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Geometric morphometrics as a tool for understanding *Dactylorhiza* (Orchidaceae) diversity in European Russia

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Geometric morphometric techniques were employed to assess the diversity of lip shapes (305 samples from 83 populations) in flowers of European Russian *Dactylorhiza* (Orchidaceae: Orchidinae). We found significant agreement between the results from geometric morphometrics, classic morphometrics and the distribution of certain nuclear DNA markers. The lip shapes from Arctic *Dactylorhiza* samples occupied an intermediate position between *D. maculata* and *D. fuchsii* samples from Central Russia, supporting a hybrid origin of 'northern tetraploids'. Lip shapes of the taxonomically controversial allotetraploid *D. baltica* were found to form a distinct group, with members having definite relationships with diploid *D. incarnata* samples from the same localities, indicating either their local origin or introgression with *D. incarnata*. In addition to demonstrating the value of geometric morphometric methods in studies of plant taxonomy and hybridization, we suggest future applications designed to explore pollinator-driven directional selection, developmental constraints and fluctuating asymmetry. © 2005 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2005, **85**, 1–12.

ADDITIONAL KEYWORDS: developmental constraints – diploid – directional selection – fluctuating asymmetry – Internal Transcribed Spacer – landmarks – tetraploid.

INTRODUCTION

Geometric morphometry (e.g. Bookstein, 1991; Pavlinov, 2001) is a relatively new technique that has generated valuable results in many fields of classic morphometry. The major difference from classic methods is the ability to understand the form of an object directly, as a cohesive whole, rather than indirectly via fragmentary measurements. Thus, the chosen geometric morphometric variables allow complete reconstruction of the shape. Two different kinds of geometric morphometry are most widely used: Fourier analysis of shape curves, and landmark-based methods such a Thin Plate Splines (TPS) analysis (Adams, Rohlf & Slice, 2002; Jensen, Ciofani & Miramontes, 2002).

Fourier analysis calculates several 'shape curves' from the object outline and then derives 'Fourier coefficients' that represent these curves; the coefficients can be used as variables for multivariate analysis of the objects investigated (Jensen *et al.*, 2002).

The landmark approach is based on placing on the shape image several so-called 'landmarks' pinpointing the most important locations on the object. These points are assumed to be homologous, at least in a geometric sense, because landmark-based methods operate only with the coordinates of these reference points, so the objects studied should be directly comparable (Pavlinov, 2001). TPS reveals the degree of 'bending energy' necessary to transform a rectangular grid superimposed on one shape to fit another shape (Rohlf & Slice, 1990, developing much earlier qualitative work by Thompson, 1917). Multivariate methods can also be applied to the results of TPS (Rohlf, Loy & Corti, 1996; Baylac, Villemant & Simbolotti, 2003).

There are many taxonomic applications of TPS and other geometric morphometry methods (e.g. Ray, 1992; Kores, Molvray & Darwin, 1993; Pavlinov, 2000; Rueber & Adams, 2001; Jensen *et al.*, 2002; Guill, Heins & Hood, 2003), but most of them have hitherto been performed on zoological and/or palaeontological objects

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(MacLeod & Forey, 2002). Botanical investigations using TPS (or other geometric morphometry methods) are sparse, despite the fact that many botanical features of taxonomic importance (e.g. leaves, sepals, petals) fit well with typical geometric morphometry conditions. There are many groups of plants that still require more intensive taxonomic treatments, especially those that apply new methods.

One such group is *Dactylorhiza* Necker ex Nevski, an orchid genus that is common and widespread in temperate Eurasia. The level of taxonomic investigation of western European dactylorchids has been relatively high (Nelson, 1976; Pedersen, 1998; Bateman, 2001), but nevertheless many questions remain unresolved. Furthermore, the eastern *Dactylorhiza* species, especially those from European Russia and Siberia, have been little investigated. Many unanswered questions concern allotetraploid species aggregates such as *D. majalis*, *D. traunsteineri* and *D. baltica* (Hedrén, 2002). Another problem is elucidating the relationships within the *D. fuchsii–D. maculata* species complex, which includes both diploids and autotetraploids (e.g. Bateman & Denholm, 2003). Earlier morphometric analyses of these problems have often pointed to the form of the lip (= the labellum: the insect landing stage in the flower) as a particularly valuable source of diagnostic characters (e.g. Heslop-Harrison, 1948, 1954; Bateman & Denholm, 1985; Reinhard, 1990; Tyteca & Gathoye, 2000). In *Dactylorhiza*, unlike some other orchids, this structure is relatively flat (and can be fully flattened without serious damage), contains no holes and is of macroscopic size (usually around 1 cm maximum in diameter). Thus, the dactylorchid lip is potentially an excellent model for investigations using geometric morphometry.

MATERIAL AND METHODS

Putative species analysed from Russia were the diploids *D. incarnata* and *D. fuchsii*, the autotetraploid *D. maculata* and the allotetraploid *D. baltica*. Also included were placeholders for allotetraploids from western Europe: *D. purpurella*, *D. praetermissa*, *D. majalis s.s.*, *D. traunsteineri s.s.*, *D. lapponica*, *D. russowii* and *D. sphagnicola*, together with the diploid *D. euxina*. The more distantly related Russian species *D. flavescens* was also measured.

We used the flattened and dried flowers from 305 plants representing 83 populations of *Dactylorhiza* species and hybrids from European Russia and the British Isles (for sampling details see Shipunov *et al*., 2004). The lips were scanned on a table scanner (HP Scanjet 7400c) into digital bitmap images with a resolution of 200 dpi, and then the computer digitizer program tpsDig (Rohlf, 2003a) was used to plot appropriate landmarks. We tested several landmark sets before choosing a nine-landmark configuration for the whole investigation (Fig. 1). This set of landmarks was based on points commonly used as the basis for multiple linear metric measurements in 'classic' morphometric analyses (e.g. Bateman & Denholm, 1983, 1985; Tyteca & Gathoye, 2000). Most of the landmarks chosen were homologous in a biological sense, though landmarks 2 and 8 had only geometric support. Following the procedure, we obtained a large TPS-file with 18 (9 landmarks × 2 dimensions) landmark coordinates for each specimen.

The next step used two approaches: (1) the first, group-based approach aimed to describe all the data simultaneously, so for each population we used a group average configuration revealed from the consensus configurations in the separate population analyses (Rohlf *et al*., 1996). These average configurations were returned by the tpsRelw program (Rohlf, 2003b) and were based on the generalized orthogonal least-squares Procrustes (GPA) procedures described by Rohlf & Slice (1990). (2) The second approach consisted of specifying reasonable subsets of data that could shed light on selected problems in Russian *Dactylorhiza* taxonomy; in this case, the data matrix based on individual plants was used.

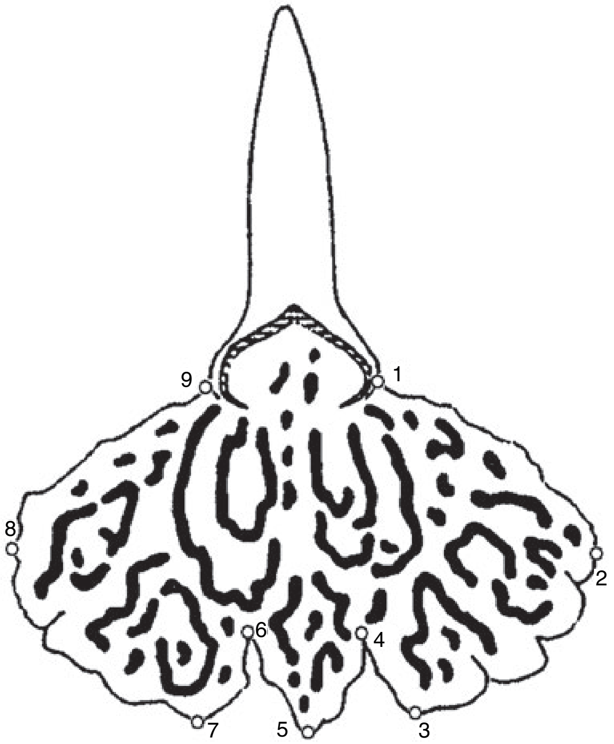

Figure 1. Relative positions on the lip of the nine selected landmark points for geometric morphometric analysis of *Dactylorhiza* species.

The tpsRelw program was then employed to calculate principal warps – the orthogonal vectors that reflect all possible transformations from the consensus configuration to each individual configuration (Fig. 2). The partial warps were calculated as coordinates of projections of specimens on each of the vectors, and the resulting matrix ('weight matrix' of Rohlf *et al.*, 1996) was used for principal components analysis (PCA) and multidimensional scaling (MDS). All 'traditional' statistic calculations used the R program, version 1.71 for Windows (Venables *et al.*, 2002).

In order to obtain taxonomic 'anchors', illustrations of mounted flowers in Reinhard (1990) were digitized and included in the analysis, in which they were denoted by upper-case letters. Sadly, the many hundreds of flowers mounted by Bateman & Denholm (e.g. 1985, 1989) could not be used satisfactorily due to the dissection of the spur away from the labellum during mounting, which marginally excised landmarks 1 and 9.

RESULTS

GROUP-BASED ANALYSIS

PCA analysis of the group-based data matrix revealed three most important principal components that encompassed 75.9% of the total variance. Although the second and third components accounted for similar amounts of variance, the ordination of PC1 and PC3 returned more taxonomic structure (Fig. 3) than did PC1 vs PC2, which served only to separate *D. fuchsii* samples.

In general, clustering occurred between putative species according to a priori taxonomic assumptions, though gaps were not discernible between any putative species. There was also some overlap of taxa in the centre of the graph, where several samples of *D. incarnata*, *D. fuchsii*, *D. baltica* and *D. purpurella* were indistinguishable. At same time, the discriminant analysis of this data matrix fully supported species assignment ($\chi^2 = 1269.3$, $P << 0.05$). Also of interest was the extensive dispersion of *D. incarnata* specimens, which overlapped both with *D. praetermissa* samples and with the *D. russowii*, *D. lapponica* and *D. praetermissa* 'anchors'. Four *D. baltica* populations formed a group located within *D. fuchsii* but far from *D. praetermissa*. In contrast, the *D. euxina* sample was placed close to *D. praetermissa*. The *D. maculata* and *D. fuchsii* populations from the Russian Arctic occupied a position intermediate between the centres of these two species. The hybrids (presumably triploids) between the diploid *D. fuchsii* and the tetraploid

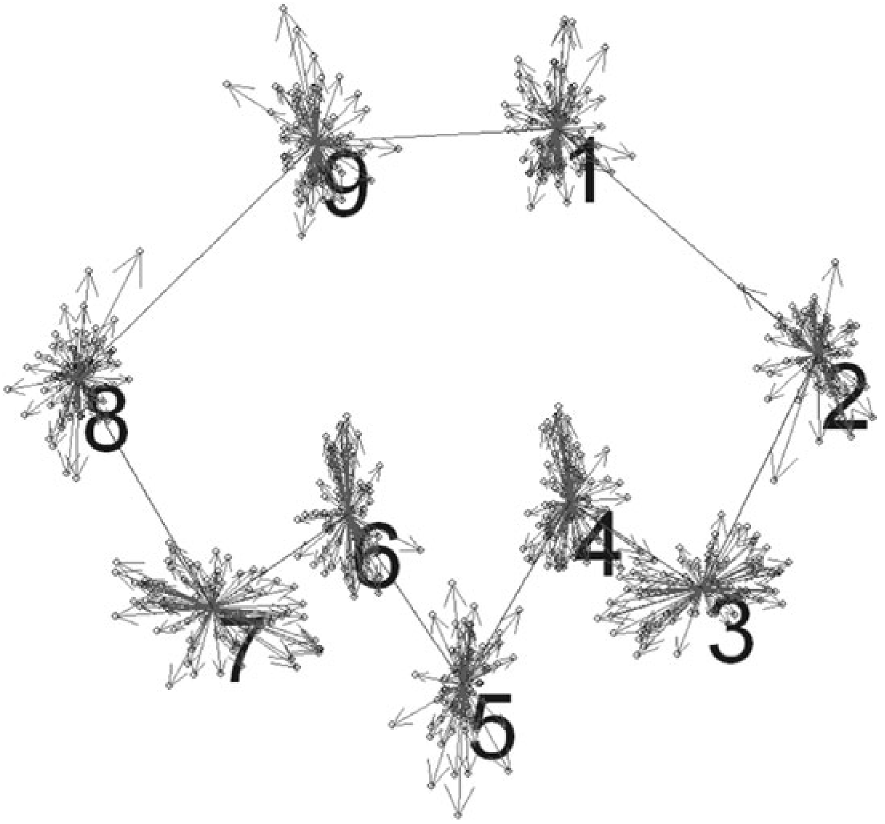

Figure 2. Overall consensus configuration showing all possible transformations given our dataset of *Dactylorhiza* species.

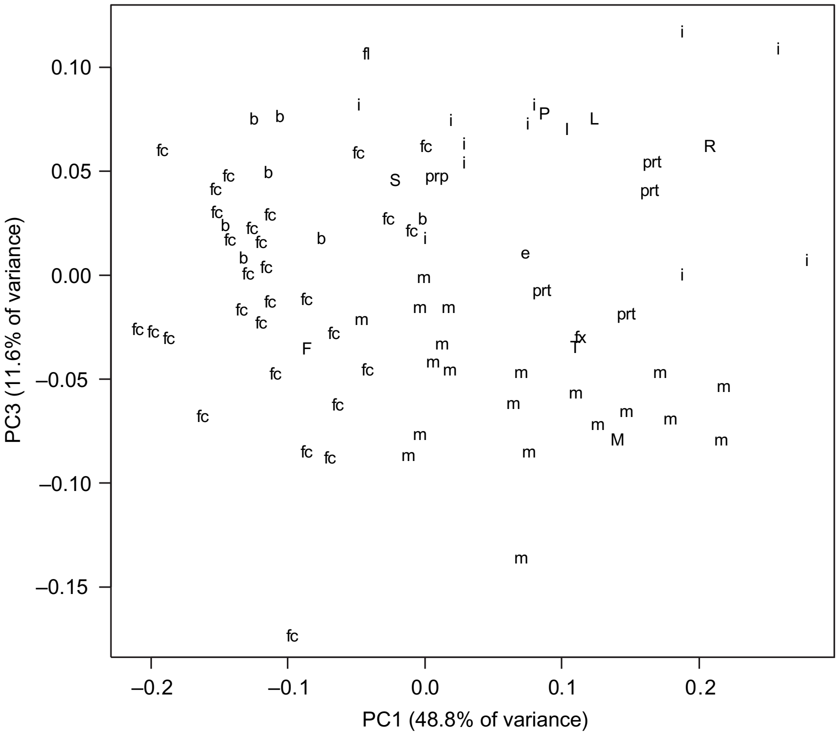

Figure 3. Principal components analysis ordination in *Dactylorhiza* species of all lip shapes generated via relative warps returned from principal warps analysis. b, *D. baltica*; e, *D. euxina*; fc and F, *D. fuchsii*; fl, *D. flavescens*; fx, hybrids *D. fuchsii* × *D. praetermissa*; i and I, *D. incarnata*; L, *D. lapponica*; m and M, *D. maculata*; prp, *D. purpurella*; prt and P, *D. praetermissa*; R, *D. russowii*; S, *D. sphagnicola*; T, *D. traunsteineri*.

D. praetermissa were close to the second parent and to the *D. traunsteineri* 'anchor'. The *D. sphagnicola* 'anchor' was located between the *D. fuchsii*, *D. maculata* and *D. incarnata* groups. Lastly, the single *D. flavescens* sample was peripherally isolated, reflecting its greater phylogenetic disparity (cf. Bateman *et al.*, 2003). Each point on the PCA graph corresponded with specific transformations of base consensus configuration (Fig. 4, outline 3); thus, the differences between species could be represented in terms of the transformations of the coordinate grids (Fig. 4).

The distribution of points revealed from the plot of PC1 and PC3 corresponded well with the variation in some molecular markers, especially ITS alleles (Shipunov *et al.*, 2004) and allowed appreciable grouping on the PCA ordination (Fig. 5). There was also good agreement (Mantel test z-statistics = 75144.31, $P < 0.05$) between distance matrices, based on the scaled geometric and classic morphometric datasets, respectively.

The 3D graph of PCA analysis proved useful for representing the results because the second and third components yielded nearly equal variance and were taxonomically discriminatory. The distinction between species increased, but still showed some overlap (Fig. 6). Each component could be characterized by specific shape changes, corresponding with given a deviation from the zero point for all axes. Positive deviation in PC1 was characterized by changes in the positions of landmarks 1, 9, 4 and 6 towards the centre of the lip (the lip becoming more rectangular), and landmarks 3 and 7 laterally (the lateral lobes becoming more prominent). These transformations corresponded with typical descriptions of *D. fuchsii* labella.

The positive deviation in PC2 reflected narrowing and lengthening of the lip which corresponded well to the typical *D. incarnata* lip shape. PC3 represented decreasing size of the lateral lobes, a feature peculiar to *D. incarnata* and *D. baltica*.

THE 'NORTHERN TETRAPLOIDS'

Dactylorhiza plants from the European Russian Arctic are tetraploids that often have morphologies intermediate between the two spotted-orchids, *D. maculata* and *D. fuchsii*; moreover, molecular markers support the putative hybrid origin of such plants (Shipunov *et al.*, 2004). We therefore analysed a subset of individuals from several populations of *D. incarnata*, *D. fuchsii* and *D. maculata* from Central Russia (Tver′ province) and from northern Karelia (near the Arctic Circle).

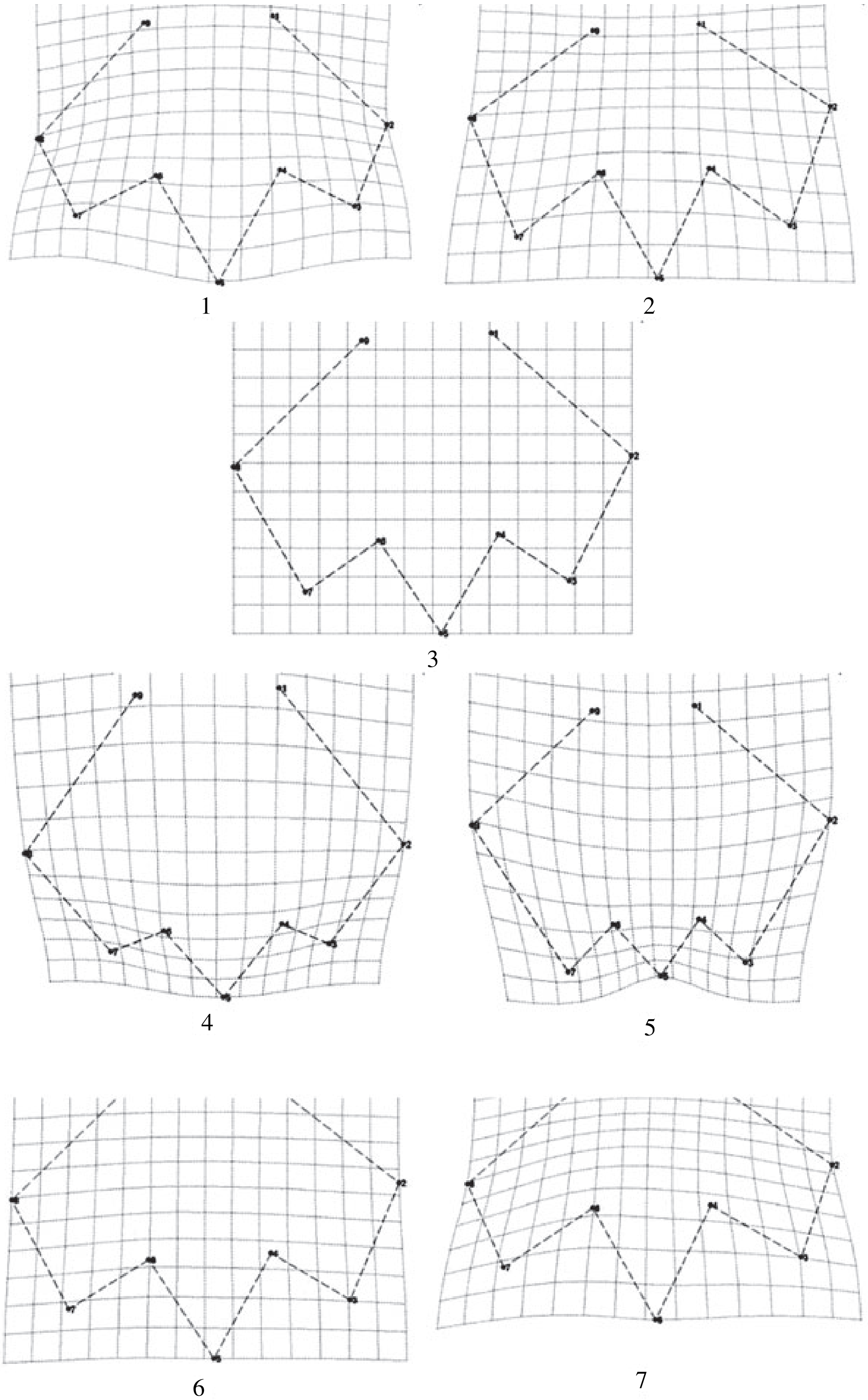

Figure 4. Cartesian transformation grids illustrating the average landmark configuration for the ordinated *Dactylorhiza* species: 1, *D. baltica*; 2, *D. fuchsii*; 3, consensus configuration; 4, *D. incarnata*; 5, *D. maculata*; 6, *D. baltica* from the second subset analysis; 7, *D. fuchsii* from the second subset analysis.

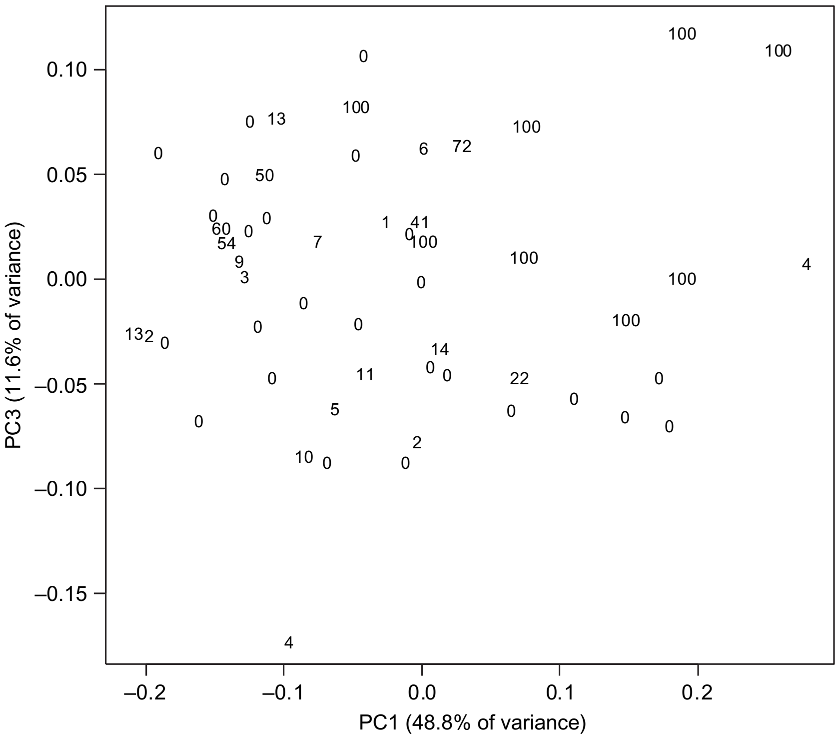

Figure 5. Principal components analysis ordination in *Dactylorhiza* species of all lip shapes, highlighting the percentage of the *D. incarnata* ITS allele observed in each sampled individual.

Both PCA and MDS gave similar results (Fig. 7). Northern plants definitely had an intermediate position between the diploid *D. fuchsii* and tetraploid *D. maculata*, far from the *D. traunsteineri* 'anchor'. Three of nine samples of northern tetraploid spotted-orchids, each from a different population, and the *D. sphagnicola* 'anchor' also fell close to *D. incarnata*. Interestingly, the northern specimens often had a lip outline that was close to the overall consensus configuration for all sampled taxa (Fig. 4).

DACTYLORHIZA BALTICA

Dactylorhiza baltica (= *D. longifolia*) represents the allotetraploid group of *Dactylorhiza*, whose members are believed to have originated from *D. incarnata*-like and *D. fuchsii*-like diploid parents (Heslop-Harrison, 1954, 1968; Hedrén, Fay & Chase, 2001). Most members of this group are apparently stable species, but some (e.g. *D. sphagnicola* and probably *D. baltica*: Hedrén, 2003) have features that suggest very recent origins. Our second subanalysis was therefore more focused, considering *D. baltica*, *D. incarnata* and *D. fuchsii* populations from western European Russia (Pskov and St. Petersburg regions).

The PCA and MDS analyses revealed similar structures (Fig. 8). Most *D. baltica* specimens were located between *D. incarnata* and *D. fuchsii* and far from the *D. praetermissa* 'anchor', whereas the *D. purpurella* sample was closer to *D. baltica*. Most *D. baltica* specimens were located close to *D. incarnata* samples from the same region. Lip transformations are summarized as a 3D graph of the three first components in Figure 9. The *D. baltica* samples formed a distinct group when projected onto the PC1/PC3 plane. The underlying positive deviation of PC3 transformations were lengthening and narrowing of the lip (landmarks 2 and 8 moving inward, and landmark 5 outward), characteristic of *D. incarnata*. The *D. baltica* 'condition' was achieved by shortening and widening the lip (negative deviation in PC3), and at the same time increasing the relative size of the lateral lobes (positive deviation in PC1) without significantly narrowing them (negative deviation in PC2).

DISCUSSION

IMPLICATIONS FOR THE SYSTEMATICS AND EVOLUTION OF *DACTYLORHIZA*

The results from classic morphometrics, geometric morphometrics and molecular markers indicates that all three techniques are in broad accordance and hence could be used jointly to further explore the systematics and evolution of *Dactylorhiza*. Geometric morphometry alone could not reveal significant inter-

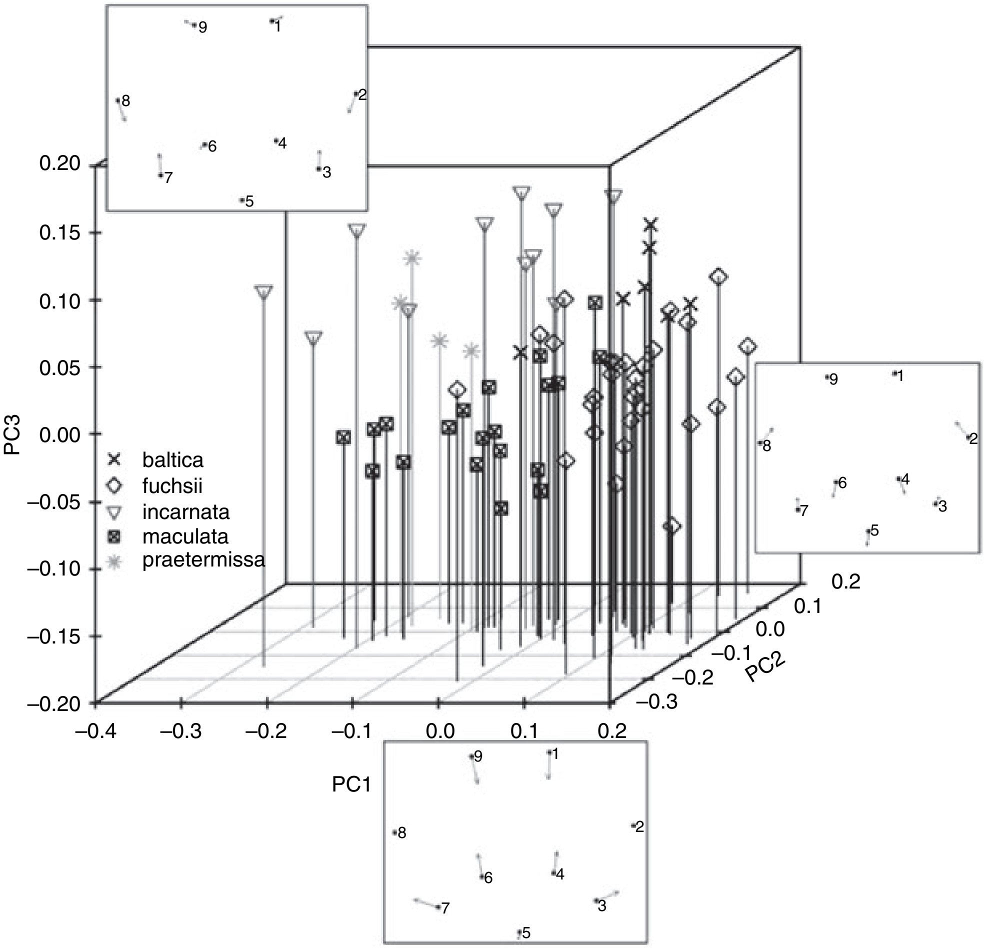

Figure 6. 3D graph of principal components analysis ordination in *Dactylorhiza* species for all lip shapes; the three insets show the directions of shape change for positive deviations of each of the first three components.

species gaps, so this method needs support from other approaches when employed at the infrageneric level. Most of the outlines in the PCA ordination for all populations belonged to northern dactylorchids that are believed to represent those lineages with on average the highest levels of introgression in their populations. The wide dispersion of *D. incarnata* samples could reflect the existing diversity of flower shapes. However, it could also be in part a methodological artefact, specifically a consequence of several difficulties in choosing the location for landmarks 3, 4, 6 and 7 on their lips due to the shallowness (and in some smaller-lipped individuals the complete absence) of the sinuses separating the median and lateral lobes.

The location of *D. flavescens* (taxonomically close to the better-known *D. romana*) supports the taxonomic position of this species, which was phylogenetically distant from all other species included in this study. The *D. euxina* sample was placed among the allotetraploids, suggesting a closer similarity than previously believed. The positions of most anchors corresponded well with known relationships among species (Bateman *et al.*, 2003; Hedrén, 2003; Shipunov *et al.*, 2004; Pillon *et al.,* in press).

'Northern tetraploids' occupied a position intermediate between typical *D. maculata* and *D. fuchsii*, offering additional support to the inferred hybrid origin of these plants. Some also resembled *D. incarnata*, perhaps showing evidence of further introgression, or perhaps indicating that in these cases *D. incarnata* was, atypically for allotetraploid dactylorchids, the maternal parent of the hybrid. There is growing evidence that in orchids the maternal parent reliably contributes considerably more than does the paternal parent to the morphology of the progeny (Bateman & Hollingsworth, 2004).

The close relationship of *D. praetermissa* and *D. baltica* advocated by some observers (e.g. Delforge, 1995) received no support from our analysis. The samples from four populations of *D. praetermissa* were located between *D. incarnata* and *D. maculata*, so the lip morphology of *D. praetermissa* (typically rather

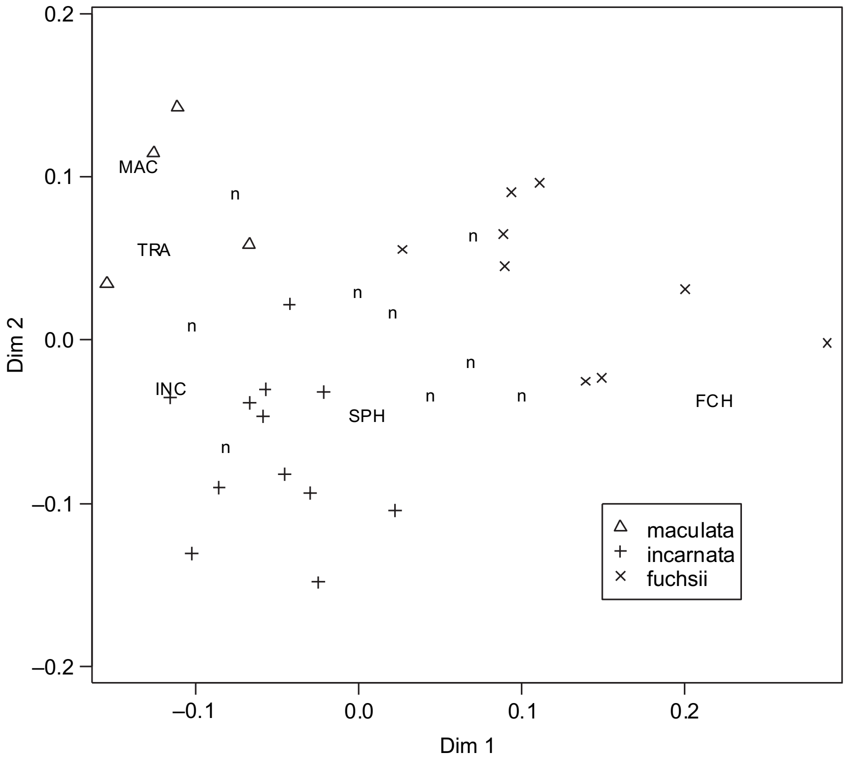

Figure 7. Multidimensional scaling ordination in *Dactylorhiza* species for lip shapes from the subset of northern plants. FCH, *D. fuchsii*; INC, *D. incarnata*; MAC, *D. maculata*; n, 'northern tetraploids'; SPH, *D. sphagnicola*; TRA, *D. traunsteineri*.

rounded and only shallowly three-lobed) was closer to that of *D. maculata* than to that of *D. fuchsii*. This interpretation also receives support from some molecular markers; for example, some *D. praetermissa* plants bear the *D. maculata* ITS allele (Pillon *et al.,* in press). The similarities observed between *D. purpurella* and *D. baltica* labella accord with the opinions of Soó (1980) and Averyanov (1990) that there is a relatively close relationship between these two species. Local resemblances between *D. baltica* and *D. incarnata* could support the hypothesis of a recent and local origin of *D. baltica*, but they could also be evidence of continuing introgression from *D. incarnata*. The separation of *D. baltica* from its supposed parental species by PC1 and PC3 reflects the specific features of its lip, which is relatively short and wide, but has only shallow notches separating the middle and lateral lobes (Fig. 4).

The ellipses of variation surrounding the mean landmark positions (Fig. 2) demonstrate that variation in dactylorchid lip shape is not merely allometric. As overall lip size increases, lip width increases on average proportionately more than does lip length (Bateman & Denholm, 1983: fig. 1), yet the majority of the ellipses of variation evident in Figure 2 were elongated longitudinally. In contrast, landmarks 3 and 7, located at the apices of the lateral lobes, showed transversely elongated ellipses, suggesting that transformations between lip shapes are more complex than simple uniform expansion analogous to that evident within a single dactylorchid inflorescence during its ontogeny (cf. Alberch *et al*., 1979).

BROADER IMPLICATIONS OF GEOMETRIC MORPHOMETRIC TECHNIQUES

The transformations among populations represented by distant points on the PCA axes show the main directions of change from one species-specific form to another. Thus, the transition between different shapes could be described readily in terms of landmark shifts, as has been done above for *D. baltica*. These shifts both visualize and summarize taxonomically valuable features, especially in the cases of suspected hybridization. There exists a wide range of possible applications of geometric morphometry, from relatively simple two-dimensional organs such as undivided leaves to complex anatomical features such as transverse sections of petioles (Volkova, Jufrjakov & Shipunov, in press).

The labella configurations for different species revealed by the PCA ordinations coincide well with tra-

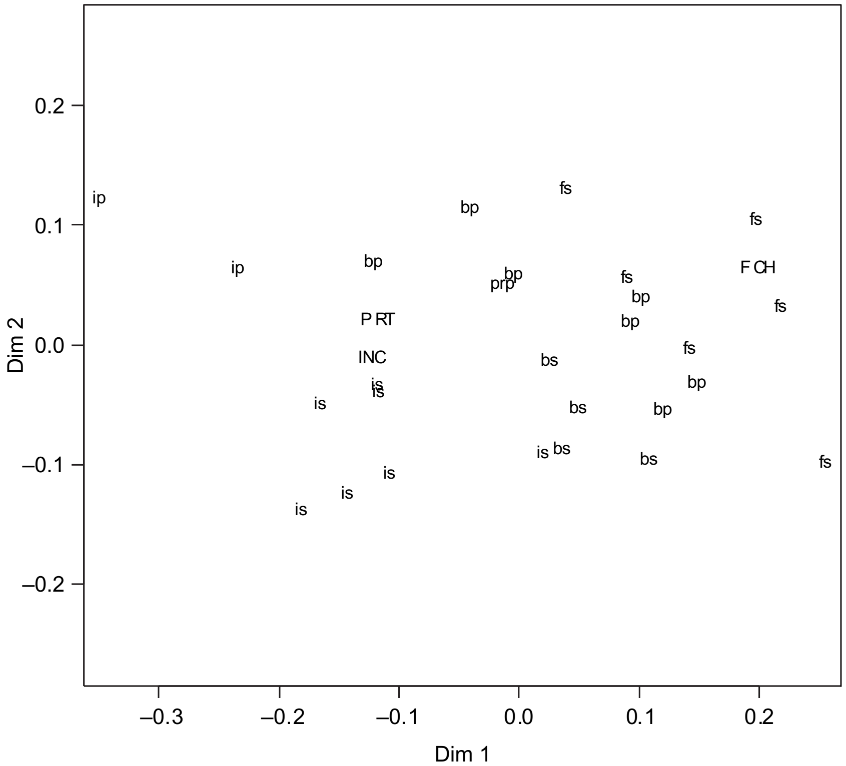

Figure 8. Multidimensional scaling ordination in *Dactylorhiza* species for lip shapes from the subset of *D. baltica* and its potential parents. bs, *D. baltica* from Saint Petersburg region; bp, *D. baltica* from Pskov region; fs, *D. fuchsii* from Saint Petersburg region; is, *D. incarnata* from Saint Petersburg region; ip, *D. incarnata* from Pskov region; prp, *D. purpurella*; FCH, *D. fuchsii* 'anchor'; INC, *D. incarnata* 'anchor'; PRT, *D. praetermissa* 'anchor'.

ditional descriptions of flower forms used in diagnostic keys and morphometric works (e.g. Stace, 1997). Such ordinations can be viewed as a 'morphological space' wherein many different flower shapes could, in theory, be realized. The most densely occupied regions of this graph might be the forms that are most highly selected (most probably via pollinator preference), whereas empty regions might represent either forms eliminated by selection or morphologically impossible forms precluded by developmental constraints.

Both of these possibilities merit further discussion and laboratory-based experimental research. There is strong evidence that many aspects of floral morphology in orchids are under strong selection pressure and/or prone to drift, and so show relatively high levels of homoplasy in phylogenetic analyses (Chase, 1999; Bateman *et al*., 2003; Tremblay *et al*., 2005). The species of *Dactylorhiza* discussed here each attract a wide range of pollinating insects by deception through mimicry of other (mostly non-orchidaceous) species of flowering plants, so there is little doubt that the combination of spur size and lip size, shape and markings is crucial to reproductive success in the group. This raises the likelihood of frequent convergences upon similar optimal labellum designs of different lineages within *Dactylorhiza*. The recent development of a strong and detailed molecular phylogenetic framework for both the diploids and the tetraploids (Bateman *et al*., 2003; Pillon *et al*., in press) allows the identification of sister species that could provide a valuable model system for exploring, via morphometric techniques, the growth patterns that permit such convergence. This work could then be followed by evolutionary–developmental genetic studies (e.g. Cronk, Bateman & Hawkins, 2002) to elucidate the underlying genetic processes.

Equally, these flowers could also constitute a useful model system for defining the nature and causes of developmental constraints. Continuing our theme of using landmark data to explore labellum shape, it would be interesting to use the labella as a rare example of a botanical study of fluctuating asymmetry (cf. Rudall *et al*., 2002). If landmarks could be established with sufficient precision (say, within a confidence interval of 0.5%), the strongly bilaterally symmetrical orchid labellum could easily be divided vertically into two portions to explore the relative degree of somatic expression of identical genomes in the aggregates of cells on either side of the mirror plane separating the right and left halves.

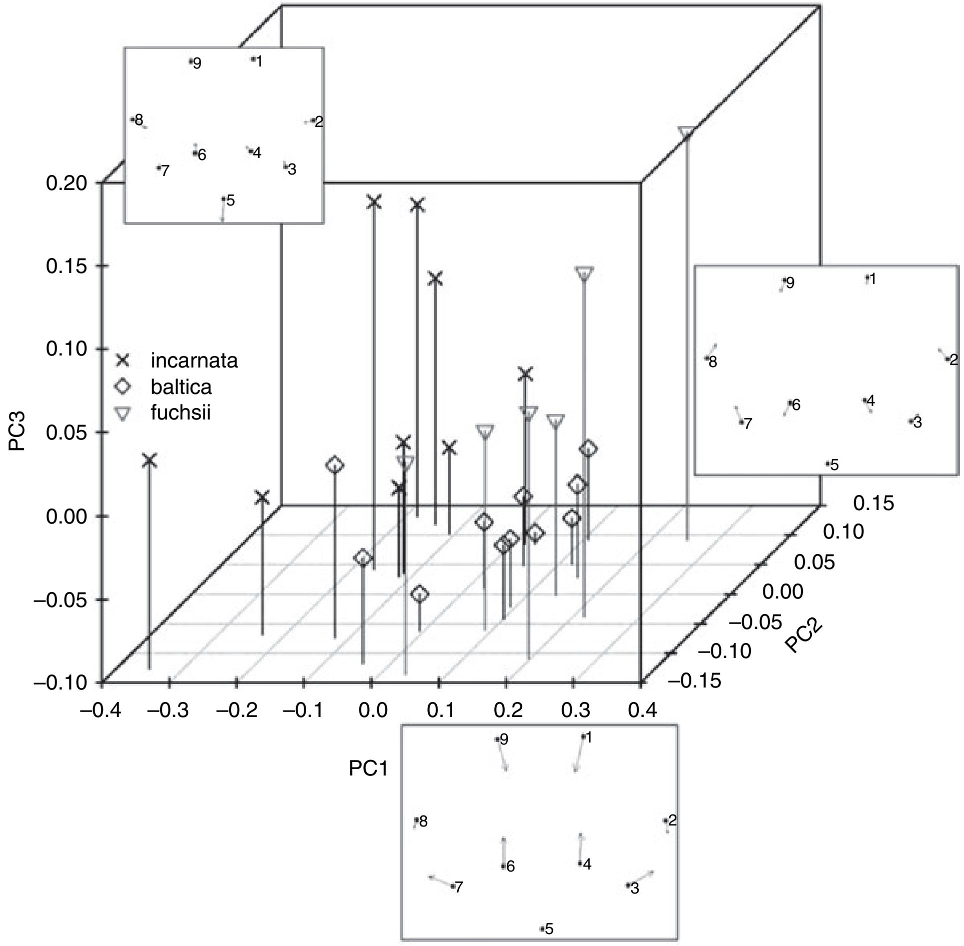

Figure 9. 3D graph of principal components analysis ordination in *Dactylorhiza* species for lip shapes from the subset including *D. baltica*; the three insets show the directions of shape change for positive deviations of each of the first three components.

It should be possible to develop additional landmark-related techniques for monitoring the ontogeny of the discrete anthocyanin markings of the labellum, and thereby relating precise attractant patterns to degree of pollinator success in these orchids. It should also prove feasible to explore the ontogeny of these markings, which in *D. fuchsii* and *D. maculata* have been said to develop outward from the vicinity of the spur entrance in parallel with development of the venation in the labellum, analogous to the ontogeny of butterfly wings (Heslop-Harrison, 1948, 1951). Although determining homologous landmark points for these complex markings would undoubtedly be challenging, the insights gained into floral evolution could justify the required effort.

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