

## Phylogenetic context, generic affinities and evolutionary origin of the enigmatic Balkan orchid *Gymnadenia frivaldii* Hampe ex Griseb.

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Although originally ascribed to the genus *Gymnadenia* R. Br. (Orchidinae: Orchidaceae), the Balkan endemic orchid *G. frivaldii* Hampe ex Griseb. has since been more frequently assigned to *Pseudorchis* Séguier (syn. *Leucorchis* E. Mey., *Bicchia* Parl.). Molecular phylogenetic analysis using the ITS region of rDNA reveals a large disparity between the two genera and demonstrates that *frivaldii* is embedded well within *Gymnadenia* s.s. Macromorphological and SEM studies further elucidate the floral and vegetative similarities between *G. frivaldii* and *Pseudorchis*, notably the heterochronically reduced gynostemium and small, short-spurred labelum; these similarities represent convergent evolutionary transitions, whereas other characters such as contrasting stigma and tuber morphologies provide stronger phylogenetic signals. The sequence-based phylogeny suggests that *G. frivaldii* represents one of three cases of independent paedomorphic floral reduction inferred in the genus; simplification has been more severe than in *G. odoratissima* but less severe than in the closely related *Gymnadenia* subgenus *Nigritella*. Alternatively, an effectively instantaneous evolutionary origin through hybridisation with a (most likely diploid) species of subgenus *Nigritella* remains possible. Reports of rare hybridisation between *G. frivaldii* and members of subgenus *Nigritella* are acceptably well documented, whereas reports of hybridisation with several other more phylogenetically distant orchid species (including the often sympatric *Pseudorchis albida*) are considered less secure.

**KEYWORDS:** *Gymnadenia*, heterochrony, hybridisation, Internal Transcribed Spacer, molecular phylogenetics, Orchidaceae, Orchidinae, paedomorphosis, *Pseudorchis*, Scanning Electron Microscopy.

### INTRODUCTION

Native orchids are used across Europe as charismatic flagships for both scientific research and motivating active conservation. Not surprisingly, they are among the most thoroughly studied and thus best understood species in the European flora. Nonetheless, there remain a few undesirably enigmatic taxa, particularly those confined to limited geographic areas in under-explored regions of Europe. One such species is *Gymnadenia frivaldii* Hampe ex Griseb., a plant more commonly referred to in late 20<sup>th</sup> century literature as *Pseudorchis frivaldii* (Hampe ex Griseb.) P.F. Hunt. This uncertain generic assignment between the trans-Eurasian genus *Gymnadenia* and the circumboreal genus *Pseudorchis* (epitomised by *P. albida*) reflects a more general collective ignorance of the nature of this ecologically specialised Balkan endemic, which resembles many other dominantly alpine orchids, including *P. albida* and *G.* subgenus *Nigritella*, in having small and unusually compact flowers and inflorescences (Fig. 1). *Pseudorchis albida* has also received relatively little attention in the literature; for example, being largely ignored in Vermeulen's (1947) otherwise excellent summary of the sys-

tematic history of subtribe Orchidinae.

In this paper we assign *G. frivaldii* to its correct molecular phylogenetic (and thus generic) placement within subtribe Orchidinae (cf. Bateman & al., 2003), compare its morphology with other species of *Gymnadenia* and *P. albida* in the light of more detailed macromorphological and micromorphological evidence, and speculate on the potential importance of heterochrony and/or allopolyploidic hybridisation in the origin of this and other species in the now well-resolved tapered-tuber clade within Orchidinae.

### MATERIALS AND METHODS

**Field observation and collection.** — Single inflorescences were sampled in silica gel from substantial populations of *G. frivaldii* in northern Greece (two populations: Kaimaktealan and Smolikas) and southwest Bulgaria (two populations: Vitosha Mts. and Pirin Mts.) by Katharine Fairhurst and Mihaela Nikolova, in June–July 2003, each supported by photographic evidence (Appendix). The rootstock of one Greek plant was also examined and photographed before being re-

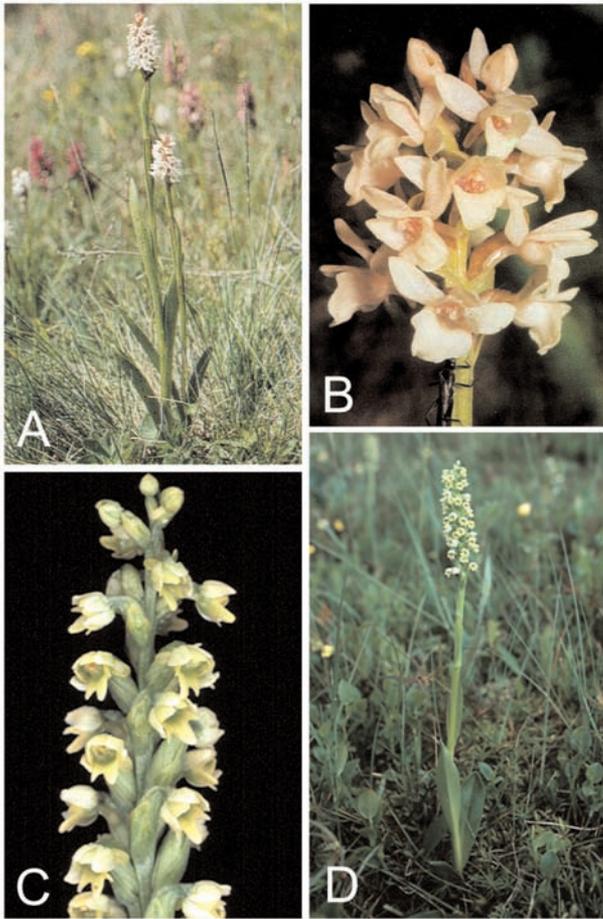


Fig. 1. A, B, *Gymnadenia frivaldii*: A, In situ at Kastoria, northern Greece; B, inflorescence, Pec, Slavic Macedonia; C, D, *Pseudorchis albida*: C, inflorescence, Keltney Burn, SC Scotland; D, In situ at Keltney Burn. Photos: A, from Delforge (2001, p. 140), B, from Baumann & Künkele (1982, p. 373); C, D, by R. Bateman.

interred. Morphological observations were supplemented with herbarium studies (notably at K) and any reliable data in the sparse existing literature.

**Scanning electron microscopy.** — Selected flowers from one Bulgarian specimen of *G. frivaldii* for SEM study were rehydrated from silica-gel dried material collected primarily for DNA extraction; they were subsequently stored in 70% ethanol. The spirit collection at RBG Kew yielded further alcohol-fixed inflorescences of *G. frivaldii* (*M. A. Clements* 2784, Greece; *E. R. S. Winter* 40140, Bulgaria) and *P. albida* (*E. M. Payne* 18500, Scotland; *J. J. Wood* 346, Italy). Selected flowers from each inflorescence were dehydrated through an alcohol series to 100% ethanol. They were then critical-point dried using a Balzer CPD 020 unit, mounted on stubs using double-sided tape, coated with gold and examined under a Hitachi cold-field emission SEM S-4700-II at 2 kV. The resulting images were recorded dig-

itally for subsequent manipulation in Adobe Photoshop. Comparison of the flowers of *G. frivaldii* originally preserved in spirit and silica gel demonstrated the absence of preservation-related artifacts.

**Molecular phylogenetic analysis.** — Base-pair sequences were obtained from the widely used, rapidly mutating ITS region of rDNA (e.g., Baldwin & al., 1995; Hershkovitz & al., 1999) using the extraction protocols of Rogers & Bendich (1994) and the analytical procedures outlined in an earlier phylogenetic analysis of Orchidinae by Pridgeon & al. (1997). DNAs were extracted from one Greek (RB 878) and one Bulgarian (RB 867) specimen of *G. frivaldii* (Appendix). Since the ITS sequences obtained from these two extremes of the Balkan distribution of the species were consistently identical in replicated analyses, no further specimens of this species were sequenced.

The approximate phylogenetic position of *G. frivaldii* was determined by inserting the Bulgarian sequence into the pre-existing ITS matrix of Bateman & al. (2003), which contained sequences for 188 accessions of tribe Orchideae plus two outgroups, together aligned in MacClade 4.05 (Maddison & Maddison, 2002). Visual inspection of the *G. frivaldii* sequence was sufficient to show that its affinities were with *Gymnadenia* s.s. rather than with *Pseudorchis* s.s. Alignment by eye was straight-forward, as *G. frivaldii* lacked any insertion-deletion events (indels) other than those already observed in other closely related species of *Gymnadenia*. Subsequent analysis of the matrix using PAUP\* 4.0b10 (Swofford, 2002) followed the heuristic procedures outlined by Bateman & al. (2003) and clearly demonstrated that *frivaldii* is indeed nested within *Gymnadenia*.

Hence, a second, more focused phylogenetic analysis was then performed, abstracting all sequences of *Gymnadenia* s.l. from the matrix of Bateman & al. (2003) and adding several further sequences from a study of *Gymnadenia* s.l. originally conducted in 1996 (Bateman & al., unpubl.). In addition, ITS sequences were obtained from accessions of *G. conopsea* s.s., sampled from sites close to the *G. frivaldii* localities in Greece and Bulgaria, and also from a Bulgarian plant tentatively assigned to *G. densiflora*. In total, 14 ingroup accessions were included (Appendix), together representing eight different ITS types. Since monophyly of *Gymnadenia* s.l. had already been confidently demonstrated by Bateman & al. (2003), a single outgroup was considered sufficient to polarise the characters; thus, a species representative of the basal group of the sister-genus, *Dactylorhiza* s.l., was selected as outgroup.

The resulting matrix contained very few missing values; moreover, the relatively strong similarity of the 15 sequences facilitated alignment, generating only four indels, three of which were located near the 3' end of ITS

2: a 1 bp indel and a 2 bp indel separated the outgroup from the ingroup, and two 1 bp indels (one of which was autapomorphic) occurred within the ingroup. Thus, it was feasible to treat these few small indels as a fifth state, rather than necessitating use of one of the several gap-coding strategies available (cf. Simmons & Ochoterena, 2000). The alignment allowed comparison of a continuous run of bases from ITS1 through 5.8S to ITS2 that totalled 643 sites; of these, 63 (10%) were variable but only 19 were parsimony-informative.

The modest number of entities analysed allowed rapid and reliable detection of all most-parsimonious trees using the branch-and-bound search strategy of PAUP. Branch support was estimated using both the decay index and fast bootstrap, via 1,000 replicates of a heuristic search employing stepwise addition.

## RESULTS AND DISCUSSION

**Morphology.** — The morphology of *Gymnadenia frivaldii* is compared with that of *Pseudorchis albida* in Figs. 1–4. Those portions of the plant given least attention by previous authors who have provided technical descriptions of *G. frivaldii* (Györfy, 1904; Camus & Camus, 1929; Baumann, 1978; Moore, 1980b; Buttler, 1991; Delforge, 2001), specifically the tubers, spur and gynostemium, have been given greatest attention here.

The fusiform tubers are divided for approximately the distal half of their length into between two and four elongate lobes narrowing into filiform roots (Fig. 2A; see also Györfy, 1904, fig. 4). The flexuous above-ground portion of the stem varies in height from a few centimetres up to 30 cm (Fig. 1A). Between two and four expanded leaves form an imperfect basal rosette only in plants occupying unusually exposed habitats; they are

more typically distributed along the lower portion of the stem, as are the one or two bract-like leaves above. The expanded leaves are broadly lanceolate, robust and fleshy, 50–90 × 8–15 mm. The inflorescence is compact and conical, reaching 20–40 mm and containing 15–40 small flowers (Fig. 1B). The membranous bracts are supplied by only a single vein and usually slightly exceed the compact ovaries.

In some individuals the flowers are creamy white, but in most plants diffuse pink anthocyanins are present; they are most intense in the gynostemium, including the pollinia, and often extend to the bracts and the upper portion of the stem. The median sepal and lateral petals are curved forward to form a loose (in some individuals very loose) hood, whereas the lateral sepals are somewhat longer, opposite and spreading. The distinctive labellum ranges from shallowly three-lobed to entire, is at least shallowly concave near its attachment and recurved toward its apex (Figs. 1B, 4C). It is approximately equidimensional (3.5–4 mm × 3–3.5 mm) when artificially flattened, and bears a small but distinct spur (Fig. 3A–C). Epidermal cells covering the thinner marginal zone appear considerably smaller than those in the fleshier main body of the labellum (Fig. 4C).

Some previous descriptions of the spur are contradictory. Our macromorphological and SEM studies (Fig. 3A–C) reveal it to be short [1.5–2(–3) mm], slender (0.3–0.5 mm), cylindrical (Fig. 3A) or slightly tapering (Fig. 3B, C), slightly downcurved and considerably shorter than the ovary. It is clearly formed by upward invagination of the labellum (Fig. 4A).

The gynostemium has rarely been described (e.g., Camus & Camus, 1929). In Fig. 4A it can clearly be seen to be unusually compact relative to most other members of subtribe Orchidinae. A pair of small pollinia (removed in Fig. 4A) are recessed in two adjacent “cowl” of thin-walled but deep thecae that are separated by a deeply invaginated, omegaform rostellum, which accommodates the two viscidia. The stigmatic surface extends laterally from immediately beneath the rostellum and above the spur entrance into two distinctive lappets.

**Distribution and ecology.** — The distribution of *G. frivaldii* is centred on the most montane portion of the Balkans, thereby encompassing parts of several countries: Greek Macedonia, Slavic Macedonia, eastern Albania and Transsylvanian Romania (e.g., Baumann, 1978; Moore, 1980b; Baumann & Künkele, 1982), with a smaller disjunct stronghold to the north in the Carpathiani Meridionalis of southwestern Bulgaria (Baumann, 1978). There is also an unconfirmed report of a single, incongruous outlying population in west-central Italy (Valentini & Montecchi, 1990).

The species is strongly altitudinally constrained, spanning a range between 1000 m and 2500 m but char-

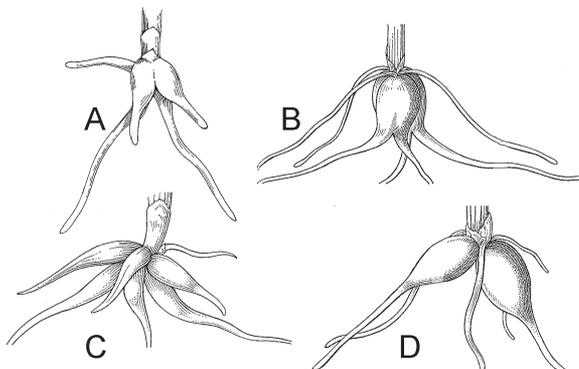


Fig. 2. Root-tubers of *Gymnadenia* and related genera. A, *Gymnadenia frivaldii*; B, *G. conopsea* s.s.; C, *Pseudorchis albida*; D, *Platanthera bifolia*. (A, drawn by the authors from photograph by K. Fairhurst; B–D, from Ross-Craig (1971, plates 28, 27, 30, respectively).

acteristically occurring at 1700–2000 m, where it preferentially inhabits wet meadows and mountain seepages (Stapperferne, 1966; Lakusic & Grgic, 1971; Baumann, 1978). These habitats and plant communities are analogous to those of the high meadows of the Alps; typical associated orchids include *Pseudorchis albida*, *Gymnadenia conopsea* s.s., species of *Gymnadenia* subgenus *Nigritella*, and *Dactylorhiza cordigera*.

*Gymnadenia frivaldii* flowers in June and July, occasionally stretching into August at higher altitudes. The flowers are occasionally reported to be fragrant, and photographic evidence suggests that they contain nectar for approximately one half of their length (e.g., Baumann & Künkele, 1982, p. 373). Evidence of pollinators is anecdotal;

small moths and butterflies are implicated (Davies & al., 1983; Cingel, 1995).

**Molecular phylogenetics.** — The molecular phylogenetic ITS studies of Pridgeon & al. (1997) and, more taxonomically comprehensively, Bateman & al. (2003) thoroughly analysed both *Pseudorchis* s.s., represented by *P. albida* and its segregate, *P. straminea*, and *Gymnadenia* s.l., represented by eight species. Together, these species spanned almost the entire morphological spectrum of this broadly delimited genus, which now encompasses both *Gymnadenia* s.s. and the morphologically distinct but molecularly very similar *Nigritella*. Generic circumscriptions of both *Gymnadenia* and *Pseudorchis* were unequivocally upheld, being mono-

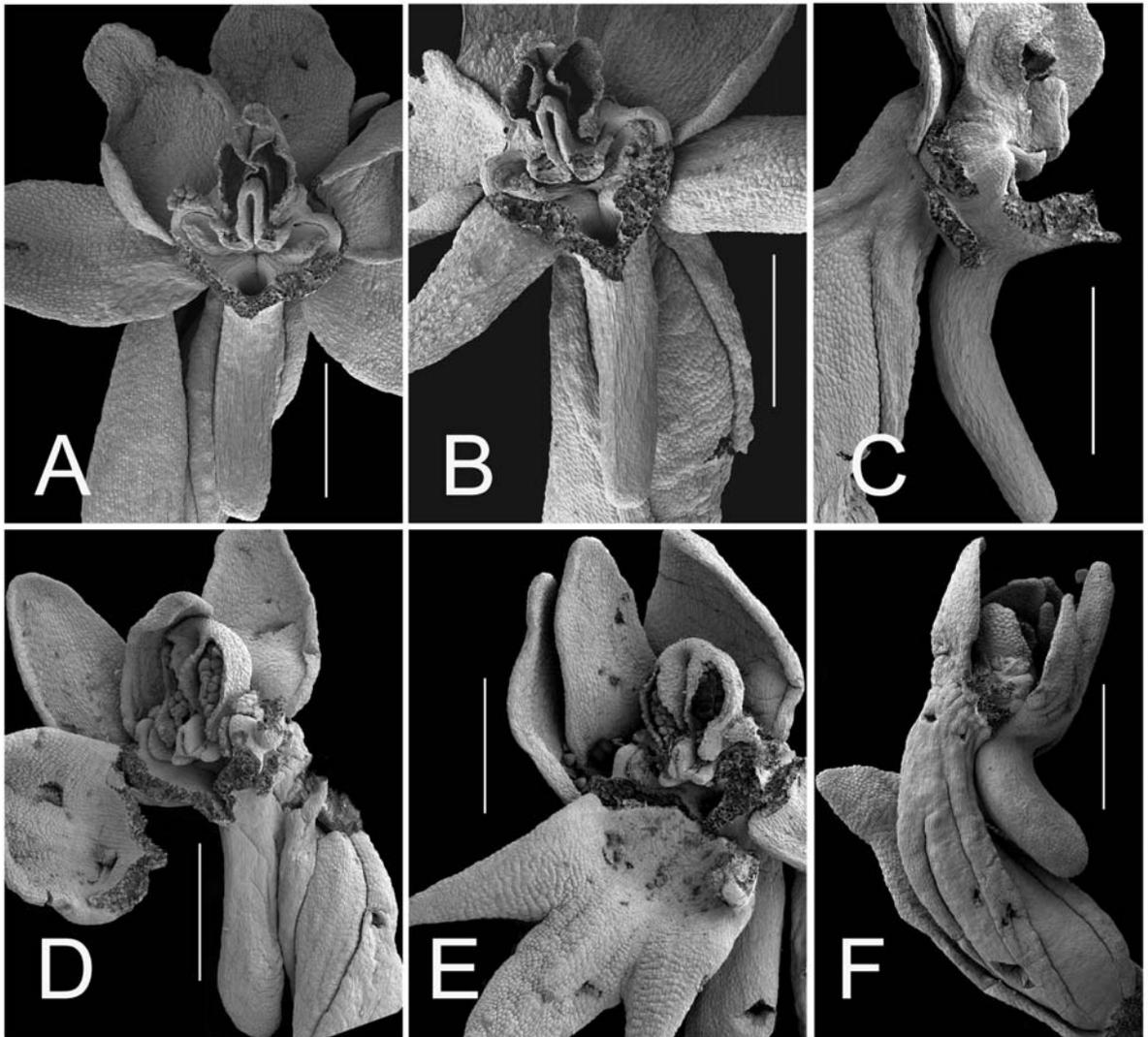


Fig. 3. Scanning electron micrographs of preserved flowers, partially dissected to reveal details of spur and gynostemium. A–C, *Gymnadenia frivaldii*, Bulgaria. A, B, Perpendicular view with labellum removed (arrow indicates right-hand lappet-like stigma lobe); C, Lateral view with labellum, left lateral petal and left lateral sepal removed; D–F, *Pseudorchis albida*, D, E, Italy, F, Scotland. D, oblique view with labellum, right lateral petal and right lateral sepal removed; E, oblique view with labellum, right lateral petal and right lateral sepal dislocated; F, lateral view of opening bud with left lateral sepal removed. Scale bars: 1 mm. Photos: P. Rudall.

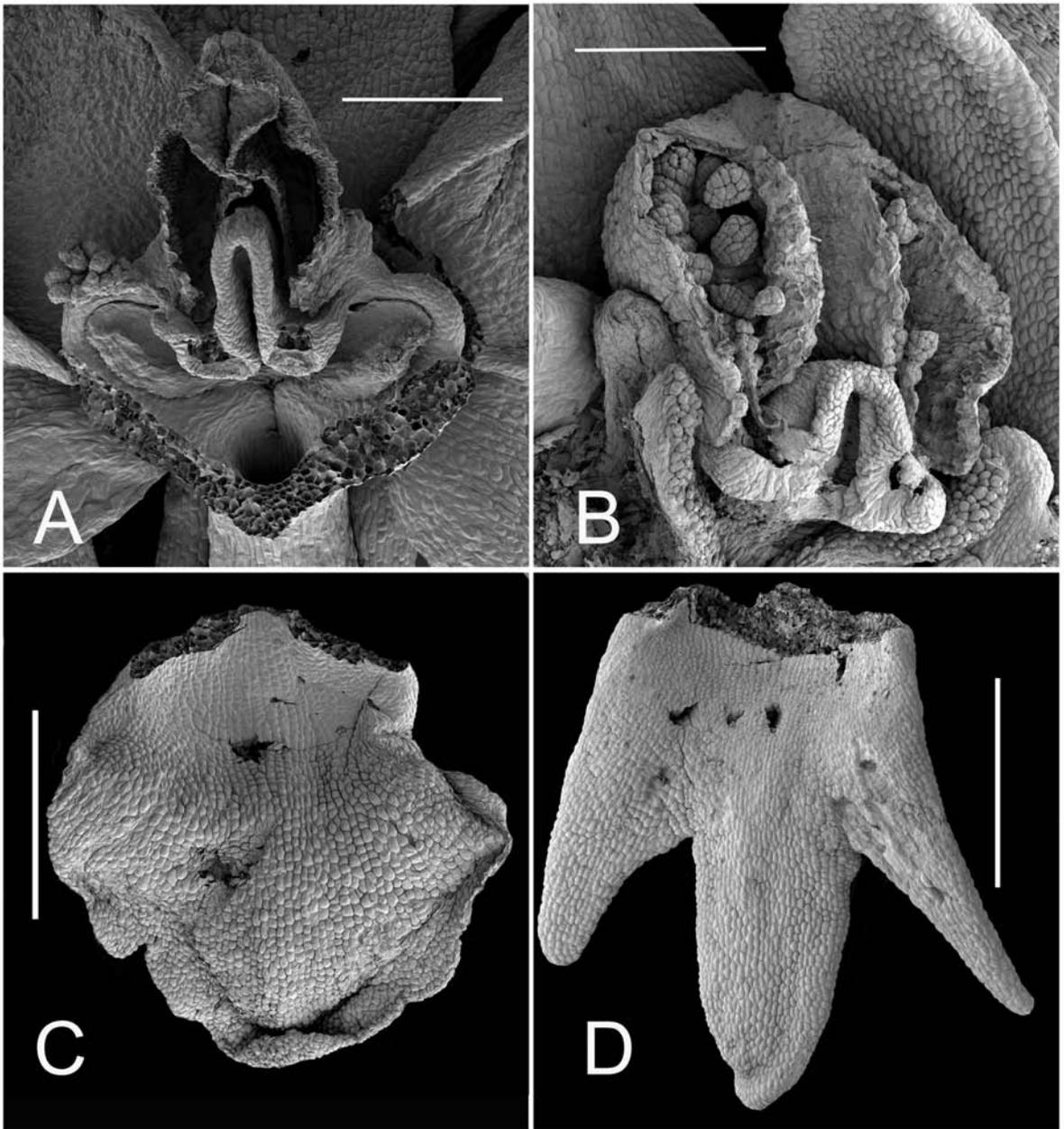
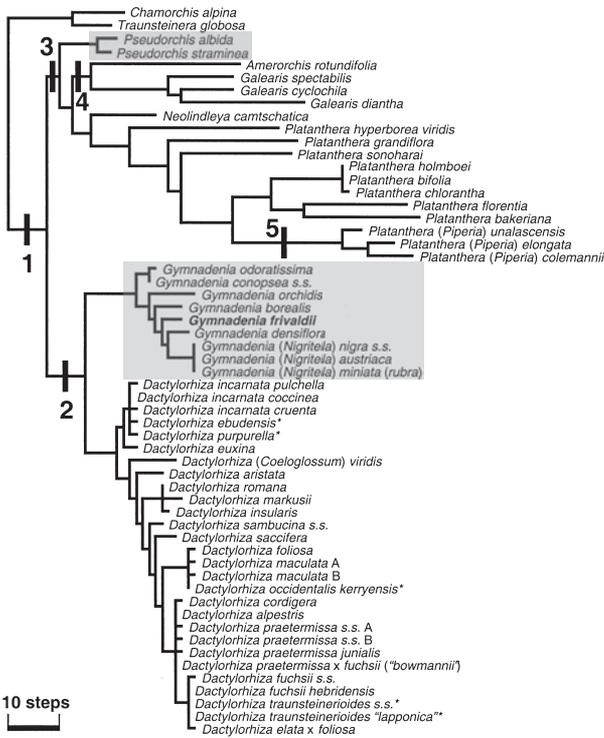


Fig. 4. Scanning electron micrographs of preserved flowers. A, C, *Gymnadenia frivaldii*, Bulgaria: A, Details of gynostemium; C, excised labellum. B, D, *Pseudorchis albida*, Italy. B, details of gynostemium; D, excised labellum. Scale bars: 0.5 mm (A, B), 1 mm (C, D). Photos: P. Rudall.

phyletic entities supported by both substantial molecular branch lengths (incorporating unique indels) and sufficient morphological synapomorphies. *Gymnadenia* s.l. is equally unequivocally sister to *Dactylorhiza* s.l., and this pair is in turn sister to the *Platanthera* clade. *Pseudorchis* is placed more ambiguously, being located between the *Gymnadenia-Dactylorhiza* and *Platanthera* clades, marginally closer to the latter. Thus, we were confident that this phylogenetic framework would prove sufficiently well-resolved to place the enigmatic *G. frivaldii*, which

as expected did not perturb the topologies obtained by Bateman & al. (2003).

Indeed, *G. frivaldii* was confidently embedded within *Gymnadenia*; when the ITS sequence from the Bulgarian accession of *G. frivaldii* was inserted into the matrix of Bateman & al. (2003), it nested firmly within *Gymnadenia* s.s., conclusively demonstrating its generic affinities (Fig. 5). Support for assignment to *Gymnadenia* rather than *Pseudorchis* from both base substitutions and indels (insertion-deletion events) is evident in



**Fig. 5. Relevant portion of representative of >10,000 most-parsimonious cladograms derived from ITS data for tribe Orchideae, highlighting (a) the considerable molecular disparity separating *Gymnadenia* from *Pseudorchis* s.s. (stippled), and (b) transitions in the morphology of the rootstock from a plesiomorphic condition of a single ovoid tuber not tapering into roots: (1) one or more tubers, distal end of each tapering into one or more roots; (2) single tuber, distal end tapering into more than one root; (3) multiple tubers, each tapering into a single root; (4) tubers absent or no longer distinguishable from roots; (5) reversion to ancestral condition of single ovoid tuber lacking roots (nb: all taxa generate multiple roots from the base of the stem). Within *Dactylorhiza*, recently formed primary hybrids and allopolyploids (asterisked) usually contain multiple ITS alleles (Pillon & al., in prep.). The tree exhibits several changes of nomenclature or specimen identification relative to fig. 2b of Bateman & al. (2003).**

the small portions of ITS1 and ITS2 selectively illustrated in Table 1.

In an attempt to obtain a clearer picture of where *G. frivaldii* is placed within the genus, a more focused

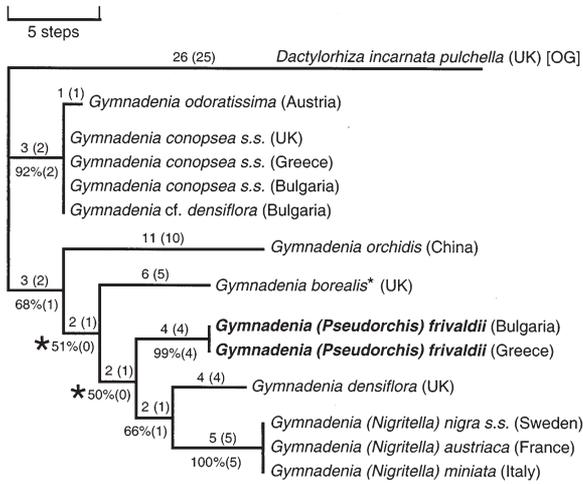
analysis was performed that assumed the monophyly of *Gymnadenia* s.l. (including *G. frivaldii*). The branch-and-bound analysis yielded three most-parsimonious trees of modest length (L = 69) and low homoplasy (CI = 0.85, RI = 0.93). The three most-parsimonious trees differed in the placement of *G. borealis*, which could equally be depicted as sandwiched stepwise between *G. orchidis* and a more derived *G. frivaldii* (Fig. 6), sister to the relatively long-branch taxon *G. orchidis*, or sister to *G. frivaldii*.

The three species of subgenus *Nigritella* yielded identical ITS sequences, as did the two accessions of *G. frivaldii* and the three accessions of *G. conopsea* s.s., despite their considerable geographic spread. However, the Bulgarian accession tentatively assigned to *G. cf. densiflora* was identical to *G. conopsea* s.s., suggesting that it was misidentified in the field (this issue is explored further by Bateman & al., unpubl.). *Gymnadenia odoratissima* differed from *G. conopsea* s.s. by only one autapomorphic substitution, a result consistent with their modest allozymic disparity (Soliva & Widmer, 1999; Hedrén & al., 2000). Otherwise, the six main genotypes represented here show considerable sequence disparities and are well-supported (even the weakest, *G. conopsea* + *G. odoratissima*, has a bootstrap vale of 92% and decay index of 2).

However, the present analysis follows that of Bateman & al. (2003) in failing to satisfactorily resolve the relationships among these disparate ITS types. Internal branches of the tree are short (two or three steps) and encompass most of what little homoplasy is evident in the matrix (Fig. 6). The nodes along the spine of the tree that straddle the node immediately subtending *G. frivaldii* collapse in the strict consensus. Even where weak support is provided by bootstrap and decay index, the relationships indicated are improbable; note, for example, the morphologically unintuitive pairing of *G. densiflora* (large, well-differentiated flowers) and subgenus *Nigritella* (small, poorly differentiated flowers). Indeed, the nesting of a monophyletic subgenus *Nigritella* well within a paraphyletic *Gymnadenia* s.s. implied by the ITS data (Fig. 6) and the intron of the plastid gene *rpl16* (Y. Pillon, pers. comm., 2004) has been challenged by results from analysis of plastid *trnL* sequences (Bateman & al., 2003, unpubl.) and allozyme analysis (Hedrén & al., 2000), which instead indicate closely

**Table 1. DNA sequences of representative portions of the ITS region of the four most relevant species included in the present molecular phylogenetic analysis, highlighting contrasting bases and demonstrating the greater overall similarity of *frivaldii* to *Gymnadenia* (5'-3').**

	ITS1 region	ITS2 region
<i>Gymnadenia frivaldii</i>	CAATTTGTTTTGTGGAGTT--GTTTGCTCCTAAAGA-	TTAGAGAATGTGTGT--ATATCCCGGGC--TATCCCAAC
<i>Gymnadenia borealis</i>	.....G	..T.....C...G.....T.....
<i>Dactylorhiza incarnata</i>	..G.....	..T.....A.G...GC.....GAG.C....T
<i>Pseudorchis albida</i>	.....GTT.....A.....	..T.....A.....GT.....A.....A.....



**Fig. 6.** Representative of three most-parsimonious cladograms derived from ITS data in the present analysis, assuming monophyly of *Gymnadenia* s.l. (Fig. 5) and using *Dactylorhiza incarnata* as outgroup. Trees were generated by parsimony using branch-and-bound; they have a length of 69 steps, CI of 0.85 (0.94 including autapomorphies) and RI of 0.93. Branch lengths include autapomorphies and reflect Acctran optimisation. Figures above each branch are the branch length and, in parentheses, the number of non-homoplastic changes; figures below each branch are fast bootstrap percentages and, in parentheses, decay index values. Asterisked branches collapsed in the strict consensus tree.

related sister groups.

Lastly, the apparent disparity between *Gymnadenia* s.l. and *Dactylorhiza* has been exaggerated by at least 20% in the analysis, due to the treatment of a single 2 bp indel as two “fifth-state” substitutions and especially to the treatment as four separate substitutions of what appears to constitute a single simultaneous duplication and substitution event in *Dactylorhiza* of the 5 bp motif “CAATG” in an exceptionally variable region of ITS2 close to 5.8S.

**Evidence from hybridisation.** — It is becoming increasingly fashionable to review and assess the credibility of records of orchid hybrids in the light of closeness of relationships among species inferred using molecular phylogenetic methods (e.g., Pridgeon & al., 1997; Bateman, 2001; Bateman & al., 2003; Bateman & Hollingsworth, 2004; H. & G. Kretzschmar, pers. comm., 2004).

Extensive lists of hybrid combinations (some decidedly uncritical) have been compiled for *Pseudorchis albida* (e.g., Hunt, 1971) and more widespread species of *Gymnadenia* subgenus *Gymnadenia* (e.g., Camus & Camus, 1929; Vermeulen, 1947; Peitz, 1972; Hunt & al., 1975; Sundermann, 1980; Delforge, 2001) and subgenus *Nigritella* (Teppner & Klein, 1985; see also Camus & Camus, 1929; Peitz, 1972; Sundermann, 1980; Gerbaud

& Schmidt, 1999; Delforge, 2001). The potential for *G. frivaldii* to hybridise is relatively restricted by a combination of its limited geographic distribution and its strong preference for alpine habitats. Nonetheless, there are several records of hybridisation involving *G. frivaldii* (cf. Stapperfenne, 1966; Hunt, 1971; Baumann, 1978; Baumann & Künkele, 1982; Davies & al., 1983), the most frequently recorded being with co-occurring populations that are most commonly (but incorrectly) referred to in the literature as “*Nigritella nigra*” (e.g., Stojanov & al., 1928; Keller & Schlechter, 1930–1940; Stapperfenne, 1966; Hunt, 1971; Peitz, 1972; Sundermann, 1980). In fact, recent integrated monographic work on subgenus *Nigritella* (e.g., Hedrén & al., 2000) suggests that *G. frivaldii* occurs as commonly with recently segregated diploids as with the tetraploid derivative of *G.* (formerly *N.*) *nigra* now known as *G. (N.) austriaca* (e.g., Teppner & Klein, 1985; Delforge, 2001). Not surprisingly, hybrids have also been reported between *G. frivaldii* and the widespread and co-occurring *G. conopsea* (Stapperfenne, 1966; Baumann & Künkele, 1982).

Moving on to reports of hybrids with other taxa, Sundermann (1980, p. 200) tantalisingly illustrated an individual of *G. frivaldii* immediately adjacent to an individual of *Pseudorchis albida*; not surprisingly, this hybrid combination has been recorded by several authors (e.g., Baumann & Künkele, 1982). Both species are supplied with both scent and nectar, and both reputedly are pollinated primarily by small moths and butterflies (Cingel, 1995), though *P. albida* is also believed to owe its high percentage fruit-set to facultative autogamy (Summerhayes, 1951; Cingel, 1995). The morphological evidence presented by Baumann (1978) for a Bulgarian hybrid of *G. frivaldii* × *P. albida* appears strong, but given the remarkable morphological convergences observed between these two species, morphological identification of any resulting intergeneric hybrids presents serious difficulties. Although other species of *Gymnadenia* frequently hybridise with species of *Dactylorhiza*, records of hybrids between *G. frivaldii* and the frequently co-occurring *D. cordigera* (e.g., Krey, 1977) similarly require confirmation, particularly given the frequent confusion of mutant non-hybrid individuals with intergeneric hybrids when morphology alone is employed (Bateman & DiMichele, 2002; Bateman & Rudall, 2006).

In such cases there is an especially strong motivation to apply modern molecular methods to disentangling the genealogy of individuals. As well as direct sequencing in search of species-specific single nucleotide polymorphisms, any species-specific indels permit a variety of short-cuts to DNA-based identification of hybrids. Moreover, contrasting phylogenetic signals from the biparentally inherited nuclear genome versus the mater-

nally inherited organellar genomes can be used to distinguish the mother from the father (cf. Bateman & Hollingsworth, 2004; Pillon & al., in prep.), often indicating a stronger contribution by the maternal parent to the phenotype of the hybrid (Bateman & Hollingsworth, 2004; Bateman & Rudall, 2006).

**Systematic implications.** — Although the orthographic preference for Grisebach's (1844) epithet *frivaldii* over Hampe's original nomen nudum of *frivaldszyána* (1837) has long been universally accepted, the taxonomic and nomenclatural history of *G. frivaldii* remains complicated by three main areas of disagreement: (1) Should *Pseudorchis*, a genus based on the widespread circumboreal *P. albida* complex, be segregated from *Gymnadenia*? (2) If the two genera are indeed best separated, should *frivaldii* be assigned to *Pseudorchis* or to *Gymnadenia*? (3) Does *Pseudorchis* Séguier (1754), *Leucorchis* E. Meyer (1848) or the less frequently used *Biccia* Parlato (1858) have nomenclatural priority for the genus that has as its type species *albida* L.?

As question (3) addresses a purely nomenclatural issue, whereas our primary interests are evolutionary, we have pragmatically accepted both the technical arguments of Dandy (1967; cf. Senghas, 1972) and the desire for stability, and so have preferred *Pseudorchis*. Although opinions expressed in orchid literature through the last century were approximately equally divided between assigning *albida* to *Leucorchis* or to *Pseudorchis*, the latter was favoured in benchmark publications such as *Flora Europaea* (Moore, 1980b) and *Genera Orchidacearum* (Pridgeon & al., 2001).

The topology in Fig. 5 firmly contradicts the majority of authors, who have assigned *P. albida* to *Gymnadenia* (e.g., Ascherson & Graebner, 1905–1907; Summerhayes, 1951; Luer, 1975; Delforge, 2001); generic separation is merited on both molecular and morphological grounds. Moreover, insertion of its ITS sequence into the matrix that generated Fig. 5 clearly demonstrated that *frivaldii* belongs in *Gymnadenia* rather than *Pseudorchis*, a fact reinforced by the subsequent, more focused phylogenetic analysis of *Gymnadenia* s.l. (Table 1, Fig. 6). Thus, questions (1) and (2) have been unequivocally answered by this study; answers that receive still further support from plastid sequences (Bateman & al., unpubl.; M. Hedrén, pers. comm., 2005).

**Comparative morphology.** — There remains an important challenge of how best to diagnose the two genera, *Pseudorchis* and *Gymnadenia*, following their recircumscriptions to respectively lose or accommodate *G. frivaldii*. We can also usefully re-examine the characters that supposedly linked *frivaldii* to genera other than *Gymnadenia*.

Some past authors attributed considerable importance to what they perceived as a loose hood formed by

the dorsal sepal and lateral petals; this character was used as a justification for placing *G. frivaldii* in *Pseudorchis* (e.g., Williams & al., 1978; Davies & al., 1983). The number of veins in the bract, a character previously used to separate *Pseudorchis albida* (three veins) from “*Pseudorchis*” *frivaldii* (one vein) (e.g., Moore 1980a, b), may prove diagnostic once all other species of *Gymnadenia* have been surveyed for consistency in this character. The labellum of true *Pseudorchis* (Figs. 3E, 4D) is more robust and more deeply incised than that of any *Gymnadenia* examined by us, as is the tuber (Fig. 2C), and the spur is more saccate (Fig. 3E, F). Both taxa have compact gynostemium with a blunt, strongly invaginated rostellum, but the rostellum of *P. albida* (Fig. 4B) is only half the relative height of that of *G. frivaldii* (Fig. 4A) and the connective is considerably wider. Crucially, *P. albida* lacks the lappet-like stigmatic surface that is a feature not only of *G. frivaldii* but also of other species of *Gymnadenia* s.s. (cf. Vermeulen, 1947; Szlachetko & Rutkowski, 2000; Pridgeon & al., 2001). Lastly, it would be helpful if the relatively subtle floral pigments of *G. frivaldii* and *P. albida* were to be analysed for comparison with the strongly orchicyanin-dominated *Gymnadenia* subgenus *Gymnadenia* and chrysanthemini/ophrysanthin-dominated *Gymnadenia* subgenus *Nigritella* (Strack & al., 1989).

This synthesis suggests that *G. frivaldii* and *P. albida* can now be viewed as a classic case of superficial floral convergence. Indeed, the convergence extends to their respective fleshy, somewhat spatulate, non-rosette forming leaves, which also characterise another specialist alpine orchid, *Traunsteinera globosa* (cf. Landwehr, 1977, p. 53; Delforge, 2001, p. 230). This genus diverged earlier in the evolutionary history of subtribe Orchidinae and now occupies a relatively long, isolated branch in the ITS phylogeny, tentatively placed (along with *Chamorchis alpina*) as sister to the tapered-tuber clade (Fig. 5). These distinctive vegetative features of the three species may reflect adaptation to their preferred alpine habitats.

The ITS phylogeny (Fig. 5) reveals as strongly synapomorphic characters such as tuber morphology and chromosome number. The clade placed immediately above *Traunsteinera-Chamorchis* that dichotomises basally into the *Platanthera-Galearis* group and the *Dactylorhiza-Gymnadenia* group is characterised by possessing tubers that taper distally into one or more roots. In members of the *Platanthera-Galearis* group other than the *Galearis* clade (loss of discernible tubers) and the “*Piperia*” clade (reversion to rootless ovoid tubers) these are multiple and divided to the stem base, so that each tapers into only a single root and is radially symmetrical in transverse section (Fig. 2C, D). By contrast, in the *Dactylorhiza-Gymnadenia* group these structures are partially congenitally fused proximal to the stem base

to generate a single composite, bilaterally symmetrical tuber that tapers into two or more roots to yield a digitate form (Fig. 2A, B). In addition, most members of the *Platanthera-Galearis* group have inherited the plesiomorphic chromosome number of  $2n = 42$ , whereas the origin of the *Dactylorhiza-Gymnadenia* clade coincides with (and indeed may have been prompted by) a single chromosomal fusion event to give  $2n = 40$  (cf. Groll, 1965; Cauwet-Marc & Balayer, 1984, 1986; Jongepierova & Jongepier, 1989; Pridgeon & al., 1997; Bateman, 2001; D'Emerico & Grünanger, 2001; Bateman & al., 2003).

Thus, both root morphology and karyotypes suggest that, whereas *G. frivaldii* is comfortably placed within *Gymnadenia*, *Pseudorchis albida* has stronger affinities with the *Platanthera-Galearis* clade than with the *Dactylorhiza-Gymnadenia* clade—a relationship rarely suggested prior to the advent of molecular phylogenetics (Lindley, 1829; Vermeulen, 1977). This insight helps to explain several divergences in aspects of life history and mycorrhizal symbiosis between *Gymnadenia* s.s. and *P. albida* that were documented (in “*Gymnadenia albida*”) by Rasmussen (1995).

**Speculative evolutionary origins: paedomorphosis and/or hybridisation?** — Despite the weakness of its internal structure, the ITS topology shown in Fig. 6 is sufficient to state with some confidence that the three lineages present that are characterised by short spurs and only shallowly three-lobed or entire labella (*G. odoratissima*, *G. frivaldii*, *G.* subgenus *Nigritella*, the former almost certainly originating in central Europe relatively recently from within *G. conopsea*) represent independent reductions in flower size and complexity consistent with one or more of the three recognised categories of paedomorphic heterochrony (e.g., Alberch & al., 1979). Careful SEM-based scrutiny of successive floral ontogenetic stages would be required to determine whether reduction occurs by delayed onset of growth of the affected organs (post-displacement), precocious offset of growth (progenesis) or decreased rate of morphological development (neoteny). Based on our current, limited, data we suspect that growth is progenetically curtailed, the effects being increasingly pronounced in a longitudinal cline from the sepal whorl to the gynostemium. It would be particularly interesting to compare the longitudinally abbreviated but laterally expansive gynostemium of *G. frivaldii* with those of closely related species of *Gymnadenia* possessing larger (though still compact) gynostemium, as well as with the similarly compact but phylogenetically convergent gynostemium of *P. albida* (Figs. 3, 4).

Also relevant to the evolutionary role of paedomorphosis is a taxon that was first segregated from *G. frivaldii* as a full species by Györfy (1904). *Gymnadenia richteri* was based on selected Hungarian populations

that tend toward even greater compactness of the inflorescence and reduction of the flower, so that the labellum is entire and only marginally larger than the other perianth segments (Györfy, 1904, figs. 1–3; see also Camus & Camus, 1929). Indeed, the entire-lipped Bulgarian plant illustrated in our Fig. 4C suggests that such morphs are not confined to Hungary. This taxon was later more appropriately reduced considerably in rank to *G. frivaldii* forma *richteri* (Györfy) Jávorka (1924–1925). It may prove to be an iterative mutant with a phenotypic expression that was termed pseudopeloric by Bateman & Rudall (2006), wherein the flower approaches but does not achieve radial symmetry. This mode of pseudopeloria was suggested as a possible origination mechanism for the even more radically altered phenotype of *Gymnadenia* subgenus *Nigritella* by Bateman & DiMichele (2002), who invoked single-gene mutation as the most likely underlying cause. Such an origin would help to explain the more ovate sepals of *G. frivaldii* relative to other species of *Gymnadenia* s.s., which are also far more likely to have recurved margins to their lateral sepals. Morphometric evidence suggests that spur shortening in *Gymnadenia* s.s. also occurs by saltational leaps rather than gradually (Bateman & al., unpubl.).

Briefly returning to the topic of hybridisation, an alternative hypothesis of the mode of origin of *G. frivaldii* is suggested by the remarkable similarity in flower morphology and, to a lesser degree, colour between *G. frivaldii* and a putative individual of the hybrid *G. odoratissima* × *G.* (= *Nigritella*) *rhellicani* from the Italian Alps illustrated by Delforge (cf. pp. 140, 144 of Delforge, 2001). Hybridisation constitutes a less direct evolutionary route by which *G. frivaldii* could acquire from its parents its distinctive paedomorphic features: specifically, its abbreviated gynostemium and poorly differentiated, funnel-shaped perianth, as well as its tightly packed inflorescence of imperfectly oriented pseudopeloric flowers.

Such heterochronic transformations in floral morphology have been attributed to gene silencing and other epigenetic effects in plants of hybrid and allopolyploid origin (e.g., Liu & Wendel, 2003). In order to test this hypothesis, we will need to compare biparentally inherited nuclear sequences such as those already obtained from ITS with uniparentally inherited plastid sequences such as *trnL* (Bateman & al., unpubl.), which would help to identify not only the potential parents but also the maternity of *G. frivaldii* (cf. Bateman & Hollingsworth, 2004). More detailed exploration of ITS sequences through cloning would also be desirable, in search of unbalanced polymorphisms that would indicate incompletely converted parental contributions, which would in turn reflect relatively recent hybridisation (cf. Pillon & al., in prep.). More simply, a karyotype from *G. frivaldii*

would be useful, given the predisposition of the digitate-tubered clade to ploidy change; polyploidy has already been observed in species of *Gymnadenia* s.s. (e.g., Mrkvicka, 1993; D'Emérico, 2001) and *G.* subgenus *Nigritella* (e.g., Teppner & Klein, 1985; Hedrén & al., 2000; D'Emérico & Grünanger, 2001), and is extensive in the sister-genus *Dactylorhiza* (Fig. 5: e.g., Heslop-Harrison, 1954; Bateman, 2001; Pillon & al., in prep.).

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## LITERATURE CITED

- Alberch, P., Gould, S. J., Oster, G. F. & Wake, D. B. 1979. Size and shape in ontogeny and phylogeny. *Paleobiology* 5: 296–317.
- Ascherson, P. & Graebner, P. 1905–1907. Orchidaceae. Pp. 612–925 in: *Synopsis der Mitteleuropäischen Flora*, vol. 3. Gebrüder Borntraeger, Leipzig.
- Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S. & Donoghue, M. J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Missouri Bot. Gard.* 82: 247–277.
- Bateman, R. M. 2001. Evolution and classification of European orchids: insights from molecular and morphological characters. *J. Europ. Orch.* 33: 33–119.
- Bateman, R. M. & DiMichele, W. A. 2002. Generating and filtering major phenotypic novelties: neoGoldschmidtian saltation revisited. Pp. 109–159 in: Cronk, Q. C. B., Bateman, R. M. & Hawkins, J. A. (eds.), *Developmental Genetics and Plant Evolution*. Taylor & Francis, London.
- Bateman, R. M. & Hollingsworth, P. M. 2004. Morphological and molecular investigation of the parentage and maternity of *Anacamptis* × *albuferensis* (*A. fragrans* × *A. robusta*), a new hybrid orchid from Mallorca, Spain. *Taxon* 53: 43–54.
- Bateman, R. M., Hollingsworth, P. M., Preston, J., Luo Yi-Bo, Pridgeon, A. M. & Chase, M. W. 2003. Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae). *Bot. J. Linn. Soc.* 142: 1–40.
- Bateman, R. M. & Rudall, P. J. 2006. The Good, the Bad, and the Ugly: using naturally occurring terata to distinguish the possible from the impossible in orchid floral evolution. *Aliso* 22: 481–496.
- Baumann, H. 1978. *Leucorchis* × *vitosa* Baumann, hybr. nat. nov.: der einzig mögliche infragenerische Bastard der Gattung *Leucorchis* E. May. *Orchidee* 29: 217–223.
- Baumann, H. & Künkele, S. 1982. *Die Wildwachsenden Orchideen Europas*. Kosmos, Stuttgart.
- Buttler, K. P. 1991. *Field Guide to Orchids of Britain and Europe*. Crowood Press, Swindon, Wilts.
- Camus, E. G. & Camus, A. 1929. *Monographie des Orchidées de l'Europe, de l'Afrique Septentrionale, de L'Asie Mineure et des Provinces Russes Trascaspiennes*. Paris.
- Cauwet-Marc, A. M. & Balayer, M. 1984. Les genres *Orchis* L., *Dactylorhiza* Necker ex Nevski, *Neotinea* Reichb. et *Traunsteinera* Reichb. Caryologie et proposition de phylogénétique et d'évolution. *Bot. Helv.* 94: 391–406.
- Cauwet-Marc, A. M. & Balayer, M. 1986. Les orchidées du bassin méditerranéen. Contribution à l'étude caryologique des espèces des Pyrénées-Orientales (France) et contrées limitrophes. II. Tribu des Ophrydæ Lindl. pro parte. *Bull. Soc. Bot. France* 133, *Lettres Bot.* 3: 265–277.
- Cingel, N. A. 1995. *An Atlas of Orchid Pollination: European Orchids*. Balkema, Rotterdam.
- Dandy, J. E. 1967. *Index of Generic Names of Vascular Plants 1753–1774*. IAPT, Utrecht.
- Davies, P., Davies, J. & Huxley, A. 1983. *Wild Orchids of Britain and Europe*, London.
- Delforge, P. 2001. *Guide des Orchidées d'Europe*, ed. 2. Lausanne, Switzerland.
- D'Emérico, S. 2001. Cytogenetics. Pp. 216–224 in: Pridgeon, A. M., Cribb, P. J., Chase, M. W. & Rasmussen, F. N. (eds.), *Genera Orchidacearum 2: Orchidoideae Part 1*. Oxford Univ. Press, Oxford.
- D'Emérico, S. & Grünanger, P. 2001. Giemsa C-banding in some *Gymnadenia* species and in *Chamorchis alpina* from Dolomites (Italy). *J. Europ. Orchid.* 33: 405–414.
- Gerbaud, O. & Schmidt, W. 1999. *Die Hybriden der Gattungen Nigritella und/oder Pseudorchis*. Orchidophile /AHO Special Volume 5. Bayern, Paris.
- Grisebach, A. H. R. 1844. *Spicilegium Florae rumelicae et bithynicae* 2. Vieweg, Brunsvigae.
- Groll, M. 1965. Fruchtansatz, Bestäubung und Merkmalsanalyse bei diploiden und polyploiden Sippen von *Dactylorchis* (*Orchis*) *maculata* und *Gymnadenia conopsea*. *Österr. Bot. Zeit.* 112: 657–700.
- Györfy, I. 1904. A Magyarföldi flóra œj *Gymnadenia-faja*. *Ann. Hist. Nat. Mus. Hungary* 2: 237–252.
- Hedrén, M., Klein, E. & Teppner, H. 2000. Evolution of polyploids in the European orchid genus *Nigritella*; evidence from allozyme data. *Phyton* 40: 239–275.
- Hershkovitz, M. A., Zimmer, E. A. & Hahn, W. J. 1999. Ribosomal DNA sequences and angiosperm systematics. Pp. 268–326 in: Hollingsworth P. M., Bateman R. M. & Gornall R. J. (eds.), *Molecular Systematics and Plant Evolution*. Taylor & Francis, London.
- Heslop-Harrison, J. 1954. A synopsis of the dactylorchids of the British Isles. *Veröff. Geobot. Institut Rübél* 1953: 53–82.
- Hunt, P. F. 1971. Taxonomic and nomenclatural notes on European and British orchid hybrids. *Orchid Rev.* 79: 138–142.
- Hunt, P. F., Roberts, R. H. & Young, D. P. 1975. Orchidaceae (624–645). Pp. 473–507 in: Stace, C. A. (ed.),

- Hybridization and the British Flora*. Academic Press, London.
- Jávorka, S.** 1924–1925. *Magyar Flora*. Stadium (Magyar Nemet Muzeum), Budapest.
- Jongepierova, I. & Jongepier, J. W.** 1989. Study on the taxonomy of *Gymnadenia conopsea* in Czechoslovakia: a preliminary report. *Mem. Soc. R. Bot. Belg.* 11: 123–132.
- Keller, G. & Schlechter, R.** 1930–1940. Monographie und Iconographie der Orchideen Europas und des Mittelmeergebietes. *Sonderbeih. Rep. Spec. Nov. Veg. A*, 1–5.
- Krey, W. D.** 1977. Unbekannter intergenerischer *Dactylorhiza cordigera* Bastard in Südjugoslawien. *Orchidee* 28: 144–145.
- Lakusic, R. & Grgic, P.** 1971. Ekologija i rasprostranjenje endemicnih vrsta *Nartheicum scordicum* Kos., *Pinguicula balcanica* Cas., *Gymnadenia frivaldii* Hampe i *Silene asterias* Griseb. *Ekologija (Beograd)* 6: 337–350.
- Landwehr, J.** 1977. *Wilde Orchideen van Europa*. Natuurmonumenten, Amsterdam.
- Lindley, J.** 1829. *Synopsis of the British Flora*. Longman, London.
- Liu, B. & Wendel, J. F.** 2003. Epigenetic phenomena and the evolution of plant allopolyploids. *Molec. Phylog. Evol.* 29: 365–379.
- Luer, C.** 1975. *The Native Orchids of the United States and Canada, Excluding Florida*. New York Botanical Garden, New York.
- Maddison, W. P. & Maddison, D. R.** 2002. *MacClade, version 4.05: Analysis of Phylogeny and Character Evolution*. Sinauer, Sunderland, Mass.
- Meyer, E. H. F.** 1848. *Flora der Provinz Preussen*, ed. 2. Grafe & Unzer, Königsberg.
- Moore, D. M.** 1980a. CCIII/15. *Gymnadenia*. P. 332 in: Tutin, T. G., Heywood, V. H., Burges, N. A., Moore, D. M., Valentine, D. H., Walters, S. M. & Webb, D. A. (eds), *Flora Europaea* 5. Cambridge Univ. Press, Cambridge.
- Moore, D. M.** 1980b. CCIII/16. *Pseudorchis*. P. 332 in: Tutin, T. G., Heywood, V. H., Burges, N. A., Moore, D. M., Valentine, D. H., Walters, S. M. & Webb, D. A. (eds.), *Flora Europaea*, vol. 5. Cambridge Univ. Press, Cambridge.
- Mrkvicka, A. C.** 1993. Statistische Untersuchungen an *Gymnadenia conopsea* (L.) R. Br. s.l. *Mitt. Arbeitskr. heim. Orchid. Baden-Württemberg* 25: 361–367.
- Parlatore, F.** 1858. *Flora Italiana III*. Le Mannier, Firenze.
- Peitz, E.** 1972. Zusammenstellung aller bisher bekannten Bastarde der in Deutschland verbreiteten Orchideen. *Jahresb. naturwiss. Ver. Wuppertal* 25: 167–200.
- Pridgeon, A. M., Bateman, R. M., Cox, A. V., Hapeman, J. R. & Chase, M. W.** 1997. Phylogenetics of subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 1. Intergeneric relationships and polyphyly of *Orchis* sensu lato. *Lindleyana* 12: 89–109.
- Pridgeon, A. M., Cribb, P. L., Chase, M. W. & Rasmussen, F. N.** (eds.). 2001. *Genera Orchidacearum*, 2. Orchi do i deae. Oxford Univ. Press, Oxford.
- Rasmussen, H. N.** 1995. *Terrestrial Orchids: From Seed to Mycotrophic Plant*. Cambridge Univ. Press, Cambridge.
- Rogers, S. O. & Bendich, A. J.** 1994. D. *Extraction of Total DNA from Plants, Algae and Fungi*. Plant Molecular Biology Manual, Labfax, Oxford.
- Ross-Craig, S.** 1971. *Drawings of British Plants*. XXVIII. Hydrocharitaceae, Orchidaceae. G. Bell, London.
- Séguier, J. F.** 1754. Orchidaceae. Pp. 243–254 in: *Plantarum quae in agro Veronesi reperitur Supplementum Seu 3*. Verona.
- Senghas, K.** 1972. *Leucorchis* versus *Pseudorchis*. *Orchidee* 23: 203–205.
- Simmons, M. P. & Ochoterena, H.** 2000. Gaps as characters in sequence-based phylogenetic analyses. *Syst. Biol.* 49: 369–381.
- Soliva, M. & Widmer, A.** 1999. Genetic and floral divergence among sympatric populations of *Gymnadenia conopsea* s.l. (Orchidaceae) with different flowering phenology. *Int. J. Plant Sci.* 160: 897–905.
- Stapperfenne, H.-J.** 1966. *Leucorchis frivaldii* (Hampe) Schltr. — eine Orchidee des südöstlichen Europa. *Orchidee* 17: 124–127.
- Stojanov, N. A., Stefanoff, B. & Georgieff, T.** 1928. Orchids new to the Bulgarian flora [In Bulgarian]. *Bull. Soc. Bot. Bulg.* 2: 35.
- Strack, D., Busch, E. & Klein, E.** 1989. Anthocyanin patterns in European orchids and their taxonomic and phylogenetic relevance. *Phytochemistry* 28: 2127–2139.
- Summerhayes, V. S.** 1951. *Wild Orchids of Britain*, ed. 1. Collins, London.
- Sundermann, H.** 1980. *Europäische und Mediterrane Orchideen — Ein Bestimmungsflora*, ed. 3. Schmiersow, Hildesheim.
- Swofford, D. L.** 2002. *PAUP\*: Phylogenetic Analysis using Parsimony (\* and Other Methods). Version 4*. Sinauer Associates, Sunderland, Massachusetts.
- Szlachetko, D. L. & Rutkowski, P.** 2000. *Gymnostemia orchidaliaum* 1. Apostasiaceae, Cypripediaceae, Orchidaceae (Thelymitroideae, Orchidoideae, Tripidioideae, Spiranthoideae, Neottioideae, Vanilloideae). *Acta Bot. Fenn.* 169: 1–379.
- Teppner, H. & Klein, E.** 1985. Karyologie und Fortpflanzungsmodus von *Nigritella* (Orchidaceae-Orchidaceae), inkl. *N. archiducis-joannis* spec. nov. und zweier Neukombinationen. *Phyton (Horn)* 25: 147–176.
- Valentini, G. & Montecchi, A.** 1990. Una nuova orchidea per l'Italia e l'Europe Occidentale — *Pseudorchis frivaldii* (Hampe ex Griseb.) P.F. Hunt. [II] *Fungo* 8: 6–7.
- Vermeulen, P.** 1947. *Studies on Dactylorchids*. Schotanus & Jens, Utrecht.
- Vermeulen, P.** 1977. Orchideen, systematisch ingedeeld. Pp. 551–557 in: Landwehr, J., *Wilde Orchideen van Europa*. Natuurmonumenten, Amsterdam.
- Williams, J. G., Williams, A. E. & Arlott, N.** 1978. *A Field Guide to the Orchids of Britain and Europe*. Collins, London.

**Appendix. Details of accessioned specimens used to generate the ITS sequences analysed in this study.****Species, Habitat/Locality: Collector, Voucher (s = silica gel, p = 1:1 photograph, h = herbarium specimen, Kew), GenBank number or previously published sequence information [P = Pridgeon & al., 1997, B = Bateman & al., 2003].**

*Dactylorhiza incarnata* subsp. *pulchella*, marsh, E Loch Kernsary, WC Scotland: *Bateman RB056* (s, p), [P]; *Gymnadenia odoratissima*, wooded marsh, NE Scharnitz, NW Innsbruck, Austria: *Bateman RB138* (s, p), [B]; *G. conopsea* s.s., limestone grassland, Box Hill, Surrey, England: *Chase MWC574* (s, h), [P]; *G. conopsea* s.s., West Balkan Mts. SW Bulgaria: *Nikolova RB868* (s) [DQ351278]; *G. conopsea* s.s., Smolikas, Macedonian Greece: *Fairhurst, RB882* (s) [DQ351281]; *G. cf. densiflora*, Vitosha Mts. S Sofia, SW Bulgaria: *Nikolova, RB869* (s) [DQ351279]; *G. densiflora*, marsh, NE Blair Atholl, NW Pitlochry, Tayside, Scotland: *Bateman RB165* (s, p) [B]; *G. orchidis*, China: *Luo078* (s, h) [B]; *G. borealis*, calcareous marsh, Applecross, WC Scotland: *Bateman RB064* (s, p) [P]; “*Pseudorchis*” *frivaldii*, Pirin Mts., Vihrenheit, S Sofia, SW Bulgaria: *Nikolova RB867* (s) [DQ351277]; “*P.*” *frivaldii*, Kaimaktealan, Macedonian Greece: *Fairhurst, RB878* (s) [DQ351280]; “*Nigritella*” *austriaca* (*nigra* s.l.), alpine meadow, Col du Menée, French Alps: *Manuel, RB067* (s) [P]; “*N.*” *nigra* s.s., Sweden: *Hedren97/322* (s, p); “*N.*” *miniata* (*rubra*), alpine meadow, E Grödner Joch, SE Brixen, Italian Alps: *Bateman RB154* (s, p) [B].