Evolutionary and Morphometric Implications of Morphological Variation Among Flowers Within an Inflorescence: A Case-Study Using European Orchids

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● *Background and Aims* This study explores the previously largely ignored morphological variation that occurs among flowers within a single inflorescence.
● *Methods* Variation in four metric parameters (labellum length and width, spur length and width) that together strongly influence pollination frequency is documented within the simple racemose inflorescences of eight individuals that represent a primary hybrid and six species of European orchids.
● *Key Results* Regression of each parameter against the location of each flower on the inflorescence, and calculation of correlation coefficients for each pair of parameters within each inflorescence, demonstrate significant decoupling of labellum and spur development, despite the fact that they are different portions of the same floral organ. Spur length and diameter are constant across inflorescences of *Dactylorhiza* other than the vestigial-spurred *D. viridis*, whereas in other genera spur length declines in parallel with labellum dimensions. These differences are likely to reflect selection pressures or developmental constraints. Strong negative deviations from the regression line for one or more parameters are evident in occasional flowers, occurring most frequently in the lowermost and uppermost one or two flowers, and so reflecting transitions in meristematic behaviour. Thus, population-level morphometric studies are best conducted on flowers taken from approximately the mid-point of the inflorescence. Moreover, in the two relatively large inflorescences where lower flowers were removed for measurement before the upper flowers had opened, labellum size increased significantly in the flowers immediately above the excisions, suggesting that excision liberated resources that were diverted into the opening buds. Repeat measurement of all flowers from one selected inflorescence demonstrated typical measurement errors of only ± 30–80 µm, irrespective of the size of the structure studied. If flowers are not mounted and measured immediately following excision, modest negative deviations of 30–50 µm result from post-mounting shrinkage; this occurs less rapidly in the spur than in the thinner labellum, which should therefore be measured first. Variation in all four parameters among all the flowers of a single inflorescence is between 42 % and 107 % of that observed between a similar number of flowers sampled from a consistent location on different (but conspecific and coexisting) inflorescences.
● *Conclusions* This result demonstrates the strong influence of epigenesis on flower morphology and further emphasizes the importance of (*a*) sampling from a consistent location within the inflorescences under comparison, (*b*) interpreting morphometric ordinations hierarchically, building from individuals to infraspecific taxa and species via populations, and (*c*) considering in any microevolutionary study the potentially profound effects of the cline in flower size within each inflorescence.

Key words: Correlation, development, epigenesis, evolution, inflorescence, labellum, measuring error, morphometrics, multivariate ordination, ontogeny, Orchidinae, orchids, regression, spur.

INTRODUCTION

Morphometric techniques have long been established as valuable tools for exploring the development, population differentiation and systematics of plants (e.g. Bookstein *et al.*, 1985; Wiens, 2000; Forey and MacLeod, 2002; Jensen, 2003). Within the systematics community, such approaches have been especially frequently deployed by students of the European orchid flora (reviewed by Pedersen, 1998; Bateman, 2001). Their popularity reflects both the intensity of research conducted on these diverse and charismatic plants and the unusually large proportion of orchid groups that challenge our ability to detect optimal boundaries between species and infraspecific taxa (e.g. Bateman, 2001).

These morphometric studies have typically employed between 20 and 50 quantified characters, generally consisting of a heterogeneous mixture of metric (continuous),

meristic, scalar and presence/absence characters. Indeed, these studies contrast less in the nature of the characters recorded than in the sampling strategy adopted in the field and the method of analysis applied to the resulting data matrices. Bateman (2001) argued that there exist two fundamental dichotomies in analytical procedures. The first dichotomy separates those studies that subject the morphometric data to multivariate ordination techniques in search of the character correlations that imply the presence of interspecific boundaries (e.g. Bateman and Denholm, 1983, 1989*a*; Tyteca and Gathoye, 1989, 2000; Pedersen, 1998; Shipunov and Bateman, 2005) from those that rely simply on univariate (character by character) comparisons (e.g. Heslop-Harrison, 1951, 1954; Roberts, 1966, 1988; Gölz and Reinhard, 1980, 1986; Reinhard, 1985, 1990). The second dichotomy distinguishes those studies that analyse data hierarchically from individuals through populations to nested sets of infraspecific taxa to species (e.g. Bateman and Denholm, 1983, 1989*a*;

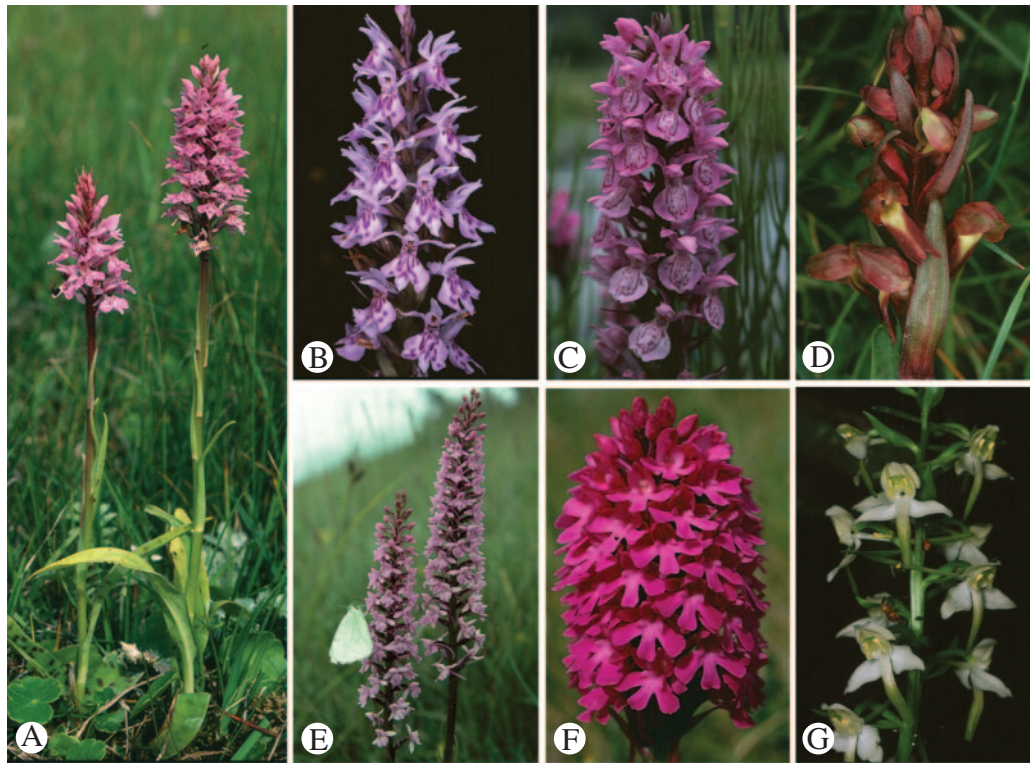* For correspondence. E-mail r.bateman@rbgkew.org

FIG. 1. Representative inflorescences of the orchid species analysed. (A) *Dactylorhiza fuchsii* (entire plant); (B) *D. fuchsii*; (C) *D. praetermissa*; (D) *D. viridis*; (E) *Gymnadenia conopsea*; (F) *Anacamptis pyramidalis*; (G) *Platanthera chlorantha*. Images by R. M. Bateman and D. M. T. Ettlinger.

Bateman, 2001) from those that do not (the vast majority of studies).

Nonetheless, the systematic botany literature contains several (sometimes heated) debates regarding how best to measure morphometric characters, and how best to mount and voucher the specimens involved (e.g. Bateman and Denholm, 1989*b* versus Roberts, 1989). In the case of conservation-sensitive species such as orchids there is a premium on causing the minimum realistic disturbance to wild-sampled plants. Thus, many studies have recorded vegetative characters in the field (some studies sampling a single leaf) and floral characters in the laboratory from a single excised flower per plant, typically mounted on a bonding surface such as double-sided adhesive tape mounted on filing cards (cf. Heslop-Harrison, 1954; Roberts, 1966; Bateman and Denholm, 1983; Gölz and Reinhard, 1986). Even within this shared protocol there is divergence between research groups in the degree to which each flower is dissected, notably whether the labellum assembly is mounted in its entirety or whether instead the spur is first excised from the remainder of the labellum.

The labellum is the relatively morphologically complex median inner perianth segment (petal) that in most orchids is the primary attractant and landing stage for pollinators, most commonly insects (Darwin, 1862; van der Pijl and Dodson, 1966; Dafni, 1992; Nilsson, 1992; van der Cingel, 1995; Rudall and Bateman, 2002). In many orchids (and many other flowering plants) the dorsiventrally flattened bulk of the labellum that acts as both visual attractant and landing stage is extended both proximally and abaxially into a conical or cylindrical structure termed the spur, which has evolved to provide (or, in many cases, to falsely appear to provide) nectar (Nieland and Wilcock, 1998; Cozzolino and Widmer, 2005). Consequently, spurs have attracted considerable interest for studies of developmental genetics (e.g. Golz *et al.*, 2002) and of microevolution, as epitomized by the genus *Platanthera* (e.g. Nilsson, 1992; Maad, 2000; Stpiczynska, 2003; Maad and Alexandersson, 2004). Not surprisingly, labellar structures are, along with the fused style and stamens of the gynostemium that is diagnostic of orchids, generally assumed to be under especially strong selection pressure (see extensive bibliography in Tremblay *et al.*, 2005).

This evolutionary interest renders the measurement of labellar structures especially critical (to evolutionary as well as systematic interpretation) but also especially problematical, as the three-dimensional spur extends more-or-less perpendicularly to the distal portion of the labellum, which in most species is more two-dimensional (Fig. 1). Once removed from the flower, the entire labellum assembly can either be mounted as a single unit (e.g. Roberts, 1966, 1989; Reinhard, 1985; Gölz and Reinhard, 1986), in which case some distortion is introduced as a result of flattening a fundamentally three-dimensional organ, or the spur and labellum can each be excised from the gynostemium and mounted separately (Heslop-Harrison, 1951, 1954; Bateman and Denholm, 1983, 1989*a*) (Fig. 2), in which case there is inevitably some inconsistency among flowers in the precise location of the requisite cuts.
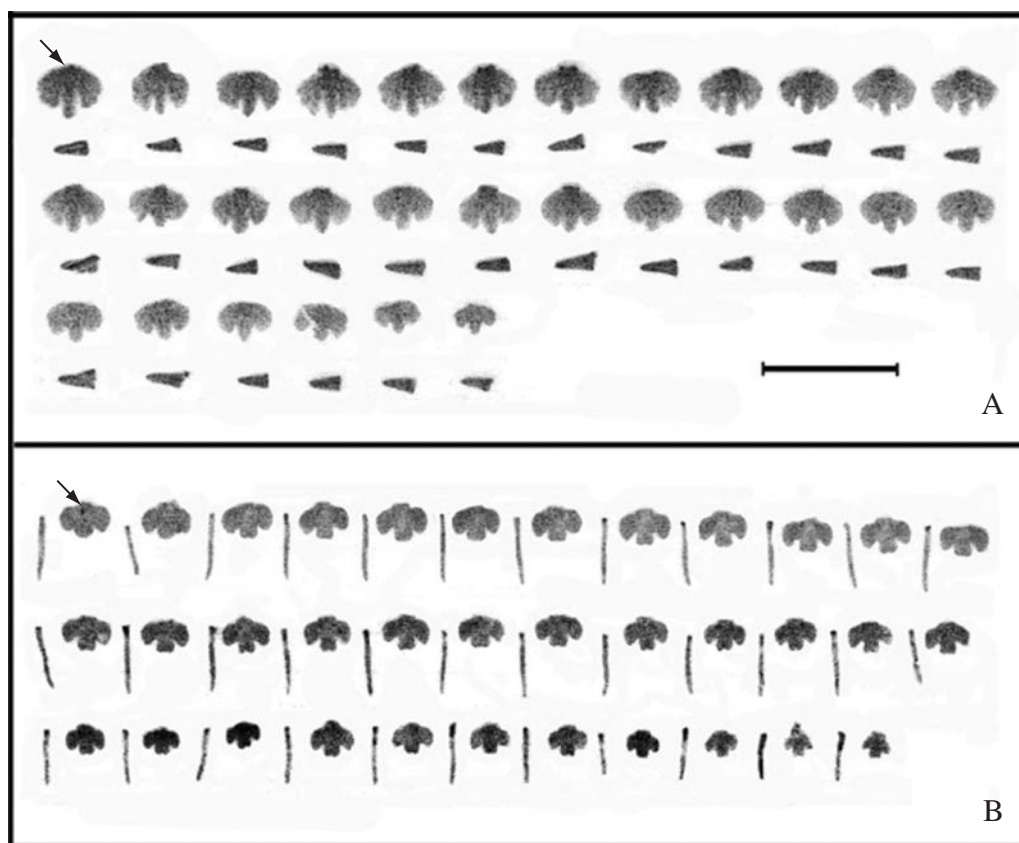
FIG. 2. Silhouette representations of the labellum and spur mounts of single inflorescences of *Dactylorhiza fuchsii* (A) and *Anacamptis pyramidalis* (B). The basalmost flower is in the top left, the apicalmost flower is in the bottom right; arrows indicate the 'shoulder' reference point in the lowermost flower of each inflorescence. Scale bar = 2 cm.

The early ontogeny of orchid flowers has been the subject of several valuable studies (e.g. Kurzweil, 1987, 1998, 2000) but, as far as is known, the patterns and relative rates of development of the spur and the remainder of the labellum have not been subject to detailed study in any species (cf. Arditti, 1992; R. M. Bateman, P. J. Rudall and B. Glover, unpubl. res.). Moreover, remarkably little attention has been paid to morphological variation among flowers within single inflorescences, either in orchids or in most other groups of flowering plants (cf. Salisbury, 1926; Remizowa *et al.*, 2005).

This paper therefore explores variation among flowers within simple, racemose inflorescences of several European native species of orchids with spur-bearing labella in order (*a*) to better understand the constraints and potential evolutionary significance of clinal variation in floral morphology within and among inflorescences, and (*b*) to assess the accuracy and reproducibility of the preferred morphometric protocol used to identify the morphological discontinuities that separate bona fide species.

MATERIALS AND METHODS

Choice of study groups

For convenience of sampling, and in order to maintain a degree of genetic (and thus hopefully developmental) cohesion, this study of clinal variation within orchid inflorescences was confined to the dominant European subtribe, Orchidinae (Pridgeon *et al.*, 2001). Recent molecular phylogenetic studies have elucidated species relationships and allowed monophyletic reclassification within the subtribe (Bateman *et al.*, 2003). Species boundaries in many groups have been thoroughly explored using morphometric (Bateman and Denholm, 1983, 1989*a*) and, increasingly, population genetic (e.g. Hedrén *et al.*, 2001; Shipunov and Bateman, 2005; Pillon *et al.*, 2006) methods, and the interaction of their floral morphology with pollinators has long been subject to detailed exploration (e.g. Darwin, 1862; van der Pijl and Dodson, 1966; Nilsson, 1992; van der Cingel, 1995; Cozzolino and Widmer, 2005; Tremblay *et al.*, 2005).

Moreover, contrasts in pollination mechanisms that are largely dictated by morphology occur among closely related species of Orchidinae. For example, most species of *Dactylorhiza* attract pollinators by deceit rather than by reward, despite possessing well-developed labellar spurs, but the near-basal diploid *D. viridis* (L.) R.M. Bateman, Pridgeon & M.W. Chase (formerly *Coeloglossum viride*) has retained only the last vestiges of its ancestral spur. The molecularly similar sister-genus, *Gymnadenia*, also maintains some species with well-developed spurs (subgenus *Gymnadenia*) and others with only vestigial spurs (subgenus, formerly genus, *Nigritella*), but all these species

TABLE 1. *Identity, sources and density of inflorescences used in this study*

Species	Source (all UK, unless otherwise stated)	Nectar reward	Density (flowers cm^{-1} ± s.d.)*
Dactylorhiza praetermissa (Druce) Soó	Rockery, RRG Kew	No	3·36 ± 1·34 (n = 50)[a]
D. cf. *foliosa* (Solander) Soó *praetermissa*	Garden of authors	No	Approx. as above
D. fuchsii (Druce) Soó	Aston Clinton, Bucks.	No	6·22 ± 2·32 (n = 90)[b]
D. (*Coeloglossum*) *viridis* (L.) Bateman *et al.*	Passo Sella, NE Italy	(Yes)	5·97 ± 2·10 (n = 10)[c]
Gymnadenia conopsea s.s. (L.) R.Br. (small)	Aston Clinton, Bucks.	Yes	5·40 ± 2·30 (n = 70)[d]
Gymnadenia conopsea s.s. (medium)	Aston Clinton, Bucks.	Yes	As above
Anacamptis pyramidalis (L.) Rich.	Aston Clinton, Bucks.	Yes	9·82 ± 3·68 (n = 20)[e]
Platanthera chlorantha (Custer) Rchb.	Aston Clinton, Bucks.	Yes	1·53 ± 0·36 (n = 39)[f]

* Sources of data: [a] Bateman and Denholm (1983), [b] Bateman and Denholm (1989*a*, plus unpubl. res.), [c] Bateman (unpubl. res.), [d] Bateman and Denholm (unpubl. res.), [e] R. H. Roberts and R. M. Bateman (unpubl. res.), [f] R. M. Bateman, P. J. Rudall and K. E. James (unpubl. res.).

offer at least some nectar reward to prospective pollinators (Rudall and Bateman, 2002; Bateman *et al.*, 2003; Cozzolino and Widmer, 2005). Such pollinator divergence helps significant numbers of species in subtribe Orchidinae to coexist, particularly in open habitats on calcareous soils (e.g. Summerhayes, 1968).

Unsurprisingly, the considerable variation in pollination syndromes is reflected not only in the dimensions of the labellar spur but more generally in substantial variation in flower size, shape and colour (Fig. 1). Nonetheless, all of the approximately 600 species in the subtribe generate flowers in spikes. These inflorescences are simple unbranched racemes generated by a single indeterminate apical meristem. In contrast with compound determinate cymes, racemes offer a relatively straightforward interpretational model (cf. Rudall and Bateman, 2003). Moreover, all of the spikes open clinally over a period of a few days or weeks and, with the exception of most populations of *Orchis simia* Lam. and its sister-species, *O. galilaea* (Bornmueller & Schulze) Schlechter, which open from the apex downward and unusually rapidly, all open in a wave that passes from the base to the apex of the inflorescence (Summerhayes, 1968). Also, all share a similar floral bauplan, including a single fertile anther that in most cases generates two hemi-pollinaria (Pridgeon *et al.*, 2001; Rudall and Bateman, 2002), though in *Anacamptis pyramidalis* the two hemi-pollinaria have become secondarily fused via a shared viscidium (e.g. Darwin, 1862; Summerhayes, 1968). In addition, the majority of the study species had already been subject to detailed morphometric systematic studies in the UK by the senior author, consistently using broadly similar suites of 40–52 characters (Bateman and Denholm, 1983, 1989*a*, unpubl. res.; R. M. Bateman, P. J. Rudall and K. E. James, unpubl. res.).

Materials

Sampling was conducted in June–July 2005 (Table 1). Two of the inflorescences selected for detailed study were already held in cultivation. A medium-sized spike of *Dactylorhiza praetermissa* was selected from a large and apparently genetically uniform and somewhat invasive population maintained in the rockery at the Royal Botanic Gardens Kew. A larger and more vigorous but otherwise rather similar *Dactylorhiza* inflorescence was sampled

in situ in the authors' garden, in order to gather data from a large spike that contained too many flowers to be measured on a single occasion (the lower flowers reliably expire before the upper flowers have opened). This particular enigmatic, but widely grown, cultivar had recently been included in a detailed genetic study of the genus (Pillon *et al.*, 2006) that showed it to possess an extraordinary combination of plastid haplotype and ITS alleles consistent with (though not conclusively demonstrating) hybrid origin as *D. praetermissa × foliosa*. This origin is also indicated by having a narrower spur and greater width : length ratio of the labellum than that of *D. praetermissa*, both of which are characteristic features of *D. foliosa*.

Five of the remaining six spikes analysed were wild-collected from a disused chalk quarry near Aston Clinton, Buckinghamshire, UK. Single medium-sized spikes were taken from large populations of four species of Orchidinae (*Dactylorhiza fuchsii*, *Gymnadenia conopsea*, *Platanthera chlorantha*, *Anacamptis pyramidalis*). In addition, a second, smaller inflorescence of *G. conopsea* was sampled in order to explore the effects on flower morphology of inflorescence size, though comparisons were not made among individuals of the same clone. All five spikes were sampled at peak flowering but, because it encompassed a relatively large number of flowers, many of the more apical buds in the spike of *A. pyramidalis* were not yet open. Consequently, the spike was maintained in water for several days, so that these further tranches of flowers could be sampled periodically as they opened, mirroring the treatment given to the garden specimen of *D. praetermissa × foliosa*. In each case, the flowers were cropped from base to apex in four successive tranches over periods of 1 or 2 d in the case of *Anacamptis* and an average of 4 d in the case of the hybrid *Dactylorhiza*. The remaining spike was sampled in the Italian Dolomites in July 2005 (Table 1). The species selected, *Dactylorhiza* (formerly *Coeloglossum*) *viridis*, was chosen because it possessed only vestigial spurs, yet was closely related to other species of *Dactylorhiza* analysed that possessed fully developed spurs (Bateman *et al.*, 2003).

Thus, altogether, the study sampled nine spikes of four genera and eight taxa: of these, three offered a nectar reward to pollinators and five did not (Table 1).

Methods

Each flower was excised from the inflorescence before the spur and that portion of the labellum distal to the spur entrance were removed by means of single scissor cuts (cf. Figs 1B and 2A). Both portions of the labellum were then mounted on double-sided adhesive tape attached to 15 cm × 10 cm filing cards, beginning in the top left-hand corner of the card with the lowermost flower and then working systematically along the phyllotactic spiral from base to apex; thus, all flowers of each inflorescence were measured. Completed mounts for *D. fuchsii* and *A. pyramidalis* are shown in Fig. 2. These vouchers are currently held in the authors' private collection, though they will eventually be deposited in the orchid herbarium at the Royal Botanic Gardens Kew.

Because of the large number of flowers scheduled for measurement, only four metric parameters were then taken from each flower mounted: the median length and maximum width of the labellum, the length of the spur, and the width of the spur when measured halfway along its length following compression. The alternative metric, spur width at its entrance, is prone to additional errors caused by inevitable variations in the precise location of the cut. This error can also affect measurement of labellum length, but this risk was largely negated by treating the 'shoulder' of the labellum (i.e. the point of maximum concavity of the margin; arrowed on Fig. 2) as its *de facto* proximal margins. In the case of *P. chlorantha*, labellum length was recorded midway along its length, to avoid the complication presented by small but variably sized lateral projections located at the base of the labellum on either side of the spur entrance. Also, measurement of spur width was made more difficult by the presence of a thickened longitudinal ridge along the lower surface of the distal half of the spur. In the case of *D. viridis*, two lateral projections of the labellum extended beyond both its length (as measured via the central lobe) and its width. Thus, length was measured to the apex of the central lobe, as in the remaining species, and width was measured halfway along the length, proximal to the level where the labellum becomes trilobate.

Almost all measurements were taken using a Leitz Wetzlar ×6 ocular magnifier, graduated in intervals of 0·1 mm over a total distance of 20 mm. Most measurements were recorded at a resolution of 0·1 mm, though this was increased to 0·05 mm (the maximum feasible resolution using the magnifier) for the widths of the especially narrow spurs of *Gymnadenia* and *Anacamptis*. Also, as the spurs of *Platanthera* exceeded 20 mm, their length was recorded using a 15-cm steel rule graduated at 0·5-mm intervals, which provided an appropriate level of resolution for describing such a large structure.

Most inflorescence sets were measured only once. However, in order to quantify errors due to mis-measurement and specimen desiccation, the mount for *D. fuchsii* was remeasured blindly, immediately after the initial set of measurements had been completed (incurring a delay of 90 min at an unusually high room temperature of approx. 27 °C) and again 5 d later, after the mount had slowly desiccated through exposure to the atmosphere.

The resulting data were input into Excel X.1 for Macintosh, and subjected to a series of straightforward statistical explorations. Mean, sample standard deviation and the resulting coefficient of variation were calculated for each variable in each inflorescence, together with correlation coefficients among each pair of variables within each inflorescence. All four variables were then subjected to regression against the relative positions of the flowers along each inflorescence.

Comparable data for the four metric measurements were also taken, using the above techniques, from ten individuals each of several naturally occurring populations of *Dactylorhiza praetermissa* (six populations), *D. fuchsii* (nine), *Gymnadenia conopsea* (seven), *Platanthera chlorantha* (six) and *D. viridis* (one); sadly, no comparative data were obtained for *Anacamptis pyramidalis*. Wherever possible, the single flower removed from each inflorescence for mounting was taken from a more-or-less consistent location, 30–50 % of the distance from the base of the inflorescence to its apex.

RESULTS

Means, sample standard deviations and coefficients of variation for each inflorescence (including the two sets of repeat measurements for *D. fuchsii*) are presented in Table 2. Coefficients of variation typically occur within the range 6–12 %. Correlation coefficients among the paired variables (almost all positive, and ranging from +0·96 to −0·30) are summarized in Table 3. Regressions for each inflorescence are shown in Figs 3–10, together with appropriate statistics. The linear regression algorithm fitted the data sufficiently well to discourage curve-fitting using algorithms that are more complex and so less readily interpreted. The regressions also constitute the source of the figures for percentage change between adjacent flowers that are summarized in Table 2.

Dactylorhiza praetermissa

The relative magnitudes of the four variables observed in this allotetraploid species are typical of the genus *Dactylorhiza*, with the width of the labellum marginally exceeding its length; the labellum is in turn a little longer than the spur, which is twice as long as wide. The regressions reveal modest parallel declines in labellum width and length from the base to the apex of the inflorescence (Fig. 3). In contrast, spur width is constant throughout the inflorescence and, extraordinarily, spur length shows a modest *increase* from base to apex (Table 2).

Coefficients of variation approximate 7 %, though spur length is a little more variable. Anomalously low values at the extremes of the inflorescence are evident in the basalmost flower 1 (labellum dimensions only) and the apicalmost flowers 34 and 35 (labellum dimensions only in 34, both labellum and spur in 35). Within the main body of the inflorescence, small (especially short) labella occur in

TABLE 2. *Statistical analysis of variation in four metric parameters within individual inflorescences (millimetres)*

Inflorescence/Parameter	Mean	s.d.	Coefficient of variation (%)	% decrease between flowers
Dactylorhiza praetermissa (n = 35)				
Labellum length	8·077	0·587	7·27	−0·40
Labellum width	8·989	0·606	6·74	−0·44
Spur length	5·917	0·551	9·31	+0·38
Spur width	3·140	0·217	6·91	+0·03
Dactylorhiza cf. *foliosa* × *praetermissa* (n = 61)				
Labellum length	8·148	0·685	8·41	−0·35
Labellum width	9·649	1·165	12·07	−0·51
Spur length	6·015	0·534	8·88	0
Spur width	2·047	0·212	10·36	−0·19
Dactylorhiza fuchsii (n = 30)				
Labellum length	6·330	0·627	9·91	−0·82
Labellum length (2)	(6·313)	(0·620)	(9·82)	
Labellum length [3]	[6·287]	[0·642]	[10·21]	
Labellum width	8·780	0·816	9·29	−0·74
Labellum width (2)	(8·757)	(0·793)	(9·06)	
Labellum width [3]	[8·740]	[0·799]	[9·14]	
Spur length	5·157	0·311	6·03	−0·11
Spur length (2)	(5·147)	(0·305)	(5·93)	
Spur length [3]	[5·107]	[0·335]	[6·56]	
Spur width	1·650	0·097	5·88	0
Spur width (2)	(1·653)	(0·082)	(4·96)	
Spur width [3]	[1·617]	[0·095]	[5·88]	
Gymnadenia conopsea (small) (n = 9)				
Labellum length	4·078	0·393	9·64	−2·50
Labellum width	5·222	0·360	6·89	−2·14
Spur length	15·656	0·532	3·40	−0·25
Spur width	0·933	0·043	4·61	−1·33
Gymnadenia conopsea (medium) (n = 15)				
Labellum length	3·933	0·424	10·78	−1·81
Labellum width	4·453	0·511	11·48	−2·04
Spur length	13·813	1·192	8·63	−1·02
Spur width	0·963	0·113	11·73	−1·02
Anacamptis pyramidalis (n = 35)				
Labellum length	4·483	0·544	12·13	−0·88
Labellum width	6·743	1·013	15·02	−1·07
Spur length	9·477	0·955	10·08	−0·67
Spur width	0·674	0·069	10·24	−0·64
Platanthera chlorantha (n = 9)				
Labellum length	13·756	1·371	9·97	−2·87
Labellum width	3·011	0·237	7·87	−2·04
Spur length	29·000	2·926	10·09	−0·75
Spur width	2·278	0·192	8·43	−1·43
Dactylorhiza (*Coeloglossum*) *viridis* (n = 16)				
Labellum length	5·419	0·544	10·04	−1·72
Labellum width	2·575	0·153	5·94	−1·04

The second (2) and third [3] data-sets for *D. fuchsii* represent two successive rounds of re-measurement.
Figures for percentage change between adjacent flowers are based on the regression lines in Figs 3−10.

flowers 7, 14 and 24. The only strong correlation evident among parameters is between labellum width and length.

Dactylorhiza foliosa × praetermissa

The presumed contribution to this hybrid from the diploid *D. foliosa* is evident in the wider labellum and narrower spur. Both of the spur dimensions are more-or-less constant through the spike. Labellum size declines apically at the same rate as *D. praetermissa*, but labellum width declines significantly more rapidly than labellum length, so that the two regression lines converge on equidimensionality in the uppermost flowers (Fig. 4).

Coefficients of variation for spur width and especially labellum width are greater than in *D. praetermissa*. Anomalously low values at the extremes of the inflorescence are evident in the labellum length of the two uppermost flowers (60, 61), whereas several interesting anomalies occur elsewhere in the spike. Three flowers (5, 6, 12) have unusually narrow spurs, and two of these (6, 12) are also unusually short. Modest increases in labellum dimensions are evident following each phase of flower removal (arrowed on Fig. 4), and a remarkable run of unusually narrow labella extends over seven flowers (12–18). Variation between the two labellum dimensions is, as usual, strongly correlated, but labellum dimensions appear uncorrelated with spur dimensions.

TABLE 3. *Correlation coefficients between the four metric parameters recorded within each inflorescence*

	Labellum length	Labellum width	Spur length
(A) *Dactylorhiza praetermissa* (n = 35)			
Labellum width	0·773		
Spur length	−0·125	−0·067	
Spur width	0·187	0·323	0·127
(B) *Dactylorhiza* cf. *foliosa* × *praetermissa* (n = 61)			
Labellum width	0·885		
Spur length	0·042	0·140	
Spur width	0·330	0·353	0·445
(C) *Dactylorhiza fuchsii* (n = 30)			
Labellum width	0·890		
Spur length	0·270	0·371	
Spur width	0·025	0·243	0·438
(D) *Gymnadenia conopsea* (small) (n = 9)			
Labellum width	0·870		
Spur length	0·186	0·176	
Spur width	0·930	0·909	0·127
(E) *Gymnadenia conopsea* (medium) (n = 15)			
Labellum width	0·918		
Spur length	0·630	0·454	
Spur width	0·454	0·490	−0·302
(F) *Anacamptis pyramidalis* (n = 35)			
Labellum width	0·964		
Spur length	0·870	0·887	
Spur width	0·670	0·742	0·607
(G) *Platanthera chlorantha* (n = 9)			
Labellum width	0·887		
Spur length	0·380	0·252	
Spur width	0·707	0·720	0·822

The correlation coefficient for labellum length versus width for *Dactylorhiza viridis* is 0·960 (n = 16).

Dactylorhiza fuchsii

The comparatively wider labellum and narrower spur are characteristic of this distinctive diploid species. Spur dimensions are remarkably constant across the spike, whereas labellum dimensions decline toward the apex at similar rates, somewhat more rapidly than in the two previous taxa (Fig. 5).

Coefficients of variation are greater for labellum than spur dimensions, and the only anomalously low values are for labellum dimensions in the two apicalmost flowers (29, 30). The strongest correlations are those between the two labellum dimensions and, to a lesser degree, between the two spur dimensions.

Gymnadenia conopsea (small inflorescence)

The labellum length : width ratio is similar to that of the above *Dactylorhiza* species but the spur is much longer and narrower, dimensions consistent with the proboscis length of its preferred lepidopteran pollinators. Unlike the non-vestigial-spurred dactylorhizas, spur length and especially width decline towards the apex (Fig. 6). In addition, the rate of decline in the labellum dimensions is much steeper.

Coefficients of variation are unusually low for spur dimensions. With the exception of a slightly short labellum in the lowermost flower there are no anomalous flowers. As usual, the two labellum dimensions are strongly correlated

with each other, but in addition they are strongly correlated with spur width.

Gymnadenia conopsea (medium inflorescence)

This plant co-occurred with the previous plant but is assumed to possess a different genotype; this is indicated by the fact that its flowers consistently have narrower labella and shorter spurs, together with a darker flower colour. Patterns of decline in size are similar to those in the smaller plant of *G. conopsea* with the exception of spur length, which declines at four times the modest rate evident in the smaller plant (Table 2 and Fig. 7).

Similarly, the coefficients of variation for spur dimensions are double. Labellum and spur lengths are slightly low in the lowermost flower, and the labellum is slightly small in the uppermost flower. A more interesting anomaly is the unusually short but wide spur of flower 10, which causes a rare negative correlation between these two parameters. As usual, there is an especially strong positive correlation between the two labellum dimensions.

Anacamptis pyramidalis

The dimension profiles for *Anacamptis* contrast substantially with those for *Gymnadenia*; the spur is shorter and the labellum is larger (especially wider). Compared with the medium-sized *Gymnadenia*, all dimensions decline less rapidly (Fig. 8).

Unusually short spurs occur in the two lowermost flowers and the uppermost flower (35); in addition, flowers 34 and 35 have unusually narrow spurs. Within the inflorescence, all dimensions but spur width increase significantly after the first two of three phases of flower removal (arrowed on Fig. 8), at least partially explaining the relatively high coefficients of variation. Correlations between parameters are unusually strong, especially those between individual labellum and spur dimensions.

Platanthera chlorantha

This species yielded the most divergent dimension profiles; the combination of a long spur and a long labellum is especially characteristic, the latter conferring on the labellum an exceptionally large length : width ratio. Spur width declines across the inflorescence at twice the rate of spur length, though the actual diminishment is small (totalling 0·6 mm across nine flowers: Fig. 9). The two labellum parameters decline more rapidly, approaching 3 % per flower for labellum length (Table 2).

Unusually short spurs are evident in the uppermost flower and in flower 2, contributing to relatively high coefficients of variation and reducing the correlation coefficients between spur length and the other three parameters.

Dactylorhiza (Coeloglossum) viridis

This species deviates considerably in floral morphology from all other species of *Dactylorhiza*. The flowers
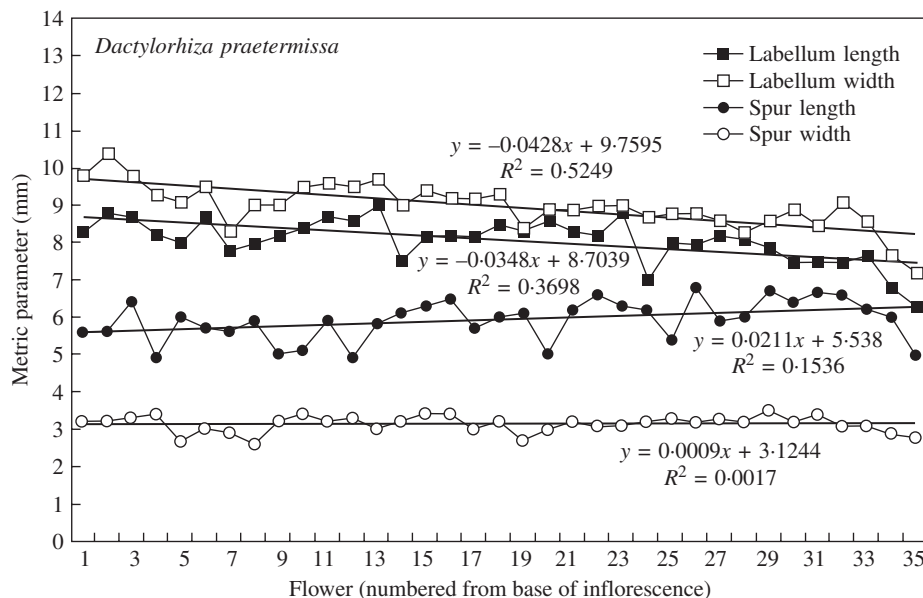
FIG. 3. Regressions of the four metric parameters (see text) against location of flower along the inflorescence of *Dactylorhiza praetermissa.* Note that the intercept, slope and *R*-squared value are given for each regression line presented in Figs 3–10.
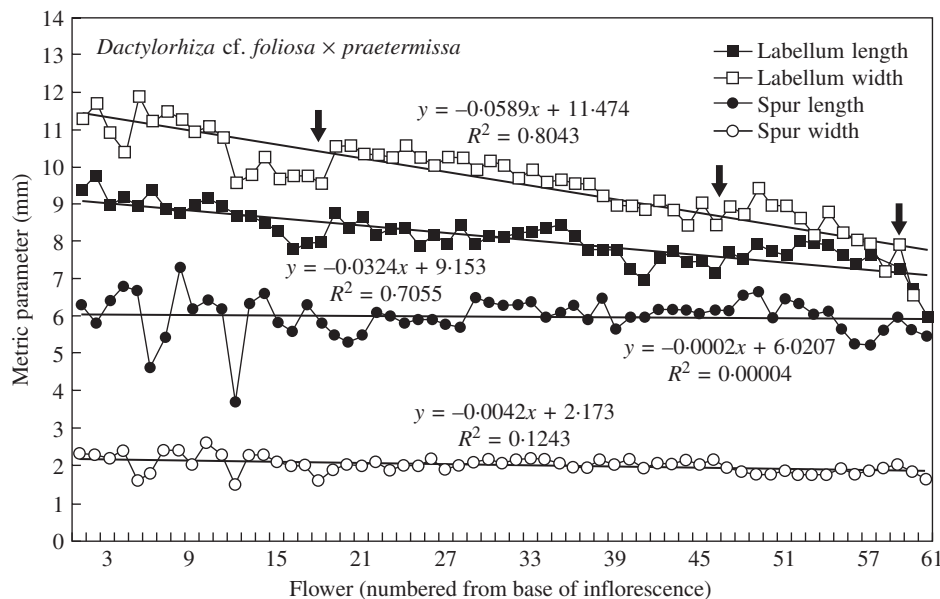

FIG. 4. Regressions of the four metric parameters against location of flower along the inflorescence of *Dactylorhiza* cf. *foliosa* × *praetermissa*. Points of flower removal are indicated by arrows.

are green, often suffused with reddish-brown. The labellum is relatively long and narrow, broadening distally into two lateral lobes that spread laterally and considerably exceed the blunt central lobe (Fig. 1D). Most notably with regard to this study, the spur is vestigial, having been evolutionarily reduced to a shallow proximal concavity containing a little nectar. Thus, the labellum dimensions can realistically be determined but the spur dimensions cannot.

The results show the largest contrast in rates of decline between labellum length and labellum width of any species studied; there is a far more modest decline in labellum

width (Fig. 10 and Table 2), which also incurs a much lower coefficient of variation.

The positive correlation between labellum length and width is exceptionally strong (0·96). Unusually, the apical-most labellum (16) is only slightly smaller than those preceding it, the most notable deviant being an atypically large flower (9) located halfway along the inflorescence.

Other analyses

Data for the repeat measurements of the 30 flowers, which constituted the inflorescence of *D. fuchsii* (Table 2
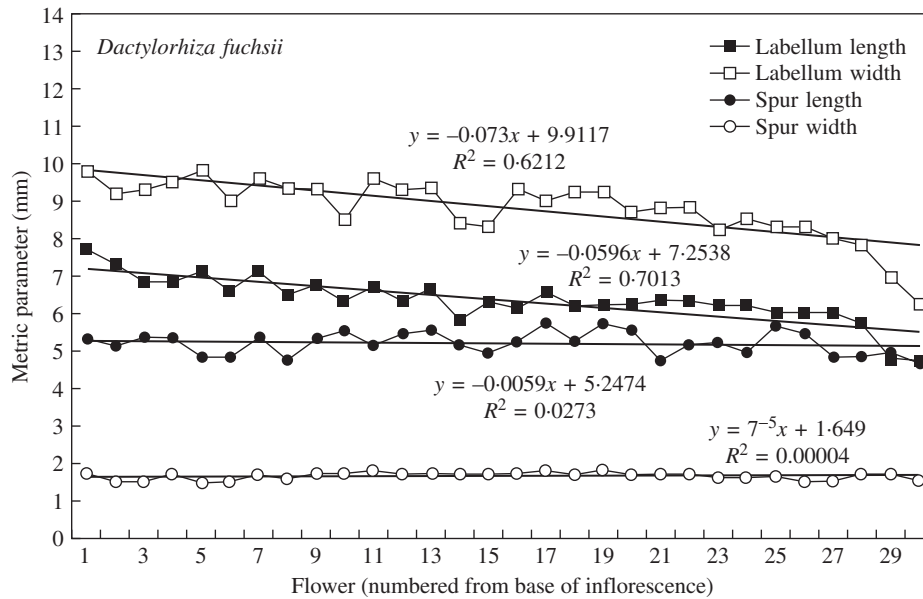
FIG. 5. Regressions of the four metric parameters against location of flower along the inflorescence of *Dactylorhiza fuchsii*.
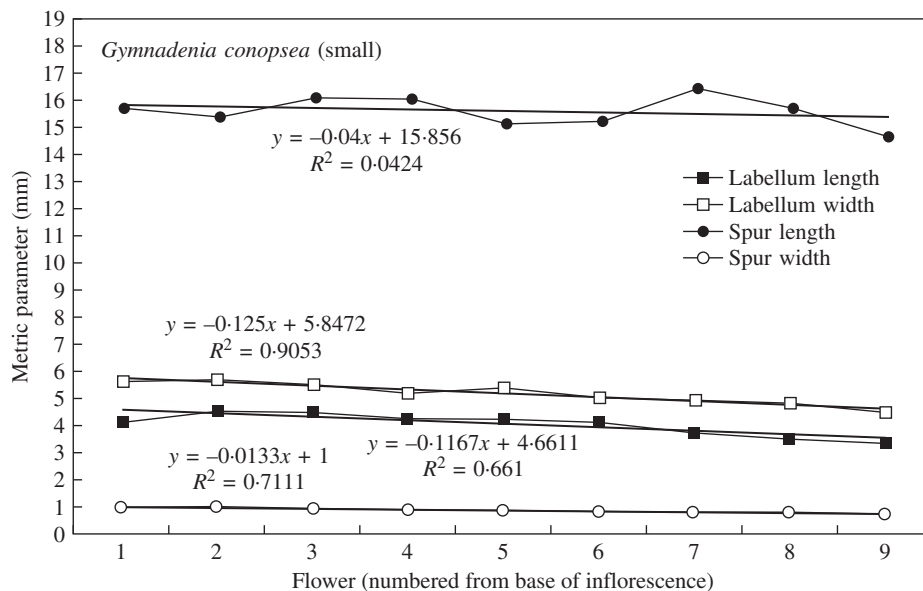

FIG. 6. Regressions of the four metric parameters against location of flower along the smaller inflorescence of *Gymnadenia conopsea*.

and Fig. 11), and for the comparisons with intra-population data-sets for genetically dissimilar flowers (Table 4) are, for convenience, presented in the Discussion.

DISCUSSION

Accuracy and repeatability of metric measurements

Repeat measurements of the 30 flowers that constituted the inflorescence of *D. fuchsii* (Fig. 2A) were designed to assess the level of both operator error and, superimposed on that operator error, shrinkage associated with desiccation following mounting.

Post-mounting shrinkage. The three sets of measurements of the 30 flowers of the *D. fuchsii* inflorescence (Fig. 2A) do not vary significantly in coefficients of variation. However, small decreases in mean values between set 1 and set 3 (obtained 5 d later) of 30–50 μm are evident for all four metrics (Table 2). The expectation was that mean values for set 2, obtained a mere 90 min after set 1, would correspond with those of set 1. This proved true for the two spur dimensions, but means for the two labellum dimensions in set 2 were intermediate between those of sets 1 and 3, suggesting that significant desiccation occurred even in the 90-min period immediately following mounting. The unusually high ambient temperature of
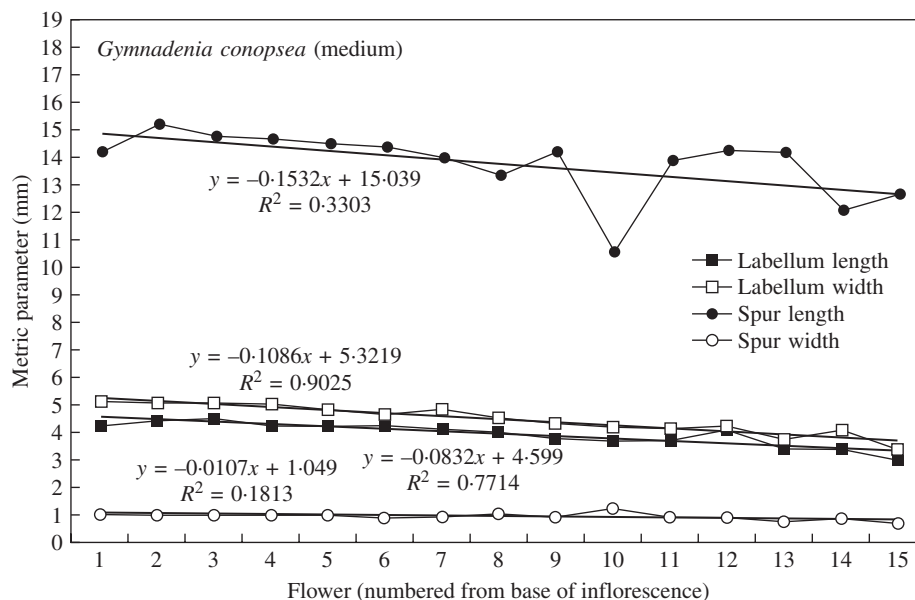
FIG. 7. Regressions of the four metric parameters against location of flower along the larger inflorescence of *Gymnadenia conopsea*.
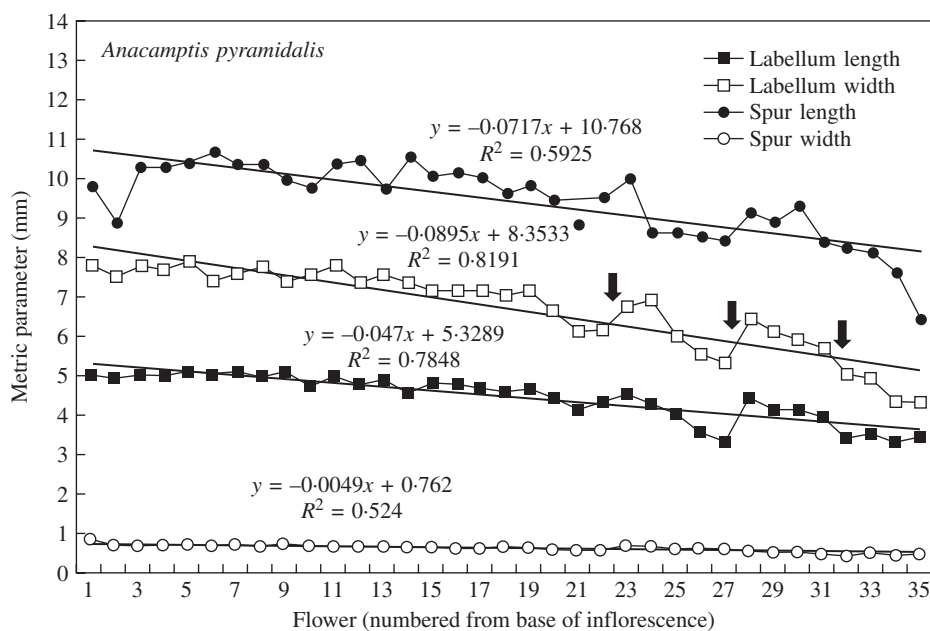
FIG. 8. Regressions of the four metric parameters against location of flower along the inflorescence of *Anacamptis pyramidalis*. Points of flower removal are indicated by arrows.

27 °C may have artificially accelerated desiccation rates. The spur desiccated far less rapidly than the planar portion of the labellum, probably because it had been laterally compressed, thereby doubling the thickness of tissue held between the adhesive and non-adhesive tapes and also trapping relatively moist air within the saccate cavity of the spur.

The similar proportional magnitudes of shrinkage in structures that differ radically in relative dimensions, and the very small absolute magnitudes of shrinkage, together suggest that shrinkage occurs only along the margins of mounted structures, where they are in imperfect contact

with the underlying adhesive tape. The observed degree of shrinkage averaged 0·7 % and in no case did it exceed 2 %.

Observer error. Measurement error by the observer was assessed primarily by determining the differential between each pair of measurements for each flower, focusing on comparison between sets 1 and 2, closely spaced in time (Fig. 11). Between one-third (labellum length) and two-thirds (spur length) of the repeat measurements are identical to the original, with most of the remaining repeat measurements deviating by only a single unit of measure-ment (i.e. by 100 μm); deviations reaching 300 μm are rare
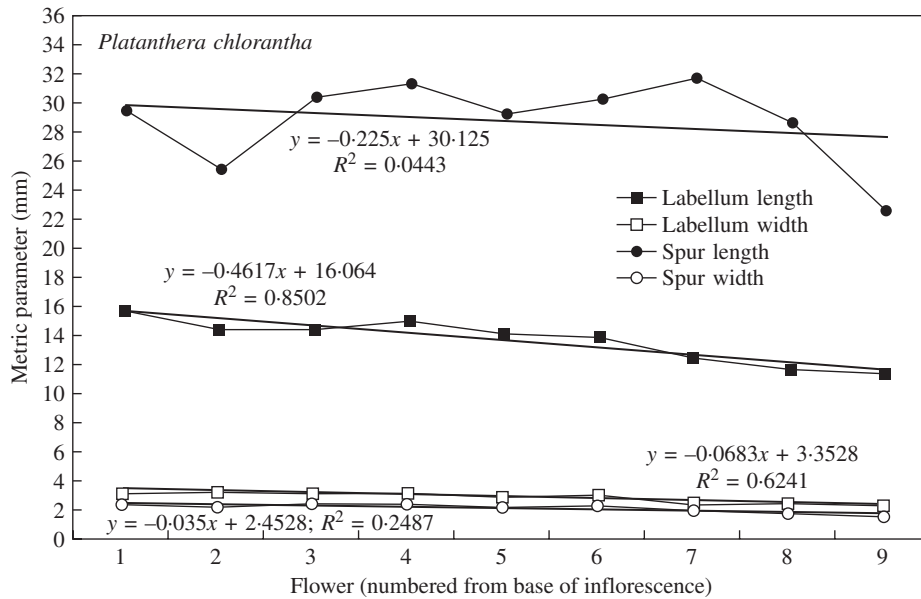
FIG. 9. Regressions of the four metric parameters against location of flower along the inflorescence of *Platanthera chlorantha*.
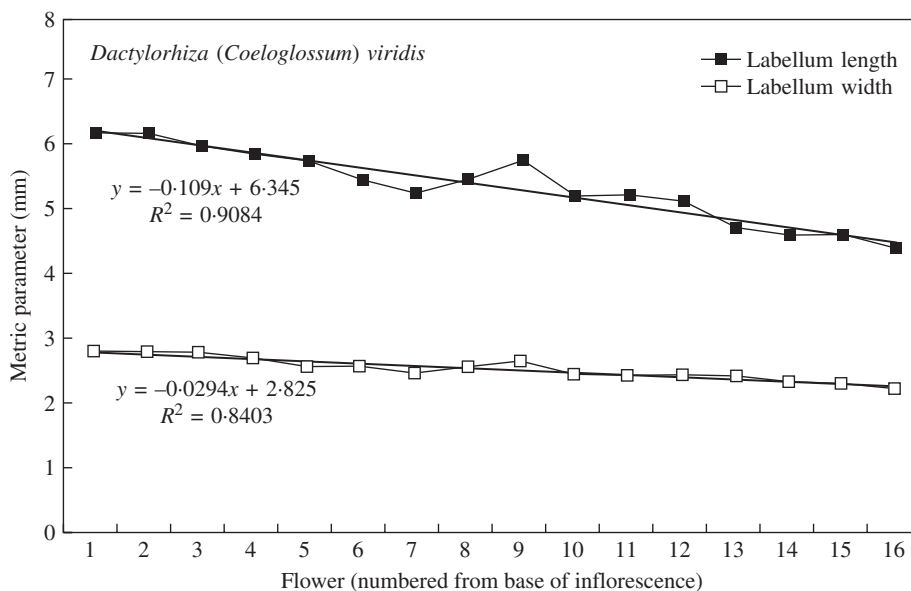
FIG. 10. Regressions of the two metric parameters describing labellum size against location of flower along the inflorescence of *Dactylorhiza viridis*.

(2·5 %). As expected, positive and negative deviations were approximately equal in number, though slight negative skewness is evident in the labellum dimensions, suggesting superimposition on observer error of additional directional error reflecting a small degree of shrinkage. As expected, the shrinkage-induced negative skew is more pronounced for all four parameters, especially spur dimensions, when sets 1 and 3 are compared rather than sets 1 and 2.

It was expected that measurement error would be greater for the two length measurements, which, unlike the two width measurements, are influenced by the precise positioning of the cut made to remove the labellum and spur from the remainder of the flower. However, this effect was not evident in either the coefficients of variation (Table 2)

or the deviations incurred during repeated measurement (Fig. 11). It appears that careful excision of the spur perpendicular to the point where it joins the base of the gynostemium, and use of the concave 'shoulder' of the labellum (rather than the cut *per se*; Fig. 2) as a reference point for the proximal end of the labellum, both offer effective methods of consistently identifying homologous points (cf. Shipunov and Bateman, 2005).

As with shrinkage, observer error appears to be largely independent of the size of the structure being measured; error appears only slightly less for spur width than for the other three parameters (Fig. 11), which are on average three to four times greater in size (Fig. 5). The absolute magnitude of observer error ranges from an estimated
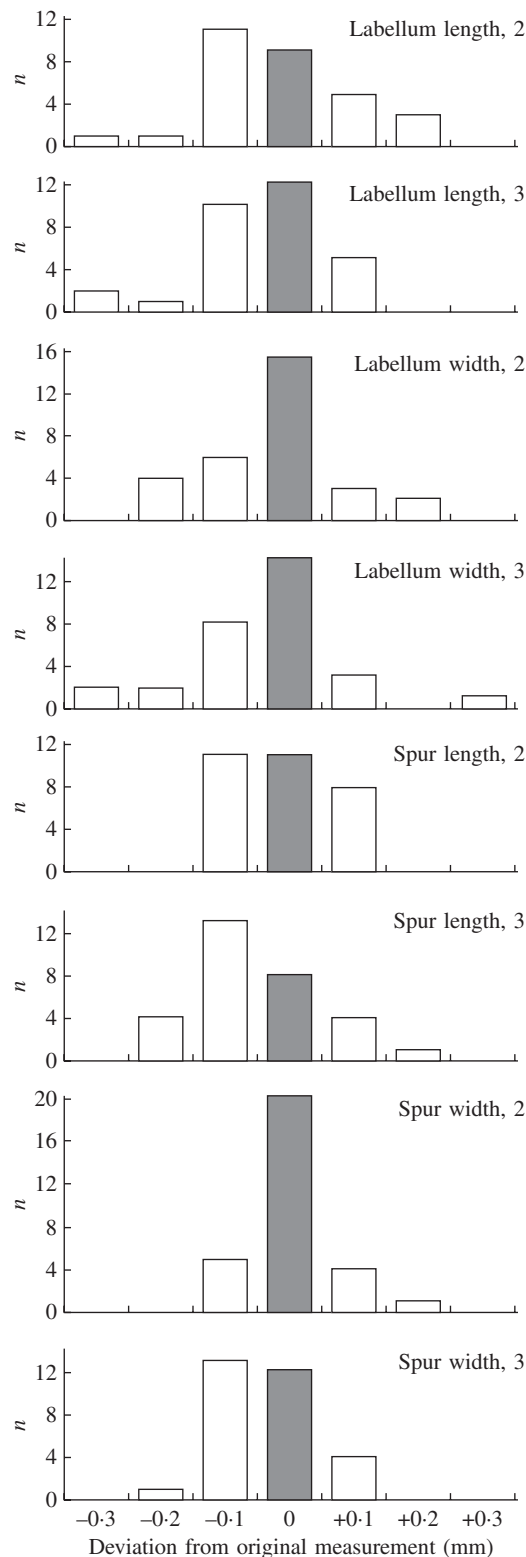
FIG. 11. Eight histograms of deviations (in millimetres) from the original measurements of the 30 flowers of *Dactylorhiza fuchsii* shown in Fig. 2A. The data describe four metric measurements (labellum length and width, spur length and width) repeated 90 min (set '2') and 5 d (set '3') after the initial 'master' set of measurements (set 1). The 'no change' column, which indicates precise consistency in measurement between temporal sets, is highlighted in each case.

$\pm 30\,\mu m$ (approximately 1·8 %) for spur width to $\pm 80\,\mu m$ (approximately 1·3 %) for labellum length.

Anomalous flowers within inflorescences

Before considering the significance of trends (almost all of which are decreases) in the sizes of at least some structures from the base to the apex of the inflorescence, we will first address the complicating issue of individual flowers that deviate significantly from the overall trend of size change within the inflorescence.

Comparison across the eight inflorescences examined shows that the flowers at the two ends of the inflorescence are the most likely to deviate negatively and substantially from the general trend; all of the eight inflorescences studied here clearly showed at least one such deviation, the possible exception being the only species with a vestigial spur, *D. viridis* (Fig. 10). Where the inflorescence contains fewer than approximately 25 flowers only one flower is likely to be affected at either end, but in larger inflorescences, two may be affected. The effects tend to be more pronounced at the apex than the base of the inflorescence, and labellum dimensions are more likely to be affected than spur dimensions.

It is tempting to ascribe these basal and apical anomalies to transient instabilities of growth associated with transitions in developmental mode: from stem elongation and bracteoidal leaf production at the base of the inflorescence (Fig. 1A), and cessation of growth of the exhausted apical meristem at the apex of the inflorescence. We have observed that the lowermost one or two flower buds on an inflorescence are frequently aborted (as was the case with the specimen of *D. praetermissa* analysed), while the apex of the inflorescence can consist of up to several tightly packed bracts that do not subtend viable flowers (six in the case of *D. praetermissa* and five in the case of *Anacamptis*).

Approximately half of the inflorescences of subtribe Orchidinae also generate at least one morphologically anomalous flower within the main body of the inflorescence (see also the Piperaceae study of Remizowa *et al.*, 2005). Often, only one parameter is affected (e.g. short labella in several flowers of *D. praetermissa*, narrow labella and narrow spurs in several contrasting flowers of *D. praetermissa × foliosa*, a short spur in flower 2 of *Platanthera*), but in other cases shifts in two or more parameters appear correlated. Positive correlation is evident in flower 9 of *D. viridis* (Fig. 10), whereas negative correlation is shown by flower 10 of the medium-sized inflorescence of *Gymnadenia*. This flower has a spur that is both unusually short and unusually wide, thereby utilising approximately the same amount of resources as the spurs of the other, more typical flowers in the spike (Fig. 7).

Further evidence that allocation of resources within an inflorescence may be a factor in its pattern of development is given by the two inflorescences that were too floriferous (or opened over too long a period) to allow simultaneous mounting of all flowers. Instead, flowers in the spikes of *Anacamptis* and *D. praetermissa × foliosa* were each

TABLE 4. *Coefficients of variation for four metric parameters among all flowers within an individual inflorescence compared with values among single flowers taken at fixed locations from ten inflorescences within each natural population (millimetres); also given are mean inflorescence densities (flowers cm^{-1})*

Species/population*	Labellum length	Labellum width	Spur length	Spur width	Spike density
Dactylorhiza praetermissa[a]					
Sawbridgeworth, Herts	6·02	5·61	5·77	11·22	2·7
Braughing, Herts	7·97	7·84	5·24	12·14	3·8
Tewinbury, Herts	7·84	9·39	11·10	16·55	3·1
Letchworth, Herts	9·53	8·78	5·58	11·18	2·9
Epsom, Surrey	6·32	14·43	12·79	9·06	4·3
Brambridge, Hants	7·33	10·60	11·53	16·83	2·8
Mean among six populations	7·50	9·44	8·67	12·85	3·3
Mean within inflorescence	7·27	6·74	9·31	6·91	
Within/among spikes (%)	97	71	107	53	
Dactylorhiza fuchsii[b]					
Axams, NW Austria	14·36	9·56	18·02	12·62	4·3
Folkestone, Kent	9·21	8·27	7·86	13·17	10·1[†]
Parham, Suffolk	9·57	10·27	18·66	8·99	5·6
St Albans, Herts	6·87	9·62	8·39	6·00	5·8
Harpenden, Herts	6·92	10·16	10·85	8·67	5·3
Tring, Herts	10·44	10·25	9·03	17·30	7·3
Pitstone, Bucks	14·44	13·06	24·53	20·99	6·4
Torrin, Skye	10·95	12·60	10·32	10·98	5·4
Castlebar, Mayo, Eire	9·77	7·32	9·80	12·41	5·8
Mean among nine populations	10·28	10·12	13·05	12·35	6·2
Mean within inflorescence	9·91	9·29	6·03	5·88	
Within/among spikes (%)	96	92	46	48	
Gymnadenia conopsea[d]					
Coulsdon, Surrey	15·84	18·85	21·05	17·72	4·9
Selbourne, Hants	17·05	19·89	15·02	11·36	4·9
Ivinghoe, Bucks	11·49	11·92	13·88	13·00	5·4
Risborough, Bucks	9·50	10·05	12·62	11·96	4·2
Selsley, Glos	14·55	11·50	14·25	23·47	4·7
Painswick, Glos	11·35	15·84	5·88	13·73	6·2
Mullagh Mor, Clare, Eire	13·03	14·82	13·56	15·24	7·6
Mean among seven populations	13·26	14·70	13·75	15·24	5·4
Mean within inflorescence (M)	10·78	11·48	8·63	11·73	
Within/among spikes (%)	83	78	63	77	
Platanthera chlorantha[e]					
Stockbury, Kent	14·52	16·01	13·79	12·20	1·6
Lavenham, Suffolk	11·14	14·20	5·48	12·94	ND
Aston Clinton, Bucks	10·15	12·02	6·99	19·43	1·4
Bix, Oxon	15·57	14·63	10·09	17·82	1·5
Badbury, Dorset	13·74	11·73	9·90	14·05	1·5
Crickhowell, Powys	11·80	4·99	11·64	13·37	ND
Mean among six populations	12·82	12·26	9·65	14·97	1·5[‡]
Mean within inflorescence	9·97	7·87	10·09	8·43	
Within/among spikes (%)	78	64	104	56	
Dactylorhiza viridis[c]					
Fifeness, Fife	15·91	14·01	NA	NA	6·0
(Mean of one population)	15·91	14·01	NA	NA	6·0
Mean within inflorescence	10·04	5·94	NA	NA	
Within/among spikes (%)	63	42	NA	NA	

NB: No comparable data were available for *Anacamptis pyramidalis* other than for spike density: 10·4±2·3 ($n = 14$) at Downe Bank, Kent; 11·2±1·6 ($n = 18$) at Devil's Dyke, Newmarket, Cambs.

*Sources of data: [a] Bateman and Denholm (1983, plus unpubl. res.), [b] Bateman and Denholm (1989a, plus unpubl. res.), [c] Bateman (unpubl. res.), [d] Bateman and Denholm (unpubl. res.), [e] R. M. Bateman, P. J. Rudall and K. E. James (unpubl. res.).

[†]High density reflects sampling early in anthesis.

[‡]Mean of four populations.

cropped in four tranches. Setting aside the terminal tranche from each inflorescence, five of the six remaining tranches appeared to cause significant *increases* in the size of the labella (and, in the case of *Platanthera*, spur width) of the flowers that opened immediately after cropping, suggesting that they had benefited from diversion of resources that would otherwise have been consumed by older flowers. In effect, flower excision reset the developmental specifications at a modestly, but nonetheless significantly, higher level (Figs 4 and 8). This phenomenon artificially inflated the coefficients of variation of the dimensions affected (Table 2).

Clines within, and contrasts between, inflorescences

Nature of the clines. If the occasional, negatively deviant, anomalous flowers are discounted, the clinal and broadly linear relationship between the four parameters measured on the flowers and their specific positions on the inflorescence appears even stronger (Figs 3–10).

Table 3 confirms that, unsurprisingly, labellum length and width reliably show a strong positive correlation, irrespective of taxonomy; this reflects the consistent declines in both parameters toward the apex of the inflorescence. This relationship is maintained in *D. viridis* in the absence of a substantial spur. In contrast, the correlation between the two spur dimensions is poor or moderate in all taxa analysed other than *Anacamptis* and *Platanthera*. Even in these taxa, the correlation between the two spur parameters is not significantly greater than those between either of the spur dimensions and the labellum dimensions. Most strikingly, correlations between spur length and either of the labellum dimensions are poor in all inflorescences except *Anacamptis* and the larger *Gymnadenia* spike.

Figures 3–9 demonstrate that spur width in particular remains constant through the inflorescence. Spur length also remains more-or-less constant in *Dactylorhiza* other than the vestigial-spurred *D. viridis*, even showing a small increase through the inflorescence of *D. praetermissa*. In contrast, in the other three genera studied, spur length declines significantly toward the apex of the spike, usually diminishing at broadly the same rate as the labellum.

Possible cause(s) of the clines. These observations suggest significant decoupling of labellum and spur development, despite the fact that these two structures are different portions of the same floral organ (e.g. Rudall and Bateman, 2002). Given the available data, attempting to explain this observation, together with the apparent preferential resourcing of the spur in the genus *Dactylorhiza* (other than *D. viridis*), carries us well into the realm of speculation. The most obvious explanations are differential selection pressures or developmental constraints.

With regard to pollination biology, most members of subtribe Orchidinae use either reward or deception to attract insects. The insects generally alight on the labellum in order to effect pollination, which is dominantly allogamous (Nieland and Wilcock, 1998; Forrest *et al.*, 2004; Cozzolino and Widmer, 2005; Tremblay *et al.*, 2005). Consequently, there is considerable functional divergence between the flat distal portion and saccate proximal portion

of the labellum. The shape and colour (and, in the case of most *Dactylorhiza* species, markings) of the distal portion act primarily to attract the pollinators and to provide them with a landing stage, whereas the location and dimensions of the spur are more critical to determining which insect visitors are capable of effecting pollination with an acceptable frequency. The visiting insect typically probes the spur in search of a reward.

Of those taxa studied here, two of the genera offer a genuine and substantial nectar reward, mainly to lepidopterans. Their visits are dominantly nocturnal in the case of *Platanthera* but dominantly diurnal in the case of *Gymnadenia* (note that few modern authors agree that *Anacamptis pyramidalis* is functionally rewarding: cf. Darwin, 1862; van der Cingel, 1995). The volume of nectar secreted by *Dactylorhiza viridis* is much smaller, being constrained by the vestigial nature of its spur, but it is sufficient to attract a variety of small beetles and ichneumons. In contrast, the remaining (and often co-occurring) dactylorhizas are food-deceptive, relying primarily on bees for pollination (Table 1) and generally achieve much lower frequencies of successful fertilization (Nieland and Wilcock, 1998; Cozzolino and Widmer, 2005). Thus, it could be argued that the spur more strongly influences pollination efficiency, and so experiences stronger selection pressures, than does the remainder of the labellum; this in turn constrains variation in spur parameters.

However, this hypothesis does not explain why spur diameter would generally be controlled more tightly than spur length in the lepidopteran-pollinated genera, nor does it explain why dactylorhizas possessing well-developed spurs achieve much greater constancy of spur length than the lepidopteran-pollinated genera, even though they are pollinated by clumsier bees each engaged in a fruitless search for food.

We therefore turn to an alternative explanation of developmental constraints. Here, one of the most critical factors could be the combination of the density (most readily measured in flowers per centimetre; Table 1) and shape (particularly the angle of the phyllotactic spiral; Fig. 1) of the inflorescence. These characteristics interact with the actual dimensions of the floral organs (particularly the labellum, spur and ovary) to determine the overall appearance of the spike. A general survey suggests that the inflorescences of Orchidinae have evolved to maximize the amount of attractive surface presented to the pollinator within the constraint of avoiding wasteful (and potentially dysfunctional) overlaps between different elements of the individual flowers. However, this inference still requires taxonomically broad, quantitative exploration.

The inflorescences of *Platanthera* present large flowers at low densities in a loose inflorescence that is cylindrical, reflecting a phyllotactic spiral that is in practice a helix (i.e. the inflorescence maintains a constant diameter over its entire length). Despite their much higher densities, the spikes of *Gymnadenia* also appear relatively open, due primarily to the small size of the labellum. *Anacamptis* produces flowers that are broadly similar in size and shape to those of *Gymnadenia*, but in contrast it packs them into an inflorescence that is much denser (typically

9–12 flowers/cm: Table 4) and has an inclined phyllotactic spiral rather than a parallel-sided helix (cf. Fig. 1E, F), especially during the first half of its flowering period when as a result it is distinctly conical (this contrasting structural organization probably explains its unusually high coefficients of variation; Table 2). Similar close packing is evident in the larger spikes of *Dactylorhiza*, wherein the larger labella tend to be closely juxtaposed, and the much broader spurs are crowded into the space between the labella and the stem. However, this apparently greater risk of over-crowding in the spike should be an argument in favour of spur length decreasing toward the apex of the spike, in parallel with the gently decreasing phyllotactic spiral, rather than dictating the remarkable constancy of dimensions observed in the spurs of the non-vestigial dactylorhizas.

In at least some species, pollinators tend to progress systematically from the base to the apex of the inflorescence, so that lower flowers have relatively high pollination frequencies (Nieland and Wilcock, 1998; Ishii and Sakai, 2001; Stpiczynska, 2003), while pollen removal tends to be greater toward the apex of the inflorescence (Tremblay, 2006). Thus, there is a potential selective advantage in preferentially resourcing lower flowers to maximize seed production. However, it becomes difficult to untangle cause and effect; does the relatively large size of the lower flowers genuinely contribute to their relative high pollination success?

Inferring the genetic control of the development of orchid inflorescences versus eudicot models

The typically short-lived eudicots *Arabidopsis* and *Antirrhinum* have provided much of our knowledge of inflorescence development and its underlying genetics (Coen and Nugent, 1994; Bradley *et al.*, 1996, 1997). Therefore knowledge of these model organisms is outlined before attention is turned to petaloid monocots. In the following discussion, putative gene orthologues are given first for *Arabidopsis* and then for *Anthirrhinum* (reviewed by Okamuro *et al.*, 1993; papers in Cronk *et al.*, 2002).

Based on these model angiosperms, the genetic control of inflorescence morphogenesis is often simplified to a balance of power (or at least of influence) that is dictated by mutual repression between *TFL1/CEN*, which encourages apical meristems, and *LFY/FLO*, which encourages the onset of flowering and retards internodal elongation (e.g. Weigel *et al.*, 1992; Bradley *et al.*, 1996; Cremer *et al.*, 2001; Baum and Donoghue, 2002; Theissen *et al.*, 2002; Rudall and Bateman, 2003). Also important are the A-class gene families *AP1/SQUA* (MADS-box) and *AP2*, which can play a wide range of roles at differing points in the development of inflorescences and the associated flowers (e.g. Ratcliffe *et al.*, 1999; Theissen *et al.*, 2002), effectively priming the subsequent activities of the diversity of floral identity genes that constitute the bulk of the MADS-box cluster. These encompass the E-class genes epitomized by *AGL2*, which include genes implicated in the initiation of inflorescences in epidendroid orchids (Yu and Goh, 2000, 2001; Johansen *et al.*, 2006). In

addition, the TCP-class genes that establish the 'dorsiventral' polarity in zygomorphic flowers have been well characterized in *Antirrhinum*, wherein the 'dorsalizing' *CYC* (= *tb1* of *Zea*), reinforced by *DICH*, is complementary to the 'ventralizing' *DIV* (Clark and Coen, 2002; Cubas, 2002, 2004). The last essential piece of the jigsaw in model organisms is provided by the physiological genes that respond to environmental cues by initiating production of inflorescences across the body of the plant by activating genes such as *TFL1* and *LFY* (e.g. Jack, 2004; thematic papers in *Science*, 12 August 2005).

However, not all gene classes follow this comfortable hierarchy of being reliably expressed in different organs of the plant, thus conferring organ identity. Expression of *CEN* in *Antirrhinum* is confined to the flowers, whereas expression of the orthologous *TFL1* in *Arabidopsis* extends to vegetative structures (Bradley *et al.*, 1997). Similarly, in contrast to *LFY* in *Arabidopsis* and *FLO* in *Antirrhinum*, the tobacco orthologue of *LFY/FLO*, *NFL*, has an expanded zone of expression that encompasses vegetative as well as floral meristems (Kelly *et al.*, 1995). Perhaps the best example of contrasting expression patterns among lineages is *KNOX* (= *knotted1* of *Zea*) (e.g. Bharathan *et al.*, 1999), which is expressed only in the apical meristem of *Arabidopsis* but has been co-opted for use in the development of the compound leaves of tomato (Tsiantis *et al.*, 2002), though not in the non-homologous compound leaves of pea (Hofer *et al.*, 2001). Plant growth is indeterminate under *KNOX*, but switches to determinate where *KNOX* is suppressed by MYB-class genes such as *PHAN*. This crucial antagonistic control of land-plant morphogenesis appears to have emerged independently in microphyllous and megaphyllous vascular plants (Harrison *et al.*, 2005). The similarly crucial antagonism between *LFY* and *TFL1*, involving mutual inhibition, operates by blocking either apical or lateral meristematic responses and by delaying up-regulation, suggesting that the relative timing of up-regulation of the interacting genes is crucial in determining the morphological outcome (Ratcliffe *et al.*, 1999). Even more provocative are the multiple roles identified for the maize gene *ifa1*, whose influences over both meristem determinacy and meristem identity only become apparent in double mutants with genes such as *zag1* and *ids1* (Laudencia-Chingcuanco and Hake, 2002), providing a potential basis for both heterochronic and heterotopic shifts (e.g. Rudall and Bateman, 2004; Baum and Hileman, 2006).

Returning briefly to *KNOX*, as well as profoundly influencing morphogenesis is stems and leaves, this gene has also been implicated in radical shifts in floral structure (Bateman and Rudall, 2006), where mutation via transposon insertion in non-coding regions had caused ectopic expression of functional transcripts of the *KNOX* homeodomain (Golz *et al.*, 2002). These transcripts in turn caused the growth of spur-like outgrowths in *Antirrhinum*, a genus that normally lacks spurs, though some of its close relatives reliably possess them.

Thus, comparison of a very few model organisms (*Arabidopsis*, *Antirrhinum*, *Solanum*, *Nicotiana*, *Zea*) is sufficient to demonstrate that at least some crucial morphogenetic genes shift expression patterns and, thereby, sometimes functions between lineages. The shifts can be physical, temporal, or both. In addition, the morphogenetic consequences of modifications to genes cannot be considered in isolation, since it is interactions among genes, and the epigenetic environment in which they are expressed, that dictate the resulting morphology. And inflorescences are especially problematic to interpret, as they combine some elements of developmental programming from the indeterminate shoot system with others from the determinate flowers (Baum and Hileman, 2006).

It is therefore questionable how much of the well-known, hard-won model of reproductive development derived from eudicots is applicable to petaloid monocots in general and European orchids in particular, especially given their classically geophytic subterranean morphology. It has been stated that the life history of such orchids means that they exist in a state of perpetual somatic youthfulness. The main perennating organ is a tuberoid (strictly a root-stem tuber) that is considered homologous with a polystelic axillary shoot (Sharman, 1939; Rasmussen, 1995). Each year the tuberoid is wholly replaced by at least one new tuberoid, while in most years the previous tuberoid generates a rosette of expanded leaves. In individuals destined to flower, an unbranched stem elongates from within the rosette and typically generates additional leaves that sequentially become smaller and increasingly bract-like. Eventually, the bracteoidal leaves are succeeded by true bracts subtending spirally arranged flower buds to generate the racemose inflorescence (Fig. 1).

Thus far, developmental-genetic studies of petaloid monocots have been few relative to those of 'suprapetaloid' grasses, and have focused on the activities of organ identity genes within the flowers of tulips (Kanno *et al.*, 2003) and orchids (e.g. Rudall and Bateman, 2004; Tsai *et al.*, 2004; Bateman and Rudall, 2006; Xu *et al.*, 2006). It seems likely that, when such studies are expanded to encompass the earlier ontogenetic phases of such plants, allowing comparison with model organisms such as *Arabidopsis* and *Antirrhinum*, significant differences in genetic control will emerge. These are likely to include strong 'pre-programming'. (*a*) There is circumstantial evidence that each individual 'decides' whether it is going to flower in the previous year, and thus in the previous tuberoid. (*b*) The growth of the flowering stem during the flowering season appears to primarily reflect elongation, its structure having been determined during early ontogeny (i.e. within the tuberoid and, in the case of geophytic orchids such as those of subtribe Orchidinae, beneath ground level). (*c*) The transition from 'vegetative' to reproductive behaviour of the stem, as marked by the transition from bracteoidal leaves to true bracts, appears subtle, and may simply reflect transgression of a size threshold as the apical meristem gradually decreases in size. As noted above ('Anomalous flowers'), the subsequent history of the inflorescence may be determined at least in part by internal allocation of resources.

Nonetheless, orchid inflorescences have advantages as potential model systems for studying morphogenesis; they

are simple racemes, and lack the blurred boundaries between inflorescence and flower that afflict many other angiosperm lineages (Rudall and Bateman, 2003, 2004; Remizowa *et al.*, 2005). We anticipate that protocols currently applied to model organisms will soon be successfully transferred to the inflorescences of Orchidaceae.

Inferring the relative proportions of floral variation attributable to genetic, ontogenetic and environmental causes

Table 4 compares coefficients of variation for the four parameters within the studied inflorescences with data for conspecific populations. Ten putatively genetically distinct individuals were analysed per population, each inflorescence yielding a single flower sampled at a more-or-less fixed location within the inflorescence.

Relative levels of variation among flowers show patterns of variation between inflorescences that are broadly similar to those observed within inflorescences. For example, there are some notable contrasts in values for measures that one might expect *a priori* to be similar (e.g. labellum length relative to labellum width in the Epsom population of *D. praetermissa* and the Crickhowell population of *P. chlorantha*, and spur length relative to spur width in several populations of *D. praetermissa* and *D. fuchsii*, the Painswick population of *G. conopsea*, and the Aston Clinton population of *P. chlorantha*). When species are compared, *D. praetermissa* is on average significantly less variable than the other orchid species in parameters other than spur width.

However, the main benefit is gained from the population studies when they are compared with values for within-spike variation for the same species. This approach provides an opportunity to estimate, for each of the five orchid species compared in Table 4, the relative proportions of the variation observed in the flowers that are due to genetic and to non-genetic causes (admittedly, values for *D. viridis* are relatively unreliable, as only one population was measured). Given that variation within an inflorescence is by definition non-genetic, any excess of variation above that baseline level for each variable can be attributed to genetic causes (arguably supplemented with some variation induced by differences in the microhabitats of individuals within the population).

Within-spike variation was lowest relative to between-spike variation in labellum width for *D. viridis* (42 %, based on an undesirably small sample) and highest in spur length for *D. praetermissa* and *P. chlorantha*, where within-spike variation marginally exceeded between-spike variation (107 % and 104 %, respectively; Table 4). Averaging the percentages for the four variables yielded values of 71 % for *D. fuchsii*, 75 % for *G. conopsea*, 76 % for *P. chlorantha* and 82 % for *D. praetermissa*. Thus, size variation in flowers within an inflorescence is typically three-quarters of that observed between inflorescences for topologically equivalent flowers; this was an unexpectedly high figure, with considerable implications for best practice in morphometric studies.

CONCLUSIONS

Implications for morphometric systematic studies

It is advisable to measure all excised structures as soon as possible after mounting, and to prioritize the measurement of thin, relatively desiccation-prone structures such as labella over thicker structures such as bilaterally flattened spurs. If such care is taken, shrinkage can effectively be eliminated as a source of measurement error. Even if less care is taken, post-mounting shrinkage is demonstrably a trivial source of error in morphometric studies if they are performed using the analytical procedures recommended here.

Moreover, like shrinkage, observer error is shown to be a trivial source of statistical noise if the recommended morphometric procedures are employed. Unlike shrinkage, observer error is random regarding whether the resulting deviation is positive or negative, and so any such error generates random noise in the data rather than spurious directional trends.

Excision can introduce errors into the measurements of the lengths of structures, but this study demonstrates that such errors can be rendered negligible by careful and consistent excision (as in the present spurs), or preferably by selecting as a baseline a landmark that is reliably present within the boundary of the cut (as in the present labella: cf. Shipunov and Bateman, 2005).

However, by far the greatest potential source of error in orchid floral morphometric studies is comparing flowers taken from different locations on the inflorescence. It is clear that the metric variation across a single inflorescence can equal that observed between the fixed-point flowers of a large sample of genetically distinct inflorescences.

Careful measurements within inflorescences demonstrate the occurrence of occasional anomalous deviations from the norm in at least one of the four floral parameters; these are concentrated in (but not confined to) the base and apex of the inflorescence. This suggests that the lowermost and uppermost flowers (at least the bottom two and top two flowers in the case of an inflorescence of subtribe Orchidinae) should be avoided during morphometric sampling. Ideally, the representative flower should be excised from a consistent location (say one-third of the distance from the base to the apex of the spike; Bateman and Denholm, 1989*b*), but even then, the adjacent flowers should be surveyed briefly to ensure that the selected flower is not developmentally anomalous within the spike.

Inferences regarding inflorescence development and evolution in Orchidinae

Modest but significant increases in labellum size in younger flowers are frequently observed to follow excision of older flowers. This suggests that the otherwise consistent acropetal decrease in labellum size, often accompanied by a reduction in spur length and occasionally in spur width, reflects differential allocation of resources across each inflorescence. There remains some flexibility in the allocation of resources across the inflorescence, and probably across the plant as a whole, even though the

approximate size of the inflorescence of an individual member of subtribe Orchidinae appears to be determined early in ontogeny. The developmental-genetic control of morphogenesis in the racemose inflorescences of such geophytes is inferred to differ substantially from that demonstrated in annual, supraterranean model organisms such as *Arabidopsis*, *Antirrhinum* and *Zea*.

Similarly, resource allocation is likely to be the cause underlying the occasional negative deviations in one or more parameters in individual flowers or small clusters of flowers. The usual concentration of such anomalous flowers toward the two extremities of the inflorescence suggests that the determinate development of the flowers is perturbed by the two most fundamental transitions in the behaviour of the stem apical meristem: from vegetative to reproductive growth at the base of the inflorescence, and the cessation of reproductive growth near the stem apex that presages exhaustion of the apical meristem.

The apparent decoupling of labellum dimensions from spur width, and also from spur length in the case of *Dactylorhiza*, are of particular interest. The original hypothesis of a structural developmental constraint imposed by the subtle variations in inflorescence architecture no longer appears valid. If the developmental constraint is not structural it may instead prove to be ontogenetic. Specifically, it would be useful to extend the few available ontogenetic studies of Orchidinae flowers (Kurzweil, 1987) to the present study species in order to determine the relative rates of expansion of the labellum and spur as the flower develops (the earlier studies focused on gynostemium development at the expense of later developing structures such as the labellum). The apparent partial decoupling of labellum dimensions, spur length and spur diameter may ultimately prove to reflect a series of heterochronic shifts (e.g. Gould, 1977; Alberch *et al.*, 1979; Bateman, 1994; Rudall and Bateman, 2002, 2003, 2004; Bateman and Rudall, 2006).

Of course, a heterochronic explanation of these trends would not necessarily preclude an adaptive explanation. Several approaches could be used to explore further the possibility of adaptive control of floral development across the inflorescence, including seeking any changes in pollinator behaviour (or in the composition of the spectrum of effective pollinators) as the wave of anthesis progresses toward the apex of the inflorescence, and observing whether there is heterogeneity of seed-set (and pollinium removal) across the inflorescence (e.g. Stpiczynska, 2003; Tremblay, 2006). In addition, detailed comparison of inflorescence development in sister pairs of allogamous and autogamous species might prove fruitful, as one would expect greater morphogenetic latitude in the autogamous taxa, which are no longer dependent for their reproductive success on either the labellum or the spur to attract pollinators (Rudall *et al.*, 2002; Bateman *et al.*, 2006). Also, the inferences on inflorescence evolution made here would benefit from observation of additional spikes of both these and other species, noting, for example, the different rates of decline in spur length observed in the two physically adjacent *Gymnadenia* spikes studied here (cf. Figs 6 and 7).

Implications for microevolutionary studies

Comparison of variation within and between inflorescences demonstrates that, on average, three-quarters of the flower size variation observed within an orchid population is mirrored across a single inflorescence. This startling figure simultaneously demonstrates (*a*) the importance of sampling from a consistent location within the inflorescences under comparison, and (*b*) the consistently strong influence on morphology of the epigenetic phenomena that biologists collectively term development.

Most previous studies of non-genetic morphological variation have focused on the modifying influences on the organism of various aspects of the external environment, but this study has placed greater emphasis on the internal physiological environment of the individual, as manifested by developmental clines within each inflorescence. No doubt the resulting morphological variation is influenced to some degree by the external environment (perhaps explaining at least some of the morphological perturbations observed within specific inflorescences), but most of the observed variation is probably programmed into the developmental trajectory of the inflorescence early in its ontogeny.

This conclusion has especially important implications for microevolutionary studies. Specifically, the surprisingly large magnitude of variation in flower size across a typical inflorescence relative to that evident between inflorescences means that there are substantial differences in resourcing between early-opening and late-opening flowers. Thus, there are likely to be corresponding differences between those flowers in biological properties. Some of these properties will be intrinsic, such as the number of seeds that can be generated by the flower, whereas others will be extrinsic, such as the relative success of the flower in donating pollen to, or receiving pollen from, potential pollinators.

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