant families. This is especially true in the genus *Dactylorhiza* Necker ex Nevski, in which deviating population groups are often recognised as separate species (see Tyteca, 2001 for review). The high number of easily observed and variable floral characters has contributed to taxonomic treatments that might not reflect evolutionary relationships. Moreover, those easily scored and variable characters are weakly discriminant, which may lead to difficult interpretation in the field, even for a specialist. *Dactylorhiza* is mainly distributed in Eurasia and is represented in North America by one species in Alaska (*D. aristata*) and one introduced species (*D. praetermissa*), which has been naturalized in the northeastern USA and Canada. In Western Europe, *Dactylorhiza* appears to have undergone extensive reticulate evolution (Hedrén, 1996a; Heslop-Harrison, 1954; Soó, 1980). The number of species, which strongly varies depending on the different authors (for review, see Pedersen, 1998 and Tyteca, 2001), ranges from 6 (Sundermann, 1980) to 49 species (Delforge, 2001) in Europe alone, and from 12 (Klinge, 1899) to 75 (Averyanov, 1990) species worldwide. Most of the species are either diploids ($2n=40$) or tetraploids ($2n=80$) and, with the exception of *D. maculata*, all the extant tetraploid species are considered as allotetraploids. These allotetraploid species have evolved by repeated hybridization between the two diploid ($2n=40$) parental groups *D. incarnata s.l.* and *D. fuchsii s.l.* (Hedrén, 1996a,b; Hedrén et al., 2001; Heslop-Harrison, 1954). In the absence of conclusive evidence for a possible molecular subdivision of the allotetraploid *Dactylorhiza* complex, Pedersen (1998) and Hedrén (2002) treated this complex as a single species with well-known, widespread, and geographically and/or ecologically separated allotetraploids recognised as subspecies. This scenario of the evolution of the *Dactylorhiza* allotetraploid complex has been proposed on the basis of allozyme data (Hedrén, 1996a), which were not found polymorphic enough for a detailed understanding of the evolution of this complex. On the basis of AFLP data (Hedrén, 2001) and more recently nrDNA sequences (Devos et al., 2005), clear genetic discontinuities have been found between four main diploid lineages (i.e., *D. fuchsii s.str.*, *D. incarnata*, *D. foliosa*, and *D. saccifera*). Devos et al. (2005) also suggested that an ancestral diploid lineage closely related to the extant endemic species *D. foliosa* has led to the speciation of the autotetraploid *D. maculata*. No allotetraploid species were included in the aforementioned study, but it would be interesting to know if the different diploid lineages could have been involved in the evolution of the *Dactylorhiza* allotetraploid complex in Western Europe or if all the allotetraploid species have been formed by hybridization events between the same pair of parents as previously proposed.

In the present study, nrDNA sequences of both internal and external transcribed spacers and cpDNA PCR-RFLP data were used for phylogenetic inference in the *Dactylorhiza* allotetraploid complex in Western Europe. The main objective of this study was to reexamine the reticulate scenario of the *Dactylorhiza* allopolyploid complex previously proposed on the basis of allozyme and nuclear AFLP data (Hedrén, 1996a,b; Hedrén et al., 2001) and to explore the possible implication of taxa other than *D. incarnata* and *D. fuchsii* in the formation of the allotetraploid species in Western Europe.

2. Materials and methods

2.1. Plant material and DNA isolation

Sequences of both ITS and ETS were obtained for several accessions of nine *Dactylorhiza* species (Table 1): *D. majalis* (12 accessions), *D. praetermissa* (3), *D. traunsteineri* (3), *D. elata* (9), *D. sambucina* (2), *D. sphagnicola* (5), *D. angustata* (5), *D. brennensis* (3), and *D. incarnata* (8). Moreover, ITS and ETS sequences previously published by Devos et al. (2005) were added to complete the data set. Those previously published sequences came from 11 accessions of *D. fuchsii*, 10 accessions of *D. maculata*, 8 accessions of *D. saccifera*, and 8 accessions of *D. foliosa* (Table 1). Fresh leaves were collected from 44 natural populations in Belgium, France (including Corsica), Italy, Germany, Portugal, and Madeira (Table 1). Our sampling also included 2 accessions that we identified as F1 hybrids between the two diploids *D. fuchsii s. str.* and *D. incarnata*. Their hybrid origin is clear because both accessions occurred with, and were morphologically intermediate between, the parents. Total genomic DNA was isolated from leaf tissue using the cetylt-

Table 1
Dactylorhiza species analyzed in this study, their origin and their ploidy level

Taxon	Origin	Accession #	Ploidy level	GenBank accession # ITS / ETS
Ingroup				
D. angustata (Arvet-Touvet) Tyteca & Gathoye	Allevard, Alps, France	1–5	Tetraploid ($2n = 80$)	DQ074242–DQ074246/DQ074317–DQ074321
D. brennensis (Nelson) Tyteca & Gathoye	Meziere en Brenne, France	1[a]	Tetraploid ($2n = 80$)	DQ074255–DQ074259/ETS not sequenced
	Meziere en Brenne, France	2[a]		DQ074260–DQ074363/ETS not sequenced
	Meziere en Brenne, France	3[a]		DQ074264–DQ074267/ETS not sequenced
D. elata (Poiret) Soo	Col de I'homme mort, France	1, 2, 3	Tetraploid ($2n = 80$)	DQ074230, DQ074231, DQ074233/DQ074305, DQ074307, DQ074308
	Mas de Combes, France	4		DQ074232/DQ074306
	Mas de Combes, France	5[a]		DQ074251–DQ074254/ETS not sequenced
	Montpaon, France	6		DQ074234/DQ074309
	St Rome de Tarn, France	7		DQ074235/DQ074310
	Soutelo, Portugal	8		DQ074236/DQ074311
	Nossa Senhora de Vagos, Portugal	9		DQ074237/DQ074312
D. foliosa (Lowe) Soo	Portela, Madeira	1–4	Diploid ($2n = 40$)	AY699480, AY699483/AY699553, AY699556
	Encumeada, Madeira	5, 6		AY699484, AY699485/AY699557, AY699558
	Queimadas, Madeira	7, 8		AY699486, AY699487/AY699559, AY699560
D. fuchsii (Druce) Soo	Tienne St Inal, Belgium	1	Diploid ($2n = 40$)	AY699423/AY699512
	Baronville, Belgium	2, 3		AY699424, AY699425/AY699513, AY699514
	De Panne, Belgium	4		AY699426/AY699515
	Kerseguenou, France	5		AY699427/AY699516
	Col du Granier, France	6		AY699428/AY699517
	Montpaon, France	7, 8		AY699431, AY699432/AY699520, AY699521
	Bievres, France	9		AY699435/AY699523
	Biscia marsh, Italy	10, 11		AY699437, AY699438/AY699525, AY699526
D.fuchsii X D. incarnata	Biscia marsh, Italy	1[a]		DQ074247–DQ074248/ETS not sequenced
	Kerseguenou, France	2[a]		DQ074249–DQ074250/ETS not sequenced
D. incarnata (L.) Soo	Platte de sous les Monts, Belgium	1, 2	Diploid ($2n = 40$)	DQ074218, DQ074219/DQ074293, DQ074294
	Latour, Belgium	3		DQ074220/DQ074295
	Kerseguenou, France	4, 5		AY699422, DQ074221/AY699511, DQ074296
	Col de Perjuret, France	6, 7		AY699510, DQ074222/AY699576, DQ074297
	Biscia marsh, Italy	8		DQ074223/DQ074298
D. maculata (L.) Soo	Wesomont, Belgium	1	Tetraploid ($2n = 80$)	AY699447/AY699534
	St-Hubert, Belgium	2–4		AY699448–AY699450/AY699535–AY699537
	Lanneanou, France	5, 6		AY699456, AY699457/AY699539, AY699540
	Guisseny marsh, France	7		AY699459/AY699545
	Dourbies, France	8		AY699460/AY699546
	Aigoual forest, France	9, 10		AY699469, AY699479/AY699550, AY699552
D. majalis (Reichenbach) Hunt & Summerhayes	Hěrock, Belgium	1–3	Tetraploid ($2n = 80$)	DQ074206–DQ074208/DQ074281–DQ074283
	Ri d'Howisse, Belgium	4, 5		DQ074209, DQ074210/DQ074284, DQ074285
	Housta, Belgium	6		DQ074211/DQ074286
	Ri d'Howisse, Belgium	4, 5		DQ074209, DQ074210/DQ074284, DQ074285
	Housta, Belgium	6		DQ074211/DQ074286

(*continued on next page*)

Table 1 (*continued*)

Taxon	Origin	Accession #	Ploidy level	GenBank accession # ITS / ETS
	Platte de sous les Monts, Belgium	7, 8		DQ074212, DQ074213/DQ074287, DQ074288
	Wesomont, Belgium	9, 10		DQ074214, DQ074215/DQ074289, DQ074290
	Lanuejols, France	11, 12		DQ074216, DQ074217/DQ074291, DQ074292
D. praetermissa (Druce) Soo	Guisseny marsh, France	1	Tetraploid ($2n = 80$)	DQ074224/DQ074299
	Keremma, France	2, 3		DQ074225, DQ074226/DQ074300, DQ074301
D. saccifera (Brongniart) Soo	Venaco, Corsica	1, 2	Diploid ($2n = 40$)	AY699488, AY699489/AY699561, AY699562
	Vizzavona, Corsica	3		AY699490/AY699563
	Zonza, Corsica	4, 5		AY699493, AY699495/AY699566, AY699568
	along the N196 road, Corsica	6		AY699496/AY699569
	Val di Varri, Italy	7, 8		AY699497, AY699498/AY699572, AY699573
D. sambucina (L.) Soo	Seoane, Spain	1		DQ073238/DQ074313
	Pedrafita, Spain	2		DQ073239/DQO74314
D. sphagnicola (Hoppner) Averyanov	Haut butě marsh, France	1[a]	Tetraploid ($2n = 80$)	DQ074268–DQ074271/ETS not sequenced
	Lagland, Belgium	2[a]		DQ074272–DQ074276/ETS not sequenced
	Lagland, Belgium	3[a]		DQ074277–DQ074280/ETS not sequenced
	Plaine-Haie, Belgium	4, 5		DQ074240, DQ074241/DQ074315, DQ074316
D. traunsteineri (Sauter) Soo	Praubert, France	1, 2	Tetraploid ($2n = 80$)	DQ074227, DQ074228/DQ074302, DQ074303
	Ettal marsh, Gernany	3		DQ074229/DQ074304
Outgroups				
Gymnadenia conopsea (L.) R. Brown	Lavaux St Anne, Belgium	1, 2	Diploid ($2n = 40$)	AY699508–AY699509/AY 699574–AY699575

Classification follows species circumscription in Delforge (2001). For the determination of the ploidy level, see Gatoye and Tyteca (1989) and Tichy and Del Prete (2001). GenBank accessions AY are those that were previously published by Devos et al. (2005).

[a] Indicates which accessions have been cloned for the ITS region, and for which multiple sequences that correspond to the different clones have been submitted to GenBank.

rimethylammonium bromide procedure outlined by Doyle and Doyle (1987) without the RNase treatment.

2.2. Nuclear ribosomal DNA, PCR amplification, cloning, and sequencing protocols

The complete ITS region was amplified in a single PCR with primers ITS5a (Stanford et al., 2000) and ITS4 (White et al., 1990) using standard procedures. Primers ETSdact1 (Devos et al., 2005) and IGS88 (Oh and Potter, 2005) were used to amplify part of the 5′ end of the 18S gene and a contiguous ETS fragment of approximately 950 bp for all sampled individuals. The PCR conditions used for amplifying the ITS and ETS regions were similar, except that the annealing temperature was 65 °C for the ETS and 55 °C for the ITS region and that the polymerase used was Advantage-GC cDNA PCR Kit (BD Biosciences) for ETS and Takara Ex Taq (Takara Mirus) for ITS. PCR products were purified with the QIAquick PCR purification kit (Qiagen) and both strands of ITS and ETS purified PCR products were sequenced directly using BigDye terminators (Applied Biosystems) and the amplification primers, i.e., ITS4 and ITS5a for the ITS region, and ETSdact1 and IGS88 for the ETS fragment.

Accessions that lacked clear base reading were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) to separate the differing nrDNA copies. When sequences are determined from cloned PCR products, errors introduced by the *Taq* DNA polymerase in PCR may yield inaccurate sequencing results. To minimize enzymatic error during the amplification stage of the nrDNA for those accessions, PfuTurbo DNA polymerase (Stratagene) was used because of its proof-reading function. Five to ten clones were randomly selected, and amplified using the M13 forward and reverse primers. The resulting cloned amplicons were sequenced in both directions, using the same conditions as for the direct PCR products.

2.3. Chloroplast DNA analysis: PCR-RFLP

A PCR-RFLP approach was used to characterize five chloroplast regions (*trn*H-*trn*K1, *trn*C-*trn*D, *psa*A-*trn*S, *trn*V-

*rbc*L, and *trn*Q-*trn*R) for each sampled accession. These five chloroplast regions were amplified and digested following the protocol described in Devos et al. (2003). The restriction products were separated on polyacrylamide gels of different concentrations of 19:1 or 29:1 acrylamide–bisacrylamide solutions depending on the size of the polymorphic restriction fragments to be resolved (Devos et al., 2003). The fragment length polymorphism detected by the restriction of the different chloroplast regions was scored as presence or absence of the different restriction fragments. The restriction site polymorphism was scored as presence or absence of the restriction sites itself, rather than of fragments, since the gain of a restriction site will replace one fragment with two.

2.4. Phylogenetic analyses

Complementary strands of both ITS and part of the ETS region were assembled using Sequencher 4.01 (Gene Codes Corporation, 1998). Contigs were aligned manually using MacClade 4.1 (Maddison and Maddison, 1992), with gaps inserted where necessary to preserve positional homology. IUPAC ambiguity codes were used to record polymorphic sites. All sequences were deposited in GenBank (see Table 1 for accession numbers) and the alignment of the ITS plus ETS sequences was deposited in TreeBase (URL: http://www.treebase.org) under the matrix accession number M2513. The data set resulting from the PCR-RFLP analysis of the five chloroplast regions can be obtained from the first author upon request.

In this study, the complete cpDNA and ITS/ETS gene trees are presented as separate analyses to investigate possible incongruence between the nuclear and chloroplast trees. Incongruence between nuclear and chloroplast tress may indicate hybridization and introgression events. *Gymnadenia conopsea* was used as the outgroup in the ITS/ETS phylogeny because of its sister relationship with *Dactylorhiza* as shown by an ITS phylogeny of the Orchidinae (Bateman et al., 2003; Pridgeon et al., 1997). The cpDNA tree is presented as an unrooted network because no cpDNA PCR-RFLP data were obtained for *G. conopsea*. The maximum parsimony method (MP) was used for the phylogenetic analysis of both the cpDNA PCR-RFPL and nrDNA data sets. For the nrDNA data set, maximum likelihood (ML) and Bayesian inference were additionally used. For the accessions that showed monomorphic sequences for both the ITS and ETS regions, the two aligned data sets were merged. Many accessions contained the same, identical sequence; therefore, only one representative of each unique sequence was used in the phylogenetic analyses.

The most parsimonious trees were searched using NONA 2.0 (Goloboff, 1998) in combination with WinClada (Beta) 0.99 (Nixon, 1999). Tree search options of HOLD 10,000, HOLD/100, and MULT*1000 were used. Nodal support was assessed using 1000 bootstrap replicates.

The ML analysis of the nrDNA (ITS plus ETS) data set was implemented in PHYML (Guindon and Gascuel, 2003). The model of sequence evolution which best fits the data was determined using Modeltest 3.06 (Posada and Crandall, 1998). The model and its parameters were chosen based on the outcomes of a hierarchical likelihood ratio test ($\alpha = 0.01$). Support for clades was assessed in PHYML using 1000 bootstrap replicates.

The Bayesian inference was performed using MrBayes v3.0B (Huelsenbeck and Ronquist, 2001). The MCMC process was set for four chains to run simultaneously for 2,000,000 generations. A tree was sampled every 100 generations. The model of sequence evolution was the same as for the ML analysis and all parameters were estimated during the analysis. Gaps were excluded from the alignment but each potentially phylogenetically informative indel was recoded as a binary character according to the 'simple indel coding' method (Simmons and Ochoterena, 2000). The first 2000 trees were discarded as burnin and the remaining trees were used to calculate a 50% majority rule consensus tree and to determine the posterior probabilities for the individual branches. Clades that were present in more than 95% of the sampled trees were considered significantly supported (Huelsenbeck and Ronquist, 2001).

3. Results

3.1. Nuclear ribosomal DNA sequence polymorphism and phylogenetic analysis

The data set comprised 80 *Dactylorhiza* accessions with no intra-accession nrDNA polymorphism, 1586 characters (ITS plus ETS) from which 235 were parsimony-informative. Six accessions of *D. fuchsii* had identical sequences. Similarly, two accessions of *D. maculata*, one accession of *D. elata*, and four accessions of *D. incarnata* had identical sequences.

Using Modeltest, the HKY 85 nucleotide substitution model (Hasegawa et al., 1985) was chosen for the ML analysis, with variable sites assumed to follow a gamma distribution (shape parameter = 0.4378). The ML analysis generated one best tree ($-\ln L = 4313.36639$) in which seven well-supported lineages were identified within the ingroup (Fig. 1; clades A–G). The best tree sampled during the Bayesian analysis was found in generation 1,993,700 and had the log likelihood of −4589.564. The 50% majority rule consensus of the trees sampled by the Bayesian analysis did not differ significantly from the best ML tree. The maximum parsimony analysis of the nrDNA data set yielded five equally most parsimonious trees of 328 steps (CI = 0.91; RI = 0.97). The different methods of phylogenetic reconstruction (likelihood, parsimony, and Bayesian) yielded best trees with the same major topology (MP and Bayesian trees not shown).

Each allotetraploid accession was nested within a clade that includes either a diploid species (Fig. 1; clades A, B, and G) or the autotetraploid *D. maculata* (Fig. 1; clade C). The diploid *D. incarnata* along with two of three accessions of *D. traunsteineri* formed a strongly supported monophyletic group (Fig. 1; clade G), which was sister to the rest of the ingroup. *D. traunsteineri* was identified as a transclade species, i.e., its accessions were not constrained to a single clade.

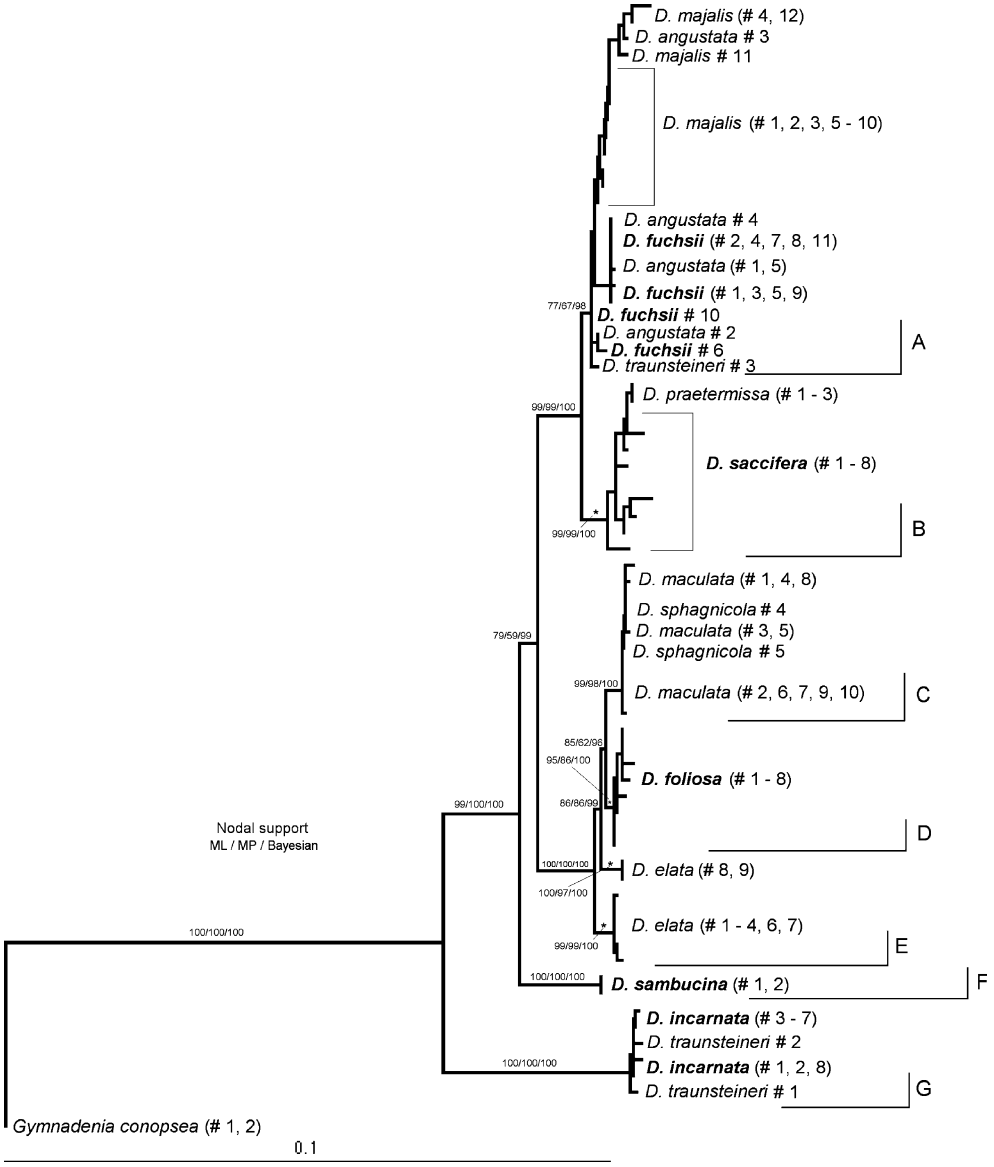

Fig. 1. Maximum-likelihood tree resulting from the analysis of ITS and ETS sequences from diploid and tetraploid Western European *Dactylorhiza* species. Nodal support is indicated above branches. Numbers after species names refer to the accession number (Table 1). *Dactylorhiza* accessions not included in this tree because of their recombinant nature are *D. sphagnicola* # 1, 2, 3; *D. brennensis* # 1, 2, 3 ; and *D. elata* # 5. Clades representing the main lineages are indicated by a letter from A to G and are discussed in the text. Terminals in bold are diploid ($2n = 40$).

Accessions of *D. traunsteineri* were nested within clades A and G. On the other hand, all the accessions of the allotetraploid species *D. majalis* and *D. angustata* grouped within clade A, while accessions of *D. sphagnicola* grouped within clade C and accessions of *D. praetermissa* segregated into clade B. Interestingly, accessions of *D. elata* were not grouped with any of the diploid species nor with the autotetraploid *D. maculata*. Instead, *D. elata* formed two well-supported clades by itself (both included in clade E), which are sister to clades C and D representing *D. maculata* and *D. foliosa*, respectively.

3.2. ITS cloned sequences and recombination

Seven *Dactylorhiza* accessions, which displayed intragenomic polymorphism for the ITS region, were cloned to separate the different ITS copies. Most of the ITS sequences obtained from those cloned accessions were apparent recombinants between ITS sequences identical from those found in either clade A, B, C or G (Fig. 2), and therefore they were not included in the phylogenetic analysis. For example, *D. sphagnicola* # 1 clone 'a' displayed, for the first nine polymorphic sites, the nucleotide composition of the consensus ITS sequence that corresponds to clade C and then conformed to the nucleotide composition of the consensus ITS sequence that corresponds to clade G in the nrDNA tree. One clone of this accession (*D. sphagnicola* # 1 clone 'b') displayed a non-recombinant ITS sequence matching the consensus ITS sequence of clade G. All cloned accessions displayed at least one non-recombinant ITS sequence matching the consensus ITS sequence of either clade A, B, C or G (Fig. 2). All the ITS

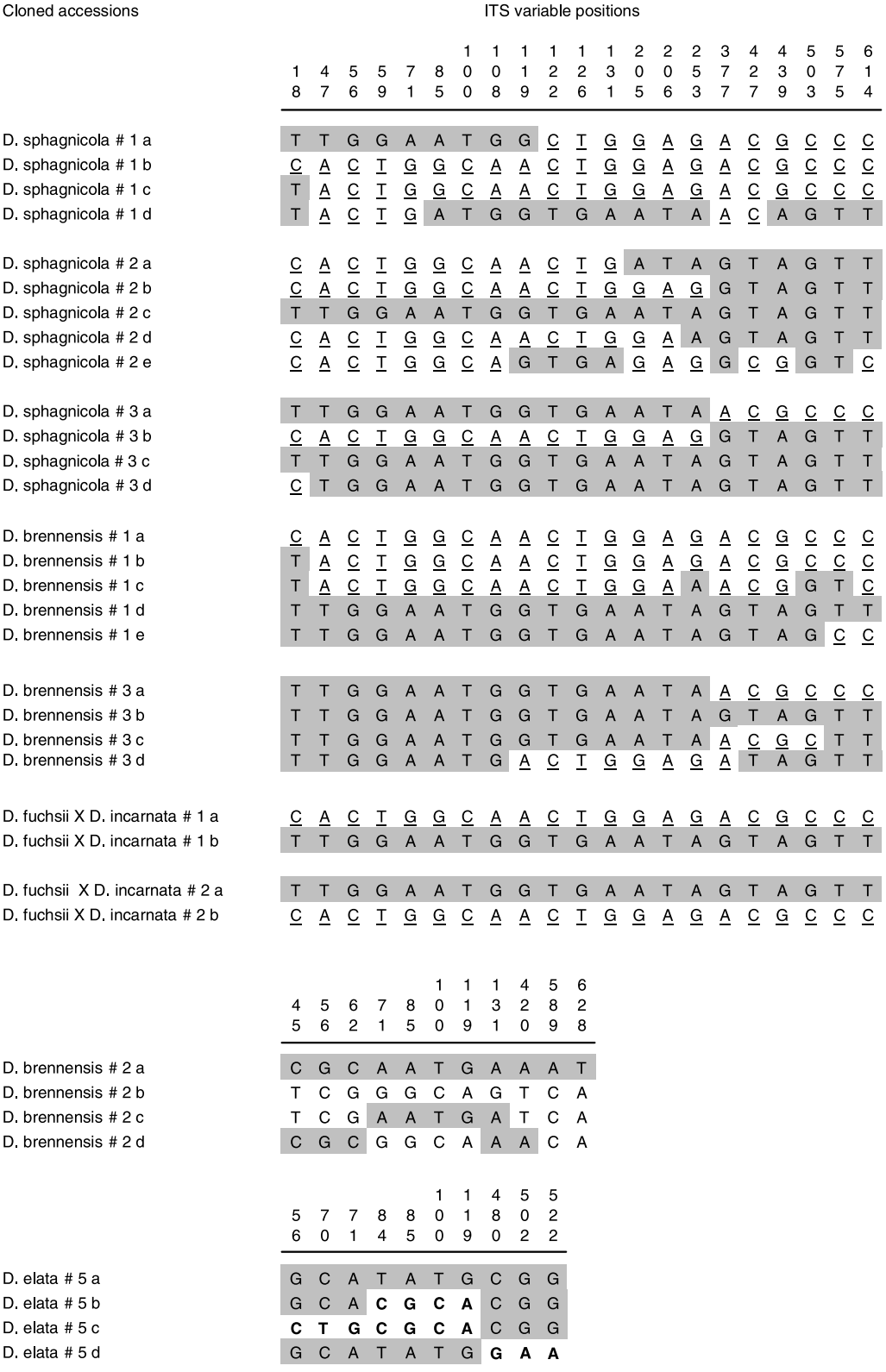

Fig. 2. Variable nucleotide positions of cloned ITS sequences obtained from three *D. sphagnicola*, three *D. brennensis*, and one *D. elata* accession. Number after species name refers to the accession number (Table 1) and the letter refers to the different clones. To illustrate putative recombination, nucleotides representing polymorphic positions and corresponding to the consensus sequence of clade C are highlighted, nucleotides representing polymorphic positions and corresponding to the consensus sequence of clade B are bolded, nucleotides representing polymorphic positions and corresponding to the consensus sequence clade G are underlined and nucleotides representing polymorphic positions and corresponding to the consensus sequence from clade A are in no format. ITS positions are numbered as in the ITS/ETS final aligned data set.

sequences identified as recombinant and recovered from the three *D. sphagnicola* cloned accessions, i.e., *D. sphagnicola* # 1, 2, 3, appears to be recombinant between clades C and G. The ITS sequences identified as recombinant and recovered from the cloning of three *D. brennensis* accessions appeared to be recombinant between clades C and G (accessions # 1 and # 3) or between clades C and A (accession # 2). ITS sequences identified as recombinant and recovered from the cloning of *D. elata* # 5 appear to be recombinant between clade C and B. Two putative F1 hybrids between *D. fuchsii* and *D. incarnata* were cloned for the ITS region. All sequences recovered displayed a nucleotide composition identical to the ITS consensus sequence of clade C or G but no clones displayed recombination between the parental ITS sequences as observed within the ITS sequences recovered from the allotetraploid accessions cloned (Fig. 2).

3.3. cpDNA tree and congruence with nuclear ribosomal tree

The PCR-RFLP survey of the chloroplast DNA from the 12 *Dactylorhiza* species investigated revealed 48 polymorphic fragments, which together allowed the identification of 19 haplotypes. The maximum parsimony analysis of these 19 haplotypes yielded 32 most parsimonious trees of 70 steps (CI = 0.69; RI = 0.82). The strict consensus unrooted tree is presented in Fig. 3.

Four main groups with moderate bootstrap support were resolved in the cpDNA tree. Most *D. fuchsii* and all *D. saccifera* accessions formed one group (group I; Fig. 3). *D. maculata* and *D. foliosa* accessions were all grouped within group II. *D. sambucina* and *D. incarnata* accessions formed two groups, group III and IV, respectively. The relationship within each group was virtually completely unresolved because of the relatively small number of informative characters. However, *D. saccifera* (group I) and *D. foliosa* (group II) displayed unique haplotypes, while *D. fuchsii* (group I) and *D. maculata* (group II) shared some of their haplotypes with the allotetraploid species investigated.

4. Discussion

4.1. ITS/ETS phylogeny and the evolution of the Dactylorhiza allotetraploid complex

In Western Europe all the allotetraploid *Dactylorhiza* species were thought to have originated from hybridization events between the *D. incarnata s. l.* lineage and the *D. fuchsii s.l.* (including the autotetraploid *D. maculata*) lineage (Hedrén, 1996a,b). These two parental lineages appeared to be genetically coherent and well distinct from each other (Hedrén, 1996a, 2003; Hedrén et al., 2001). Recently, nuclear ITS and ETS markers allowed us to further subdivide Hedrén's *D. fuchsii* lineage into four independent and well-supported lineages, i.e., *D. fuchsii s.str.*, *D. saccifera*, *D. maculata*, and *D. foliosa* (Devos et al., 2005). As shown by the results of our phylogenetic analysis of the ITS and ETS data sets (Figs. 1 and 2), three of these lineages have been involved in the evolution of the *Dactylorhiza* allotetraploid complex.

The *D. fuchsii s.str.* lineage has been identified, based on our ITS and ETS tree (Fig. 1), as one parental lineage in the formation of the allotetraploids *D. majalis* and *D. angustata*. This is suggested by the fact that all *D. majalis* and *D. angustata* accessions are grouped in the ITS/ETS phylogenetic tree within clade A, which represents the diploid *D. fuchsii s. str.* lineage. *D. fuchsii* may also be one of the parental lineages of the allotetraploid *D. traunsteineri*. *D. traunsteineri* accessions have been found within both clade A (*D. fuchsii* lineage) and clade G (*D. incarnata* lineage). The presence of *D. traunsteineri* in these two clades suggests that this species could have evolved from an allopolyploidization event between *D. incarnata* and *D. fuchsii*. In that case, concerted evolution

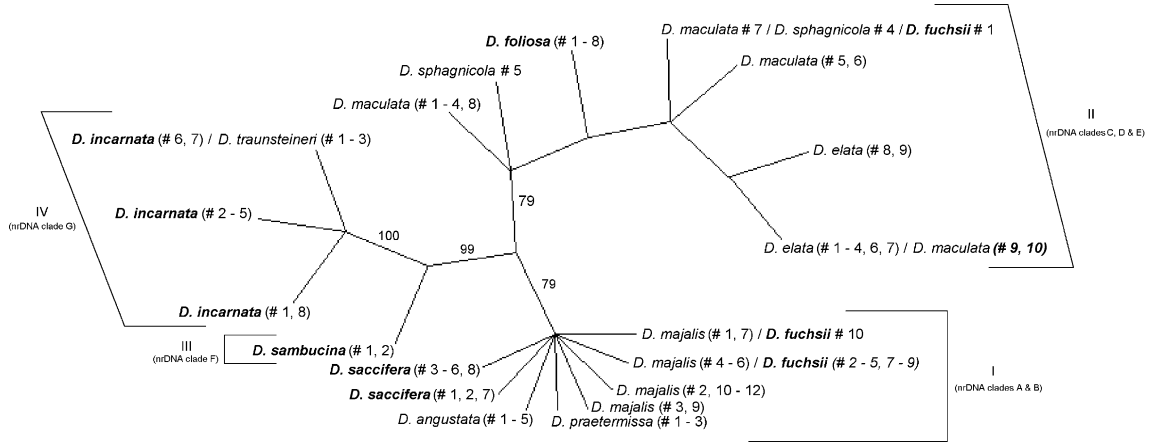

Fig. 3. Strict unrooted consensus of 32 equally most-parsimonious trees recovered from the analysis of cpDNA PCR-RFLP data from diploid and tetraploid Western European *Dactylorhiza* species. Numbers above branches give percentage occurrence of a group in 1000 bootstrap replicates. Terminals in bold are diploid ($2n = 40$). Accessions clustered within group I were found within clades A and B in the nuclear ribosomal tree (Fig. 1) while the accessions clustered within group II were found within clades C, D, and E in the same ITS/ETS phylogeny (Fig. 1). Accessions that fell within groups III and IV were found in clades F and G of the ITS/ETS phylogeny, respectively.

could have acted in opposite directions so that some accessions contain the *D. fuchsii* (A) nrDNA haplotype, while others contain the *D. incarnata* (G) nrDNA haplotype. All *D. traunsteineri* accessions shared cpDNA haploptypes with *D. incarnata* including accession #3 (Fig. 2), whose nrDNA sequence was nested in the clade A in the ITS/ETS tree (Fig. 1), suggesting that the ovulate parent was *D.incarnata* and that the pollen parent was *D. fuchsii*.

The second diploid lineage, *D. saccifera* (Fig. 1; clade B), appeared to have played an important role in the evolution of the *Dactylorhiza* tetraploid complex in Western Europe. *D. saccifera*, closely related to *D. fuchsii s. str.*, has been shown to be involved in the formation of an allotetraploid species in Turkey based on allozyme data (Hedrén, 2001) but was never shown as one of the progenitors of any Western European allotetraploid species. However, we identified this lineage as one of the progenitors of *D. praetermissa* (Fig. 1). All three accessions of this species have been found in the ITS/ETS tree within clade B, which represents the *D. saccifera* lineage (Fig. 1).

Finally, the autotetraploid *D. maculata* lineage (Fig. 1; clade C) appeared to have been involved in the formation of *D. sphagnicola*, *D. elata*, and *D. brennensis*. Accessions of *D. sphagnicola* that did not show intragenomic polymorphism for the nrDNA grouped with *D. maculata* accessions in the ITS/ETS tree (Fig. 1), while polymorphic accessions of *D. sphagnicola* and *D. brennensis* displayed ITS recombinant sequences between *D. maculata* and either *D. incarnata* or *D. fuchsii* (Fig. 2). Allotetraploid taxa can evolve by hybridization between a tetraploid and a diploid species by means of unreduced gametes (deWet, 1980; Ramsey and Schemske, 1998). Based on AFLP data, Hedrén et al. (2001) suggested that most allotetraploids in the *Dactylorhiza* polyploid complex evolved by hybridization between the autotetraploid *D. maculata* and the diploid *D. incarnata*.

It is also possible that the allotetraploid *D. sphagnicola*, *D. brennensis*, and *D. elata* originated from the same group of diploids that gave rise to the autotetraploid *D. maculata*. This scenario is most likely for the allotetraploid *D. elata*. As indicated by the ITS/ETS phylogenetic tree (Fig. 1), none of the *D. elata* accessions were grouped with the accessions of *D. maculata* within clade C. All the accessions of this species that did not present intragenomic polymorphism for the nrDNA did not group together but branched as successive sister lineages to the clades C and D in the ITS/ETS tree (Fig. 1). This pattern may indicate a more ancient origin compared to the other allotetraploid species for which the ITS and ETS sequences are almost identical to the sequences displayed by the diploid and the autotetraploid species that occur today. In this case, an ancestral diploid species that would have given rise to the extant *D. maculata* lineage by autopolyploidization and to the Madeiran diploid species, *D. foliosa*, could have been involved in the formation of *D. elata*.

4.2. Congruence between ITS/EST and cpDNA phylogenies

In hybrids, concerted evolution may lead to the conversion of one nrDNA parental type into the other nrDNA parental type, making the recovery of both lineages that took part in the formation of an allopolyploid species difficult. In the *Glycine tomentella* allopolyploid complex, concerted evolution was found to favor one parental genome in some allopolyploid race and the other parental genome in others (Rauscher et al., 2004; Wendel et al., 1995). In this allopolyploid complex, different repeat types were even favored among different accessions of the same allopolyploid lineage (Rauscher et al., 2004), making the recovery of the hybrid history of this allopolyploid complex possible. In the *Dactylorhiza* allopolyploid complex, concerted evolution has favored the same parental genome making the recovery of the hybrid history of this complex difficult. However, when concerted evolution has occurred and the hybrid history has been lost, incongruence between nrDNA and cpDNA based phylogenies may still provide important evidence of hybridization and the detection of both progenitors of allopolyploid species (Wendel and Doyle, 1998). This is unfortunately not the case in our study since the phylogenetic tree of the cpDNA haplotypes (Fig. 3), even if not completely resolved, was largely consistent with the ITS/ETS tree (Fig. 1). The accessions that formed clades A and B in the ITS/ETS tree were clustered together in the chloroplast tree (group I, Fig. 3), while all the accessions that formed clades C and D of the ITS/ETS tree fell within group II in the same chloroplast tree. Only two incongruences between the cpDNA tree and the ITS/ETS tree were observed. One *D. traunsteineri* accession (*D. traunsteineri* # 3) resolved in clade A in the ITS/ETS tree with *D. fuchsii* but was found within group IV in the cpDNA haplotypic tree, which represents *D. incarnata*. The other incongruent accession, i.e., *D. fuchsii* # 1, segregated within clade A of the ITS/ETS tree but within group II of the chloroplast tree.

The almost perfect congruence between the *Dactylorhiza* cpDNA phylogenetic tree and the ITS/ETS phylogeny suggests that, in the *Dactylorhiza* polyploid complex, ITS and ETS evolution is predominantly driven in favor of the nuclear ribosomal DNA type brought by the maternal parent. Considerable attention has been paid to understand patterns of molecular evolution and genomic interactions following polyploidy (Osborn et al., 2003; Skalicka et al., 2005; Song et al., 1995; Wendel et al., 1995). Directional changes in which the paternal genome of an allotetraploid underwent greater allopolyploid-associated modification than the maternal genome were observed in some synthetic allotetraploids (Skalicka et al., 2005; Song et al., 1995). Those directional genome changes, possibly influenced by cytoplasmic–nuclear interactions (Song et al., 1995), have been observed by analyzing low copy genes or gene expression but such observations are still limited to highly repetitive satellite DNA and rDNA (Skalicka et al., 2005).

In some allotetraploid accessions, the homogenization of the parental nrDNA repeats has not gone to completion,

which can give us some hints about the paternal lineages that might have been involved in the allopolyploidization process. Concerted evolution has not completely homogenized the ITS parental nrDNA sequences in three accessions of *D. sphagnicola*, three accesssions of *D. brennensis* and one accession of *D. elata* as shown by the intra-accession ITS sequence polymorphism observed (Fig. 2). This intra-accession ITS polypmorphism suggested that the *D. incarnata* lineage (clade G) could have played the role of the paternal progenitor in the formation of the allotetraploid *Dactylorhiza* species. However, this intra-accession ITS polymorphism could also be due to a recent hybridization subsequent to the formation of the allopolyploid species. This is probably the case for the *D. elata* cloned accessions and the third cloned accession of *D. brennensis*. Previous studies using allozyme markers identified *D. incarnata* as one of the progenitors that has led to the formation of all the *Dactylorhiza* allotetraploid species in Western Europe (Hedrén, 1996a). It is thus more probable that the presence of the *D. saccifera* ITS sequence type within the *D. elata* accession and the presence of the *D. fuchsii s. str.*. ITS type within the *D. brennensis* accession were caused by a local hybridization event. *D. brennensis* and *D. fuchsii* grow in sympatry and some individuals could be of recent hybrid origin.

Most of the *Dactylorhiza* ITS cloned sequences appeared to be recombinant. Such chimeric nrDNA sequences have been reported in other polyploid species (Andreasen and Baldwin, 2003; Baldwin et al., 1995; Campbell et al., 1997; Sang et al., 1995; Van Houten et al., 1993; Wendel et al., 1995) and were thought to represent transition stages in the homogenization process of the nrDNA region (Campbell et al., 1997; Strachan et al., 1985; Wendel et al., 1995). PCR jumping in the early stages of PCR amplification can also yield chimeric patterns similar to those observed in our study (Bradley and Hillis, 1997; Cronn et al., 2002; Pääbo et al., 1990). In hybrids of the first generation, in vivo recombination between the parental genomes should not be observed. As expected, putative F1 hybrids between *D. fuchsii* and *D.incarnata* presented cloned sequences corresponding to the two parental ITS sequences and no recombinant sequences were observed (Fig. 2). The absence of recombination after PCR in those putative F1 hybrids is a strong evidence against possible PCR artifact. It is therefore likely that most of the recombinant cloned sequences observed in the polyploid taxa sampled in this study are not PCR artifacts but true in vivo recombinants resulting from incomplete concerted evolution.

4.3. Taxonomic notes

Pedersen (1998) and Hedrén (2002) proposed a conservative taxonomic treatment of the *Dactylorhiza* polyploid complex, in which polyploids were grouped into species according to their genome composition and in spite of the fact that a polyploid lineage may have evolved independently more than once.

In the absence of conclusive evidence for a possible molecular subdivision of the polyploid *Dactylorhiza* complex, Pedersen (1998) and Hedrén (2002) treated the allotetraploid group as one single species with well-known, widespread, and geographically and/or ecologically separated allopolyploids recognised as subspecies. However, our molecular study of the *Dactylorhiza* allotetraploid complex allowed us to distinguish at least three molecular subgroups, which could thus be treated as distinct species. The first one of those species includes the well-known *D. ajalis* along with *D. angustata* and *D. traunsteineri*. The second one is *D. praetermissa*, for which we identified an origin linked with the *D. saccifera* lineage as the maternal parent and the *D. incarnata* lineage as the paternal parent. Nowadays, the contact area between both progenitors of *D. raetermissa* (i.e., *D. incarnata* and *D. saccifera*) is narrow, but still exists at least in central Italy, where both species have been observed to hybridize (Rossi et al., 1983). Moreover, the distribution of both species might have overlapped more extensively in the past, for example during the last glaciation or the post-glacial recolonization.

The last allotetraploid "species" identified in our study would include *D. sphagnicola*, *D. elata*, and *D. brennensis*, all three involving the *D. maculata* lineage or its extinct diploid ancestral progenitor as the maternal progenitor. However, we also noted that *D. elata* showed a more ancient origin as evidenced by its more basal position in regard to the *D. maculata* and *D. foliosa* lineages. This might make sense from a biogeographical point of view. *D. elata* is the allotetraploid entity that shows the most extreme southwestern distribution, while *D. foliosa* is nowadays restricted to Madeira. It can therefore be hypothesized that a common ancestor of the latter and *D. maculata*, now extinct, might have given rise to *D. elata* through allopolyploidization, before both taxa separated and *D. foliosa* retreated to the island of Madeira while *D. maculata* evolved as a widespread European taxon. In this scenario, *D. sphagnicola* would appear as a more recent taxon that involves only the *D. maculata* lineage. Such a history might give an argument towards maintaining *D. elata* as a separate species from, at least, *D. sphagnicola*.

Although morphometric studies developed by, e.g., Tyteca and Gathoye (1993, 1999, 2000) for *Dactylorhiza* allotetraploid populations in Western Europe inclined their authors to distinguish many more specific entities than those that would be accepted on molecular grounds, the four entities suggested above may be corroborated by some morphological characters and biogeographic evidence. *D. ajalis* with its satellites such as indicated above, i.e., *D. traunsteineri* and *D. angustata*, are represented by rather short, early flowering plants, with few [i.e., (2) 3–6 (10)] generally spotted leaves and dark red flowers (Tyteca, 2000). They are essentially found in mountainous regions, in middle and northern Europe, and are seldom observed in purely Atlantic regions. The second species represented in our data set by *D. praetermissa*, could be related to a morphological group depicted by Delforge (2001) as the *D. praetermissa* group. This group is composed of relatively

late flowering species with an essentially Atlantic distribution and mainly represented in lowland regions. Common morphological traits are a tall habit, unspotted or more rarely spotted, more numerous leaves [4–9 (10)], and brighter flowers than in the *D. majalis* group.

The third molecular species includes *D. elata* and *D. brennensis*. This group of taxa displays a marked south-western European and northwestern African distribution, almost totally disjoint from the other groups except for small contact zones in France (Tyteca and Gathoye, 1999). In this group, the plants flower later than the other species and show a tall, sometimes slender, habit, with numerous [5–10], mostly unspotted, leaves, and a loose inflorescence. The considerations developed above about the more ancient origin of that group would allow us to distinguish it from the fourth species, i.e., *D. sphagnicola*. This species has been described as one of the most characteristic and easily identified members of the allotetraploid complex (Tyteca and Gathoye, 2000). Its flowers are generally bright pink, while its leaves are mostly unspotted, linear to narrowly lanceolate, with an erect position (whereas the leaves of the other taxa of the complex are generally spread). Its distribution (northern Europe, reaching Belgium, and northern France towards the south-west) and ecology (acid *Sphagnum* bogs and moors) also render this species quite characteristic.

Thus, morphological characteristics as well as biogeographical and ecological aspects relatively well support the molecular groups/lineages defined in this study, and would allow for the recognition of four species among our sampled populations, i.e., *D. majalis*, *D. praetermissa*, *D. elata*, and *D. sphagnicola*. The other investigated taxa, which have probably arisen through independent hybridisation events between the same parents, might be considered as subspecies of those species. However, further investigations would be required to clarify the status of non-sampled taxa and to establish more systematically the links between molecular and morphological data.

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