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Systematics and evolution of the *Dactylorhiza romana/sambucina* polyploid complex (Orchidaceae)

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The European–Mediterranean–Oriental *Dactylorhiza romana*/*sambucina* polyploid complex was studied with regard to genetic and morphological variation patterns. Allozyme and morphometric data were collected from 24 and 19 populations, respectively, initially identified as *D. flavescens*, *D. insularis*, *D. markusii*, *D. romana*, *D. sambucina*, and an indeterminate taxon. Genetic distances were calculated and illustrated by an unweighted pair-group method using arithmetic averages (UPGMA) dendrogram, and principal components analyses (PCAs) were used to summarize morphological variation patterns. Another PCA was performed on combined allozyme and morphometric data. On the basis of the dendrogram and the PCA plots, main groups of populations were delimited, and the probability that each morphological character would distinguish correctly between these groups was estimated. After combining morphometric interpretations with studies of herbarium material and information from the literature, the following taxa were confidently accepted: *D. romana* ssp. *romana*, ***D. romana* ssp. *guimaraesii* (comb. et stat. nov.)**, *D. romana* ssp. *georgica*, *D. sambucina*, ***D. cantabrica* (sp. nov.)**, and *D. insularis*. Levels of genetic diversity suggest that *D. romana s.s.* is the least derived member of the complex. The evolutionary divergence of the diploid species, *D. romana* and *D. sambucina*, was probably the outcome of vicariant speciation, whereas *D. romana* ssp. *georgica* and *D. romana* ssp. *guimaraesii* appear to have evolved from *D. romana s.s.* through incomplete vicariant and peripheral isolate speciation events, respectively. In some populations of the diploid taxa, a significant deficiency in heterozygotes was found at one to three loci. It is proposed that this pattern may indicate a Wahlund effect, hypothesizing that local populations are subdivided into demes determined by the commonly sympatric occurrence of two distinct colour morphs combined with partial morph constancy of individual pollinators (bumblebees). Several pathways are possible for the origin of the allotriploid *D. insularis* and the apparently allotetraploid *D. cantabrica*. A taxonomic revision is provided. *Botanical Journal of the Linnean Society*, 2006, **152**, 405–434.

ADDITIONAL KEYWORDS: allopolyploidy – biogeography – Mediterranean flora – polymorphism – speciation – taxonomy – Wahlund effect.

INTRODUCTION

The northern hemisphere genus *Dactylorhiza* Neck. ex Nevski (Orchidaceae) is notoriously taxonomically difficult, and neither Nelson's (1976) traditional monograph nor the treatment in *Flora Europaea* (Soó, 1980) have been widely accepted as standard amongst contemporary botanists. Rather, the response has been a massive approach by systematists, who have started to study *Dactylorhiza* by advanced methods, including cytogenetic analysis (e.g. Vöth & Greilhuber, 1980; Gathoye & Tyteca, 1989; D'Emerico *et al*., 2002), multivariate morphometric methods (e.g. Bateman & Denholm, 1983, 1985, 1989; Jagiello, 1988; van Straaten *et al*., 1988; Dufrêne, Gathoye & Tyteca, 1991; Andersson, 1995; Pedersen, 1998b, 2001, 2004a; Shaw, 1998; Shipunov *et al*., 2004), landmark analysis (Shipunov & Bateman, 2005), allozyme analysis (e.g. Hedrén, 1996, 2001a; Pedersen, 1998a, 2004a; Bullini *et al*., 2001), amplified fragment length polymorphism

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(AFLP) analysis (Hedrén, Fay & Chase, 2001), analysis of haplotype diversity (e.g. Bullini *et al*., 2001; Devos *et al*., 2003; Hedrén, 2003; Shipunov *et al*., 2004; Pillon *et al*., 2006a), and sequencing of nuclear ribosomal internal transcribed spacer (ITS) regions and of the intron of the plastid gene *rpl*16 (e.g. Bateman, Pridgeon & Chase, 1997; Pridgeon *et al*., 1997; Bateman *et al*., 2003; Shipunov *et al*., 2004; Pillon *et al*., 2006a, 2006b). During the last 25 years, application of these methods has amply demonstrated how genetic diversity, morphological plasticity, hybridization, introgression, and polyploidy contribute to the systematic complexity of *Dactylorhiza*. Until now, most studies have dealt with the *D. incarnata* (L.) Soó *s.l./maculata* (L.) Soó *s.l.* polyploid complex in Europe and Anatolia, whereas less effort has been assigned to other parts of the genus.

Dactylorhiza romana (Sebast.) Soó *s.l.*, *D. sambucina* (L.) Soó, and *D. insularis* (Sommier) Landwehr make up a group that is frequently recognized taxonomically as sect. *Sambucinae* (Parl.) Smoljan. It is morphologically well defined (e.g. Vöth, 1971; Nelson, 1976; Tyteca & Gathoye, 1988). Data from allozyme markers (Rossi *et al*., 1995), karyotype structure, and heterochromatin distribution (D'Emerico *et al*., 2002), as well as cpDNA haplotype diversity (Devos *et al*., 2003), demonstrate significant differences between the only examined member of the *D. romana*/*sambucina* group, on the one hand, and other *Dactylorhiza* species on the other. Judging from ITS and *rpl*16 intron sequence data (Bateman *et al*., 2003; Pillon *et al*., 2006a, 2006b), the phylogenetic distinction of the group seems questionable, but AFLP data (Hedrén *et al*., 2001) and preliminary sequence data from the cpDNA region *trnL* (Bateman & Denholm, 2003) suggest a monophyletic group. Its members are not considered to be involved in the *D. incarnata s.l.*/*maculata s.l.* polyploid complex (e.g. Hedrén, 2002; Devos *et al*., 2003). Natural hybridization involving one parent from each group has been demonstrated (e.g. Hansson, 1986; Rossi *et al*., 1995), but it appears to be rare and unrelated to speciation events. Altogether, it seems appropriate to analyse the *D. romana*/*sambucina* group as a morphologically and biologically distinct (and presumably monophyletic) complex.

The geographical distribution of the group ranges from Portugal in the west to northern Iran in the east, and from southern Scandinavia in the north to northern Morocco, Algeria, and Lebanon in the south. It is absent from the British Isles and western Siberia. A fairly accurate map of the total range was provided by Meusel, Jäger & Weinert (1965: Map 110d, sub nom. *D. sambucina s.l.*). Estimates of the number of taxa vary from one species with five subspecies (Sundermann, 1980), through five species (Delforge, 2001), to nine species without infraspecific taxa (Averyanov, 1990). The disagreement mainly reflects different species concepts.

In an earlier paper (Pedersen, 1998b), I sought taxon concepts applicable for *Dactylorhiza* in general, aiming to establish a hierarchical system that would simultaneously reflect differing degrees of morphological distinction, phylogenetic relationship, and reproductive isolation amongst taxa. On the basis of empirical data, I eventually recognized three hierarchical levels that could be matched with general taxon concepts from the literature, leading to the recommendation of three general taxon definitions for *Dactylorhiza*. Morphologically well-defined taxa complying with the biological species concept (Mayr, 1940) in a modern, botanically focused sense (Jonsell, 1984; Raven, 1986) should be designated as 'species'; as a result of mutual reproductive isolation, species are characterized by basically different genome compositions (usually with unique alleles). Morphologically well-defined taxa complying with the ecological species concept (Van Valen, 1976), but not with the biological species concept, should be designated as 'subspecies'; all subspecies of the same species have basically similar genome compositions (rarely with unique alleles), but their ploidy levels may differ. Taxa complying with the phenetic species concept (Sneath, 1976), but not with the biological or ecological species concept, should be designated as 'varieties'; all varieties of the same species have identical ploidy levels and very similar genome compositions. In two later studies (Pedersen, 2001, 2004a), these general guidelines have been utilized to revise minor complexes in the genus.

It has been advocated that the most promising approach to disentangle the complex evolution in *Dactylorhiza*, and at the same time provide operational classifications, would be studies that integrate morphological and molecular data (Bateman, 2001; Pedersen, 2004b; Shipunov *et al*., 2004; Shipunov & Bateman, 2005). The present approach to the *D. romana*/*sambucina* polyploid complex integrates information from allozyme and morphometric data. On this basis, I attempt to delimit and classify the members of the group in accordance with the conceptual system proposed previously (Pedersen, 1998b), and to deduce likely scenarios of evolution in the complex.

MATERIAL AND METHODS

Study taxa

Delforge's (2001) classification of the complex was tentatively adopted, and the study populations were identified accordingly as the Anatolian–Caucasian *D. flavescens* (C. Koch) Holub (abbreviated 'F'), the

west Mediterranean *D. insularis* (abbreviated 'I'), the west Mediterranean *D. markusii* (Tineo) H. Baumann & Künkele (abbreviated 'M'), the central Mediterranean–Pontic *D. romana* (abbreviated 'R'), and the European *D. sambucina* (abbreviated 'S'). Two study populations from Spain did not unequivocally match any species recognized by Delforge (2001), and so were treated as an unidentified taxon, '*D. indet.*' (abbreviated 'A'). In some of the tables below, the identifications according to the finally accepted classification are also indicated by abbreviations (C, *D. Cantabrica* H. A. Pedersen; R_{ge}, *D. romana* ssp. *georgica* (Klinge) Renz & Taubenheim; R_{gu}, *D. romana* ssp. *guimaraesii* (E. G. Camus) H. A. Pedersen; R_{ro}, *D. romana* ssp. *romana*).

Dactylorhiza sambucina is generally diploid with $2n = 40$ (exceptionally $2n = 42$) chromosomes (Hagerup, 1938; Heusser, 1938; Del Prete, Garbari & Giordani, 1980; Gathoye & Tyteca, 1989). Likewise, $2n = 40$ chromosomes are reported from *D. romana s.s.* (Del Prete *et al*., 1980; Bianco *et al*., 1987; Alba *et al*., 2003) and Iberian *D. markusii* (Bernardos *et al*., 2002; Bernardos, García-Barriuso & Amich, 2005), whereas no counts exist for *D. flavescens*. However, because of the very close allozymic relations between *D. romana s.s.*, *D. markusii*, and *D. flavescens* found in the present study (see below), it can reasonably be assumed that the latter is also diploid ($2n = 40$). It should be added that some individuals of both *D. romana s.s.* and Portuguese *D. markusii* have been observed to possess a supernumerary chromosome (D'Emerico *et al*., 2002; Bernardos, Tyteca & Amich, 2004), and that triploid individuals of *D. sambucina* ($2n = 60$) have been encountered in two Spanish populations (Bernardos *et al*., 2004).

Dactylorhiza insularis is triploid with $2n = 60$ (Scrugli, 1977; Bernardos *et al*., 2002, 2005) or $2n = 60 + 1B$ (Bernardos *et al*., 2004) chromosomes. No chromosome counts on *D. indet*. have been published but, judging from the fact that all individuals in this study were found to be balanced heterozygotes at both loci that were not monomorphic (see below), and from the banding intensity at the same loci, there is strong circumstantial evidence that *D. indet*. is allotetraploid ($2n = 80$).

Allozyme markers

Six hundred and seventy-seven individuals from 24 populations representing the *Dactylorhiza romana*/*sambucina* complex were sampled in 1999–2001 (Table 1). From each plant, the distal 1–2 cm^2 of the stem leaf first or second from the top was collected. In the field, the tissue samples were placed in an insulated bag (*c*. 5 °C), and later the same day they were transferred to a refrigerator where they were kept at the same temperature for a few days. As soon as possible, the samples were transferred to a –80 °C freezer where they were kept until extraction (approximately 6 months later).

For each individual *c*. 0.5 cm^2 of the frozen tissue sample was ground with a small amount of washed sea-sand in 80 μL of a Tris-HCl grinding buffer, slightly modified from Soltis *et al*. (1983). The homogenates were absorbed into paper wicks, and the allozymes were separated through horizontal starch gel electrophoresis. This was conducted at 50–90 mA and 200–400 V over 4 h, and the gels contained 12% starch. A discontinuous lithium-borate–Tris-citrate buffer system at pH 8.1 (Ashton & Braden, 1961) was used to separate the allozymes of diaphorase (DIA, E.C. 1.6.99-), phosphoglucoisomerase (PGI, E.C. 5.3.1.9), and phosphoglucomutase (PGM, E.C. 5.4.2.2). A histidine–citrate buffer system (Wendel & Weeden, 1989), adjusted to pH 6.0 (Ellstrand, 1984), was used to separate the allozymes of phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44), shikimate dehydrogenase (SKD, E.C. 1.1.1.25), and UTP-glucose-1-phosphate uridylyltransferase (UGPP, E.C. 2.7.7.9). Staining recipes followed Manchenko (1994) for UGPP and, with minor modifications, Wendel & Weeden (1989) for DIA, PGD, PGI, PGM, and SKD.

For each enzyme locus, the alleles were denoted by letters of the alphabet in order of migration distance of the corresponding allozymes. Relative migration distances (R_m values) were calculated relative to the common allele in European *D. incarnata* at each locus; four samples of *D. incarnata* from Hedeland in Denmark (Pedersen, 1998a) were run as standards on each gel. For each enzyme locus and population sample, the various genotypes were quantified and the allele frequencies were calculated.

Genetic distances were calculated for each pair of populations by the algorithms ARC and CHORD (both Cavalli-Sforza & Edwards, 1967), Hillis (Hillis, 1984), Hillis-UB (Swofford & Olsen, 1990), Nei-72 (Nei, 1972) and Nei-UB (Nei, 1978). The resulting distance matrices were used to construct dendrograms describing the genetic similarity amongst populations. The dendrograms were constructed by means of the unweighted pair-group method using arithmetic averages (UPGMA) algorithm (Legendre & Legendre, 1983). UPGMA is a polythetic agglomerative technique that appears to maximize the cophenetic correlation, and its use is recommended when there is no specific reason for choosing another clustering technique (Sneath & Sokal, 1973). For each cluster analysis, goodness of fit was assessed by calculating the cophenetic correlation coefficient (r), i.e. the product moment correlation between the entries of the dissimilarity matrix and those of the cophenetic matrix (Rohlf & Fisher, 1968).

Table 1. Survey of the populations examined, with indications of population number, identification at the sampling time (ID_1, based on Delforge, 2001), final classification (ID_2, according to the Taxonomy section of this paper), locality, sample size for electrophoresis (number of individuals), and sample size for morphometric analysis (number of individuals, with the number of plots given in parentheses)

No.	ID_1	ID_2	Locality	Date	$N_{electrophoresis}$	$N_{morphometry}$
1	R	R_{ro}	Cyprus, southern part: Paphos Forest	20.iii.2001	29	–
2	R	R_{ro}	Greece, Samos: Manolates	9.iv.1999	30	31 (10)
3	R	R_{ro}	Greece, Samos: Messogia	11.iv.1999	28	22 (8)
4	R	R_{ro}	Greece, Samos: Moni Vrontiani	8.iv.1999	40	39 (14)
5	R	R_{ro}	Greece, Samos: Spathareoi	10.iv.1999	30	34 (13)
6	R	R_{ro}	Italy, Sicily: Castiglione	19.iv.1999	30	24 (3)
7	R	R_{ro}	Italy, Toscana: Monte Argentario	8.iv.2001	14	–
8	F	R_{ge}	Turkey, Artvin: Artvin	17.v.2000	32	22 (1)
9	F	R_{ge}	Turkey, Artvin: Kafkasör	17.v.2000	14	30 (4)
10	F	R_{ge}	Turkey, Gümüshane: Hamsiköy S of Zigana Gecidi	25.v.2000	6	–
11	F	R_{ge}	Turkey, Gümüshane: Zigana Gecidi	24.v.2000	21	21 (10)
12	M	R_{ro}	Italy, Sicily: Bosco di Ficuzza	21.iv.1999	30	24 (5)
13	M	R_{gu}	Spain, Galicia: Covas	24.iv.2001	14	–
14	M	R_{gu}	Spain, Galicia: Doade	22.iv.2001	32	32 (10)
15	M	R_{gu}	Spain, Galicia: Lumeares	23.iv.2001	32	16 (8)
16	M	R_{gu}	Spain, Galicia: Xiras	23.iv.2001	32	31 (2)
17	I	I	Spain, Andalucia: Refugio de Juanar	17.iv.2001	20	18 (11)
18	I	I	Spain, Galicia: Monte do Cido	29.iv.2001	42	19 (12)
19	I	I	Spain, Galicia: Vilar de Silva	25.iv.2001	50	42 (20)
20	S	S	Denmark, Zealand: Rævebjerg	18.v.1999	30	30 (3)
21	S	S	Denmark, Bornholm: Kåsegaard	23.v.1999	30	28 (4)
22	S	S	Denmark, Bornholm: Langebjerg	22.v.1999	30	30 (7)
23	A	C	Spain, Galicia: Campelo	27.iv.2001	33	31 (17)
24	A	C	Spain, Galicia: O Couto	27.iv.2001	28	–

All statistical operations were performed using the program NTSYSpc 2.0 (Rohlf, 1998).

Measures of genetic variation, gene diversity, and gene differentiation were calculated according to Nei (1975). The magnitude of genetic variation in each local population was assessed as the proportion of polymorphic loci, average number of alleles per locus, and gene diversity. The estimates of gene diversity were calculated as $H_e = 1 - \Sigma p_i^2$ for each locus and averaged across loci (p_i being the frequency of the ith allele). For each taxon, the gene diversity in the total population was estimated as $H_T = 1 - \Sigma p_{\cdot k}^2$ (where $p_{\cdot k} = \Sigma_i p_{ik} s^{-1}$ and s is the number of local populations). Similarly, for each taxon, the relative magnitude of gene differentiation amongst local populations was assessed as $G_{ST} = (H_T - H_S)H_T^{-1}$ (where H_S is the average gene diversity within local populations), and the absolute degree of gene differentiation was assessed as $D_m = s(H_T - H_S)(s - 1)^{-1}$.

For each local population of the diploid taxa, the quantitative representation of genotypes at each allozyme locus was tested against the proportions expected under Hardy–Weinberg equilibrium. This was performed by chi-squared tests in the program SigmaStat.

The geographical distance was established for all pairwise combinations of populations of *D. romana s.s.* (including population 12, originally identified as *D. markusii*, but see taxonomic conclusions below). Applying the program SigmaStat, the Pearson product moment correlation procedure was used to test for correlation of the geographical distance with the Nei-72 genetic distance between populations of this taxon.

Morphology and combined data

In 1999–2001, 21 morphological characters (Table 2, characters 1–21; Fig. 1) were scored from a total of 524 individuals in 19 populations belonging to the *Dactylorhiza romana*/*sambucina* complex (Table 1). In each population, 1–20 circular plots (diameter, 3 m) were demarcated (Table 1), attempting a placing of the plots that would adequately reflect the patterns of morphological variation in the population. All undamaged flowering *Dactylorhiza* individuals within the plots were included in the study. Floral features were

Table 2. List of morphometric bona fide characters (1–21) and ratios (a–e). Binary characters are indicated with an asterisk. Based on previous examination of herbarium material, all leaves appearing from below ground level were considered as sheathing

No.	Character (and unit of measurement)
1	Height of plant, measured from ground level to apex of inflorescence (cm)
2*	Node of uppermost sheathing leaf below (0) or above (1) ground level
3	Stem diameter immediately below inflorescence (mm)
4	Number of sheathing leaves, excluding cataphylls
5	Number of non-sheathing leaves below inflorescence
6	Length of longest leaf, measured along the adaxial surface (cm)
7	Maximum width of longest leaf when flattened (cm)
8	Orientation of longest leaf relative to stem (adaxial angle, degrees)
9	Length of inflorescence, measured from node of the lowermost flower to apex of inflorescence (cm)
10	Number of flowers (including buds and fruits)
11*	Flower yellowish (0) or purplish (1), scored from the peripheral zone of the labellum lamina
12*	Presence (1) or absence (0) of markings on the proximal third of the labellum lamina
13*	Presence (1) or absence (0) of markings beyond the proximal third of the labellum lamina
14	Abaxial angle separating the lateral parts of labellum, viewed from the front (degrees)
15	Length of labellum lamina, measured along the midline (mm) (cf. Fig. 1, measure '*A*')
16	Maximum width of labellum when flattened (mm)
17*	Lamina of labellum widest in its proximal to middle part (0) or in its distal part (1)
18*	Spur straight to downcurved (0) or spur upcurved (1)
19	Length of spur (mm)
20	Vertical diameter of spur entrance (mm)
21	Vertical diameter of spur 1 mm from apex (mm)
a	Length of longest leaf (character 6) in relation to its width (character 7)
b	Length of longest leaf (character 6) in relation to height of plant (character 1)
c	Length of inflorescence (character 9) in relation to height of plant (character 1)
d	Labellum shape index (cf. Fig. 1)
e	Length of spur (character 19) in relation to length of labellum lamina (character 15)

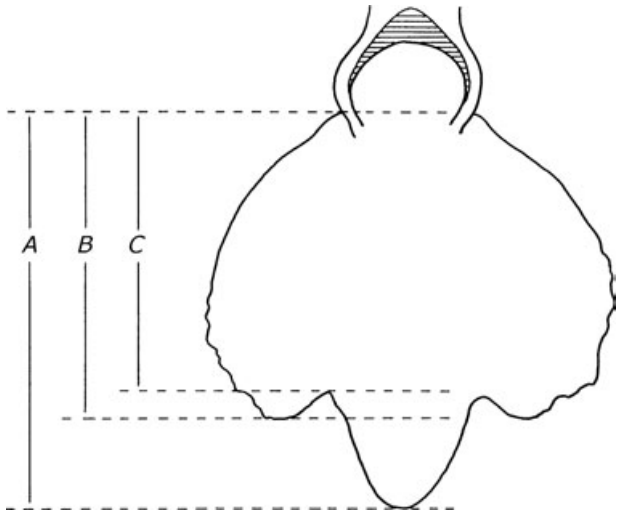

Figure 1. Outline of the lamina of a *Dactylorhiza* labellum with some important distances indicated. The labellum shape index was calculated as $2C/(A + B)$.

scored from one fully expanded flower in the mid-portion of the spike. Size measurements were taken by means of an object micrometer and a low-power binocular microscope.

Principal components analysis (PCA; Sneath & Sokal, 1973) has previously been demonstrated to be useful in morphometric studies of *Dactylorhiza* (e.g. Jagiello, 1988; van Straaten *et al*., 1988; Dufrêne *et al*., 1991; Andersson, 1994; Shaw, 1998; Foley, 2000; Pedersen, 2001, 2004a; Shipunov *et al*., 2004), and was chosen to summarize the morphological variation patterns. PCA is suited for the first iteration of analyses, because each character is given the same a priori weight, whereas intergroup distances are not taken into account. PCA was originally developed for quantitative characters, but can also be used on binary characters (Gower, 1966; Dunn & Everitt, 1982). All characters were standardized prior to the analyses (Gower, 1971). Using NTSYSpc 2.0 (Rohlf, 1998), separate analyses were conducted for populations and individuals. In the former, population means were calculated for each of the quantitative characters,

whereas the binary characters were treated as frequencies. For each binary character, only the frequency of the state coded '1' was included, in order to avoid weighting such characters more strongly than the quantitative characters.

First, analyses incorporating all populations were conducted. Subsequently, separate analyses were performed on the two main groups of populations/individuals (designated according to the initial analyses) in order to enhance the resolution in these groups.

In addition to the PCAs performed on morphometric data only, a PCA was performed on combined morphometric and allozyme data from all populations for which dual data sets were available (Table 1). Individual allozyme alleles were treated as binary characters. Thus, for each population, the frequency of each allele was entered into the analysis.

In order to assess whether the main groups of populations (designated according to the PCA results) fulfilled the criterion of morphological distinction in Pedersen's (1998b) taxon definitions, a search was made for characters that would distinguish reliably between these main groups of populations. Thus, the discriminatory power was assessed for each of the bona fide characters (Table 2, characters 1–21) and for five ratios (Table 2, characters a–e). Following Pedersen (2001), the probability that each binary character, with the states 0 and 1, would distinguish correctly between two taxa was estimated as $^*Q = 1/2 + 1/2\,|p_{A,1} - p_{B,1}|$, where $p_{A,1}$ and $p_{B,1}$ designate the frequencies of character state 1 in taxon A and taxon B, respectively. The probability (Q) that each quantitative character would distinguish correctly between two taxa was estimated by calculating the coefficient of discrimination, $K = (\mu_A - \mu_B)^2 2v^{-1}$ (Lubischew, 1962), where μ_A and μ_B are the sample means for taxon A and taxon B, respectively, and v is the pooled estimate of within-taxon variance. The probability of misclassification is approximately equal to the probability that a random normal deviate will exceed $(K/2)^2$, and so can be obtained from tables of normal distribution (Lubischew, 1962). The probability (Q) of correct identification follows directly. It is an arbitrary decision as to how high a value of Q or $^*Q$ should be for a character to be considered sufficiently reliable in distinguishing between two taxa. Based on Pedersen (2001), the critical value is fixed at 90% in the present paper.

To establish correct synonymies, protologues were consulted, and nomenclatural types were sought at relevant herbaria. The individual distributions of the finally recognized subspecies of *D. romana* in the western Mediterranean were resolved by examining representative herbarium material of *D. romana s.l.* from Portugal, Spain, Algeria, and Sicily. Similarly, the individual distributions of *D. sambucina* and *D. cantabrica* (sp. nov., see below) in the Iberian Peninsula were resolved by examining herbarium material from various parts of Spain originally assigned to *D. sambucina*. In both cases, the following herbaria were consulted: BM, C, FI, G, K, L, LD, LISU, M, MAF, MSB, PO, RO, SANT. Finally, identification keys and short descriptions of the accepted taxa were compiled on the basis of the morphometric data.

RESULTS

Allozyme markers

It was possible to interpret each of the enzymes DIA, PGD, PGI, PGM, SKD, and UGPP at one allozyme locus. DIA, PGM, SKD, and UGPP were interpreted as monomeric enzymes, whereas PGD and PGI were interpreted as dimeric enzymes. The frequencies of the various alleles in the population samples are given in Table 3.

At *dia*, six alleles were found, of which dia^d was the most frequent in all populations. *D. insularis* and *D. indet*. were consistently monomorphic at this locus, and the same was almost the case for *D. flavescens* and *D. sambucina*. In contrast, *D. markusii* and *D. romana s.s.* were more variable, although one and three populations, respectively, were monomorphic.

At *pgd*, six alleles were found, of which pgd^b was the most frequent in *D. romana s.s.*, *D. flavescens*, and *D. markusii*. Indeed, all populations of *D. markusii* and all but one of *D. romana s.s.* were monomorphic. All populations of *D. sambucina*, on the other hand, were fixed for pgd^d. In *D. insularis* and *D. indet*., all individuals were heterozygotes. In the former, apparently two copies of pgd^b were combined with one copy of pgd^d, whereas, in the latter, all individuals were balanced heterozygotes for the same alleles, apparently combining two copies of each.

At *pgi*, four alleles were found, of which pgi^b was the most frequent in all populations. Indeed, *D. insularis*, *D. indet*., one population of *D. flavescens*, and three populations of *D. markusii* were monomorphic.

At *pgm*, five alleles were found, of which pgm^c was the most frequent in *D. sambucina*, *D. flavescens*, the Sicilian population of *D. markusii*, and, particularly, in *D. romana s.s.*, where all but one population was fixed at this allele. The allele pgm^d, on the other hand, was by far the most frequent in the Spanish populations of *D. markusii*, for which all but one population was monomorphic. In *D. insularis* and *D. indet*., all individuals were heterozygotes. In the former, apparently two copies of pgm^c were combined with one copy of pgm^d, whereas, in the latter, all individuals were balanced heterozygotes for the same alleles, apparently combining two copies of each.

At *skd*, two alleles were found. All populations but one, however, were fixed for the allele skd^b. The only

Table 3. Allele frequencies (%) in the 24 population samples. Original (ID_1) and final (ID_2) identifications are indicated. For each locus, the various alleles are designated by letters of the alphabet and specified by R_m values (numbers below letters)

			dia						*pgd*						*pgi*				*pgm*					*skd*		*ugpp*						
Pop. no.	ID_1	ID_2	a 106	b 100	c 91	d 84	e 74	f 58	a 79	b 62	c 56	d 46	e 35	f 26	a 112	b 108	c 100	d 91	a 100	b 97	c 94	d 91	e 88	a 93	b 86	a 131	b 122	c 110	d 103	e 100	f 91	g 80
1	R	R_{ro}	0	17	5	67	11	0	0	88	2	10	0	0	2	76	5	17	0	0	100	0	0	2	98	0	2	88	2	8	0	0
2	R	R_{ro}	0	0	0	100	0	0	0	100	0	0	0	0	32	68	0	0	0	0	100	0	0	0	100	0	12	58	0	30	0	0
3	R	R_{ro}	0	0	0	100	0	0	0	100	0	0	0	0	27	71	2	0	0	0	100	0	0	0	100	2	39	32	0	23	4	0
4	R	R_{ro}	0	0	0	96	4	0	0	100	0	0	0	0	26	68	6	0	0	0	100	0	0	0	100	10	29	38	0	22	1	0
5	R	R_{ro}	0	0	0	100	0	0	0	100	0	0	0	0	44	56	0	0	0	0	100	0	0	0	100	5	34	48	0	13	0	0
6	R	R_{ro}	0	0	14	83	3	0	0	100	0	0	0	0	0	98	0	2	3	0	97	0	0	0	100	0	6	55	0	39	0	0
7	R	R_{ro}	0	0	0	89	11	0	0	100	0	0	0	0	0	68	32	0	0	0	100	0	0	0	100	0	0	21	0	79	0	0
8	F	R_{ge}	0	0	0	100	0	0	6	78	0	11	2	3	17	83	0	0	0	0	91	6	3	0	100	0	9	2	0	86	0	3
9	F	R_{ge}	0	0	0	100	0	0	4	92	0	4	0	0	25	75	0	0	0	0	89	11	0	0	100	0	0	7	0	93	0	0
10	F	R_{ge}	0	0	0	100	0	0	0	92	0	8	0	0	0	100	0	0	0	0	58	42	0	0	100	0	0	25	0	67	0	8
11	F	R_{ge}	0	0	0	98	2	0	5	50	0	38	5	2	5	90	0	5	0	0	81	14	5	0	100	0	0	2	0	91	2	5
12	M	R_{ro}	0	0	0	82	18	0	0	100	0	0	0	0	2	98	0	0	0	0	97	3	0	0	100	0	0	43	0	57	0	0
13	M	R_{gu}	4	0	0	96	0	0	0	100	0	0	0	0	11	68	21	0	0	0	0	100	0	0	100	0	0	7	0	93	0	0
14	M	R_{gu}	0	0	0	95	3	2	0	100	0	0	0	0	0	100	0	0	0	0	0	100	0	0	100	0	0	0	0	100	0	0
15	M	R_{gu}	0	0	0	100	0	0	0	100	0	0	0	0	0	100	0	0	0	0	0	100	0	0	100	0	0	0	0	100	0	0
16	M	R_{gu}	0	0	0	96	2	2	0	100	0	0	0	0	0	100	0	0	0	0	2	98	0	0	100	0	0	0	0	100	0	0
17	I	I	0	0	0	100	0	0	0	67	0	33	0	0	0	100	0	0	0	0	67	33	0	0	100	0	0	0	0	100	0	0
18	I	I	0	0	0	100	0	0	0	67	0	33	0	0	0	100	0	0	0	0	67	33	0	0	100	0	0	0	0	100	0	0
19	I	I	0	0	0	100	0	0	0	67	0	33	0	0	0	100	0	0	0	0	67	33	0	0	100	0	0	0	0	100	0	0
20	S	S	0	0	0	100	0	0	0	0	0	100	0	0	35	65	0	0	0	17	83	0	0	0	100	0	0	0	0	100	0	0
21	S	S	0	0	2	98	0	0	0	0	0	100	0	0	5	93	2	0	0	38	62	0	0	0	100	0	0	0	0	100	0	0
22	S	S	0	0	0	100	0	0	0	0	0	100	0	0	5	95	0	0	0	0	72	28	0	0	100	0	0	0	0	100	0	0
23	A	C	0	0	0	100	0	0	0	50	0	50	0	0	0	100	0	0	0	0	50	50	0	0	100	0	0	0	0	100	0	0
24	A	C	0	0	0	100	0	0	0	50	0	50	0	0	0	100	0	0	0	0	50	50	0	0	100	0	0	0	0	100	0	0

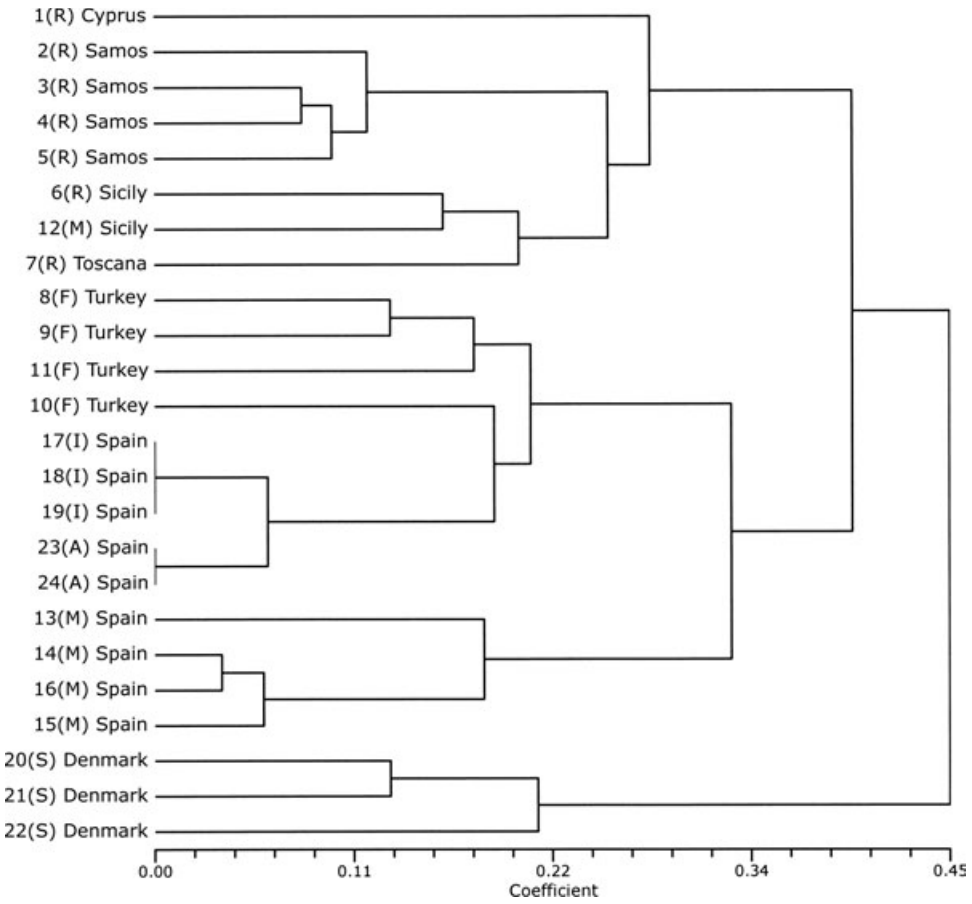

Figure 2. Dendrogram illustrating the overall genetic relationships between the 24 study populations identified previously, using morphology, according to the criteria of Delforge (2001) (Table 1). The dendrogram is based on ARC genetic distances, and was constructed employing the unweighted pair-group method using arithmetic averages (UPGMA) method of clustering.

exception was the Cypriot population of *D. romana s.s.*, in which skd^a and skd^b occurred with frequencies of 2% and 98%, respectively.

At *ugpp*, seven alleles were found, of which $ugpp^c$ was the most frequent in all populations but two of *D. romana s.s.* The exceptions were Messogia on Samos and Monte Argentario in Toscana, where $ugpp^b$ and $ugpp^e$, respectively, were found to be more frequent. In all other taxa, $ugpp^e$ was the most frequent allele. Indeed, *D. indet.*, *D. sambucina*, *D. insularis*, and all but one of the Spanish populations of *D. markusii* were fixed for this allele.

All but one of the clustering analyses gave poor to very poor fits ($r < 0.80$). The only analysis resulting in a good fit was that based on genetic distances calculated by the ARC algorithm ($r = 0.82$). A UPGMA dendrogram based on the ARC genetic distances is shown in Figure 2. There are two main clusters: one comprising *D. sambucina* and one comprising all other analysed taxa. The latter cluster is primarily composed of two subclusters: one comprising *D. romana s.s.* and the Sicilian population of *D. markusii*, and one comprising all of the remaining populations. The latter subcluster is primarily composed of two secondary subclusters: one comprising the Spanish populations of *D. markusii* and one comprising *D. flavescens*, *D. insularis*, and *D. indet*. In the latter subcluster, *D. insularis* and *D. indet*. form separate subclusters, which have evidently closer genetic affinities with each other than with any population of *D. flavescens*.

The magnitude of genetic variation in each population sample is shown in Table 4. In the following summary population 12, although originally identified as *D. markusii*, is included in *D. romana s.s.* (cf. taxonomic conclusions below). The proportion of polymorphic loci was 50–83% (mean, 67%) in *D. flavescens*, 33–83% (mean, 52%) in *D. romana s.s.*, 33–50% (mean, 39%) in *D. sambucina*, 33% in all populations of *D. insularis* and *D. indet.*, and 0–50% (mean, 25%) in *D. markusii*. The average number of alleles per locus

Table 4. Magnitude of genetic variation in the population samples assessed (over six allozyme loci) as the proportion of polymorphic loci, average number of alleles per locus, and gene diversity (H_e). Original (ID_1) and final (ID_2) identifications are indicated

Pop. no.	ID_1	ID_2	N	Polym. loci (%)	Alleles/locus	H_e
1	R	R_{ro}	29	83	3.00	0.229
2	R	R_{ro}	30	33	1.50	0.166
3	R	R_{ro}	28	33	2.00	0.186
4	R	R_{ro}	40	50	2.17	0.209
5	R	R_{ro}	30	33	1.67	0.188
6	R	R_{ro}	30	67	2.00	0.155
7	R	R_{ro}	14	50	1.50	0.160
8	F	R_{ge}	32	67	2.67	0.179
9	F	R_{ge}	14	67	1.83	0.141
10	F	R_{ge}	6	50	1.67	0.186
11	F	R_{ge}	21	83	3.00	0.219
12	M	R_{ro}	30	67	1.67	0.147
13	M	R_{gu}	14	50	1.67	0.115
14	M	R_{gu}	32	17	1.33	0.016
15	M	R_{gu}	32	0	1.00	0.000
16	M	R_{gu}	32	33	1.50	0.019
17	I	I	20	33	1.33	0.147
18	I	I	42	33	1.33	0.147
19	I	I	50	33	1.33	0.147
20	S	S	30	33	1.33	0.122
21	S	S	30	50	1.67	0.107
22	S	S	30	33	1.33	0.083
23	A	C	33	33	1.33	0.167
24	A	C	28	33	1.33	0.167

Table 5. Survey of gene diversity and degree of gene differentiation (assessed over six allozyme loci) amongst local populations of the study taxa (identified according to the finally accepted classification)

Taxon	H_T	H_S	G_{ST}	D_m
Diploid taxa				
D. romana ssp. *georgica* (4 populations)	0.203	0.181	0.108	0.029
D. romana ssp. *guimaraesii* (4 populations)	0.043	0.038	0.116	0.007
D. romana ssp. *romana* (8 populations)	0.212	0.180	0.151	0.037
D. sambucina (3 populations)	0.119	0.104	0.126	0.023
Polyploid taxa				
D. cantabrica (2 populations)	0.167	0.167	0.000	0.000
D. insularis (3 populations)	0.147	0.147	0.000	0.000

was 1.67–3.00 (mean, 2.29) in *D. flavescens*, 1.50–3.00 (mean, 1.94) in *D. romana s.s.*, 1.33–1.67 (mean, 1.44) in *D. sambucina*, 1.00–1.67 (mean, 1.38) in *D. markusii*, and 1.33 in all populations of *D. insularis* and *D. indet*. The gene diversity (H_e) was 0.141–0.219 (mean, 0.181) in *D. flavescens*, 0.147–0.229 (mean, 0.180) in *D. romana s.s.*, 0.167 in both populations of *D. indet*., 0.147 in all populations of *D. insularis*, 0.083–0.122 (mean, 0.104) in *D. sambucina*, and 0.000–0.115 (mean, 0.038) in *D. markusii*. A survey of gene diversity and degree of gene differentiation amongst local populations of each accepted taxon is given in Table 5.

For all the population samples of diploid taxa, deviations in the number of heterozygotes observed relative to the number expected under Hardy–Weinberg equilibrium are given in Table 6. A significant deficiency of heterozygotes was found in populations 4 and

Table 6. Excess or deficiency of heterozygotes (relative to Hardy–Weinberg proportions) in the study populations of diploid taxa. Original (ID_1) and final (ID_2) identifications are indicated

Pop. no.	ID_1	ID_2	*N*	*dia*	*pgd*	*pgi*	*pgm*	*skd*	*ugpp*
1	R	R_{ro}	29	0.28^{ns}	0.78^{ns}	3.17^{ns}	m	-0.14^{ns}	0.67^{ns}
2	R	R_{ro}	30	m	m	-0.06^{ns}	m	m	1.22^{ns}
3	R	R_{ro}	28	m	m	-3.83^{ns}	m	m	2.67^{ns}
4	R	R_{ro}	40	0.00^{ns}	m	-10.51‡	m	m	-4.42^{ns}
5	R	R_{ro}	30	m	m	-8.90†	m	m	1.90^{ns}
6	R	R_{ro}	30	-2.32^{ns}	m	-0.10^{ns}	m	m	-0.60^{ns}
7	R	R_{ro}	14	0.26^{ns}	m	0.91^{ns}	m	m	1.35^{ns}
8	F	R_{ge}	32	m	-3.99‡	-6.03‡	-3.36‡	m	-2.03^{ns}
9	F	R_{ge}	14	m	-0.11^{ns}	-0.25^{ns}	0.29^{ns}	m	0.18^{ns}
10	F	R_{ge}	6	m	0.12^{ns}	m	-1.92^{ns}	m	-0.89^{ns}
11	F	R_{ge}	21	0.18^{ns}	-1.60^{ns}	-0.89^{ns}	-1.76^{ns}	m	0.46^{ns}
12	M	R_{ro}	30	2.14^{ns}	m	-0.18^{ns}	0.25^{ns}	m	7.29*
13	M	R_{gu}	14	-0.08^{ns}	m	0.26^{ns}	m	m	-1.82‡
14	M	R_{gu}	32	-0.08^{ns}	m	m	m	m	m
15	M	R_{gu}	32	m	m	m	m	m	m
16	M	R_{gu}	32	-0.48^{ns}	m	m	-0.25^{ns}	m	m
20	S	S	30	m	m	-8.65†	-8.47‡	m	m
21	S	S	30	-0.18^{ns}	m	-1.97*	-7.14*	m	m
22	S	S	30	m	m	-2.85‡	-9.10‡	m	m

m, population was monomorphic for a single allele; ns, not significant, $P > 0.05$.
*$P < 0.05$.
†$P < 0.01$.
‡$P < 0.001$.

5 (*pgi*) of *D. romana s.s.*, in population 8 (*pgd*, *pgi*, *pgm*) of *D. flavescens*, in the Spanish population 13 (*ugpp*) of *D. markusii*, and in all three populations (*pgi*, *pgm*) of *D. sambucina*. A significant excess of heterozygotes was found only in the Sicilian population 12 (*ugpp*) of *D. markusii*.

The geographical distance and the Nei-72 genetic distance between populations of *D. romana s.s.* were found to be positively correlated ($P < 0.001$). The correlation is visualized by linear regression in Figure 3.

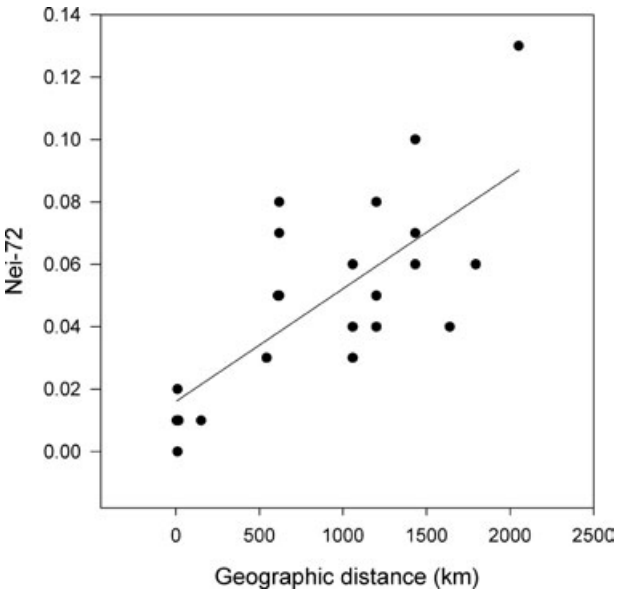

Figure 3. Linear regression showing the positive correlation of geographical distance with the Nei-72 genetic distance between populations of *Dactylorhiza romana s.s.* Each dot represents one pair of populations.

Morphology and combined data

Variation along the first two axes from the PCAs incorporating all populations, and performed on morphometric data only, is illustrated in Figure 4. In the plot of the analysis conducted on population values (Fig. 4A), *D. insularis* and *D. sambucina* form separate clusters that are fairly close to the sole analysed population of *D. indet*. The Spanish populations of *D. markusii* form another separate cluster. The Sicilian population of *D. markusii* is placed in between two very close clusters comprising all populations of *D. flavescens* and *D. romana s.s*. In the plot of the analysis based on specimen values (Fig. 4B), a similar pattern is seen, except that clusters now overlap. A

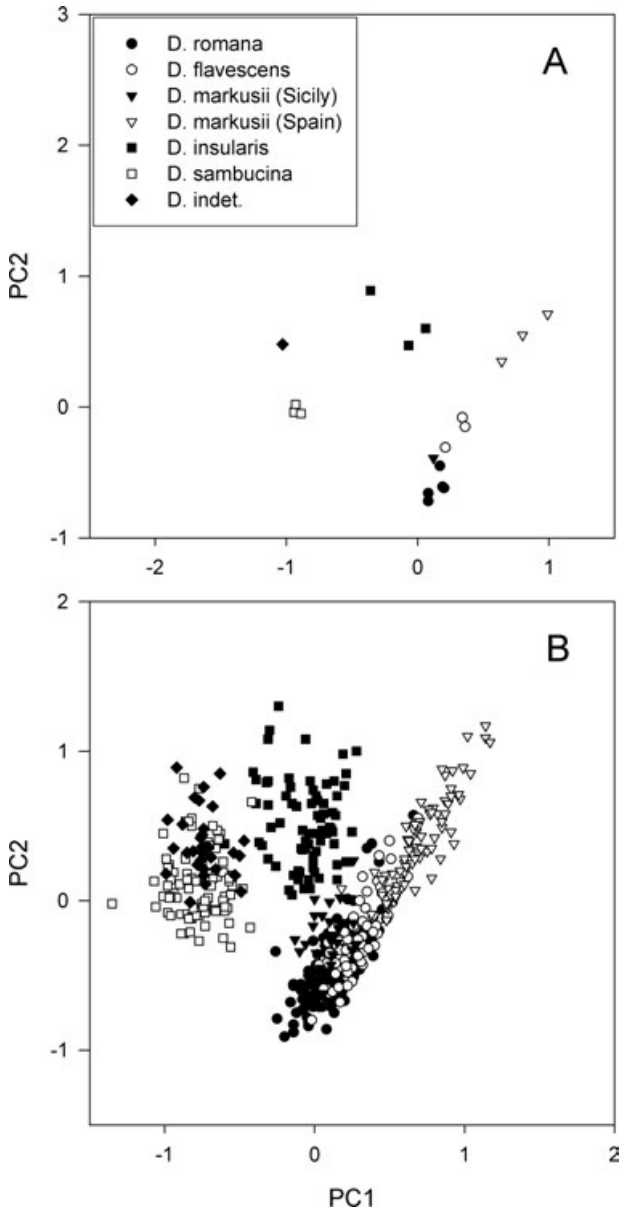

Figure 4. Plots from the first two principal components (PCs) of the principal components analysis (PCA) incorporating population samples of all study taxa (identifications based on Delforge, 2001) and performed on morphometric data only. A, Plot from the analysis conducted on population values; the variation was 34.4% along PC axis 1 and 26.4% along PC axis 2. B, Plot from the analysis conducted on specimen values; the variation was 24.8% along PC axis 1 and 20.9% along PC axis 2.

particularly strong overlap is exhibited by *D. sambucina* and *D. indet*., as well as by *D. romana s.s.*, *D. flavescens*, and the Sicilian population of *D. markusii*. A relatively clear distinction is evident between *D. sambucina*, *D. indet*., and *D. insularis*, on the one hand, and *D. romana s.s.*, *D. markusii*, and *D. flavescens* on the other. Consequently, these two main groups were selected for separate PCAs, aiming at a higher resolution of the variation in both groups. For each plot in Figure 4, the contributions of individual characters to each multivariate axis are listed in Table 7.

Table 7. Contributions of individual characters to the first two multivariate axes of the principal components analyses (PCAs) performed on morphometric data from all study taxa. Characters are numbered according to Table 2

	Population values		Specimen values	
Character	PC1	PC2	PC1	PC2
1	0.62	0.62	0.61	0.66
2	–0.84	0.30	–0.76	0.34
3	0.10	0.83	0.20	0.85
4	–0.20	–0.84	–0.18	–0.50
5	0.75	0.62	0.73	0.54
6	0.59	–0.01	0.51	0.25
7	–0.73	0.50	–0.53	0.60
8	0.00	–0.76	–0.01	–0.47
9	0.63	0.42	0.50	0.57
10	0.39	0.51	0.36	0.60
11	–0.41	–0.33	–0.35	–0.16
12	–0.83	0.47	–0.77	0.47
13	–0.76	0.19	–0.62	0.21
14	–0.30	0.39	–0.15	0.28
15	–0.47	–0.32	–0.36	–0.06
16	–0.23	–0.55	–0.20	–0.19
17	–0.76	0.19	–0.42	0.12
18	0.80	–0.47	0.73	–0.49
19	–0.05	–0.87	–0.07	–0.65
20	–0.51	–0.03	–0.48	0.15
21	–0.89	0.27	–0.71	0.36

Variation along the first two axes from the PCAs incorporating *D. romana s.s.*, *D. flavescens*, and *D. markusii* only is illustrated in Figure 5. In the plot of the analysis conducted on population values (Fig. 5A), *D. flavescens* and the Spanish populations of *D. markusii* both form loose clusters that are well separated from a tight cluster comprising *D. romana s.s.* and the Sicilian population of *D. markusii*. In the plot of the analysis conducted on specimen values (Fig. 5B), a similar pattern is evident. However, some overlap can be observed between the *D. flavescens* cluster and the cluster comprising *D. romana s.s.*, and the Sicilian population of *D. markusii*, and particularly between the *D. flavescens* cluster and the cluster comprising Spanish *D. markusii*. For each plot in Figure 5, the contributions of individual characters to each multivariate axis are listed in Table 8.

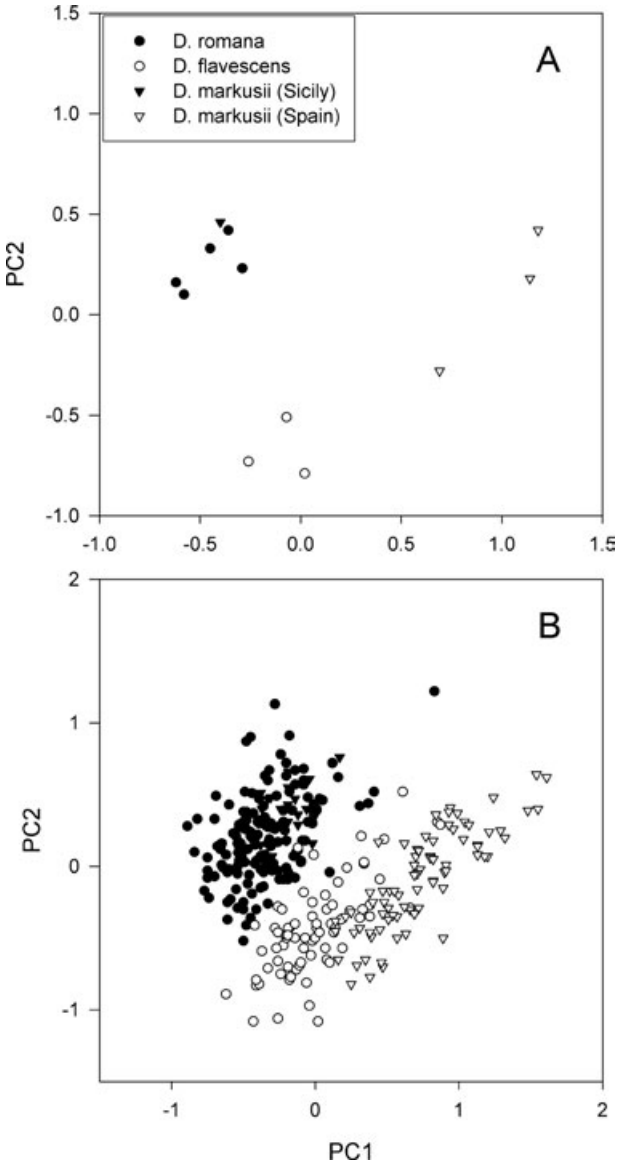

Figure 5. Plots from the first two principal components (PCs) of the principal components analysis (PCA) incorporating population samples of *Dactylorhiza flavescens*, *D. markusii*, and *D. romana s.s.* only (identifications based on Delforge, 2001). A, Plot from the analysis conducted on population values; the variation was 37.4% along PC axis 1 and 18.9% along PC axis 2. B, Plot from the analysis conducted on specimen values; the variation was 24.7% along PC axis 1 and 15.3% along PC axis 2.

Variation along the first two axes from the PCAs incorporating *D. sambucina*, *D. insularis*, and *D. indet*. only is illustrated in Figure 6. In the plot of the analysis conducted on population values (Fig. 6A), *D. sambucina* and *D. insularis* are widely separated along the first axis, the former forming a tight cluster to the left, and the latter forming a very loose cluster to the right. The sole population of *D. indet*. assumes a position intermediate between these clusters. In the plot of the analysis conducted on specimen values (Fig. 6B), a corresponding variation along the first axis can be seen. However, all three clusters are fairly loose, and there is slight overlap between *D. sambucina* and *D. indet*., and between *D. indet*. and *D. insularis*. For each plot in Figure 6, the contributions of individual characters to each multivariate axis are listed in Table 9.

Table 8. Contributions of individual characters to the first two multivariate axes of the principal components analyses (PCAs) performed on morphometric data from *Dactylorhiza romana s.s.*, *D. flavescens*, and *D. markusii* only. Characters are numbered according to Table 2; invariant characters have been deleted

	Population values		Specimen values	
Character	PC1	PC2	PC1	PC2
1	0.96	0.18	0.92	0.19
2	0.56	0.12	0.13	0.01
3	0.84	0.37	0.84	0.40
4	–0.90	0.07	–0.51	0.30
5	0.98	0.12	0.91	0.02
6	0.25	0.78	0.41	0.55
7	–0.07	0.31	0.23	0.44
8	–0.76	0.35	–0.40	0.39
9	0.87	0.33	0.79	0.41
10	0.89	0.05	0.84	0.21
11	–0.28	–0.74	–0.15	–0.22
14	–0.59	–0.14	–0.16	–0.04
15	–0.62	0.68	–0.41	0.75
16	–0.58	0.70	–0.39	0.77
17	0.27	0.31	–0.01	–0.03
18	0.21	–0.23	0.06	–0.16
19	–0.77	0.45	–0.65	0.57
20	–0.13	0.88	–0.15	0.71
21	–0.26	–0.43	0.03	0.09

Variation along the first two axes from the PCA incorporating all populations, and performed on combined morphometric and allozyme data, is illustrated in Figure 7. The populations of *D. insularis* and *D. sambucina* form separate clusters with the sole analysed population of *D. indet*. in a more or less intermediate position. The populations of *D. flavescens* and Spanish *D. markusii* form separate tight clusters. A cluster comprising all analysed populations of *D. romana s.s.* is slightly looser, and the Sicilian population of *D. markusii* assumes an intermediate position between *D. flavescens* and *D. romana s.s.* The contributions of individual characters to each multivariate axis are listed in Table 10.

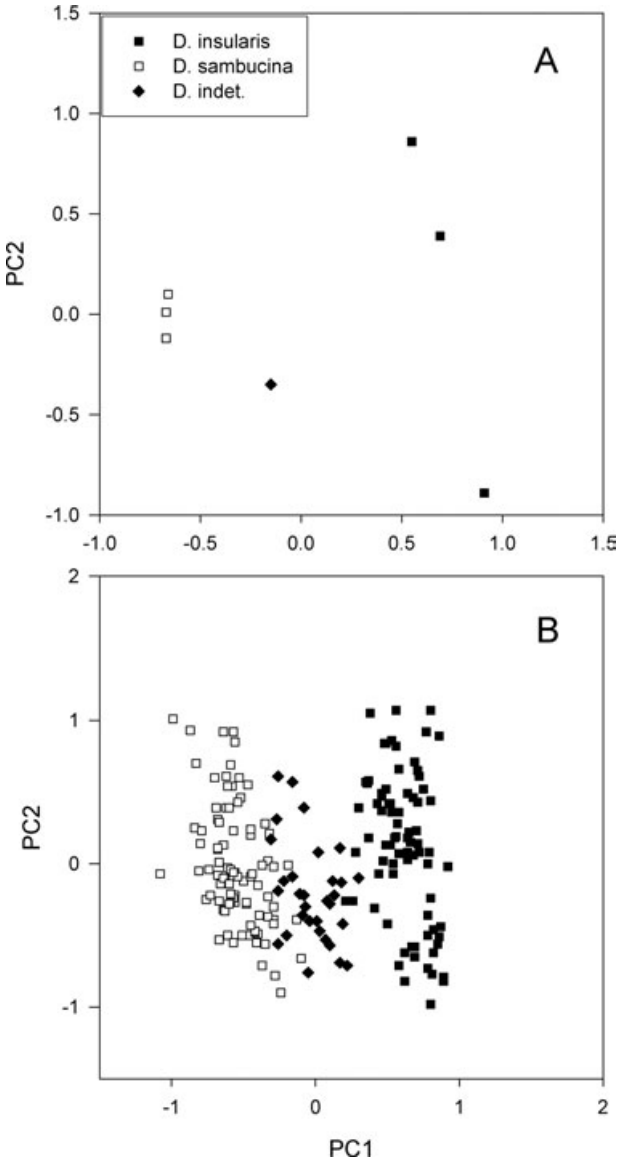

Figure 6. Plots from the first two principal components (PCs) of the principal components analysis (PCA) incorporating population samples of *Dactylorhiza insularis*, *D. sambucina*, and *D. indet*. only (identifications based on Delforge, 2001). A, Plot from the analysis conducted on population values; the variation was 49.5% along PC axis 1 and 30.6% along PC axis 2. B, Plot from the analysis conducted on specimen values; the variation was 31.5% along PC axis 1 and 22.0% along PC axis 2.

Table 9. Contributions of individual characters to the first two multivariate axes of the principal components analyses (PCAs) performed on morphometric data from *Dactylorhiza sambucina*, *D. insularis*, and *D. indet*. only. Characters are numbered according to Table 2

	Population values		Specimen values	
Character	PC1	PC2	PC1	PC2
1	0.56	0.82	0.52	0.76
2	–0.84	0.46	–0.72	0.17
3	0.47	0.84	0.33	0.85
4	–0.89	–0.15	–0.77	0.05
5	0.91	0.40	0.82	0.34
6	0.50	0.24	0.56	0.40
7	–0.14	0.75	–0.13	0.71
8	–0.83	0.31	–0.68	0.15
9	0.19	0.94	0.14	0.86
10	–0.75	0.61	–0.26	0.71
11	–0.88	–0.02	–0.73	–0.05
12	–0.61	–0.75	–0.33	–0.20
13	–0.59	–0.30	–0.40	–0.22
14	0.97	0.12	0.79	0.14
15	–0.45	0.87	–0.30	0.62
16	–0.62	0.63	–0.43	0.64
17	–0.64	–0.27	–0.26	–0.17
18	0.35	0.68	0.10	0.16
19	–0.96	0.19	–0.89	0.21
20	–0.83	0.48	–0.70	0.41
21	–0.91	0.28	–0.70	0.25

The probabilities of each character distinguishing correctly ($^{(*)}Q \geq 90\%$) between the various main groups of populations (defined according to the PCA results) are given in Table 11.

Within the group comprising *D. romana s.s.*, *D. flavescens*, and *D. markusii* (i.e. *D. romana s.l.*), characters 1 (plant height), 5 (number of non-sheathing leaves), and 19 (length of spur) were all found to distinguish reliably between *D. romana s.s.* (including Sicilian *D. markusii*) and Spanish *D. markusii*, whereas character 20 (vertical diameter of spur entrance) was the only character to distinguish reliably between *D. flavescens*, on the one hand, and *D. romana s.s.* (including Sicilian *D. markusii*) and Spanish *D. markusii* on the other.

Within the group comprising *D. sambucina*, *D. insularis*, and *D. indet*., characters 4 (number of sheathing leaves), 5 (number of non-sheathing leaves), 14 (abaxial angle separating lateral parts of labellum), 19 (length of spur), and e (length of spur relative to length of labellum lamina) were all found to distinguish reliably between *D. insularis* and *D. sambucina*. On the other hand, character 8 (orientation of longest leaf) was the only character found to distinguish reliably between *D. indet*. and *D. sambucina*, and only character 13 (presence vs. absence of markings beyond proximal third of labellum) distinguished reliably between *D. indet*. and *D. insularis*.

A clear distinction was found between *D. romana s.l.* and the group comprising *D. insularis*, *D. sambucina*, and *D. indet*. Thus, characters 12 (presence vs.

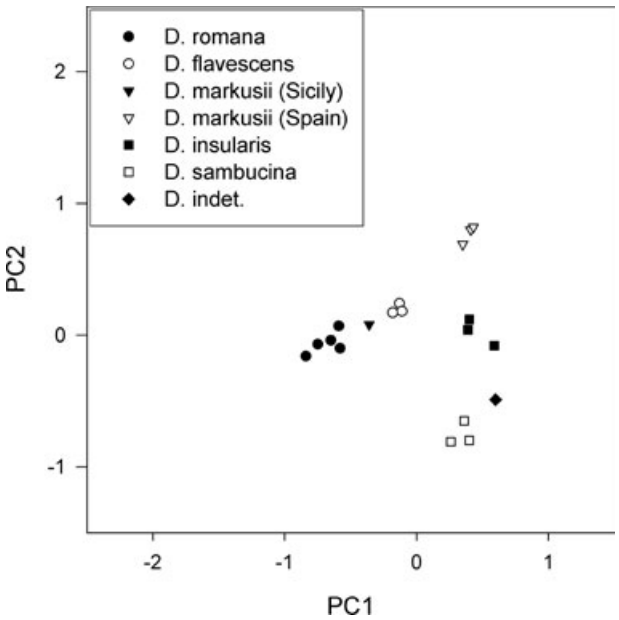

Figure 7. Plot from the first two principal components (PCs) of the principal components analysis (PCA) incorporating population samples of all study taxa (identifications based on Delforge, 2001) and performed on combined morphometric and allozyme data. The variation was 24.7% along PC axis 1 and 21.9% along PC axis 2.

absence of markings on proximal third of labellum) and 18 (spur straight to downcurved vs. spur upcurved) were found to distinguish reliably between *D. romana s.l.* and each member of the latter group. In addition, character 21 (vertical diameter of spur 1 mm from apex) distinguished reliably between *D. romana s.l.*, on the one hand, and both *D. sambucina* and *D. indet*. on the other. Finally, characters 7 (maximum width of longest leaf) and 13 (presence vs. absence of markings beyond proximal third of labellum) distinguished reliably between *D. romana s.l.* and *D. indet*., whereas character 2 (node of uppermost sheathing leaf below vs. above ground level) distinguished reliably between *D. romana s.l.* and *D. sambucina*.

DISCUSSION

Delimitation and ranking of diploid taxa

The allozyme data suggest a primary distinction between *D. sambucina*, on the one hand, and *D. romana s.s.*, *D. flavescens*, and *D. markusii* (i.e. *D. romana s.l.*) on the other (Fig. 2). The differences between these main groups are particularly pronounced at *pgd*, but not even at this locus are the alleles completely different between the two groups (Table 3). However, Bullini *et al*. (2001) found a complete distinction between *D. sambucina* and *D. romana s.s./D. markusii* at six of 19 allozyme loci examined in Italian material, and calculated Nei's (1972) genetic distance to average 0.59. Given this background, and assuming the introgression detected by Bullini *et al*. (2001) to be a fairly restricted phenomenon, there is little doubt that *D. sambucina* and *D. romana s.l.* comply with the biological species concept (Mayr, 1940) in a modern, botanically focused sense (Jonsell, 1984; Raven, 1986). Consequently, they should be recognized as separate species (Pedersen, 1998b). This view is supported by the morphometric and combined data. A very clear distinction between the populations of *D. sambucina* and *D. romana s.l.* is evident from Figures 4A, 7, and, even in the plot based on specimen morphometric values (Fig. 4B), there is no overlap between the two taxa. Furthermore, four characters distinguish reliably between *D. romana s.l.* and *D. sambucina* (Table 11). These findings are consistent with a PCA conducted by Tyteca (1997: fig. 8).

Table 10. Contributions of individual characters to the first two multivariate axes of the principal components analysis (PCA) performed on combined morphometric and allozyme data from all study taxa (analysis conducted on population values). Morphological characters are numbered according to Table 2, and allozyme alleles are designated according to Table 3

Morphology			Allozymes		
Character	PC1	PC2	Allele	PC1	PC2
1	0.40	0.74	dia^c	–0.26	–0.02
2	0.54	–0.75	dia^d	0.33	–0.16
3	0.70	0.30	dia^e	–0.27	0.15
4	–0.68	–0.41	dia^f	0.27	0.56
5	0.35	0.88	pgd^a	–0.12	0.19
6	–0.25	0.52	pgd^b	–0.50	0.76
7	0.59	–0.57	pgd^d	0.52	–0.77
8	–0.67	–0.24	pgd^e	–0.11	0.13
9	0.23	0.69	pgd^f	–0.10	0.16
10	0.46	0.49	pgi^a	–0.64	–0.30
11	–0.04	–0.52	pgi^b	0.69	0.31
12	0.66	–0.68	pgi^c	–0.43	–0.21
13	0.43	–0.62	pgi^d	–0.19	0.10
14	0.27	–0.20	pgm^a	–0.29	0.04
15	–0.29	–0.56	pgm^b	0.23	–0.56
16	–0.53	–0.37	pgm^c	–0.70	–0.53
17	0.44	–0.62	pgm^d	0.62	0.65
18	–0.64	0.65	pgm^e	–0.11	0.14
19	–0.86	–0.31	$ugpp^a$	–0.60	–0.09
20	0.06	–0.50	$ugpp^b$	–0.77	–0.07
21	0.53	–0.79	$ugpp^c$	–0.87	–0.04
			$ugpp^e$	0.93	0.05
			$ugpp^f$	–0.42	0.00
			$ugpp^g$	–0.11	0.14

Table 11. The probability $^{(*)}Q$ of each character distinguishing correctly between various groups of populations ('RR', *Dactylorhiza romana s.l.*, i.e. F/M/R). Note that 'M' in this table includes only the Spanish study populations of *D. markusii*, whereas the Sicilian population 12 is included in 'R'. Characters are numbered according to Table 2, and binary characters are indicated by an asterisk

Char. no.	*R* > < F	*R* > < M	*F* > < M	I > < S	I > < A	S > < A	RR > < I	RR > < S	RR > < A
1	62	92	85	78	77	54	56	64	65
2*	50	51	51	84	59	74	66	100	76
3	52	80	79	69	65	55	69	53	57
4	56	87	80	90	79	64	76	55	56
5	66	96	84	97	83	74	54	74	64
6	63	56	67	75	69	59	56	72	66
7	51	52	52	52	59	58	82	85	94
8	63	75	61	87	60	92	79	55	83
9	50	75	72	59	70	62	52	58	65
10	62	81	68	59	52	56	56	50	55
11*	72	57	79	88	50	88	61	77	61
12*	50	50	50	59	59	50	91	100	100
13*	50	50	50	69	100	81	50	69	100
14	52	63	69	93	76	75	82	51	67
15	79	81	51	56	50	57	54	60	54
16	84	83	55	63	50	61	57	53	57
17*	50	50	51	60	73	63	50	59	72
18*	52	54	51	51	51	50	96	97	97
19	88	95	73	95	70	89	77	54	71
20	97	67	92	78	72	58	54	74	69
21	61	54	67	79	80	56	58	91	90
a	62	56	68	74	73	51	68	87	86
b	72	87	80	51	63	66	56	55	55
c	61	75	67	77	68	60	63	63	53
d	57	52	59	74	63	82	67	84	58
e	73	85	71	95	75	86	84	61	77

Tyteca found that populations of *D. sambucina* from Italy, France, and Spain form a tight cluster distinctly separated from populations of Iberian *D. markusii* and *D. romana s.s.* from mainland Italy. In a relatively narrow zone of geographical overlap, *D. romana* and *D. sambucina* often occur sympatrically, and the interspecific hybrid *D. romana s.s.* × *sambucina* (*D.* × *fasciculata* (Tineo) H. Baumann & Künkele; Fig. 8A) is not rare.

Within *D. romana s.l.*, the allozyme data indicate a relatively clear distinction between the groups of populations tentatively referred to as *D. romana s.s.*, *D. flavescens*, and *D. markusii* (Fig. 2). However, it appears that the Sicilian study population of *D. markusii* belongs together with *D. romana s.s.* rather than with Spanish *D. markusii* (Fig. 2), and this finding is further supported by the morphological patterns of variation (Fig. 5). Accepting this widened circumscription of *D. romana s.s.*, the main differences in allele frequencies between this taxon and Spanish *D. markusii* are found at *pgm*, although noticeable differences can also be observed at *pgi* and *ugpp* (Table 3). *D. flavescens* assumes a somewhat intermediate position at *pgm* and *ugpp*, and has more alleles than both of the others at *pgd* (Table 3).

Bullini *et al*. (2001) examined 19 allozyme loci in eight Italian populations of *D. romana s.s.* (three from Sicily) and three Sicilian populations of *D. markusii*. They found the two taxa to be virtually identical and, consequently, concluded that *D. markusii* was synonymous with *D. romana s.s.* As long as *D. markusii* is considered to be endemic to Sicily, both the allozyme and morphometric data of the present study support this view. At the same time, however, they strongly indicate that the Spanish populations tentatively identified as *D. markusii* should be recognized as a taxon distinct from *D. romana s.s.*

On the basis of morphometric data, Wucherpfennig (2004) suggested widespread co-occurrence and introgressive hybridization between *D. romana s.s.* and *D. markusii* in Sicily. In the present study, the only Sicilian population tentatively assigned to *D. markusii* (population 12) was also the only population to show a significant excess of heterozygotes (at

Figure 8. A, *Dactylorhiza romana s.s.* × *sambucina*, Italy, Sicily, Etna, 19.iv.1999; B, C, *D. romana* ssp. *romana* (probably influenced by introgression from ssp. *guimaraesii*), Italy, Sicily, Bosco di Ficuzza, 20.iv.1999; D, E, *D. cantabrica*, Spain, Galicia, Lugo, Campelo, 28.iv.2001; F, *D. sambucina*, Denmark, Bornholm, Kåsegård, 23.v.1999. Photographs by H. Æ. Pedersen.

ugpp, Table 6). Taking into account the allele frequencies at *ugpp* in *D. romana s.s.* and Spanish *D. markusii* (Table 3), the observed heterosis in population 12 might indeed rely on introgression from 'Spanish *D. markusii*' into *D. romana s.s.* Judging mainly from the variation in spur length, Wucherpfennig (2004) considered populations in western Sicily to be pure *D. markusii*. In the present study, however, both allozyme data and the overall patterns of morphological differentiation suggest that population 12 (also from western Sicily), despite probable introgression from 'Spanish *D. markusii*', is much closer to pure *D. romana s.s.* than to the latter. Considering this evidence, it seems most suitable to (somewhat

pragmatically) classify all Sicilian populations of *D. romana s.l.* as *D. romana s.s.*, in accordance with Bullini *et al*. (2001). Alternatively, plants from a large proportion of populations could be treated as hybrids, but, taking into account the clinal variation in spur length from western to eastern Sicily (Wucherpfennig, 2004), presumably indicating a cline in degree of introgression, it would be extremely difficult to distinguish between 'hybrids' (Fig. 8B, C) and pure *D. romana s.s.* in Sicily. According to Rossi & Maury (2002), records of *D. markusii* from Sardinia are most likely misidentifications of *D. insularis*.

The morphometric data support the recognition of *D. romana s.s.* (including Sicilian *D. markusii*), *D. flavescens*, and Spanish *D. markusii* as separate taxa. Thus, a clear distinction of the three groups of populations is evident from Figure 5A, and, in the plot based on specimen values (Fig. 5B), the same clusters are recognizable (although they are less distinct). Furthermore, for each pair of these taxa, at least one reliable distinguishing character was identified (Table 11).

Judging from the six allozyme loci examined in this study, only allele frequencies separate the three diploid taxa constituting *D. romana s.s.*, and, on this basis, it seems inappropriate to recognize them as separate species (cf. the general species concept of Pedersen, 1998b). The dendrogram based on genetic distances (Fig. 2) and the overall morphological variation patterns (Fig. 5) together indicate that *D. flavescens* and Spanish *D. markusii* are closer to each other than to *D. romana s.s.* (including Sicilian *D. markusii*). One possible outcome would be to treat *D. romana s.s.* as one subspecies, and *D. flavescens* and Spanish *D. markusii* as two varieties constituting another subspecies – a solution, however, that appears excessively hierarchical and also unparsimonious (*D. markusii* and *D. flavescens* occurring to the west and east, respectively, of the central Mediterranean–Pontic *D. romana s.s.*). Indeed, being fairly well separated geographically, the three taxa comply with the ecological species concept (Van Valen, 1976) and should, consequently, be recognized as three distinct subspecies (Pedersen, 1998b). This decision is consistent with the PCA performed on combined morphometric and allozyme data (Fig. 7). Throughout the rest of this paper, the central, western, and eastern subspecies of *D. romana* are recognized as ssp. *romana*, ssp. *guimaraesii* (comb. et stat. nov.) and ssp. *georgica*, respectively (for nomenclatural details, see the 'Taxonomy' section).

Delimitation and ranking of polyploid taxa

In the present study, the triploid *D. insularis* was found to be monomorphic at *dia*, *pgi*, *skd*, and *ugpp*, but showed fixed heterozygosity at *pgd* and *pgm* (Table 3). At *dia*, *pgi*, and *skd*, the sole allele found in *D. insularis* was the most frequent allele in all study populations. Its only allele at *ugpp* (*ugpp*^{e}) was exclusive in *D. sambucina* and *D. indet*., but was also found in *D. romana s.l.*, particularly in ssp. *guimaraesii* (Table 3). At *pgd*, *D. insularis* combined two copies of *pgd*^{b} with one copy of *pgd*^{d}. The former allele occurs with frequencies of 50–100% in all study populations of *D. romana s.l.* and 50% in both populations of *D. indet*., whereas the latter allele occurs with frequencies of 100% in all study populations of *D. sambucina*, 50% in both populations of *D. indet*., 4–38% in the four populations of *D. romana* ssp. *georgica*, and 10% in the Cypriot population of *D. romana s.s.* (Table 3). At *pgm*, *D. insularis* combined two copies of *pgm*^{c} with one copy of *pgm*^{d}. The former allele occurs with frequencies of 58–100% in all study populations of *D. romana* ssp. *romana* and ssp. *georgica*, 62–83% in the three populations of *D. sambucina*, 50% in both populations of *D. indet*., and 2% in one of the four populations of *D. romana* ssp. *guimaraesii*, whereas the latter allele occurs with frequencies of 98–100% in all study populations of *D. romana* ssp. *guimaraesii*, 50% in both populations of *D. indet*., 6–42% in all study populations of *D. romana* ssp. *georgica*, 28% in one of the three study populations of *D. sambucina*, and 3% in one Sicilian population of *D. romana s.s.* (Table 3).

Bullini *et al*. (2001) examined 19 allozyme loci in one Spanish and six Italian populations of *D. insularis*, as well as in nine Italian populations of *D. sambucina* and 11 Italian populations of *D. romana s.s.* They convincingly demonstrated that *D. insularis* is an allotriploid, combining two alleles from *D. romana s.l.* with one from *D. sambucina*. Bullini *et al*. (2001) did not consider that the alleles from *D. romana s.l.* could be contributed by the western or eastern subspecies rather than the central Mediterranean–Pontic *D. romana s.s.* Under all circumstances, however, the allozyme data of the present study are consistent with the hypothesis that *D. insularis* is an allotriploid taxon that combines genomes from two valid parental species.

No chromosome counts on *D. indet*. have been published; however, judging from the fact that all individuals in this study were found to be balanced heterozygotes at both loci that were not monomorphic (Table 3), and from the banding intensity at the same loci, there is strong, if circumstantial, evidence to indicate that *D. indet*. is allotetraploid. At the six allozyme loci examined in this study, *D. indet*. is similar to *D. insularis*, except for the circumstance that it has two copies of both alleles at *pgd* and *pgm* (Table 3). Taking the above considerations concerning *D. insularis* into account, it therefore seems evident

that it universally combines two alleles from *D. romana s.l.* with two alleles from *D. sambucina*.

Having genome compositions that are basically different from any of the diploid species, and, furthermore, with *D. insularis* being isolated through its apparently parthenogenetic mode of reproduction (Diana, 1997), *D. indet*. and *D. insularis* comply with the biological species concept (Mayr, 1940) in a modern, botanically focused sense (Jonsell, 1984; Raven, 1986). Consequently, both should be recognized as separate species (Pedersen, 1998b).

The morphometric and combined data are consistent with the decision to treat *D. insularis* and *D. indet*. as distinct species. In the PCAs incorporating all study populations, they were both found to be distinct from *D. romana s.l.* (Figs 4, 7), and in the PCAs incorporating only *D. sambucina*, *D. insularis*, and *D. indet*., these taxa formed individual clusters that were distinctly separated in the plot based on population values (Fig. 6A) and only slightly overlapped in the plot based on specimen values (Fig. 6B). Two and five characters were found to distinguish reliably between *D. romana s.l.*, on the one hand, and *D. insularis* and *D. indet*., respectively, on the other (Table 11). Five characters were found to distinguish reliably between *D. sambucina* and *D. insularis*, whereas only one reliable character was found to distinguish between *D. indet*. and *D. insularis*, and one other to distinguish between *D. sambucina* and *D. indet*. (Table 11). In the latter case, however, character 19 (length of spur) was almost reliable (rate of success, 89%). All in all, the three biological species, *D. sambucina*, *D. insularis*, and *D. indet*., are morphologically similar, but can be identified on the basis of key characters.

Throughout the rest of the paper, the newly recognized allotetraploid taxon (Fig. 8D, E) is named *D. cantabrica* (sp. nov., diagnosis provided below).

Evolution of the diploid taxa

The degree of genetic differentiation between populations generally increases with the occupation time of a particular area by a species (Barrett & Husband, 1989). This also seems to be true for *Dactylorhiza*. Thus, Andersson (1995) studied the morphological variation of *D. majalis* (Rchb.) P. F. Hunt & Summerh. ssp. *traunsteineri* (Saut. ex Rchb.) H. Sund. on the background of land uplift data, and demonstrated that variation between populations was greater in the older region examined than in the younger region. Similarly, allozyme data (Hedrén, 1996, 2001a; Pedersen, 1998a) clearly showed that Anatolian populations of *D. incarnata s.l.* were far more genetically differentiated than populations from northern Europe – the species appears to have lost genetic variation during its recolonization from southern refugia after the Weichselian glaciation (Hedrén, 2002). In a taxonomically and geographically more comprehensive study of *Dactylorhiza* using plastid and nuclear DNA sequence data, Pillon *et al*. (2006b) found a higher phylogenetic and genetic diversity in the Caucasus and the Mediterranean Basin than in western Europe, thus supporting Hedrén's (2002) findings on a wider scale.

Changes in breeding system also have a great potential impact on genetic differentiation between populations, but this does not seem to be important for the *D. romana*/*sambucina* complex. Thus, both *D. sambucina* and *D. romana s.l.* act by deceit, as their flowers are pollinated by bees probing the empty spur in search of nectar (Nilsson, 1980; Pettersson & Nilsson, 1983; Lagutova, Nazarov & Shevchenko, 1996; Vöth, 1999; Kropf & Renner, 2005).

Both the relative genetic differentiation between local populations (G_{ST}) and the absolute degree of gene differentiation (D_m) were found to be higher in *D. romana s.s.* than in any other of the diploids (Table 5). Consequently, it might be hypothesized that this complex originated somewhere within the present range of *D. romana s.s.* (an area extending from Sicily to the Crimea), and that *D. romana s.s.* is the least derived modification of the first common ancestor of the complex. According to this scenario, *D. sambucina*, *D. romana* ssp. *guimaraesii*, and *D. romana* ssp. *georgica* evolved in connection with the migration of their common ancestor towards the north, west, and east, respectively. The significant positive correlation of the geographical distance and the Nei-72 genetic distance between populations of the widely distributed (and amply sampled) *D. romana s.s.* (Fig. 3) indicates that isolation by distance may have been an important factor for the differentiation of the diploid complex. However, this still leaves us to consider two possible modes of speciation (Lynch, 1989): vicariant speciation or peripheral isolate speciation.

The wide geographical range of the northern *D. sambucina* exhibits a narrow overlap with that of *D. romana s.l.* In addition, the study populations of *D. sambucina* contain relatively high levels of gene diversity (H_T, H_S), although the levels are slightly lower than in *D. romana* ssp. *romana* and ssp. *georgica* (Table 5). Altogether, it seems reasonable to consider the evolutionary separation of *D. romana s.l.* and *D. sambucina* as the outcome of a vicariant speciation event.

The geographical range of the eastern *D. romana* ssp. *georgica* is juxtaposed to that of the central ssp. *romana*, and the study populations of both taxa exhibit relatively high levels of gene diversity (H_T, H_S; see Table 5). The most parsimonious conclusion would be that the mutual divergence of these taxa resulted from an event of (incomplete) vicariant speciation. The west-

ern *D. romana* ssp. *guimaraesii*, on the other hand, occupies a geographical range that is slightly disjunct from that of ssp. *romana* (except perhaps for a hybrid zone in Sicily; see above). Furthermore, very low levels of genetic variation were found in the study populations of the former (Tables 3–5). These findings suggest that ssp. *guimaraesii* originated through (incomplete) peripheral isolate speciation as a response to westward long-distance dispersal. Thus, the low levels of genetic variation might be explained by a population bottleneck effect caused by a single founder event.

A significant deficiency in heterozygotes at one to three loci was found in populations 4, 5, 8, 13, and 20–22 (Table 6). A pronounced deficiency in heterozygotes in northern European populations of *D. incarnata* is explained by excessive inbreeding, in accordance with the extremely low levels of allozyme variation observed in these populations (Pedersen, 1998a; Hedrén, 2001b, 2002). In the diploid study populations of the *D. romana*/*sambucina* complex, however, the deficiency in heterozygotes is less consistent (Table 6), and the level of allozyme variation is almost universally higher (Table 4). In this case, therefore, a more plausible explanation might be a Wahlund (1928) effect, that is, the inclusion of two or more genetically distinct units in each population sample. Thus, the local populations might be further subdivided into demes determined by the commonly sympatric occurrence of two distinct colour morphs (plants with predominantly yellowish and purplish flower colours, respectively; Fig. 8F). This hypothesis cannot be tested within the present data set, but is consistent with the circumstance that deficiency in heterozygotes appears to be less common in *D. romana* ssp. *guimaraesii*, in which only the yellowish colour morph is known, than in the other diploid taxa (Table 6). Nilsson (1980) and Gigord *et al*. (2002) observed an imperfect morph constancy by bumblebees in natural and experimental populations of *D. sambucina*, respectively. The general flower constancy of individual bumblebees is well documented (e.g. Free, 1970; Heinrich, 1976; Thomson, 1981; Gumbert, 2000), and, as the colours of the morphs in *D. sambucina* are expected to fall close to the optical sensitivity maxima in bumblebees (Nilsson, 1980), it might be hypothesized that the partial morph constancy observed is related to the innate colour preferences of bumblebees. At least, this would be in agreement with the fact that deficiency in heterozygotes seems to be most pronounced in *D. sambucina* (Table 6), the species most consistently pollinated by bumblebees (Nilsson, 1980; Pettersson & Nilsson, 1983; Lagutova *et al*., 1996; Vöth, 1999; Kropf & Renner, 2005). However, it is important to note that the morph constancy of bumblebees in populations of *D. sambucina* is imperfect (Pellegrino *et al*., 2005), which may explain why the putative Wahlund effect is only of moderate magnitude (Table 6). In addition to the partial morph constancy of bumblebees, other mechanisms have been suggested as being responsible for the maintenance of colour dimorphism (or polymorphism) in *D. sambucina*. These include negative frequency-dependent selection (Gigord, Macnair & Smithson, 2001) and partial post-pollination barriers detected through differential viability and germinability of seeds from experimental crosses (Pellegrino, Bellusci & Musacchio, 2005; Jersákova, Kindlmann & Renner, 2006).

Evolution of the polyploid taxa

As a result of the general correspondence in distribution of allozyme markers in the diploids and polyploids (Table 3), and the lack of genome reorganization in the polyploids, a relatively recent origin of the polyploids seems likely. Consequently, present geographical distributions might well be expected to reflect spatial aspects of speciation (Levin, 1993).

Dactylorhiza insularis and *D. cantabrica* consistently share the same alleles at all loci examined, and their allele frequencies only deviate according to the allotriploid and presumably allotetraploid nature, respectively, of the two species (Table 3). Taking into account the features of the diploid taxa as well, this leaves us with several possible origins of the polyploids.

Bullini *et al*. (2001) proposed that *D. insularis* originated by fusion of an unreduced (diploid) gamete from *D. romana s.s.* with a normal (haploid) gamete from *D. sambucina*. This hypothesis is consistent with the allozyme data obtained in the present study (Table 3). Tentatively accepting an origin of *D. insularis* from two diploid taxa, however, also involves other possibilities than that proposed by Bullini *et al*. (2001). According to the data presented in Table 3, the most likely alternative seems to be an origin from hybridization between *D. romana* ssp. *guimaraesii* and *D. sambucina*. An origin from *D. romana* ssp. *georgica* × *sambucina* appears highly unlikely according to current distributions (see the 'Taxonomy' section). The same can be said about an origin from hybridization between any pair of subspecies under *D. romana s.l.*, and this possibility is also contradicted by the morphological variation patterns observed (Fig. 4).

The apparently allotetraploid *D. cantabrica* may have originated from the same combinations of diploid taxa as discussed for *D. insularis* above, but the exact pathway leading to an allotetraploid instead of a triploid must have been different.

Accepting *D. sambucina*, *D. romana s.s.*, and *D. romana* ssp. *guimaraesii* as the most likely diploid candidates to have taken part in the evolution of the

polyploids, either of the polyploids may have participated as well. One possibility is that *D. insularis* ($2n = 60$) originated by fusion of a normal gamete ($n = 20$) of *D. romana s.l.* with a normal gamete ($n = 40$) of *D. cantabrica*. Alternatively, *D. cantabrica* ($2n = 80$) may have originated by fusion of an unreduced gamete ($n = 60$) of *D. insularis* with a normal gamete ($n = 20$) of *D. sambucina*.

TAXONOMY

KEY TO THE SPECIES

1. Labellum devoid of markings; spur upcurved ..... 1. *D. romana*
1. Labellum provided with markings (sometimes only few and confined to its extreme base); spur straight to downcurved 2
2. Longest leaf describing an adaxial angle of more than 55° relative to the stem. Spur more than 11 mm long, more than 1.4 times as long as the lamina of labellum ..... 2. *D. sambucina*
2. Longest leaf describing an adaxial angle of less than 55° relative to the stem. Spur less than 11 mm long, less than 1.4 times as long as the lamina of labellum 3
3. Usually more than three non-sheathing leaves below the inflorescence. Labellum devoid of markings beyond its proximal one-third ..... 4. *D. insularis*
3. Usually fewer than three non-sheathing leaves below the inflorescence. Labellum bears markings beyond its proximal one-third ..... 3. *D. cantabrica*

1. *DACTYLORHIZA ROMANA* (SEBAST.) SOÓ

For synonyms and typifications, see under the subspecies.

Description: PLANT (6.8–)12.6–38.9(–45.4) cm high with the node of the uppermost sheathing leaf almost always placed below ground level. STEM (2.0–)2.3–5.6(–8.0) mm in diameter immediately below the inflorescence. SHEATHING LEAVES (excluding cataphylls) (1–)2–6(–9); the longest one (4.9–)8.1–13.1(–20.8) × (0.7–)0.9–1.3(–1.9) cm, (4.2–)6.9–13.2(–17.8) times as long as wide, (0.2–)0.3–0.8(–1.1) times as long as the height of the plant, describing an adaxial angle of (30–)39–83(–90)° to the stem. NON-SHEATHING LEAVES below the inflorescence (0–)1–8(–11). INFLORESCENCE (2.3–)3.5–9.8(–14.5) cm long, (0.1–)0.2–0.4(–0.6) times as long as the height of the plant, carrying (3–)6–23(–37) yellow or purple flowers. LAMINA OF LABELLUM devoid of markings, (5.2–)6.4–9.3(–10.8) × (7.3–)8.1–13.6(–16.5) mm, almost always slightly three-lobed [shape index (0.5–)0.7–0.8(–1.0)], almost always widest in its proximal to middle part, with the sidelobes describing an abaxial angle of (10–)32–144(–270)° to each other. SPUR almost always upcurved, (7.4–)9.3–19.2(–23.0) mm long, (1.0–)1.3–2.3(–2.7) times as long as the lamina of labellum, (1.5–)1.8–3.3(–3.6) mm in diameter at the entrance, (0.6–)0.8–1.3(–1.8) mm in diameter 1 mm below the apex.

KEY TO THE SUBSPECIES

1. Vertical diameter of spur entrance less than 2.5 mm ..... 1c. ssp. *georgica*
1. Vertical diameter of spur entrance more than 2.5 mm 2
2. Plant less than 23 cm high; fewer than four non-sheathing leaves below the inflorescence. Spur more than 12 mm long ..... 1a. ssp. *romana*
2. Plant more than 23 cm high; more than four non-sheathing leaves below the inflorescence. Spur less than 12 mm long ..... 1b. ssp. *guimaraesii*

1a. ssp. romana

Dactylorhiza romana (Sebast.) Soó, Nom. Nov. Gen. *Dactylorhiza*: 3 (1962) (non in Ann. Univ. Sci. Budapest. Rolando Eötvös, Sect. Biol. 3: 337 (1960), comb. inval., cf. Saint Louis code: Art. 33.3); *Orchis romana* Sebast., Roman. Pl. 1: 12, Pl. 3 (1813); *Orchis sambucina* (L.) Soó ssp. *romana* (Sebast.) Bornm. in Bot. Jahrb. Syst. Beibl. 140: 126 (1927); *Dactylorchis romana* (Sebast.) Verm., Stud. Dactylorch.: 65 (1947). *Type*: Italy, Roma, 'Pigneto di Sacchetti', sine anno, *Sebastiani, Gismondo & Mauri* s.n. (not located).

Orchis bracteata Ten., Fl. Napol. I: LII (1811), nom. illeg. (non Willd.). *Type*: not designated.

Orchis lucana Spreng., Pl. Min. Cogn. Pug. 2: 79 (1815). *Type*: Italy, 'Lucania Calabriae', sine coll. et no. (not located).

Orchis pseudosambucina Ten., Syn. Nov. Pl. Prodr. Fl. Neap. Descr. 72 (1815); *Orchis mediterranea* Klinge ssp. *pseudosambucina* (Ten.) Klinge in Trudy Imp. S.-Peterburgsk Bot. Sada XVII(1): 164 (1898), comb. inval. (cf. Saint Louis code: Art. 43.1); *Dactylorhiza sulphurea* (Link) Franco ssp. *pseudosambucina* (Ten.) Franco in Bot. J. Linn. Soc. 76: 366 (1978), comb. inval. (cf. Saint Louis code: Art. 43.1); *Dactylorhiza sambucina* (L.) Soó ssp. *pseudosambucina* (Ten.) H. Sund., Eur. Medit. Orchid., ed. 3: 40 (1980). *Type*: Italy, Napoli, Monte Nuovo near Pozzuoli, sine anno, *Tenore* s.n. (syntypes BM! G! ?M ?NAP).

Orchis fasciculata Tineo var. *obtusifolia* Tineo in Guss., Fl. Sicul. II: 875 (1845); *Orchis romana* Sebast. var. *fasciculata* (Tineo) Soó f. *obtusifolia* (Tineo) Soó in Repert. Spec. Nov Regni Veg. XXIV: 30 (1927). *Type*: not designated.

Orchis markusii Tineo, Pl. Rar. Sicil. 9 (1846); *Orchis pseudosambucina* Ten. var. *markusii* (Tineo) Nyman, Consp. Fl. Eur. 693 (1882); *Orchis romana* Sebast. var. *fasciculata* (Tineo) Soó f. *markusii* (Tineo) Soó in Repert. Spec. Nov Regni Veg. XXIV: 30 (1927); *Orchis sulphurea* Link var. *markusii* (Tineo) Jahand. & Maire, Cat. Pl. Maroc 1: 151 (1931), comb. inval. (cf. Saint Louis code: Art. 43.1); *Orchis romana* Sebast. var. *markusii* (Tineo) Rivas Goday in Farmacognosia IV(6): 194–195 (1945); *Dactylorhiza romana* (Sebast.) Soó ssp. *siciliensis* (Klinge) Soó var. *markusii* (Tineo) Soó, Nom. Nov. Gen. *Dactylorhiza*: 3 (1962) (non in Ann. Univ. Sci. Budapest. Rolando Eötvös, Sect. Biol. 3: 337 (1960), comb. inval., cf. Saint Louis code: Art. 33.3); *Dactylorhiza markusii* (Tineo) H. Baumann & Künkele in Mitt. Arbeitskreis Heimische Orchid. Baden-Württemberg 13: 461 (1981); *Dactylorhiza romana* (Sebast.) Soó ssp. *markusii* (Tineo) Holub in Folia Geobot. Phytotax. 19: 214 (1984). *Type*: Italy, Sicily, Palermo, Gibilrossa, 1827, *Tineo* s.n. (holotype ?PAL, isotype FI (photo seen)).

Orchis sicula Tineo, Pl. Rar. Sicil. 8 (1846); *Orchis pseudosambucina* Ten. var. *sicula* (Tineo) K.Richt., Pl. Eur. 1: 271 (1890); *Dactylorhiza romana* (Sebast.) Soó ssp. *siciliensis* (Klinge) Soó var. *sicula* (Tineo) Soó, Nom. Nov. Gen. *Dactylorhiza*: 3 (1962) (non in Ann. Univ. Sci. Budapest. Rolando Eötvös, Sect. Biol. 3: 337 (1960), comb. inval., cf. Saint Louis code: Art. 33.3); *Dactylorhiza sicula* (Tineo) Aver. in Bot. Zhurn. (Moscow & Leningrad) 69: 875 (1984). *Type*: Italy, Sicily, Caronie, Monte Pizzu di l'Ursu, sine anno, *Tineo* s.n. (not located).

Orchis flavescens C. Koch in Linnaea 22: 281 (1849), p.p.; *Orchis romana* Sebast. ssp. *georgica* (Klinge) Soó lus. *flavescens* (C. Koch) Soó in Repert. Spec. Nov. Regni Veg. XXIV: 30 (1927), p.p.; *Dactylorchis flavescens* (C. Koch) Verm., Stud. Dactylorch. 65 (1947), p.p.; *Dactylorhiza flavescens* (C. Koch) Holub in Folia Geobot. Phytotax. 11: 83 (1976), p.p. (non (C. Koch) H. Baumann & Künkele in Mitt. Arbeitskreis Heimische Orchid. Baden-Württemberg 13: 237 (1981), comb. superfl.). *Type*: not designated.

Orchis mediterranea Klinge ssp. *siciliensis* Klinge in Trudy Imp. S.-Peterburgsk Bot. Sada XVII(1): 165 (1898), nom. inval. (cf. Saint Louis code: Art. 43.1); *Orchis siciliensis* (Klinge) A. W. Hill in Index Kew., suppl. IX: 194 (1938); *Dactylorhiza romana* (Sebast.) Soó ssp. *siciliensis* (Klinge) Soó, Nom. Nov. Gen. *Dactylorhiza*: 3 (1962) (non in Ann. Univ. Sci. Budapest. Rolando Eötvös, Sect. Biol. 3: 337 (1960), comb. inval., cf. Saint Louis code: Art. 33.3); *Dactylorhiza sulphurea* (Link) Franco ssp. *siciliensis* (Klinge) Franco in Bot. J. Linn. Soc. 76: 367 (1978), comb. inval. (cf. Saint Louis code: Art. 43.1); *Dactylorhiza sambucina* (L.) Soó ssp. *siciliensis* (Klinge) H. Sund., Eur. Medit. Orchid., ed. 3: 40 (1980). *Type*: not designated.

Orchis pseudosambucina Ten. [unranked] *ficuzzae* Gand., Nov. Consp. Fl. Eur. 462 (1910), nom. nud. *Type*: not designated.

Orchis pseudosambucina Ten. [unranked] *inarimensis* Gand., Nov. Consp. Fl. Eur. 462 (1910), nom. nud. *Type*: not designated.

Orchis pseudosambucina Ten. [unranked] *spruneri* Gand., Nov. Consp. Fl. Eur. 462 (1910), nom. nud. *Type*: not designated.

Orchis romana var. *incarnata* E. G. Camus & A. Camus, Iconogr. Orchid. Europe: 214 (1928). *Type*: '*Heldr*., Exsicc., n°. 2202' (not located, and it is not known to which of Heldreich's exsiccates the number refers).

Orchis romana var. *lutea* E. G. Camus & A. Camus, Iconogr. Orchid. Europe: 214 (1928). *Type*: not designated.

Orchis romana lus. *bicolor* G. Keller in Repert. Spec. Nov. Regni Veg. Sonderbeih. A(II): 206 (1933). *Type*: Italy, Napoli, Solfatara near Pozzuoli, sine anno, coll. et no. (not located).

Orchis romana Sebast. ssp. *libanotica* Mouterde, Nouv. Fl. Liban. Syrie 1: 342, Pl. CXII(4) (1966); *Dactylorhiza libanotica* (Mouterde) Aver. in Bot. Zhurn. (Moscow & Leningrad) 69: 876 (1984). *Type*: Lebanon, Jabal Lubān, Bayt Miri, Aïn Cheik, 25 February 1936, *Mouterde* 4630 (holotype G!).

Description: PLANT (6.8–)12.6–21.0(–30.6) cm high. STEM (2.0–)2.4–3.9(–6.5) mm in diameter immediately below the inflorescence. SHEATHING LEAVES (excluding cataphylls) (3–)4–6(–9); the longest one (4.2–)7.7–12.3(–15.8) times as long as wide, (0.4–)0.5–0.8(–1.1) times as long as the height of the plant, describing an adaxial angle of (40–)59–83(–90)° to the stem. NON-SHEATHING LEAVES below the inflorescence (0–)1–3(–5). INFLORESCENCE (2.5–)3.8–6.8(–12.2) cm long, (0.2–)0.3–0.4(–0.6) times as long as the height of the plant, carrying (3–)6–14(–27) yellow or purple

flowers. LAMINA OF LABELLUM (6.6–)7.6–9.3(–10.8) × (7.5–)10.4–13.6(–16.5) mm, with the sidelobes describing an abaxial angle of (10–)33–144(–270)° to each other. SPUR (11.3–)14.4–19.2(–23.0) mm long, (1.1–)1.7–2.3(–2.7) times as long as the lamina of labellum, (2.4–)2.9–3.3(–3.6) mm in diameter at the entrance.

Good illustrations: Nelson (1976: Pl. 77a, b, d), Landwehr (1977: Pls 18–20, 21(1–2)), Baumann & Künkele (1982: 91; 1988: 32–33 centre), Buttler (1986: 71 top), Mossberg & Nilsson (1987: 105), Kreutz (1998: 164–167), Delforge (2001: 168), Kretzschmar, Kretzschmar & Eccarius (2002: 87–89), Rossi & Maury (2002: Pl. XXIX).

Distribution: Baumann & Künkele (1982: 90) provided a fairly accurate map of the total range of this taxon. *D. romana s.s.* is distributed from Sicily (Appendix 1) and the Mediterranean parts of mainland Italy (see map in Rossi & Maury, 2002: 43), across the southern parts of the Balkan Peninsula, the Greek islands, and (sub)oceanic parts of western/central Anatolia (see map in Kreutz, 1998: 167), to Cyprus, northern Lebanon, and the Crimea.

1b. ***ssp.* guimaraesii** *(E. G. Camus) H. A. Pedersen,* ***comb. et stat. nov.***

Orchis romana Sebast. var. *guimaraesii* E. G. Camus in E. G. Camus & A. Camus, Iconogr. Orchid. Europe: 216 (1928); *Orchis guimaraesii* (E. G. Camus) Rivas Goday in Farmacognosia IV(6): 197 (1945). *Type*: E. G. Camus & A. Camus, Iconogr. Orchid. Europe: Pl. 33(14–15), 1921 (lectotype, designated here).

Orchis sulphurea Link [*s.s.*] in J. Bot. (Schrader) 1(3): 132 (1806), nom. nud.; *Orchis romana* Sebast. lus. *sulphurea* (Link) Soó in Bot. Arch. 23: 63 (1929), comb. inval.; *Dactylorhiza sulphurea* (Link) Franco [*s.s.*] in Bot. J. Linn. Soc. 76: 366 (1978), comb. inval. *Type*: not designated.

Description: PLANT (13.6–)24.8–38.9(–45.4) cm high. STEM (3.0–)3.6–5.6(–8.0) mm in diameter immediately below the inflorescence. SHEATHING LEAVES (excluding cataphylls) (1–)2–4(–5); the longest one (6.2–)8.2–13.2(–17.8) times as long as wide, (0.2–)0.3–0.5(–0.8) times as long as the height of the plant, describing an adaxial angle of (30–)39–68(–85)° to the stem. NON-SHEATHING LEAVES below the inflorescence (3–)5–8(–11). INFLORESCENCE (3.9–)5.4–9.8(–14.5) cm long, (0.1–)0.2–0.3(–0.4) times as long as the height of the plant, carrying (8–)12–23(–37) yellow flowers. LAMINA OF LABELLUM (5.2–)6.5–7.7(–8.5) × (7.3–)8.5–10.2(–10.8) mm, with the sidelobes describing an abaxial angle of (10–)32–83(–130)° to each other. SPUR (7.4–)9.3–11.1(–12.1) mm long, (1.1–)1.3–1.6(–1.8) times as long as the lamina of labellum, (2.2–)2.6–3.1(–3.5) mm in diameter at the entrance.

Good illustrations: Nelson (1976: Pl. 79a, sub nom. *D. siciliensis*), Landwehr (1977: Pl. 21(4–5), sub nom. *D. romana* ssp. *siciliensis*), Baumann & Künkele (1982: 81, sub nom. *D. markusii*; 1988: 35 top, sub nom. *D. markusii*), Buttler (1986: 73 top right, sub nom. *D. markusii*), Sánchez Pedraja (2005: fig. 5c–c_1, Phot. 41, sub nom. *D. sulphurea*).

Distribution: According to the present genetic and morphometric findings, combined with examination of herbarium material of *Dactylorhiza romana s.l.* from the western Mediterranean (Appendix 1), ssp. *guimaraesii* is restricted to the Iberian Peninsula and the Atlas Mountains of northern Morocco and Algeria (see also Tyteca, 1997: 315, sub nom. *D. markusii*; Sánchez Pedraja, 2005: 100, sub nom. *D. sulphurea*).

1c. ssp. georgica (Klinge) Renz & Taubenheim

In Notes Roy. Bot. Gard. Edinburgh 41: 271 (1983); *Orchis mediterranea* Klinge ssp. *georgica* Klinge in Trudy Imp. S.-Peterburgsk Bot. Sada XVII(1): 166 (1898), nom. inval. (cf. Saint Louis code: Art. 43.1); *Orchis georgica* (Klinge) Lipsky, Fl. Kavk. 457 (1899); *Orchis romana* Sebast. ssp. *georgica* (Klinge) E. G. Camus, Monogr. Orchid. 172 (1908); *Orchis romana* Sebast. var. *georgica* (Klinge) Schltr. in Repert. Spec. Nov. Regnum Veg. Sonderbeih. A(I): 187 (1927); *Orchis romana* Sebast. ssp. *georgica* (Klinge) Soó in Repert. Spec. Nov. Regni Veg. XXIV: 30 (1927); *Dactylorhiza romana* (Sebast.) Soó ssp. *georgica* (Klinge) Soó, Nom. Nov Gen. *Dactylorhiza*: 3 (1962) (non in Ann. Univ. Sci. Budapest. Rolando Eötvös, Sect. Biol. 3: 337 (1960), comb. inval., cf. Saint Louis code: Art. 33.3); *Dactylorhiza sambucina* (L.) Soó ssp. *georgica* (Klinge) H. Sund., Eur. Medit. Orchid., ed. 3: 40 (1980). *Type*: Rchb.f., Icon. Fl. Germ. Helv. XIII/XIV: Pl. 62(I), 1851 (lectotype, designated here).

Orchis flavescens C. Koch in Linnaea 22: 281 (1849), p.p.; *Orchis romana* Sebast. ssp. *georgica* (Klinge) Soó lus. *flavescens* (C. Koch) Soó in Repert. Spec. Nov. Regni Veg. XXIV: 30 (1927), p.p.; *Dactylorchis flavescens* (C. Koch) Verm., Stud. Dactylorch. 65 (1947), p.p.; *Dactylorhiza flavescens* (C. Koch) Holub in Folia Geobot. Phytotax. 11: 83 (1976), p.p. (non (C. Koch) H. Baumann & Künkele in Mitt. Arbeitskreis Heimische Orchid. Baden-Württemberg 13: 237 (1981), comb. superfl.). *Type*: not designated.

Orchis tenuifolia C. Koch in Linnaea 22: 281 (1849); *Orchis romana* Sebast. ssp. *georgica* (Klinge) Soó lus. *tenuifolia* (C. Koch) Soó in Repert. Spec. Nov. Regni Veg. XXIV: 30 (1927). *Type*: Azerbaijan, Gyandzha, sine anno, *C. Koch* s.n. (holotype destroyed at B).

Dactylorhiza ruprechtii Aver. in Bot. Zhurn. (Moscow & Leningrad) 68: 537 (1983). *Type*: Georgia, Kaischaur between Tblisi and Vladikavkaz, 11.v.1861, *Ruprecht* s.n. (holotype LE).

Description: PLANT (7.4–)13.5–23.9(–33.5) cm high. STEM (2.0–)2.3–3.9(–5.5) mm in diameter immediately below the inflorescence. SHEATHING LEAVES (excluding cataphylls) (2–)3–6(–8); the longest one (5.4–)6.9–10.4(–14.5) times as long as wide, 0.4–0.6(–0.8) times as long as the height of the plant, describing an adaxial angle of (40–)46–77(–90)° to the stem. NON-SHEATHING LEAVES below the inflorescence 1–4(–8). INFLORESCENCE (2.3–)3.5–7.0(–9.7) cm long, 0.2–0.3(–0.5) times as long as the height of the plant, carrying (5–)7–18(–28) yellow or purple flowers. LAMINA OF LABELLUM (5.6–)6.4–7.9(–9.4) × (7.4–)8.1–10.0(–11.8) mm, with the sidelobes describing an abaxial angle of (10–)50–138(–180)° to each other. SPUR (7.8–)10.2–13.3(–15.5) mm long, (1.0–)1.4–1.9(–2.2) times as long as the lamina of labellum, (1.5–)1.8–2.4(–2.9) mm in diameter at the entrance.

Good illustrations: Renz (1978: Pl. 49), Baumann & Künkele (1982: 67, sub nom. *D. flavescens*; 1988: 32–33 bottom), Buttler (1986: 71 bottom, sub nom. *D. flavescens*), Kreutz (1998: 161–163, sub nom. *D. flavescens*) Delforge (2001: 167, sub nom. *D. flavescens*).

Distribution: This subspecies ranges from eastern Anatolia (see map in Kreutz, 1998: 163, sub nom. *D. flavescens*) across Transcaucasia and the Caucasus to the Elburz Mountains of northern Iran (Renz, 1978).

2. *DACTYLORHIZA SAMBUCINA* (L.) SOÓ

Nom. Nov. Gen. *Dactylorhiza*: 3 (1962) (non in Ann. Univ. Sci. Budapest. Rolando Eötvös, Sect. Biol. 3: 336 (1960), comb. inval., cf. Saint Louis code: Art. 33.3); *Orchis sambucina* L., Fl. Suec., ed. 2: 312 (1755); *Orchis mixta* Retz. var. *sambucina* (L.) Retz., Prodr. Fl. Scand. 167 (1779), comb. inval. (cf. Saint Louis code: Art. 43.1); *Dactylorchis sambucina* (L.) Verm., Stud. Dactylorch. 65 (1947). *Type*: Sine loco, coll., no. et anno (probably collected in Sweden by *Tuwén*) (lectotype LINN-1054.34!, designated by H. Baumann *et al*. Mitt. Arbeitskreis Heimische Orchid. Baden-Württemberg 21: 468 (1989)).

Orchis latifolia L., Sp. Pl. 2: 941 (1753), p.p., nom. rej.; *Dactylorhiza latifolia* (L.) Soó, Nom. Nov. Gen. *Dactylorhiza*: 4 (1962) (non in Ann. Univ. Sci. Budapest. Rolando Eötvös, Sect. Biol. 3: 341 (1960), comb. inval., cf. Saint Louis code: Art. 33.3), p.p. *Type*: uncertain, cf. H. A. Pedersen in Taxon 49: 299–301 (2000).

Orchis sambucina L. var. *ochroleuca* Winterl, Index Horti Bot. Univ. Hung. Pest. [unpaginated] (1788), nom. nud. *Type*: not designated.

Orchis sambucina L. var. *rubra* Winterl, Index Horti Bot. Univ. Hung. Pest. [unpaginated] (1788); *Orchis sambucina* L. lus. *rubra* (Winterl) Soó in Repert. Spec. Nov. Regni Veg. Sonderbeih. A(II): 203 (1933); *Dactylorhiza sambucina* (L.) Soó f. *rubra* (Winterl) Hyl., Nord. Kärlväxtfl. II: 387 (1966); *Dactylorhiza latifolia* (L.) Soó f. *rubra* (Winterl) D. Tyteca & Gathoye in Orchidophile (Asnières) 92: 112 (1990). *Type*: not designated.

Orchis schleicheri Sweet, Brit. Fl. Gard. II: sub Pl. 199 (1827) (non Hort. Brit.: 382 (1826), nom. nud.). *Type*: Switzerland, sine loco et anno, *Schleicher*/cult. Colvill s.n. (not located).

Orchis sambucina L. var. *incarnata* Gaudin, Fl. Helv. V: 441 (1829); *Dactylorhiza sambucina* (L.) Soó lus. *incarnata* (Gaudin) Soó, Nom. Nov. Gen. *Dactylorhiza*: 3 (1962). *Type*: not designated.

Orchis sambucina L. var. *purpurea* W. D. J. Koch, Syn. Fl. Germ. Helv., ed. 2: 792 (1837); *Orchis sambucina* L. f. *purpurea* (W. D. J. Koch) Neuman, Sver. Fl. 629 (1901); *Orchis sambucina* L. subvar. *purpurea* (W. D. J. Koch) Rouy, Fl. France XIII: 155 (1912). *Type*: not designated.

Orchis lutea Dulac, Fl. Hautes-Pyrénées: 125 (1867). *Type*: not designated.

Orchis laurentina R. Bolos ex Vayr. in Anales Soc. Esp. Hist. Nat. IX: 96 (Sept. 1879) (non Pl. Notabl. 160–161 (Oct. 1879)); *Orchis sambucina* L. [unranked] *laurentina* (R. Bolos ex Vayr.) Willk., Suppl. Prodr. Fl. Hispan. 42 (1893); *Orchis sambucina* L. f. *laurentina* (R. Bolos ex Vayr.) Soó in Repert. Spec. Nov. Regni Veg. XXIV: 29 (1927); *Dactylorhiza sambucina* (L.) Soó var. *laurentina* (R. Bolos ex Vayr.) Soó, Nom. Nov. Gen. *Dactylorhiza*: 3 (1962) (non in Ann. Univ. Sci. Budapest. Rolando Eötvös, Sect. Biol. 3: 336 (1960), comb. inval., cf. Saint Louis code: Art. 33.3); *Orchis sambucina* L. ssp. *laurentina* (R. Bolos ex Vayr.) Malag. in Acta Phytotax. Barcinon. 1: 64 (1968). *Type*: Spain, 'inmediaciones del lago de Laurenti', vii.1801, '*Platraver* (Bolós hb.)' (not located).

Orchis sambucina L. f. *bracteata* Schulze in Mitt. Bot. Vereins Gesamtthüringen 1889: 26 (in Mitt. Geogr. Ges. Jena VII(3–4)) (1889); *Orchis sambucina* L. var. *bracteata* (Schulze) Harz in Schltdl *et al.*, Fl. Deutschl., jubilee ed., IV: 271 (1895). *Type*: not designated.

Orchis sambucina L. f. *rubrobracteata* Harz in Schltdl *et al.*, Fl. Deutschl., jubilee ed., IV: 271 (1895); *Dactylorhiza sambucina* (L.) Soó f. *rubrobracteata* (Harz) Soó in Acta Bot. Acad. Sci. Hung. 16: 368 (1971). *Type*: not designated.

Orchis sambucina L. var. *robusta* Neuman, Sver. Fl.: 629 (1901); *Dactylorhiza sambucina* (L.) Soó f. *robusta* (Neuman) Soó in Acta Bot. Acad. Sci. Hung. 16:

368 (1971). *Type*: Sweden, Östergötland, Jonsberg, Gränsö, 26.vi.1884, *Elmqvist* s.n. (holotype LD!).

Orchis sambucina L. [unranked] *asturica* Gand., Nov. Consp. Fl. Eur.: 462 (1910), nom. nud. *Type*: Spain, Burgos, Soncillo, 1880, *Estebanez* s.n. (holotype LY (photo seen)).

Orchis sambucina L. [unranked] *neapolitana* Gand., Nov. Consp. Fl. Eur.: 462 (1910), nom. nud. *Type*: Italy, Napoli, v.1888, *Altobelli* s.n. (holotype LY (photo seen)).

Orchis sambucina L. [unranked] *tergestina* Gand., Nov. Consp. Fl. Eur.: 462 (1910), nom. nud. *Type*: Italy, Trieste, v.1879, *Marchesetti* s.n. (holotype LY (photo seen)).

Orchis sambucina L. [unranked] *alandica* Gand., Nov. Consp. Fl. Eur.: 462 (1910), nom. nud. *Type*: Finland, Åland, Geta, Bolsteholm, 21.v.1878, *Arrhenius* s.n. (holotype LY (photo seen)).

Orchis sambucina L. subvar. *luteopurpurea* Rouy, Fl. France XIII: 155 (1912); *Orchis sambucina* L. lus. *luteopurpurea* (Rouy) G. Keller & Soó in Repert. Spec. Nov. Regni Veg. Sonderbeih. A(II): 203 (1933). *Type*: not designated.

Orchis sambucina L. f. *barlae* Rouy, Fl. France XIII: 155 (1912). *Type*: not designated.

Orchis sambucina L. lus. *lutea* Walther Zimm., Formen Orchid. Deutschl. 38 (1912). *Type*: not designated.

Orchis sambucina L. lus. *hybrida* Walther Zimm. in Allg. Bot. Z. Syst. XXII: 129 (1916); *Dactylorhiza sambucina* (L.) Soó var. *hybrida* (Walther Zimm.) Peitz in Jahresber. Naturwiss. Vereins Wuppertal 25: 178 (1972). *Type*: not designated.

Orchis sambucina L. f. *lanceolata* Walther Zimm. in Allg. Bot. Z. Syst. XXII: 129 (1916). *Type*: not designated.

Orchis sambucina L. f. *obovata* Walther Zimm. in Allg. Bot. Z. Syst. XXII: 129 (1916). *Type*: not designated.

Orchis sambucina L. f. *hungarica* Soó in Repert. Spec. Nov. Regni Veg. XXIV: 29 (1927); *Orchis sambucina* L. var. *hungarica* (Soó) A. Camus in E. G. Camus & A. Camus, Iconogr. Orchid. Europe: 212 (1928); *Dactylorhiza sambucina* (L.) Soó var. *hungarica* (Soó) Soó, Nom. Nov. Gen. *Dactylorhiza*: 3 (1962) (non in Ann. Univ. Sci. Budapest. Rolando Eötvös, Sect. Biol. 3: 337 (1960), comb. inval., cf. Saint Louis code: Art. 33.3). *Type*: not designated.

Orchis sambucina L. var. *lutea* E. G. Camus in E. G. Camus & A. Camus, Iconogr. Orchid. Europe: 212 (1928). *Type*: not designated.

Orchis sambucina L. var. *zimmermannii* A. Camus in E. G. Camus & A. Camus, Iconogr. Orchid. Europe: 212 (1928). *Type*: not designated.

Orchis sambucina L. monstr. *subregalis* Soó in Repert. Spec. Nov. Regni Veg. Sonderbeih. A(II): 203 (1933). *Type*: not designated.

Description: PLANT (9.0–)11.7–18.9(–25.0) cm high with the node of the uppermost sheathing leaf placed above ground level. STEM (2.0–)2.8–4.5(–6.0) mm in diameter immediately below the inflorescence. SHEATHING LEAVES (excluding cataphylls) (3–)4–6(–7); the longest one (4.8–)6.1–9.6(–12.3) × (0.9–)1.2–2.1(–2.9) cm, (2.1–)3.6–6.3(–8.7) times as long as wide, (0.3–)0.4–0.6(–0.8) times as long as the height of the plant, describing an adaxial angle of (40–)56–80(–85)° to the stem. NON-SHEATHING LEAVES below the inflorescence 0–1(–2). INFLORESCENCE (3.2–)3.8–6.2(–9.8) cm long, (0.2–)0.3–0.4(–0.6) times as long as the height of the plant, carrying (6–)8–17(–32) yellow or purple flowers. LAMINA OF LABELLUM provided with markings in its proximal one-third (and fairly often beyond) (6.8–)7.6–9.1(–10.0) × (7.7–)9.4–12.5(–14.6) mm, almost always slightly three-lobed [shape index (0.7–)0.8–0.9(–1.0)], usually widest in its proximal to middle part, with the sidelobes describing an abaxial angle of (10–)47–113(–130)° to each other. SPUR straight to downcurved, (9.8–)12.0–14.7(–17.7) mm long, (1.2–)1.4–1.8(–2.1) times as long as the lamina of labellum, (2.5–)3.1–3.7(–4.0) mm in diameter at the entrance, (1.1–)1.4–2.0(–2.9) mm in diameter 1 mm below the apex.

Good illustrations: Figure 8F; Nelson (1976: Pl. 76a–e), Landwehr (1977: Pls 17(1–5), 18), Hansson (1985: 88), Buttler (1986: 75 top), Mossberg & Nilsson (1987: 103), Baumann & Künkele (1988: 34–35 centre), Reinhard *et al*. (1991: 173), Johnsen (1994: Pl. 28), Bournérias (1998: 155–156), Presser (2000: 98–99), Rossi & Maury (2002: Pl. XXVII), Souche (2004: 84), Baumann, Kretzschmar & Blatt (2005: figs 336–339), Sánchez Pedraja (2005: fig. 5b–b_1, Phots 38–89).

Distribution: Baumann & Künkele (1982: 96) provided a fairly accurate map of the total range of this species. The main area covers southern, central, and eastern parts of Europe. In the south, it ranges to central Spain (Appendix 2; Sánchez Pedraja, 2005: 98), through mainland Italy to Sicily, and across the Balkan Peninsula to the Peloponnese. In the east, the main area reaches a line from eastern Bulgaria to Kiev, and in the north it reaches central Germany (Baumann *et al*., 2005: Map 341/1), southern Poland (see map in Zajac & Zajac, 2001: 188) and Belarus. An area slightly disjunct from the main area ranges from southern Norway across eastern Denmark to south-eastern Sweden, southernmost Finland, and the Estonian island of Saaremaa.

3. *DACTYLORHIZA CANTABRICA* H. A. PEDERSEN, **SP. NOV.**

Type: Spain, Galicia, Lugo, Caurel, O Couto, 13.v.2003, *Larrinaga* s.n./Herb. Sant. 50170 (holotype SANT!).

Diagnosis: Species haec *D. sambucina*e (L.) Soó affinis, sed foliis erectioribus calcarique labelli breviori distinguitur.

Description: PLANT (10.1–)11.7–17.7(–19.5) cm high with the node of the uppermost sheathing leaf placed below or above ground level. STEM (3.0–)3.2–4.5(–5.5) mm in diameter immediately below the inflorescence. SHEATHING LEAVES (excluding cataphylls) 3–5(–6); the longest one (6.1–)7.2–10.0(–11.4) × (1.1–)1.4–2.2(–2.8) cm, (2.9–)4.0–5.8(–7.5) times as long as wide, (0.4–)0.5–0.7 times as long as the height of the plant, describing an adaxial angle of (10–)23–47(–60)° to the stem. NON-SHEATHING leaves below the inflorescence (0–)1–3. INFLORESCENCE (3.0–)3.4–5.3(–6.6) cm long, 0.2–0.4(–0.6) times as long as the height of the plant, carrying (6–)8–14(–16) yellow flowers. LAMINA OF LABELLUM provided with markings below its proximal one-third and beyond (6.7–)7.5–8.7(–9.6) × (7.8–)8.7–11.4(–12.9) mm, slightly three-lobed [shape index 0.7–0.8], widest in its proximal to distal part, with the sidelobes describing an abaxial angle of (60–)89–161(–80)° to each other. SPUR straight to downcurved, (9.1–)9.7–11.0(–11.6) mm long, (1.0–)1.2–1.4(–1.5) times as long as the lamina of labellum, (2.9–)3.0–3.5(–3.8) mm in diameter at the entrance, (1.3–)1.4–1.8(–2.2) mm in diameter 1 mm below the apex (Fig. 8D, E).

Distribution: According to the present genetic and morphometric findings, combined with an examination of representative Spanish herbarium material originally assigned to the morphologically similar *D. sambucina* (Appendix 2), *D. cantabrica* is endemic to Sierra del Caurel in the western part of Cordillera Cantabrica, northern Spain.

4. *DACTYLORHIZA INSULARIS* (SOMMIER) LANDWEHR

In Orchidee (Hamburg) 20: 128 (1969) (non (Sommier) E. Nelson, Monogr. Ikonogr. Orchid. Gattung *Dactylorhiza*: 104 (1976), comb. inval. (cf. Saint Louis code: Art. 33.3) et non (Sommier) Ó. Sanchez & Herrero in Castrov *et al*. (eds), Fl. Iber. 21: 98 (2005), comb. superfl.); *Orchis insularis* Sommier in Boll. Soc. Bot. Ital. 1895: 247 (1895); *Orchis sambucina* L. var. *insularis* (Sommier) Fiori, Fl. Italia I: 245 (1896); *Orchis romana* Sebast. var. *insularis* (Sommier) E. G. Camus, Monogr. Orchid.: 172 (1908); *Orchis sambucina* L. ssp. *insularis* (Sommier) Briq., Prodr. Fl. Corse I: 371 (1910); *Dactylorhiza sambucina* (L.) Soó ssp. *insularis* (Sommier) Soó in Ann. Univ. Sci. Budapest. Rolando Eötvös, Sect. Biol. 3: 337 (1960), comb. inval. (cf. Saint Louis code: Art. 33.3). *Type*: Italy, Grosseto, Isola del Giglio, 22.v.1894, *Sommier* s.n. (lectotype FI (photo seen), designated here but already labelled as lectotype by L. Hautzinger in 1974, isolectotype FI!).

Orchis pseudosambucina Ten. ssp. *castellana* Rivas Goday in Farmacognosia IV(6): 197 (1945); *Orchis romana* Sebast. var. *castellana* (Rivas Goday) Rivas Goday in Farmacognosia IV(6): 197 (1945); *Orchis sambucina* L. var. *castellana* (Rivas Goday) Rivas Goday in Farmacognosia IV(6): 197 (1945); *Orchis sulphurea* Link ssp. *castellana* (Rivas Goday) Rivas Goday in Anales Jard. Bot. Madrid 21(2): 306 (1963), comb. inval. (cf. Saint Louis code: Art. 43.1); *Dactylorhiza insularis* (Sommier) Landwehr ssp. *castellana* (Rivas Goday) D. Rivera & López Vélez, Orquíd. Prov. Albacete: 82 (1987). *Type*: not designated.

Dactylorhiza sambucina (L.) Soó ssp. *insularis* Soó, Nom. Nov. Gen. *Dactylorhiza*: 3 (1962), nom. nud. *Type*: not designated.

Dactylorhiza romana (Sebast.) Soó ssp. *bartonii* Huxley & P. F. Hunt in J. Roy. Hort. Soc. XCII: 309 (1967); *Dactylorhiza insularis* (Sommier) Landwehr var. *bartonii* (Huxley & P. F. Hunt) Landwehr in Orchidee (Hamburg) 20: 128 (1969); *Dactylorhiza bartonii* (Huxley & P. F. Hunt) Aver. in Bot. Zhurn. (Moscow & Leningrad) 69: 876 (1984); *Dactylorhiza insularis* (Sommier) Landwehr var. *bartonii* (Huxley & P. F. Hunt) D. Rivera & López Vélez, Orquíd. Prov. Albacete: 82 (1987); *Dactylorhiza insularis* (Sommier) Landwehr f. *bartonii* (Huxley & P. F. Hunt) Gathoye & D. Tyteca in Lejeunia n.s. 143: 52 (1994). *Type*: Spain, Cuenca, Montes Universales, Tragacete, 3.vi.1966, *Huxley, Gorer & Barton* 115 (holotype K!).

Dactylorhiza insularis Englmaier in Abh. Zool. Bot. Ges. Österr. 22: 105 (1984), nom. nud. *Type*: not designated.

Description: PLANT (10.8–)16.9–29.6(–35.7) cm high with the node of the uppermost sheathing leaf usually placed below ground level. STEM (3.0–)3.6–5.4(–6.5) mm in diameter immediately below the inflorescence. SHEATHING LEAVES (excluding cataphylls) (1–)2–3(–4); the longest one (4.8–)8.2–14.8(–17.7) × (1.0–)1.1–2.1(–3.1) cm, (2.7–)5.0–10.3 (–14.4) times as long as wide, (0.2–)0.4–0.6(–0.8) times as long as the height of the plant, describing an adaxial angle of (5–)29–53(–70)° to the stem. NON-SHEATHING LEAVES below the inflorescence (1–)3–5(–6). INFLORESCENCE (3.3–)4.3–7.0(–8.9) cm long, 0.2–0.3(–0.6) times as long as the height of the plant, carrying (5–)8–13(–18) yellow flowers. LAMINA OF LABELLUM usually provided with markings (but not beyond its proximal one-third), (6.3–)7.2–8.9(–9.9) × (7.6–)9.2–11.0(–11.9) mm, slightly three-lobed [shape index (0.7–)0.8(–0.9)], widest in its proximal to middle part, with the sidelobes describing an abaxial angle of (130–)141–180° to each other. SPUR nearly always straight to downcurved, (7.5–)8.3–10.4(–12.5) mm long, (1.0–)1.1–1.2(–1.4) times as long as the lamina of labellum, (2.0–)2.6–3.2(–3.4) mm in diameter at the

entrance, (0.9–)1.0–1.4(–1.8) mm in diameter 1 mm below the apex.

Good illustrations: Nelson (1976: Pl. 78), Landwehr (1977: Pls 22–23), Baumann & Künkele (1982: 75; 1988: 32–33 top), Buttler (1986: 73 bottom), Giotta & Piccitto (1990: 70–71), Bournérias (1998: 157), Delforge (2001: 165), Rossi & Maury (2002: Pl. XXVIII), Souche (2004: 85), Sánchez Pedraja (2005: fig. 5a–a_1, Phot. 40).

Distribution: This species is mainly distributed in the Iberian Peninsula (Tyteca, 1997: 314; Sánchez Pedraja, 2005: 99) and in Corsica, Sardinia, and central Italy (Rossi & Maury, 2002: 37). Isolated occurrences are found in the Corbieres in southern France (Bournérias, 1998: 157) and in the Rif Mountains of northern Morocco (Baumann & Baumann, 2005: 920).

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APPENDIX 1

LIST OF EXAMINED HERBARIUM MATERIAL OF *DACTYLORHIZA ROMANA S.L.* FROM PORTUGAL, SPAIN, ALGERIA, AND SICILY

Dactylorhiza romana ssp. guimaraesii

ALGERIA. El Asnam: Hammam Righa, v.1899 (*Mason* s.n. K); Theniet El Had, iv.1852 (*Munby* s.n. K), iv.1870 (sine coll. et no. C), 29.iv.1873 (*Durando* s.n. FI, G), v.1887 (*Battandier & Trabut* 494 G, L, RO), 16.v.1888 (*Letourneux* s.n. C, FI).

PORTUGAL. Beira Alta: Matança, v.1887 (*de Mariz* s.n. LISU); Quinta do Cidro, 30.iv.1979 (*Roseira & Sena* s.n. PO), 15.v.1984 (*Sena et al.* s.n. PO). – Estremadura: Monte Junto, 20.v.1888 (*Murray* s.n. BM). – Trás-o-Montes: Torre de Dona Chama, 15.iv.1943 (*Banos Cameiro* s.n. PO). – Province unknown: Sine loco, iii–iv.1879 (*Pereiraloutinho* 310 LISU).

SPAIN. Albacete: Calar del Mundo, 3.v.1975 (*Lopez* s.n. MAF). – Cáceres: Castañar de Ibor, 16.iv.1967 (*Ladero* s.n. MAF); Cerro Carbonero, 16.iv.1967 (*Ladero* s.n. MAF); Hospital del Obispo, 10.iv.1961 (*Rivas Goday* s.n. MAF); Romangordo, iv.1946 (*Rivas Goday* s.n. MAF). – Huelva: Aracena, 1968 (*Sanchez Jurado* s.n. MAF).

Dactylorhiza romana ssp. romana

ITALY, SICILY. Catania: Etna, 16.iv.1965 (*Merxmüller & Wiedmann* 20138 M), 14.iv.1973 (*Schmidt* s.n. M),

16.iv.1988 (*Förther* s.n. M; sine coll. et no. M). – Messina: Capizzi, 1847 (*Tineo* s.n. K). – Palermo: Castelbuono, 6.iv.1874 (*Strobl* s.n. K), 10.iv.1952 (*Merxmüller & Wiedmann* 384/52 M); Collesano, 16.iv.1980 (*Wood* 383 K, 385 K); Ficuzza, iii–iv.1871 (*Todaro*/Fl. Sic. Exicc. 965 BM, FI, K), iv.1877 (*Lojacono* s.n. FI), 1881 (*Todaro* s.n. FI), iv.1885 (*Lojacono* 296 BM, G, LD); Gibilrossa, 1847 (*Tineo* s.n. K), sine anno (*Tineo* s.n. RO; sine coll. et no. RO); Misilmeri, 20.iii.1855 (*E. & A. Huet du Pavillon* s.n. G, K); Palermo, iv.1845 (sine coll. et no. K), v.1853 (sine coll. et no. K); along track to Portella di Vento and Giardinello, 17.iv.1980 (*Wood* 394 K). – Siracusa: W of Siracusa, 11.iv.1979 (*Vegener* s.n. C).

APPENDIX 2

List of examined herbarium material from Spain, originally assigned to *Dactylorhiza sambucina*

Dactylorhiza cantabrica

Spain. Lugo: O Couto, 13.v.2003 (*Larrinaga* s.n. SANT); Pedrafita do Caurel, 3.vi.1981 (*Amigo* s.n. SANT); Taro Blanco, 3.vi.1981 (*Izco et al.* s.n. SANT).

Dactylorhiza sambucina

Spain. Burgos: Domingo de Silos, 12.vi.1970 (*Rivas Goday & Izco* s.n. MAF); Montes Obarenes, 24.v.1909 (*Elias* s.n. M), 19.v.1910 (*Elias* s.n. M), 19.v.1912 (*Elias* s.n. M). – Gerona: 3 km W of Collado de Coubet, 23.v.1975 (sine coll. et no. M); Riutes S of Latour-de-Carol, 25.v.1972 (*J. Koch* s.n. M). – Guadaljara: Orea, 29.v.1972 (*J. Koch* s.n. M). – Huesca: Candanchu S of Col de Somport, 8.vi.1972 (*J. Koch* s.n. M); Panticosa, vii.1946 (*Rivas Goday* s.n. MAF); Somport, 11.v.1961 (*Stace* 558 BM, 559 BM; *Walker* 178 BM). – León: Barrios de Luna, 5.v.1992 (*Romero & Cortizo* s.n. SANT); Busdongo, 25.v.1962 (*Rivas Goday* s.n. MAF); 15 km W of La Pola, 20.v.1998 (*Podlec* 54746 MSB); 1 km SE of Oseja de Sajambre, 17.v.1972 (*Brummitt & Chater* 164 K); Puebla de Lillo, 7.vi.1985 (*Guitian* s.n. SANT); Torrestio, 12.vi.1970 (*Rivas Goday et al.* s.n. MAF); Valporquero, 6.vi.1985 (*Pablo* s.n. SANT). – Madrid: Puerto de Samosierra, 29.iv.1961 (*Rivas Goday & Galiano* s.n. MAF); Sierra de Guadarrama, 16.v.1912 (*Vicioso* s.n. LISU). – Navarra: Collado de Uztarroz, 25.v.1960 (*Sandwith* 5828 K). – Oviedo: Picos de Europa, vii.1879 (*Boissier* s.n. G), vi.1960 (*B. & R. Martinez* s.n. MAF). – Teruel: Grigos, vi.1895 (*Reverchon* s.n. G), sine anno (*Fuchs* s.n. M). – Zaragoza: Moncayo, 7.vi.1946 (*Rivas & Madueño* s.n. MAF).