Floral ontogenetic evidence of repeated speciation via paedomorphosis in subtribe Orchidinae (Orchidaceae)

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Received 21 August 2007; accepted for publication 23 November 2007

Thoroughly sampled molecular phylogenies of the dominantly European orchid subtribe Orchidinae were used to identify a pair and a triplet of recently diverged species in which: (1) divergence involved substantial changes in floral morphology, particularly in the labellar lobes and spur; and (2) the polarity of those changes could be inferred phylogenetically. Floral ontogeny in the selected species was documented in detail through macromorphological, light microscopic, and scanning electron microscopic study of a wide range of ontogenetic stages. All study species showed differentiation of perianth segments earlier than the gynostemium. Unsurprisingly, component parts of the basic floral organs (gynostemial auricles and rostellum, labellar lateral lobes, and spur) were initiated relatively late, the spur and ovary continuing to expand beyond anthesis. The predominant evolutionary pattern identified in the two case studies was paedomorphosis via progenesis (earlier offset of growth); this credibly explained the reduction in spur size and lateral lobing of the labellum in Gymnadenia odoratissima and, especially, G. austriaca relative to G. conopsea. Loss of resupination in G. austriaca was best viewed as the deletion of a formerly terminal ontogenetic stage. Radical reduction of the spur of Dactylorhiza viridis relative to D. fuchsii was also attributed to progenesis, although the long, narrow outline and relatively short central lobe of its labellum were attributed to increased growth of the lateral lobes (i.e. hypermorphosis resulting in peramorphosis). Microscopic study of epidermal cell types on the labellum and spur suggested a degree of decoupling of micromorphological from macromorphological transitions, although both were subject to heterochronic shifts. Each of the two case studies was consistent with, but not proof of, saltational macroevolution operating via functional changes in one or more key developmental genes. © 2008 The Linnean Society of London, Botanical Journal of the Linnean Society, 2008, 157, 429–454.


INTRODUCTION

Orchidaceae is arguably the largest of all the monocot families, containing an estimated 20 000 species divided amongst approximately 800 genera (Dressler, 1993; Pridgeon et al., 1999, 2001). A characteristic feature of orchids is their elaborate flowers, commonly considered to be a consequence of their close ecological and co-evolutionary ties to a vast assemblage of vertebrate and invertebrate pollinators. The unique and complex floral morphology and consequent diverse pollination biology of orchids have fascinated botanists and evolutionary biologists since their discovery (for example, Darwin, 1877). However, with the exception of Kurzweil’s ontogenetic studies (summarized by Kurzweil, 1998), there are relatively little comparative developmental data on this phylogenetically well-resolved group of flowering plants.

Despite the unusual terminology associated with orchid floral morphology and the dramatic phenotypic diversity in orchid floral form (Rudall & Bateman, 2002), the basic arrangement of floral parts in orchid flowers is typical of ‘normal’ monocot flowers in that

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they possess a trimerous organization that consists of five successive floral whorls, each with three segments (Fig. 1A, B). Travelling from the base to the apex of the floral axis, we encounter the outer perianth, inner perianth, outer stamens, inner stamens, and, lastly, the carpels (Benzing, 1987). However, in many other respects, orchid flowers are relatively highly modified, specifically: (1) the presence of a gynostemium; (2) strong floral zygomorphy reflecting (a) the presence of staminodes and/or complete suppression of abaxial stamens, and (b) the presence of a median petaloid labellum, which is usually accompanied by various structures including cylindrical nectar spurs; and (3) resupination of the ovary and/or pedicel (i.e. the ovary and/or pedicel undergo 180° torsion that shifts the labellum from the uppermost to the lowermost position by anthesis).

The structures associated with the gynostemium show the greatest deviation from the basic monocotyledonous condition (Dressler, 1993; Rudall & Bateman, 2002). The gynostemium parts are notoriously difficult to interpret, but detailed ontogenetic investigations have helped to clarify their homologies (for example, Kurzweil, 1998). In addition to the fertile components, many of the gynostemium parts represent sterile reproductive structures. For example, in orchidoid orchids, the two small structures, termed auricles, on either side of the fertile anther are commonly interpreted as two sterile adaxial stamens (staminodes). Carpel apices and lateral receptive stigmatic carpel lobes also form part of the gynostemium. The median carpel is the most prominent, forming a largely non-receptive tongue-like organ that develops between the two locules of the anther, the rostellum, or, more specifically, the rostellar projection if referring to only the non-receptive parts. This structure is thought to help prevent self-pollination (Szlachetko & Rutkowski, 2000). In orchidoid orchids, the rostellum usually encloses the viscidia, sometimes in a thin-walled pouch derived from the apical portion of the rostellum, termed the bursicula. The remaining two lateral carpel apices generate the receptive stigmatic surfaces (Kurzweil, 1987).

**PAEDOMORPHOSIS AND SPECIATION IN ORCHIDS**

Morphological and ontogenetic analyses of orchid flowers can be particularly informative with regard to floral evolution and speciation in some orchid groups. The former genus *Nigritella* has been depicted phylogenetically as either sister to (*Hedrén, Klein & Teppner, 2000) or nested within (Pridgeon et al., 1997; Bateman et al., 2003) *Gymnadenia s.s.* Such molecular evidence led to the taxonomic inclusion of *Nigritella* within *Gymnadenia*, despite the substantial morphological differences that distinguish the two fundamental phenotypes. Species of the former genus, such as *G. (N.) austriaca*, are believed to be pseudopeloric, having evolved through floral simplification and diminished floral zygomorphy. They subsequently radiated to generate several distinct species (Hedrén et al., 2000; Bateman & DiMichele, 2002). The phylogenetic position of the former genus *Nigritella* – tentatively nested within *Gymnadenia* (Fig. 1C) – makes this group an ideal candidate for evolutionary studies, as it provides the polarity necessary to infer the direction of character evolution.

Bateman & DiMichele (1994, 2002) proposed that heterochrony – a change in the timing of development of one or more organs between putative ancestor and descendant (Alberch et al., 1979) – might have instantaneously triggered the origin of the former genus *Nigritella* from within *Gymnadenia*, resulting in paedomorphosis of the flower and explaining the great morphological differences amongst these species in the absence of significant internal transcribed spacer (ITS) sequence variation (Bateman et al., 1997, 2003; Bateman, Rudall & James, 2006).

Paedomorphosis is defined as the displacement of ancestral features to later stages in the ontogeny of the descendants (Gould, 1977; Alberch et al., 1979; Fink, 1982). The apparently juvenile simple-lipped, non-resupinate, short-spurred ‘*Nigritella*’ flower exemplifies such paedomorphic features. Such a flower is thought to be derived from the more complex, resupinate flower of *Gymnadenia s.s.*, which is characterized by a deeply trilobed labellum bearing a nectariferous spur (Bateman & DiMichele, 2002).
Such transitions most probably influenced speciation via switches in host–pollinator interactions. Bateman et al. (2006) extended this study to several additional species within the Gymnadenia clade in order to explain the potentially instantaneous reduction in spur length and lateral labellum lobes successively from G. odoratissima, G. frivaldii, and the former genus Nigritella. Based on scanning electron micrographs of mature orchid flowers, Bateman et al. (2006) hypothesized early cessation of growth in the spur and labellum of the presumed descendant orchid species relative to that of the equivalent organs in the ancestor, and hence concluded that 'nigritellan' flowers, such as those of G. austriaca, owed their origin to paedomorphosis.

In this paper, we test this hypothesis by investigating floral structure and ontogeny in three closely related species within the Gymnadenia clade (Fig. 1C), seeking paedomorphic characters in the mature flowers. Using scanning electron microscopy of successive floral ontogenetic stages, we follow the ontogeny of the labellum, spur, and ovary to determine the order of initiation and cessation of development of specific shared structures, and identify the mode(s) of heterochrony most likely to be operating. We also expand this theory to Dactylorhiza, the sister genus to Gymnadenia (Fig. 1C), which constitutes a second possible example of speciation resulting from heterochronic shifts.

MATERIAL AND METHODS

PHYLOGENETIC CONTEXT

In this paper, we focus on two closely related groups of orchidoid species within the subtribe Orchidinae (Orchidaceae). This clade has a particularly well-resolved phylogeny (Fig. 1C). All species have been studied morphologically (Dressler, 1993; Freudenstein & Rasmussen, 1999), and nuclear ITS rDNA data are available for at least 190 species (Bateman et al., 2003). Phylogenetic reconstruction has revealed several examples of molecularly closely related species with clear gross morphological differences in the length of the floral nectar spur, the shape of the labellum, and the structure of the ovary. This makes subtribe Orchidinae highly amenable to evolutionary developmental studies (Bateman & DiMichele, 2002; Bateman et al., 2003). Within this subtribe, we focus on Gymnadenia s.l. and Dactylorhiza s.l. (Fig. 1C).

PLANT MATERIAL

The plant material examined was obtained either from the extensive collections of fixed material at the Royal Botanic Gardens, Kew (K) or collected from the wild by the authors in the Dolomites of northern Italy and three sites in the Chiltern Hills. Material collected by the authors: Gymnadenia conopsea (L.) R.Br. (two samples: Dolomites 2005; Buckinghamshire, UK 2006); G. odoratissima (L.) Rich. (Dolomites 2005); G. austriaca (Teppner & Klein) Delforge (Dolomites 2005); Dactylorhiza fuchsii (Drue) Soó (Hertfordshire, UK 2006); D. viridis (L.) R.M. Bateman, Pridgeon & M.W. Chase (formerly Coeloglossum viride [L.] Hartm.) (Hampshire, UK 2006). Material obtained from K: D. fuchsii (three samples: Chesterman 217, Italy 1981; Wood 474, Italy 1982; collector unknown, 0-1375); D. viridis (two samples: Jeans s.n., UK 1963; collector unknown, 0-1396); G. conopsea (Mason s.n., UK); G. densiflora (Wahlenb.) Aver. (Wood 534, France 1982); G. odoratissima (three samples: Milne-Redhead 2238, Austria 1936; Barneby s.n., France 1963; Wood 358, Italy 1979); G. austriaca (three samples: Barneby 21, Italy 1968; Wood 355, Italy 1979; collector unknown, 0-1397).

Inflorescences showing flowers at various stages of development were fixed using formalin–acetic acid–alcohol (85% ethanol [70%], 10% formaldehyde, and 5% glacial acetic acid) for a minimum of 72 h. Material obtained from K was stored in Kew Mix (53% industrial methylated spirit [IMS], 28% water, 5% glacial acetic acid) for a minimum of 72 h. Material obtained from K was stored in Kew Mix (53% industrial methylated spirit [IMS], 28% water, 5% glacial acetic acid) for a minimum of 72 h. Material obtained from K was stored in Kew Mix (53% industrial methylated spirit [IMS], 28% water, 5% glacial acetic acid) for a minimum of 72 h. Material obtained from K was stored in Kew Mix (53% industrial methylated spirit [IMS], 28% water, 5% glacial acetic acid) for a minimum of 72 h. Material obtained from K was stored in Kew Mix (53% industrial methylated spirit [IMS], 28% water, 5% glacial acetic acid) for a minimum of 72 h. Material obtained from K was stored in Kew Mix (53% industrial methylated spirit [IMS], 28% water, 5% glacial acetic acid) for a minimum of 72 h.

For scanning electron microscopy, floral buds and inflorescences were dissected in 70% ethanol to expose floral organs and organ primordia, before being dehydrated in an ethanol series to absolute ethanol. Dehydrated plant material was processed using a Tousimis Supercritical Autosamdr 815B critical-point drier, mounted onto stubs with double-sided adhesive pads, and coated with platinum using an Emitech K550 sputter coater for two periods of 4 min. Material was visualized using a Hitachi S-4700-II cold field emission scanning electron microscope at 2.0 kV.

LIGHT MICROSCOPY

For light microscopy, flower buds were dissected in 70% ethanol, dehydrated, and embedded in Paraplast wax using a Leica TP 1010 Tissue Processor. Serial sections were cut at a thickness of 14 μm using a Leica RM 2155 rotary microtome, mounted onto Polyamine glass slides, and allowed to adhere in a slide oven at 35 °C for 24 h. They were then stained with Safranin and Alcian blue using a Leica AutoStainer XL automated slide stainer, and mounted perma-
RESULTS

FLORAL ONTOGENY: GYMNASDIA CONOPSEA/ODORATISSIMA VS. G. AUSTRIACA

Macromorphology

In both G. conopsea and G. odoratissima (Fig. 2A, B, E), flowers are borne on a cylindrical many-flowered inflorescence. Flowers of G. conopsea and G. odoratissima are small and scented, often a violet-pink, lilac, pale pink, or white (Fig. 2A, B, E). The lateral sepals are spreading, whereas the dorsal sepal converges with the lateral petals to form a hood that covers the short gynostemium (Figs 3A, 4A).

The inflorescences of G. austriaca are hemispherical, or sometimes ovoid and almost conical (Fig. 2C). Numerous flowers are borne on a compact inflorescence in all former Nigritella species, subtended by a glabrous, slightly papillate bract. Flowers of G. austriaca are typically very dark reddish-purple (Fig. 2C). All of the perianth segments are acute; the lobe is wider, obtuse, and longer than the lateral lobes (Fig. 3D). All of the petals are clearly resupinate (Figs 3B, C, 4B, D). Numerous papillae are present in the lower two-thirds of the spur, where they are more numerous on the adaxial epidermis of the spur interior (Fig. 4E); they are typically bulbous at their tips (Figs 4G, 6D, E).

Both the spur and labellum of G. austriaca differ considerably from those of G. conopsea and G. odoratissima (Fig. 5A). In G. austriaca, the labellum is relatively similar to the other petals; it is lanceolate and subtily trilobed with a rolled margin, and is oriented upwards (Fig. 5B, C). In addition, the labellar spur is broad, short, and slightly reflexed, reaching only 1–1.5 mm in length (Figs 5A, D, H, 6H).

However, the spur and labellum of G. austriaca show similarities to those of G. conopsea and G. odoratissima at the cellular level. The interior surface of the spur contains numerous papillae, each with a bulbous – possibly secretory – tip (Figs 5D, G, 6H, M, N). The papillae are distributed evenly, in contrast with G. conopsea and G. odoratissima (Figs 5D, G, 6M), where they are prevalent only in the lower two-thirds of the spur and on the adaxial epidermis. In all three species the adaxial epidermis of the labellum is characterized by domed to conical cells (Figs 3H, 4A, C, 5E) that may function as osmophores (cf. Stpiczynska, 2001).

Labellum and spur

The Gymnadenia labellum is most commonly trilobed with a long- to moderate-length, filiform, nectariferous spur. In G. conopsea, the labellum is typically wider than long (Fig. 3H) and the lobes are more or less equally sized. The spur is usually more than twice as long as the ovary (Fig. 3A), 10–20 mm in length. The interior surface of the mature spur is covered with papillae in the lower two-thirds of the spur and on the adaxial inner epidermis, whereas the epidermis in the upper third of the spur consists exclusively of smooth epidermal cells (Fig. 3C). Closer examination of the papillae shows that they possess a bulbous tip (Fig. 3D), suggesting that they may be secretory.

The labellum of G. odoratissima is also typical of other Gymnadenia species, but differs subtly from that of G. conopsea. It is significantly longer than wide. The lateral lobes are rounded and the median lobe is wider, obtuse, and longer than the lateral lobes (Fig. 4A, C). The spur is much shorter than that of G. conopsea (3–6 mm), approximating the ovary, which is clearly resupinate (Figs 4A, C, D, 7G). The spur entrance is relatively narrow, as is the case with G. conopsea (Figs 3B, C, 4D). Numerous papillae are present in the lower two-thirds of the spur, where

nation in the ovary of *G. conopsea*, which is also apparent on the interior of the ovary (Figs 4C, F, H, 6F, G). In both *G. conopsea* and *G. odoratissima*, the direction of resupination is apparently random; 180° torsion can be either clockwise or anticlockwise, with no apparent pattern to the organization of clockwise/anticlockwise flowers in the inflorescence (e.g. *G. conopsea*; Fig. 10I, J).

Figure 2A–F. Photographs of flowers and inflorescences, showing long floral spurs (A, D), short spurs (C, F) and medium-length spur (E). Spurs indicated by arrows. Fig. 1A. *Gymnadenia conopsea* flowers. Fig. 1B. *G. conopsea* inflorescence. Fig. 1C. *G. austriaca* (formerly *Nigritella austriaca*) inflorescence. Fig. 1D. *Dactylorhiza fuchsii* flowers. Fig. 1E. *G. odoratissima* flowers. Fig. 1F. *D. viridis* flowers. Photographs: R. M. Bateman.
Figure 3A–H. Gymnadenia conopsea, floral morphology and ontogeny (SEM). Fig. 3A. Opening flower bud. Fig. 3B. Detail of mature gynostemium of developing bud. Fig. 3C. Epithelial surface of mature spur interior. Fig. 3D. Detail of spur papillae. Fig. 3E. Spur initiation at base of developing labellum. Fig. 3F. Lateral view, spur and gynostemium development. Fig. 3G. Interior detail of developing spur showing absence of papillae. 3H. Near-mature labellum showing detail of the crenulate margin of the lateral labellum lobes and the development of domed cells on the surface. Scale bars = 1 mm in A–C; 50 μm in D; 500 μm in E, F, H; 200 μm in G. Figure abbreviations: * – lateral petals, A1 – median adaxial fertile stamen, au – auricle, b – bract, co – anther connective, ds – dorsal sepal, la – labellum (lip), lc – lateral carpel lobe, Ll – lateral labellum lobe, ls1 – lateral sepal 1, ls2 – lateral sepal 2, ov – ovary, ro – rostellum, sp – spur, th – anther thecae.
In contrast, *G. austriaca* has a sessile, non-resupinate ovary (Figs 5A, F, 6M). Like *G. conopsea* and *G. odoratissima*, the ovary contains numerous small ovules that have parietal placentation. Examination of the interior of the ovary by light and electron microscopy has confirmed that the absence of resupination, evident from examination of the exterior of the ovary, is echoed throughout the interior structure (Figs 5F, 6H, M).

**FLORAL MORPHOLOGY: D. FUCHSII VS. D. VIRIDIS**

**Macromorphology**

In *Dactylorhiza* (Fig. 2D, F), the inflorescence is usually densely flowered, as in *D. fuchsii* (Fig. 2D), with leafy floral bracts often approximating the flowers in length (Figs 2D, 7A–C). The inflorescence is compact to lax, conical to subcylindrical, varies considerably in height, and bears numerous medium-sized pink flowers (Fig. 2D). Lateral sepals are ovate to lanceolate, asymmetrical, often spotted on the inner surface, spreading to suberect. The dorsal sepal is a stout, narrowly conical, apart from the very base of the labellum surrounding the entrance to the spur (Fig. 9F–H).

The spur of *D. fuchsii* is a stout, narrowly conical structure approximately as long as the ovary, 6–10 mm in length (Fig. 7A–D). Electron and light micrographs of the interior epidermis reveal numerous well-developed papillae (Figs 7F, 9G); these are distributed along the entire length of the spur, although they are concentrated towards the apex. In *D. fuchsii*, the papillae have a bulbous tip, suggesting that they are secretory (Fig. 7D, F).

The elongate labellum of *D. viridis* (typically 10 mm long and 3 mm wide) is flat, penduncled, or sometimes bent backwards, with a median longitudinal groove (Fig. 8A, C–E). Like the other perianth parts, the labellum is mostly green with a brown–purple margin (Fig. 2F). The elongate, parallel-sided labellum terminates in three lobes, the lateral lobes exceeding the tooth-like median lobe (Fig. 8C). Prior to anthesis, the lateral lobes form a tight-fitting cover over the short, erect gynostemium (Figs 9A, B, F, 14G, H). Numerous domed cells, visible even by eye, are evenly distributed over the labellum surface. These cells are less elaborate in structure than those that characterize the labellum of *D. fuchsii* (Figs 7E, 8C, 9A, B, 14H), and are unlikely to be osmophores.

The labellum possesses a globose (almost spherical), basally bilobed spur (Figs 8A, B, E, 9C, D). Cells of the interior epidermis of the spur form a smooth surface with no evident papillae, even in mature spurs dissected from flowers at anthesis (Fig. 8F, 14I).

**Labellum and spur**

The labellum of *D. fuchsii* is large (Figs 2D, 7A–D), 6–10 mm long and 8–16 mm wide; it is deeply trilobed, variously patterned with purple loops, spots, and dashes (Fig. 2D). The lateral lobes of the labellum are wrapped around the sides of the gynostemium in bud, but are broadly spread at anthesis (Fig. 7A–D); they are rounded, occasionally with a moderately crenulate margin. The triangular median lobe is almost as wide as, and typically longer than, the lateral lobes.

Detailed examination of the interior surface of the *D. fuchsii* labellum reveals that the cells are distinctly conical, apart from the very base of the labellum surrounding the entrance to the spur (Fig. 9F–H).

In contrast, *D. fuchsii* has a short, erect gynostemium (Figs 7G, 9E). The anther is erect, tapering towards the base, consisting of a pair of large anther locules united by a broad and pointed anther connective; towards its base, the viscidia are formed in the pouch bursicula (Fig. 7A). The gynostemium has two small, but rather prominent, lateral auricles, located on either side of the fertile stamen above two relatively undifferentiated...
basal bodies, to which they have become fused (Fig. 9F). The median carpel forms the three-lobed rostellum, the median lobe of which is pleated and usually situated between the large parallel anther loculi (Fig. 9E, F). The receptive lateral carpels that form the broad, bilobed stigmatic surface are clearly visible within the neck of the broad spur entrance (Fig. 7G).

Figure 5A–H. Gymnadenia austriaca, floral morphology (SEM). Fig. 5A, B. Mature flower at anthesis, showing the erect, lanceolate labellum, short globose spur and sessile, non-resupinate ovary. Fig. 5C. Mature labellum dissected to show the rolled margin formed by the reduced lateral labellum lobes. Fig. 5D. Mature spur dissected; arrow indicates numerous papillae in the lower two thirds of the spur interior. Fig. 5E. Domed cells of labellum surface. Fig. 5F. Longitudinally split ovary, clearly demonstrating non-resupination. Fig. 5G. Details of spur papillae. Fig. 5H. Mature flower longitudinally split to show the labellum, gynostemium and spur. Scale bars = 1 mm in A–C, F, H; 300 μm in D; 100 μm in E; 50 μm in G. Figure abbreviations: b – bract, ds – dorsal sepa, dp – dorsal petal, Gy – gynostemium, la – labellum (lip), Ll – lateral labellum lobe, ls1 – lateral sepal 1, ls2 – lateral sepal 2, mL – median labellum lobe, ou – ovule, ov – ovary, pl – placenta, sp – spur, th – anther thecae.
The gynostemium of *D. viridis* is similar to that of *D. fuchsii* in terms of gross structural organization, although superficially it looks substantially different (Fig. 8A, B, F). The anther is broader and square in shape; the anther loculi are relatively small and shallow (Fig. 9A, B). Below the anther, the median carpel lobe consists of a trilobed rostellum, the median part of which is located between the anther loculi in a fairly open pleat. Clearly visible within the median part of which is located between the anther loculi is a trilobed rostellum, the receptive stigmatic surface (Fig. 8A, F). The stigmatic surface is a broad bilobed structure, similar in shape to that of *D. fuchsii* but smaller (Fig. 8A, F).

In both *D. fuchsii* and *D. viridis*, the ovary is cylindrical-fusiform, sessile, resupinate, and glabrous, the single locule containing numerous small ovules with parietal placentation (Figs 7A, 8B, E, 9D, I). The three parietal placentas twist around the interior surface of the ovary, providing a further demonstration of the extent of 180° torsion in the ovary and resupination of the flower (Figs 8B, D, E, 9C, D, I).

**FLORAL ONTOGENY: GYMNADENIA CONOPSEA/ODORATISSIMA VS. G. AUSTRIACA**

**Ontogeny of the perianth**
The first perianth organs to be initiated are the sepals, which are notably larger than the structures observable in the inner perianth whorls (Fig. 10A, B). The primordium of the labellum is clearly visible between the lateral sepals and the slightly larger lateral petals. Based on the relative size of the organs, we conclude that the lateral petals are initiated after the lateral sepals, but prior to the labellum. The gynostemium develops subsequent to the perianth segments (Fig. 10C) and appears to follow a developmental programme typical of other orchidoid orchids (cf. Kurzweil, 1987, 1998).

**Labellum, spur, and ovary development:** the primitive (ancestral) condition
In both *G. conopsea* and *G. odoratissima*, the spur is initiated at the base of the labellum, at approximately the same time as the anther is completing its development, but prior to, or concurrent with, the initiation of later developing gynostemium parts; these include the auricles, basal bulges, rostellum, and the receptive stigmatic lobes (Figs 3E, F, 10C, D). Thus, spur initiation should be attributed to mid-stage development of the flower, following the terminology of Kurzweil (1987). The lateral lobes of the labellum develop shortly after, prior to the extension of the spur and ovary and the initiation of the auricles, which occurs afterwards (Figs 3E, F, 10C–H). Coincident with initiation of the lateral lobes of the labellum and the auricles, the spur begins to elongate by intercalary growth somewhat later than the extension of the ovary, subsequently catching up prior to resupination, which seems to occur during extension of the ovary (Figs 3F, 10F–J). In *G. conopsea*, the spur continues to elongate after the ovary has completed its development until its mature size is reached during, or shortly after, anthesis (Fig. 3A).

Dissection of the early spur to reveal the spur interior epidermis demonstrates the later development of the papillae that characterize the distal two-thirds of the spur in both species (Figs 3C, F, 4E, G, 10K). Such floral details are amongst the last structures to develop. Similarly, examination of a dissected labellum in *G. conopsea* (Fig. 3H) and a young labellum of *G. odoratissima* reveals that the dome-shaped cells that characterize the inner surface of the labellum are amongst the last structures to develop, probably just prior to the spur papillae.

**Labellum, spur, and ovary development: the derived (descendant) condition**
Initiation of the spur from the base of the labellum occurs in much the same way in *G. australis* as in the other two *Gymnadenia* species examined (Fig. 11A, B), prior to the initiation of later developing gynostemium parts, such as the auricles and basal bulges. In contrast with the other *Gymnadenia* species, the spur ceases development early, quickly attaining its mature size prior to the extension of the ovary. The developing ovary never shows any sign of 180° torsion related to floral resupination, thus resembling the juvenile ovaries of *G. conopsea* and *G. odoratissima* (Figs 5A, 11C–H).

Dissection of the spur at this stage of development reveals that, as is the case for *G. conopsea* and *G. odoratissima*, the papillae that characterize the epidermis of the mature spur interior are not yet evident; they form last of all, immediately prior to anthesis (Figs 5D, G, 11H). The presence of these papillae within the reduced spur immediately prior to anthesis shows that, despite the early cessation of growth, the spur is a mature organ.

The labellum of *G. australis* is initiated complete, lacking the lateral lobes seen in most *G. odoratissima* and all *G. conopsea*. Despite the lanceolate appearance of the mature labellum, close examination of the developing labellum clearly shows the presence of two narrow, elongate lateral lobes that seem to arrest their development relatively early, contributing to the rolled lateral margin of the otherwise apparently entire labellum (Figs 5A–C, 11F).

**FLORAL ONTOGENY: D. FUCHSII VS. D. VIRIDIS**

**Ontogeny of the perianth**
Early floral ontogeny in both species is mostly similar to that of other orchidoid species (cf. Kurzweil, 1987),
as described earlier for Gymnadenia. In flowers of D. fuchsii, the spur is initiated from the base of the labellum at approximately the same time that auricles are initiated on either side of the developing anther. At this point, the lateral lobes of the labellum have yet to form (Fig. 12D, E), whereas, in Gymnadenia, the lateral labellum lobes form later, shortly after the auricles have been initiated. Interestingly, in D. fuchsii, auricles are initiated whilst the gynostemium is still relatively immature (Fig. 12F), as revealed by the dissection of young buds with already initiated spurs (Fig. 12B, C).

Although the auricles are relatively well differentiated in these buds, the median carpel apex has yet to extend into the deeply pleated rostellum; nonetheless, it has already extended between the anther thecae to form a deeply trilobed structure typical of orchidoids (cf. Kurzweil, 1987). Lateral carpel apices are also relatively unelaborated at this point (Fig. 12B, C), but resemble the broadly spread, bilobed receptive structure that sits within the spur entrance at maturity (Figs 7A, 12C, 13D, E).

Labellum, spur, and ovary development: the primitive (ancestral) condition

Later development of the labellum proceeds by intercalary growth of the lateral lobes, coincident with extension of the spur and ovary (Figs 12D–F, 13A–C). The conical cells that characterize the mature labellum surface are initially absent, forming later by elaboration of the domed cells, which extend apically throughout the duration of organ maturation (Fig. 13H, I). The ovary initially elongates at a greater rate than the spur, but later the spur catches up, prior to resupination of the ovary (Fig. 13E). As the spur elongates, details of the accessory organs characteristic of the mature gynostemium become elaborated, particularly the developing rostellum, auricles, and the anther, which itself increases considerably in girth, generating the steeply tapering anther towards its base (Fig. 13D). Dissection of the developing spur (Fig. 13D–G) reveals that papillae are absent from D. fuchsii prior to spur elongation, although they develop much sooner than in Gymnadenia, long before resupination (Fig. 13E, F).

Labellum, spur, and ovary development: the derived (descendant) condition

In D. viridis, spur development ceases relatively early, compared with the ovary and the gynostemium elements, as indicated by the size of the spherical spur and the semi-mature nature of the lateral stigma lobes, which typically characterize the latest stages of floral development in orchidoids (Fig. 14B–G). Comparison of different stages of spur development suggests that the interior of the mature spur retains immature features, such that no papillae develop (Fig. 14I).

Interestingly, floral development in D. viridis includes resupination of the ovary, despite the apparent lack of maturity in the spur (Figs 8B, D, E, 14G, I). The lateral labellum lobes and spur are initiated at approximately the same time, at a point at which the gynostemium is approaching maturity (Fig. 14B–F). The differential size of the lateral and median labellum lobes, which are initiated coincident with the development of the spur, is caused by extended growth of the lateral labellum lobes (Fig. 14E–G). The domed cells that characterize the labellum of D. viridis (Fig. 14H) resemble those of the early developing D. fuchsii labellum, suggesting that the labellum of D. viridis also experiences premature arrest of cellular differentiation.

DISCUSSION

Paedomorphic features in orchid flowers

We have identified several distinctly paedomorphic features in the flowers of the derived member of each species pair compared with the presumed ancestral form. That is, features of the flowers of the derived species resemble earlier developmental stages of their respective ancestors. Reduction of the labellar spur, simplification of the labellum, and non-resupination of the ovary are all apparently paedomorphic features that are likely to have played key roles in the
Figure 7A–G. Dactylorhiza fuchsii, floral morphology (SEM). Fig. 7A. Mature flower at anthesis, showing deeply trilobed labellum and stout, conical spur. Fig 7B. Mature bud prior to anthesis and resupination showing stout, conical spur. Fig. 7C. Mature bud prior to anthesis, dissected to show link between labellum and gynostemium; the lateral lobes of the labellum are wrapped around the thecae of the gynostemium. Fig. 7D. Dissected spur/labellum shortly prior to anthesis, showing conical cells (arrowed) on labellum surface. Fig. 7E. Conical cells on labellum surface. Fig. 7F. Papillae on the internal surface of the spur. Fig. 7G. Mature gynostemium, showing elongate, tapering anther with lanceolate connective, sculptured auricles, rostellum and the receptive lateral carpel lobes in the neck of the spur. Scale bars = 1 mm in A–D; 50 μm in E, F; 500 μm in G. Figure abbreviations: * – lateral petals, A1 – median adaxial fertile stamen, au – auricle, b – bract, bu – bursicula, co – anther connective, Gy – gynostemium, la – labellum (lip), lc – lateral carpel lobe, Ll – lateral labellum lobe, ls1 – lateral sepal 1, ls2 – lateral sepal 2, mL – median labellum lobe, ov – ovary, ro – rostellum, se – spur entrance, sp – spur.
**Figure 8A–F.** *Dactylorhiza viridis*, floral morphology and ontogeny (SEM). Fig. 8A. Mature flower, perianth parts dissected, showing details of elongate labellum, gynostemium, receptive stigmatic surface and spur entrance (arrow); the ovary is resupinate. Fig. 8B. Lateral view of mature column just prior to anthesis; the spur is reflexed and globose. Fig. 8C. Dissected mature labellum; note lateral labellum lobes are much longer than median one, which is reduced and rounded to triangular in shape. Fig. 8D. Details of 180° torsion of the ovary, which had been split longitudinally to reveal three parietal placentas bearing numerous small ovules; the dorsal and lateral sepals are erect, forming a hood that encloses the gynostemium. Fig. 8E. Lateral view of mature flower, lateral sepals removed, showing linear lateral sepals enclosed in hood formed by erect petals. Fig. 8F. Details of mature gynostemium showing prominent auricles and basal bulges; the anther is short and square, as is the pleat of the rostellum; dissection of the spur in this mid-mature bud has revealed the absence of papillae. Scale bars = 1 mm. Figure abbreviations: * - lateral petals, **A1** – median adaxial fertile stamen, **au** – auricle, **b** – bract, **bb** – basal bulges, **ds** – dorsal sepal, **Gy** – gynostemium, **la** – labellum (lip), **lc** – lateral carpel lobe, **lc1** – lateral carpel lobe 1, **Ll** – lateral labellum lobe, **ls1** – lateral sepal 1, **mL** – median labellum lobe, **ou** – ovule, **ov** – ovary, **pl** – placenta, **ro** – rostellum, **sp** – spur, **th** – anther thecae.
Figure 9A–I. *Dactylorhiza viridis* (A–D), *D. fuchsii* (E–I), transverse sections of flowers (LM). Fig. 9A, B. TS mature gynostemium. Fig. 9C. TS spur and ovary; papillae absent from interior spur surface. Fig. 9D. TS ovary showing parietal placentation and numerous small ovules. Figs 9E, F. TS mature gynostemium. Fig. 9G. TS spur, showing numerous bulbous papillae lining interior of spur (arrow). Fig. 9H. TS mature labellum showing conical cells. Fig. 9I. TS ovary showing parietal placentation and numerous small ovules. Scale bars = 10 μm. Figure abbreviations: *– lateral petals, au – auricle, ds – dorsal sepal, la – labellum (lip), ls1 – lateral sepal 1, ls2 – lateral sepal 2, ou – ovule, ov – ovary, pl – placenta, po – pollinium, ro – rostellum, sp – spur, th – anther thecae.

Figure 10A–K. Gymnadenia odoratissima, floral ontogeny (SEM). Fig. 10A. Young bud. Fig. 10B, Labellum development. Fig. 10C. Spur initiation at base of labellum (arrow). Fig. 10D. Young spur in early bud emerging between the lateral sepals. Figs 10E–J. Growth and elongation of spur and ovary prior to resupination, coincident with development of lateral labellum lobes; the auricles of the gynostemium are already initiated (F, G). Initiation of resupination can be either anticlockwise (I), or clockwise (J). Fig. 10K. Developing spur dissected to reveal interior epidermis; note the absence of papillae. Scale bars = 100 μm in A, K; 200 μm in B, C, E; 500 μm in D, F; 1 mm in G–J. Figure abbreviations: * – lateral petals, A1 – median adaxial fertile stamen, au – auricle, b – bract, ds – dorsal sepal, Gy – gynostemium, la – labellum (lip), Li – lateral labellum lobe, ls1 – lateral sepal 1, ls2 – lateral sepal 2, ov – ovary, sp – spur, th – anther thecae.
Figure 11A–H. Gymnadenia austriaca, floral ontogeny (SEM). Fig. 11A, B. Details of spur initiation (A) front, (B) reverse, of the same bud, showing spur initiation prior to initiation of auricles; arrow indicates the position of the initiating spur. Fig. 11C. Later spur development and elongation prior to extension of the ovary. Figs 11D, E. Spur and gynostemium attain maturity relatively early, prior to preanthetic enlargement of ovary. Fig. 11F. Extension of ovary and labellum and initiation of lateral labellum lobes. Figs 11G, H. Details of mature gynostemium, (H) dissected, apparently mature spur; note absence of papillae. Scale bars = 300 μm in A, E; 500 μm in B–D, F. Figure abbreviations: A1 – median adaxial fertile stamen, au – auricle, b – bract, co – anther connective, la – labellum (lip), lc1 – lateral carpel lobe 1, lc2 – lateral carpel lobe 2, Ll – lateral labellum lobe, ov – ovary, ro – rostellum, se – spur entrance, sp – spur, th – anther thecae.
speciation of *G. austriaca* and *D. viridis* from their respective, presumably long-spurred ancestors. The ontogenetic series suggests that, in each case, growth of these organs ceases prematurely, although to significantly different degrees for each organ in the two species comparisons considered here. The only likely exception is the elongated labellum of *D. viridis*, which probably represents over-development of the lateral lobes via hypermorphic peramorphosis, achieving a final morphology apparently more advanced than that of the ancestor through a later cessation of growth (Gould, 1977).

**Figure 12A–F.** *Dactylorhiza fuchsii*, early floral ontogeny (SEM). Fig. 12A. Young bud. Figs 12B, C. Details of late gynostemium development, the anther, auricles and lateral carpel lobes are already formed; meanwhile, the median carpel lobe grows between the anther thecae and differentiates into the rostellum. Fig. 12D. Spur initiation at base of labellum; lateral labellum lobes are yet to be initiated. Fig. 12E. Auricles are initiated at approximately the same time as the spur. Fig. 12F. Lateral lobes of the labellum are initiated and grow prior to further extension of the initiated spur and ovary. Scale bars = 100 μm in A; 200 μm in B–F. Figure abbreviations: * – lateral petals, A1 – median adaxial fertile stamen, au – auricle, co – anther connective, ds – dorsal sepal, la – labellum (lip), lc – lateral carpel lobe, Ll – lateral labellum lobe, ls1 – lateral sepal 1, mC – median carpel lobe, mL – median labellum lobe, ov – ovary, sp – spur, th – anther thecae.
Figure 13A–I. Dactylorhiza fuchsii, late floral ontogeny (SEM). Fig. 13A. Details of spur initiation in a non-dissected bud, the spur is initiated between the lateral sepals at the base of the labellum. Fig. 13B. The labellum continues to develop prior to elongation of the spur, lateral lobes of the labellum are visible and the median lobe has extended. Fig. 13C. Auricles are visible at the side of the fertile anther. Figs 13D–G. Spur extension occurs relatively late after development of the gynostemium (D); numerous developing papillae visible on the inner epidermis (G), as the spur elongates, papillae become more pronounced over the entire inner epidermis of the spur (E, F), arrows indicate spur papillae. Figs 13H–I. The conical cells of the labellum surface develop late by apical extension of the dome-shaped cells that cover the labellum surface at an early stage of its development. Scale bars = 1 mm in A; 50 μm in G, H, 500 μm in B–F, I. Figure abbreviations: A1 – median adaxial fertile stamen, au – auricle, co – anther connective, la – labellum (lip), lc – lateral carpel lobe, LL – lateral labellum lobe, mL – median labellum lobe, ov – ovary, ro – rostellum, sp – spur, th – anther thecae.

PROGENESIS CONTRIBUTES SUBSTANTIALLY TO FLORAL EVOLUTION IN ORCHIDINAE

Paedomorphosis is the displacement of ancestral features to later stages in the ontogeny of presumed descendants (Gould, 1977; Alberch et al., 1979). It is a concept that is commonly referred to in animal evolutionary studies and, although it may be a frequent occurrence in plant evolution, there are considerably fewer examples of its discussion in the plant literature (Li & Johnston, 2000). Mechanistically, paedomorphosis can occur via one of three routes: (1) neoteny, (2) progenesis; or (3) post-displacement. In the zoological literature, paedomorphosis is usually determined by reference to the relative timing of somatic and reproductive development. Thus, neoteny is defined as a delay in somatic development with respect to reproductive maturity, and progenesis is defined as the early onset of reproductive maturity, often resulting in an early cessation of somatic growth and development (Gould, 1977; Alberch et al., 1979). Post-displacement paedomorphosis occurs when the onset of development of a trait is delayed, resulting in incomplete trait development (Alberch et al., 1979).

However, in organisms that do not sequester their germline and in which the timing of reproductive maturity may be different for different branches or modules, such definitions are unhelpful. Using a qualitative model of heterochrony involving size, shape, and age as independent variables (Alberch et al., 1979), more botanically relevant definitions have been sought (for example, Bateman, 1994). In plants, neoteny is best defined as a reduction in the rate of development of an organ, relative to the equivalent organ in an ancestor, whereas progenesis is characterized by premature cessation of growth of an organ in the descendant relative to the equivalent organ in the ancestor (Bateman, 1994). The definition of post-displacement paedomorphosis is the late onset of organ growth relative to unaltered offset time.

The patterns of floral paedomorphosis observed in G. austriaca – spur reduction and labellum simplification – and in D. viridis – spur reduction only – appear to be generated by premature cessation of growth in these organs relative to the ontogeny of the presumed ancestor (Fig. 15). Thus, the development of these organs appears to be progenetically curtailed. By contrast, non-resupination of the ovary in G. austriaca appears to lie outside the realms of bona fide heterochrony, but rather results from the deletion of an entire, arguably terminal, developmental stage from the putative descendant relative to the putative ancestor. Loss of this developmental stage is not a necessary consequence of floral progenesis, as resupination has been retained in the ontogeny of D. viridis.

DEGREE OF HETEROCHRONY DIFFERS BETWEEN G. AUSTRIACA AND D. VIRIDIS

In the present study, the extent of paedomorphosis differs between G. austriaca and D. viridis. In contrast with the reduced spur of G. austriaca, which is characterized internally by papillae, D. viridis does not possess these structures lining the interior of the spur at maturity. In fact, these structures are never initiated in D. viridis, perhaps reflecting deletion from the ancestral ontogeny rather than true heterochrony. Alternatively, the absence of papillae in D. viridis could be the result of a loss of these structures, independent of the larger scale trend for spur reduction.

Other paedomorphic characters are more obviously divergent between the two paedomorphic events considered. Simplification of the labellum is more apparent in G. austriaca, which has a lanceolate, barely trilobed labellum, quite different from the deeply trilobed labellum characteristic of long-spurred members of the Gymnadenia clade. In contrast, although clearly very different from the labellum of longer spurred members of the genus Dactylorhiza, the labellum of short-spurred D. viridis retains its trilobed structure and appears to be peramorphic, rather than paedomorphic. In addition, the ovary of G. austriaca is non-resupinate, whereas the ovary of
Figure 14A–I. Dactylorhiza viridis, floral morphology and ontogeny (SEM). Fig. 14A. Earliest floral ontogeny. Fig. 14B. Spur initiation at base of labellum, coincident with lateral labellum lobe development. Fig. 14C. Elaboration of lateral labellum lobes, spur and ovary. Fig. 14D. Later elaboration and enlargement of spur into a broad saccate structure. Figs 14E, F. The spur rapidly attains its mature size and shape and the lateral labellum lobes elongate. Fig. 14G. Semi-mature bud, the labellum forms a tight-fitting cap that sits on top of the anther, the spur has completed its development prior to 180° torsion of the ovary; the auricle and basal bulges are almost fully differentiated. Fig. 14H. Young labellum showing dome-shaped epidermal cells; the lateral lobes form a cap that fits neatly over the anther. Fig. 14I. Mature spur dissected to show the absence of papillae. Scale bars = 100 μm in A; 500 μm in B–F, I; 1 mm in G, H. Figure abbreviations: * — lateral petals, A1 — median adaxial fertile stamen, au — auricle, la — labellum (lip), Ll — lateral labellum lobe, ov — ovary, ro — rostellum, sp — spur, th — anther thecae.

D. viridis retains the ancestral resupinate condition. Although not clearly the result of bona fide heterochrony, this is a further example of the different extent to which floral ontogeny has been modified in each of the descendant species considered with respect to their putative ancestor.

DECOUPLING OF MICROMORPHOLOGICAL FROM MACROMORPHOLOGICAL HETEROCHRONIC SHIFTS

Assuming that the loss of papillae in D. viridis results from heterochronic shifts rather than independent loss, it appears that, in addition to considerable variation in the extent of heterochronic change between the species comparisons considered, heterochrony could operate independently at different scales (organs/part organs/cells) in the flowers of each of the descendant species. Examination of the spur ultrastructure in G. austriaca reveals that the paedomorphosis so evident in the gross structure of the spur is not reflected at the cellular level. Although the reduced spur of G. austriaca is clearly paedomorphic at a macromorphological level, the interior of the spur retains secretory papillae — the ancestral character state — at maturity. 

Previous ultrastructural studies of nectar secretion in the spurs of G. odoratissima (Stpiczynska & Matusiewicz, 2001), which has a longer spur than G. austriaca, have suggested that structures identical to those lining the spur of G. austriaca may be required for nectar secretion in G. odoratissima. Although the spur has become reduced, it seems that it may not have lost the ability to function as a nectar-secreting organ, as suggested by earlier reports of small amounts of nectar occurring in flowers of G. austriaca (van der Pijl & Dodson, 1966; van der Cingel, 1995; Delforge, 2006). Further anatomical and histological studies are required to clarify the precise functional role (if any) of the papillae in the nectar spurs of these species.

These observations suggest that cellular maturity and organ maturity need not necessarily be concordant, and that paedomorphosis can operate differently at both the macromorphological and micromorphological levels.

CONSEQUENCES OF HETEROCHRONIC SHIFTS

Spurs and speciation

Despite substantial differences in floral morphology and ontogeny, there is little variation in ITS sequence data amongst long- and short-spurred representatives in both the Gymnadenia and Dactylorhiza clades (Bateman et al., 2003). This suggests that such paedomorphic features may be responsible for the recent radiation of short-spurred species amongst Orchidaceae in general and within the Gymnadenia clade in particular (Bateman et al., 2006) (Fig. 1C).

The morphology of the nectar spur has been intimately tied to reproduction in several angiosperm groups. Simple differences in the length, shape, orientation, and coloration of spurs are commonly associated with different pollinators and affect reproductive isolation (Hodges & Arnold, 1995; Hodges, 1997a; Whittall & Hodges, 2007). Phylogenetic evidence shows that nectar spurs have evolved on multiple occasions in a diverse range of angiosperms (Hodges, 1997b), especially within Orchidaceae (Smets et al., 2000; Rudall, Manning & Goldblatt, 2003), and spur evolution has frequently been linked to the unusually high species richness of these groups of flowering plants.

Many of the authors investigating the relationship between the possession of spurs and species diversification rates have focused on Aquilegia species (for example, Hodges & Arnold, 1994, 1995). In the Disa draco complex (Orchidaceae), similar results have been obtained from a variety of natural populations (Johnson & Steiner, 1997). Experimental shortening of nectar spurs in the flowers of members of the Disa complex, and in another orchid, Platanthera (Nilsson, 1988), significantly reduced the frequencies of both pollen deposition and subsequent fruit set, by restricting crucial access needed by a specific long-tongued pollinator. This appears to be a convincing demonstration of the role of spurs in promoting reproductive isolation (but see Bateman & Sexton, 2008).
previous studies are too parochial to be of great help in understanding orchid evolution, wherein the importance of co-evolution with pollinators has been hugely exaggerated. In the absence of concrete evidence for or against shifts in pollinators or switches from allogamy to autogamy, we cannot conclude whether this phenomenon explains the origin of the ‘Nigritella’ floral phenotype.

In addition, if spur reduction also resulted in functional vestigiality, a switch to deceptive pollination may have been the predominant driver of such a radiation; this shift has been shown to radically and repeatedly alter pollinator behaviour and the genetic profiles of orchid populations (Cozzolino & Widmer, 2005). In the case of the former genus Nigritella, at least, we find this hypothesis unlikely in the presence of good micromorphological evidence and observations of nectar in the spur, suggesting that nectar production survived radical spur reduction. This implies that the pollinators are still rewarded, albeit to a lesser degree (Fig. 1C). However, the presence of papillae is not a guaranteed indicator of nectar secretion, as D. fuchsii, with prominent papillae-like cells lining the interior epidermis, does not secrete nectar. By contrast, D. viridis, whilst lacking any obvious nectar-secreting structures inside the spur, has been reported to secrete small amounts of nectar that may originate from sources other than the spur. This may well represent a shift from a deceptive to rewarding pollination strategy.

A further possible explanation for the presence of paedomorphic orchid flowers is in keeping with zoological considerations of the ecological and evolutionary consequences of paedomorphosis (Gould, 1977). Progenetic paedomorphosis in the spur may increase the range of animals able to access nectar, and thus reduce pollinator specificity. However, although many of these explanations seem plausible, seeking adaptive significance in spur reduction may be wholly unnecessary (Bateman & Sexton, 2008). We have observed all study taxa flourishing at a single high-altitude site, although with some differences regarding prevalence with respect to altitude. Both short- and long-spurred representatives occur alongside one another, with no apparent evidence to suggest any difference in competitiveness or reproductive success. In the absence of field observations or genetic studies to suggest any competitive advantage of flowers with short vs. long nectar spurs, we are unable to conclude that there are any adaptive advantages associated with spur reduction. Consequently, there may be no adaptive consequences for spur reduction in Orchidinae; rather, this phenomenon would exemplify a case of making do with whatever mixed hand a lineage is dealt by Nature.

POTENTIAL INSIGHTS FROM SPUR 
EVOLUTIONARY-DEVELOPMENTAL GENETICS: 
SALTATIONAL MACROEVOLUTION IN ORCHIDINAE

Bateman & DiMichele (2002) proposed that a single mutation in a critical developmental gene might have instantaneously triggered the origin of the 'Nigritella' floral phenotype from within the Gymnadenia clade. Dramatic alterations in floral morphology that might have resulted from mutation in a single key developmental gene exemplify a possible case of post-saltational radiation (i.e. species radiation following a dramatic evolutionary leap in phenotype) amongst members of the subtribe Orchidinae. Our data are consistent with this suggestion, as the observed heterochrony is most probably the result of changes in timing of expression of a single master regulatory gene.

Further insights into the role of heterochrony in orchid floral evolution should be gained through future investigations of the molecular developmental genetics of nectar spurs in Orchidinae and other relevant taxa. Previous studies of spur genetics have focused largely on the inheritance of spurs in Aquilegia species, where a single gene is demonstrably critical to the inheritance of the nectariferous spur (Prazmo, 1965). Only recently have clues as to the identity of the gene(s) involved in spur development become apparent. The isolation of two independent spontaneous dominant mutations in Antirrhinum majus (Golz, Keck & Hudson, 2002), which possesses ectopic 'spur-like' petal tubes similar to those that characterize other spurred members of the Antirrhineae, may provide clues to the identity of these genes. We hope that our present evolutionary–developmental genetic investigations into the genetic regulation of spur development amongst key members of the subtribe Orchidinae will provide further insights into the role of heterochrony in orchid floral evolution.

ACKNOWLEDGEMENTS

We thank the members of the Micromorphology Section, Royal Botanic Gardens, Kew and the Molecular Development Group, University of Cambridge for valuable advice and discussion. Thanks are also due to Peter Endress and Louis Ronse De Craene for critically reviewing the manuscript. RMB is grateful to the Botanical Research Fund for a small fieldwork grant.

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