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Nuclear ribosomal DNA sequence variation and evolution of spotted marsh-orchids (*Dactylorhiza maculata* group)

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Abstract

Sequences of both internal and external transcribed spacers of nuclear ribosomal DNA were sequenced for four species belonging to the *Dactylorhiza maculata* group or "spotted marsh-Orchids". These four species are *D. fuchsii*, *D. saccifera*, *D. foliosa*, and *D. maculata*. Extensive nuclear ribosomal DNA polymorphism was uncovered within the diploid *D. fuchsii* and the putative autotetraploid *D. maculata*. Within the phylogenetic trees reconstructed using parsimony and Bayesian analyses, four main lineages (A, B, C, and D) were well supported. While *D. saccifera*, *D. maculata*, and *D. foliosa* were confined to clades B, C, and D, respectively, *D. fuchsii* accessions were spread over three clades (A, B, and C). Lineage C, which included accessions of the diploid *D. fuchsii* and the tetraploid *D. maculata*, was closely related to the lineage of *D. foliosa* (lineage D), an endemic diploid species from Madeira. Moreover, intra-individual polymorphism was found within accessions of *D. maculata*, *D. fuchsii*, and *D. saccifera*. It is shown that in some instances two lineages, contributed to the observed intra-individual polymorphism (C and A in *D. maculata*, A and B in *D. fuchsii* and *D. saccifera*). Evolutionary scenarios leading to this extensive nuclear ribosomal DNA polymorphism are discussed in the light of results from maternally inherited chloroplast DNA markers and an autopolyploid origin of *D. maculata* from a *D. foliosa*-like ancestor is postulated.

Keywords: Orchidaceae; *Dactylorhiza maculata*; ITS; ETS; Polyploidy; nrDNA polymorphism; 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., Alvarez and Wendel, 2003; Baldwin and Markos, 1998; Baldwin et al., 1995; Hershkovitz et al., 1999; Kelch and Baldwin, 2003; Lee et al., 2002) 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). Although nrDNA consists of thousands of copies within a genome, intra-genomic diversity is generally low (Baldwin et al., 1995). This homogeneity among nrDNA repeats is attributed to concerted evolution (Ainouche and Bayer, 1997; Baldwin et al., 1995; Dover, 1982; Fuertes Aguilar et al., 1999; Zimmer et al., 1980), a process that acts through gene conversion and unequal crossing over. Despite the homogenization between the nrDNA repeats, extensive intra-individual and intra-specific

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variation has been observed in various plant species (Campbell et al., 1997; Gaut et al., 2000; Hughes et al., 2002; Sang et al., 1995). Concerted evolution may indeed in some cases be disrupted or its rate reduced by allopolyploidization, apomixis, or long generation time, allowing the retention of polymorphic sequences within individuals and the detection of hybridogenous taxa (Campbell et al., 1997; Suh et al., 1993; Zhang and Hewitt, 2003). When concerted evolution has not reached completion, resolving patterns of reticulation may be possible mostly through detection of additive nucleotide sites in direct sequences of PCR products (Aguilar and Feliner, 2003; Feliner et al., 2004; Sang et al., 1995; Whittall et al., 2000) and/or detection of parental sequences among clones from PCR products (Andreasen and Baldwin, 2003; Campbell et al., 1997; Feliner et al., 2004; Rauscher et al., 2002; Widmer and Baltisberger, 1999). Nevertheless, processes other than hybridization need to be considered when interpreting polymorphic sequence patterns of nrDNA. Incomplete lineage sorting and divergence among paralogous loci 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 polymorphic nrDNA sequence patterns can be aided by other lines of evidence such as sequence data from unlinked chloroplast DNA (Doyle, 1992; Sang and Zhong, 2000; Wendel and Doyle, 1998).

The *Dactylorhiza* Necker ex Nevski genus is mainly distributed in Eurasia. In North America the genus is represented by a native species (*D. aristata*) in Alaska and one introduced species (*D. praetermissa*), which is naturalized in the north part of the East American cost. This orchid genus is taxonomically complex and appears to have undergone extensive reticulate evolution (Hedrén, 1996; Heslop-Harrison, 1954; Soó, 1980). 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 6 (Sundermann, 1980) to 49 species (Delforge, 1994). Most of the species are either diploids ($2n = 40$) or tetraploids ($2n = 80$) and, with the exception of *D. maculata*, all the extant European tetraploid species are considered as allotetraploids having evolved by repeated hybridization between two main diploid lineages ($2n = 40$), one consisting of the diploid *D. incarnata* and the other consisting of the diploid *D. fuchsii* (Hedrén, 1996; Hedrén et al., 2001; Heslop-Harrison, 1954). Within the *Dactylorhiza* polyploid complex, the three diploid species *D. fuchsii*, *D. saccifera*, *D. foliosa*, and the tetraploid species *D. maculata* belong to the *Dactylorhiza maculata* group or "spotted marsh-Orchids" (Bateman and Denholm, 1989). While *D. fuchsii* is widespread throughout Western and Nothern Europe, *Dactylorhiza foliosa* is endemic to the Island of Madeira, situated in the Atlantic Ocean, ≈1000 km off the European coast. *Dactylorhiza saccifera*, a southeastern European diploid species, is sister to the more western diploid *D. fuchsii*, and both species are considered by some authors as the same taxon (e.g. Rossi and Eldredge Maury, 2002). *Dactylorhiza maculata*, widespread throughout Western and Nothern Europe, is considered as an autopolyploid having evolved from the diploid *D. fuchsii* (Hedrén, 1996). The autopolyploid origin of *D. maculata* is supported by a close morphological similarity with *D. fuchsii* (Bateman and Denholm, 1989; Heslop-Harrison, 1954), but also by a close correspondence in both allele composition and allele frequencies for seven allozyme loci (Hedrén, 1996). However, AFLP fingerprints (Hedrén et al., 2001) and an ITS-based phylogeny of the Orchidinae (Bateman et al., 2003) indicate that *D. maculata* is somewhat differentiated from *D. fuchsii* and more closely related to the diploid Madeiran endemic species *D. foliosa*, with which it shares a distinctive ITS type. It is difficult to consider *D. foliosa* as a potential parental species in the formation of *D. maculata* because of its very limited and isolated distribution (Hedrén et al., 2001). However, the fact that only one accession of *D. foliosa* was sampled and the fact that the accessions of *D. fuchsii* and *D. maculata* were sampled in Sweden, at the nothern edge of their range, leave the question of the origin of the tetraploid *D. maculata* open to further investigations.

In this paper, we focus on the evolution of the spotted marsh-orchids with an emphasis on the diploid *D. fuchsii* and its putative autotetraploid *D. maculata* in Western Europe. In preliminary tests, intra-individual polymorphism in nrDNA sequences was observed for these two species. The goal of this paper is to readdress the origin of the tetraploid *D. maculata* and the likely source of the ITS and ETS sequence polymorphism observed within the studied species. We also examined PCR-RFLP data from five chloroplastic regions for the same samples in order to infer the causes of nrDNA polymorphism with more confidence.

2. Material and methods

2.1. Sampling and DNA isolation

For this study, fresh leaves from 15 accessions of *D. maculata*, 8 accessions of *D. foliosa*, 13 accessions of *D. saccifera*, 19 accessions of *D. fuchsii*, 2 accessions of *D. incarnata*, and 2 accessions of *Gymnadenia conopsea* were collected from 23 natural populations in Belgium, France (including Corsica), Italy and Madeira (Table 1). Total genomic DNA was isolated from leaf tissues using the cetyltrimethylammonium bromide procedure outlined by Doyle and Doyle (1987) without RNase treatment.

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

Taxon	Origin	Accession No.	Ploidy level	GenBank Accession No. ITS/ETS
Ingroup				
D. foliosa (Lowe) Soó	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. saccifera (Brongniart) Soó	Venaco, Corsica	1, 2	Diploid ($2n = 40$)	AY699488–AY699489/AY699561–AY699562
	Vizzavona, Corsica	3–5		AY699490–AY699492/AY699563–AY699565
	Zonza, Corsica	6–8		AY699493–AY699495/AY699566–AY699568
	Along the N196 road, Corsica	9		AY699496/AY699569
	Along the N196 road, Corsica	10[a]		AY699499–AY699502/AY699571
	Along the N196 road, Corsica	11[a]		AY699503–AY699507/AY699570
	Val di Varri, Italy	12, 13		AY699497–AY699498/AY699572–AY699573
D. fuchsii (Druce) Soó	Tienne St. Inal, Belgium	1	Diploid ($2n = 40$)	AY699423/AY699512
	Baronville, Belgium	2, 3		AY699424–AY699425/AY699513–AY699514
	De Panne, Belgium	4		AY699426/AY699515
	Kerséguénou, France	5		AY699427/AY699516
	Col du Granier, France	6–8		AY699428–AY699430/AY699517–AY699519
	Montpaon, France	9, 10		AY699431–AY699432/AY699520–AY699521
	Bièvres, France	11		AY699433/ not sequenced for the ETS
	Bièvres, France	2–13		AY699434–AY699435/AY699522–AY699523
	Col de Biscia, Italy	14–19		AY699436–AY699441/AY699524–AY699529
D. maculata (L.) Soó	Wésomont, Belgium	1[a]	Tetraploid ($2n = 80$)	AY699442–AY699446/AY699530–AY699533
	Wésomont, Belgium	2		AY699447/AY699534
	St-Hubert, Belgium	3–5		AY699448–AY699450/AY699535–AY699537
	Lagland, Belgium	6[a]		AY699451–AY699455/AY699538
	Lannéanou, France	7, 8		AY699456–AY699457/AY699539–AY699540
	Guissény marsh, France	9[a]		AY699458/AY600541–AY699544
	Guissény marsh, France	10		AY699459/AY699545
	Dourbies, France	11		AY699460/AY699546
	Dourbies, France	12[a]		AY699461–AY699468/AY699547–AY699549
	Aigoual forest, France	13		AY699469/AY699550
	Aigoual forest, France	14[a]		AY699470–AY699478/AY699551
	Aigoual forest, France	15		AY699479/AY699552
Outgroups				
D. incarnata (L.) Soó	Kerséguénou, France	1	Diploid ($2n = 40$)	AY699422/AY699511
	Col du Perjuret, France	2		AY699510/AY699576
Gymnadenia conopsea (L.) R. Brown	Lavaux St. Anne, Belgium	1, 2	Diploid ($2n = 40$)	AY699508–AY699509/AY699574–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).

[a] This indicates which accessions have been cloned for ITS and/or ETS regions, and for which multiple sequences that correspond to the different clones have been submitted to GenBank.

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

The complete ITS region was amplified in a single PCR with primers ITS-5a (Stanford et al., 2000) and ITS-4 (White et al., 1990) using standard procedures. The ETS region is located between the 18S and 26S rDNA genes, adjacent to the 5′ end of the 18S gene. To design a suitable primer to amplify it, we initially used primers IGS1 and IGS88 (Oh and Potter, 2005) to amplify the whole intergenic spacer of the 18S – 26S nuclear ribosomal DNA (IGS) for a subset of ten individuals belonging to six *Dactylorhiza* species. The resulting IGS fragment, was then sequenced for ≈800 bp directly upstream from the 18S gene using primer IGS88. An internal sequencing primer (ETSdact, 5′-GGGATC AAAATGCAAGCAACATG-3′) was then designed to further sequenced ≈700 bp of the IGS region. A region of complete nucleotide identity was found in the six species and a primer was designed within it (ETSdact1, 5′-T TTGCGTTGCTTGCTAGTGCTTG-3′). This primer, located ≈1200 bp directly upstream from the 18S gene, was then used together with primer IGS88 to amplify part of the 5′ extremity of the 18S gene and one ETS fragment of ≈950 bp for all sampled individuals. The PCR conditions used for amplifying the ITS and ETS regions are 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 showed double peaks or lacked clear base reading were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) in order to separate the differing ITS and/or ETS copies. In order to minimize the enzymatic error in the amplification stage of ITS and/or ETS for those accessions, PfuTurbo DNA polymerase (Stratagene) was used because of its proofreading function. Five to ten clones per cloning reaction 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.

Some accessions showed a few double peaks on the electropherogram and/or a series of superimposed peaks downstream from a given nucleotide. These superimposed peaks, resolved when sequencing in the opposite direction indicate the presence of two sequence types differing by nucleotide insertions or deletions (indels). Those accessions that seemed to bear more than one ITS and/or ETS repeat types were not cloned but the two contributing sequences were inferred by comparison to monomorphic sequences from the same data set.

2.3. Chloroplast DNA analysis: PCR-RFLP

A PCR-RFLP approach was used to characterize five chloroplastic 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 accessions. Amplicons of these five chloroplastic regions were amplified and digested following the same protocol as described in details by 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. Each polymorphic fragment has been scored as binary characters.

2.4. Phylogenetic analyses

Complementary strands of 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 determined for this study were deposited in GenBank (see Table 1 for accession numbers) and the alignment of the ITS plus ETS sequences has been deposited in TreeBase URL: (http://www.treebase.org) under the matrix accession number M2284. The data set resulting from the PCR-RFLP analysis of the five chloroplastic regions can be obtained from the first author upon request.

In this study, we present the complete cpDNA and ITS/ETS gene trees as separate analyses to investigate possible incongruences between the nuclear and chloroplastic trees that may indicate hybridization and introgression events.

For accessions that showed monomorphic sequences for both ITS and ETS regions, the two aligned data sets were merged. Identical sequences were identified and all but one representative of each unique sequence were removed prior to phylogenetic analyses. The phylogenetic trees were constructed for both the ITS/ETS and the chloroplastic PCR-RFLP data sets by unweighted parsimony using PAUP* 4.0b10 (Swofford, 2002). The shortest trees were searched by the heuristic method with 1000 replications of random addition of sequences, TBR branch swapping and ACCTRAN optimization. For the ITS plus ETS data set, gaps were excluded from the alignment but were recoded as binary character according to the ‘simple indel coding’ method (Simmons and Ochoterena, 2000). Support for clades were estimated with 1000 bootstrap replicates using TBR branch swapping of the heuristic search.

In addition to this classic phylogenetic method of tree reconstruction, a Bayesian analysis using Metropolis-coupled Markov chain Monte Carlo (MCMC) was implemented on the ITS/ETS data set using MrBayes software (Huelsenbeck and Ronquist, 2001). The Bayesian analysis consisted of four chains with random starting trees, the GTR + Γ model of nucleotide substitution and uniform prior distribution of parameters. This evolutionary model, which best fits the data, was determined by the hierarchical likelihood ratio test using MrModeltest 2.1 (Nylander, 2002). The four chains were run for two millions generations and trees were sampled every 100 generations. Stationarity was determined by visual examination of the log-likelihood plots and burn-in trees were discarded. Clades that were present in more than 95% of the sampled trees were considered as significantly supported (Huelsenbeck and Ronquist, 2001).

Two accessions of *Dactylorhiza incarnata* and two accessions of *Gymnadenia conopsea* were used as outgroups. *Dactylorhiza incarnata* has a basal position in the phylogeny of the genus *Dactylorhiza* while the genus *Gymnadenia* is sister to the genus *Dactylorhiza* as inferred from a phylogeny of the Orchidinae (Bateman et al., 2003; Pridgeon et al., 1997). Pairwise sequence divergence for the ITS/ETS sequences were calculated in PAUP* 4.0b10 (Swofford, 2002) using the same GTR + Γ model as for the Bayesian analysis.

3. Results

3.1. ITS/ETS sequence polymorphism and phylogenetic analysis

The total aligned length of the ITS1 + 5.8S + ITS2 regions was 652 bp in the 89 sequences obtained (direct and cloned, outgroups included). The whole ITS region contained eight indels, 47 parsimony informative and 10 parsimony uninformative variable sites. Gap coding resulted in four additional characters, bringing the total number of parsimony informative characters to 51. The total aligned length of part of the ETS region (directly upstream of the 18S gene) was 935 bp in the 66 sequences obtained (direct and cloned, outgroups included). It contained 12 indels, 163 parsimony informative sites, and 29 parsimony uninformative sites. Five indels were recoded bringing the total number of parsimony informative characters for the ETS region to 168. The average GC content for all sequences (direct and cloned) was 49% for ITS and 46% for ETS.

Six identical sequences were observed in *D. fuchsii*, of which five were removed from further analyses. Similarly, two identical sequences were observed in *D. maculata*, one of which was removed. No other ITS/ETS sequence was observed more than once. Maximum parsimony analysis of the 36 unique ITS/ETS sequences of *D. fuchsii*, *D. saccifera*, *D. foliosa*, and *D. maculata* accessions yielded 325 equally most parsimonious trees of 310 steps (CI = 0.945; RI = 0.981). A strict consensus tree with a Consistency Index of 0.933 and a Retention Index of 0.977 was generated (Fig. 1a). The burn-in in the Bayesian analysis performed occurred after 15000 generations. The mean $\ln L$ score for all the trees sampled at stationary was of −4090.34. The 50% majority rule consensus tree recovered by the Bayesian analysis is presented in Fig. 1b.

The topologies of the consensus trees derived from the two analyses were similar, both presenting four highly diverged clades (named A, B, C, and D), which were strongly supported by bootstrap and Bayesian posterior probabilities values (Figs. 1a and b, respectively). Surprisingly, multiple accessions of the diploid *D. fuchsii* from which the sequences were obtained did not form a monophyletic group in the phylogenetic trees. Sequences from this species were distributed among three of the four divergent clades (clades A, B, and C). *Dactylorhiza maculata* and *D. foliosa* were restricted to clades C and D, respectively. Pairwise sequence divergence between outgroups and ingroup ranged from 0.05093 (*D. incarnata* # 1 and *D. fuchsii* # 15) to 0.09227 (*Gymnadenia conopsea* and *D. saccifera* # 4). Pairwise sequence divergence ranged from 0.00062 to 0.00627 within clade A, from 0.00062 to 0.01204 within clade B, from 0.00063 to 0.00444 within clade C and from 0.00063 to 0.0038 within clade D. Average sequence divergence was 0.01169 between clades A and B, 0.02674 between clades A and C, 0.02683 between clades A and D, 0.00728 between clade C and D, 0.03254 between clade C and B, and finally, 0.03257 between clade D and B. Separate analyses of ITS and ETS sequences produced consistent relationships, with more resolving power when combining the two data sets. The relationship between clades A and clade B was not fully resolved when using the ETS data alone, while the relationship between clade C and clade D was unresolved when using the ITS data alone.

Direct sequences of ten out of 19 *D. fuchsii* accessions showed polymorphic ITS and ETS sites occurring at some of the positions that were synapomorphic for either of the well-supported clades A, B, or C (Fig. 2). Those accessions were identified as reflecting intra-genomic polymorphism, i.e., the co-occurrence of more than one nrDNA repeat type within the same individual. Out of these ten accessions of *D. fuchsii*, four accessions (*fuchsii* # 11, 7, 8, 12) had 12 polymorphic ITS sites, 6 of them being synapomorphic for either clades A or B, and 6 ETS polymorphic sites, none of them occurring at synapomorphic positions for either clade A or B. These four accessions were interpreted as involving nrDNA repeats from both clades A and B. Those sequences were excluded from the phylogenetic analysis because they

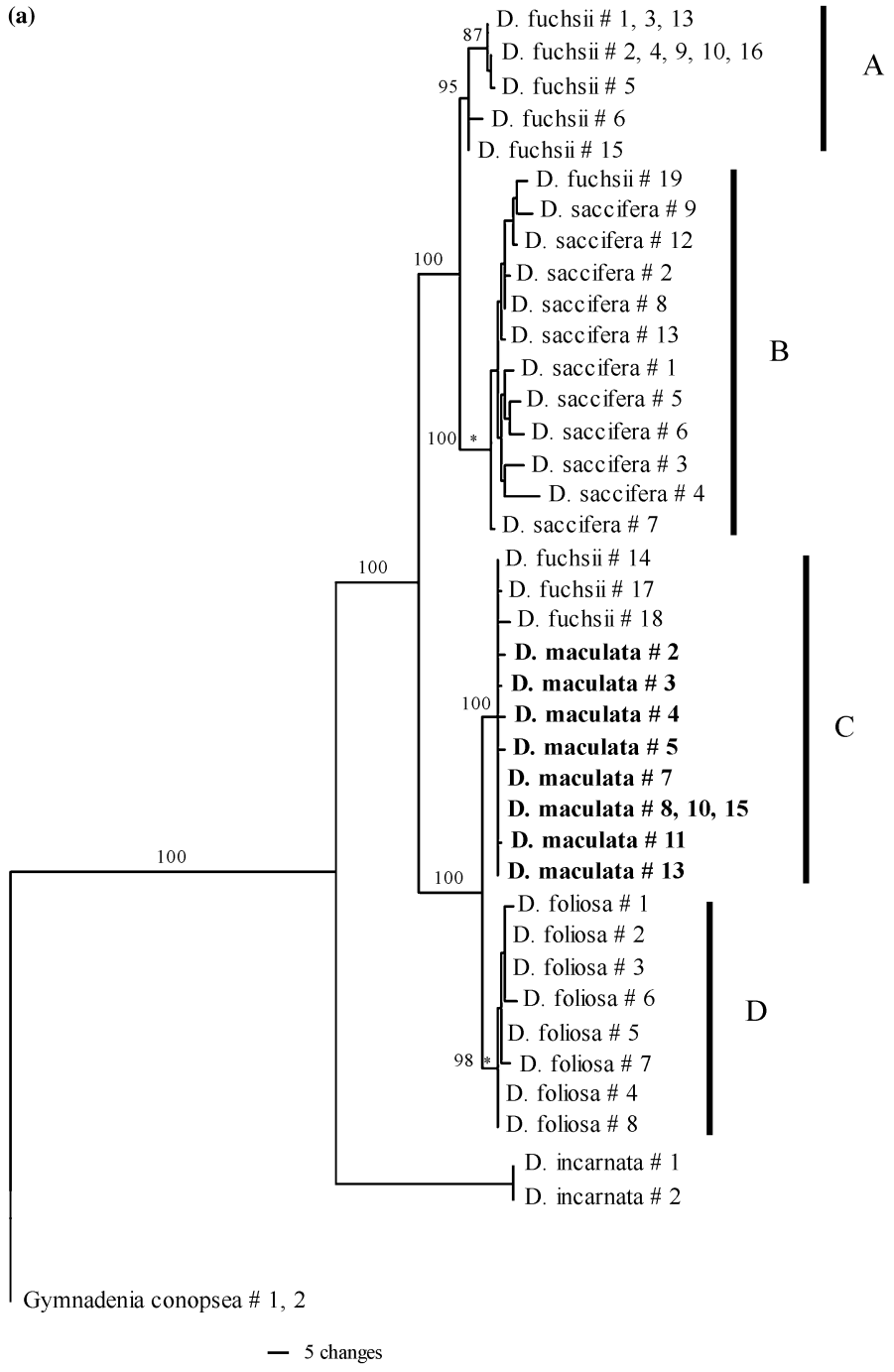

Fig. 1. Phylogenetic trees based on ITS and ETS sequences of five *Dactylorhiza* species (a) Strict consensus of 325 equally most parsimonious trees. Number above branches give percentage occurrence of a group in 1000 bootstrap replicates. (b) Fifty percent majority rule consensus of the trees recovered in Bayesian analysis. Bayesian posterior probabilities values are given for the major clades and indicated above branches. The four major clades (bold lines) are discussed in the text. Terminals in bold are tetraploid ($2n = 80$). Number after species name refers to the accession number (see Table 1). *Dactylorhiza* accessions not included in the trees (*D. maculata* # 1, 6, 9, 12, 14, and *D. fuchsii* # 7, 8, 11, 12, and *D. saccifera* # 10, 11) are putative interclade hybrids.

presented polymorphism for synapomorphic positions of both different well-supported clades A and B. Five accessions (*fuchsii* # 1, 3, 5, 13, and 15) had four polymorphic ITS sites, none of them being synapomorphic for either clade A, B, or C. These accessions were interpreted as involving mixture of intra-clade A nrDNA repeats (Fig. 2) and were resolved in the phylogenetic tree in clade A (Fig. 1). Finally, one accession (*fuchsii* # 14) which resolved in clade C was interpreted as involving mixture of intra-clade C nrDNA repeats.

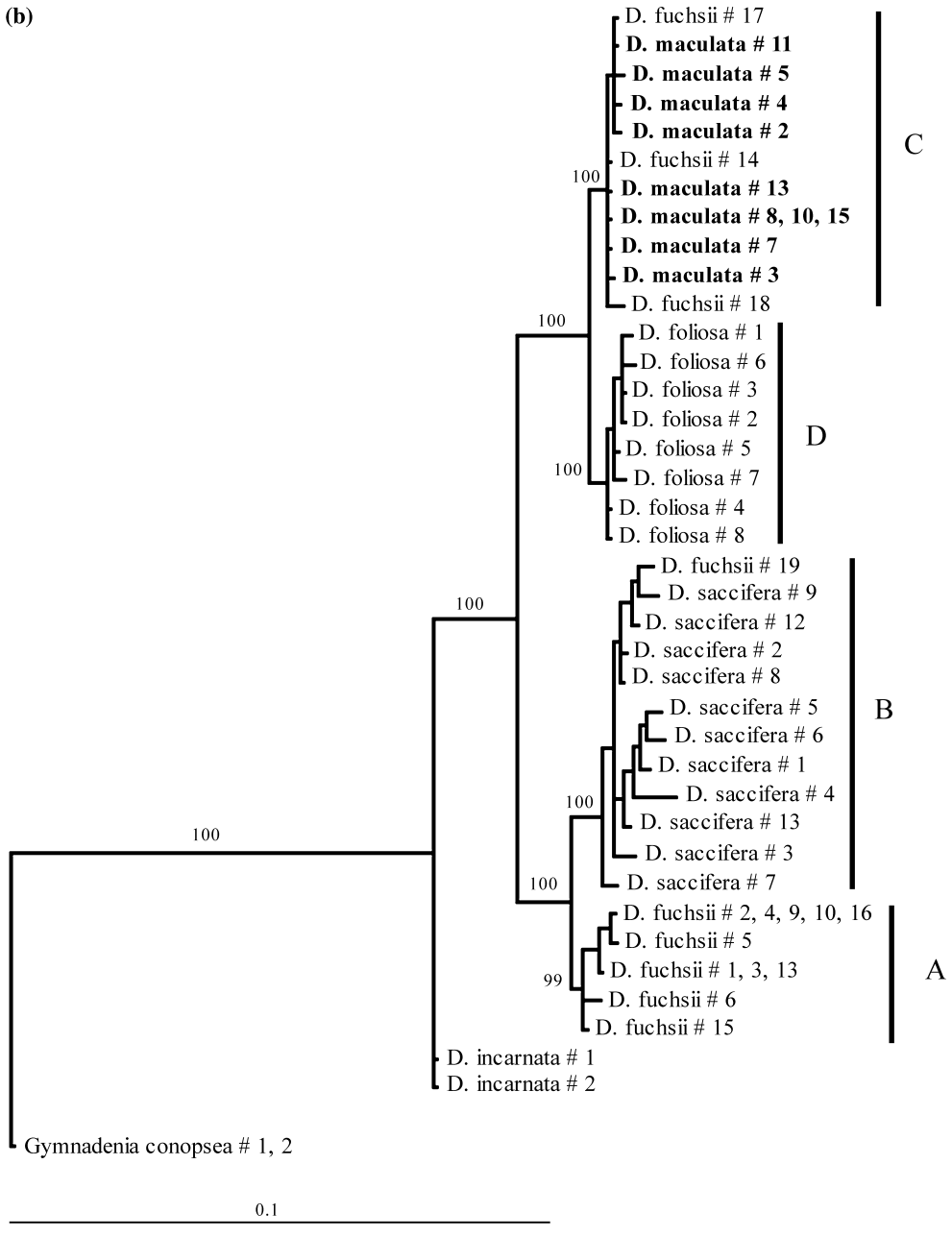

Fig. 1 (*continued*)

3.2. ITS/ETS cloned sequences and recombination

For five accessions of *D. maculata* and two accessions of *D. saccifera* (footnote 'a' in Table 1), direct sequences of the ITS and/or ETS region displayed a series of uninterpretable superimposed peaks and were subsequently cloned in order to separate the differing intra-individual nrDNA copies.

Out of the five *D. maculata* accessions cloned, two were cloned for both ITS and ETS regions, two others were cloned for the ITS region only because the ETS region did not show intra-genomic polymorphism, while the fifth accession was cloned for the ETS region only, because the ITS regions did not present any problem when sequenced directly. Fig. 3 displays the distribution of polymorphic positions in ITS and ETS clones of the five *D. maculata* accessions cloned. Most of the polymorphic positions correspond to the synapomorphies distinguishing clades A and C. Clones that may have undergone extensive recombination between sequences typical of clades A and C were detected for both ITS and ETS regions (Fig. 3). For each cloned accession, some clones were found to be almost identical to sequences from clades A or C but no accession showed both ITS and ETS sequences identical to clade A sequences and therefore were not included in the phylogenetic tree. The

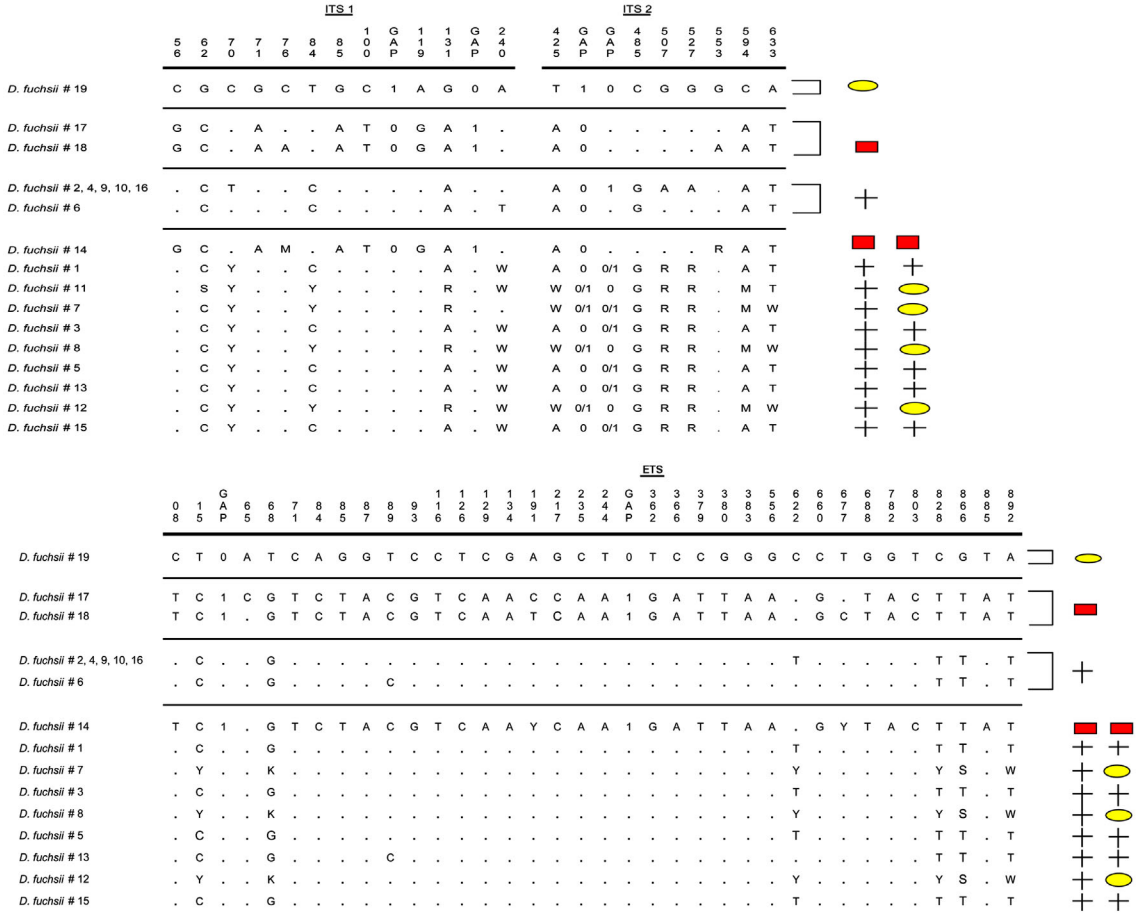

Fig. 2. Variable nucleotide sites for both ITS and ETS sequences of direct PCR products from multiple accessions of *D. fuchsii*. Dots indicate sequence matches to the sequence of *D. fuchsii* # 19. Symbols on the right represent genotypes of the three major Clades (⬭ clade B; ▬ clade C and + clade A) and their combinations in multiple accessions of *D. fuchsii*. ETS region for *D. fuchsii* # 11 was unable to amplify. Site numbers correspond to site position in the ITS and ETS aligned data sets. M = A & C; R = A & G; S = C & G; W = A & T; Y = C & T; K = G & T.

number of putative crossing-over events was one (in most of the clones) or two (in three ITS clones and two ETS clones from *D. maculata* # 1, one ETS clone from *D. maculata* # 6 and one clone from *D. maculata* # 12). For instance, *D. maculata* #12 clone 'f' displayed the ITS nucleotide composition of the clade A for the first six polymorphic sites and then conformed to the nucleotide composition of the clade C. *D. maculata* # 12 clone 'c' displayed the ITS nucleotide composition of the clade C for the first six polymorphic sites, switched to the clade A for five polymorphic sites (including two indels) and then reverted to the composition of the clade C for the rest of the region.

Fig. 3 displays also the distribution of polymorphic positions in ITS clones of the two cloned accessions of *D. saccifera* (*D. saccifera* # 10 and 11) from Corsica. Those 2 accessions were not cloned for the ETS region because this region did not show intra-genomic polymorphism when sequenced directly. Sequences of the ETS region recovered typical ETS sequences from clade B for both accessions. ITS sequences recovered by cloning were identical to ITS sequences typical of either clade A or B. No accession showed, as for *D. maculata*, both ITS and ETS sequences identical to clade A sequences and therefore were not included in the phylogenetic tree. Only one clone recovered from *D. saccifera* # 11 displayed recombination between sequences from clades A and B.

A number of methods have been developed for the accurate detection of recombination from DNA sequences (e.g. Mcguire et al., 1997; Mcguire et al., 2000; Posada, 2002). However, a minimum sequence divergence of 5% seems necessary for most methods to be effective (reviewed by Posada and Crandall, 2001). Because of this requirement the use of a software for detecting recombination has been avoided and we had to detect possible recombination by eye counting on our detailed knowledge of ITS and ETS variation in the four *Dactylorhiza* species studied.

3.3. cpDNA tree and congruence with nuclear ribosomal tree

The survey by PCR-RFLP of the chloroplast DNA from the four *Dactylorhiza* species investigated revealed 37 polymorphic fragments, which together allowed the

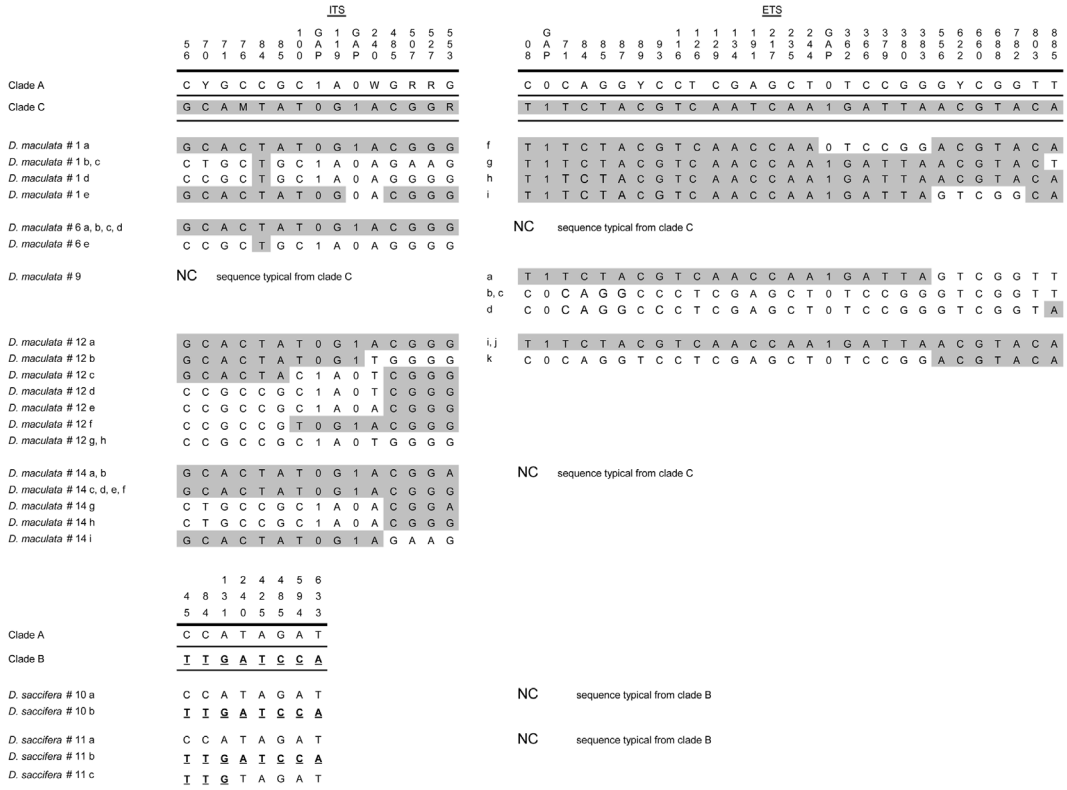

Fig. 3. Variable nucleotide positions of ITS and ETS cloned sequences of 5 *D. maculata* and 2 *D. saccifera* accessions. Number after species name refers to the accession number (see Table 1) and the letter after the accession number refers to the different clones. Variable nucleotide states between sequences representing clades A and C are included for comparison with cloned sequences from *D. maculata* accessions. Variable nucleotide states between sequences representing clades A and B are included for comparison with cloned sequences from *D. saccifera* accessions. To illustrate putative recombinant clones, nucleotides corresponding to the sequence representing clade C are highlighted, clade B are underlined while nucleotide representing clade A are not formated. ITS and ETS positions are numbered as in the ITS and ETS final aligned data sets. NC means that the ITS or ETS region was not cloned and sequenced directly with good results.

identification of 19 haplotypes. The maximum parsimony analysis of these 19 haplotypes yielded 27 most parsimonous trees of length 58 (CI = 0.63; RI = 0.796). The strict consensus tree is presented in Fig. 4.

Two main clades with moderate bootstrap support were resolved in the cpDNA phylogenetic tree. The relationship within these two clades was virtually completely unresolved because of the relatively small number of informative characters. However, *D. fuchsii* and *D. maculata* haplotypes were mostly distributed in clades AB and CD, respectively. *D. saccifera* and *D. foliosa* accessions displayed specific haplotypes, resolved in clades AB and CD, respectively. This relationship between the cpDNA haplotypes, even if not completely resolved, was largely consistent with the ITS/ETS tree (Fig. 1), clade AB in the chloroplastic tree corresponding to clades A and B of the ITS/ETS tree and clade CD corresponding to clades C and D of the ITS/ETS tree. However, a few incongruences between the cpDNA tree and the ITS/ETS tree have been observed. Three *D. fuchsii* accessions (*D. fuchsii* # 14, 17, and 18) were resolved in clade C with accessions of *D. maculata* in the ITS/ETS tree, while distributed within clade AB in the cpDNA tree. Conversely, one *D. fuchsii* accession (*D. fuchsii* # *1*) resolved in clade A in the ITS/ETS tree together with the other sequences of *D. fuchsii* was found in clade CD in the cpDNA haplotypic tree. A fifth accession (*D. fuchsii* # 19) presented a typical *D. fuchsii* cpDNA haplotype but resolved in clade B with *D. saccifera* in the ITS/ETS tree. Moreover, it is important to note that two of the five *D. maculata* accessions observed as recombinant for their ITS and/or ETS sequences presented a cpDNA haplotype typical of *D. fuchsii* (clade AB in the cpDNA haplotypic tree).

4. Discussion

4.1. Coexistence of diverged lineages within D. fuchsii

Extensive ITS and ETS sequence polymorphism has been uncovered within the widespread diploid species *D. fuchsii*. Three divergent and well-supported nrDNA lineages (clades A, B, and C) were represented in this species which could be explained by hybridization. The incongruent position of the five *D. fuchsii* accessions between the ITS/ETS and cpDNA trees is supportive of

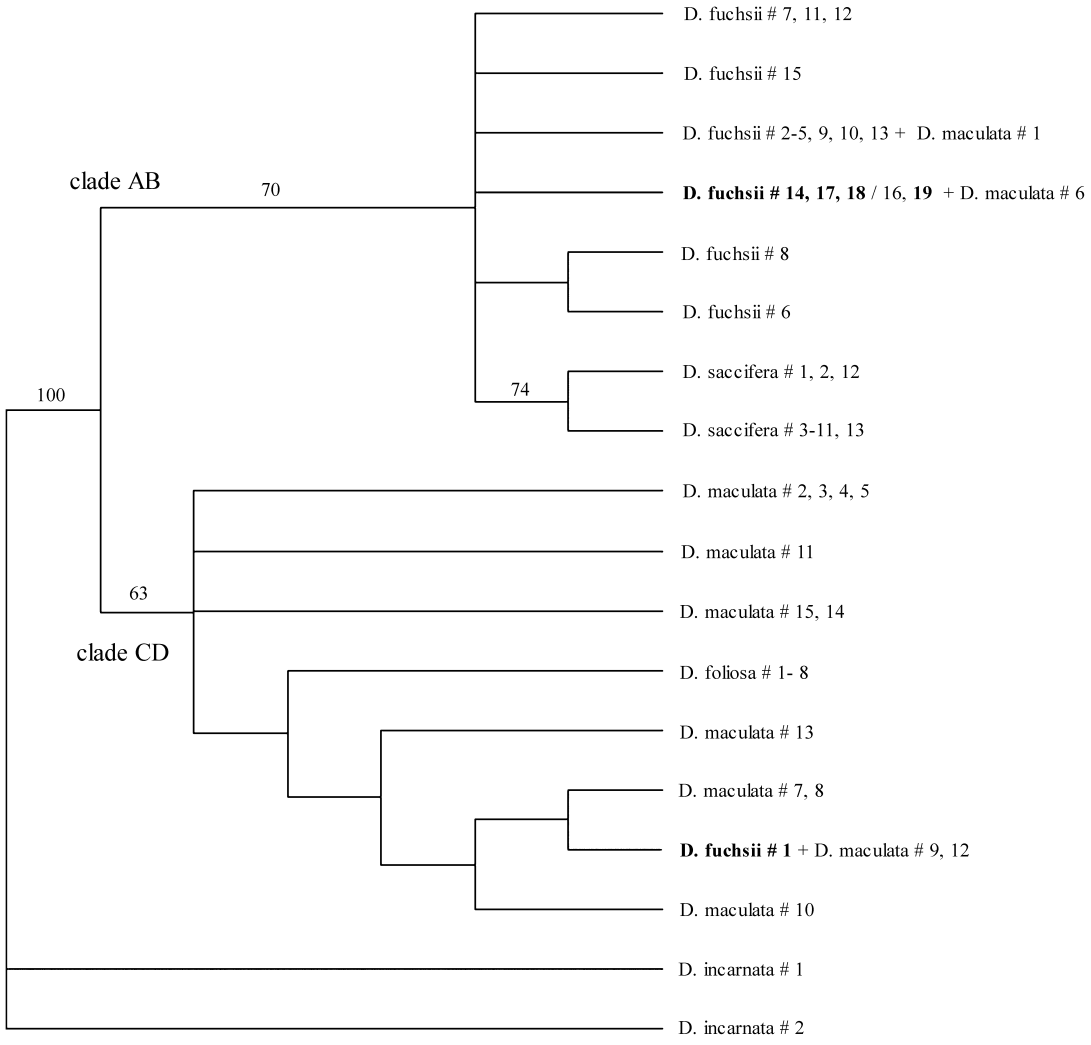

Fig. 4. Strict consensus of 27 equally most parsimonious trees recovered from the analysis of 19 chloroplastic haplotypes observed in the *Dactylorhiza* accessions studied. Number above branches give percentage occurrence of a group in 1000 bootstrap replicates. Clade AB corresponds to clades A and B in the nuclear ribosomal tree (see Fig. 1) while clade CD corresponds to clades C and D in the same nuclear ribosomal tree. The positions of five *D. fuchsii* accessions, in bold in the tree, are incongruent with the ITS/ETS tree.

a hybridization scenario. One of these five accessions (*D. fuchsii* # 19) branched in clade B of the ITS/ETS tree with all the accessions of *D. saccifera* but showed a cpDNA haplotype typical of *D. fuchsii*. This incongruence could be due to introgression of nrDNA sequence into *D. fuchsii* from *D. saccifera*. Hybridization between *D. fuchsii* and *D. saccifera* is possible or even likely because (1) *D. fuchsii* and *D. saccifera* are both diploid and closely related (some authors do not differentiate the two); (2) their geographic distributions, even if not overlapping, are in contact in the north of Italy, where the analyzed samples come from; (3) based on intra-individual polymorphic sites, four accessions of *D. fuchsii* were interpreted as hybrids between lineage A and B. These four accessions were not polymorphic for all the ITS positions where clades A and B differ. However, polymorphisms do not have to be present at each site in which the two hybridizing nrDNA repeat types differ. Homogenization acting through concerted evolution can indeed act in a biased direction only for some nucleotides (Hess et al., 1984). A similar pattern of partial conversion was found by Sang et al. (1995) within the ITS region of various *Paeonia* species. These authors suggested that the observed homogenization pattern was due to the fact that certain regions within a gene are more likely to undergo gene conversion than others.

Similarly, the presence of the three *D. fuchsii* accessions (D. fuchsii # 14, 17, and 18) in clade C, together with accessions of *D. maculata*, could be the result of hybridization. Hybridization and gene flow from tetraploid to diploid levels was documented by Stebbins (1971) as a rare process in plants because it would require either repeated hybridization between the two taxa via the production of unreduced gametes by the diploid species, or introgression through an intermediate triploid hybrid. However, introgression of the tetraploid

D. maculata into the diploid *D. fuchsii* seems to be frequent. The three *D. fuchsii* accessions, branched within the *D. maculata* lineage in the ITS/ETS tree, displayed a cpDNA haplotype found in clade AB of the cpDNA tree, i.e., the clade within which most *D. fuchsii* accessions branched. Such incongruence between cpDNA and nrDNA trees is typically interpreted as the result of hybridization and thus suggests introgression between *D. fuchsii* and *D. maculata*. In this case, the three introgressed *D. fuchsii* individuals could have kept the typical *D. fuchsii*-like cpDNA haplotype while concerted evolution would have acted in favor of the *D. maculata* ITS/ETS type. Moreover, this scenario of hybridization between *D. maculata* and *D. fuchsii* is even more likely when we consider the fact that the ITS and ETS sequences from five *D. maculata* accessions showed recombination between ITS and ETS sequences typical of *D. fuchsii* accessions from clade A and sequences typical of clade C. The study by Devos et al. (2003) of the chloroplast DNA of 27 accessions of *D. fuchsii* and 35 accessions of *D. maculata* also suggests frequent hybridization between *D. fuchsii* and *D. maculata*. In the latter study, two haplotypic lineages were also found, one of which was represented by the vast majority of the *D. fuchsii* analyzed while the other clade was represented by the majority of the *D. maculata* accessions sampled. Some overlap between *D. fuchsii* and *D. maculata* haplotypes was however observed such that the most common *D. fuchsii* haplotypes were found in a few accessions of *D. maculata* and vice versa, which can be explained by a secondary contact (hybridization) between those taxa. A similar pattern was also found by Hedrén (2003) for Scandinavian populations of *D. fuchsii* and *D. maculata*.

4.2. On the origin of D. maculata

On the basis of cytogenetic evidence and allozyme banding patterns, *D. maculata* has always been considered as an autotetraploid species that evolved from the diploid *D. fuchsii* (Hedrén, 1996; Heslop-Harrison, 1954). The close relationship between *D. maculata* and *D. foliosa* in both the ITS/ETS and the cpDNA trees is not consistent with the close relationship between *D. maculata* and *D. fuchsii* inferred from allozymes. However, no allozymic data are available for *D. foliosa* and we cannot exclude that *D. maculata* allozymes could be as close to *D. foliosa* than to *D. fuchsii*. Our results suggest that the tetraploid *D. maculata* could have originated once by autopolyploidization from a *D. foliosa*-like ancestor that probably went extinct, and not as commonly believed from a diploid taxon closely related to the extant *D. fuchsii*. It could have been suggested that *D. maculata* originated from *D. fuchsii* by autopolyploidization since three accessions of this species were found in clade C with all the accessions of *D. maculata*. However, as discussed above, the hybrid origin of those three *D. fuchsii* accessions does not support this hypothesis. Similarly, the ITS or ETS sequences typical of clade A that were found in some cloned accessions of *D. maculata* (e.g., #9 and #12) could have been interpreted as evidence for an autopolyploid origin of *D. maculata* from *D. fuchsii*. However, those two *D. maculata* accessions showed intra-individual polymorphism along with a cpDNA haplotype typical of *D. maculata* and may thus be considered to be of hybrid origin. A close relationship between *D. maculata* and *D. foliosa* was also pointed out by Bateman et al. (2003) and Hedrén et al. (2001), but *D. foliosa* was never thought to be a potential parental species because of its restricted distribution.

Recombinant sequences observed in the five cloned *D. maculata* accessions can be the result of either real in vivo recombination or PCR artefacts (Bradley and Hillis, 1997; Cronn et al., 2002; Gandolfi et al., 2001). Either way, recombinant cloned sequences are the evidence of the presence of two divergent nrDNA repeat types within an individual. While single recombination events may be due to in vitro recombination, double in vitro recombination seems unlikely (Gandolfi et al., 2001). Autapomorphic T (thymine) and A (adenine) states were found in areas of putative sequence recombination and could be due to PCR jumping in early stage of PCR amplification (Paabo et al., 1990). However, this is probably not the case in the present study because the DNA polymerase used before the cloning reaction (PfuTurbo DNA polymerase) lacks the terminal deoxynucleotidyltransferase activity. Therefore, it is likely that most of the recombinant cloned sequences are not PCR artefacts but true in vivo recombinants. Concerted evolution clearly has not homogenized the parental ITS and ETS sequences for those five accessions of *D. maculata* which might indicate a recent hybrid origin.

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