

DACTYLORHIZA (ORCHIDACEAE) IN EUROPEAN RUSSIA: COMBINED MOLECULAR AND MORPHOLOGICAL ANALYSIS¹

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Four plastid and two nuclear (internal transcribed spacer [ITS] ribosomal DNA) markers were used in this study of the *Dactylorhiza maculata* and *D. incarnata* complexes (Orchidaceae: Orchidinae) to determine diversity and taxonomic distribution of haplotypes, hybridization frequencies, and maternal parentage of hybrids in 125 samples from 78 populations from European Russia and the Caucasus. A morphometric study of all populations revealed significant correspondence between morphological and plastid DNA data. Most *D. maculata* sensu stricto (s.s.) specimens from Russia have *D. fuchsii* haplotypes; this could be evidence for introgression or widespread hybridization between these species in northern Russia. Heterogeneity within populations is much higher for ITS data and is strongly correlated with latitude. Both plastid and nuclear data are significantly correlated with distribution along a south–north axis. Several haplotypes and ITS alleles uncommon in western Europe are more widely distributed in Russia, whereas some frequent haplotypes from western Europe are absent.

Key words: combined analysis; *Dactylorhiza*; microsatellites; Orchidaceae; systematics.

Dactylorhiza Necker ex Nevski is a moderately species-rich orchid genus distributed across subtropical to Arctic Eurasia and North Africa; one species (*D. aristata*) also occurs in Alaska and one (*D. praetermissa*) in Newfoundland. Unfortunately, a stable infrageneric classification of *Dactylorhiza* does not yet exist. The most recent monographic work (Averyanov, 1988, 1989, 1990a, b, 1991, 1992) based on previous classifications (Vermeulen, 1947; Senghas, 1968), contained a system in which the genus is divided into four sections and seven subsections. Many specialists (e.g., Delforge, 1995) broadly accept the species placement of Averyanov's system but use informal "groups" instead of formal "sections" and "subsections."

Subsequent investigations of allozyme markers (e.g., Hedrén, 2002) confirmed that some species of *Dactylorhiza* are diploid (e.g., *D. fuchsii* and *D. incarnata*), at least one other is apparently autopolyploid (*D. maculata* sensu stricto [s.s.]), and a third group of species has an allotetraploid origin (*D. majalis* and others). Recent molecular studies, based on internal transcribed spacer (ITS) sequencing (Bateman et al., 1997, 2003) and amplified fragment length polymorphism (AFLP) analysis (Hedrén et al., 2001; Hedrén, 2002), have shown that the genus is sister to *Gymnadenia* sensu lato (s.l.) (Bateman et al., 1997) and consists of five groups (Bateman and Denholm, 2003; Bateman et al., 2003): "*Dactylorhiza incarnata* group," including *D. euxina* and *D. umbrosa* (diploids); "*Dac-*

tylorhiza maculata group," including *D. fuchsii*, *D. saccifera*, *D. foliosa* (diploids), and *D. maculata* (autotetraploid); "*Dactylorhiza majalis* group," including the allotetraploid species *D. traunsteineri*, *D. baltica*, *D. russowii*, *D. praetermissa*, *D. purpurella*, and associated infraspecific taxa; "*Dactylorhiza sambucina* group," including *D. romana* and *D. flavescens* (all diploid); and putatively "primitive" diploids, such as *Dactylorhiza aristata*, *D. viridis* (= *Coeloglossum viride*), and *D. iberica*.

The number of species (12–75) recognized varies significantly among authors (Pedersen, 1998). Their underlying taxonomic concepts differed, and many species are poorly defined. Some authors, for example, accepted morphologically different allotetraploid forms as different species (Averyanov, 1988, 1989, 1990a, b, 1991, 1992; Tyteca and Gathoye, 1999, 2000), but others (Bateman and Denholm, 1983; Hedrén et al., 2001) insist that most of them are subspecies (or even varieties) of *D. majalis* s.l. Another example is *D. "cruenta"*, which we treated only as a form of *D. incarnata* in this study. This situation can be explained by a high frequency of hybridization and polyploidization events (Vermeulen, 1947; Heslop-Harrison, 1968) and significant polymorphism of most morphological characters widely used in diagnoses.

Smoljaninova (1976) listed nine species of *Dactylorhiza* from European Russia, whereas Averyanov (1988, 1989, 1990a, b, 1991, 1992) recognized 14 species plus a further eight from the Russian part of the Caucasus. Averyanov united these species in "species aggregata," six in European Russia and five in the Caucasus. According to Cherepanov (1995), there are 13 species of *Dactylorhiza* from European Russia and six from the Russian Caucasus. Later, Averyanov (2000) accepted only seven species from European Russia.

The main taxonomic problems of Russian dactylorchids are similar to those previously elucidated for western Europe: (1) relations within and between members of the *Dactylorhiza maculata* complex, (2) taxonomic status of allotetraploids (notably *D. traunsteineri* s.l. and *D. baltica*) and their origin, and

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TABLE 1. Classification of European Russian and Russian Caucasus *Dactylorhiza* species according to Averyanov (1988, 1989, 1990a, b, 1991, 1992).

Sections	Subsections	"Species aggregata"	Species
<i>Dactylorhiza</i>	<i>Dactylorhiza</i>	<i>incarnata</i>	<i>D. incarnata</i>
		<i>olocheilos</i>	<i>D. cruenta</i>
			<i>D. euxina</i>
			<i>D. markowitschii</i>
			<i>D. salina</i>
			<i>D. amblyopoda</i>
	<i>Maculatae</i>		<i>D. urvilleana</i>
			<i>D. fuchsii</i>
			<i>D. hebridensis</i>
			<i>D. psychrophila</i>
			<i>D. maculata</i>
			<i>D. elodes</i>
<i>Latifoliae</i>		<i>D. sudetica</i>	
		<i>D. kolaensis</i>	
		<i>D. baltica</i>	
		<i>D. traunsteineri</i>	
		<i>D. russowii</i>	
		<i>D. curvifolia</i>	
<i>Sambucinae</i>		<i>D. sambucina</i>	
		<i>D. flavescens</i>	
		<i>D. ruprechtii</i>	
		<i>D. romana</i>	

(3) taxonomic status of different forms of the *D. incarnata* aggregate. Here we use the separate names for members of the *D. maculata* complex, and where we state *D. maculata*, we are referring to the narrowly defined autotetraploid rather than the whole complex (i.e., including *D. fuchsii* and other diploids).

Unfortunately, there are fewer taxonomic studies of dactylorhizids in Russia than in western Europe. Most investigators have used the so-called "classical" method, based on analysis of herbarium specimens, which over the last 30 years has been challenged by the adoption of morphometric approaches and more recently by the advent of multivariate statistical methods. Fortunately, molecular methods are also fully quantitative and therefore can be used in conjunction with morphometry. For Russian material, morphometric studies are rare, and molecular analyses have never been performed. The goal of our work was to establish a morphometric and molecular framework for an extensive investigation of European Russian *Dactylorhiza*, simultaneously utilizing both morphological and molecular data in the "demographic" approach advocated by Bateman (2001; i.e., limits of taxa are established by looking at variation within and between populations).

Choice of molecular marker—Plastid DNA is widely used in molecular phylogenetic and phylogeographic studies. These regions evolve relatively slowly and hence can help to determine patterns of geographical distribution, although some polymorphic regions have proved useful for distinguishing among closely related species and even populations (Soliva and Widmer, 1999; Fay and Cowan, 2001). Plastid DNA also offers the ability to determine the maternal parent of an allopolyploid because orchids have uniparental (maternal) inheritance of plastids (Corriveau and Coleman, 1988).

The internal transcribed spacer (ITS) region of nuclear ribosomal DNA is another valuable tool for understanding infrageneric taxonomy in orchid systematics (Bateman et al., 1997, 2003), but polyploidy, hybridization, and gene conversion (Franzke and Mummenhoff, 1999; Chase et al., 2003) in

Dactylorhiza can lead to undesirable complexity (Bateman et al., 2003). On the other hand, ITS offers the possibility of elucidating hybrid origins and maternity when combined with plastid sequences (e.g., Bateman and Hollingsworth, 2004). In this study, we use a combined approach based on all three data types: morphometric, plastid haplotypes (from four regions), and ITS alleles (assessed on the basis of two length variable regions).

MATERIALS AND METHODS

Molecular material—Tissue samples from 125 individual plants representing 78 populations were collected in European Russia and the western Caucasus, from Krasnodar to the Murmansk region (more than 3000 km from south to north). The samples were dried in silica gel. DNA was extracted by the 2× cetlytrimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1987), but without an RNA treatment. Polymerase chain reactor (PCR) was performed with a set of primers (Y. Pillon, M. F. Fay, and M. W. Chase, unpublished data) designed to amplify four polymorphic plastid loci (Table 2) in three plastid DNA regions: the *trnS-trnG* spacer, the *trnL* intron, and the *trnL-trnF* spacer.

The ITS sequences (including the 5.8S rDNA gene) were determined for representatives of 35 species of *Dactylorhiza*, following the sequencing protocol described by Pridgeon et al. (1997), but with the addition of dimethyl sulfoxide (DMSO) in the PCR reactions to reduce problems in primer annealing caused by the typical secondary structure (stems and loops) of ribosomal DNA. Sequence editing and assembly were done with Sequence Navigator and AutoAssembler (Applied Biosystems [ABI] Warrington, Cheshire, UK). Alignment was performed by eye because the level of sequence divergence was low and insertions/deletions (indels) were rare. As a result, we observed that *Dactylorhiza* maintains several ITS alleles (Bateman et al., 2003) that differed from one another by both base substitutions and characteristic indels; these indels alone are good markers for these alleles (Fig. 1). Two pairs of primers were designed to amplify length-polymorphic regions and permit us to detect the presence of the different ITS alleles (Table 3). Thus, the characterization of particular ITS alleles was first accomplished via direct sequencing of PCR products containing these amplified regions and cloning if more than one allele was present, but then in the later stages by amplification of the two length-variable fragments described above, which permitted us to determine which alleles were present in each plant accession.

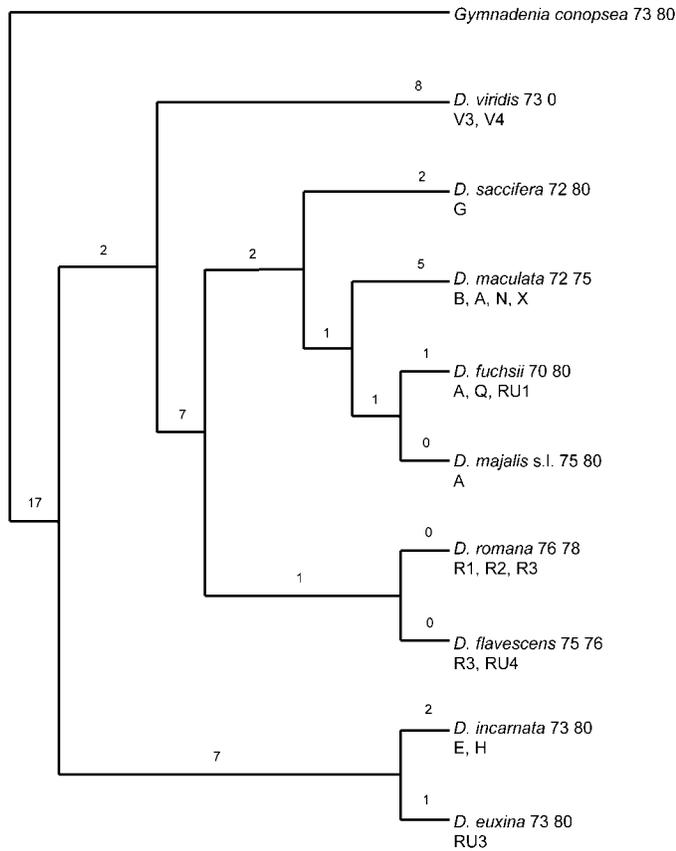


Fig. 1. The cladogram (branch lengths shown) of some of *Dactylorhiza* ITS alleles. The sizes of “D_fuch” and “D_mac” fragments, respectively (see Table 3), are the two numbers after each species allele.

All samples for the length-variable fragment analyses were run on a 3100 genetic analyzer (ABI), and the size of fragments was determined using GeneScan and Genotyper software (ABI). A minimum spanning network (MSN) for plastid haplotypes was constructed via Arlequin version 2.0 using a matrix of Kronecker distances (Schneider et al., 2000). This network was visualized using Graphviz version 1.10 (Gansner and North, 2000).

Morphometric material—Many previous investigators have used a set of morphological characters in alliance with multivariate statistical analysis for characterization of *Dactylorhiza* accessions (Bateman and Denholm, 1983, 1985, 1989, 2003; Reinhard, 1990; Dufrière et al., 1991; Pedersen, 1998; Tyteca and Gathoye, 1999, 2000; Foley, 2000). Principal components analysis (PCA) was used in a majority of these studies and also several more recent papers in which morphological and DNA data were combined at the population level, as was recently done for pit vipers (Puerto et al., 2001). Principal components analysis is a well-known technique that uses eigenvalue matrix calculations to visualize multidimensional data and compute loadings for each character. Another useful approach is principal coordinates analysis (PCoA),

TABLE 3. Internal transcribed spacer (ITS) polymorphic fragments used.

Primer name	Locus	Typical length (base pairs) for alleles		
		<i>Dactylorhiza incarnata</i>	<i>Dactylorhiza fuchsii</i>	<i>Dactylorhiza maculata</i>
D_fuch	ITS	73	70	75
D_mac	ITS	80	80	72

which uses a distance matrix instead of the data matrix for PCA. We chose 14 morphological characters (Table 4), most of which were measured in nature on the same plants subsequently used for DNA extractions (in a few cases we measured neighboring plants occurring in the same population). We analyzed these data using both PCA and multidimensional scaling (MDS, the expanded variant of PCoA). All statistical calculations used the R program, version 1.71 for Windows and Linux (Venables et al., 2002).

RESULTS

Species-level taxonomy—Plastid DNA—We found 15 combinations of plastid fragment lengths, henceforth termed haplotypes (Table 5), which were assigned to the samples without initial reference to the species designations. The minimum spanning network (Fig. 2) reflects the relationships and distances between haplotypes. There are several groups of haplotypes: (1) *D. fuchsii* group (A, G, RU1, Q), (2) *D. maculata* group (B, N, X), (3) *D. incarnata* group (E, H, RU3, RU5), and (4) *D. flavescens* group (R3, RU4, V3, V4). Among these, the *D. fuchsii* (A) haplotype is the most frequent and widespread in Europe (Y. Pillon, M. F. Fay, and M. W. Chase, unpublished data).

We also observed several haplotypes specific to Russia, namely RU1, RU3, RU4, and RU5 (the first was from northern Russia, the others from the western Caucasus). The first is similar to the typical A haplotype. The RU3 haplotype found in *D. euxina* (= *D. caucasica*) is close (but not identical) to the K haplotype reported from samples of this species in Turkey (Y. Pillon, M. F. Fay, and M. W. Chase, unpublished data). The RU4 haplotype belongs to *D. flavescens* and has a wider distribution in the western Caucasus than the R3 haplotype typical of this species. Some of these vouchers, however, were labeled as “*D. romana*” by the collector, but neither the ITS data nor the morphology of these specimens show any differences from *D. flavescens*. Haplotype RU5 is intermediate between the *D. incarnata* and *D. flavescens* groups, more closely resembling *D. incarnata*.

There is a clear association between species and haplotype, with only a few exceptions. Most northern Russian *D. maculata* specimens share their haplotype with *D. fuchsii* and do not have haplotype B (or N or X), which is typical for *D. maculata* from central Russia and western Europe. All samples collected as “*D. cruenta*” have haplotype E, which is typical

TABLE 2. Plastid microsatellites and polymorphic fragments used.

Region name	Locus	Typical length (base pairs)	
		<i>Dactylorhiza incarnata</i>	<i>Dactylorhiza fuchsii</i>
Orch1 microsatellite	<i>trnL</i> intron	84	85
Msf polymorphic fragment	<i>trnL-trnF</i> spacer	163	159
Msl1 polymorphic fragment	<i>trnS-trnG</i> spacer 5' region	232	234
Msl2 microsatellite	<i>trnS-trnG</i> spacer 3' region	226	236

TABLE 4. Morphological characters used for *Dactylorhiza*.

Label	Description (all in millimeters)
PHIGH	Plant height, from the ground to the top of inflorescence
LEAFL	Length of longest leaf
LEAFW	Width of longest leaf
L.WPOS	Position of maximal width (the distance from leaf base to the place of maximal width)
LEAFSP	Leaf spot presence (1 none, 2 weak, 3 heavy)
SPOTS	Leaf spot shape (1 elongated, 2 rounded)
INFL.L	Length from the lowest bract to the top of inflorescence
SPUR.L	Spur length, measured underneath the spur
LIPL	Lip length, from the base to the top of middle lobe
LIPW	Lip width
MIDD.L	Length of middle lobe of the lip, from the base to the top of lobe
LATER.L	Length of lateral lobe of the lip, from the base to the top of lobe
LIPDRW	Lip marking (0 none, 1 spotted, 2 striate, 3 other)
LIPCOL	Lip color (1 white or nearly white, 2 pink, 3 dark pink)

of *D. incarnata*. Haplotype H (initially found in Georgian material) was also found in several *D. incarnata* samples from central Russia. *Dactylorhiza saccifera* (= *D. amblyopoda*) and some of the *D. flavescens* samples from western Caucasus have haplotypes (G and R3, respectively) typical of these species. *Dactylorhiza viridis* (= *Coeloglossum viride*) samples have haplotypes V3 and V4.

Our putative tetraploid specimens (*D. traunsteineri*, *D. russovii*, and *D. baltica*) had the A haplotype most commonly found in *D. fuchsii*. Some northern plants with the A and Q haplotypes (collected as either “*D. maculata*” or “*D. fuchsii*”) could also in fact be allotetraploids (see Discussion).

ITS—The data from nuclear ITS fragments are much more diverse, as expected for a DNA region with biparental inheritance (Table 5). In addition, tetraploids can possess up to four alleles, and we observed the conversion of the ITS allele to the parental type in many plants (some fragments were much lower in intensity than would otherwise have been expected

in hybrids). We again immediately noted that the distribution of ITS alleles and previously used species concepts (Table 1) were highly correlated.

Nearly all specimens with the plastid A haplotype are polymorphic for ITS alleles. Most of them, especially from northern Russia, have both the *D. maculata* and *D. fuchsii* alleles. Specimens with the A haplotype from central Russia often have some copies of the *D. incarnata* allele, which is similar to many specimens of western European *D. traunsteineri* and some other allotetraploids (e.g., *D. praetermissa* and *D. purpurella*). The 77-base-pair (bp) “D_{mac}” fragment (nearly identical to 75-bp “D_{mac}” fragment) is rare in western European populations, but it is frequent among Russian *D. maculata*. Fragment lengths of *D. incarnata* are stable among different populations. Two Caucasian *D. flavescens* samples with the R3 haplotype have traces of a *D. fuchsii* allele, but other samples of this species have only the pure *D. flavescens* allele. An analogous situation exists for *D. saccifera*, but in this case there are traces of *D. incarnata* alleles. *Dactylorhiza euxina* (haplotype RU3) and putative *D. armeniaca* (haplotype RU5; see later) both have only the *D. incarnata* allele.

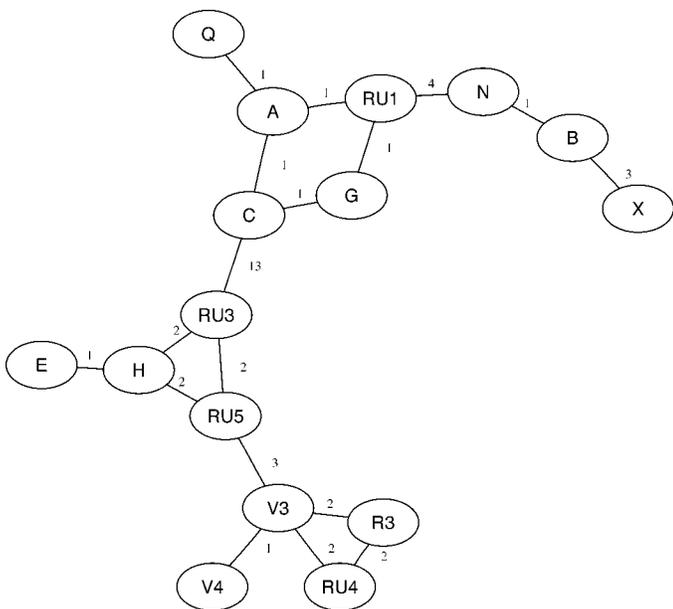


Fig. 2. Minimum spanning network for observed haplotypes of *Dactylorhiza*. Numbers are connection lengths calculated from the matrix of Kronecker distances.

Morphology—Both PCA and MDS of the morphological data revealed similar structure that corroborates haplotype distribution (Fig. 3); the samples form four groups. Most samples belong to group I in the upper left (one member of this group falls into group II), which contains *D. maculata*, *D. fuchsii*, and *D. traunsteineri* samples with haplotypes A, Q, and RU1, mostly from the Russian North. Others are group II in the lower left (one member falls into group IV), which contains *D. incarnata* with haplotype E; group III with two subgroups in upper right and center of the ordination, which contains *D. maculata* with haplotypes B and X; and group IV in lower right, which contains *D. fuchsii*, *D. baltica*, and putative allotetraploids with haplotype A, most from central Russia.

The most important morphological characters distinguishing these four groups are reflected in their loadings on the first two (or in some cases three) principal components. In this case, the first two principal components encompass more than 53% of variation, so use of lower order components is not necessary. The highest loadings are: plant height, leaf length, length of lateral lobes of the lip, and position of maximal leaf width for PC1, and leaf spot shape, spike length, lip width, and leaf width for PC2 (Table 6).

Adding other data can enhance the quality of grouping ob-

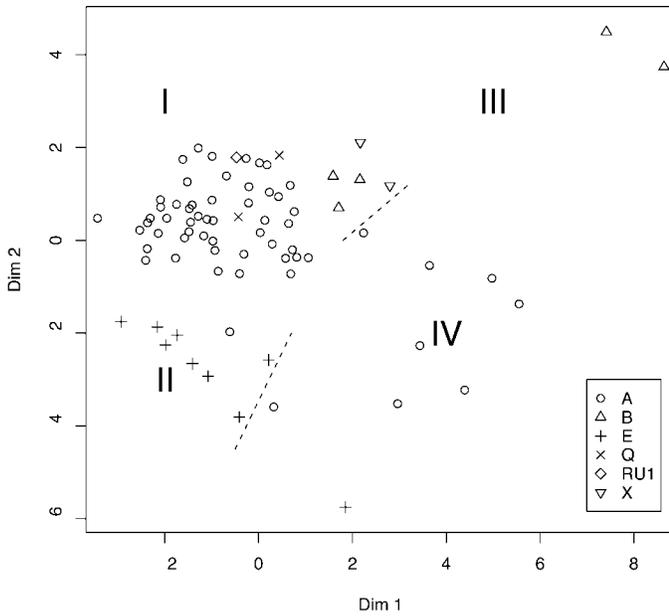


Fig. 3. Multidimensional scaling of *Dactylorhiza* morphological data (plastid haplotypes of these plants are indicated).

served in PCA. If these data are congruent, the distinction of groups usually becomes clearer. In this study, patterns become more structured after addition of the haplotype data to the source matrix for PCA; in contrast, the addition of the ITS data reveals somewhat less structure (Fig. 4). The addition of both plastid and ITS data revealed distinctive groups without outliers (Fig. 5).

TABLE 6. Character loadings in first two principal components for the analysis of *Dactylorhiza* morphological data only (high loadings are highlighted in boldface type).

Character abbreviation	Principal component 1 (34.2% of variance)	Principal component 2 (19.2% of variance)
PHIGH	-0.362	-0.190
LEAFL	-0.348	-0.278
LEAFW	-0.309	-0.337
L.WPOS	-0.344	0
LEAFSP	0	0.373
SPOTS	-0.117	0.403
SPUR.L	-0.313	0.232
LIPL	-0.305	0.217
LIPW	-0.279	0.347
MIDDLE.L	-0.202	0.190
LATER.L	-0.352	0.118
LIPDRW	0	0.168
LIPCOL	0.141	-0.200
INFL.L	-0.240	-0.362

Groups I and IV—There are two different groups of samples with the A haplotype: (I) “*D. maculata*-like” forms, mainly from northern Russia, and (IV) typical *D. fuchsii* and some putative allotetraploid forms, mainly from southern areas of central Russia. Group IV have either *D. incarnata* alleles or are “pure” *D. fuchsii*, but they never have *D. maculata* alleles.

With the aim of understanding morphological differences between these groups, we analyzed morphometric information from samples with the A haplotype by discriminant analysis and regression trees. Discriminant analysis of the morphological characters supported both group I and group IV with 100% probability ($\chi^2 = 36$, $P \ll 0.01$). The most important discriminant characters were (a) leaf width and (b) plant height. Regression trees (Breiman et al., 1984) can describe

TABLE 5. Combinations of plastid haplotypes and ITS DNA alleles of *Dactylorhiza* (*D. viridis* excluded).

Plastid microsatellites (lengths, bp)				Plastid haplotype	ITS alleles					Species assignment
<i>orch1</i>	<i>Msf</i>	<i>ms1</i>	<i>ms2</i>		<i>D. fuchsii</i> (70, 80) ^a	<i>D. incarnata</i> (73, 80)	<i>D. maculata</i> 1 (75, 72)	<i>D. maculata</i> 2 (75, 77)	<i>D. flavescens</i> (75, 76)	
85	159	234	236	A	+	-	-	-	-	<i>D. fuchsii</i> , <i>D. maculata</i>
85	159	234	236	A	+	-	+	-	-	<i>D. fuchsii</i> , <i>D. maculata</i> , <i>D. traunsteineri</i>
85	159	234	236	A	+	+	-	-	-	<i>D. baltica</i> , <i>D. fuchsii</i>
85	159	234	236	A	+	+	+	-	-	<i>D. fuchsii</i> , <i>D. maculata</i>
85	159	238	236	B	+	-	-	-	-	<i>D. maculata</i>
85	159	238	236	B	+	-	+	-	-	<i>D. maculata</i>
85	159	238	236	B	+	-	+	+	-	<i>D. maculata</i>
85	159	238	236	B	+	+	+	+	-	<i>D. maculata</i>
84	163	232	226	E	-	+	-	-	-	<i>D. incarnata</i>
86	159	234	235	G	-	-	+	-	-	<i>D. saccifera</i>
86	159	234	235	G	-	+	+	-	-	<i>D. saccifera</i>
84	163	232	227	H	-	+	-	-	-	<i>D. incarnata</i> (including “ <i>cruenta</i> ”)
86	159	238	236	N	+	-	+	-	-	<i>D. maculata</i>
84	159	234	236	Q	+	-	+	-	-	<i>D. fuchsii</i>
84	159	234	236	Q	+	+	+	-	-	<i>D. fuchsii</i>
85	163	227	228	R3	-	-	+	-	+	<i>D. flavescens</i>
85	163	227	228	R3	+	+	+	-	+	<i>D. flavescens</i>
86	159	234	236	RU1	+	-	+	-	-	<i>D. fuchsii</i>
85	163	232	228	RU3	-	+	-	-	-	<i>D. euxina</i>
85	163	226	227	RU4	-	-	-	-	+	<i>D. flavescens</i>
85	163	226	227	RU4	-	-	+	-	+	<i>D. flavescens</i>
85	163	231	227	RU5	-	+	-	-	-	<i>D. armeniaca</i>
85	159	238	233	X	+	-	+	+	-	<i>D. maculata</i>
85	159	238	233	X	+	+	+	-	-	<i>D. maculata</i>

^a The lengths (base pairs) of “D-fuch” and “D-mac” fragments, respectively.

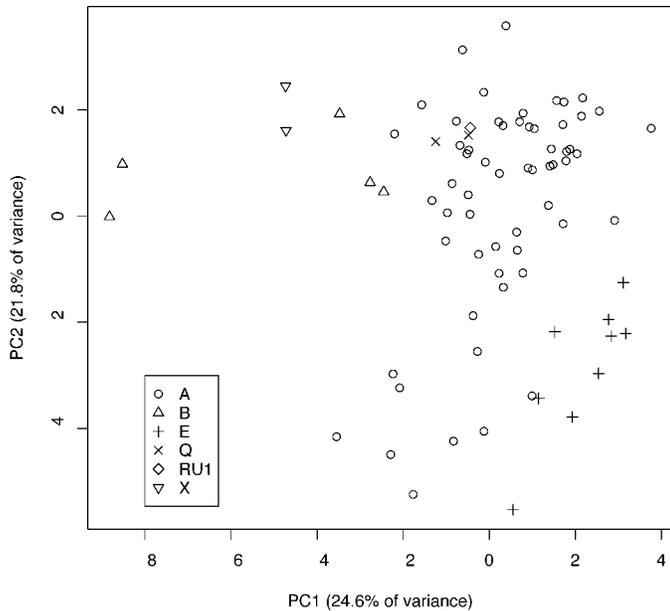


Fig. 4. Simultaneous principal components analysis of *Dactylorhiza* morphological and ITS allele data (plastid haplotypes of these plants are indicated).

the character values that predict classification, and in this case leaf width (“less than” vs. “more than 22.5 mm”) clearly divides the existing samples.

Geographic patterns—Plastid data—We found a significant geographical pattern of haplotype distribution from south to north (Kruskal-Wallis $\chi^2 = 21.4529$, $P \ll 0.05$). The distribution of haplotypes in Russia also has similarities to patterns observed in the western European flora. For example, the dactylorhiza flora of central Russia has some links with Sweden (haplotype X), but rarer haplotypes (N, Q) are distributed only in central Europe (Y. Pillon, M. F. Fay, and M. W. Chase, unpublished data). The Caucasian haplotype RU3 is similar to Turkish *D. euxina* haplotypes K and I, and RU4 is similar to the Mediterranean R3 haplotype.

The ITS data—When the ITS data were used for PCA analysis alone, the structure of the graph was consistent only with distribution by latitudinal zones (i.e., with the sites categorized according to latitude in 5° intervals: from 50° to 55°, 55° to 60°, and so on). We used linear regression analysis to estimate the relationship between latitudinal zone and heterogeneity within populations (the measure of heterogeneity was the standard deviation of fragment length types) as a dependent variable, and a significant relationship was found ($F = 24.46$ on 64 df, $P \ll 0.05$; see also Fig. 6).

DISCUSSION

The haplotype network (Fig. 2) is largely consistent with most previous classifications of *Dactylorhiza* despite the fact that it was first constructed without reference to previous taxonomic concepts. Haplotype H, typical for *D. incarnata*, is frequent in European Russia and has a wider distribution than was imagined from patterns observed in western European populations (Y. Pillon, M. F. Fay, and M. W. Chase, unpublished data). Some rare haplotypes (RU3, RU5) may have origi-

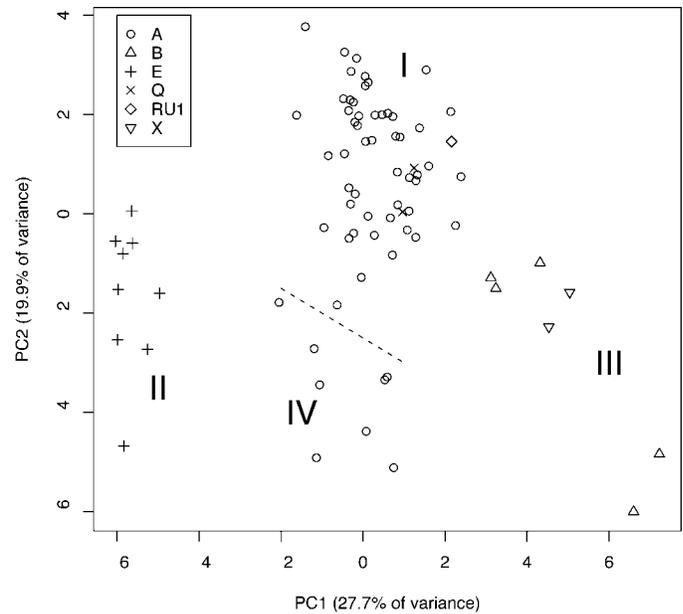


Fig. 5. Simultaneous principal components analysis of *Dactylorhiza* morphological, plastid microsatellites, and ITS allele data (plastid haplotypes of these plants are indicated).

inated in the region where they were found. Haplotype RU4 (and its specific ITS allele), however, is characteristic of *D. flavescens* in the western Caucasus, and it is likely that this genotype has a wider distribution encompassing all regions of the Caucasus. The long-standing floristic record of *D. romana* in the western Caucasus (Kodosh Cup near Tuapse: M. Vakhrameeva, Moscow University, personal communication; Zernov, 2002) is probably an identification error because both populations of supposed *D. romana* sampled have the morphology, ITS alleles, and haplotype typical of *D. flavescens*.

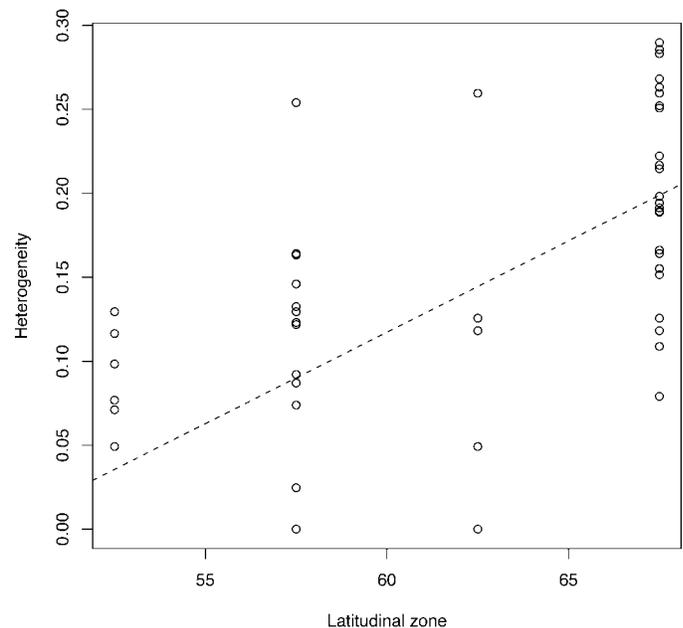


Fig. 6. Linear regression between within-population heterogeneity and latitude.

Thus, we conclude that *D. romana* does not exist in the Russian Federation, and its distribution in eastern Europe is restricted to the Crimea. In the light of the position of haplotype RU5 in the network, one can hypothesize that this specimen belongs to a group with characteristics of both *D. incarnata* and *D. euxina*. Hedrén (2001) showed that some plants intermediate in morphology between *D. incarnata* and *D. euxina* from Turkey should be referred to a separate tetraploid species, *D. armeniaca*. The diagnosis of *D. armeniaca* is congruent with morphometric data from our specimen (voucher from Tuapse, Krasnodar region), so we have probably here the first record of this species in Russia.

Dactylorhiza maculata and *D. fuchsii* from northern Russia are difficult to distinguish (L. Averyanov, Botanical Institute, Saint-Petersburg, personal communication), so the possession of A, Q, and RU1 haplotypes (all from the *D. fuchsii* group; Fig. 2) for all of these plants requires an explanation. Plants initially identified as *D. traunsteineri* (an allotetraploid) from these sites have similar morphologies and genotypes (some Scandinavian *D. traunsteineri* specimens also have the A haplotype: Y. Pillon, M. F. Fay, and M. W. Chase, unpublished data). Most of these plants grow in *Sphagnum* bogs, the typical habitat of *D. maculata*. On the other hand, the morphology of these specimens differs from that of typical *D. maculata* (Fig. 3). The discriminant and regression analyses show that morphologically our specimens with the A haplotype are clearly divided into northern plants with narrow leaves (group I; Figs. 3, 5) and southern plants with broad leaves (group IV). The ITS alleles from the first group are those of both *D. fuchsii* and *D. maculata*. All of these northern plants are tetraploids according to several cytological observations (Averyanov, 1990a). This leads us to speculate that most northern Russian specimens with A, Q, and RU1 haplotypes are allotetraploids formed by *D. maculata* and *D. fuchsii*, probably with the participation of unreduced gametes from the *D. fuchsii* parent (Ramsey and Schemske, 1998).

The C haplotype, found in many of the western European and Turkish allotetraploids (Y. Pillon, M. F. Fay, and M. W. Chase, unpublished data), is absent from Russia. Many of these plants also have the *D. incarnata* ITS allele. Our *D. russowii* (= *D. traunsteineri* s.l.) specimen from South Karelia has the *D. incarnata* allele but combined with the haplotype A. Other putative allotetraploids (including several specimens referred to *D. baltica*) with *D. incarnata* alleles also have the A haplotype and are morphologically similar to typical *D. fuchsii* (group IV; Fig. 3). However, this tentative conclusion could reflect the limited range of morphometric characters used.

Two group III samples are distant from the remainder on the PCA plot (Fig. 3). The basis for such a position is their wide lip, but this character is often found in typical *D. maculata* (Bateman and Denholm, 1989). The ITS alleles of all specimens in group III are similar (Table 5).

Dactylorhiza "cruenta" is now often accepted as a form of *D. incarnata* (e.g., Bateman and Denholm, 1985; Hedrén et al., 2001), a view supported by our data. This "taxon" only has the E haplotype and an ITS allele consistent with *D. incarnata*; it also belongs to the same group (group II; Fig. 3) as *D. incarnata*. This group is homogeneous with two exceptions: first (the point in the right top of the group) is the "northern tetraploid" with extremely small flowers, but all other plants measured (but not sampled for DNA) from this population have flowers typical of group I. The second excep-

tion (the point on the bottom of the graph) is a mammoth plant of *D. incarnata* with unusually large flowers, but again all other plants from this population have typical *D. incarnata* morphology. Both of these outliers fall in their expected groups in the analysis containing all three data matrices (Fig. 5).

We did not find heterogeneity in plastid haplotypes within populations, even though for more than a half of them several samples were examined. The intrapopulation diversity of ITS alleles is considerable and shows a clear south-north geographic cline (Fig. 6), with the latter being significantly more heterogeneous.

The correspondence of the morphological PCA graph to haplotypes and a priori delimited species clearly shows that the morphological data correlate with plastid haplotypes better than with standard taxonomic circumscriptions that have been used for species description. This also indicates that characters used in descriptions and keys for this species require further revision. The agreement between different categories of data is greater in simultaneous analysis of morphology and haplotypes than for morphology and ITS data, which supports the use of plastid data as a good species marker in this group of plants in spite of its strictly maternal inheritance. Most workers would not expect plastid DNA patterns and morphological variation to be better correlated than a biparentally inherited region like ITS, but the congruence of plastid haplotypes and morphology in this case could be due to the fact that the location of plants during the last glacial maximum (their refugium) is more important to their morphological characteristics than their present location and with which other plants they are interacting at present.

This study is the first of which we are aware in plants to sample DNA and measure morphological characters for the same individuals. Because of the high level of congruence between the two, relatively robust statements can be made about which characters are reliable for distinguishing taxa and which are not helpful for these purposes. In addition, a strong case is made for collecting morphological data from plants destined to be used in molecular studies and for combining these categories of data to produce clearer ideas about how taxa should be circumscribed and identified in the field.

The geographical patterns revealed by this study of plastid DNA are straightforward because most of European Russia has a typical postglacial flora (e.g., Tikhomirov et al., 1987–1988), which means that immigration was likely from refugia in western Europe and/or the Caucasus and Crimea. The clear correlation between latitude and heterogeneity of the ITS data demonstrates that either ITS gene conversion in northern regions of European Russia is much slower or (more probably) the time since hybridization occurred in northern *Dactylorhiza* populations is less than in southern populations.

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