Evolution and temporal diversification of western European polyploid species complexes in *Dactylorhiza* (Orchidaceae)

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Patterns of polyploid evolution in the taxonomically controversial *Dactylorhiza incarnata*/*maculata* groups were inferred genetically by analyzing 399 individuals from 177 localities for (1) four polymorphic plastid regions yielding aggregate haplotypes and (2) nuclear ribosomal ITS allele frequencies. Concordance between patterns observed in distributions of plastid haplotypes and ITS alleles renders ancestral polymorphism an unlikely cause of genetic variation in diploids and allopolyploids. Combining the degree of concerted evolution in ITS alleles (thought to reflect gene conversion) with inferred parentage provides support for a quadripartite classification of western European allopolyploid dactylorchids according to their respective parentage and relative dates of origin. The older allotetraploids that generally exhibit only one parental ITS allele can be divided into those derived via hybridization between the divergent complexes we now call *D. incarnata* s.l. and *D. fuchsii* (e.g., *D. majalis*) and those derived via hybridization between *D. incarnata* s.l. and *D. maculata* (e.g., *D. elata*). Similarly, the younger allotetraploids that maintain evidence of both parental ITS alleles can be divided into those derived from hybridization between *D. incarnata* s.l. and *D. fuchsii*, or perhaps in some cases a diploid species resembling *D. saccifera* (e.g., *D. praetermissa*, *D. purpurella*, *D. traunsteineri* s.l., *D. baltica*), and those derived from hybridization between the *D. incarnata* s.l. and *D. maculata* groups (e.g., *D. occidentalis*, *D. sphagnicola*). Older allotetraploids are inferred to have passed through glacially induced migration bottlenecks in southern Eurasia, whereas at least some younger allotetraploids now occupying northern Europe are inferred to have originated post-glacially and remain sympatric with their parents, a scenario that is largely in agreement with the morphology and ecology of these allotetraploids. ITS conversion is in most cases biased toward the maternal parent, eventually obscuring evidence of the original allopolyploidization event because plastid haplotypes also reflect the maternal contribution. Gene flow appears unexpectedly low among allotetraploids relative to diploids, whereas several mechanisms may assist the gene flow observed across ploidy levels. There is good concordance between (1) the genetically delimited species that are required to accurately represent the inferred evolutionary events and processes and (2) morphologically based species recognized in certain moderately conservative morphological classifications previously proposed for the genus. Further research will seek to improve sampling, especially in eastern Eurasia, and to develop more sensitive markers for distinguishing different lineages within (1) the remarkably genetically uniform *D. incarnata* group (diploids) and (2) locally differentiated populations of (in some cases unnamed) allotetraploids.

KEYWORDS: allopolyploid, autopolyploid, concerted evolution, *Dactylorhiza*, ecological differentiation, ITS ribosomal DNA, phylogeny, plastid microsatellites, speciation

INTRODUCTION

***Dactylorhiza* is a taxonomically problematic, evolutionarily complex genus. —** *Dactylorhiza* Necker ex Nevsky (1937) is a genus of terrestrial orchids with a circumboreal to warm-temperate distribution and centres of diversity in Europe and the Near East. Taxonomy of these dactylorchids is widely considered to have been complicated by relatively great morphological variability within species and high frequency of hybridization between species. Recent opinions expressed on the total number of species occurring in Europe, North

Africa/Macaronesia and the Near East range from six (Sundermann, 1980) to 61 (Delforge, 2005), whereas Averyanov (1990) estimated the number of species at 75 world-wide. The mean number of species recognized in published studies has increased progressively through time (reviewed by Pedersen, 1998) and is epitomized by the jump from 49 to 61 species between the first and third editions of Delforge (1993, 2005).

Refining, with justification, the taxonomy of the genus has become increasingly important because many putative *Dactylorhiza* species are declining and others have probably always been endangered narrow endemics. Reconciling morphologically and genetically circumscribed entities (recognized at whatever taxonomic level) is a necessary pre-requisite for a meaningful taxonomic hierarchy, which in turn is needed to accurately characterize their biogeography, ecology and conservation status. It is therefore cause for concern that Bateman & al. (2003: 22) concluded that "For the present (and despite considerable research effort), *Dactylorhiza* remains perhaps the most tantalizing of the dominantly European clades of Orchidinae, its phylogenetic history obscured partly by a combination of iterative hybridization and chromosomal instability, and partly by suboptimal species delimitation and misidentifications of chosen study organisms."

Many of the European dactylorchids belong to the *D. incarnata*/*maculata* polyploid complex, as defined by Hedrén (2001a, 2002), which is best viewed as consisting of three groups of species: *D. incarnata* s.l., *D. maculata* s.l., and allotetraploids formed by crosses between species of the first two complexes (Table 1). *Dactylorhiza incarnata* s.l. is an aggregate of diploid taxa (eight or more named taxa, depending on the author) that is morphologically variable but genetically homogenous according to data from isozymes (Hedrén, 1996) and amplified fragment length polymorphisms (AFLPs; Hedrén & al., 2001). *Dactylorhiza euxina*, endemic to the Near East, is a close relative of *D. incarnata*, albeit clearly distinct (Bateman, 2001; Hedrén, 2001b; Bateman & al., 2003), which is also involved in allopolyploidization (Hedrén, 2001b). These species are hereafter termed the *D. incarnata* group.

Dactylorhiza maculata s.l. (hereafter termed the *D. maculata* group) is a heterogeneous set of diploid (*D. fuchsii*, *D. saccifera*) and tetraploid (*D. maculata*) species that are more readily distinguished in peripheral portions of their respective ranges (e.g., Heslop-Harrison, 1951; Dufrêne & al., 1991; Bateman & Denholm, 2003). For example, in the British Isles, *D. fuchsii* and *D. maculata* are easily separated using floral and vegetative characters and have distinct ecological preferences: the former grows on alkaline to neutral soils that vary from unusually dry habitats to marshland, whereas the latter is an acid-heath specialist. In contrast, in Germany, Austria and eastern France some taxonomists wholly reject the distinction between *D. fuchsii* and *D. maculata* (e.g., Baumann & Künkele, 1988), whereas others identify as *D. maculata* plants that grow in habitats that in the British Isles would be occupied strictly by *D. fuchsii*. Hybridization, resulting in the reputedly near-sterile triploid *D.* ×*transiens*, occurs occasionally, especially where the two taxa have been brought into unusually close proximity, often by anthropogenic habitat disturbance (Bateman & Haggar, in press).

The importance of polyploidy. — *Dactylorhiza maculata* has long been viewed as an autotetraploid derivative of the diploid *D. fuchsii* (e.g., Heslop-Harrison, 1951, 1954), a view more recently given credence by their similar allozyme profiles (Hedrén, 1996). Nonetheless, nuclear

Table 1. General taxonomy and distribution of Western European species of *Dactylorhiza* discussed in this paper.

Ploidy	Species	Distribution
Diploid	*D. foliosa*	Madeira
Diploid	*D. fuchsii*	Western Europe, North Africa and Western and Central Asia; in the east replaced by *D. saccifera*
Diploid	*D. saccifera*	Italy, Greece, the Balkans
Diploid	*D. incarnata*	Western Europe, North Africa and Western and Central Asia
Diploid	*D. euxina*	Near East
Diploid	*D. sambucina*	Sweden to southern France, east to Greece and Eastern Europe
Diploid	*D. (Coeloglossum) viridis*	North Temperate Zone
Autotetraploid	*D. maculata*	Western Europe, but difficult to separate from *D. fuchsii* in Central and Eastern Europe and rare in southern Europe
Allotetraploid[a]	*D. majalis* s.l. (including *alpestris*, *elata*, *occidentalis*, *praetermissa*, *purpurella*, *sphagnicola*, *traunsteineri*)	Broadly distributed in Europe and Asia, with isolated occurrences in North America and Iceland; some taxa with localized distribution (e.g., *occidentalis* confined to Ireland)

[a]Thought to have originated as crosses between the *Dactylorhiza incarnata* group and the *D. maculata* group, but exact parentage highly speculative and the primary focus of this study.

ribosomal spacer DNA sequences (ITS nrDNA: Pridgeon & al., 1997; Bateman & al., 2003), AFLPs (Hedrén & al., 2001) and nuclear chalcone synthetase (Inda & al., submitted) clearly distinguish between these two taxa, indicating that they are better viewed as independent evolutionary units. *Dactylorhiza maculata* is more closely related to other, more clearly distinct diploids, such as *D. foliosa* (from Madeira) than it is to *D. fuchsii* (Hedrén & al., 2001; Bateman & al., 2003; Inda & al., submitted). Moreover, this relationship is reinforced by genetic data obtained in this study, which allowed us to distinguish easily between allopolyploids parented by the diploid *D. fuchsii* from rarer allopolyploids such as *D. sphagnicola* (Hedrén, 2003) and certain populations in northern Russia (Shipunov & al., 2004) that were parented by the tetraploid *D. maculata* s.str. Hence, *D. maculata* s.str. and *D. fuchsii* are here treated as separate species, although rank is a matter of choice and some of us (MH) would prefer to recognise these as subspecies because of the evidence found in this and other studies that they often hybridize to such an extent that distinguishing them becomes difficult.

Allotetraploid taxa possess a mixture of characters derived from members of the *D. maculata* and *D. incarnata* groups and are typically referred to as the *D. majalis* aggregate (sensu latissimo). Although allozymes (McLeod, 1995; Hedrén, 1996, 2001b) and AFLPs (Hedrén & al., 2001) confirmed their hybrid origin(s), neither technique identified precisely the parental lineages involved in polyploid formation. Multiple origins of allotetraploids have long been suspected (Heslop-Harrison, 1954, 1968). More recently, they have been demonstrated for some taxa in Sweden using allozymes (Hedrén, 1996) and PCR-RFLPs (Hedrén, 1996, 2003; Devos & al., 2003) in European Russia by combining ITS sequences with plastid and nuclear microsatellites (Shipunov & al., 2004), and by conventional and landmark-based morphometrics (Shipunov & Bateman, 2005). However, to obtain a more complete picture, these intentionally parochial integrated studies need to be expanded to a Europe-wide scale.

Relevance of plastid microsatellite and ITS sequences. — Repeating units of short DNA motifs termed microsatellites are abundant in the plastid genome of higher plants (e.g., Powell & al., 1995). Their variability makes them useful markers to study patterns of diversity within and between closely related species (Powell & al., 1995; Provan & al., 2001), particularly with respect to biogeography. They have already demonstrated their usefulness in genetic studies of orchids (Fay & Cowan, 2001; Cozzolino & al., 2003a, b; Forrest & al., 2004; Shipunov & al., 2004). Furthermore, they are easy to develop and can be used on degraded DNA, such as that typically extracted from herbarium specimens (Fay & Cowan, 2001). In orchids and most other angiosperms, the plastid genome is exclusively maternally inherited (Corriveau & Coleman, 1988), so these markers have the potential to identify the maternal parents of hybrids and thus to indicate the direction of the crosses between the *D. incarnata* and *D. maculata* groups that underpin allopolyploid events. They can also provide information on identity and geographical origin of maternal parents and number of times allotetraploid lineages of similar parentage have succeeded in becoming established.

The internal transcribed spacers (ITS) of nuclear ribosomal DNA have been widely used to reconstruct phylogenetic relationships because of their variability and ease of amplification with nearly universal primers (cf. Baldwin & al., 1995). They have also proved useful in the detection of hybrids because for a period following the hybridization event hybrids are likely to display both parental alleles; examples include *Paeonia* (Paeoniaceae: Sang & al., 1995), *Miscanthus* (Poaceae: Hodkinson & al., 2002) and *Anacamptis* s.l. (Orchidaceae: Bateman & Hollingsworth, 2004). However, this region can undergo concerted evolution (Hillis & Dixon, 1991), resulting in loss of one parental allele from taxa of hybrid origin (Wendel & al., 1995; Alvarez & Wendel, 2003; Chase & al., 2003). Previous molecular phylogenetic studies indicated that this phenomenon occurs in *Dactylorhiza*; only a single ITS allele was recovered by PCR from taxa known to be allotetraploids (Pridgeon & al., 1997; Bateman & al., 2003). Considering that ITS sequences from *D. incarnata*, *D. fuchsii*, *D. saccifera* and *D. maculata* differ by both substitution and length polymorphisms (Bateman & al., 2003), more detailed study could distinguish relative contributions of putative parents to at least some allotetraploids.

Important contrasts have been observed among groups of flowering plants in patterns of ITS evolution. For instance, loss of one parental allele occurred in polyploids estimated to have formed about 100 years ago in *Cardamine* (Brassicaceae: Franzke & Mummenhoff, 1999), whereas both parental types have been maintained in older, putatively Plio-Pleistocene allotetraploids of *Amelanchier* (Rosaceae: Campbell & al., 1997) and *Paeonia* (Paeoniaceae: Sang & al., 1995). Concerted evolution of ITS is consistently biased towards one parent in some cases (e.g., in *Cardamine*: Franzke & Mummenhoff, 1999), whereas in others it can convert in opposite directions in different allotetraploids formed within the same genus (e.g., *Gossypium*: Wendel & al., 1995; *Nicotiana*: Chase & al., 2003; Clarkson & al., 2004, 2005).

Fortunately, comparison of patterns derived from ITS sequences with those derived from plastid microsatellites can reveal such biases. In this study, we use these two categories of marker to study a large number of *Dactylorhiza* accessions sampled across a wide geographical area. Sampling was designed to determine whether we can discriminate among putative species in the *D. maculata* group, explore the extent to which they hybridize and

identify which of them has hybridized with a member of the *D. incarnata* group during formation of each identifiable allotetraploid. Additional goals were to investigate possible multiple origins of particular allotetraploid taxa and determine which of the two species was the maternal parent of each allotetraploid lineage. We also explored patterns of gene conversion in ITS nrDNA and assessed the degree to which it can provide a relative time frame for dating formation of these allotetraploid lineages.

MATERIALS AND METHODS

Sampling and DNA extraction. — A total of 399 accessions was analyzed, representing 177 localities and 44 named taxa, together sampled widely across the range of genus *Dactylorhiza* (Electronic supplement). Many of the populations were analyzed for more than one accession, particularly if suspected hybrids were observed in the field. Vouchers for many of the accessions consist of pickled flowers (most deposited in the Herbarium at the Royal Botanic Gardens, Kew). Samples of *Gymnadenia* s.l. (including *Nigritella*), which is the undoubted sister genus of *Dactylorhiza*, and *Pseudorchis*, which is a member of the *Platanthera* clade that is sister to *Dactylorhiza* plus *Gymnadenia* (Pridgeon & al., 1997; Bateman & al., 2003, 2006), constituted outgroups in the phylogenetic analysis of ITS sequences.

DNA was extracted from leaves or, more often, flowers using a 2× CTAB extraction protocol (Doyle & Doyle, 1987), but with some modifications. Although most DNA extractions were taken from either fresh or silica gel-dried materials (Chase & Hills, 1991), a few herbarium specimens up to 100 years old were also used. Most DNAs were further cleaned on a caesium chloride/ethidium bromide gradient (1.55 $g \cdot ml^{-1}$) or with QIAquick columns (Qiagen Ltd, Crawley, West Sussex, U.K., following the manufacturer's protocol for PCR reactions), although some were simply precipitated with ethanol and resuspended in 0.25× TE buffer without further cleaning.

Plastid microsatellites. — We first examined seven plastid regions in search of length variation (e.g., microsatellites or larger insertions/deletions) by sequencing these from a carefully selected reference set of species that included *D. fuchsii*, *D. maculata* and *D. incarnata*. This permitted us to select for further study four length-variable sites in three regions: the *trnL* intron, the intergenic spacer (IGS) between *trnL* and *trnF*, and the spacer between *trnS* and *trnG*. We then developed new primers that closely flanked the length-variable regions producing fragments less than 250 base-pairs (bp) in length. For sequencing, the PCR mix included 45 µL of 1.5 mM $MgCl_2$ Reddy PCR Master Mix 1.1X (ABgene Ltd, Epsom, Surrey, U.K.), 2 µL of 25 mM $MgCl_2$, 1 µL of 0.4% bovine serum albumin (BSA), 0.5 µL of each primer (100 ng/µL), and 2 µL of template DNA. The *trnL* intron and the *trnL-trnF* IGS were amplified using the primers c/d and e/f of Taberlet & al. (1991), whereas the *trnS-trnG* IGS was amplified using the primers of Hamilton (1999). The following PCR program was used: 4 min at 94°C, 28 cycles of 1 min at 94°C, 1 min at 48°C and 1 min at 72°C, with a final extension of 5 min at 72°C.

PCR products were purified using QIAquick columns (Qiagen Ltd) following the manufacturer's protocols. Both strands were sequenced using Big Dye Terminator 3.0 (Applied Biosystems Inc., ABI, Warrington, Cheshire, U.K.), and cycle sequencing products were run on an ABI 3100 Prism genetic analyzer, all following the manufacturer's protocols. Sequences were edited in Sequence Navigator and assembled in Autoassembler (both ABI). Alignments were performed manually in PAUP* 4.01b10 (Swofford, 2001), following the recommended procedures of Kelchner (2000).

The four length-variable plastid regions are either short, tandem, mixed-base repeats (two) or microsatellites (two with homopolymer repeats). More closely spaced primers were then designed to amplify these four regions: Dact Ms1, Dact Ms2 (both in the *trnS-trnG* IGS), Orch1 (*trnL-F* IGS) and Dact Msf (*trnL* intron; all in Table 2). One of each pair of primers was labelled with a fluorescent dye. The four length-variable plastid fragments were then amplified cheaply and efficiently in a single 10 µL PCR reaction; fragments were separated on an ABI 3100 genetic analyzer, and the length of each amplified fragment was determined with Genescan 3.1 and Genotyper version 2.0 (ABI). Each reaction contained 9.2 µL 2.5 mM $MgCl_2$ PCR Master Mix (ABgene), 0.2 µL of 0.4% BSA, 0.1 µL of each of the eight primers (100 ng/µL), and 0.4 µL of template DNA. The program used was 4 min at 94°C, 26 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final elongation of 10 min at 72°C. The four target microsatellites were then combined to define a number of plastid haplotypes. A minimum spanning tree was drawn by hand to summarize the relationships between the haplotypes found in the *D. maculata* group; no variation was discovered in the *D. incarnata* group, but this haplotype is so divergent from the others that it was excluded from the minimum spanning tree.

nrDNA markers. — First, the entire ITS region (ITS1 spacer plus 5.8S rDNA gene plus ITS2 spacer) was amplified using the primers 17SE and 26SE (Sun & al., 1994). Each 50 µL volume PCR reaction comprised 45 µL of 1.5 mM $MgCl_2$ Reddy PCR Master Mix 1.1X (ABgene), 1 µL of 0.4% BSA, 1 µL of dimethylsulphoxide (DMSO), 0.8 µL of ddH_2O, 0.6 µL of each primer (100 ng/µL), and 1 µL of template DNA. The program used was: 2 min at 94°C, 26 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min 30 s at 72°C, with a final extension of 5 min at

Table 2. List of the primers used in the study. All primer sequences read 5′→3′.

Fragment amplified	Primer	Sequence or reference
Dact Ms1	trnS	Hamilton (1999)
	Dact Ms1	CGT TGG AAC AAA AGA AGT AC
Dact Ms2	Dact Ms2	GAG TAA TAG TGT TTC TAA GAG
	trnG	Hamilton (1999)
Orch1	Orch1 F	Fay & Cowan (2001)
	Orch1 R	Fay & Cowan (2001)
Dact Msf	Dact Msf	CTA AGA AAT TAA GGG GGC TA
	trnL f	Taberlet & al. (1991)
Dfuch	ITS.dact.fuch F	ATT GAA TCG CTC CAT AAG AC
	ITS.dact.fuch R	ACC GCA TGA CGG GCC ATT CT
Dmac	ITS.dact.mac F	TGT GCC AAG GTA AAT ATG CA
	ITS.dact.mac R	TAG GAG CAA ACA ACT CCA CA

72°C. Sequencing procedures and sequence analysis were identical to those applied to the plastid regions, except for the addition of DMSO to the former (to reduce the effects of paired-stem formation on strand extension). Cloning using standard recombinant DNA techniques was required for ITS whenever direct sequencing revealed heterogeneity ascribed to the presence of multiple alleles. Although several thousand copies of nrDNA ITS are present in each individual dactylorchid, we will nonetheless employ the term allele in its broadest sense to describe each characteristic, collective nrDNA sequence (i.e., individual repeats are most unlikely to be identical, so this term is used to refer to the consensus sequence). To establish the relationships of each allele, phylogenetic analysis was performed in PAUP*4.01b10 using maximum parsimony; heuristic searches employed 200 replicates of random taxon entry order with tree bisection-reconnection (TBR) swapping, and no tree limit per replicate. These complete ITS sequences were submitted to GenBank (DQ022863 to DQ022894).

Length variation was observed in the alignment; moreover, some of the underlying insertions/deletions (indels) clearly distinguished among *D. incarnata*, *D. fuchsii*, *D. saccifera* and *D. maculata*. In our second phase of analysis, we therefore designed primers to amplify two short, length-variable fragments that taken together would be diagnostic of the alleles present in each accession analyzed (Table 3). These markers are expected to be codominant (unless gene conversion is complete) and thus are useful for determining the parental taxa involved in hybridization. The two polymorphic fragments in the ITS regions were amplified in a single tube. The PCR reaction contained 18 µL of 1.5 mM $MgCl_2$ PCR Master Mix (ABgene), 0.4 µL of 0.4% BSA, 0.4 µL of DMSO, 0.32 µL of H_2O, 0.24 µL of each of the four primers (100 ng/µL), and 0.4 µL template DNA. The PCR program followed that for the plastid regions but with an annealing temperature of 52°C. As in the plastid analysis, ITS fragments were run on a 3100 genetic analyzer, and fragment lengths for each accession were determined using Genescan 3.1 and Genotyper 2.0. When multiple alleles were found in a single accession, their relative proportions were estimated using the signal strength (peak height) on the original electropherograms. Although we recognize that these proportions are not rigorously defined, exact ratios are highly influenced by gene conversion and so are not relevant to our conclusions. However, the fact that the ratios observed are not consistent with expected simple ratios demonstrates that conversion has occurred.

RESULTS

Plastid haplotypes. — The four plastid regions were successfully amplified in all samples, including several herbarium samples collected up to 100 years ago. Only the two longer (approximately 220 bp) microsatellites, Dact Ms1 and Dact Ms2, failed to amplify from most DNAs extracted from herbarium specimens. Full data for the plastid microsatellites and ITS markers are presented in the Electronic supplement.

When analyzed in combination, the four plastid fragments defined 34 haplotypes. All species of the *D. maculata* s.l. group are characterized by a unique deletion in the *trnL-trnF* IGS; consequently, the Dact Msf fragment is 4 bp shorter than that of any other *Dactylorhiza* species. Haplotypes recorded in the *D. incarnata* group differ so much from those of other *Dactylorhiza* species that it is difficult to assess how they are related to those recovered from the *D. maculata* group. The *D. incarnata* group has been shown to be distantly related within *Dactylorhiza* to the *D. maculata* group in analyses of both ITS and plastid DNA data (Pridgeon & al., 1997; Pillon & al., 2006). Hence, they are omitted from the minimum spanning tree

Table 3. Summary of the main plastid haplotypes and ITS alleles found in the sampled taxa.

Plastid haplotype	ITS allele	Taxa
Diploid and autotetraploid		
A	IIIb-V	*fuchsii* (incl. *okellyi*, *cornubiensis*)
B	I	*maculata* (incl. *ericetorum*, *islandica*)
D	I	*foliosa*
A, C, G, W	VI-IIIb-V	*saccifera*
E	Xa	*incarnata* (incl. *cruenta*, *pulchella*, *coccinea*, *ochroleuca*, *borealis*)
K, Y	Xb	*euxina*
S1, S2	IIIc	*sambucina*
R1, R2, R3	IX	*romana*
F	VIIIa	*aristata*
J	XI	*iberica*
V1, V2, V3, V4, V5, V6	VIIIb, IX	*viridis* (formerly *Coeloglossum*)
Allotetraploid *incarnata* × *maculata* group		
A	IIIb-V	*majalis* s.str., *praetermissa* (incl. *junialis*), *traunsteineri* (incl. *lapponica*), *baltica*, *purpurella* (inc. *cambrensis*)
C	IIIb-V	*majalis* s.str., *praetermissa* (incl. *junialis*), *traunsteineri* (incl. *lapponica*), *alpestris*, *nieschalkiorum*
B	I	*elata* (Europe), *occidentalis* (incl. *kerryensis*), *sphagnicola*
O	IIIb	*elata* (North Africa)
Allotetraploid *euxina* × *maculata* group		
C	IIIb	*urvilleana*
Allotetraploid *euxina* × *incarnata*		
E	Xa	*armeniaca*

Note: For clarity, only the more common combinations of markers are shown, and rare individuals that are genetically atypical of their morphologically circumscribed species are omitted. In the case of allotetraploids the paternal ITS allele is excluded, as it was missing from many accessions.

that shows relationships inferred among the haplotypes; instead, this focuses on the *D. maculata* group (Fig. 1), which provides nearly all of the information that allows genetic differentiation of allopolyploid taxa. When we were assigning individual plants to a particular haplotype, we did not initially characterize them according to which species they had originally been assigned. Nonetheless, it soon became clear that most haplotypes could readily be ascribed to a particular species epithet.

***Diploids and autopolyploids*.** Haplotype A occurred in most accessions of *D. fuchsii* throughout its range, including the anthocyanin-deficient *D. fuchsii* var. *okellyi* from the western seaboard of Ireland and the anthocyanin-rich *D. fuchsii* var. *cornubiensis* from Cornwall (south-western England; cf. Bateman & Denholm, 1989). In contrast, our sole accession of a similar anthocyanin-rich form from the western seaboard of Scotland, *D. fuchsii* var. *hebridensis*, contained the B haplotype. This is the most common haplotype in *D. maculata* s.str., including putative varieties *ericetorum* from the British Isles and *islandica* from Iceland. Haplotypes of the B group all differ from the A haplotype by a 4 bp insertion in the Dact Ms1 fragment. Several other haplotypes (F, M, N, T) derived from the B haplotype were also found in *D. maculata*. The N haplotype is of particular interest as it was also found in *D. fuchsii* in many parts of its range (occurring in 14 out of the 108 samples of *D. fuchsii* examined). The N haplotype differs from the B haplotype by only a single change and from the A haplotype by two changes. Among rarer haplotypes, only the two samples of *D. maculata* from Ireland had the M haplotype; only our single accession from Portugal, *D. maculata* var. *caramulensis*, had the P haplotype, and just one accession of *D. maculata* from Sweden yielded the X haplotype (Fig. 1). In a few cases, plants initially identified as *D. fuchsii* on morphological criteria were shown to contain not the characteristic A haplotype but rather the B haplotype, more typical of *D. maculata*, and vice versa.

Dactylorhiza saccifera, a diploid long considered on morphological grounds to be closely related to *D. fuchsii* (cf. Landwehr, 1977), frequently contained the A haplotype typical of *D. fuchsii*. This was particularly true of samples from Croatia, where the two species are known to form intermediates, and Turkey. However, many accessions of *D. saccifera* contained one of the rarer haplotypes, G and W, which were not found in any other diploids and thus are probably more characteristic of this species. One accession of *D. saccifera* from Croatia had the E haplotype characteristic of *D. incarnata* (see below). Although the C haplotype differs only by a single base in one of the microsatellite markers (homopolymer; Dact Ms2) from the A haplotype, it was never found in *D. fuchsii*. However, the C haplotype was found in a single Greek individual of *D. saccifera*, the only putatively diploid accession to exhibit this haplotype. Otherwise, the C haplotype was found only in allotetraploids, predominating in some populations (see below and Electronic supplement).

In contrast with the *Dactylorhiza maculata* group, the diploid *D. incarnata* group maintained its already well-established record for genetic homogeneity (cf. Hedrén, 1996; Bateman, 2001; Hedrén & al., 2001; Bateman & al., 2003). Although our 55 accessions of the *D. incarnata* group encompassed six putative taxa and a wide geographical range, almost all yielded the characteristic E haplotype. The notable exception was *D. euxina* from the Near East, which had haplotypes K and Y, most similar to haplotype E of *D. incarnata*. Diploid species phylogenetically interpolated between the *D. incarnata* and *D. maculata* groups (Bateman & al., 2003), such as *D.* (formerly *Coeloglossum*) *viridis*, *D. aristata*, *D. sambucina* and *D. romana*, maintain distinct (and, in some cases, diverse) haplotypes.

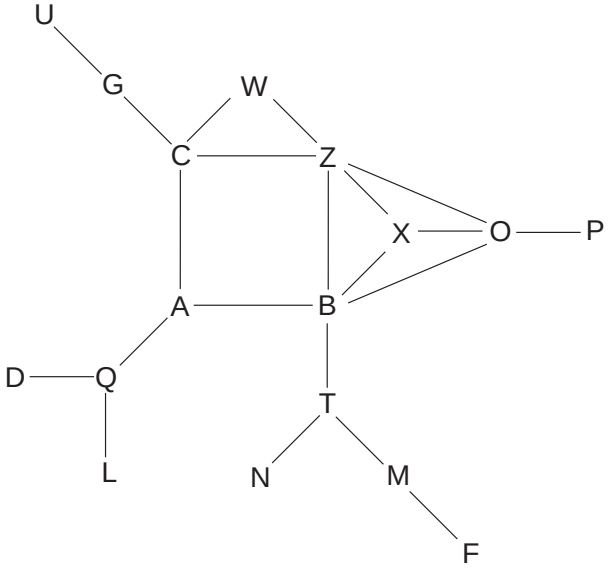

Fig. 1. Minimum spanning tree showing the relationships of the plastid haplotypes of the *Dactylorhiza maculata* group. All lines indicate single-step transitions. A is the most common haplotype in the diploid species *D. fuchsii*, whereas B is most common in the autotetraploid *D. maculata*. Haplotype C was found only once in a diploid (a single accession of *D. saccifera*), but it is common in several allotetraploids.

Allopolyploids. Allotetraploids yielded ten haplotypes, of which the most common, A, B and C, occupy central positions in the minimum spanning tree of haplotypes derived from the *maculata* group (Fig. 1). These haplotypes allow division of allotetraploids into two major categories that largely correspond to groups of morphologically defined taxa:

(1) The *Dactylorhiza majalis* group has predominantly the A (*fuchsii*-derived) and C haplotypes. It includes accessions from several morphologically defined species groups—the *majalis* group (also including *D. alpestris* and probably *D. pratermissa*), the *traunsteineri* group (also including *lapponica*), the *purpurella* group (also including *D. cambrensis*)—as well as the more geographically isolated *D. baltica* (southeastern Baltic region and northwestern Russia) and *D. nieschalkiorum* (Turkey).

Except for one accession of *D. saccifera* from Greece, the C haplotype was found only in some of the taxa in this category of allotetraploids: it was present in the majority of accessions of *D. majalis* (16 of 25 samples), *D. alpestris* (4 of 6) and *D. traunsteineri* (20 of 32). The C haplotype was rarely found in *D. praetermissa* (only 4 of 19) and never in *D. purpurella* (20). A geographical split was evident within *D. lapponica*; the single British sample had the C haplotype, whereas the three Swedish accessions had the A haplotype typical of *D. fuchsii*. The N haplotype, which occurred in a minority of populations of both *D. maculata* and *D. fuchsii*, was also found in three accessions of *D. majalis* from two populations in France, located close to populations of *D. fuchsii* that also contained this unusual haplotype.

(2) The *Dactylorhiza elata* group predominantly exhibits the *maculata* B or similar haplotypes. The group is geographically disparate, including not only the widespread western Mediterranean *D. elata* but also the Irish endemic *D. occidentalis* (incorporating *D. kerryensis*) and the northwest European *D. sphagnicola*. Western European *D. elata* reliably has the B haplotype, whereas most North African accessions yielded either the O or Z haplotypes. The similar X haplotype was found in one unnamed allotetraploid putatively locally synthesized in Sweden (M. Hedrén, unpublished data); otherwise it was found only in a single accession of *D. maculata*, also from Sweden.

The plastid haplotypes were sufficiently discriminating to allow "forensic horticulture" in the *Dactylorhiza elata* group. Apparently clonal clusters of plants labelled

D. elata that have long been cultivated at the Royal Botanic Garden Edinburgh (U.K.) and the National Botanical Garden Glasnevin (Ireland) have the D haplotype typical of *D. foliosa*, a commonly cultivated endemic from the isolated island of Madeira. These plants are most likely hybrids between *D. elata* and *D. foliosa* that were created in cultivation. Several other samples of unknown origin collected in gardens, such as the misnamed *D.* "*fuchsii* cv. Bressingham Bonus", also presented evidence of hybridization, and thus ultimately proved to be of little value in this study. The E haplotype characteristic of the *D. incarnata* group was rarely found in allotetraploids. Unsurprisingly, it occurred in the only analyzed sample of *D. armeniaca*, an allotetraploid derived from *D. euxina* × *incarnata* (Hedrén, 2003), although it was also found in some accessions (4 of 20) of the northwestern European *D. purpurella*. Figure 2 shows the geographical distribution of haplotypes found in the allotetraploids.

ITS alleles. — ITS fragments were successfully amplified for each accession, including most targeted herbarium samples. Sequencing of the complete ITS region was only undertaken if a potentially new allele was expected based on a novel fragment length because we were able to use length to distinguish among the ITS alleles of all parental diploids and *D. maculata* s.str. Relationships among the eleven ITS alleles detected in *Dactylorhiza*

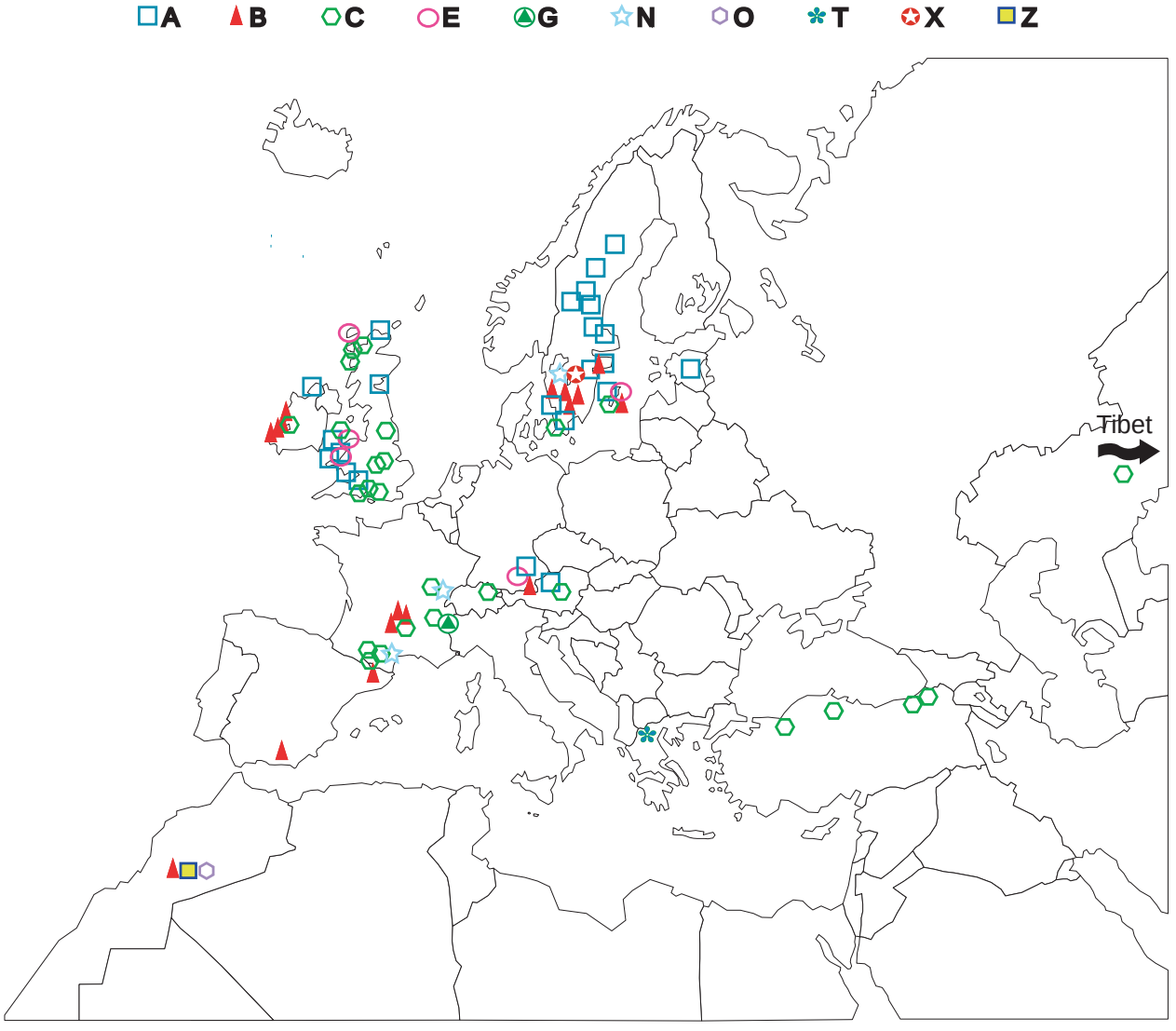

Fig. 2. Distribution of plastid haplotypes found in the allotetraploid taxa of *Dactylorhiza*. Each population is represented by at least one dot, although polymorphic populations are represented by as many dots as the number of haplotypes they contained. To aid presentation, populations from Anglesey (northern Wales) and Gotland (southeastern Sweden) are each represented as single populations. Symbols for haplotypes are explained on top of the figure.

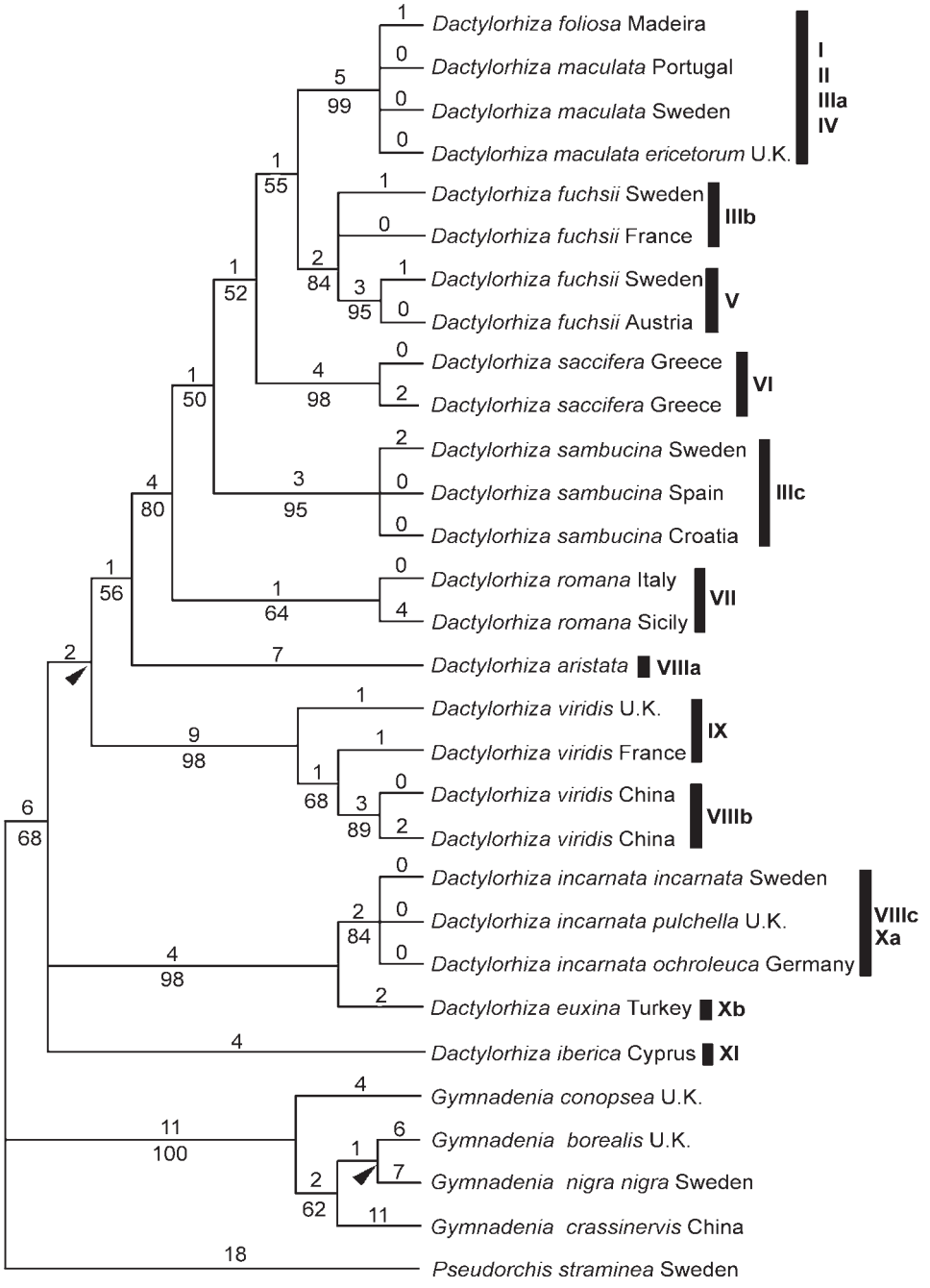

Fig. 3. Phylogenetic tree showing the relationships among the different ITS alleles found in diploid and autotetraploid *Dactylorhiza* based on DNA sequences of the entire ITS region. All allotetraploid taxa were excluded from this tree. Alleles occurring at different positions in the tree but represented by the same Roman numeral exhibit the same indel patterns and so these produced fragments of the same size differing in base substitutions. Numbers above and below branches are branch lengths and bootstrap percentages, respectively. Arrows indicate clades that collapse in the strict consensus tree.

are summarized in Fig. 3 (cf. Bateman & al., 2003), and examples of trace files for one ITS fragment obtained from five contrasting species are shown in Fig. 4.

Diploids and autopolyploids. Many putatively diploid samples yielded two, or even three, ITS alleles. Although presence of three alleles in a diploid appears unintuitive, theoretically it could occur if conversion had not yet reached completion when a plant crossed with another plant that possessed yet another allele. Less surprisingly, some of the allotetraploids were able to maintain three or occasionally four ITS alleles. We assigned an approximate ratio (1 : 1, 1 : 2 or 1 : 3) to accessions maintaining two or

more high-frequency alleles (Fig. 4). In addition, we noted cases when only a trace of an allele was present, defining a minor allele as one at least three times less frequent than the most abundant major allele present. No PCR bias was observed when we performed amplifications of the ITS fragments on samples of known ratios (i.e., when we used a mixture of the entire ITS regions previously cloned as template DNA; data not shown). We estimate from the above mixing experiments that we can begin to detect the presence of a minor allele at a frequency somewhere between 5% and 10%. However, because the alleles overlap in their respective fragment-length patterns, in some cases it was not possible to exclude the presence of a small amount of one additive type. For instance, when the 72 + 75 bp and 70 + 80 bp fragment pairs were both found in a single accession, we could not wholly reject the potential presence of a small proportion of the mutually overlapping 75 + 80 bp pattern.

Most accessions of *D. fuchsii* had either allele V or IIIb or both, whereas most accessions of *D. maculata* had the I allele (Fig. 3). In most *D. maculata* var. *islandica* accessions examined (4 out of 5), we found evidence for *fuchsii* ITS alleles as well as the typical *maculata* allele I. Allele VI was found in most samples of *D. saccifera*, but in many it was mixed with allele V or allele IIIb, indicating a close relationship with *D. fuchsii*. The Xa allele was found in all samples of *D. incarnata* s.l., rarely associated with the VIIIc allele, whereas the similar Xb allele was found in the closely related *D. euxina*.

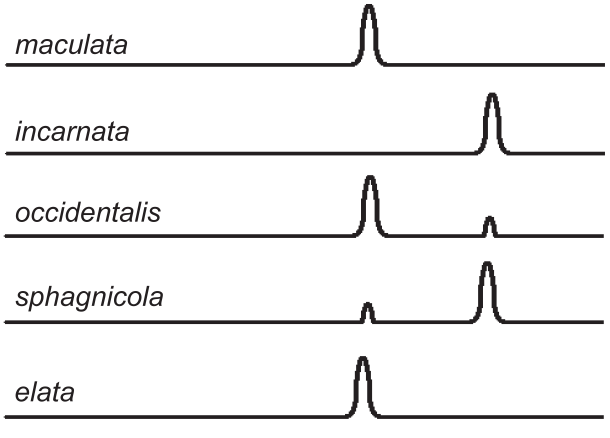

Fig. 4. Examples of traces obtained with one ITS fragment (Dmac). The autotetraploid *Dactylorhiza maculata* generally displays a 72-bp long fragment and the diploid *D. incarnata* an 80-bp long fragment. *Dactylorhiza occidentalis* (Ireland), *D. sphagnicola* (northwestern Europe) and *D. elata* (southwestern Europe and northwestern Africa) are all allotetraploids formed by hybridization between *D. maculata* (the maternal parent) and *D. incarnata*. Both parental alleles are present in both *D. occidentalis* and *D. sphagnicola*, but the maternal allele is dominant in the former and the paternal allele is dominant in the latter. In contrast, the paternal allele has been lost from *D. elata*.

Allotetraploids. In allotetraploids, several patterns of ITS alleles were observed. Overall, they either possessed between one and three alleles of the *D. maculata* group plus that of the *D. incarnata* group or they maintained alleles of only one of these two parental groups; in most cases it was the *D. incarnata* group that was not represented. Fig. 5 shows the geographical distribution of allotetraploids displaying ITS types from either one or both parental lineages. This demonstrates that complete loss of one parental allele is more common in North Africa and southern Europe, particularly in the characteristic allotetraploids of these regions, *D. elata* and *D. majalis* s.str. Alleles I, III and V of the *D. maculata* group were all frequent in the allotetraploids, whereas allele VI characteristic of *D. saccifera* was rare.

Among allotetraploids, ITS alleles mirrored the plastid haplotypes in revealing structured patterns that corresponded well with groups of named taxa:

(1) Members of the *Dactylorhiza majalis* group (characterized by *fuchsii* plastid haplotypes) had predominantly the *fuchsii* alleles V or IIIb, but degrees of evidence of the presumed *incarnata* parent varied both between and within species. Some *D. alpestris* possessed only the two *fuchsii* alleles, V and IIIb, whereas in others these were balanced by equal copy frequencies of *incarnata* allele Xa. The single sample of *D. baltica* had only the *fuchsii* allele V. Most of the 25 *D. majalis* samples had *fuchsii* V and IIIb alleles, only one exhibiting a trace of the *incarnata* Xa allele. The four specimens of *D. lapponica* had predominantly the *fuchsii* allele V, but the three accessions from Sweden also exhibited a trace of the *incarnata* Xa allele. The closely related *D. traunsteineri* was especially heterogeneous. A few individuals had predominantly *incarnata* Xa alleles (some also possessed the *incarnata* haplotype E), and only five accessions lacked any trace of *incarnata* Xa; nonetheless, in most individuals the *fuchsii* alleles V and IIIb were dominant. Another variable tetraploid, *D. praetermissa*, was distinguished mainly by the presence in half the accessions of the *saccifera* allele VI, which ranged in frequency from dominance in two accessions, to presence as just a trace in four. Otherwise, this species contained a mix of the two *fuchsii* alleles (V and IIIb) and occasionally the *incarnata* allele Xa, although this was rarely equal or dominant. *Dactylorhiza purpurella/cambrensis* combined the *incarnata* Xa allele with the *fuchsii* V allele, the former always with at least comparable frequency with the latter. Shifting the geographic focus to Turkey, our limited samples of both *D. urvilleana* and *D. nieschalkiorum* exhibited only the *fuchsii* IIIb allele.

(2) Members of the *Dactylorhiza elata* group (characterized by *maculata* B or related plastid haplotypes) had dominantly *maculata* alleles (I, or more rarely its variant, IV). The notable exception was *D. sphagnicola*,

which dominantly had the *incarnata* allele Xa, most often occurring in a 3:1 ratio with the *maculata* I allele. The single analyzed sample of *D. kerryensis* had no detectable copies of the *incarnata* allele Xa, whereas the morphologically similar *D. occidentalis* s.str. showed a 3:1 ratio of *maculata* to *incarnata* alleles. As with plastid haplotypes, a clear distinction was evident between accessions of *D. elata* from North Africa versus those from southwestern Europe. The nine samples from North Africa exhibited only the *maculata* IIIa allele (a fragment equal in length to one of the common *fuchsii* alleles but showing a different set of substitutions) or the *maculata* IV allele, with no trace of the *incarnata* X allele. In contrast, the six samples of *D. elata* from Europe had the *maculata* allele I, occasionally supplemented with a trace of the *incarnata* Xa allele.

Correlation between patterns in ITS alleles and plastid haplotypes. — There was a significant correlation between plastid haplotypes and ITS types found in the allotetraploids ($p < 0.001$; χ^2 test). Most notably, haplotypes A and C were most frequently associated with ITS alleles IIIb, V and VI (*fuchsii* and *saccifera* markers, respectively), whereas the B haplotype was most frequently associated with ITS allele I (*maculata* markers).

The *Dactylorhiza maculata* group proved far more genetically diverse than *D. incarnata* s.l., containing four common ITS alleles and 16 haplotypes. Many samples displayed both *D. fuchsii* and *D. maculata* alleles. The A haplotype typical of *D. fuchsii* was found in a few accessions that had been designated at the time of collection as *D. maculata*, and the B haplotype typical of *D. maculata*

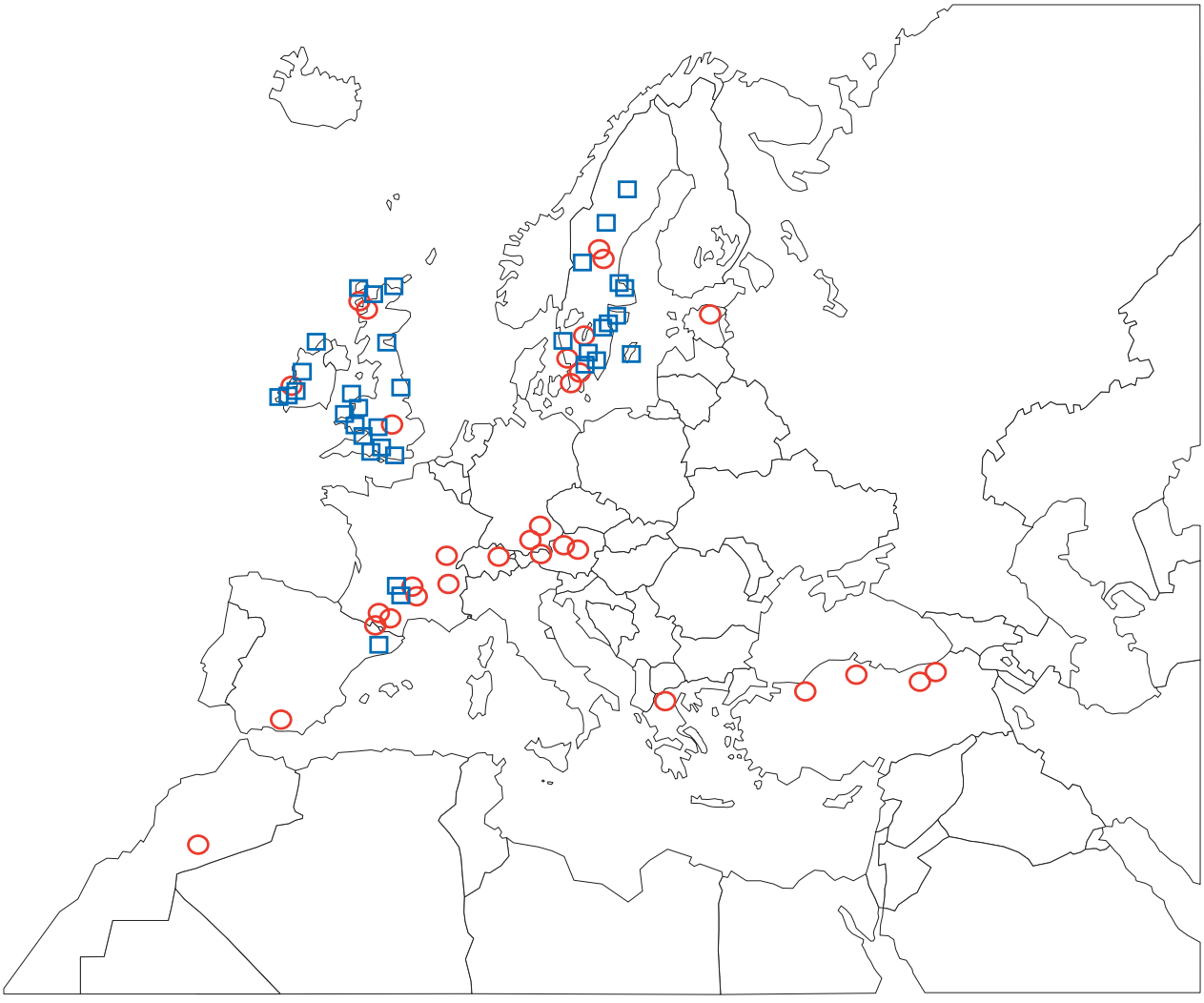

Fig. 5. Concerted evolution of ITS in allotetraploids across Europe and adjacent regions. Populations of allotetraploids containing ITS alleles of both parental groups, *D. incarnata* s.l. and *D. maculata* s.l., are represented by blue squares. Populations from which one parental allele has been lost are represented by red circles. To aid presentation, populations from Anglesey (northern Wales) and Gotland (southeastern Sweden) are each represented as single populations.

was similarly found in a few samples initially identified as *D. fuchsii*. The frequency of individuals that combined *fuchsii* and *maculata* ITS alleles showed a geographical trend, being frequent in Austria, occasional in France and rare in the British Isles (Fig. 6). Most *D. saccifera* exhibited the A haplotype shared with *D. fuchsii* and had a mixture of ITS alleles characteristic of both *D. fuchsii* and itself (i.e., alleles III and V versus VI). However, some Greek samples contained only the typical *D. saccifera* allele VI, combined with plastid haplotypes G and W that were not found in any other diploid species. The *D. saccifera* allele VI was also found in a few *D. fuchsii* from Croatia and the British Isles. In Britain, allele VI was detected only in *D. fuchsii* individuals cohabiting with allotetraploid *D. praetermissa*, many of which also exhibited the VI allele. This indicated introgression between the two species, which differ in ploidy.

DISCUSSION

Comparison with previous studies of *Dactylorhiza* that used similar molecular markers. — Our study of plastid haplotypes shares several accessions with a plastid PCR-RFLP study by Hedrén (2003), which in turn overlapped taxonomically with the PCR-RFLP study by Devos & al. (2003). Not surprisingly, all three plastid investigations yielded broadly similar results. Our ITS work built on previous molecular phylogenetic studies by Pridgeon & al. (1997) and Bateman & al. (2003), but here we have sampled far more extensively within the target species. The main advances in our study are dense sampling of individuals and amalgamation of the two previously separate lines of evidence from maternally inherited plastid regions and a biparentally inherited nuclear region.

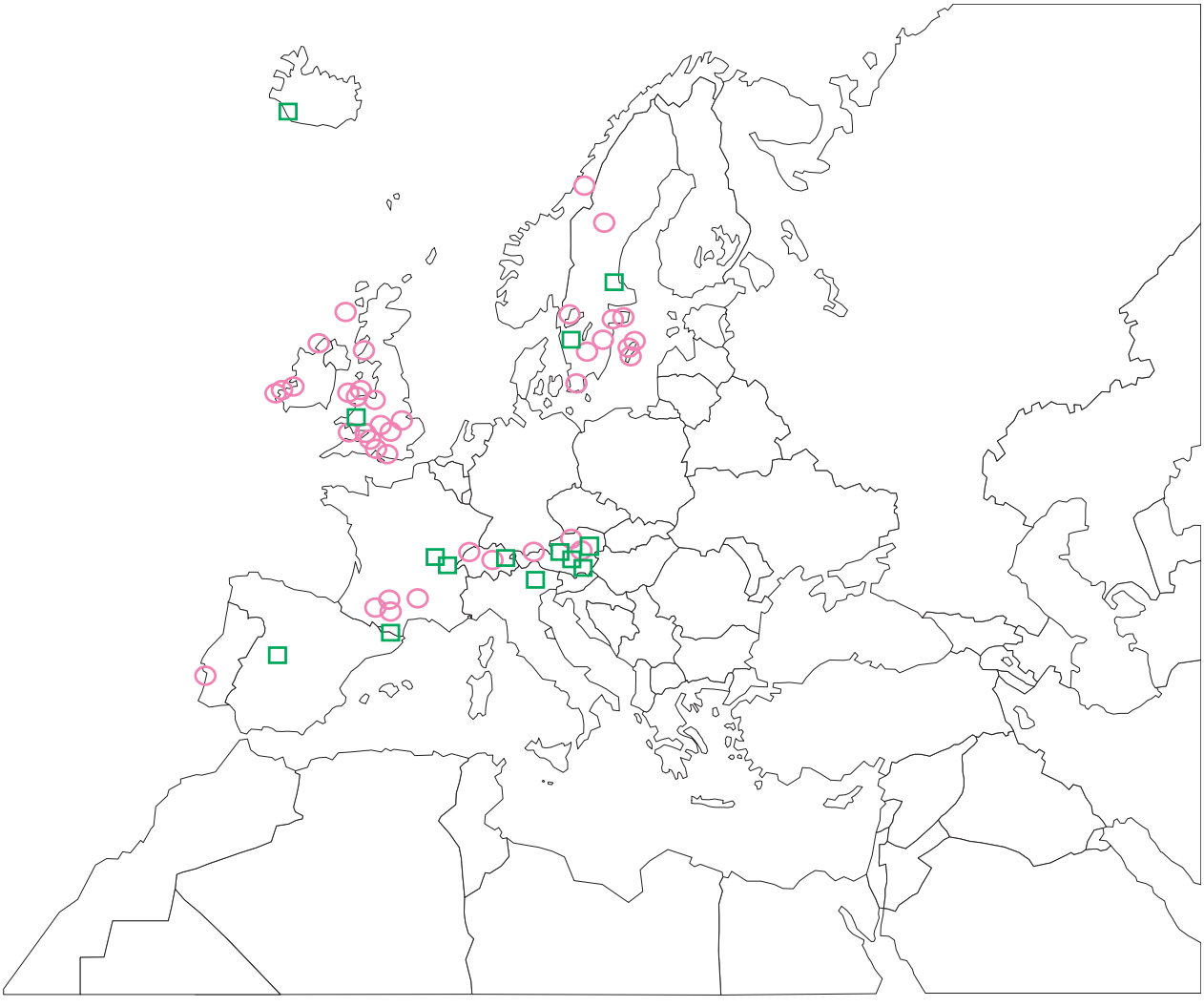

Fig. 6. Mixing of *Dactylorhiza maculata* s.str. and *D. fuchsii* ITS alleles across Europe. Populations of the *D. maculata* group that contained ITS alleles characteristic of *D. fuchsii* and those characteristic of *D. maculata* are represented by green squares, whereas populations that contained alleles of only one of the two species are represented by purple circles.

In all accessions examined in our study, a strong positive correlation was observed between particular plastid haplotypes and particular ITS alleles. This allows us to address Hedrén's (2003) concern that, on the basis of plastid data alone, he could not distinguish between ancestral polymorphisms and those due to recent hybridization. Our interpretation is predicated on the assumption that if ancestral polymorphisms were segregating then no strong correlation would be expected between markers from plastid and nuclear genomes (cf. Ramsey & Schemske, 1998; Morjan & Rieseberg, 2004). Thus, for the parental taxa *D. incarnata*, *D. fuchsii* and *D. maculata*, most populations in which more than one marker was detected are assumed to result from hybridization rather than retention of ancestral polymorphism.

The positive correlation between ITS alleles and plastid haplotypes constitutes further evidence that the diploid *D. fuchsii* and autotetraploid *D. maculata* should be regarded as distinct species, even though there is clearly current gene flow across the divide of their different ploidy levels in many parts of their shared range (Fig. 6: cf. Devos & al., 2003; Hedrén, 2003; Shipunov & al., 2005). These species undoubtedly experienced a period of isolation in the past that was sufficient to allow them to develop their distinct morphologies and ecological preferences; some of their secondary contact has resulted from post-glacial migration patterns and also recent human disturbance of the landscape. Evidence that hybridization between these two taxa is a recent phenomenon comes from the fact that the mixing of haplotypes and ITS alleles now observed in these species rarely occurs in allotetraploids. If local populations of *D. fuchsii* show evidence of introgression with *D. maculata*, then locally formed allotetraploids should show similar mixtures of markers, contrary to our observations. Locally formed allotetraploids could have formed from single hybridizations, so it might be expected that such allotetraploid populations would be genetically consistent, but it would be highly unlikely that all parental *D. fuchsii/maculata* parents of such hybrids would themselves never be introgressed individuals. Most authors are also happy to treat *D. foliosa* (from Madeira) as a distinct species. This species is more closely related to *D. maculata* than the latter is to *D. fuchsii*, so if *D. foliosa* is recognized as a distinct species, then so must *D. fuchsii* and *D. maculata* (if DNA data are considered to have a bearing on which taxa are to be recognized).

Molecular markers developed for this study were subsequently employed in the same laboratory as part of a more geographically constrained study that focused on dactylorchids of European Russia (Shipunov & al., 2004, 2005; Shipunov & Bateman, 2005). This demonstrated that in Russia the N haplotype, which is most similar to the B haplotype of *D. maculata* but is also commonly found in *D. fuchsii*, occurred only in *D. maculata*. This observation supports our inference that the N haplotype originated in *D. maculata* and subsequently became introgressed into *D. fuchsii*. In European Russia and Georgia, populations of the *D. incarnata* group contain not only the typical E haplotype but also the similar H haplotype. Allotetraploids occurring in northern Russia, most notably *D. baltica*, exhibited a mixture of *fuchsii* and *maculata* markers, but lacked the C haplotype commonly found in southern Europe. Overall, the patterns observed in European Russian *Dactylorhiza* revealed few new haplotypes and are highly congruent with those reported here for western European *Dactylorhiza*.

Employing ITS alleles as nuclear markers. — In this study we took advantage of indels in ITS to amplify length-variable fragments that are usually codominant, unless alleles are lost through concerted evolution/gene conversion. Our technique allowed us to screen a large number of samples, including herbarium specimens that typically yield highly degraded DNA. We amplified both fragments in the same reaction in small volumes, thereby establishing a highly efficient screening method that did not require cloning when two or more alleles were present. Analysis of electropherograms from direct sequencing of PCR products containing two or more alleles was made difficult due to this length variation. However, we found that coincidence of these indels with alleles discovered earlier through sequencing (and in some cases cloning) was sufficient to identify each allele when the two fragments were considered together. Thus, we made the conservative assumption that neither fragment alone was diagnostic.

No apparent bias was detected when evaluating mixtures of DNA of known ratio, as previously observed by Rauscher & al. (2002). However, we observed only infrequently the simple allelic ratios expected from hybrids, indicating that in many cases copies of some alleles were being eliminated or back-crosses were occurring; both processes "reinforce" one allele relative to others. For example, although one accession of *D. fuchsii* from Sorvilier, Switzerland, had the expected diploid chromosome number of $2n = 40$ (L. Hanson, M. Fay & M. Chase, unpublished data), it also had the *D. fuchsii* IIIb and V alleles in a 1 : 2 ratio, which would have been most parsimoniously interpreted as indicating that this plant was triploid rather than diploid. It also proved difficult to analyze patterns from individuals that yielded more than two alleles because one particular fragment length can sometimes be attributed to more than one allele (Fig. 3). Nonetheless, we could routinely detect alleles representing only 10% of the total copies present and could reach 5% with reasonable confidence, showing that this technique is more sensitive than direct sequencing (Rauscher & al., 2002).

In summary, ITS fragments were a useful tool for determining parentage of many accessions, but observed

ratios of alleles were not reliably informative about ploidy levels, nor could complete absence of an allele be conclusively demonstrated. Reinforcement of an allele due to backcrossing of the progeny with one parent could also be masked by effects of concerted evolution and/or gene conversion.

Significance of concerted evolution in ITS. — In allotetraploids, a degree of homogenization occurred in the great majority of cases because parental ITS alleles were generally detected in non-Mendelian proportions. In many cases, one parental allele was apparently "lost" (or, more accurately, was reduced to less than 5%–10% of all copies present: Fig. 5). Comparison with maternally inherited plastid microsatellites showed that in most cases the missing allele was the paternal one, generally that derived from the *D. incarnata* group. For example, examination of a single accession of *D. armeniaca*, a recently described allotetraploid derived from hybridization between *D. euxina* and *D. incarnata* (Hedrén, 2001b), showed that it lacked the *euxina* ITS allele but possessed a typical *incarnata* plastid haplotype, indicating conversion to the ITS allele of the maternal parent. Among allotetraploids there were two main exceptions to the rule of maternal conversion. *Dactylorhiza sphagnicola* always had a majority of the *D. incarnata* (Xa) ITS allele in spite of having the B haplotype characteristic of *D. maculata*, whereas the majority of samples of *D. purpurella* (including *D. cambrensis*) combined the A haplotype of *D. fuchsii* with the *incarnata*-derived Xa ITS allele, which occurred in equal or more often greater frequency than the *fuchsii*-derived V allele. In these two unusual cases concerted evolution appears to be favouring the paternal ITS allele rather than the maternal allele.

Two contrasting mechanisms are most often proposed for concerted evolution of rDNA: unequal crossing-over and gene conversion (Hillis & Dixon, 1991). The strong parental bias observed in *Dactylorhiza* favours the hypothesis of gene conversion (Hillis & al., 1991) because it is an unlikely outcome of unequal crossing-over, nor is it clear how a maternal effect could persist across the several generations needed to reduce one allele to an undetectable level. Studies of nuclear ribosomal ITS and the 18S rDNA intron in the marine macroalga *Caulerpa* (Durand & al., 2002) and of ITS and IGS in *Drosophila* (Polanco & al., 1998) similarly revealed differential evolution in both directions and rates of concerted evolution that are incompatible with the unequal crossing-over model. Moreover, homogenization of ITS demonstrably occurs in flowering plants even when multiple ribosomal clusters occupy different chromosomes (Wendel & al., 1995; Chase & al., 2003).

Exploring origins and migration patterns of allotetraploids. — One important goal of this study was to evaluate the possibility of using ITS conversion rates to estimate the period of time elapsed since the initial hybridization event that preceded successful establishment of each allotetraploid lineage and thereby to explore likely implications of contrasting dates of origin for subsequent evolutionary histories of the resulting lineages. It soon became apparent from our data that most of the allotetraploid samples from southern Europe had lost one of their parental ITS alleles, whereas both parental alleles were typically still detectable in allotetraploids from the British Isles and Scandinavia (both examined in this study) and from northern Russia (reported by Shipunov & al., 2004; Fig. 5). Although acknowledging limits of sensitivity of our technique for detecting low-frequency alleles, our results were highly internally consistent; within most study populations, either all individuals had retained both parental types or all had lost one. Because most northern allotetraploids have retained at least some evidence of both parental alleles, indicating that concerted evolution has not reached completion, we hypothesize that they are younger than southern allotetraploids (in making this assumption we recognize that several other factors, most notably contrasts in effective population sizes, can also influence rates of change in the frequency of ITS alleles). During the Quaternary, northern Europe experienced several cycles of thick ice cover followed by recolonization. The whole of Scandinavia and much of the British Isles were covered with ice 18,000 years ago, and periglacial conditions persisted until 11,700 years ago. Thus, it is tempting to speculate that in Europe the northern allotetraploids became established post-glacially, whereas the southern allotetraploids that have largely eliminated one parental ITS allele may antedate the last glacial maximum.

Combining the degree of gene conversion with inferred parentage suggests a quadripartite classification of western European allopolyploid dactylorchids according to their respective parentages and putative relative dates of origin. Older allotetraploids that lack one parental ITS allele can be divided into those derived from hybridization between *D. incarnata* s.l. and *D. fuchsii* (*D. majalis*) and those derived from hybridization between *D. incarnata* s.l. and *D. maculata* (*D. elata*). Similarly, younger allotetraploids that maintain evidence of both parental ITS alleles can be divided into those derived from hybridization between *D. incarnata* s.l. and *D. fuchsii* (e.g., *D. praetermissa*, *D. purpurella*, *D. traunsteineri* s.l., *D. baltica*) and those derived from hybridization between *D. incarnata* s.l. and *D. maculata* (e.g., *D. occidentalis*, *D. sphagnicola*). Application of a range of molecular techniques has further teased apart the two categories of younger allotetraploids (e.g., Hedrén, 1996, 2001, 2002, 2003; Hedrén & al., 2001; Devos & al., 2003) and is particularly effective when combined with morphometric data gathered from the same individuals collected for genetic

analyses (McLeod, 1995; Bateman, 2001; Shipunov & al., 2004; Shipunov & Bateman, 2005).

Local origins have already been inferred for some of the younger allotetraploids, indicating that they evolved in situ in northern regions (Hedrén & al., 2001; Hedrén, 2002; Shipunov & al., 2004; Bateman, 2006, in prep.). However, we infer that the majority of allotetraploids have recolonized northern areas by relatively recent migration from the south because southern markers such as the C haplotype are widespread in many allotetraploids but almost completely absent from their presumed progenitors among diploids and autopolyploids.

If so, the pattern of colonization inferred for the *D. incarnata/maculata* complex is unusual. In most other temperate clades that admix diploid and polyploid species, polyploids have proven to be strong colonizers of Arctic regions (Abbott & Brochmann, 2003), whereas their diploid progenitors have remained much further south. Although we differ in our respective opinions regarding the relative average colonization abilities of the diploids, autopolyploids and allopolyploids, it is evident that one category is not clearly superior to the others, whereas in many other groups polyploids are competitively superior in the boreal zone. Possible reasons for this observation include the fact that, contrary to many other polyploid groups such as the fern *Asplenium* (Vogel & al., 1999), polyploid species in the *Dactylorhiza* complex do not have colonization ability enhanced by apomixis. Also, Dijk & Grootjans (1998) argued that, at least in the Netherlands, *D. majalis* s.str. and *D. praetermissa* prefer more fertile soils than do *D. maculata* and *D. incarnata*, perhaps indicating that these allotetraploids are only able to successfully colonize a narrower range of habitats.

***Dactylorhiza incarnata* group. —** As previously observed with allozymes (Hedrén, 1996), AFLPs (Hedrén & al., 2001), PCR-RFLPs (Hedrén, 2003) and ITS sequencing (Pridgeon & al., 1997; Bateman & al., 2003), remarkably little genetic variation was observed within the *D. incarnata* group (here defined broadly to include *D. euxina*), even though we sampled several morphologically circumscribed taxa that together spanned a large part of its overall geographical range. Each individual analyzed yielded only the plastid haplotype E, and most individuals contained only the ITS allele Xa. However, allele VIII, which differs from allele Xa only in possessing a 2 bp deletion, occurred alongside allele Xa in a few samples from Wales and Ireland. The only sample from Turkey analyzed had two ITS alleles, both broadly resembling allele Xa but distinguishable by several substitutions. Extended analysis of *D. incarnata* s.l. from Turkey for allozymes and plastid markers indicated that the species is much more molecularly variable in southeastern Europe than in northern and western Europe (Hedrén, 2001b, in prep.). The endemic Turkish diploid *D. euxina* had two unique haplotypes and a distinct ITS allele, although as expected both were most similar to those found in *D. incarnata*.

Dactylorhiza incarnata is also profoundly distinct from the *D. maculata* group as observed with allozymes (Hedrén, 1996, 2001b), but a few cases of possible introgression have been observed. The plastid haplotype and ITS allele X of *D. incarnata* were found in a few specimens of *D. saccifera* from Croatia. We also found on single occasions the *fuchsii* ITS allele V and *fuchsii* haplotype A in two samples of *D. incarnata* subsp. *pulchella*. Furthermore, Shipunov & al. (2004) demonstrated that the *fuchsii* V allele is widespread in some populations of *D. incarnata* in Russia. Using RFLPs, Hedrén (2003) and Devos & al. (2003) also revealed some evidence of hybridization and/or subsequent introgression between *D. incarnata* and members of the *D. maculata* group in Sweden. Thus, limited gene flow may still be possible between the two divergent parental groups, either directly or via allotetraploids as a bridge (a decidedly less readily detected process).

***Dactylorhiza maculata* group. —** Although morphologically based studies are divided on whether to recognize *D. maculata*, *D. fuchsii* and *D. saccifera* as separate species (cf. Dufrêne & al., 1991; Bateman & Denholm, 1989, 2003; Ståhlberg, 2007), there is growing molecular evidence that the former two represent lineages evolved in isolation for a considerable period of time, and most of us (not MH) argue that all three are best regarded as distinct species. They have distinct ITS sequences (Fig. 3: Pridgeon & al., 1997; Bateman & al., 2003) and, at least in Sweden, are readily distinguished using AFLP data (Hedrén & al., 2001). In this study we found markers in both the plastid and the nuclear genomes that clearly distinguish among the three taxa. Admittedly, we have also observed mixing of these markers in several accessions, but we believe that this is due to secondary hybridization and/or introgression rather than incomplete lineage sorting. In this context, an introgression zone including two distinct genotypes within *D. maculata* s.str. has recently been documented in Sweden (Ståhlberg, 2007). As regards *D. saccifera*, increased sampling is required from the Balkans to decide whether the taxon is distinct from *D. fuchsii*; the distinctive ITS alleles (Fig. 3) found in most accessions of *D. saccifera* indicates that it too existed in isolation from both *D. fuchsii* and *D. maculata* for a significant period of time. Another line of evidence for their distinctiveness comes from the allotetraploids, which allow us to infer genetic content of their parents. In allotetraploids, markers characteristic of *D. fuchsii* and *D. maculata* are rarely combined, indicating that at the time and place of the formation of the allotetraploids *D. fuchsii* and *D. maculata* were clearly distinct and not hybridizing as extensively as they are today.

A few samples identified as *Dactylorhiza maculata* that had some *D. fuchsii* markers (ITS type and haplotype) or vice versa provide circumstantial evidence of hybridization or introgression. Artificial hybridization of these two species has revealed substantially greater fertility in back-crosses than in the first generation (Bateman & Haggar, in press). Distinguishing between *D. fuchsii* and *D. saccifera* also proved challenging. The most problematic situation was encountered in the Alps region (eastern France, Switzerland and Austria: Fig. 6), where many samples exhibited both *fuchsii* and *maculata* markers, whereas such cases are rarer in the British Isles and southern Scandinavia. A recent set of 20 accessions of *Dactylorhiza* sampled from acidic sites that should have been assigned to *D. maculata* if they were found in western Europe (M. Chase, G. Fischer & D. Dockrel, unpubl.) showed that every one of them was a recent hybrid of *D. fuchsii* and *D. maculata* (recent because they all exhibited both ITS alleles). These genetic observations correlate well with morphology, as field botanists regard the two species as more difficult to distinguish in central Europe (Heslop-Harrison, 1951; Dufrêne & al., 1991; Bournérias & Prat, 2005) than in marginal areas in the northwestern part of the Continent (Bateman & Denholm, 1989, 2003; Pedersen, 1998, 2004). Furthermore, putative species morphologically intermediate between *D. fuchsii* and *D. maculata*, such as *D. savogiensis* and *D. sudetica*, have been described in the Alps and contiguous uplands (e.g., Delforge, 2001). Unfortunately, the ploidy of these plants remains unknown.

The fact that several ITS types were still detected in some accessions of the *D. majalis* group indicated that gene conversion had not reached completion, in contrast with southern allotetraploids. If taken together with the absence of mixtures of *fuchsii* and *maculata* markers in allotetraploids, this indicates that most hybridization/introgression events involving allotetraploids occurred recently, after the formation of at least most allotetraploids. They most likely originated soon after the re-establishment of sympatry between these species when they expanded postglacially out of separate glacial refugia. In this context, it is noteworthy that the alpine region, where the relationship between *D. fuchsii* and *D. maculata* appears especially complex, is considered as an important zone of secondary contact among post-glacial migrants (termed a "suture zone" by Hewitt, 2000). However, all markers used in this study are susceptible to rapid fixation, either because they are maternally inherited (plastids) or because they are subject to concerted evolution (ITS). Such markers can reveal ancient genetic exchange between species even though the species themselves apparently remain morphologically distinct, as has recently been observed among the anthropomorphic species group within the genus *Orchis* s.str. (M. Fay & al., unpublished data). Such complex situations are best explored further by examination of multi-locus markers such as AFLPs and/or a selection of biparentally inherited nuclear microsatellites/introns in low-copy genes.

The occurrence of some populations that combine markers typical of *Dactylorhiza fuchsii* and *D. maculata* can be explained by recent local hybridization, despite their contrasting ecological preferences. For example, the karstic landscape of the Burren in western Ireland supports mainly the calcicole *D. fuchsii*, but small pockets of peat-rich residual soils dotted across the limestone support the calcifuge *D. maculata*, bringing the two species into intimate proximity. They also frequently meet in mixed habitats in Scandinavia (Ståhlberg, 2007). Admittedly, many other sites where the two species co-occur reflect recent anthropogenic disturbance. Nonetheless, combinations of markers were also found in several populations where only one of the two species was found, most notably in Iceland where *D. fuchsii* is not known to now occur. Some samples confidently identified as either *D. fuchsii* or *D. maculata* were found to contain markers characteristic of the other species. For example, near Llangurig, Wales, plants with morphology characteristic of *D. maculata* and growing in typically acid soils yielded markers of both species, even though *D. fuchsii* was not observed growing in the immediate vicinity. Introgression between the two taxa is a more likely explanation of such observations, probably occurring in both directions.

In terms of likely underlying processes, transfer of markers from the diploid *Dactylorhiza fuchsii* to the tetraploid *D. maculata* is possible via unreduced gametes in *D. fuchsii*. Although *D. maculata* is generally accepted to be an autotetraploid (Hagerup, 1944; Heslop-Harrison, 1951), few reliable chromosome counts are available (confusion regarding which morphological characters best distinguish between *D. fuchsii* and *D. maculata* casts doubts on some determinations; e.g., Tanako & Kamenoto, 1984). Thus, *D. maculata* may still be diploid in some parts of its range. Conversely, other observations indicate that in central Europe tetraploidy may occur in the typically diploid *D. fuchsii* (e.g., Hedrén, 2002; Bournérias & Prat, 2005; Ståhlberg, 2007). Also, Hagerup (1944) noted the occasional development of haploid embryos without fertilization in both *D. fuchsii* and *D. maculata* s.str., results later confirmed by Heslop-Harrison (1957). If such embryos were viable they could permit gene flow from (auto)tetraploids to diploids. Thus, the theoretical barrier to gene flow between the putatively diploid *D. fuchsii* and tetraploid *D. maculata* is probably a less profound obstacle than is generally supposed; moreover, such a barrier has been overcome in *Taraxacum*, for example (Menken & al., 1995).

Except for a unique ITS allele, *Dactylorhiza saccifera* appears to be connected to *D. fuchsii* by populations with

atypical combinations of plastid markers. *Dactylorhiza fuchsii* ITS alleles were found in *D. saccifera* in Croatia, Turkey and Greece, which was the only region where some samples of *D. saccifera* (1) did not have markers typically found in *D. fuchsii* and (2) exhibited distinct haplotypes. We also detected variable populations of *D. saccifera* from Greece. In addition, we found the *D. saccifera* ITS allele VI in a few samples of *D. fuchsii*, not only from Croatia where the two species co-occur, but also in Britain, 600 km from the nearest extant populations of *D. saccifera* (as explained below, this apparent enigma could reflect occasional hybridization between *D. fuchsii* and *D. praetermissa*).

Several observations support the origin of *D. maculata* in North Africa or the Iberian Peninsula. The first is the occurrence only in the Macaronesian island of Madeira of *D. foliosa*, a diploid species that resembles *D. maculata* both morphologically and especially genetically (Pridgeon & al., 1997; Hedrén & al., 2001). We also found a distinct ITS allele in the samples of *D. elata* from Morocco (one parent of which was probably *maculata*-like); this differs from the common *D. maculata* allele only in lacking a distinctive 8 bp indel and thus appears to be plesiomorphic relative to all ITS alleles recovered from the *D. maculata* group. We also identified several unique but *maculata*-like plastid haplotypes in dactylorchids from this region. Corresponding morphological diversity is indicated by recognition by some authorities of three segregates from *D. maculata* s.str. that are endemic to Morocco, Algeria and the Iberian Peninsula—*D. maurusia*, *D. battandieri* and *D. caramulensis*, respectively (Delforge, 2005)—although they are at best only subtly morphologically distinct.

Dactylorhiza saccifera may have had a refugium in Greece because this is the only area where genetically it is both diverse and relatively distinct from *D. fuchsii*; also, Greece is the centre of the present range of *D. saccifera*. Although we did not include samples of *D. saccifera* from Italy, another refugial candidate, preliminary results from another study have not revealed unusual genetic diversity in this region (M. Hedrén, unpublished data). We have obtained even less evidence regarding possible refugia for *D. fuchsii* because this species showed little variation in plastid microsatellites (A or, less frequently, N). The fact that an additional haplotype, Q, is common in Russia (Shipunov & al., 2004) tentatively indicates an eastern refugium, although the Balkans and Italy also remain credible candidates.

Allotetraploids. — It is noteworthy that we detected little evidence of gene flow between allotetraploids, indicating presence of effective barriers to gene exchange. This is perhaps not surprising, given that extensive artificial crosses conducted among Swedish dactylorchids by Malmgren (1992) yielded fertile F2 plants only when one of the parents was *Dactylorhiza incarnata* s.l. or *D. sphagnicola*, the latter with the *D. incarnata* ITS alleles predominating instead of the typical (for allotetraploids) alleles of the *D. maculata* group. Bateman & Haggar (in press) created artificial hybrids between *D. praetermissa* and *D. purpurella* that showed high fertility in both the first generation and backcrosses. Accessions of *D. majalis* s.str. or *D. traunsteineri* exhibiting *maculata* markers were rare, and no *fuchsii* markers were observed in *D. elata* or *D. occidentalis* accessions, even though allotetraploids of these two categories often grow sufficiently close to each other to expect occasional cross-pollinations. However, allotetraploid populations that mix *fuchsii* and *maculata* haplotypes have recently been reported from Sweden (Hedrén, 2003) and are also suspected to occur in Scotland (R.M. Bateman, unpub.).

Species in the *Dactylorhiza maculata* group, most commonly *D. fuchsii*, were maternal parents of the great majority of allotetraploids. As observed in polyploids of other families (Soltis & Soltis, 1999), allotetraploid dactylorchids of western Europe have several origins; the number of plastid haplotypes indicates at least ten independent allopolyploid events. However, three haplotypes occurred in most allotetraploids, having successfully spread across most of the range of the genus: the most common *fuchsii* haplotype (A), the most common *maculata* haplotype (B), and the C haplotype, the last concentrated in the south and of an uncertain parental derivation. In addition, it is clear that, although it is always reported to be a tetraploid, *D. maculata* (or a genetically similar entity) was the maternal parent of several allotetraploid taxa.

The C haplotype was found in only one putatively diploid individual, a Greek *Dactylorhiza saccifera*. However, this accession also contained the common *saccifera* ITS allele VI, whereas most allotetraploids that possess the C haplotype have the *fuchsii* allele IIIb. Thus, our current (albeit limited) sampling suggests that *D. saccifera* is not likely to be a parent of these allotetraploids. It seems more likely that the diploid species that originally donated the C haplotype to *D. majalis* and similar allotetraploids is extinct or at least has become sufficiently rare to escape our Europe-wide sampling effort. The C haplotype has a central position in the minimum spanning tree between the A haplotype characteristic of *D. fuchsii* and the G and W haplotypes found in some *D. saccifera* from the Greek mainland (Fig. 1). The presumed diploid species that once exhibited the C haplotype was probably formerly widespread, considering that the C haplotype has been found in allotetraploid samples stretching from the Pyrenees to the Tibetan plateau. However, this haplotype declines in frequency northward, being rare in Scandinavia and absent from European Russia (Shipunov & al., 2004). This suggests either that the hypothetical ancestral diploid became rare before the end of the last glaciation or that it failed to migrate northward following glaciation.

In either case, its contribution to formation of relatively young northern allotetraploids was less important, being replaced in this role by typical *D. fuchsii*. Virtual absence of the C haplotype from sampled diploids and its preponderance in several allotetraploids suggests either that these allotetraploids were not formed in their present geographical locations or (less likely) raises the possibility that older allotetraploids may have contributed to the origin of younger allotetraploids (e.g., *D. majalis* s.str. to *D. alpestris*).

Most allotetraploids with A or C haplotypes had *fuchsii* or *saccifera* ITS alleles, and most possessing the B haplotype had the *maculata* ITS allele; the only exceptions to this pattern were a few samples combining *maculata* haplotypes with *fuchsii* ITS alleles. Putative allotetraploid accessions containing both *fuchsii* and *maculata* ITS alleles were rarely detected, indicating that introgression between *D. maculata* and allotetraploids is an infrequent event, even though they share the same ploidy level. The only exceptions found in this study were from Iceland, where four out of five accessions contained both *maculata* and *fuchsii* markers, despite the supposed absence from Iceland of *D. fuchsii*. Also, Shipunov & al. (2004) reported the presence in northern Russia of allotetraploid populations that do not have *D. incarnata* as one of their parents but rather appear to be derived from hybridization between *D. fuchsii* and *D. maculata*. Nonetheless, almost all allotetraploids lack evidence of prior hybridization or introgression between *D. fuchsii* and *D. maculata*, and allotetraploids do not currently appear to be operating as a genetic bridge linking *D. fuchsii* and *D. maculata*.

Moreover, the characteristic *Dactylorhiza saccifera* ITS allele VI was rarely found in allotetraploids examined here, indicating a limited contribution of *D. saccifera* to their formation. However, the distribution of this allele is unusual. It also occurs sporadically and typically at low frequencies across the range of *D. fuchsii* (e.g., Croatia, U.K.), frequently in *D. praetermissa* (U.K.; slightly more than 50% of the accessions sampled) and is present in single populations of *D. majalis* (France) and *D. purpurella* (Wales). Thus, although *D. saccifera* is presently limited to the eastern Mediterranean and the Near East, it is possible that it once extended into western Europe. Alternatively, presence of the *saccifera* allele in other taxa, particularly *D. fuchsii*, could be the result of local hybridization with sympatric *D. praetermissa*. However, this hypothesis similarly requires a subsequent major contraction in the range of *D. praetermissa* to its present northwestern European enclave, after presumably originating in, and migrating out of, the Mediterranean region. This seems unlikely, given that *D. praetermissa* is here characterized as a young allotetraploid.

Hedrén (2001b) inferred that *Dactylorhiza saccifera* or a closely related taxon was one parent of the allotetraploids characteristic of Turkey because it is the only member of the *D. maculata* group that currently occurs in the region. However, different ITS alleles were found in *D. fuchsii* and *D. saccifera*, suggesting that the actual parent of these allotetraploids may instead be a hypothesized diploid that is either extinct or as yet undiscovered.

Our data suggest that several widely recognized allotetraploid taxa have multiple origins, including the exceptionally widely distributed *Dactylorhiza majalis*. In the case of *D. purpurella*, presence of both *D. fuchsii* and *D. incarnata* haplotypes indicates that hybridization events that accompanied polyploidization occurred in both directions or that introgression with its parents has contributed to additional haplotypes after the original allotetraploid was formed.

The case of *D. traunsteineri* and the closely related *D. lapponica* is especially instructive. Samples of each taxon from the British Isles, Scandinavia (the type region for *D. lapponica*) and the Alps (the type region for *D. traunsteineri*) are readily distinguished using either haplotypes or ITS alleles, but within each region, there are no significant differences between the two supposed species, conclusions previously indicated by studies of allozymes (Hedrén, 1996, 2002, 2003; Bateman, 2001) and AFLPs (Hedrén & al., 2001). It is clearly advisable to synonymize *D. lapponica* with *D. traunsteineri* across their respective (and virtually coincident) ranges. However, the systematist must then make the difficult decision of whether to (1) recognize a single allopolyploid species that has at least three independent evolutionary origins (Table 3) or (2) recognize three separate species that are putatively distinct, one species located in each of the three geographical regions. Perhaps the most appropriate arbiter is whether putative segregated species can be recognized using morphological characters with an acceptable level of confidence. On this basis, Bateman (2006) assigned to *D. traunsteinerioides* those dactylorchid populations in the British Isles that had received considerable conservation attention because they had previously been ascribed to *D. traunsteineri* and/or to *D. lapponica*.

However, even given extensive population genetic data and focusing on a restricted geographical area, it can prove challenging to determine with sufficient confidence the number of origins of a particular allotetraploid taxon. For example, Swedish populations of *D. sphagnicola* collectively have only one origin according to plastid markers (see also Hedrén, 2003), but allozyme data indicate at least two origins (Hedrén, 1996), and fine-scale analysis of additional plastid markers indicates multiple origins (Hedrén, Nordström & Ståhlberg, unpub.).

Inferring the current evolutionary status of allotetraploids. — One of the most important questions raised by the *Dactylorhiza incarnata/maculata* complex is why allotetraploids that we can demonstrate to have the

same pair of parental species can exhibit substantially different morphological, ecological and distributional properties. Examples of such contrasts include *D. sphagnicola* versus *D. occidentalis* versus *D. elata* (all derived from hybridization between *D. maculata* and *D. incarnata*) and *D. traunsteineri* s.l. versus *D. purpurella* versus *D. majalis* (all derived from hybridization between *D. fuchsii* and *D. incarnata*). There are two contrasting hypotheses that could, either separately or in combination, explain differentiation and specialization among allopolyploids.

(1) Post-origin differentiation of allotetraploids. This hypothesis is predicated on (1) presumed ability of differential directional or disruptive selection to fine-tune to contrasting ecologies the products of different polyploidization events between the same two parental species, and (2) using contrasting degrees of ITS gene conversion to provide relative dates of different polyploidization events occurring between the same pair of parental species.

For example, the Irish endemic *D. occidentalis* is a recently synthesized allotetraploid, whereas the more widespread Iberian/North African *D. elata* is judged to be substantially older. Its greater age since formation offers selection more time to operate on the *D. elata* phenotypes and thereby to mould them to fit a distinct set of ecological parameters. This hypothesis predicts that *D. occidentalis* (a taxonomically controversial species, once tentatively misidentified as an autopolyploid: cf. Bateman & al., 2003; Bateman, 2006) should still exhibit a blend of parental traits, whereas the longer existence of *D. elata* should have allowed it sufficient time to diverge from parental traits, thereby becoming more specialized and thus more readily recognizable as a bona fide species, a process perhaps assisted by a greater degree of genomic re-organization and integration of the two parental genomes (Parakonny & Kenton, 1995). Furthermore, if older allotetraploids, such as *D. elata* and *D. majalis* s.str., did indeed originate before the last glacial maximum, then they would have responded to profound climate change by migrating first southward and then northward, presumably alongside their progenitors. If so, they would likely have passed through at least one genetic bottleneck, which would have further homogenized their genetic, morphological and ecological characteristics (cf. Cozzolino & al., 2003b). In contrast, the more recently synthesized allotetraploids such as *D. occidentalis* and *D. praetermissa*, hypothesized to have originated during the Holocene, should appear more heterogeneous.

An analogous but probably older case is provided by allotetraploid species complexes in *Nicotiana* (Solanaceae). In section *Polydicliеae* (sensu Knapp & al., 2004), evidence from plastid (Chase & al., 2003) and ITS (Clarkson & al., 2004) DNA sequences indicated that two allotetraploid species, *N. clevelandii* and *N. quadrivalvis*, were generated from the same parental lineages at different times in their history. These two species now exhibit contrasting floral morphologies and ecologies and have only a slight range overlap in southwestern North America. Given sufficient time, some such entities become distinct evolutionary lineages that can undergo subsequent phyletic radiations; examples include *Nicotiana* section *Repandae*, which consists of four species with a common origin, and section *Suavolentes*, which consists of approximately 25 species with a common origin (Chase & al., 2003; Clarkson & al., 2004).

(2) Pre-origin differentiation of parents of allotetraploids. A contrasting hypothesis can also explain our ability to distinguish morphologically and ecologically most of the independent lineages resulting from separate polyploidization events between the *Dactylorhiza incarnata* and *D. maculata* groups, as indicated by genetic data. This focuses more on the considerable degrees of morphological, ecological and, at least in the case of the *D. maculata* group, genetic differentiation that is evident among various named infraspecific taxa *within* the two parental groups (Bateman, 2001, 2006, in prep.).

Within the British Isles alone, *D. incarnata* is represented by at least six named infraspecific entities: one a specialist of sphagnum bogs and another favouring depressions in dune systems, whereas the remaining four are characteristic of alkaline fens and marshes, occasionally extending into alkaline/neutral meadows (Heslop-Harrison, 1953; Bateman & Denholm, 1985). Moreover, *D. fuchsii* exhibits the widest habitat tolerance of any British orchid species. In addition to named infraspecific specialists of upland and coastal pastures, populations inhabiting chalk and limestone grasslands, alkaline/neutral pastures, and alkaline/neutral marshes and woodland can all be distinguished by subtle morphological differences (Bateman & Denholm, 1989). This degree of largely correlated variation in morphology and habitat preference offers much potential for iteratively generating contrasting allotetraploid lineages from within the same pair of parental species.

Consider, for example, the three moisture-loving allotetraploids that are shown by genetic data to be the progeny of *D. incarnata* and *D. maculata* s.str. As its name suggests, *D. sphagnicola* preferentially inhabits acid sphagnum bogs in Scandinavia and northwestern Continental Europe, where the most likely maternal parent is the sphagnum bog specialist *D. maculata elodes*, inheriting from it not only morphologies subtly distinct from those of the parental nominate race but also its extreme ecological preference (cf. Hedrén, 2003). In contrast, the Irish endemic *D. occidentalis* tolerates soils varying from slightly acidic to slightly alkaline, especially when subject to anthropogenic disturbance. Both its morphology and ecology suggest that it is more

likely derived from hybridization between *D. incarnata incarnata* and *D. maculata ericetorum*. Lastly, the relatively poorly researched, putatively older allotetraploid *D. elata* from southwestern Iberia and northwestern Africa prefers alkaline pastures and seepages and is hypothesized to represent hybridization between *D. incarnata* s.l. (this species also has been under-researched in the southwestern extreme of its range) and one or more of the regional segregates of *D. maculata* (*D. caramulensis*, *D. battandieri* or *D. maurusia*). The rare Moroccan endemic *D. maurusia* is of particular interest in this context, as it is morphologically reminiscent of *D. elata* (e.g., Landwehr, 1977) and, unusually for *D. maculata* s.str., it inhabits alkaline soils. Separate evolutionary origins are likely for *D. elata* populations in Iberia and North Africa (the source of the holotype; Pedersen & al., 2003), given their distinct haplotypes and contrasting converted ITS alleles (Table 4), and the tendency to taxonomically separate French and Spanish populations from the nominate race as infraspecific taxa on the grounds of their subtly distinct morphologies (e.g., Nieschalk & Nieschalk, 1972; Landwehr, 1977; Delforge, 2005).

This contrasting hypothesis thus relies on the assumption that these allotetraploids originated locally, in sympatry with their parents, and reflect both the detailed morphology and habitat preferences of those parents. This scenario implies that our ability to distinguish subtly genetically distinct lineages derived repeatedly from the same two parental species relies more on selection honing the parents prior to polyploid formation than post-derivational selection honing allotetraploid lineages, thereby down-playing the evolutionary (and taxonomic) importance of relative periods elapsed since the initiating hybridization event. This hypothesis is best evaluated by studying morphologically and genetically diagnosable allotetraploids that show unusually restricted distributions and so are assumed to be of recent origin (Bateman, 2006; Bateman & al., in prep.).

CONCLUSIONS

Systematic implications of the genetic patterns. — Of the three species aggregates considered here, the least taxonomically controversial within western Europe, at least at the species level, has been the *Dactylorhiza incarnata* group. With few exceptions (notably Delforge, 2005), authorities have been inclined to award species status only to *D. cruenta* among the named taxa within this group, and this elevation is not upheld by genetic data (Hedrén, 1996; Hedrén & al., 2001; Bateman & al., 2003). Treatments of the *D. maculata* group have historically ranged from recognition as a single species (most common if study focuses on regions suspected of sustaining relatively high levels of introgression) through frequent recognition of three core species (*D. maculata*, *D. fuchsii* and *D. saccifera*) to further division into local endemic species (Delforge, 2005). Not surprisingly, classification of allotetraploids has been most controversial, varying from most (Sundermann, 1980) or many (Soó, 1980) infraspecific taxa allocated to a single aggregate species, *D. majalis*, through to highly divided treatments recognizing many species, most poorly morphologically differentiated (Averyanov, 1991; Delforge, 2005).

Our own framework taxonomy (Table 4), which currently excludes local endemics, is a compromise between these extremes. It represents an attempt to synthesize previous, morphology-based taxonomic circumscriptions (and associated knowledge of ecological preferences and geographical distributions) with more process-oriented

Table 4. Recommended framework classification of European members of the *Dactylorhiza incarnata* and *D. maculata* groups and their derived polyploid complex. The plastid haplotype and ITS allele(s) given here are considered typical of each taxon. This summary focuses on well-established species, incorporating regional endemics but excluding local endemics.

Taxon	Ploidy and parentage	Plastid haplotype	ITS allele(s)
D. fuchsii (incl. *cornubiensis*, *okellyi*)	2X	A	V, IIIb
D. maculata (incl. *ericetorum*, *elodes*)	4X (autotetraploid)	B	I
D. saccifera	2X	C, G, W	VI
D. incarnata s.l. (all W European taxa)	2X	E	Xa
D. euxina	2X	Y, K	Xb
D. elata (North Africa)	*maculata* × *incarnata*	O	IIIa, completely converted
D. elata (Europe)	*maculata* × *incarnata*	B	I, most accessions completely converted
D. occidentalis (incl. *kerryensis*)	*maculata* × *incarnata*	B	I dominant, X in 1/3 or fewer copies
D. sphagnicola	*maculata* × *incarnata*	B	Xa dominant, I in 1/3 or fewer copies
D. majalis (incl. *alpestris*)	*fuchsii* × *incarnata*	A, C	V, IIIb, most accessions completely converted
D. praetermissa (incl. *junialis*)	*fuchsii/saccifera* × *incarnata*	A, C	V, IIIb, VI
D. traunsteineri (incl. *lapponica*)	*fuchsii* × *incarnata*	A, C	V, IIIb, rarely with Xa dominant
D. purpurella (incl. *cambrensis*)	*fuchsii* × *incarnata*	A	V, IIIb, rarely with Xa dominant

data on occurrence of gene flow (hybridization/introgression), both currently and, by inference, in the past. Dividing species more finely risks generating taxa that cannot reliably be distinguished using morphology or, in many cases, DNA data, thereby hampering communication and undermining conservation initiatives (the conservation implications of our data are explored elsewhere; Pillon & al., 2006). Alternatively, further amalgamating species into "super-species", in response to evidence of past or present gene flow among component species, obscures our hard-won knowledge of evolutionary processes operating within this allopolyploid complex. In particular, morphological, genetic and ecological differentiation evident among both diploids and tetraploids, and evolutionary causes of that differentiation, would no longer be represented in an unnecessarily crude taxonomy (consider the extreme cases of lumping certain highly calcicolous lineages of *D. fuchsii* with the highly calcifugic *D. maculata elodes* or combining ecologically contrasting allopolyploids *D. traunsteineri* and *D. sphagnicola*). Our insights into the probable contrasting ages of different allotetraploid lineages, and their consequently contrasting genetic compositions and evolutionary trajectories, would also be ignored.

Current co-existence of various diploids and tetraploids in at least some regions without genetic mixing indicates that barriers to gene flow are operating. Moreover, differentiation of the taxa has strong ecological as well as geographical components, suggesting that these taxa are likely to operate as distinct evolutionary units and hence supporting our use of the species category. Even where significant gene flow is evident between species in portions of their present ranges (e.g., between *D. fuchsii* and *D. maculata* in the Alps), there is evidence that these species have in the past experienced periods of isolation that allowed them to develop substantially different plastid haplotypes and ITS alleles. Furthermore, the significant correlation between ITS alleles and plastid haplotypes in both diploids and tetraploids means it is unlikely that lineage sorting rather than hybridization/introgression is responsible for the heterogeneity of these markers observed in *Dactylorhiza*.

With regard to future field collecting, our primary objectives are to intensify sampling in likely glacial refugia in Iberia/North Africa, Italy, Greece and the Caucasus and to extend application of our markers eastward into Asia. Novel haplotypes were found in material from the Russian Caucasus (Shipunov & al., 2004), and a set of just four probable allotetraploids collected in Georgia revealed no less than three unique haplotypes (albeit clearly related to those previously found in *Dactylorhiza fuchsii*). Only one of our 399 samples was located east of the Urals; this sample, from the Tibetan Plateau, reassuringly yielded a haplotype and ITS profile typical of the dominantly European *D. majalis*.

Limitations to the application of the genetic markers used in this study. — The two sets of genetic markers used here, plastid DNA fragment length variants and ITS nrDNA alleles, are both subject to being "captured": plastid DNA due to its uniparental (maternal) pattern of inheritance and ITS because of concerted evolution/gene conversion, which over time erodes evidence of its original biparental inheritance. Although parentage of young hybrids can be determined with confidence using these markers, older hybrids will not appear to be hybrids because of conversion of one ITS allele. Moreover, because conversion usually favours the maternal allele, maternally inherited plastid DNA markers are likely to generate sets of relationships concordant with those derived from maternally biased converted ITS alleles, thereby further camouflaging evidence of past hybridization events. However, for the majority of accessions studied here, we were able to use these markers to identify hybrids and determine which species was the maternal parent. An unexpected benefit of quantifying ITS frequencies was that the degree of loss of the less favoured ITS allele indicates relative ages of allotetraploid taxa. This is especially advantageous when, as here, the same pair of parental taxa has generated multiple allotetraploid lineages at different times in the past.

Admittedly, even these two complementary sets of markers appear too conservative to adequately interpret some fine-scale patterns. For example, the considerable morphological variation evident within the *D. incarnata* group has proven invisible to most markers used so far, with the exception of a single allozyme locus (cf. Hedrén, 1996; Bateman, 2001) and one promising plastid region (M. Hedrén, unpublished data). Better markers within the *D. incarnata* group are essential if we are to evaluate our hypothesis that various allotetraploids are still being synthesized locally (e.g., Hedrén, 2003: Bateman, 2006; Bateman & al., in prep.). Similarly, if parental markers are too highly conserved we cannot detect cases of local hybridization and introgression. The results of this study provide a tantalizing glimpse into the complex evolution and ecology of these widespread European orchids, which nonetheless remain a serious challenge to the taxonomist.

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Appendix. Accessions sampled in this study and their ITS alleles and plastid haplotypes.

Species	Number	Origin	Country[a]	Voucher[b]	Haplotype	Gel reading of the ITS fragments (lengths in bp)[c]		Alleles[c]		
						Dfuch	Dmac	Major	Minor	Ratio
D. fuchsii	11861	Below Schneealpen	Au	*Schönswetter & Tribsch 603A*	A	70(1)75(1)	“72”80	V-III	I	1:1
D. fuchsii	11862	Below Schneealpen	Au	*Schönswetter & Tribsch 603B*	N	“70”75	72(1)80(1)	I-III	V	1:1
D. fuchsii	11863	Below Schneealpen	Au	*Schönswetter & Tribsch 603C*	A	70(1)75(1)	72(1)80(2)	V-I-III		
D. fuchsii	11864	Below Schneealpen	Au	*Schönswetter & Tribsch 603D*	N	~70(2)75(3)	72(1)80(2)	V-I	(III)	
D. fuchsii	11865	Below Schneealpen	Au	*Schönswetter & Tribsch 603E*	N	70(1)75(2)	72(1)80(1)	V-I-III		
D. fuchsii	15166	NE Scharnitz	Au	*Bateman 139*	A	70	80	V		
D. fuchsii	14618	Paklenica National Park	Cr	*Schönswetter & Tribsch 6301* WU	A	72(1)75(1)	80	VI-III		2:1
D. fuchsii	14619	Paklenica National Park	Cr	*Schönswetter & Tribsch 6301* WU	A	70“72”	80	V-	VI	
D. fuchsii	14620	Paklenica National Park	Cr	*Schönswetter & Tribsch 6301* WU	A	70(2)72(1)	80	V-VI		2:1
D. fuchsii	14621	Paklenica National Park	Cr	*Schönswetter & Tribsch 6301* WU	A	70(3)72(1)	80	V-VI		3:1
D. fuchsii	12124	Pyrenees, Nalzen	Fr	*Civeyrel & al. 574B*	A	70(3)75(1)	80	V-III		3:1
D. fuchsii	12125	Pyrenees, Nalzen	Fr	*Civeyrel & al. 574C*	A	70	80	V		
D. fuchsii	12139	Pyrenees, Pla des Ails	Fr	*Civeyrel & al. 583*	A	70“75”	80	V	III	
D. fuchsii	12140	Pyrenees, Pla des Ails	Fr	*Civeyrel & al. 584*	A	70“75”	80	V	III	
D. fuchsii	12157	Pyrenees, Espezel	Fr	*Civeyrel & al. 590*	A	“70”75	80	III	V	
D. fuchsii	13813	Bourgogne, Sagy	Fr	*Chase 13813a* (spirit)	N	70(1)75(3)	72(3)80(1)	I-V	(III)	3:1
D. fuchsii	13814	Bourgogne, Sagy	Fr	*Chase 13813b* (spirit)	B	70(1)75(1)	72(1)80(3)	V-I-III		2:1:1
D. fuchsii	13817	Bourgogne, Flacey	Fr	*Chase 13817a* (spirit)	A	“70”75	72“80”	I	V (III)	
D. fuchsii	13818	Bourgogne, Flacey	Fr	*Chase 13817b* (spirit)	A	“70”75	72(1)80(1)	I-III	V	1:1
D. fuchsii	13819	Bourgogne, Flacey	Fr	*Chase 13819* (spirit)	A	75	72“80”	I	III	
D. fuchsii	15189	Massif Central, Vallee de la Dourbie	Fr		B	75	72“77”	I	II	
D. fuchsii	15190	Massif Central, Vallee de la Dourbie	Fr		B	75	72	I		
D. fuchsii	14607	Sorvilier	He	*Fay K1874.3* (spirit)	N	70(2)75(1)	80	V-III		2:1
D. fuchsii	14699	NNW Braunwald	He	*Bateman 738*	A	~70(1)>75(1)	72(1)80(2)	V-I-III		
D. fuchsii	14606	Birr Castle	Ir	No voucher	A	“70”75	80	III	V	

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. fuchsii	3877	Gotland, Boge	Sw	*Hedrén 97108*	A	"70"75	80	V	III	
D. fuchsii	3971	Skane, Maryd	Sw	*Hedrén 97037*	A	75	80	III		
D. fuchsii	3976	Gotland, Gerum	Sw	*Hedrén 97096*	N	70(1)75(1)	80	V-III		1:1
D. fuchsii	H356	Ostergotaland, Karna	Sw	*Hedrén 97184*	A	70"75"	80	V	III	
D. fuchsii	3989	Ostergotaland, Karna	Sw	*Hedrén 97186*	A	70(1)75(1)	80	V-III		1:1
D. fuchsii	3996	Södermanland, Svarta	Sw	*Hedrén 97221*	A	70	80	V		
D. fuchsii	5547	Jämtland, Hammerdal	Sw	*Hedrén 97282*	A	70(3)75(1)	80	V-III		3:1
D. fuchsii	H174	Västragotaland, Jattened	Sw		A	70"72""75"	80	V	VI-III	
D. fuchsii	H175	Västragotaland, Jattened	Sw		N	70	80	V		
D. fuchsii	O-1375	Gotland, Fide Parish	Sw		A	70	80	V		
D. fuchsii	10240	Nr. Cardiff	UK	*Chase 10240*(*1*)	N	70(1)75(1)	80	V-III		1:1
D. fuchsii	10241	Nr. Cardiff	UK	*Chase 10240*(*2*)	A	70(1)75(1)	80	V-III		1:1
D. fuchsii	10242	Nr. Cardiff	UK	*Chase 10240*(*3*)	A	75	80	III		
D. fuchsii (hyperchromic)	13810	Nr. Cardiff	UK	*Chase 13810* (spirit)	A	70(2)72(1)75(1)	80	V-III-VI		2:1:1
D. fuchsii? hybrid	14075	Nr. Cardiff	UK	*Chase 14075* (spirit)	A	70(2)75(1)	80	V-III		2:1
D. fuchsii	10243	Uffington	UK	*Chase 10243*(*1*)	A	70(1)75(1)	80	V-III		1:1
D. fuchsii	10244	Uffington	UK	*Chase 10243*(*2*)	A	70(1)75(1)	80	V-III		1:1
D. fuchsii	10245	Uffington	UK	*Chase 10243*(*3*)	A	~70(1)75(1)	80	V-III		
D. fuchsii	10246	Uffington	UK	*Chase 10243*(*4*)	A	70	80	V		
D. fuchsii	10247	Uffington	UK	Chase 10243(5)	A	70(3)75(1)	80	V-III		3:1
D. fuchsii	13526	Anglesey, Rhos-y-gard	UK	*Fay 635a* no voucher	A	70	80	V		
D. fuchsii	13527	Anglesey, Rhos-y-gard	UK	*Fay 635b* no voucher	A	70	80	V		
D. fuchsii	13746	Anglesey, Malltraeth	UK	*Fay 670a* (spirit)	A	70	80	V		
D. fuchsii	13786	Avon, Cuckoo Lane	UK	*Chase 13786a* (spirit)	A	~"70"72(1)75(2)	80	III-VI	V	
D. fuchsii	13787	Avon, Cuckoo Lane	UK	*Chase 13786b* (spirit)	A	70(2)75(1)	80	V-III		2:1
D. fuchsii	13788	Avon, Cuckoo Lane	UK	*Chase 13786c* (spirit)	A	70(3)75(1)	80	V-III		3:1

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. fuchsii	13789	Avon, Cuckoo Lane	UK	*Chase 13786d* (spirit)	A	70“75”	80	V	III	
D. fuchsii	13791	Avon, Cuckoo Lane	UK	*Chase 13786f* (spirit)	A	70(2)75(1)	80	V-III		2:1
D. fuchsii	13792	Avon, Cuckoo Lane	UK	*Chase 13786g* (spirit)	A	70>75	80	V>III		
D. fuchsii	13793	Avon, Cuckoo Lane	UK	*Chase 13786h* (spirit)	A	70	80	V		
D. fuchsii	14195	Sussex, Cradle Hill	UK	*Chase 14195a* (spirit)	A	70“75”	80	V	III	
D. fuchsii	O-784	Box Hill	UK		A	70(1)75(1)	80	V-III		1:1
D. fuchsii “*Rachel*”	10284	Kew 1997-6379, Surrey, North Downs	UK	*Chase 10284*	A	“70”75	80	III	V	
D. fuchsii	11794	Kew 1998-2727		*Chase 11794*	A	70(1)75(1)	80	V-III		1:1
D. fuchsii	O-1123	Bateman isozyme			A	70	80	V		
D. fuchsii (collected as *maculata* s.l.)	14593	Steiermark, Rottenmanner Tauern	Au	*Schönswetter & Tribsch 3065* herbario Gutermann	A	~70(1)>75(1)	72(1)80(2)	V-I	(III)	
D. fuchsii (collected as *maculata* s.l.)	14594	Steiermark, Rottenmanner Tauern	Au	*Schönswetter & Tribsch 3065* herbario Gutermann	A	70(1)<75(1)	~72(1)80(2)	V-I	(III)	
D. fuchsii (collected as *maculata* s.l.)	14595	Steiermark, Rottenmanner Tauern	Au	*Schönswetter & Tribsch 3065* herbario Gutermann	A	“70”75	80	III	V	
D. fuchsii (collected as *maculata* s.l.)	14596	Steiermark, Rottenmanner Tauern	Au	*Schönswetter & Tribsch 3065* herbario Gutermann	A	75	~72(2)80(1)	I-III		
D. fuchsii (collected as *maculata* s.l.)	14597	Oberösterreich, Mollner Voralpen	Au	*Schönswetter & Tribsch 3053-3055* WU	N	70(3)75(1)	80	V-III		3:1
D. fuchsii (collected as *maculata* s.l.)	14598	Oberösterreich, Mollner Voralpen	Au	*Schönswetter & Tribsch 3053-3055* WU	N	70	80	V		
D. fuchsii (collected as *maculata* s.l.)	14599	Oberösterreich, Mollner Voralpen	Au	*Schönswetter & Tribsch 3053-3055* WU	A	70	80	V		
D. fuchsii (collected as *maculata* s.l.)	14600	Oberösterreich, Mollner Voralpen	Au	*Schönswetter & Tribsch 3056* WU	N	70	80	V		
D. fuchsii (collected as *maculata* s.l.)	14747	Steiermark, Hochschwab-gebiet	Au	*Schönswetter & Tribsch 6290* WU	A	70	80	V		
D. fuchsii (collected as *maculata* s.l.)	14748	Steiermark, Hochschwabgebiet	Au	*Schönswetter & Tribsch 6290* WU	A	70“75”	80	V	III	

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. fuchsii (collected as *maculata* s.l.)	14749	Steiermark, Schladminger Tauern	Au	*Schönswetter & Tribsch 3069* herbario W. Gutermann	Q	70(1)75(1)	80	V-III		1:1
D. fuchsii (collected as *maculata* s.l.)	14750	Steiermark, Schladminger Tauern	Au	*Schönswetter & Tribsch 3069* herbario W. Gutermann	A	70(1)75(3)	“72”80	III-V	I	3:1
D. fuchsii (collected as *maculata* s.l.)	14751	Steiermark, Kalkalpen	Au	*Schönswetter & Tribsch 3047* WU	A	75	~72(1)80(1)	I-III		
D. fuchsii (collected as *maculata* s.l.)	14692	Pyrenees, Pic du Canigou	Fr	*Schönswetter & Tribsch 6403*	N	75	72	I		
D. fuchsii (collected as *maculata* s.l.)	14693	Pyrenees, Pic du Canigou	Fr	*Schönswetter & Tribsch 6403*	N	75	“72”80	III	I	
D. fuchsii (collected as *maculata* s.l.)	14622	Trento, Lagorai	It	*Schönswetter & Tribsch 6331*	A	“70”75	“72”80	III	V-I	
D. fuchsii (collected as *maculata* s.l.)	14623	Trento, Lagorai	It	*Schönswetter & Tribsch 6331*	A	~70(1)75(2)	“72”80	V-III	I	
D. fuchsii (collected as *maculata* s.l.)	14624	Trento, Lagorai	It	*Schönswetter & Tribsch 6331*	A	70(2)75(1)	72(1)80(2)	V-I	(III)	2:1
D. fuchsii (collected as *maculata* s.l.)	14625	Trento, Lagorai	It	*Schönswetter & Tribsch 6331*	A	~ 70(1)75(1)	~72(1)80(1)	V-I	(III)	1:1
D. fuchsii	10285	Bix Bottom	UK	No voucher	A	70(1)“72”75(2)	80	III-V	VI	2:1
D. fuchsii	10286	Bix Bottom	UK	No voucher	A	70(3)75(1)	80	III-VI		3:1
D. fuchsii	10287	Bix Bottom	UK	No voucher	A	70“72“	80	V	VI	
D. fuchsii	10288	Bix Bottom	UK	No voucher	A	70(2)72(1)75(3)	80	V-III-VI		
D. fuchsii	10289	Bix Bottom	UK	No voucher	A	“72”70	80	V	VI	
D. fuchsii	10290	Bix Bottom	UK	No voucher	A	72(1)75(3)	80	III-VI		3:1
D. fuchsii?	13750	Anglesey, Malltraeth	UK	No voucher	N	~70(1)75(1)	80	V-III		
D. fuchsii (collected as *maculata* s.l.)	14697	Steingletscher	He	*Bateman 738*	A	70(2)75(1)	80	V-III		2:1
D. fuchsii v. *cornubiensis*	17040	Cornwall, St. Ives	UK	*Bateman & Rudall s.n.*	A	75	80	III		
D. fuchsii v. *cornubiensis*	17042	Cornwall, St. Ives	UK	*Bateman & Rudall s.n.*	A	75	80	III		
D. fuchsii v. *cornubiensis*	17043	Cornwall, St. Ives	UK	*Bateman & Rudall s.n.*	A	75	80	III		

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. fuchsii v. *cornubiensis*	17045	Cornwall, Tintangel	UK	*Bateman & Rudall s.n.*	A	75	80	III		
D. fuchsii v. *cornubiensis*	17046	Cornwall, Tintangel	UK	*Bateman & Rudall s.n.*	A	75	80	III		
D. fuchsii v. *cornubiensis*	17047	Cornwall, Tintangel	UK	*Bateman & Rudall s.n.*	A	75	80	III		
D. okellyi	14604	Carron, Co. Clare, Co. Clare	Ir	No voucher	A	70(1)75(1)	80	V-III		1:1
D. okellyi	15554	Mullach Mor, Co. Clare	Ir	*Waldren 02-05*	A	70(1)75(1)	80	V-III		1:1
D. okellyi	15555	Carron, Co. Clare	Ir	*Waldren 02-19* TCD	A	70(1)75(1)	80	V-III		1:1
D. okellyi?	14605	Burren, Co. Clare	Ir	No voucher	A	70(1)75(1)	80	V-III		1:1
D. hebridensis	15162	Barra, Eoligarry	UK	*Bateman 583*	B	75	72	I		
D. bythinica	11797	40°39′87″ N, 31°25′52″ E	Tu	*Hedrén 98080*	A	70“72”“75”	80	V	III-VI	
D. saccifera	11800	41°06′25″ N, 33°44′89″ E	Tu	*Hedrén 98066*	A	72(1)75(3)	80	III-VI		3:1
D. saccifera	H201	SW Pavliani	Gr	*Hedrén 010619*	G	72	80	VI		
D. saccifera	H202	SW Pavliani	Gr	*Hedrén 010619*	W	72(1)75(1)	80	III-VI		1:1
D. saccifera	H204	SW Pavliani	Gr	*Hedrén 010619*	G	72	80	VI		
D. saccifera	15986	Kastoria	Gr	*Bateman 74*	C	72	80	VI		
D. cf. *saccifera*	14614	Paklenica National Park	Cr	*Gutermann*	E	~70(1)73(1)	80	V-X		
D. cf. *saccifera*	14615	Paklenica National Park	Cr	*Gutermann*	A	~70(1)72(2)75(1)	80	V-III-VI		
D. cf. *saccifera*	14616	Paklenica National Park	Cr	*Gutermann*	A	“72”73	80	X	VI	
D. cf. *saccifera*	14617	Paklenica National Park	Cr	*Gutermann*	A	~70(1)72(2)75(1)	80	V-III-VI		
D. maculata (*islandica*)	11798	Lambafell	Ic	*Hollingsworth 268*	B	70(1)75(3)	72(3)80(1)	I-V	(III)	3:1
D. maculata (*islandica*)	14608	Lambafell	Ic	*Hollingsworth 267*	B	“70”75	72“80”	I	V (III)	
D. maculata (*islandica*)	14609	Lambafell	Ic	*Hollingsworth 269*	B	75	72“80”	I	III	
D. maculata (*islandica*)	14701	Lambafell	Ic	*Hollingsworth 273*	B	75	72	I		
D. maculata (*islandica*)	14702	Lambafell	Ic	*Hollingsworth 274*	B	70(1)75(3)	72(3)80(1)	I-V	(III)	3:1
D. maculata (*ericetorum*)		Donegal	Ir	*Hodkinson 1*	M	75	72	I		
D. maculata (*ericetorum*)		Donegal	Ir	*Hodkinson 3*	M	75	72	I		
D. maculata	5553	Värntresk	No	*Hedrén 97037*	A	70(1)75(1)	80	V-III		1:1

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. maculata (*caramulensis*)	11795	Vila Nova de Mil Fontes	Po	*Pinto s.n.*	P	75	72	I		
D. maculata	10684	Between Bejar and Barca d'Àvila (Avila)	Sp	*Cotrim & Pinto*	B	"70"75	72"80"	I	V	
D. maculata	3995	Södermanland, Svarta	Sw	*Hedrén 97214*	N	75	72	I		
D. maculata	H38	Gotland, Kauparve	Sw		B	75	72	I		
D. maculata	H192	Gotland, Kauparve	Sw		B	75	72	I		
D. maculata	H365	Småland, Sjomaden	Sw	*Hedrén 97261*	A	75	72	I		
D. maculata	H367	Småland, Sjomaden	Sw	*Hedrén 97264a*	X	75	72(1)77(1)	I-II		1:1
D. maculata	H371	Västragotaland, Karshult	Sw	*Hedren 980711*	A	70(1)75(1)	72(1)75(1)	V-I	(III)	1:1
D. maculata	H375	Uppland, Langbromossen	Sw	*Hedrén 97235*	B	70(1)75(3)	~72(1)80(1)	V-I-III		
D. maculata (*ericetorum*)	13500	Anglesey, Nant Isaf	UK	*Fay 659* (spirit)	B	75	72	I		
D. maculata (*ericetorum*)	13528	Anglesey, Rhos-y-gard	UK	*Fay 655a* (spirit)	B	75	72	I		
D. maculata (*ericetorum*)	13528	Anglesey, Rhos-y-gard	UK	*Fay 655a* (spirit)	B	75	72	I		
D. maculata (*ericetorum*)	13529	Anglesey, Rhos-y-gard	UK	*Fay 655b* (spirit)	B	75	72	I		
D. maculata (*ericetorum*)	13530	Anglesey, Rhos-y-gard	UK	*Fay 655c* (spirit)	B	75	72	I		
D. maculata (*ericetorum*)	13780	Great Orme, Llandudno	UK	*Fay 628a* no voucher	B	75	72	I		
D. maculata (*ericetorum*)	13781	Great Orme, Llandudno	UK	*Fay 628b* no voucher	B	75	72	I		
D. maculata (*ericetorum*)	14070	Hertfordshire, Bricketwood common	UK	*Bateman s.n.* (spirit)	B	75	72	I		
D. maculata (*ericetorum*)	14071	Hertfordshire, Bricketwood common	UK	*Bateman s.n.* (spirit)	B	75	72	I		
D. maculata (*ericetorum*)	14072	Hertfordshire, Bricketwood common	UK	*Bateman s.n.* (spirit)	B	75	72	I		
D. maculata (*ericetorum*)	14073	Hertfordshire, Bricketwood common	UK	*Bateman s.n.* (spirit)	B	75	72	I		
D. maculata (*ericetorum*)	14074	Hertfordshire, Bricketwood common	UK	*Bateman s.n.* (spirit)	B	75	72	I		
D. maculata (*ericetorum*)	6505	Lake District, Duddon Valley	UK	*Fay s.n.* (no voucher)	B	75	72	I		

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. maculata (*ericetorum*)	6506	Lake District, Duddon Valley	UK	*Fay s.n.* (no voucher)	B	75	72	I		
D. maculata (*ericetorum*)	15191	Llangurig, Ponterwyd	UK	*Chase 15191* (spirit)	N	75	72	I		
D. maculata (*ericetorum*)	15192	Llangurig, Ponterwyd	UK	*Chase 15192* (spirit)	N	75	72(2)80(1)	I-V		2:1
D. maculata (*ericetorum*)	15552	Llangurig, Ponterwyd	UK	*Chase 15552* (spirit)	B	75	72“80”	I	V	
D. maculata (*ericetorum*)	15553	Llangurig, Ponterwyd	UK	*Chase 15553* (spirit)	B	75	72“80”	I	V	
D. maculata (*ericetorum*; coll. as a potential hybrid)	13501	Anglesey, Nant Isaf	UK	*Fay 660* (spirit)	N	75	72	I		
D. foliosa	537	Madeira	Po	*Chase 0-537*	D	75	72	I		
D. foliosa	14695	Pico de Jorge, Madeira	Po	*Bateman 608*	D	75	72	I		
D. aristata	15160	From cultivation		*Bateman 366*	F	73	78	VIII		
D. cordigera	15156	Kastoria	Gr	*Bateman 108*	T	75	80	III		
D. ebudensis	994	W Newtonferry, North Uist	UK	*Bateman 55*	E	70(1)73(2)	80	X-V		2:1
D. elata	15121	Western Atlas	Mo	*Bateman 760*	O	75	80	III*		
D. elata	15122	Western Atlas	Mo	*Bateman 760*	Z	75	80	III*		
D. elata	15123	Western Atlas	Mo	*Bateman 760*	O	75	80	III*		
D. elata	15124	Western Atlas	Mo	*Bateman 760*	B	75	80	III*		
D. elata	15125	Western Atlas	Mo	*Bateman 760*	O	75	78(1)80(2)	III*-IV		2:1
D. elata	15126	Western Atlas	Mo	*Bateman 760*	O	75	80	III*		
D. elata	15127	Western Atlas	Mo	*Bateman 760*	O	75	80	III*		
D. elata	15128	Western Atlas	Mo	*Bateman 760*	O	75	80	III*		
D. elata	Herb5	Tashdiert	Mo	*Balls B2991*	?	75	80	III*		
D. cf. *elata*	Herb1	Cherfchaouene	Mo	*Ferguson & al. 6374195*	O	75	80	III*		
D. elata	Herb6	gue de Constantine	Al	*Joad 1882*	?	75	80	III*		
D. elata	15159	Barcelona, Vic Parador	Sp	*Bateman 322*	B	“73”75	72“80”	I	X (III*)	
D. elata	718	Sierra de Nevada	Sp	*Chase O-718*	B	75	72	I		
D. elata	Herb3	Jaen	Sp	*Sandwitch 6304*	?	75	72	I		
D. elata	H346	Massif Central, Val de Trebans	Fr	*Hedrén 990610*	B	“73”75	72(3)80(1)	I	X (III*)	

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. elata	H66	Massif Central, Compregnac	Fr	*Hedrén 990610*	B	75	72	I		
D. elata	Herb2	Aveyron	Fr	*Wood 559*	?	75	72	I		
D. occidentalis	10155	Moher	Ir	*Chase 10155*	B	73(1)75(3)	72(3)80(1)	I-X		3:1
D. occidentalis	10156	Poulsallagh	Ir	*Chase 10156*	B	“73”75	72“80”	I	X	
D. occidentalis	10157	Poulsallagh	Ir	*Chase 10157*	B	“73”75	72“80”	I	X	
D. occidentalis	10158	Poulsallagh	Ir	*Chase 10158*	B	“73”75	72“80”	I	X	
D. occidentalis	10159	Lough Bunny	Ir	*Chase 10159*	B	73(1)75(3)	72(3)80(1)	I-X		3:1
D. occidentalis	10160	Lough Bunny	Ir	*Chase 10160*	B	73(1)75(3)	72(3)80(1)	I-X		3:1
D. occidentalis	10161	Lough Bunny	Ir	*Chase 10161*	B	73(1)75(1)	72(1)80(1)	I-X		3:1
D. kerryensis	15157	W Galway	Ir	*Bateman 118*	B	75	72	I		
D. sphagnicola	3978	Småland, S. Ljuna	Sw	*Hedrén 97152*	B	73(3)75(1)	72(1)80(3)	X-I		3:1
D. sphagnicola	3999	Småland, Madesjo	Sw	*Hedrén 97260*	B	73(3)75(1)	72(1)80(3)	X-I		3:1
D. sphagnicola	H293	Småland, Sjomaden	Sw	*Hedrén 97259*	B	73(3)75(1)	72(1)80(3)	X-I		3:1
D. sphagnicola	3992	Södermanland, Kila	Sw	*Hedrén 97204*	B	73“75”	“72”80	X	I	
D. sphagnicola	H101	Västragotaland, Karshult	Sw	*Hedrén 980711*	B	73(3)75(1)	~72(1)80(3)	X-I		3:1
D. majalis	12158	Pyrenees, Espezel	Fr	*Civeyrel & al. 591A*	C	75	80	III		
D. majalis	12159	Pyrenees, Espezel	Fr	*Civeyrel & al. 591B*	C	75	80	III		
D. majalis	12160	Pyrenees, Espezel	Fr	*Civeyrel & al. 591C*	N	70(2)75(1)	80	V-III		2:1
D. majalis	12161	Pyrenees, Espezel	Fr	*Civeyrel & al. 591D*	N	75	80	III		
D. majalis	14567	Oberösterreich, Kirchdorf	Au	*Schönswetter & Tribsch 3059* WU	A	70(1)75(1)	80	V-III		1:1
D. majalis	14568	Oberösterreich, Kirchdorf	Au	*Schönswetter & Tribsch 3059* WU	A	70(1)75(1)	80	V-III		1:1
D. majalis	14569	Oberösterreich, Kirchdorf	Au	*Schönswetter & Tribsch 3059* WU	A	70(1)75(1)	80	V-III		1:1
D. majalis	14570	Steiermark, Hochschwabgebiet	Au	*Schönswetter & Tribsch 6294* WU	C	“70”75	80	III	V	
D. majalis	14571	Steiermark, Hochschwabgebiet	Au	*Schönswetter & Tribsch 6294* WU	C	“70”75	80	III	V	
D. majalis	14572	Steiermark, Hochschwabgebiet	Au	*Schönswetter & Tribsch 6294* WU	C	“70”75	80	III	V	
D. majalis	14573	Steiermark, Hochschwabgebiet	Au	*Schönswetter & Tribsch 6294* WU	C	“70”75	80	III	V	

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. majalis	14574	Steiermark, Hochschwabgebiet	Au	*Schönswetter & Tribsch 6294* WU	C	“70”75	80	III	V	
D. majalis	14592	Steiermark, Hochschwabgebiet	Au	*Schönswetter & Tribsch 6294* WU	C	“70”75	80	III	V	
D. majalis	12144	Pyrenees, Pla des Ails	Fr	*Civeyrel & al. 586A*	C	75	80	III		
D. majalis	12146	Pyrenees, Pla des Ails	Fr	*Civeyrel & al. 586C*	C	75	80	III		
D. majalis	12147	Pyrenees, Pla des Ails	Fr	*Civeyrel & al. 586D*	C	75	80	III		
D. majalis	12149	Pyrenees, Pla des Ails	Fr	*Civeyrel & al. 596F*	C	75	80	III		
D. majalis	12150	Pyrenees, Pla des Ails	Fr	*Civeyrel & al. 596G*	C	75	80	III		
D. majalis	16543	Bourgogne, St Martin	Fr	*Chase 16543* (spirit)	N	70(1)75(1)	80	V-III		1:1
D. majalis	16544	Bourgogne, St Martin	Fr	*Chase 16544* (spirit)	C	70(1)75(1)	80	V-III		1:1
D. majalis	H65	Massif Central, La Batie I	Fr	*Hedrén 990606*	B	70(1)“72”“73”75(1)	80	V-III	X-VI	
D. majalis	15170	Bayern	Ge	*Bateman 459*	A	75	80	III		
D. majalis	3969	Skane, Saxtorp	Sw	*Hedrén 97028*	C	“70”75	80	III	V	
D. majalis	O-1382	Skane, Torma-Hallestad	Sw		A	75	80	III		
D. majalis	8039	Nyingch, southeastern Xizhang (Tibet)	Ch	*Luo 9*	C	75	80	III		
D. alpestris	O-963	Andorra	An	*Bateman 48*	C	75	80	III		
D. alpestris	14698	Braunwald	He	*Bateman 727*	C	75	80	III		
D. alpestris	H382	Massif, Central, La Batie II	Fr	*Hedrén 990606*	B	70(2)73(1)75(1)	80	V-III-X		
D. alpestris	H40	Massif Central, Eygas	Fr	*Hedrén 990611*	C	70(1)75(1)	80	V-III		1:1
D. alpestris	15983	Alpes, Col du Sarenne, Top	Fr	*Bateman 69*	C	70(3)75(1)	80	V-III		3:1
D. alpestris	15984	Alpes, Col du Sarenne, Bottom	Fr	*Bateman 70*	G	70(3)73(1)75(3)	78(1)80(2)	V-III-X-?		
D. traunsteineri	15158	Kitzbühel [type]	Au	*Bateman 144*	B	75	80	III		
D. traunsteineri	15171	Murnauer Moos	Ge	*Bateman 461*	E	73	80	X		
D. traunsteineri	15165	Ballindooly, N Galway	Ir	*Bateman 117*	C	70(1)73(2)75(2)	80	III-X-V		
D. traunsteineri	3972	Gotland, Hall	Sw	*Hedrén 97074*	C	70(1)75(1)“73”	80	V-III	X	1:1
D. traunsteineri	3979	Ostergotaland, Karna	Sw	*Hedrén 97189*	A	70(1)“73”75(1)	80	V-III	X	1:1

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. traunsteineri	3980	Västragotaland, Radane	Sw	*Hedrén 98165*	A	70(1)75(1)	80	V-III		1:1
D. traunsteineri	3993	Södermanland, Svarta	Sw	*Hedren 97210*	A	70(3)73(1)75(2)	80	V-III-X		
D. traunsteineri	3998	Uppland, Ed	Sw	*Hedrén 97250*	A	70(2)73(1)75(1)	80	V-III-X		2:1:1
D. traunsteineri	5554	Medelpad, Ange	Sw	*Hedrén 97313*	A	70(2)75(1)	80	V-III		2:1
D. traunsteineri	H327	Medelpad, Granboda	Sw	*Hedrén 97310*	A	~70(3)75(1)	80	V-III		
D. traunsteineri	H413	Gotland, Lojsthajd	Sw	*Hedrén 010716*	B	70(2)73(1)75(2)	80	V-III-X		
D. traunsteineri	13493	Anglesey, Nant Isaf	UK	*Fay 658a* (spirit)	E	70>73>75	80	V-III-X		
D. traunsteineri	13494	Anglesey, Nant Isaf	UK	*Fay 658b* (spirit)	E	73	80	X		
D. traunsteineri	13495	Anglesey, Nant Isaf	UK	*Fay 658c* (spirit)	A	70“73”	80	V	X	
D. traunsteineri	13496	Anglesey, Nant Isaf	UK	*Fay 658d* (spirit)	C	“70”73(3)75(1)	80	X-III	V	3:1
D. traunsteineri	13497	Anglesey, Nant Isaf	UK	Fay 658e (spirit)	C	70(1)73(2)“75”	80	X-V	III	2:1
D. traunsteineri	13498	Anglesey, Nant Isaf	UK	*Fay 658f* (spirit)	C	70(3)73(2)75(1)	80	V-X-III		
D. traunsteineri	13499	Anglesey, Nant Isaf	UK	*Fay 658g* (spirit)	C	70“73”“75”	80	V	III-X	
D. traunsteineri	13517	Anglesey, Rhos-y-gard	UK	*Fay 633a* (spirit)	C	70(3)73(2)75(1)	80	V-X-III		
D. traunsteineri	13518	Anglesey, Rhos-y-gard	UK	*Fay 633b* (spirit)	C	70“73”“75”	80	V	X-III	
D. traunsteineri	13519	Anglesey, Rhos-y-gard	UK	*Fay 633c* (spirit)	C	70(2)73(1)	80	V-X		2:1
D. traunsteineri	13520	Anglesey, Rhos-y-gard	UK	*Fay 633d* (spirit)	C	70(3)73(1)	80	V-X		3:1
D. traunsteineri	13521	Anglesey, Rhos-y-gard	UK	*Fay 633e* (spirit)	C	70“73”	80	V	X	
D. traunsteineri	13522	Anglesey, Rhos-y-gard	UK	*Fay 633f* (spirit)	C	~70(1)“73”75(1)	80	V-III	X	
D. traunsteineri	13523	Anglesey, Rhos-y-gard	UK	*Fay 633g* (spirit)	C	70(3)73(1)	80	V-X		3:1
D. traunsteineri bowmanii	15161	Hants, Exbury	UK	*Bateman 468*	C	70(1)“73”75(2)	80	III-V	X	2:1
D. traunsteineri	15167	NE Yorks	UK	*Bateman 300*	C	70(1)“73”75(1)	80	V-III	X	1:1
D. traunsteineri	15169	Hants, Mapledurwell fen	UK	*Bateman 325*	C	70“73”“75”	80	V	X-III	
D. traunsteineri	15988	Oxfordshire, Cothill fen	UK	*Bateman*	C	70(1)73(1)75(2)	80	III-V-X		2:1:1
D. traunsteineri	15989	Oxfordshire, Cothill fen	UK	*Bateman*	C	“70”“73”75	80	III	V-X	

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. traunsteineri	O-990	Wester Ross, Loch Kersary	UK	*Bateman 53*	C	70“73”“75”	80	V	III-X	
D. traunsteineri	O-996	Wester Ross, Loch a Nhuilinn	UK	*Bateman 52*	C	70(3)75(1)	80	V-III		3:1
D. lapponica	989	E Skye, Raasay	UK	*Bateman 54*	C	70“75”	80	V	III	
D. lapponica	4000	Hürjdalen, Linsell, Hamra	Sw	*Hedrén 97269*	A	70“73”	80	V	X	
D. lapponica	5550	Jamtland, Hammerdal	Sw	*Hedrén 97297*	A	70(3)“73”75(1)	80	V-III	X	3:1
D. lapponica	5552	Lycksele	Sw	*Hedrén 97305*	A	70(1)“73”75(2)	80	III-V	X	2:1
D. baltica (*longifolia*)	9485	Tartu	Es	*Chase 9485*	A	70	80	V		
D. purpurella	987	Caithness, Thurso East	UK	*Bateman 51*	A	70(1)73(1)	80	V-X		1:1
D. purpurella	13748	Anglesey, Malltraeth	UK	*Fay 671* (spirit)	A	~70(1)73(1)	80	V-X		
D. purpurella	13763	Nr Llanfrothen, Pont Croesor	UK	*Fay 677a* (spirit)	A	“70”73	80	X	V	
D. purpurella	13764	Nr Llanfrothen, Pont Croesor	UK	*Fay 677b* (spirit)	A	70(1)73(1)	80	V-X		1:1
D. purpurella	13765	Nr Llanfrothen, Pont Croesor	UK	*Fay 677c* (spirit)	A	70(1)73(3)	80	X-V		3:1
D. purpurella	13766	Nr Llanfrothen, Pont Croesor	UK	*Fay 677d* (spirit)	A	70(1)73(3)	80	X-V		3:1
D. purpurella	13767	Nr Llanfrothen, Pont Croesor	UK	*Fay 677e* (spirit)	A	~70(1)73(2)	80	X-V		
D. purpurella	13768	Nr Llanfrothen, Pont Croesor	UK	*Fay 677f* (spirit)	A	70(1)73(1)	80	V-X		1:1
D. purpurella	15193	Ynyslas, Dyfed	UK	*Chase 15193* (spirit)	A	72(1)73(1)	80			
D. purpurella	15194	Ynyslas, Dyfed	UK	*Chase 15194* (spirit)	E	73	80	X		
D. purpurella	16547	Ynyslas, Dyfed	UK	*Chase 16547* (spirit)	A	72(1)75(1)	80	X-VI		1:1
D. purpurella	16549	Ynyslas, Dyfed	UK	*Chase 16549* (spirit)	E	“70”73	80	X	V	
D. purpurella	16550	Ynyslas, Dyfed	UK	*Chase 16550* (spirit)	E	73	80	X		
D. purpurella	964	Aberlady Bay, East Lothian	UK	*Bateman 46*	A	70(1)73(3)	80	X-V		3:1
D. purpurella	13754	Anglesey, Newborough Warren	UK	*Fay 668a* (spirit)	A	“70”73	80	X	V	
D. purpurella	13755	Anglesey, Newborough Warren	UK	*Fay 668b* (spirit)	E	73	80	X		
D. purpurella	13756	Anglesey, Newborough Warren	UK	*Fay 668c* (spirit)	A	“70”73	80	X	V	
D. purpurella	13757	Anglesey, Newborough Warren	UK	*Fay 668d* (spirit)	A	~70(1)73(1)“75”	80	V-X	III	
D. purpurella	13758	Anglesey, Newborough Warren	UK	*Fay 669* (spirit)	A	~70(1)73(1)“75”	80	V-X	III	

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. purpurella		Donegal	Ir	*Hodkinson 2*	A	70“73”“75”	80	V	X-III	
D. nieschalkiorum	11801	40°36′37 N, 31°16′17″ E	Tu	*Hedrén 98081*	C	75	80	III		
D. nieschalkiorum (*ilgazica*)	11793	41°02′81″ N, 34°04′11″ E	Tu	*Hedrén 98057*	C	75	80	III		
D. praetermissa	1124	Hertfordshire, Blagrove common	UK	*Denholm s.n.*	C	75	80	III		
D. praetermissa	10235	Nr. Cardiff	UK	*Chase 10235* (*1*)	A	7273	80	X-VI		
D. praetermissa	10236	Nr. Cardiff	UK	*Chase 10235* (*2*)	A	73(2)75(1)	80	X-III		2:1
D. praetermissa	10237	Nr. Cardiff	UK	*Chase 10235* (*3*)	A	“72”75	80	III	VI	
D. praetermissa	10238	Nr. Cardiff	UK	*Chase 10235* (*4*)	A	“72”75	80	III	VI	
D. praetermissa	10239	Nr. Cardiff	UK	*Chase 10235* (5)	A	“72”75	80	III	VI	
D. praetermissa	14052	Nr. Cardiff	UK	*Chase 14052* (spirit)	A	70(1)“75”73(1)	80	V-X	III	1:1
D. praetermissa	14053	Nr. Cardiff	UK	*Chase 14053* (spirit)	A	70(1)“72”73(1)	80	V-X	VI	1:1
D. praetermissa	14054	Nr. Cardiff	UK	*Chase 14054* (spirit)	A	72“73”	80	VI	X	
D. praetermissa	14056	Nr. Cardiff	UK	*Chase 14056* (spirit)	A	75	80	III		
D. praetermissa	14057	Nr. Cardiff	UK	*Chase 14057* (spirit)	A	72“73”	80	VI	X	
D. praetermissa	14059	Nr. Cardiff	UK	*Chase 14059* (spirit)	A	~70(1)72(1)75(1)	80	V-III-VI		
D. praetermissa	15173	Hants, Brambridge	UK	*Bateman 469*	C	70(1)“73”75(3)	80	III-V	X	3:1
D. praetermissa	15175	Dorset, Hinton St Mary	UK	*Bateman 471*	A	“73”75	80	III	X	
D. praetermissa junialis	15174	Hants, Brambridge	UK	*Bateman 470*	C	70(1)“73”75(3)	80	V-III	X	1:3
D. praetermissa junialis	15176	Dorset, Hinton St Mary	UK	*Bateman 472*	A	73(1)75(3)	80	III-X		3:1
D. praetermissa junialis	6098	Novia Scotia, St Johns	Ca	*Bateman 76*	C	72(3)73(1)	80	VI-X		3:1
D. praetermissa?	16548	Ynyslas, Dyfed	UK	*Chase 16548* (spirit)	A	“72”73	80	X	VI	
D. praetermissa? (collected as *maculata* s.l).	12122	Pyrenees, St Marcel-Aulon	Fr	*Civeyrel & al. 567*	A	70(3)72(1)75(2)	80	V-III-VI		
D. urvilleana	11799	40°44′49″ N, 39°33′51″ E	Tu	*Hedrén 98020*	C	75	80	III		
D. urvilleana	H31	Trabzon, Uzungol	Tu		C	75	80	III		

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. sp. (*allotet*)	3985	Gotland, Viklau	Sw	*Hedrén 97098*	A	70	80	V		
D. sp. (*allotet*)	3997	Uppland, Frotuna	Sw	*Hedrén 97226*	A	70(1)“73”75(1)	80	V-III	X	1:1
D. sp. (*allotet*)	H134	Gotland, Gylvik	Sw		A	~73(1)75(1)	80	III-X		
D. sp. (*allotet*)	H436	Vastragotaland, Dimbo	Sw	*Hedrén 010710*	N	75	72	I		
D. sp. (*allotet*)	H440	Vastragotaland, Dimbo	Sw	*Hedrén 010710*	X	75	72	I		
D. sp. (*allotet*)	H135	Gotland, Gylvik	Sw		E	70(1)73(1)	80	V-X		1:1
D. incarnata	11857	Burgenland, Zitzmannsdorfer Wiese	Au	*Fischer & al.* no voucher	E	73	80	X		
D. incarnata	11858	Burgenland, Zitzmannsdorfer Wiese	Au	*Fischer & al.* no voucher	E	73	80	X		
D. incarnata	12141	Pyrenees, Pla des Ails	Fr	*Civeyrel & al. 585A*	E	73	80	X		
D. incarnata	12142	Pyrenees, Pla des Ails	Fr	*Civeyrel & al. 585B*	E	73	80	X		
D. incarnata	12143	Pyrenees, Pla des Ails	Fr	*Civeyrel & al. 585C*	E	73	80	X		
D. incarnata	16545	Jura, Sarvagnat	Fr	*Chase 16545* (spirit)	E	73	80	X		
D. incarnata	16546	Jura, Sarvagnat	Fr	*Chase 16546* (spirit)	E	73	80	X		
D. incarnata	15179	Roscommon, Lough Rea	Ir		E	73	80	X		
D. incarnata	15180	Roscommon, Lough Rea	Ir		E	73	“78”80	X	VIII	
D. incarnata	15181	Roscommon, Lough Rea	Ir		E	73	78(1)80(3)	X-VIII		3:1
D. incarnata	3970	Skane, Troll-Ljungby	Sw	*Hedrén 97034*	E	73	80	X		
D. incarnata	3975	Gotland, Hall	Sw	*Hedrén 97082*	E	73	80	X		
D. incarnata	3986	Uppland, Bladaker	Sw	*Hedrén 97132*	E	73	80	X		
D. incarnata	3987	Västragotaland, Radane	Sw	*Hedrén 97164*	E	73	80	X		
D. incarnata	3991	Ostergotaland, Slaka	Sw	*Hedrén 97194*	E	73	80	X		
D. incarnata	3994	Södermanland, Svarta	Sw	*Hedrén 97213*	E	73	80	X		
D. incarnata	0-1378	Gotland, Harudden	Sw		E	73	80	X		
D. incarnata	H4	Erzurum, E Erzurum	Tu		E	73(1)77(1)	80	X	X-XII	1:1
D. incarnata	13502	Anglesey, Nant Isaf	UK	*Fay 661a* (spirit)	E	73	78(1)80(3)	X-VIII		3:1

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. incarnata	13503	Anglesey, Nant Isaf	UK	*Fay 661b* (spirit)	E	73	80	X		
D. incarnata	13504	Anglesey, Nant Isaf	UK	*Fay 661c* (spirit)	E	73	80	X		
D. incarnata	13516	Anglesey, Rhos-y-gard	UK	Fay 632 (spirit)	E	73	80	X		
D. incarnata	13751	Anglesey, Newborough Warren	UK	*Fay 666* (spirit)	E	73	80	X		
D. incarnata	13759	Nr. Llanfrothen	UK	*Fay 675* (spirit)	E	73	80	X		
D. incarnata	15178	Barra, Eoligarry	UK	*Bateman 582*	E	73	80	X		
D. incarnata	15185	Dyfed, Ynyslas	UK	*Chase 15185* (spirit)	E	73	80	X		
D. incarnata	15186	Dyfed, Ynyslas	UK	*Chase 15186* (spirit)	E	73	80	X		
D. incarnata	15187	Dyfed, Ynyslas	UK	*Chase 15187* (spirit)	E	73	80	X		
D. incarnata s.l.	O-1385	Gotland	Sw		E	73	80	X		
D. incarnata s.l.	O-1388	Gotland	Sw		E	73	80	X		
D. cruenta	3974	Gotland, Hall	Sw	*Hedrén 97078*	E	73	80	X		
D. cruenta	3988	Ostergotaland, Karna	Sw	*Hedrén 97178*	E	73	80	X		
D. cruenta	5546	Hürjedalen, Linsell	Sw	*Hedrén 97272*	E	73	80	X		
D. cruenta	5549	Jämtland, Hammerdal	Sw	*Hedrén 97287*	E	73	80	X		
D. cruenta	6097	Wester Ross, Lochdroma	UK	*Bateman 57*	E	73	80	X		
D. cruenta	10163	Mulloch Mor	Ir	*Chase 10163*	E	73	80	X		
D. cruenta	10165	Lough Bunny	Ir	*Chase 10165*	E	73	80	X		
D. cruenta	15163	Lough Carra, Mayo	Ir	*Bateman 115*	E	73	80	X		
D. cruenta	15164	Lough Bunny	Ir	*Bateman 116*	E	73	80	X		
D. ochroleuca	3973	Gotland, Hall	Sw	*Hedrén 97075*	E	73	80	X		
D. ochroleuca	3990	Ostergotaland, Kaga	Sw	*Hedrén 97192*	E	73	80	X		
D. ochroleuca	14696	Cambs, Chippenham fen	UK	*Bateman 612*	E	73	80	X		
D. ochroleuca	15172	Murnauer Moos	Ge	*Bateman 462*	E	73	80	X		
D. pulchella	O-988	Wester Ross, Kernsary	UK	*Bateman 56*	E	73	80	X		

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. pulchella	13505	Anglesey, Nant Isaf	UK	*Fay 662* (spirit)	A	73	80	X		
D. pulchella	13783	Surrey, Thursley Heath	UK	*Chase 13783a* (spirit)	E	73	80	X		
D. pulchella	13784	Surrey, Thursley Heath	UK	*Chase 13783b* (spirit)	E	73	80	X		
D. pulchella	13785	Surrey, Thursley Heath	UK	*Chase 13783c* (spirit)	E	73	80	X		
D. pulchella	15182	Dyfed, Ynyslas	UK	*Chase 15182* (spirit)	E	“70”73	80	X	V	
D. coccinea	O-965	East Lothian, Aberlady Bay	UK	*Bateman 45*	E	73	80	X		
D. coccinea	13752	Anglesey, Newborough	UK	*Fay 667a* (spirit)	E	73	80	X		
D. coccinea	13753	Anglesey, Newborough	UK	*Fay 667b* (spirit)	E	73	80	X		
D. coccinea	15183	Dyfed, Ynyslas	UK	*Chase 15183* (spirit)	E	73	80	X		
D. coccinea	15184	Dyfed, Ynyslas	UK	*Chase 15184* (spirit)	E	73	80	X		
D. borealis	5548	Jämtland, Hammerdal	Sw	*Hedrén 97286*	E	73	80	X		
D. borealis	5551	Lycksele	Sw	*Hedrén 97303*	E	73	80	X		
D. armeniaca	H39	Artvin, Savsat	Tu	*Hedrén 000609*	E	73	80	X		
D. euxina	12821	40°41′52″ N, 40°41′64″ E	Tu	*Hedrén 98025*	K	73	80	X		
D. euxina	H37	Trabzon, Zigana Pass	Tu		Y	73	80	X		
D. iberica	O-960	Kew 1982-1515, Troodos Monts	Cy	*Chase O-960*	J	73	73	XI		
D. iberica	12822	40°36′37″ N, 31°16′17″ E	Tu	*Hedrén 98078*	E	73	80	X		
D. sambucina	O-1373	Gotland, Valar	Sw		S1	75	80	III		
D. sambucina	O-1374	Gotland, Valar	Sw		S2	75	80	III		
D. sambucina	O-1377	Gotland, Hejnum	Sw		S1	75	80	III		
D. sambucina	14610	Paklenica National Park	Cr	No voucher	S1	75	80	III		
D. sambucina	14611	Paklenica National Park	Cr	No voucher	S1	75	80	III		
D. sambucina	14612	Paklenica National Park	Cr	No voucher	S1	75	80	III		
D. sambucina	14613	Paklenica National Park	Cr	No voucher	S1	75	80	III		
D. sambucina	15168	Huesca, Valle de echo	Sp	*Bateman 315*	S1	75	80	III		

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. sambucina	15188	Massif Central, Vallee de la Dourbie	Fr		S1	75	80	III		
D. romana	O-760	Siena	It	*Rossi*	R1	76	78	IX		
D. romana	15177	Sicily, NE Etna	It	*Bateman 522*	R2	76	78	IX		
D. romana	15987	Crete, NW Spili	Gr	*Bateman 34*	R3	76	78	IX		
D. viridis	13070	Li Xian County, northwestern Sichuan	Ch	*Luo & Luo 657*	V3	73	78	VIII		
D. viridis	13074	Hongyuan County, northwestern Sichuan	Ch	*Luo & Luo 667*	V5	73	78	VIII		
D. viridis	13075	Hongyuan County, northwestern Sichuan	Ch	*Luo & Luo 668*	V2	73	78	VIII		
D. viridis	13079	Jinchuan County, northwestern Sichuan	Ch	*Luo & Luo 677*	V5	73	78	VIII		
D. viridis	15985	Alpes, Col du Sarenne	Fr	*Bateman 71*	V1	73	_	IX		
D. viridis	15982	Fifeness, Fife	UK	*Bateman 66*	V1	73	_	IX		
D. viridis	O-576	UK	UK	*Hartwell s.n.*	V3	73	_	IX		
D. viridis	15979	40°40′37″ N, 40°42′59″ E	Tu	*Hedrén 98033*	V6	73	78	VIII		
D. viridis	15980		Sw	*Hedrén 980706*	V1	73	_	IX		
D. viridis	O-1396				V4	73	_	IX		
D. sp.	14602	Georgia	Go	*Albach 430*	H	73	80	X		
D. sp.	14603	Georgia	Go	*Albach 430*	H	73	80	X		
D. sp.	15155	Georgia	Go	*Albach 431*	U	75	80	III		
D. sp.	14691	Georgia	Go	*Albach 431*	I	73(1)75(1)	80	X-III		1:1
D. transiens	10283	Kew 1980-2844, W Suffolk, Elmswell	UK	*Chase 10283*	C	“72”“73”75	80	III	VI-X	
D. hybrid	13507	Anglesey, Nant Isaf	UK	*Fay 664* (spirit)	C	70(3)73(1)	80	V-X		3:1
D. maculata × *traunsteineri*	13508	Anglesey, Nant Isaf	UK	*Fay 665* (spirit)	C	75>72	72>80	I-III		
D. fuchsii × *maculata*?	13513	Nr Llanfrothen	UK	*Fay 679* (spirit)	A	73(1)75(2)	72(2)80(1)	I-X	(III)	2:1

Appendix. Continued.

Species	Number	Origin	Country	Voucher	Haplotype	Gel reading of the ITS fragments (lengths in bp)		Alleles		
						Dfuch	Dmac	Major	Minor	Ratio
D. majalis × *incarnata*	12145	Pyrennees, Pla des Ails	Fr	*Civeyrel & al. 586B*	E	73(2)75(1)	80	X-III		2:1
D. majalis × *incarnata*	12148	Pyrennees, Pla des Ails	Fr	*Civeyrel & al. 596E*	E	73(1)75(2)	80	III-X		2:1

Note: Empty cells denote unavailable information.

[a]Abbrevations: Al, Algeria; An, Andorra; Au, Austria; Ch, China; Cr, Croatia; Cy, Cyprus; Es, Estonia; Fr, France; Ge, Germany; Go, Georgia; Gr, Grecce; He, Switzerland; Ic, Iceland; Ir, Ireland; It, Italy; Mo, Morocco; No, Norway; Po, Portugal; Sp, Spain; Sw, Sweden; Tu, Turkey; UK, United Kingdom

[b]All in K unless otherwise noted.

[c]A band was considered as minor (indicated between quotation marks “ ”), if it was more than three times weaker than the strongest band. Similarly an allele is minor if its quantity is at least three times smaller than the one of the main allele, otherwiese it was considered as major.
Number in brackets for the gel reading indicate the relative proportion of a given band.
Brackets are used for alleles that were not observed but for which we could not reject the presence due to band overlap.
The ratio given only takes into account the major alleles.

*Same length as III, but this allele has substitutional differences when sequenced.