

Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae)

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Internal transcribed spacer (ITS nuclear rDNA) data have been obtained from 190 terrestrial orchid species, encompassing all genera and the great majority of the widely recognized species of Orchidinae, a heterogeneous selection of species of Habenariinae, and single species of Satiyriinae and Disinae (the latter serving as outgroup). The resulting parsimony-based phylogeny reveals 12 well-resolved clades within the Orchidinae, based on *Anacamptis s.l.*, *Serapias*, *Ophrys*, *Steeniella*–*Himantoglossum s.l.* (including ‘*Comperia*’ and ‘*Barlia*’, most species being $2n = 36$), *Neotinea s.l.*, *Traunsteinera*–*Chamorchis*, *Orchis s.s.*, *Pseudorchis*–*Amerorchis*–*Galearis*–*Neolindleya*–*Platanthera s.l.* (most $2n = 42$), *Dactylorhiza s.l.*, *Gymnadenia s.l.* (most $2n = 40, 80$), *Ponerorchis s.l.*–*Hemipilia s.l.*–*Amitostigma*–*Neottianthe*, and *Brachycorythis* (most $2n = 42$). Relationships are less clearly resolved among these 12 clades, as are those within Habenariinae; the subtribe appears either weakly supported as monophyletic or as paraphyletic under maximum parsimony, and the species-rich genus *Habenaria* is clearly highly polyphyletic. The triphyly of *Orchis* as previously delimited is confirmed, and the improved sampling allows further generic transfers to *Anacamptis s.l.* and *Neotinea s.l.* In addition, justifications are given for: (1) establishing *Steeniella* as the basally divergent member of an appreciably expanded *Himantoglossum* that incorporates the former genera ‘*Barlia*’ and ‘*Comperia*’, (2) reuniting ‘*Piperia*’ with a broadly defined *Platanthera* as section *Piperia*, necessitating ten new combinations, (3) broadening *Ponerorchis* to include *Chusua*, and *Hemipilia* to include single ‘orphan’ species of *Ponerorchis* and *Habenaria*, and (4) recognizing ‘*Gymnadenia*’ *camtschatica* as the monotypic *Neolindleya camtschatica* within the *Pseudorchis*–*Platanthera* clade. Few further generic transfers are likely in Orchidinae *s.s.*, but they are anticipated among habenariid genera, on acquisition of additional morphological and molecular evidence; one probable outcome is expansion of *Herminium*. Species-level relationships are also satisfactorily resolved within most of the major clades of Orchidinae, with the notable exceptions of *Serapias*, the derived sections of *Ophrys*, *Himantoglossum s.s.*, some sections within *Dactylorhiza*, the former genus ‘*Nigritella*’ (now tentatively placed within *Gymnadenia s.l.*), *Hemipilia s.l.*, and possibly *Ponerorchis s.s.* Relationships among the 12 major clades broadly accord with bona fide records of intergeneric hybridization. Current evidence supports the recently recognized $2n = 36$ clade; it also indicates a $2n = 40$ clade that is further diagnosed by digitate root-tubers, and is derived relative to the recently recognized clade of exclusively Asian genera (*Ponerorchis s.l.*–*Hemipilia s.l.*–*Amitostigma*–*Neottianthe*). This in turn appears derived relative to the Afro-Asiatic *Brachycorythis* group; together, these two clades identify the plesiomorphic chromosome number as $2n = 42$. If the African genus *Stenogolottis* is correctly placed as basally divergent within a monophyletic Habenariinae, the tribe Orchideae and subtribes Orchidinae and Habenariinae could all have originated in Africa, though in contrast the Asiatic focus of the basally divergent members of most major clades of Orchidinae suggests an Asiatic radiation of the subtribe. Morphological characters informally ‘mapped’ across the

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molecular phylogeny and showing appreciable levels of homoplasy include floral and vegetative pigmentation, flower shape, leaf posture, gynostemium features, and various pollinator attractants. Qualitative comparison of, and reciprocal illumination between, degrees of sequence and morphological divergence suggests a nested set of radiations of progressively decreasing phenotypic magnitude. Brief scenarios, both adaptive and non-adaptive, are outlined for specific evolutionary transitions. Recommendations are made for further species sampling, concentrating on Asian Orchidinae (together with the Afro-Asiatic *Brachycorythis* group) and both Asian and Southern Hemisphere Habenariinae, and adding plastid sequence data. Taxonomic changes listed are: *Anacamptis robusta* (T. Stephenson) R.M. Bateman, **comb. nov.**, *A. fragrans* (Pollini) R.M. Bateman, **comb. nov.**, *A. picta* (Loiseleur) R.M. Bateman, **comb. nov.**, *Neotinea commutata* (Todari) R.M. Bateman, **comb. nov.**, *N. conica* (Willdenow) R.M. Bateman, **comb. nov.**, *Platanthera elegans* Lindley ssp. *maritima* (Rydberg) R.M. Bateman, **comb. nov.**, *P. elegans* Lindley ssp. *decurtata* (R. Morgan & Glicenstein) R.M. Bateman, **comb. nov.**, *P. elongata* (Rydberg) R.M. Bateman, **comb. nov.**, *P. michaelii* (Greene) R.M. Bateman, **comb. nov.**, *P. leptopetala* (Rydberg) R.M. Bateman, **comb. nov.**, *P. transversa* (Suksdorf) R.M. Bateman, **comb. nov.**, *P. cooperi* (S. Watson) R.M. Bateman, **comb. nov.**, *P. colemanii* (R. Morgan & Glicenstein) R.M. Bateman, **comb. nov.**, *P. candida* (R. Morgan & Ackerman) R.M. Bateman, **comb. nov.** and *P. yadonii* (R. Morgan & Ackerman) R.M. Bateman, **comb. nov.** © 2003 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2003, **142**, 1–40.

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INTRODUCTION

During the last decade, molecular phylogenetics has undergone an exponential increase in both the range of techniques available (e.g. Soltis, Soltis & Doyle, 1998; Hollingsworth, Bateman & Gornall, 1999; Chase, Fay & Savolainen, 2000a) and the spectrum of topics to which they are applied. Early studies understandably addressed broader phylogenetic questions, notably the origins of major clades of land-plants (summarized in Kenrick & Crane, 1997) or the relationships among, and re-delimitation of, angiosperm families (Soltis *et al.*, 2000; see also Angiosperm Phylogeny Group, 1998). Predictably, early molecular studies of the Orchidaceae sought to discover the closest sister-group to the family (e.g. Chase *et al.*, 1994; Dressler & Chase, 1995) or the relationships of major groups within the family (e.g. Kores *et al.*, 1997). In our opinion, despite much progress, both issues have yet to be unequivocally resolved (cf. Rasmussen, 1999; Rudall & Bateman, 2002).

More recently, it became clear that evolutionary interpretations of plant groups would benefit from more focused phylogenetic surveys that maximized species-level coverage within specific clades at lower taxonomic levels. The Orchidaceae is a prime candidate for such studies (e.g. Chase, 1999; Pridgeon *et al.*, 1999, 2001; Bateman, 2001), given that it is arguably (a) the second-largest extant plant family and (b) one of the most recent species-rich plant families to undergo a major evolutionary radiation. However, Chase (2001) recently postulated a rather earlier origin (c. 100 Ma), an assertion based largely on molecular clock evidence (e.g. Sanderson, 1997).

Here, we greatly extend the sampling of recent pioneering nrDNA ITS sequence analyses of the subtribe Orchidinae (Bateman, Pridgeon & Chase, 1997; Pridgeon *et al.*, 1997; Bateman, 1999a, 2001; Bateman *et al.*, 2001). Although this is the most species-rich and ecologically dominant subtribe of Orchidoideae in the much-studied European orchid flora, those clades more basally divergent within the subtribe also have representative species in Asia and North America. Our objective is to encompass all of the widely recognized extant species assigned to the subtribe, with the intention of gaining a better understanding of patterns of molecular and morphological character change and, ultimately, of the underlying evolutionary processes.

REVIEW OF PREVIOUS PHYLOGENETIC STUDIES OF THE ORCHIDOIDEAE

MORPHOLOGY

Thus far, morphological cladists have focused on broad-brush (and typically non-algorithmic) analyses of the entire orchid family. Sampling was therefore rather sparse, and the coded taxa were required to represent high taxonomic levels in Burns-Balogh & Funk (1986) and Dressler (1993). Dressler's (1993: fig. 7.6) benchmark, but rather character-deficient, phylogeny of the Orchidoideae and Discoideae at subtribal level yielded a polytomy of the Orchidinae, Habenariinae and Discoideae. The genus-based phylogenetic analysis of Freudenstein & Rasmussen (1999: fig. 1) generated a fairly well-supported, derived Orchidoideae–Discoideae clade, but no meaningful resolu-

tion among the four representative genera that were included (*Disa*, *Satyrium*, *Platanthera*, *Dactylorhiza*). Sadly, despite extensive morphological study over two centuries, there has been no work on the Northern Hemisphere Orchidinae to match the morphological phylogenetic monographs of South African orchids by H. P. Linder and H. Kurzweil (e.g. Linder & Kurzweil, 1994, 1999; Kurzweil & Linder, 1999).

ALLOZYME AND RFLP ANALYSES

Schlegel *et al.* (1989) obtained allozyme data from a range of members of the genus *Orchis* as then conceived. In retrospect, the incongruity of *Anacamptis morio* and *Neotinea ustulata*, both traditionally ascribed to *Orchis s.l.*, is clearly evident in their much longer terminal branches relative to those subtending the six species of *Orchis s.s.* that were analysed. In a more detailed analysis, Rossi *et al.* (1994) then correctly inferred considerable genetic distances among four species groups: the derived portion of our *Anacamptis s.l.* clade (e.g. *A. morio*), the basal portion of our *Anacamptis* clade (e.g. *A. laxiflora*), the anthropomorphic members of *Orchis s.s.* (including the former monotypic genus '*Aceras*') and the more derived portion of that clade (represented by *O. quadripunctata* and *O. brancifortii*). However, Rossi *et al.* did not examine members of the third clade that was formerly included in *Orchis*, namely *Neotinea s.l.*

Cozzolino *et al.* (1998) then performed an RFLP (restriction fragment length polymorphism) study of plastid DNA that examined nine members of the former *Orchis s.l.* plus the related '*Aceras*' *anthrophorum* and *Anacamptis pyramidalis* (but again lacking *Neotinea s.l.*). Also included in the ingroup was *Dactylorhiza saccifera*, and the outgroup consisted of *Serapias vomeracea* and *Cephalanthera rubra*. However, various sequencing studies show that *Cephalanthera* is far too distant from *Orchis s.l.* to form a reliable outgroup (a point noted by Cozzolino *et al.*, 1998; Aceto *et al.*, 1999), and that the second 'outgroup', *Serapias*, is in fact more closely related to *Orchis s.l.* than is one of the supposed ingroups, *Dactylorhiza* (cf. Pridgeon *et al.*, 1997). Not surprisingly, the resulting preferred most-parsimonious tree (Cozzolino *et al.*, 1998: fig. 1b) deviates considerably from our own recent research (Bateman, 2001; Bateman *et al.*, 2001), especially near the base, yielding congruent topologies only with the pairing of *Orchis mascula* with *O. pauciflora* and of species within our *Anacamptis s.l.* clade. Moreover, *Anacamptis pyramidalis* was subsequently suspected by Aceto *et al.* (1999) of having undergone plastid capture, though recent unpublished *trnL* analyses suggest that this assumption

was probably incorrect (R. Bateman and P. Hollingsworth, unpubl. obs.).

PLASTID DNA SEQUENCING

Early molecular phylogenetic studies of the Orchidaceae that included representative species of Orchideae used the plastid genes *rbcL* and *ndhF*. The first *rbcL* study (Chase *et al.*, 1994: fig. 2) analysed *Disa tripetaloides* and *Platanthera ciliaris*, which proved to be well-supported sister-genera functioning as an aggregate placeholder for the Orchideae. Three approximately contemporaneous studies of *ndhF* used *Habenaria repens* as the placeholder for the Orchideae (Neyland & Urbatsch, 1995, 1996a,b). This species has since proved a less than ideal representative (see 'Habenariids' below), and was replaced by *Orchis quadripunctata* as the placeholder for Orchideae in an *nad1* analysis of epidendroids (Freudenstein, Senyo & Chase, 2000a).

Extending earlier studies of *rbcL*, with its limited number of variable sites, Kores *et al.* (1997: fig. 1) added to *D. tripetaloides* and *H. repens* the newly sequenced *Satyrium nepalense* and *Orchis quadripunctata*. The resulting tree gave strong jack-knife support to monophyly of the Orchideae but no support to the preferred pectinate topology within the clade of (((*Satyrium*, *Disa*) *Habenaria*) *Orchis*). The next, more densely sampled *rbcL* study of the Orchidaceae (Cameron *et al.*, 1999: fig. 4) restored to the analysis *P. ciliaris* and added *Ophrys apifera*, overturning all of the relationships inferred by Kores *et al.* to yield the topology (((*Satyrium*, *Platanthera*) *Habenaria*) (*Ophrys*, *Orchis*) *Disa*), albeit again without strong support for the internal nodes. Kores *et al.* (2000: fig. 1) analysed ten species (identities not specified) of nine genera of Orchideae in a *matK* study focusing on Diurideae, again yielding a unique and highly contrasting topology of (((((*Gymnadenia*, *Platanthera*) *Orchis*) (*Stenoglottis*, *Holothrix*)) (*Habenaria*, *Cynorkis*) *Gennaria*)) *Satyrium*), nested within five Deseae. Kores *et al.* (2001) further explored these relationships by combining the plastid regions *matK* and *trnL*, again including ten species of Orchideae and generating the topology (((((*Ophrys*, *Holothrix*) (*Habenaria*, *Peristylus*)) *Satyrium*) ((*Disa*, *Monadenia*) (*Corycium*, *Pterigodium*))) *Disperis*).

Thus, plastid sequences have contributed only modestly and equivocally to understanding of the phylogeny of the Orchideae, the primary advance being confirming the morphologically inferred monophyly of the Orchideae as a whole rather than gaining reliable insights on relationships within the group. This limitation largely reflected inadequate sampling of Orchideae in studies designed primarily to address relationships of orchids outside the subtribe.

NUCLEAR DNA SEQUENCING

Nuclear ribosomal ITS DNA of Orchidinae was first sequenced by Cozzolino *et al.* (1996); five species of *Orchis* s.l. were resolved using ITS1 into two of the three groups subsequently recognized by Pridgeon *et al.* (1997): '*O.* *coriophora*', '*O.* *morio*' and '*O.* *laxiflora*' (our *Anacamptis* s.l.) vs. *O. simia* and *O. purpurea* (our *Orchis* s.s.).

A greatly increased scale of sampling for molecular analysis in the Orchidinae was heralded by the nuclear ribosomal DNA matrix of Pridgeon *et al.* (1997) and Bateman *et al.* (1997). The 88 sequences of the ITS1–5.8S–ITS2 assembly (cf. Baldwin, 1992; Baldwin *et al.*, 1995; Hershkovitz, Zimmer & Hahn, 1999) provided the forerunner of the present study. Data were analysed in detail (Fig. 1) and subsequent discussion topics included sequence evolution, inter- and infrageneric relationships and hybridization frequencies, comparison with the results of past studies (both non-phylogenetic and phylogenetic), key synapomorphies (including tubers and karyotypes), homoplastic characters (including floral morphology and pigmentation), and misleading information

detected in the extensive literature on the group. Their primary taxonomic conclusion was that *Orchis* as then widely delimited was triphyletic. After discussing levels of sequence disparity necessary for particular taxonomic ranks and reviewing the taxonomic history of the genus, the authors controversially but logically reassigned the majority of species in the former *Orchis* to expanded concepts of *Neotinea* and especially of *Anacamptis*. Other taxonomic rationalizations were the inclusions of (a) *Nigritella* within an otherwise apparently paraphyletic *Gymnadenia*, (b) the monotypic *Aceras* within *Orchis* s.s., and (c) the monotypic *Coeloglossum* within *Dactylorhiza* (which required subsequent nomenclatural conservation: Cribb & Chase, 2001) (Fig. 1).

Bateman (1999a: fig. 19.3; see also Bateman *et al.*, 1998) presented a preliminary ITS tree derived from an expanded matrix of 129 species, including five genera absent from the previous ITS studies. The short discussion focused on patterns of morphological homology within the clade, the wisdom (or otherwise) of 'mapping' such characters across molecular trees, and the potential of comparing morphological and molecular trees to detect saltational evolutionary

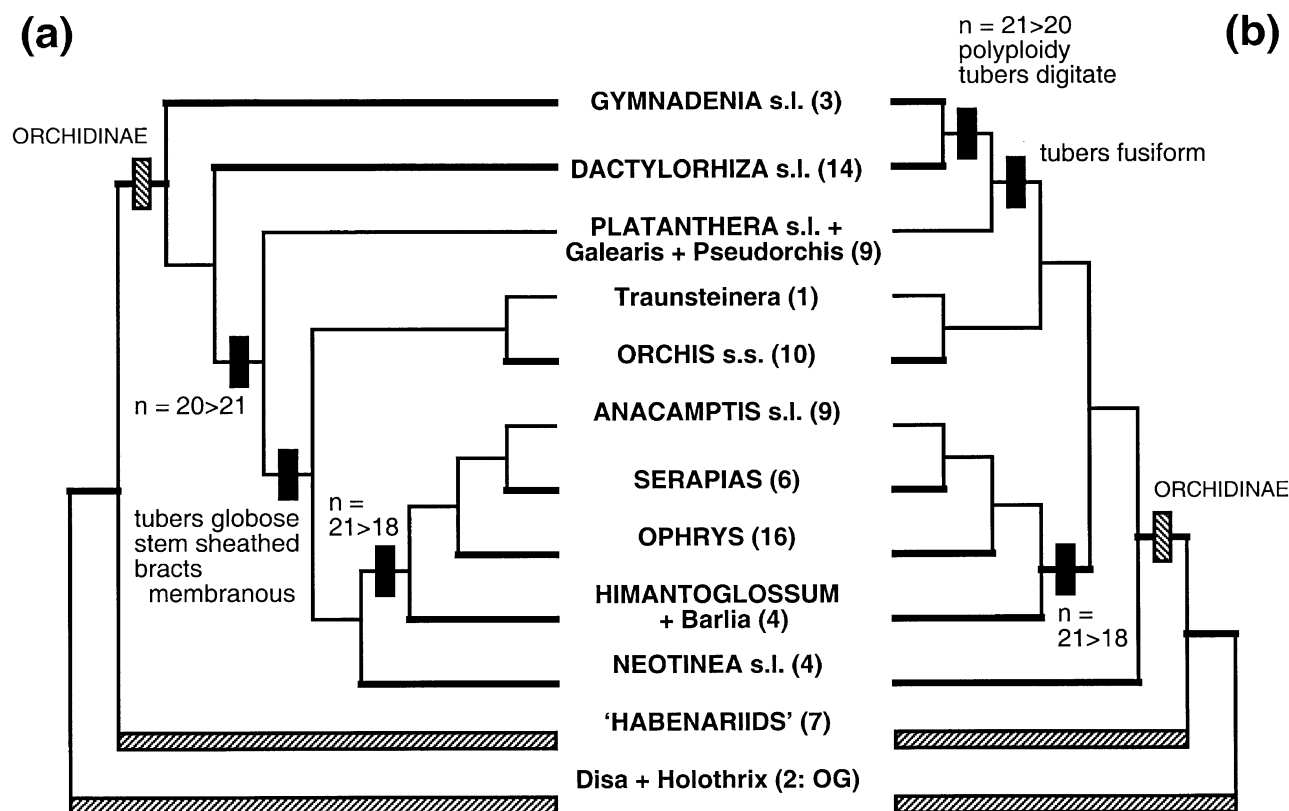


Figure 1. Summary of the ITS phylogenies of the Orchidinae presented by Pridgeon *et al.* (1997: figs 1–4), as estimated (a) without and (b) with indels. Thick branches indicate >80% bootstrap support; cross-hatched groups are paraphyletic (see also Bateman *et al.*, 1997: fig. 1). The number of named taxa analysed for each major clade is indicated in parentheses.

events. The matrix was upgraded to 172 species of Orchideae for inclusion in *Genera Orchidacearum* 2 (Pridgeon *et al.*, 2001), and further upgraded to the current matrix (together with *Dactylorhiza* cf. *hata-girea*) by Bateman (2001) in order to generate a Neighbour Joining tree.

An independent study of ITS1 and ITS2 by Aceto *et al.* (1999) presented trees for 30 species of Orchidinae (all but *Orchis patens* s.s. were analysed independently for the present study), confirming the triphyly of *Orchis* s.l. and the existence of a $2n = 36$ clade already elucidated by Pridgeon *et al.* (1997). However, the placements of the *Neotinea* and *Himantoglossum* clades, and of 'Aceras', differed, perhaps because *Genaria diphylla*, a highly divergent long-branch taxon (Pridgeon *et al.*, 1997; Bateman *et al.*, 2001), was chosen by Aceto *et al.* (1999) as the sole outgroup. This choice also gave ambiguous polarization for crucial characters such as karyotype.

The six genera of Orchidinae analysed by Cameron *et al.* (1999) for *rbcL* (albeit most represented by different species) were also incorporated in a later and taxonomically broader molecular study focusing on Diseae and using nuclear ITS sequences (Douzery *et al.*, 1999: fig. 1). Nine sequences of Orchidinae and five of Habenariinae carried forward from the earlier study (Bateman *et al.*, 1997; Pridgeon *et al.*, 1997) were combined with a further six habenariids and 30 Diseae, revealing a paraphyletic Diseae s.l. (Diseae s.s. sandwiched within a polyphyletic Coryciinae, with Satyriinae relatively derived) subtending a monophyletic Orchidinae as sister to a monophyletic Habenariinae. Support for monophyly of Orchidinae is strong, but that for Habenariinae is very weak (bootstrap values <50%), causing Pridgeon *et al.* (2001) to sink Habenariinae into Orchidinae. Interestingly, the topology of Cameron *et al.* (1999) resembles the morphological subtribal phylogeny of Dressler (1993), but with a highly contrasting rooting that reverses the polarity of most key characters. However, except for the near-basal divergence of *Disa*, the results of this study differed strongly from those of the preceding, less well-sampled *rbcL* analyses. Within the Orchidinae/Habenariinae, with the exception of the pairings of *Pseudorchis albida* plus *Platanthera chlorantha* and *Habenaria sagittifera* plus *Herminium lanceum*, the tree showed disconcertingly little topological similarity to the preceding, more intensively sampled ITS analysis (Bateman *et al.*, 1997, 1998; Pridgeon *et al.*, 1997; Bateman, 1999a).

Luo Y.-B. and A. Pridgeon (pers. comm., 2000) similarly used the ITS framework of Pridgeon *et al.* as the basis of an unpublished study to examine in greater detail single additional sequences from south-east Asian representatives of the Habenariinae genera *Habenaria* and *Peristylus*, and the Orchidinae genera

Platanthera, *Galearis*, *Gymnadenia*, *Neottianthe*, *Amitostigma* and *Ponerorchis* (including 'Chusua'), together with eight putative species of *Hemipilia*. Relationships among the south-east Asian clade, consisting of *Amitostigma*–*Hemipilia*, resemble those observed in the present study, but generic relationships within the ostensible sister group of *Platanthera*–*Gymnadenia*–*Dactylorhiza*–*Pseudorchis*–*Orchis* s.s.–*Galearis* again are strongly contradicted by the earlier ITS study of Pridgeon *et al.* (1997). Cozzolino *et al.* (2001) further developed their ITS matrix to preferentially encompass Middle Eastern segregate species previously encompassed by *Orchis* s.l., focusing their interpretations on pollination biology. Lastly, ITS sequences have proved to be sufficiently good taxonomic markers to identify to species level individual pollinia found attached to pollinating insects (Widmer *et al.*, 2000).

Other recent studies have investigated additional regions of nuclear rDNA. Focusing on higher taxonomic levels, an analysis of representatives of c. 60 genera spanning the Orchidaceae (Freudenstein, Senyo & Chase, 2000b) compared sequences for ITS2 with those for 26S rDNA, noting that sequence alignment is more challenging for ITS. The results strongly supported the monophyly of five subfamilies (including Orchidoideae s.l., incorporating the former Spiranthoideae: Salazar, 2003) and many tribes, but also emphasized the difficulty of resolving relationships among the tribes. An 18S study of the Orchidaceae focusing on identifying multiple origins of mycoheterotrophy (Molvray, Kores & Chase, 2000: fig. 1) included four genera of Orchideae in an apparently weakly supported topology of (((*Habenaria*, *Platanthera*) *Orchis*) *Satyrium*), the sister-group relationship of *Habenaria* and *Platanthera* differing from the various topologies generated using ITS.

MATERIAL AND METHODS

A total of 190 sequences was analysed, representing 187 named taxa. Most of the 88 ITS sequences analysed by Pridgeon *et al.* (1997; sample details given in their table 2) were carried forward, via Bateman (1999a), into this more detailed analysis. The few exclusions included two of three apparently contrasting ITS sequences extracted from a single individual of *Dactylorhiza praetermissa* and one of the pair of molecularly identical individuals of *Anacamptis laxiflora*. Also, the putative Southern Hemisphere outgroup *Holothrix scopularia* was deemed too difficult to align (as well as being viewed as a doubtful bona fide member of the Orchidinae–Habenariinae clade), and *Habenaria quinqueseta* was judged to possess an unacceptably large proportion of missing values. In addition, a new sequence was substituted for the original *Orchis pauciflora* due to its surprising (though admittedly ulti-

Table 1. Plant materials of Orchidaceae tribe Orchideae analysed in this study in addition to those listed by Pridgeon *et al.* (1997: table 2). ¹ = new combination made in this paper; ² = name currently illegitimate, new combination yet to be made; ³ = supposed species here demonstrated to be a hybrid

Taxon	Reference/voucher
Tribe Orchideae	
Subtribe Orchidinae	
<i>Amerorchis rotundifolia</i> (Banks ex Pursh) Hultén	Chase O-0850
<i>Amitostigma gracile</i> Schlechter	Bateman 488 (Lee)
<i>Anacamptis (Orchis) fragrans</i> (Pollini) R.M.Bateman, Pridgeon and M.W.Chase ¹	Bateman 282
<i>Anacamptis (Orchis) israelitica</i> (H.Baumann and Dafni) R.M.Bateman, Pridgeon and M.W.Chase	Bateman 107 (Tattersall)
<i>Anacamptis (Orchis) longicornu</i> (Poiret) R.M.Bateman, Pridgeon and M.W.Chase	Bateman 105 (Tattersall)
<i>Anacamptis (Orchis) palustris</i> (Jacquin) R.M.Bateman, Pridgeon and M.W.Chase	Hedré 5557
<i>Anacamptis (Orchis) picta</i> (Loiseleur) R.M.Bateman, Pridgeon and M.W.Chase ¹	Bateman 430 (Lowe)
<i>Anacamptis (Orchis) robusta</i> (Stephenson) R.M.Bateman, Pridgeon and M.W.Chase ¹	Bateman 281
<i>Anacamptis (Orchis) syriaca</i> (Boissier ex H.Baumann & Künkele) R.M.Bateman, Pridgeon and M.W.Chase	Bateman 106 (Tattersall)
<i>Chamorchis alpina</i> (L.) L.C.M.Richard	Bateman 182 (Gössmann)
<i>Chusua (Ponerorchis) cf. chidori</i> (Makino) P.F.Hunt	Bateman 437
<i>Chusua donii</i> Nevski	Luo 053
<i>Chusua (Ponerorchis) jooiokiana</i> (Makino) P.F.Hunt	Bateman 587 (Lee)
<i>Dactylorhiza 'bowmanii'</i> ³ (= <i>D. traunsteineri</i> ssp. <i>bowmanii</i> M.Jenkinson)	Bateman 468
<i>Dactylorhiza cordigera</i> (Fries) Soó	Bateman 108 (Manuel)
<i>Dactylorhiza euxina</i> (Nevski) Czerepanov	Hedré s.n.
<i>Dactylorhiza fuchsii</i> (Druce) Soó var. <i>hebridensis</i> (Wilmott) Heslop-Harrison f.	Bateman 341
<i>Dactylorhiza cf. hatagirea</i> (D.Don) Soó [excluded from analysis]	Bateman 191 (Long)
<i>Dactylorhiza incarnata</i> (L.) Soó ssp. <i>cruenta</i> (O.F.Möller) P.D.Sell	Bateman 057
<i>Dactylorhiza insularis</i> (Sommier) Landwehr	Bateman 431 (Lowe)
<i>Dactylorhiza markusii</i> (Tineo) H.Baumann and Künkele	Bateman 109 (Manuel)
<i>Dactylorhiza occidentalis</i> (Pugsley) Delforge var. <i>kerryensis</i> (Wilmott) R.M.Bateman and Denholm	Bateman 118
<i>Dactylorhiza praetermissa s.s.</i> (Druce) Soó	Bateman 469
<i>Dactylorhiza praetermissa</i> (Druce) Soó var. <i>junialis</i> (Vermeulen) Senghas	Bateman 470
<i>Dactylorhiza saccifera</i> (Brongniart) Soó	Bateman 172 (Manuel)
<i>Dactylorhiza sambucina</i> (L.) Soó	Bateman 112 (Manuel)
<i>Dactylorhiza traunsteineri</i> (Sauter ex Reichenbach p.) Soó ssp. <i>ebudensis</i> (Wiefelspütz) ²	Bateman 055
<i>Galearis diantha</i> (Schlechter) P.F.Hunt	Luo 074
<i>Gymnadenia (Nigritella) nigra s.s.</i> (L.) Reichenbach f.	Hedré 97322
<i>Gymnadenia densiflora</i> (Wahlenberg) Dietrich	Bateman 165
<i>Gymnadenia (Nigritella) miniata</i> (Crantz) Hayek	Bateman 154
<i>Gymnadenia odoratissima</i> (L.) L.C.M.Richard	Bateman 138
<i>Gymnadenia orchidis</i> Lindley	Luo 078
' <i>Habenaria</i> ' <i>purpureopunctata</i> Lang	Luo 014
<i>Hemipilia crassicalcarata</i> Chien	Luo 245
<i>Hemipilia cruciata</i> Finet	Luo 055
<i>Hemipilia flabellata</i> Bureau and Franchet	Luo 228
<i>Hemipilia henryi</i> Rolfe	Luo 186
<i>Hemipilia kwangsiensis</i> Tang and Wang ex Lang	Luo s.n.
<i>Hemipilia limprichtii</i> Schlechter	Luo 123
<i>Hemipilia quinquangularis</i> Tang and Wang	Luo 221
<i>Himantoglossum (Comperia) comperianum</i> (Steven) P.Delforge	Bateman 114 (Tattersall)
<i>Himantoglossum calcaratum</i> (G.Beck) Schlechter	Bateman 170 (Manuel)
<i>Neolindleya camtschatica</i> (Chamisso) Nevski	Bateman 578 (Lee)
<i>Neotinea (Orchis) commutata</i> (Todaro) R.M.Bateman, Pridgeon and M.W.Chase ¹	Bateman 547
<i>Neotinea (Orchis) conica</i> (Willdenow) R.M.Bateman, Pridgeon and M.W.Chase ¹	Bateman 195 (Phillips)

Table 1. Continued

Taxon	Reference/voucher
<i>Neotinea (Orchis) tridentata</i> (Scopoli) R.M.Bateman, Pridgeon and M.W.Chase (replaces Chase O-0914 of Pridgeon <i>et al.</i> , 1997)	Bateman 548
<i>Neottianthe calcicola</i> Schlechter	Luo 178
<i>Neottianthe cucullata</i> (L.) Schlechter	Bateman 177 (Manuel)
<i>Ophrys atlantica</i> Mumby	Bateman 208 (Phillips)
<i>Ophrys benacensis</i> (Reisigl) O. and E.Danesch and Ehrendorfer	Bateman 571
<i>Ophrys biancae</i> (Todaro) Macchiati	Bateman 527
<i>Ophrys candica</i> W.Greuter, Matthäs and Risse	Bateman 037
<i>Ophrys dyris</i> Maire	Bateman 093 (Courtis)
<i>Ophrys elegans</i> (Renz) H.Baumann and Künkele	Bateman 201 (Rowland)
<i>Ophrys exaltata</i> Tenore	Bateman 365 (Manual)
<i>Ophrys fuciflora</i> (F.W.Schmidt) Moench	Bateman 167 (Manuel)
<i>Ophrys iricolor</i> Desfontaines	Bateman 033
<i>Ophrys levantina</i> Gözl and Reinhard	Bateman 230 (Rowland)
<i>Ophrys lunulata</i> Parlato	Bateman 528
<i>Ophrys oxyrrhynchos</i> Todaro	Bateman 540
<i>Ophrys pallida</i> Rafinesque	Bateman 553
<i>Ophrys sphegifera</i> Willdenow	Bateman 211 (Phillips)
<i>Ophrys spruneri</i> Nyman	Bateman 001
<i>Ophrys umbilicata</i> Desfontaines	Bateman 203 (Rowland)
<i>Ophrys vasconica</i> (O. and E.Danesch) P.Delforge	Bateman 316 (Lowe)
<i>Orchis brancifortii</i> Bivona-Bernardi	Bateman 103 (Tattersall)
<i>Orchis canariensis</i> Lindley	Bateman 131 (Welsh)
<i>Orchis cazorlensis</i> Lacaita	Bateman 479 (Lowe)
<i>Orchis galilaea</i> (Bornmüller and Schulze) Schlechter	Bateman 098 (Tattersall)
<i>Orchis ichnusae</i> (Corrias) J. and P.Devillers-Terschuren	Bateman 102 (Tattersall)
<i>Orchis langei</i> K.Richter	Chase O-0921
<i>Orchis olbiensis</i> Reuter ex Grenier	Bateman 271
<i>Orchis ovalis</i> F.W.Schmidt ex Mayer	Bateman 156
<i>Orchis pallens</i> L.	Bateman 314 (Lowe)
<i>Orchis patens</i> Desfontaines	Cozzolino Z94097-8
<i>Orchis pauciflora</i> Tenore (replaces Chase O-0710 of Pridgeon <i>et al.</i> , 1997)	Bateman 005
<i>Orchis prisca</i> Hautzinger	Bateman 286 (Clarke)
<i>Orchis provincialis</i> Balbis ex Lamarck and DC.	Bateman 100 (Tattersall)
<i>Orchis punctulata</i> Steven ex Lindley	Bateman 099 (Tattersall)
<i>Orchis scopulorum</i> Summerhayes	Bateman 104 (Tattersall)
<i>Orchis spitzelii</i> Sauter ex W.D.J.Koch	Bateman 168 (Manuel)
<i>Orchis troodii</i> (Renz) Delforge	Bateman 200 (Rowland)
<i>Platanthera bakeriana</i> Kraenzlin	Luo 035
<i>Platanthera (Piperia) colemanii</i> (R.Morgan & Glicenstein) R.M.Bateman ¹	Temple <i>s.n.</i>
<i>Platanthera (Piperia) elongata</i> (Rydberg) R.M.Bateman ¹	Temple <i>s.n.</i>
<i>Platanthera (Piperia) foetida</i> Geyer ex Hooker	Chase O-0930 (Hapeman)
<i>Platanthera holmboei</i> H.Lindberg f.	Bateman 443 (Lowe)
<i>Ponerorchis brevicarata</i> (Finet) Soó	Luo 161
<i>Pseudorchis straminea</i> (Fernald) Sojak	Liden <i>s.n.</i>
<i>Serapias bergonii</i> E.G.Camus	Bateman 294 (Ettlinger)
<i>Stenisiella satyrioides</i> (Steven) Schlechter	Güner 10238 (K)
Subtribe Habenariinae Benth	
<i>Bonatea speciosa</i> Willdenow	Roux <i>s.n.</i>
<i>Brachycorythis macrantha</i> (Lindley) Summerhayes	Chase O-0593
<i>Cynorkis 'lowiana'</i> Reichenbach f. ²	Chase O-0583
<i>Cynorkis</i> sp.	Bateman 372
<i>'Habenaria' repens</i> Nuttall	Chase O-0381

Table 1. *Continued*

Taxon	Reference/voucher
<i>Habenaria arenaria</i> Lindley	Chase O-1135
<i>Habenaria delavayi</i> Finet	Luo 161
<i>Habenaria socotrana</i> Balfour f.	Bateman 199
<i>Habenaria tibetica</i> Schlechter	Bateman 186 (Long)
<i>Habenaria tridactylites</i> Lindley	Bateman 194 (Ettlinger)
<i>Herminium alaschianum</i> Maximowicz	Bateman 188 (Long)
<i>Herminium (Peristylus) coeloceras</i> Schlechter	Luo 052
<i>Herminium (Platanthera) latilabris</i> (Lindley) ²	Bateman 392 (Noltie)
<i>Herminium monorchis</i> (L.) R.Brown	Bateman 121
<i>Stenoglottis longifolia</i> Hooker f.	Bateman 373
<i>Stenoglottis woodii</i> Schlechter	Bateman 374
Tribe Diseae Dressler	
Subtribe Satyriinae Schlechter	
<i>Satyrium nepalense</i> D.Don	Chase O-0539

mately upheld) phylogenetic position, separate from the other two yellow-flowered *Orchis* species.

A further 109 sequences were added during the present study. Accession numbers of these samples additional to Pridgeon *et al.* (1997: table 2) are given here in Table 1. Some sequences were derived from plant material supplied by several collaborators, and the ITS sequence for *Orchis patens* s.s. was downloaded from GenBank deposits made by Aceto *et al.* (1999: ITS1 = Z94097, ITS2 = Z94098; data are not available for the intervening 5.8S region). Together, these sequences encompass all well-founded genera of the re-delimited Orchidinae, and for most genera they incorporate the majority of the species widely recognized by terrestrial orchid specialists (cf. Delforge, 1995, 2001). Coverage of the newly expanded concept of the Habenariinae is broad at the generic level but remains undesirably sparsely sampled at the species level (cf. Dressler, 1993; Linder & Kurzweil, 1999). Taxonomy for Orchidinae largely follows the classification of Delforge, unashamedly a 'splitters' classification at the species level.

The complete ITS1–5.8S–ITS2 assembly (e.g. Hershkovitz *et al.*, 1999) was determined for each species, following extraction and sequencing protocols described by Pridgeon *et al.* (1997). Sequence editing employed Sequence Navigator (Applied Biosystems, Inc.), and alignment was performed by several pairs of eyes. After considerable experimentation, *Disa uniflora* was eventually designated as the outgroup. A single outgroup is acceptable in this case, given that the monophyly of the re-delimited Orchidinae had already been thoroughly tested (Pridgeon *et al.*, 1997; Cameron *et al.*, 1999; Bateman *et al.*, 2001). Although *Satyrium nepalense* was added to the matrix as a

potential intermediate outgroup, following the recognition on ITS evidence of a monophyletic Satyriinae immediately above the Diseae by Douzery *et al.* (1999) and Kores *et al.* (2000), its placement in some resulting trees above the long-branch Habenariinae *Gen-naria diphylla* (cf. Bateman, 2001) rendered it unsatisfactory as an outgroup, since it demonstrated the topological instability characteristic of a 'wildcard' taxon *sensu* Nixon & Wheeler (1992).

In total, 708 base positions were considered in the analysis. Short regions close to the priming sites in 18S and especially 26S were excluded due to the relatively poor quality of the sequence reads. Ambiguous gaps were scored as missing data, though 36 small but unambiguous indels were included in the analysis as bistate characters, following the 'simple coding' procedure of Simmons & Ochoterena (2000; cf. Giribet & Wheeler, 1999).

After some ultimately unproductive experimentation, tree-building followed a relatively simple and rapid protocol. A heuristic search was performed using PAUP* 4 (Swofford, 2000). All characters were treated as unordered and of equal weight. The initial heuristic search was 1000 random addition replicates with TBR branch swapping, saving a maximum of ten trees per replicate. The resulting 730 most-parsimonious trees were then used as starting trees for a further search, again using TBR swapping. Memory constraints prevented the second search from swapping to completion, limiting the number of trees saved to 10 000. Standard tree descriptors and the strict consensus tree were then obtained. To assess the support for individual nodes, a heuristic fast bootstrap search was subsequently performed for 10 000 replications using PAUP*.

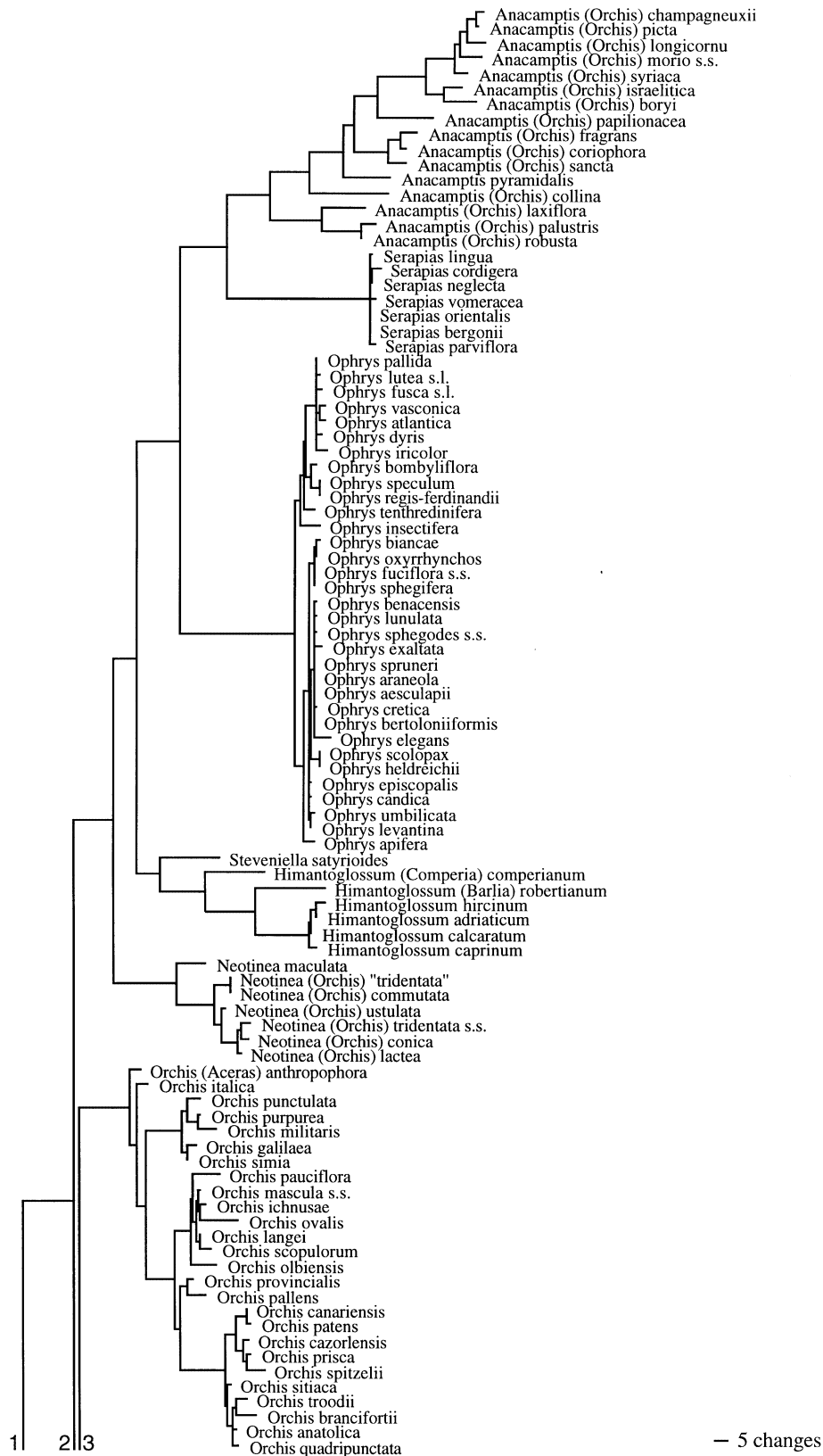


Figure 2. Selected most-parsimonious ITS tree, presented as a phylogram under Acctran optimization, for 186 species of Orchidinae and selected Habenariinae plus a Disease outgroup.

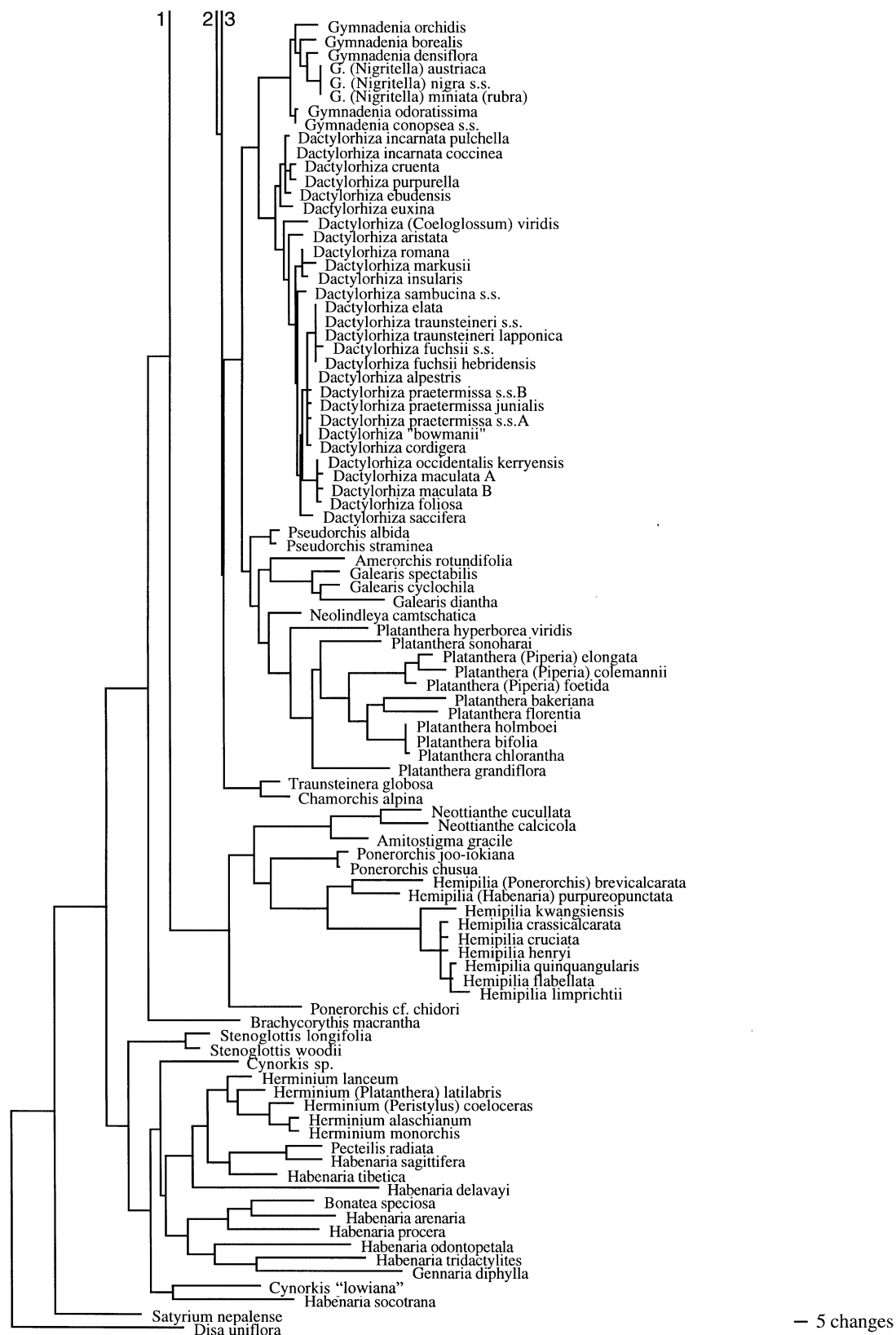


Figure 2. Continued

RESULTS

Of the 744 molecular characters included, 214 were constant. A further 87 bases were variable but uninformative, leaving 443 parsimony-informative characters. A heuristic search of the 190 coded taxa resulted in >10 000 trees of a Fitch (1971) length of 2886 (including autapomorphies), Consistency Index of 0.35 (0.32 excluding uninformative characters) and Retention Index of 0.81. A corresponding Neighbour Joining tree based on the same alignment was published by Bateman (2001: fig. 15; see also Temple, 2001).

Base composition, compositional bias and transition/transversion ratios of the original matrix were discussed by Pridgeon *et al.* (1997) and will be further reviewed by us elsewhere (R. Bateman *et al.*, unpubl. data); here, we will instead focus on perceived relationships inferred from the phylogeny, discussed in the context of mapped morphological and other non-sequence characters. To this end, a randomly selected most-parsimonious tree, optimized under Acctran, is presented as Figure 2, and the strict consensus tree bearing fast bootstrap percentages constitutes Figure 3.

RELATIONSHIPS WITHIN THE MAJOR CLADES

OVERVIEW

The ITS phylogenies, plus as yet unpublished *trnL* phylogenies for a subset of the coded taxa (R. Bateman and P. Hollingsworth, unpubl. obs.), delimit 12 well-supported clades of unequivocal Orchidinae (Fig. 2), each encompassing from one to five genera as circumscribed by Bateman *et al.* (1997) and further modified in the present study. There is also a relatively poorly resolved basal habenariid group, the two subtribes being separated by the ambiguously placed genus *Brachycorythis*, which is unusual in occurring in both the Northern (Chen, Tsi & Luo, 1999) and Southern (Linder & Kurzweil, 1999) Hemispheres. In contrast, most of the perceived relationships among those major clades receive weaker bootstrap support.

Here, we consider briefly each of those 12 clades in turn, beginning with *Anacamptis s.l.* (arbitrarily depicted here as the 'apex' of the tree), and concluding with the more ambiguous *Brachycorythis* and Habenariinae plus *Satyrium* and *Disa*. We then summarize higher level relationships and broader patterns of character distribution. A few of the clades are discussed in greater detail, where further generic transfers are required, notably (1) upgrading to species level some subspecies of *Anacamptis s.l.* and *Neotinea s.l.* recognized by Bateman *et al.* (1997), (2) incorporating the former monotypic genera '*Comperia*' and '*Barlia*' into an expanded *Himantoglossum*, (3) incor-

porating '*Piperia*' into a yet further expanded *Platanthera*, and (4) returning '*Gymnadenia camtschatica*' to the monotypic genus *Neolindleya*. Also, *Ponerorchis s.l.* appears triphyletic; *P. cf. chidori* is retained in *Ponerorchis* pending further species sampling, but '*P. brevicarata*' is transferred to the morphologically similar *Hemipilia*, along with '*Habenaria purpureopunctata*' (for alternative taxonomic views see Bateman *et al.*, 2001; Luo & Chen, in press). In contrast, we maintain *Chamorchis* and *Traunsteinera* as separate (arguably monotypic) genera, despite their surprising relationship as sister taxa. Suggested taxonomic rearrangements of the Habenariinae are not formalized here, as current taxonomic sampling is viewed as insufficiently dense. More detailed accounts of the remaining clades of Orchidinae (albeit discussing far fewer coded taxa) were given by Bateman *et al.* (1997).

Within the text, the symbol '-' is used to indicate an inclusive clade of three or more species shown on the figures that is bracketed by the two explicitly stated end-members (e.g. the clade of 21 closely related species of *Ophrys* bracketed by *O. apifera*-*O. biancae* on Fig. 2). Levels of bootstrap support for particular clades on Figure 3 are summarized as poor (<50%), moderate (51-80%) or strong (>80%). Comments on the correlation between degrees of ITS sequence divergence and possible species-level status are highly tentative, and should be considered carefully in conjunction with discussions placed under 'Species delimitation' and 'Future research'.

ANACAMPTIS (16 CODED TAXA)

This moderately supported clade encompasses all species of the former *Orchis* that possess 36 (or, in the case of *A. papilionacea*, 32) chromosomes (for a more detailed account see Tichy & Del Prete, 2001). Since the analysis of Pridgeon *et al.* (1997), it has attracted further attention from Cozzolino *et al.* (2001) and R. Bateman and P. Hollingsworth (unpubl. obs.). Molecular, morphological and cytological (D'Emérico, Pignone & Bianco, 1996; D'Emérico, Pignone & Scrugli, 2000) divergences are on average high within the clade (Fig. 2), which shows clear karyological differences from both *Serapias* and *Ophrys* (D'Emérico *et al.*, 2000a).

Groups that are monophyletic on molecular evidence and also possess morphological cohesion include *champagneuxii-boryi* (including the pairing of *A. boryi* with *A. israelitica*, which share the characteristic of basipetal flowering: Cozzolino *et al.*, 2001), *fragrans-sancta* and *laxiflora-robusta*; the latter group, and the group immediately above *A. papilionacea*, are each supported by single indels. The more morphologically individualistic *A. pyramidalis*, *A. papilionacea*

Table 2. Modifications to the species-level revisions of the expanded genera *Anacamptis s.l.* and *Neotinea s.l.* presented in Bateman *et al.* (1997)***Anacamptis robusta* (T.Stephenson) R.M.Bateman, *comb. nov.****Basionym:* *Orchis palustris* Jacquin var. *robusta* T.Stephenson, *J. Bot. (Lond.)* 69: 179 (1931).*Synonyms:* *Orchis laxiflora* Lamarck ssp. *robusta* (T.Stephenson) H.Sundermann, *Europ. Medit. Orch.* 40 (1980); *Orchis robusta* (T.Stephenson) Gözl and Reinhard, *Ber. Schweiz. Bot. Ges.*, **85**: 288 (1975); *Anacamptis palustris* (Jacquin) R.M.Bateman, Pridgeon and M.W.Chase ssp. *robusta* (T.Stephenson) R.M.Bateman, Pridgeon and M.W.Chase, *Lindleyana* 12: 120 (1997).***Anacamptis fragrans* (Pollini) R.M.Bateman, *comb. nov.****Basionym:* *Orchis fragrans* Pollini, *Elem. Prov. Ver.* 2: 155 (1811)*Synonyms:* *Orchis coriophora* L. ssp. *fragrans* (Pollini) Sudre, *Fl. Toulous.* 187 (1907); *Anacamptis coriophora* (L.) R.M.Bateman, Pridgeon and M.W.Chase ssp. *fragrans* (Pollini) R.M.Bateman, Pridgeon and M.W.Chase, *Lindleyana* 12: 120 (1997).***Anacamptis picta* (Loiseleur) R.M.Bateman, *comb. nov.****Basionym:* *Orchis picta* Loiseleur, *Mem. Soc. Linn. Paris* 6: 431 (1827).*Synonym:* *Orchis morio* L. ssp. *picta* (Loiseleur) Arcangeli, *Comp. Fl. Ital.* ed. 2: 167 (1894) [placed under *Anacamptis morio* (L.) R.M.Bateman, Pridgeon and M.W.Chase by Bateman *et al.*, 1997: 122].***Neotinea commutata* (Todari) R.M.Bateman, *comb. nov.****Basionym:* *Orchis commutata* Todari, *Orch. Sic.* 24 (1842).*Synonyms:* *Orchis tridentata* Scopoli ssp. *commutata* (Todari) Nyman, *Consp.* 691 (1882); *Neotinea tridentata* (Scopoli) R.M.Bateman, Pridgeon and M.W.Chase ssp. *commutata* (Todari) R.M.Bateman, Pridgeon and M.W.Chase, *Lindleyana* 12: 122 (1997).***Neotinea conica* (Willdenow) R.M.Bateman, *comb. nov.****Basionym:* *Orchis conica* Willdenow, *Spec.* 4: 14 (1805).*Synonyms:* *Orchis tridentata* Scopoli ssp. *conica* (Willdenow); *Neotinea tridentata* (Scopoli) R.M.Bateman, Pridgeon and M.W.Chase ssp. *conica* (Willdenow) R.M.Bateman, Pridgeon and M.W.Chase, *Lindleyana* 12: 122 (1997).

and *A. collina* are, unsurprisingly, more ambiguously placed (*A. collina* also yielded strongly divergent allozyme patterns according to Rossi *et al.*, 1994). The new deeper placements of *A. pyramidalis* and *A. collina* undermine the 'papilionacea subgroup' tentatively recognized by Bateman *et al.* (1997). Similar positional ambiguities, and a contrasting topology, are evident in the ITS phylogenies of Aceto *et al.* (1999: fig. 1) and Cozzolino *et al.* (2001: fig. 1). The morphologically predicted placement of *A. syriaca* as unequivocal sister to the *morio* group demonstrates that the specimen analysed by Cozzolino *et al.* (2001) as '*A. syriaca*' was, as they subsequently suspected, more akin to *A. papilionacea*.

The only species of Orchidinae analysed by Cozzolino *et al.* but absent from our tree is *A. dinsmorei*; as expected, this proved to be the Asia Minor segregate of *A. palustris*. The tentative suggestion of Bateman *et al.* (1997) that the vegetatively distinct *laxiflora*–*palustris*–*robusta* clade (represented in their tree only by *A. laxiflora*) could merit generic segregation receives some support from the subsequent tree (Fig. 2), since their inclusion reduces bootstrap support for *Anacamptis* as currently delimited to 59%, compared with 89% for the remainder of the clade. However, *trnL* data suggest that these species are bona fide members of *Anacamptis* (R. Bateman and P. Hollingsworth, unpubl. obs.).

The eight-step ITS disparity between *A. coriophora* s.s. and *A. fragrans*, and the five-step disparity between *A. palustris* s.s. and *A. robusta*, suggest that Bateman *et al.* (1997) may have been premature in treating *fragrans* and *robusta* as subspecies rather than as bona fide species; they are therefore elevated to species level in Table 2. However, a much smaller ITS disparity was inferred in a more detailed survey of the *palustris* group conducted by Cafasso *et al.* which has in addition revealed biogeographically informative variation in plastid minisatellites (Cafasso *et al.*, 2001). Moreover, the decision of Bateman *et al.* to include *A. morio* ssp. *picta* as a synonym of *A. morio* s.s., taken in the absence of molecular data for *picta*, is not upheld; rather, *picta* appears from ITS data to be more closely related to *A. champagneuxii* and to the relatively large-spurred species *A. longicornu*, newly incorporated in our matrix (see also Cafasso *et al.*, 2000; Cozzolino *et al.*, 2001).

SERAPIAS (7 CODED TAXA)

As in *Ophrys* (below), monophyly of *Serapias* is strongly supported by ITS evidence; the large base-pair divergence, plus three synapomorphic indels, reinforce its unequivocal morphological cohesion. Although well covered taxonomically, even less molecular resolution is evident within *Serapias* than within

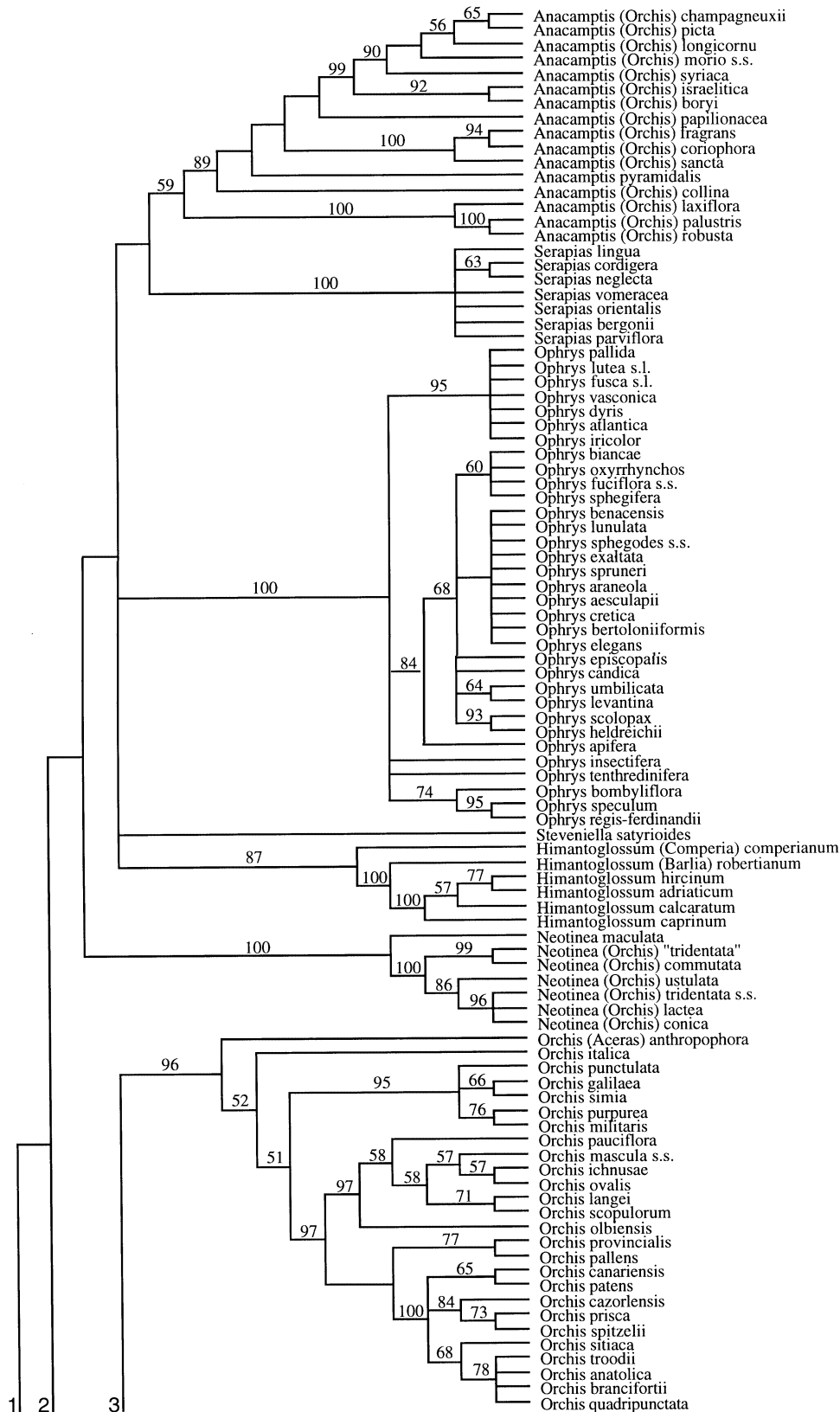
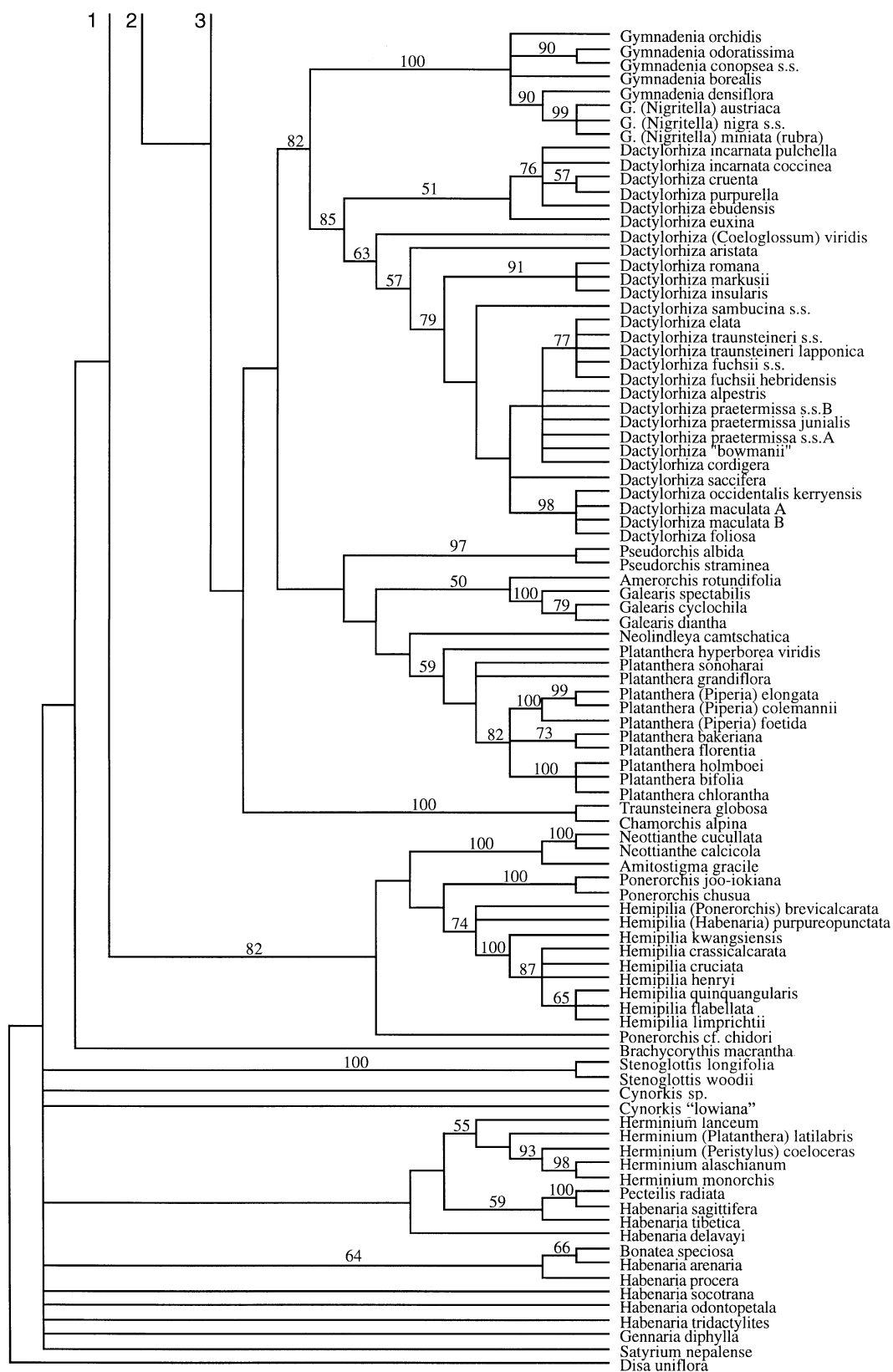


Figure 3. Strict consensus tree of the 10 000 most-parsimonious trees found when the number of trees was limited during the present analysis. Figures are all fast bootstrap support values exceeding 50%, based on 10 000 replicates.

Figure 3. *Continued*

Ophrys, and adding *S. bergonii* to the analysis failed to modify the original topology of Pridgeon *et al.* (1997). The present results cannot unequivocally distinguish the morphologically distinct tetraploid *S. lingua* (possibly an autotetraploid derivative of *S. parviflora*) from the remaining, diploid species. A recent study of heterochromatin patterns (D'Emérico *et al.*, 2000) revealed similarly low levels of divergence, though their conclusion that this indicates a recent evolutionary origin of the genus (p. 491) is inconsistent with the long ITS branch subtending the genus (Fig. 2); rather, the low divergence in karyotypic features indicates recent origin of the species within the genus, even for the more morphologically distinct species aggregates (cf. Lorenz, 2001). Hopefully, current research being pursued by S. Cozzolino and co-workers using genetic fingerprinting techniques will yield clearer phylogenetic insights into the 29 putative 'species' recognized by Delforge (2001).

OPHRYS (33 CODED TAXA)

Although totalling less than one sixth of the 215 putative species listed by Delforge (2001), the 33 species analysed here encompass all of his informal groups and subgroups; they more than double the species-level coverage achieved by Pridgeon *et al.* (1997). The monophyly of the genus is strongly supported by both base divergence and no less than five indels (Figs 2a, 3a), but most relationships within the genus are poorly resolved and interpretations are therefore tentative.

Some ITS trees (e.g. Bateman, 2001: fig. 15) suggest that a heterogeneous and apparently paraphyletic group of species with relatively simple labella gave rise to a more derived and species-rich group with complex three-dimensional labella and beaked connectives, although in Figure 2a the two groups tentatively appear monophyletic, each supported by an indel. Two distinctive and enigmatic species, *O. insectifera* and *O. tenthredinifera*, vary considerably in placement, effectively acting as 'wild-card' taxa within the genus (Fig. 3a: Nixon & Wheeler, 1992). Notable features of Figure 2a are the monophyly of the morphologically distinct *fusca-lutea* group (including the controversial *Ophrys pallida*, which superficially resembles *O. bombyliflora* as well as the *O. fusca* aggregate) and the basal divergence of *Ophrys apifera* within the group rich in morphologically similar 'species' that is defined by possessing a strongly three-dimensional labellum bearing a complex speculum.

Within this group, the tree tentatively suggests non-monophyly of several species groups recognized by Delforge (1995), including the *tenthredinifera*, *scolopax*, *bornmuelleri* and *fuciflora* groups. It also fails to separate the groups progressively segregated from *Ophrys sphegodes sensu latissimo*, not only from each

other but also from the *Ophrys bertolonii* group (including *O. bertoloniiformis* and *O. benacensis*). Some groups suggest a strong geographical influence, such as the pairing of the Sicilian species *O. oxyrrhynchos* (*fuciflora* group) and the very similar *O. biancae* (supposedly *bornmuelleri* group).

Soliva, Kocyan & Widmer (2001) combined ITS and *trnL-F* data for 32 species of *Ophrys* (15 shared with our set of 33 species) and six outgroups (surprisingly excluding the sister genus of *Ophrys*, *Anacamptis*). Analysis of *trnL-F* yielded even fewer phylogenetically informative characters than ITS, so not surprisingly Soliva *et al.* similarly detected limited phylogenetic structure. Their maximum parsimony topology differed from Figure 2 in two features: (1) the *O. fusca-lutea* group was subtended first by *O. tenthredinifera*, then by *O. bombyliflora*, then by *O. speculum*, a morphologically improbable topology (cf. Devillers & Devillers-Terschuren, 1994); interestingly, the maximum likelihood tree of Soliva *et al.* (2001) more closely resembled our parsimony tree in this respect. (2) *Ophrys insectifera* was weakly supported as being associated with the *O. apifera* clade rather than the *O. fusca* clade. Moreover, Soliva *et al.* suggested that the 14 species analysed by them that yielded sequences identical to those of other species reflected hybridization (i.e. sequence divergence followed by homogenization), in contrast to Bateman (2001) who invoked conspecificity (i.e. a historical absence of divergence).

STEVENIELLA-HIMANTOGLOSSUM S.L., INCLUDING 'COMPERIA' AND 'BARLIA' (7 CODED TAXA)

In our previous study (Pridgeon *et al.*, 1997) we analysed four putative species of *Himantoglossum* s.s., together with the widespread Mediterranean species *Barlia robertiana*. In this study we have added a fifth species of *Himantoglossum* s.s., *H. calcaratum*, which as expected yielded similar ITS sequences to the other eastern Mediterranean segregates of *H. hircinum*. The substantial separation of these segregates from *H. hircinum* s.s. envisaged by Teschner (1980) is not supported. All four of these taxa, plus *H. affine*, have been given subspecific status in some treatments (e.g. Sundermann, 1973, 1980; Moore, 1980). Interestingly, *H. caprinum* and *H. calcaratum* were the only taxa segregated from *H. hircinum* by Moore (1980), yet in Delforge's (1995, 1999, 2001) more finely divided reclassification of the genus, *calcaratum* was treated as a synonym of *caprinum*. Here, *H. calcaratum* is depicted as moderately supported sister to *H. hircinum* plus *H. adriaticum*, differing from its Adriatic companion *H. adriaticum* by only two ITS substitutions but from the more geographically widespread *H. caprinum* by four substitutions.

The rare Canary Island endemic '*Barlia metlesicsiana*' was sampled and sequenced too late for inclusion in the present parsimony analysis. It showed a surprisingly high divergence from its 'mainland' European sister-species '*B. robertiana*' of 13 substitutions plus three indels, suggesting longer isolation than for Macaronesian endemics of other clades of Orchideae such as *Orchis canariensis* (see '*Orchis*' below).

Of even greater interest are the results obtained from adding to the matrix two widely recognized monotypic genera, *Comperia comperiana* and *Steveniella satyrioides*, which have attracted considerable speculation regarding both their systematic / phylogenetic position and their nomenclature (e.g. Delforge, 1999, 2000, 2001; Baumann, Künkele & Lorenz, 2000; Bateman, 2001). Both putative genera were originally described (albeit illegitimately) by von Steven (1809) as members of an exceptionally broad concept of *Orchis* but were later erected as the largely undisputed monotypic genera *Comperia* (illegitimately by Koch, 1849; then legitimately by Ascherson & Graebner, 1907) and *Steveniella* (by Schlechter, 1918; see also Sundermann, 1972; Baumann, Künkele & Lorenz, 2000; Delforge, 2000). In the case of *Steveniella satyrioides*, this taxonomic odyssey occurred via an impressive series of 'stepping stone' genera; the species was first transferred (with some prescience) to *Himantoglossum* by Sprengel (1826) before passing through, in chronological order, *Peristylus*, *Platanthera*, *Coeloglossum*, *Habenaria* (Baumann *et al.*, 2000) and the *Orchis* (*Anacamptis*) *coriophora* group *sensu* Pridgeon *et al.* (1997) (Delforge, 2000, 2001).

The majority of recent treatments have placed *Steveniella* and *Comperia* together, and *Barlia* and *Himantoglossum s.s.* together, but have not recognized a close relationship between the two pairs of genera (Moore, 1980; Sundermann, 1980; Buttler, 1991). This approach is epitomised by *Flora Europaea* (ed. Moore, 1980), wherein the two pairs of genera were described by different contributing authors, further decreasing the probability of recognizing any morphological similarities between them. *Himantoglossum s.s.* and *Barlia* were linked to 'higher' Orchidinae, typically *Orchis s.l.* (especially '*Aceras*') and *Serapias* and/or *Anacamptis pyramidalis*. In contrast, *Steveniella* and *Comperia* were compared with the 'lower' Orchidinae, typically *Neotinea maculata* (often with *Traunsteinera globosa*) and *Dactylorhiza s.s.* (often with '*Coeloglossum viride*').

An alternative hypothesis grouped *Comperia* with *Barlia* and *Himantoglossum s.s.*, once again placed close to *Orchis* / '*Aceras*' and *Serapias* / *Anacamptis s.s.* (Landwehr, 1977; Delforge, 1995, 1999). This treatment left *Steveniella* isolated and again viewed as relatively primitive. Landwehr (1977) placed *Steveniella*

between *Neottianthe cucullata* / *Neotinea maculata* and *Traunsteinera globosa*. Delforge (1995) similarly placed *Steveniella* between *Neotinea s.s.* / *Traunsteinera* and *Anacamptis pyramidalis*, but in his text he noted especially a similarity to the *Anacamptis* (formerly *Orchis*) *coriophora* group (a comparison echoed by Pridgeon *et al.*, 1997: 101). He subsequently transferred *Steveniella* to that group as '*Orchis prosteveniella*' (Delforge, 2000, 2001).

Our intriguing results reveal that these four genera constitute a probable clade, with the three putative monotypic genera diverging sequentially, in the order *Steveniella*–*Comperia*–*Barlia*, subtending a far less resolved group of less morphologically differentiated species of *Himantoglossum s.s.* Moreover, *Comperia*–*Barlia*–*Himantoglossum* appears to be a well-resolved clade (87% bootstrap) supported by two indels (one shared with *Serapias* plus *Anacamptis s.l.*) and morphologically defined by vegetative vigour, toothed lateral petals and large, elongate, trilobed labella. In contrast, the smaller, 1–2-leaved, small-flowered *Steveniella* superficially resembles members of the *Neotinea s.l.* and *Neottianthe*–*Hemipilia* clades (with which it shares an indel), though its gynostemium structure, purplish-brown galea, strongly three-lobed labellum and short, robust spur do, as Sprengel perceived, suggest some similarities to the more derived himantoglossids. Although the position of *Steveniella* in the ITS tree is tenuous, collapsing to a polytomy in the strict consensus tree (Fig. 3a), we can confidently reject the many previous hypotheses of its relationship with other genera that were advanced in the absence of phylogenetic data.

Chromosomally, *Himantoglossum s.s.* and *Barlia* have recently been carefully examined and shown to have the 36 chromosomes expected of members of the large $2n = 36$ clade identified by Pridgeon *et al.* (1997), though *H. hircinum* possesses an additional B-chromosome (Carpineri & Rossi, 1987; D'Emérico, Bianco & Medagli, 1992). *Steveniella* has been reported to have the improbable karyotype of $2n = 38$ by Sundermann & von der Bank (1977), but this assertion may simply reflect the possible presence of another B-chromosome analogous to that of *H. hircinum*. More difficult to explain is the count of $2n = 30$ reported for *Comperia* by Ströhlein & Sundermann (1972), which may represent a counting error (Delforge, 1999) or a true autapomorphic reduction in chromosome number by fusion, analogous to the decrease from $2n = 36$ to $2n = 32$ unequivocally observed in *Anacamptis* (formerly *Orchis*) *papilionacea* in the morphologically heterogeneous *Anacamptis* clade (D'Emérico *et al.*, 1990; Pridgeon *et al.*, 1997).

Steveniella and '*Comperia*' are confined to Asia Minor where they have almost mutually exclusive distributions: *Steveniella* to the north and east, and

'Comperia' to the south and west (e.g. Baumann & Künkele, 1982; see also Baumann *et al.*, 2000). *Barlia* and *Himantoglossum* s.s. similarly occur in Asia Minor, but also include species that extend west throughout the Mediterranean and north-west into Continental Europe.

Each of the four genera as previously conceived possesses morphological autapomorphies within the clade: note for example the single fully expanded leaf, flat labellum and bilobed spur of *Steveniella*, the four filiform labellar extensions and elongate spur of 'Comperia', the well-developed lateral labellum lobes and early flowering period of 'Barlia', and the extremely elongate, helically twisted central labellum lobe of *Himantoglossum* s.s. (admittedly, *H. formosum* is intermediate between the *H. hircinum* aggregate and 'Barlia' in these characters, e.g. Delforge, 2001).

Taxonomically, the topology shown in Figure 2a presents a similar dilemma to that posed to Pridgeon *et al.* (1997) by that of *Neotinea maculata* vs. the more derived species of *Neotinea* formerly attributed to

Orchis s.l. (i.e. the *tridentata-lactea* group: see below). Monophyly permits continued recognition of the three more-or-less monotypic genera; also, each possesses morphological (and supposed chromosomal) autapomorphies and is subtended by a substantial ITS branch length of c. 25 steps. Branches of similar or lesser lengths subtend the genera *Pseudorchis*, *Amerorchis*, *Galearis* and *Neolindleya* at the base of the *Platanthera* clade, *Ponerorchis*, *Amitostigma* and *Neottianthe* at the base of the *Hemipilia* clade, and separate *Traunsteinera* from the highly morphologically dissimilar *Chamorchis*. On the other hand, monotypic genera by definition contain no phylogenetic structure, and the phylogenetic structure among the three more derived genera is reflected in the morphological synapomorphies outlined above. More pragmatically, amalgamating the genera nullifies some of the nomenclatural controversies that survived even the formal conservation of *Himantoglossum* against *Loroglossum* in 1972 (cf. Garay, 1997; Delforge, 1999; Baumann *et al.*, 2000; Bateman, 2001).

Table 3. Revised classification of the *Steveniella*–*Himantoglossum* s.l. group (see also Nelson, 1968; Delforge, 1999; Baumann *et al.*, 2000). Asterisked taxa have not yet generated ITS sequences

- (1) *Himantoglossum hircinum* (L.) Sprengel, *Syst. Veg.* 3: 694 (1826).
Basionym: *Satyrium hircinum* L., *Sp. Plant.* 944 (1753).
Synonym: *Orchis hircina* (L.) Crantz, *Stirp. Austr.* 2: 484 (1769).
- (2) *Himantoglossum adriaticum* H.Baumann, *Orchidee* 29: 171 (1978).
Synonym: *Himantoglossum hircinum* (L.) Sprengel ssp. *adriaticum* (H.Baumann) Sundermann, *Europ. medit. Orchideen*: 40 (1980).
- (3) *Himantoglossum caprinum* (M.-Bieb.) Sprengel, *Syst. Veg.* 3: 694 (1826).
Basionym: *Orchis caprina* M.-Bieb., *Fl. Taurico-Caucasica* 3: 602 (1819).
Synonym: *Himantoglossum hircinum* (L.) Sprengel ssp. *caprinum* (M.-Bieb.) K.Richter, *Pl. Europ.* 1: 276 (1890).
- (4) *Himantoglossum calcaratum* (G.Beck) Schlechter, *Fedd. Repert.* 15: 287 (1918).
Basionym: *Aceras calcaratum* G.Beck, *Ann. Hofmus. Wien* 2: 55 (1887).
Synonym: *Himantoglossum hircinum* (L.) Sprengel ssp. *calcaratum* (G.Beck) Soó, *Bot. Arch. (Berlin)* 23: 90 (1929, published as '1928').
- (5) *Himantoglossum affine** (Boissier) Schlechter, *Fedd. Repert.* 15: 287 (1918).
Basionym: *Aceras affinis* Boissier, *Fl. Orientalis (Orchidaceae)* 5: 56 (1884).
Synonym: *Himantoglossum hircinum* (L.) Sprengel ssp. *affine* (Boissier) Sundermann, *Europ. medit. Orchideen*: 40 (1980).
- (6) *Himantoglossum formosum** (Steven) K.Koch, *Linnaea* 22: 287 (1849).
Basionym: *Orchis formosa* Steven, *Mém. Soc. Nat. Mosc.* 4: 66 (1813).
- (7) *Himantoglossum robertianum* (Loiseleur) P.Delforge, *Nat. Belg.* 80: 401 (1999).
Basionym: *Orchis robertiana* Loiseleur, *Fl. Gallica* 1: 606 (1807, as '1806').
Synonym: *Barlia robertiana* (Loiseleur) W.Greuter, *Boissiera* 13: 192 (1967).
- (8) *Himantoglossum metlesicsianum* (Teschner) P.Delforge, *Nat. Belg.* 80: 401 (1999).
Basionym: *Barlia metlesicsiana* Teschner, *Orchidee* 33: 117 (1982).
- (9) *Himantoglossum comperianum* (Steven) P.Delforge, *Nat. Belg.* 80: 401 (1999).
Basionym: *Orchis comperiana* Steven, *Mém. Soc. Nat. Mosc.* 8: 259 (1829).
Synonym: *Comperia comperiana* (Steven) Ascherson and Graebner, *Syn. Mitteleur. Fl.* 3: 620 (1907).
- (10) *Steveniella satyrioides* (Steven) Schlechter, *Fedd. Repert.* 15: 295 (1918).
Basionym: *Orchis satyrioides* Steven, *Mém. Soc. Nat. Mosc.* 2: 176 (1809), *nom. illegit.*
Synonym: *Himantoglossum satyrioides* (Steven) Sprengel, *Syst. Veg.* 3: 694 (1826), *nom. illegit.* (for full synonymy see Baumann *et al.*, 2000).

Prompted in part by ITS results already published by ourselves (Bateman *et al.*, 1997; Pridgeon *et al.*, 1997), Delforge (1999, 2001) recently stimulated further controversy by incorporating *Barlia* and *Comperia* into *Himantoglossum* and subsequently *Steveniella* into *Orchis s.l.* (= our *Anacamptis*) as *Orchis prosteveniella* (Steven) Delforge, noting the apparent illegitimacy of '*Orchis satyrioides*' von Steven (1809) (Delforge, 2000, 2001). On the current balance of evidence, and on phylogenetic logic, we concur with his decisions regarding '*Barlia*' and '*Comperia*', but our data clearly reject his treatment of *Steveniella*. Similarly, the molecular isolation and morphological distinctiveness of *Steveniella* suggest that it is better left as a monotypic genus than sunk into a further expanded *Himantoglossum* as *H. satyrioides* (Steven) Sprengel (1826: see the revised classification in Table 3).

After presenting much well-informed discussion, Delforge (1999; fig. 10) followed Teschner (1980) in producing a wholly speculative 'phylogeny' and a derived 'natural' key that were not based on any data matrix but rather were extrapolated from our original tree (Pridgeon *et al.*, 1997). In the absence of relevant ITS sequences, we share Delforge's speculation that *H. affine* is placed below the *H. hircinum-caprinum* group; we also agree that the rare and elusive *H. formosum* could be placed below *H. affine*, although on morphological evidence it is equally likely to be placed on the *Barlia* lineage rather than that of *Himantoglossum s.s.* However, we defer formal judgement on these species pending acquisition of molecular data. Here, ITS sequences conclusively refute Delforge's (1999, 2001) speculative *Barlia-Comperia* clade, which appears to have been based on the shared characteristic of a stigmatic surface that is higher than wide. Our data suggest that this is a shared primitive character (symplesiomorphy) rather than a shared derived character (synapomorphy) and thus is unsuitable for grouping these taxa; it renders Delforge's speculative phylogeny and his related key unequivocally 'unnatural'.

Delforge (1995, 1999, 2001) further speculated that his *Barlia-Comperia* alliance was 'probably derived from a taxon of the *Orchis spitzelii* species group', but our ITS tree reveals a vast disparity of 109 substitutions (= c. 16% total ITS sequence divergence) between *Himantoglossum* (formerly *Comperia*) *comperiana* and *Orchis spitzelii*, clearly demonstrating that several floral similarities between these species (e.g. elongate robust labella, broad conical spurs, pale purple flowers bearing darker purple markings) are evolutionary convergences and hence are unsuitable for classification at this level (cf. Bateman *et al.*, 1997; Chase, 1999; Bateman, 2001).

NEOTINEA S.L.

(7 SEQUENCES REPRESENTING 6 CODED TAXA)

The formerly monotypic *Neotinea* was expanded by Pridgeon *et al.* (1997) and Bateman *et al.* (1997) to encompass the small number of small-flowered, essentially trilobed-lipped species of the *ustulata* group that were formerly included in *Orchis s.l.* A similar topology separating the *Neotinea* group was subsequently obtained by Aceto *et al.* (1999: fig. 1). These former members of *Orchis* could in theory have been treated as a genus separate from the more narrowly delimited original concept of *Neotinea*, given the relatively long molecular branch, distinct vegetative markings and reputed autogamy of *N. maculata*, but there are clear similarities in the size and morphology of the flowers. The clade has 100% bootstrap support and two synapo-morphic indels.

New insights have also been generated among closely related species. Firstly, as expected, *N. conica* proved similar to *N. lactea*, though the four-step disparity tentatively suggests that Bateman *et al.* (1997) may have erred in awarding subspecific status to the former. Secondly, *Neotinea commutata* and the characteristically smaller-flowered but otherwise morphologically similar *N. tridentata* were both sampled from a mixed population on Sicily. Surprisingly, they proved to be highly divergent on ITS evidence (see also Cafasso *et al.*, 2000), apparently refuting the suggested origin of *N. commutata* as an autotetraploid of *N. tridentata* (Delforge, 1995). Even more surprisingly, *N. commutata* yielded an identical sequence to the specimen of supposed *O. tridentata* from west-central Italy analysed by Pridgeon *et al.* (1997), rather than with the coexisting Sicilian *tridentata* which resolved as sister-species to the morphologically similar *N. conica* and *N. lactea* (they also share a characteristic indel). This result indicates that the specimen of *N. 'tridentata'* originally published by Pridgeon *et al.* (1997) was attributable to *N. commutata*. *Neotinea commutata* and *N. conica* are upgraded to species status in Table 2. The Table excludes the taxonomically contentious late-flowering form of *N. ustulata*, 'ssp.' *aestivalis* (Kümpel, 1988; Kümpel & Mrkvicka, 1990; Tali & Kull, 2001), which is currently the subject of detailed molecular investigations (K. Tali, R. Bateman, M. Chase and M. Fay, unpubl. obs.). Preliminary results, including identical ITS sequences, suggest that its original status as a variety is more appropriate.

ORCHIS S.S. (26 CODED TAXA)

Sampling within true *Orchis* has been much improved since the publication of Pridgeon *et al.* (1997). The

main remaining omission is *O. stevenii*, which on recent thin-layer chromatographic evidence may best be treated as a subspecies of *O. militaris* (Breiner, 1999). The basic structure originally observed within the clade by Pridgeon *et al.* is upheld. The clade benefits from 97% bootstrap support, aided by an indel. *Orchis* (formerly *Aceras*) *anthropophora* and then *O. italica* are basally divergent (in contrast, *O. anthropophora* diverged above *O. italica* in the tree of Aceto *et al.*, 1999; the two were shown as sisters in the tree of Cozzolino *et al.*, 2001); in our tree they render paraphyletic the more primitive 'anthropomorphic' group. Similar relationships were discerned by Qamaruz-Zaman (2000) using AFLP data. The remaining five anthropomorphic species, sampling now including the often dominantly yellow-flowered eastern Mediterranean species *O. punctulata* and *O. galilaea*, form a well-supported clade (*punctulata-militaris*), which is sister to the species-rich group of broad-lipped *Orchis* that are morphologically convergent (and were formerly classified) with the *Anacamptis morio* group (*A. longicornu-boryi*).

This clade in turn reveals a near-trichotomy of three well-supported clades: (1) the yellow-flowered *O. pallens* group, (2) *O. mascula* and its segregates, and (3) the *O. spitzelii-anatolica-quadrupunctata* complex (100% bootstrap support, including an indel). This topology confirms the placement predicted by Bateman *et al.* (1997) for *O. spitzelii* and its relatives, but shows less molecular divergence than might be expected from the considerable morphological diversity evident within the group. *Orchis patens* s.s. also belongs to this clade, as sister to the morphologically similar *O. canariensis* (see also Aceto *et al.*, 1999). The substantial allozymic divergence between *O. quadrupunctata* and the morphologically similar *O. brancifortii* reported by Rossi *et al.* (1994) is reflected in our ITS tree, though only a single base substitution was detected by Cafasso *et al.* (2000).

The most surprising result, first noted by Bateman (1999a), was the inclusion of the yellow-flowered *O. pauciflora* in the otherwise purple-flowered *O. mascula* group, reliably separated from the other yellow-flowered species *O. provincialis* and *O. pallens*. We further investigated this unexpected placement by sequencing a second specimen of *O. pauciflora* from a different country. Nonetheless, Aceto *et al.* (1999) and Cozzolino *et al.* (2001) obtained the converse placement, *provincialis* rather than *pauciflora* grouping with *O. mascula*, suggesting that further accessions of both species should be sequenced for ITS (see also hybridization evidence in Pellegrino *et al.*, 2000, 2001). Nonetheless, either topology refutes Bateman *et al.*'s (1997) treatment of *pauciflora* as a subspecies of the morphologically similar *O. provincialis*. This may help explain the substantial anatomical differences noted

between *O. provincialis* and *O. pauciflora* by Del Prete & Miceli (1999), although our tree does not support the sectional/subsectional re-arrangements of either these authors or Hautzinger (1978). Further complicating the issue of yellow-flowered *Orchis*, Vermeulen (1977) earlier had erroneously argued that it is *O. pallens* that should be classified with *O. mascula*, separately from the remaining yellow-flowered species, which were placed by Vermeulen in their own subsection, *Provinciales*.

Also within the *mascula* group, the ITS divergence of some recent species-level segregates of *O. mascula*, such as *O. langei* (syn. *O. mascula* ssp. *hispanica*, a name that may in fact encompass two distinct genetic entities), *O. scopulorum* and *O. olbiensis*, was supported by relatively substantial AFLP divergence documented by Qamaruz-Zaman (2000), and at least one Portuguese population of *O. olbiensis* has since proven to be tetraploid (Bernardos *et al.*, 2002). This evidence suggests that Bateman *et al.* (1997) may have been in error to award these taxa only subspecific status. However, the relatively long ITS branch subtending *O. ovalis* is inconsistent with the more modest divergence evident in the AFLP data, which show it as negligibly distinct from *O. mascula* s.s., as is *O. ichnusae* (see also Cafasso *et al.*, 2000) and probably also the as-yet unsequenced *O. tenera*. The morphology of the *mascula* group was recently reviewed by Essink *et al.* (1999).

TRAUNSTEINERA-CHAMORCHIS (2 CODED TAXA)

Perhaps the most surprising outcome of this expanded matrix is the pairing of two arguably monotypic alpine/boreal specialists, *Traunsteinera* and *Chamorchis*. *Traunsteinera* is tall with large leaves evenly spaced along the stem, which terminates in a compressed conical spike of pale pink flowers with trilobed labella and short spurs that superficially resemble those of *Neotinea* s.l. In contrast, *Chamorchis* has a short stem that is subtended by basally concentrated filiform leaves and bears a more elongate spike of pale green flowers and entire, spur-less labella; superficially they are reminiscent of the flowers of *Dactylorhiza* (formerly *Coeloglossum*) *viridis* or *Pseudorchis albida*.

Among recent authors, only Landwehr (1977), benefiting from the shrewd taxonomic advice of P. Vermeulen, implied a close relationship between *Chamorchis* and *Traunsteinera*, by depicting them on adjacent plates in his iconograph. Most authors understandably placed *Chamorchis* adjacent to *Platanthera* and/or *Pseudorchis* (e.g. Moore, 1980; Sundermann, 1980; Buttler, 1991; Delforge, 1995), though others implied affinities with '*Coeloglossum*' (Buttler, 1991) and, less intuitively, with *Neottianthe* (Moore, 1980; Delforge, 1995). In contrast, *Traunsteinera* was

most frequently linked to *Orchis* s.s. (often including 'Aceras': Moore, 1980; Sundermann, 1980; Buttlar, 1991) and *Neotinea maculata* (Moore, 1980; Sundermann, 1980; Buttlar, 1991; Delforge, 1995); also less plausibly to *Steveniella* by Landwehr (1977).

In fact, the sister-group relationship of these two morphologically distinct but co-occurring genera is strongly upheld by the ITS data (bootstrap support 100%); moreover, their ITS sequences are sufficiently similar to be more typical of distantly related species within a single genus than of separate but closely related genera (cf. Bateman *et al.*, 1997), and they have similar karyotypes distinct from those of other Orchidinae (D'Emérico & Grünanger, 2001). The main argument for maintaining *Chamorchis* and *Traunsteinera* as separate genera lies in morphological characters, notably the paucity of obvious synapomorphies relative to autapomorphies of the existing genera.

PSEUDORCHIS–*AMERORCHIS*–*GALEARIS*–
NEOLINDLEYA–*PLATANThERA* S.L. (INCLUDING
'*PIPERIA*') (18 CODED TAXA)

This clade receives less bootstrap support (<50%) than the preceding clades, with the geographically widespread, short-branch genus *Pseudorchis* being the most tenuous, basally divergent genus in the group (Fig. 2). The negligible ITS divergence between *Ps. albida* and the recently segregated *Ps. straminea* lends little support to recent morphometric (Reinhammar, 1995) and allozyme (Reinhammar & Hedrén, 1998) studies indicating that the latter should be treated as a full species, segregated from *Ps. albida*, though there is evidence of ecological segregation (Reinhammar, Olsson & Sørmealand, 2002).

The next group to diverge consists of the monotypic *Amerorchis*, which as anticipated by Pridgeon *et al.* (1997) is placed (albeit weakly; cf. Bateman, 2001) as sister to *Galearis*; these taxa are barely sufficiently divergent, both morphologically and molecularly (it has one autapomorphic indel), to justify Hultén's (1968) generic segregation of *Amerorchis*. The two Asian species of *Galearis* form a derived pairing relative to the North American *G. spectabilis* and to *Amerorchis*, surprisingly indicating that the North American lineage gave rise to the East Asian species; further sampling of *Galearis* is desirable to test this hypothesis.

The relationship among *Galearis*–*Amerorchis*, *Neolindleya* and *Platanthera* is essentially unresolved (Fig. 3). *Neolindleya camtschatica* (Kränzlin, 1899 in 1897–1904) is more often attributed to *Gymnadenia* (e.g. Chen *et al.*, 1999), but clearly it has no close relationship to that genus. It is sufficiently distinct from *Platanthera* on both morphological and molecular

grounds to merit its status as a monotypic genus, analogous to *Amerorchis*. This result was a surprise, as '*G. camtschatica*' has a pink-purple flower colour and narrow spur in accordance with *Gymnadenia* but has vegetative characters more consistent with one of the more robust species of *Dactylorhiza* (albeit also possessing the autapomorphy of crenulate leaf margins), and a distinct elongate, notched labellum resembling that of *D.* (formerly *Coeloglossum*) *viridis*. A confirmatory chromosome count for *Neolindleya* is desirable for comparison with *Galearis*, *Amerorchis* and *Platanthera* (all $2n = 42$; Pridgeon *et al.*, 1997).

Although sampling of *Platanthera* s.l. is sparse compared with the detailed ITS analysis of 36 species presented by Hapeman & Inoue (1997), the few new additions reveal some interesting relationships. For example, the clade immediately above *P. sonoharai* is supported by 82% bootstrap and two indels. At the species level, the Chinese *Platanthera bakeriana* is shown to be sister to the Japanese *P. florentia*, and the previously noted negligible divergence between *P. bifolia* and *P. chlorantha* is extended to include the questionably distinct green-flowered species *P. holmboei*, which characterizes montane regions of the eastern Mediterranean (its western Mediterranean equivalent, *P. algeriensis*, has yet to be sequenced successfully but also appears questionably distinct from *P. chlorantha*).

The supposed North American genus '*Piperia*' is, as we anticipated, nested well within *Platanthera* s.l. (Figs 2, 3). The three species analysed show appreciable ITS divergence but nonetheless constitute a tight monophyletic group (100% bootstrap plus an indel) that is more appropriately recognized as a section *Piperia* within the genus *Platanthera*. It can therefore be viewed as analogous to other former genera now encompassed by *Platanthera* s.l. such as *Limnorchis*, *Lysiella* and *Tulotis* (cf. Rydberg, 1901a,b; Landwehr, 1977; Hapeman & Inoue, 1997; Lee, 1998; Chen *et al.*, 1999). Admittedly, of these former genera, *Piperia* is perhaps the most morphologically distinct (to quote Luer, 1975: 162, 'the species of *Piperia* differ distinctly from those of *Platanthera* or *Habenaria*. As much justification exists to maintain the genus *Piperia* as to maintain the genera *Amerorchis*, *Dactylorhiza* and *Galearis* distinct from *Orchis*'). Section *Piperia* is separated from the remaining five sections of *Platanthera* (as outlined by Hapeman & Inoue, 1997) by its very short, inconspicuous caudicles, leaves typically senescing during, rather than subsequent to, anthesis, and most notably by tubers that are globose (albeit somewhat elongate) rather than fusiform (Ackerman, 1977: table 1). Section *Piperia* appears to represent the most derived species group of *Platanthera* found in North America; the other, more derived species of the genus shown in Figure 2 either occur in Asia or Europe.

Table 4. Revised classification of *Platanthera* section *Piperia* (formerly the genus *Piperia*). Asterisked taxa have not yet generated ITS sequences

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- (1a) *Platanthera elegans* Lindley ssp. *elegans**, *Gen. Spec. Orchid.* 285 (1835).
 Synonym: *Piperia elegans* (Lindley) Rydberg, *Bull. Torrey Bot. Club* **28**: 270 (1901a).
- (1b) *Platanthera elegans* Lindley ssp. *maritima** (Rydberg) R.M.Bateman, **comb. nov.**
 Basionym: *Piperia maritima* Rydberg, *Bull. Torrey Bot. Club* **28**: 641 (1901b).
- (1c) *Platanthera elegans* Lindley ssp. *decurtata** (R.Morgan and Glicenstein) R.M.Bateman, **comb. nov.**
 Basionym: *Piperia elegans* Rydberg ssp. *decurtata* R.Morgan and Glicenstein, *Lindleyana* **8**: 93 (1993).
- (2) *Platanthera elongata* (Rydberg) R.M.Bateman, **comb. nov.**
 Basionym: *Piperia elongata* Rydberg, *Bull. Torrey Bot. Club* **28**: 270 (1901a).
 Synonym: *Piperia elegans* (Lindley) Rydberg var. *elata* (Jepson) Luer, *Native Orchids of North America and Canada*: 167 (1975).
- (3) *Platanthera michaelii** (Greene) R.M.Bateman, **comb. nov.**
 Basionym: *Habenaria michaeli* Greene, *Bull. Calif. Acad. Sci.* **1**: 282 (1885).
 Synonyms: *Piperia michaeli* (Greene) Rydberg, *Bull. Torrey Bot. Club* **28**: 640 (1901b); *Piperia elongata* Rydberg ssp. *michaelii* (Greene) Ackerman, *Bot. J. Linn. Soc.* **75**: 266 (1977).
- (4) *Platanthera leptopetala** (Rydberg) R.M.Bateman, **comb. nov.**
 Basionym: *Piperia leptopetala* Rydberg, *Bull. Torrey Bot. Club* **28**: 637 (1901b).
- (5) *Platanthera transversa** (Suksdorf) R.M.Bateman, **comb. nov.**
 Basionym: *Piperia transversa* Suksdorf, *Allg. Bot. Zeit. Syst.* **12**: 43 (1906).
- (6) *Platanthera foetida* Geyer ex Hooker f., *J. Bot. Kew Misc.* **7**: 376 (1855).
 Basionym: *Spiranthes unalascensis* Sprengel, *Systema Vegetabilium* **3**: 708 (1826).
 Synonyms: *Herminium unalasc(h)ense* (Sprengel) Reichenbach.f., *Icon. Fl. Germ. Helv.* **13–14**: 107 (1838), *Habenaria unalasc(h)ensis* (Sprengel) S.Watson, *Proc. Amer. Acad. Arts* **12**: 277 (1877); *Platanthera unalascensis* (Sprengel) Kurtz, *Bot. Jahrb.* **19**: 408 (1894); *Piperia unalasc(h)ensis* (Sprengel) Rydberg, *Bull. Torrey Bot. Club* **28**: 270 (1901a).
- (7) *Platanthera cooperi** (S.Watson) R.M.Bateman, **comb. nov.**
 Basionym: *Habenaria cooperi* S.Watson, *Proc. Amer. Acad. Arts* **12**: 276 (1877).
 Synonym: *Piperia cooperi* (S.Watson) Rydberg, *Bull. Torrey Bot. Club* **28**: 636 (1901b).
- (8) *Platanthera colemanii* (R.Morgan and Glicenstein) R.M.Bateman, **comb. nov.**
 Basionym: *Piperia colemanii* R.Morgan and Glicenstein, *Lindleyana* **8**: 89 (1993).
- (9) *Platanthera candida** (R.Morgan and Ackerman) R.M.Bateman, **comb. nov.**
 Basionym: *Piperia candida* R.Morgan and Ackerman, *Lindleyana* **5**: 207 (1990).
- (10) *Platanthera yadonii** (R.Morgan and Ackerman) R.M.Bateman, **comb. nov.**
 Basionym: *Piperia yadonii* R.Morgan and Ackerman, *Lindleyana* **5**: 209 (1990).
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'*Piperia*' was both established and monographed by Rydberg (1901a,b), who recognized ten species; one further species was soon added by Suksdorf (1906). More conservative treatments by Luer (1975) and Ackerman (1977) listed totals of three and four species, respectively, the latter figure being echoed by Dressler (1993). Ackerman (1977) thoroughly revised morphological, taxonomic and biogeographical knowledge of the genus. He also noted chromosome counts of $2n = 42$ for all four species, a value that similarly characterizes *Platanthera* s.s. (Pridgeon *et al.*, 1997), and he reported thin-layer chromatography data that suggested low degrees of divergence among the species. He recognized only one species that possessed short (≤ 5 mm) spurs, '*P.*' *unalascensis*, but soon acknowledged that this represented a highly morphologically heterogeneous species aggregate (cf. Coleman, 1995). Hence, two short-spurred species of '*Piperia*' were segregated by Morgan & Ackerman (1990), and a further two species by Morgan & Glicenstein (1993).

Largely following these authors, we provisionally recognize ten species in *Platanthera* section *Piperia*, together with two additional subspecies of *P. elegans* (Table 4). Species 1–5 have spurs >5 mm long and species 6–10 have spurs ≤ 5 mm long, although these are not natural groups; the long-spurred *P. elongata* is sandwiched between the short-spurred *P. colemanii* and the more widely distributed short-spurred *P. foetida* (formerly *Piperia unalascensis*). Spur length is a highly evolutionarily malleable character in the Orchidaceae. The section *Piperia* is largely confined to the states of the western seaboard of North America (*P. elegans* ssp. *decurtii* and *P. yadonii* being especially local and rare), though the most widely distributed species, *P. foetida*, has also established outliers in the extreme north-west and north-east of North America. Ackerman (1977) suggested that the close morphological similarity among '*Piperia*' species reflects both their relatively recent origins and lack of extinction of intermediates, plausible hypotheses that are best

tested by further molecular investigations of the section.

Our revised classification of *Platanthera* section *Piperia* (Table 4) requires ten new combinations, although fortunately the generitype, *Platanthera elegans*, is one of the two exceptions.

DACTYLORHIZA S.L. (28 SEQUENCES REPRESENTING
26 CODED TAXA)

Sampling of the *Dactylorhiza* clade (which includes the former monotypic genus *Coeloglossum*: Bateman *et al.*, 1997; Pridgeon *et al.*, 1997) has been doubled relative to our previous study. The previous pattern of relationships is retained here, though the positions of the additional taxa provide further fascinating insights that will be discussed in greater detail elsewhere. Interpretation of the genus is complicated by relatively low levels of ITS divergence, and by extensive and iterative polyploidy (e.g. Averyanov, 1990; Hedrén, 1996; Hedrén, Fay & Chase, 2001; Tyteca, 2001; Bateman & Denholm, 2003; R. Bateman and P. Hollingsworth, unpubl. obs.; all taxa above *D. saccifera* in Fig. 2 except *D. foliosa* and *D. fuchsii* are polyploid).

Firstly, the analysis of Bateman (2001) suggested that the south-east Asian *D. hatagirea* is the basally divergent member of the genus, and moreover is sufficiently distinct to appreciably weaken (from 89% to <50%) the formerly strong bootstrap support for the monophyly of the genus (cf. Pridgeon *et al.*, 1997); this and other similar species (e.g. '*Orchis*' *wardii* of Chen *et al.*, 1999) warrant more detailed study. However, incongruities in the sequence data obtained by us for *D. cf. hatagirea* encouraged us to omit the taxon from the present analysis, leaving a more cohesive *Dactylorhiza* with a synapomorphic indel and bootstrap support of 85%. The addition of *D. euxina* below the relatively primitive diploids of the *D. incarnata* aggregate (albeit a relationship poorly supported by bootstrap) is no surprise, unlike the inclusion in this clade of the Scottish allotetraploids *D. purpurella* and *D. ebudensis*, which cluster with the *traunsteineri* complex on morphometric (McLeod, 1995) and AFLP (Hedrén, Fay & Chase, 2001) evidence.

Next to branch off is *D. iberica*, which was incorrectly represented by an additional sequence for *D. maculata* in the analyses of Pridgeon *et al.* (1997) and Bateman (2001). Re-analysis of the species, conducted too late for inclusion in the present tree, places *D. iberica* in the weakly resolved basal portion of *Dactylorhiza* alongside other diploids such as *D.* (formerly *Coeloglossum*) *viridis*, *D. aristata* and the questionably distinct *D. romana*-*D. sambucina* aggregate, which includes the triploid *D. insularis*. The phylogenetic position of *D. romana* gives little support to

arguments that this species has a primitive karyotype (cf. D'Emérico, Pignone & Scrugli, 2002). Within the remaining, well-supported clade, *D. saccifera* does not group with the morphologically similar *D. fuchsii*. *Dactylorhiza occidentalis*, now viewed by us as a probable autopolyploid, falls into the morphologically heterogeneous but indel-supported clade of the diploid *D. foliosa* plus the presumed autopolyploid *D. maculata* (cf. Hedrén, Fay & Chase, 2001; Bateman & Denholm, 2003). Out of a plexus of the *D. majalis* complex of allopolyploids (*D. cordigera*-*bowmanii*) emerges a well-supported clade containing the diploid *D. fuchsii* aggregate, plus allopolyploids of the *D. traunsteineri* complex and *D. elata* from the western Mediterranean.

Credible interpretation of these intriguing patterns will require careful integration of ITS sequences with *trnL* sequences (e.g. Bateman & Denholm, 2003; R. Bateman and P. Hollingsworth, unpubl. obs.), AFLPs (Hedrén *et al.*, 2001), allozymes (e.g. Hedrén, 1996; R. Bateman and P. Hollingsworth, unpubl. obs.), plastid microsatellites (Y. Pillon, M. Chase and M. Fay, unpubl. obs.), and morphometric ordinations (e.g. Bateman & Denholm, 1989 *et pro*, also unpubl. obs.; Tyteca, 2001). For the present (and despite considerable research effort) *Dactylorhiza* remains perhaps the most tantalizing of the dominantly European clades of Orchidinae, its phylogenetic history obscured partly by a combination of iterative hybridization and chromosomal instability and partly by suboptimal species delimitation and misidentifications of chosen study organisms.

GYMNADENIA S.L. (8 CODED TAXA)

As in *Dactylorhiza*, sampling in *Gymnadenia s.l.* has doubled since the publication of Pridgeon *et al.* (1997), who took the controversial step of sinking the two most widespread species of the morphologically distinct '*Nigritella*' back into synonymy with *Gymnadenia s.s.*, which would otherwise have been unequivocally paraphyletic. Further species of '*Nigritella*' were transferred to *Gymnadenia* by Delforge (1998) and Teppner & Klein (1998), although this was done without the benefit of additional sequence data. Indeed, the decision to synonymise these genera has since been challenged by contradictory data from allozymes (Hedrén, Teppner & Klein, 2000) and *trnL* sequences (Bateman & Denholm, 2003; R. Bateman, P. Hollingsworth and M. Hollingsworth, unpubl. obs.).

The strongly supported *Gymnadenia s.l.* clade is presently the subject of several research projects, including morphometrics (R. Bateman and I. Denholm, unpubl. obs.), population-level ITS and *trnL* sequences (R. Bateman, P. Hollingsworth and M. Hollingsworth, unpubl. obs.), allozymes (K. Marhold, pers.

comm., 1999; Hedrén, Klein & Teppner, 2000), karyotypes (E. Klein, pers. comm., 1997; K. Marhold, pers. comm., 1999; D'Emérico & Grünanger, 2001) and nuclear microsatellites (Campbell, 2000; V. Campbell *et al.*, unpubl. obs.). All available evidence reinforces the then tentative decision of Bateman *et al.* (1997) to separate *G. densiflora* and *G. borealis* from *G. conopsea* as full species on the basis of significant and consistent ITS divergence (albeit less divergence than was originally inferred by Bateman *et al.*). Although tetraploid karyotypes have been reported in both *G. densiflora* (Mrkvicka, 1993) and *G. conopsea* s.s. (K. Marhold, pers. comm., 1999), *Gymnadenia* s.s. appears dominantly diploid in the U.K. (e.g. J. Bailey, pers. comm., 1998) and elsewhere in western Europe.

In contrast, the more widely distributed species of 'Nigritella' analysed by us are triploids and tetraploids (e.g. Teppner & Klein, 1985, 1998); only recently have we sequenced more localized diploids such as 'N.' *lithopolitana* and 'N.' *rhellicani* (Teppner & Klein, 1985; D'Emérico, 2001). Although not included in the present analysis, they yielded ITS sequences identical to those of the tetraploids (R. Bateman *et al.*, unpubl. obs.). Moreover, Hedrén (1999) recently demonstrated that the Scandinavian endemic apomict \times 'Gymnigritella' *runei* Teppner & Klein (1989) arose by stabilized hybridization between the triploid 'Nigritella' *nigra* ssp. *nigra* and the diploid *Gymnadenia conopsea* s.l., suggesting genetic compatibility and thereby further justifying the incorporation of 'Nigritella' into *Gymnadenia*. Putative species of the 'Nigritella' complex remain undifferentiable on ITS (Bateman, 2001; R. Bateman *et al.*, unpubl. obs.) and *trnL* (R. Bateman *et al.*, unpubl. obs.) evidence, despite recent recognition of clear thin-layer chromatography differences separating the *nigra* and *miniata* (= *rubra*) complexes (Breiner, 1999).

On present evidence, most of the *Gymnadenia* s.s. species form a near-polytomy. The often sympatric *G. conopsea* s.s. and *G. odoratissima* have identical or near-identical ITS sequences, despite their clear and reliable morphological differences, and the most robust species, *G. densiflora*, appears somewhat unintuitively to be the sister-species of the relatively diminutive members of the well-supported 'Nigritella' complex (cf. Bateman & DiMichele, 2002), a position contradicted by evidence from allozymes (Hedrén *et al.*, 2000) and *trnL* sequences (Bateman & Denholm, 2003; R. Bateman *et al.*, unpubl. obs.).

PONERORCHIS S.L.–HEMIPILIA S.L.–AMITOSTIGMA– NEOTTIANTHE (15 CODED TAXA)

No members of this archetypal Asian clade were sequenced by Pridgeon *et al.* (1997), constituting the most important sampling gap in the original study.

The group has since been actively targeted for study by us, most notably by Luo Yi-Bo; our results can be compared with the strict consensus ITS trees of Luo (1999; also taken into account are unpublished trees prepared by Y.-B. Luo and A. Pridgeon).

Both these studies, together with the present investigation and Bateman (2001), agree on the existence of a major dichotomy separating *Neottianthe* plus *Amitostigma* from *Hemipilia* s.s. plus 'Habenaria' *purpureopunctata*. However, considerable topological instability is conferred upon the clade by *Ponerorchis* s.l. (including *Chusua*). Luo (1999) tentatively found *Ponerorchis* to be paraphyletic relative to the *Amitostigma*–*Neottianthe* clade. Reworking of the data matrix with additional species (Y.-B. Luo and A. Pridgeon, unpubl. obs.) showed *Ponerorchis* to be diphylectic; a tight cluster of species surrounded the type species, *P. graminifolia* (not included in the present study but similar to *P. jooiokiana*), with 'P.' *brevicalcarata* more closely associated with *Hemipilia*. A similar topology resulted from Bateman's (2001) Neighbour Joining tree, with *P. cf. chidori* (distinguished by two indels) linked to the *P. jooiokiana* group. However, the maximum parsimony tree of the same matrix presented here (Fig. 2b) shows *P. cf. chidori* as basal within the entire group, though other most-parsimonious trees from the same analytical set placed this species immediately above *P. jooiokiana*; it appears to be a 'wildcard' taxon.

Sampling for other genes and careful re-examination of morphology are desirable to clarify relationships in this group. It seems most likely that this will lead to a narrower concept of *Ponerorchis* constructed around the type species, possibly with new genera being erected around *P. cf. chidori* and *P. brevicealcarata*. A further complication is provided by the Tibetan endemic 'Habenaria' *purpureopunctata sensu* Lang & Tsi (1978), which molecularly resembles *P. brevicealcarata*, being depicted either as sisters (Fig. 2b) or as potentially paraphyletic to *Hemipilia* s.l. (Bateman, 2001). Bateman provisionally assigned both species to *Hemipilia*, although a long branch that includes three indels separates them from *Hemipilia* s.s.

In contrast, Luo & Chen (in press) controversially argued that 'Habenaria' *purpureopunctata* should become a new monotypic genus, *Hemipiliopsis* Y.B. Luo and S.C. Chen, distinguished primarily by its unusually poorly developed rostellar lobes with laterals that extend directly forward and by its naked viscidia positioned directly above the entrance of the large, saccate spur. However, these are all evolutionarily malleable characters within Orchidinae (see below); moreover, this taxonomic option does not take adequate account of the close relationship with *P. brevicealcarata* evident in Figure 2, which contradicts the suggestions of Luo & Chen (in press) that

'*Habenaria*' *purpureopunctata* is most closely related to *Brachycorythis*.

Hemipilia s.s. has 100% bootstrap support and three non-homoplastic indels. With the exception of the clear basal divergence of *H. kwangsiensis*, relationships within the well-sampled *Hemipilia* s.s. are poorly resolved and terminal branches are short, suggesting recent species-level divergence.

Further sampling and morphological study are prerequisites for eventual taxonomic revision of this interesting and under-researched clade. Overall, the majority of the genera currently recognized in this wholly Asiatic group survive the test of monophyly, despite the long-term taxonomic controversies that they continue to engender (cf. Vermeulen, 1972; von Soó, 1974; Chen, 1982; Banjeri & Pradham, 1984; Chen & Luo, 1999; Chen *et al.*, 1999; P. J. Cribb *et al.*, pers. comm., 1999; Luo, 1999; Luo & Chen, 1999, 2000; Bateman *et al.*, 2001; H. A. Pederson, pers. comm., 2001; Luo, Shen & Zhu, in press; Y.-B. Luo and A. Pridgeon, unpubl. obs.).

BRACHYCORYTHIS (1 CODED TAXON)

The single ITS sequence for *Brachycorythis*, obtained from the relatively unspecialized west-central African species *B. macrantha*, was inherited from the study of Douzery *et al.* (1999). This hydrophilic genus of c. 35 species has an unusually wide distribution that is focused on central Africa (Summerhayes, 1955; Pridgeon *et al.*, 2001) and diminishes in diversity southward toward Madagascar and the Cape (Linder & Kurzweil, 1999), and eastward as far as China and Taiwan (Chen *et al.*, 1999). The genus has previously been compared with *Platanthera* s.l., *Gymnadenia* and *Habenaria* (Summerhayes, 1955).

Although it has an elongate-globose underground tuber, the affinities of *Brachycorythis* with Orchideae are not immediately apparent from its above-ground organs. In most species, the helically arranged ovate leaves are evenly distributed along the robust monopodial stem and grade imperceptibly into exceptionally large bracts in the often lax inflorescence, although a few species, segregated by some authors as *Schwarztkopffia* (Kränzlin, 1900), are mycoheterotrophic (Summerhayes, 1955). The flowers have spreading tepals; the petal bases show varying degrees of fusion to the gynostemium, which bears two pollinia, each with a naked viscidium. The labellum is generally shallowly three-lobed and constricted at the base, where it generates a spur that varies from elongate to saccate. Asiatic species such as *B. henryi* tend to have larger, more cylindrical flowers with entire labella (Chen *et al.*, 1999). Wider geographical sampling, encompassing its Asiatic range, is highly desirable.

HABENARIINAE PLUS OUTGROUPS (22 CODED TAXA)

Our sample size of habenariids has tripled to 20 over the past five years, though it remains a minor fraction of the c. 1040 species estimated to comprise the putative subtribe (including c. 110 species previously placed within the then more broadly delimited Orchidinae by Dressler, 1993). Previous authors of morphological studies, which generally emphasized gynostemial features, have noted that relationships within the subtribe are highly equivocal (e.g. Kurzweil, 1987; Kurzweil & Weber, 1992; Luo *et al.*, in press), and they remain so given present DNA evidence. Our previous analyses (Pridgeon *et al.*, 1997; Bateman, 1999a, 2001; Bateman *et al.*, 2001) indicated varying degrees of paraphyly of the group relative to the Orchidinae, yet it was shown as weakly monophyletic (with *Stenoglottis* basally divergent) in the taxonomically broader ITS study of Douzery *et al.* (1999), implying that outgroup choice may profoundly affect the results.

Using the present data matrix and sequence alignment, Bateman's (2001) Neighbour Joining tree tentatively suggested monophyly of the habenariids, with the exception of the long-branch monotypic genus *Gennaria*, which was placed between the single representatives of Satyriinae and Diseae. However, short internal branches and long terminal branches indicated the statistical weakness of this association, given the present inadequate sampling of species. Maximum parsimony yielded some trees indicating complete monophyly of the habenariids (Fig. 2b) but others indicating substantial paraphyly relative to Orchidinae, resulting in a bootstrap consensus tree with no strongly supported relationships other than those between closely related species pairs (Fig. 3b). Moreover, the scattered indels all have multiple origins, being duplicated in other more derived groups within Orchidinae s.s.

Review of these recent studies indicates that the highly species-rich genus *Habenaria* will require extensive taxonomic fragmentation and reconstruction around monophyletic generic segregates (a parallel process to that experienced by the Orchidinae, which was progressively dismantled during the past century: Bateman *et al.*, 1997). *Herminium* appears likely to survive as a monophyletic group (with *H. lanceum* basally divergent) if the genus is expanded to incorporate some Asian habenariid species currently assigned to *Peristylus* (and, even more retrogressively, to *Platanthera* in the case of '*P.*' *latilabris*). As currently delimited, *Peristylus* contains some temperate species such as '*P.*' *coeloceras* that, on evidence of gynostemium morphology, should be transferred to *Herminium*, and other tropical species such as *P. goodyerioides* that show greater affini-

ties with some species of *Habenaria* (Luo, 1999; Luo *et al.*, in press; see also Lang, 1999).

Other putative generic segregates, notably *Pecteilis* and *Bonatea*, occur as derived members of clades otherwise consisting of species of *Habenaria s.l.*, and the current placement of *Cynorkis* is questionable, the two species sequenced (neither confidently identified to species level) only forming a monophyletic group in a minority of most-parsimonious trees. *Cynorkis* shows strong floral convergence with the bona fide Orchidinae genus *Amitostigma*; both genera possess short, broad tepals, slender spurs and planar labella divided into four lobes that expand towards their tips (cf. Vermeulen, 1972: fig. 18; Linder & Kurzweil, 1999: figs 25, 26). Once again, *Stenoglottis* is placed as basal to the habenariids, the two species sequenced showing relatively low divergence. Substantial variation in gynostemium structure recently observed within *Habenaria* should provide useful phylogenetic data (Luo *et al.*, in press).

Focusing on the base of the tree, several anomalies are evident, both between the two ITS analyses and between both molecular studies and conventional taxonomy. The most positionally unstable genera (*Gennaria*, *Satyrium*, the now excluded *Holothrix* and 'Habenaria' *repens*, which is either misnamed or misidentified) tended to be relatively difficult to align and to be subtended by comparatively long branches, raising the spectre of possible long-branch effects. However, experimentation with our matrix suggests that the range of outgroups used may be the primary factor leading to the contrasting topologies; much denser sampling of species within these genera is desirable. The ostensible basal genera of Orchidinae and Habenariinae, *Brachycorythis* and *Stenoglottis*, respectively, especially merit further study, as they appear to define the most appropriate boundary between the Habenariinae and Orchidinae (*Stenoglottis* was depicted as belonging to the Habenariinae by virtue of only a single step in the ITS tree of Douzery *et al.*, 1999).

RELATIONSHIPS AMONG THE MAJOR CLADES: CORRELATING MOLECULAR AND MORPHOLOGICAL EVIDENCE

Whereas most of the clades described in the previous section are viewed as well-supported, comparison of Figures 1–3 in general, and in particular of the selected most-parsimonious tree in Figure 2 with the strict consensus tree of Figure 3, demonstrates that the relationships among most of those clades are far more ambiguous (see also Bateman *et al.*, 1997, 2001; Pridgeon *et al.*, 1997; Bateman, 2001). Such ambiguity is also evident in the ITS phylogeny first published by Aceto *et al.* (1999) and further developed by Cozzolino *et al.* (2001; see below).

In our original analysis (Bateman *et al.*, 1997; Pridgeon *et al.*, 1997), exclusion (Fig. 1a) and inclusion (Fig. 1b) of insertion–deletion events (indels) generated profoundly contrasting topologies that agreed only in the well-supported monophyly of the recircumscribed Orchidinae and in the only moderately supported relationships within the *Anacamptis–Himantoglossum* clade, which is delimited by a chromosomal reduction from $n = 21$ to $n = 18$ and a tendency toward viscidial fusion. In Figure 1a, the sister-groups of this clade are *Neotinea s.l.* and *Orchis s.s.–Traunsteinera*, respectively, together generating a clade that is weakly supported by ITS but is well marked by morphological characters such as globose tubers, sheathed inflorescences and membranous bracts (Vermeulen, 1947; Pridgeon *et al.*, 1997).

Pridgeon *et al.* viewed this topology as more credible in overall patterns of relationship than that generated from nucleotides plus indels (Fig. 1b), wherein *Orchis–Traunsteinera* is shown as sister to a fusiform/digitate-tubered clade and *Neotinea* appears to be the most basally divergent clade of Orchidinae. In contrast, the representation of the *Platanthera*, *Dactylorhiza* and *Gymnadenia* groups was viewed as more credible in Figure 1b than in Figure 1a, with the fusiform-tubered *Platanthera* appealingly depicted as an intermediate form leading to the relatively derived digitate tubers (see also Blinova, 2000), chromosomal reduction (from $n = 21$ to $n = 20$), and tendencies for both natural hybridization and polyploidy that unite *Dactylorhiza s.l.* with *Gymnadenia s.l.* The polarity of these key characters is, of course, the converse in Figure 1a, where these genera appear primitive rather than derived (a topology admittedly somewhat more consistent with biogeographical patterns: see below).

This triumvirate of major clades (*Orchis s.s.*, *Gymnadenia s.l.–Dactylorhiza s.l.*, *Platanthera* group) is also evident in our new expanded tree (Fig. 2), with the highly ambiguously placed *Traunsteinera–Chamorchis* shown as sister to the three (i.e. no longer linked directly to *Orchis s.s.*, as was previously believed). This relationship is supported by only a single step but makes better morphological sense than their previous placement (Fig. 1a,b), as *Orchis s.s.* possesses inflorescence sheaths and membranous bracts whereas *Traunsteinera* and especially *Chamorchis* are more plesiomorphic and thus akin to *Pseudorchis* and *Platanthera* in these features. The new tree also recovers, in its original topology, the derived clade that possesses the cytological synapomorphy of $n = 18$ (albeit with only weak bootstrap support: Fig. 3a). Interestingly, D'Emérico *et al.* (2001) noted that *Himantoglossum s.l.* and *Anacamptis s.l.* have similarly symmetrical

karyotypes, whereas those of *Serapias* and *Ophrys* are highly asymmetrical and highly divergent, both from each other and from *Himantoglossum s.l.* and *Anacamptis s.l.*, which apparently represent the relatively primitive condition. Moreover, mycorrhizal inoculation patterns under cultivation also reveal the relative phylogenetic isolation of *Ophrys* and *Serapias* in particular (R. Manuel, pers. comm., 2001).

Neotinea s.l. is on ITS evidence once again depicted as sister to the $2n = 36$ clade, as in the studies of Pridgeon *et al.* (1997) and Bateman (2001), but contrary to Bateman (1999a), wherein the genus was tentatively placed as sister to *Orchis s.s.* A sister-group relationship with *Orchis s.s.* is more consistent with allozyme (W. Rossi, pers. comm., 2001) and karyotype (D'Emérico, 2001) evidence, though the more suspect placement is that of *Orchis s.s.*, which is shown, somewhat unintuitively, as sister to *Traunsteinera–Platanthera–Dactylorhiza–Gymnadenia*, thereby once again refuting Vermeulen's (1947) concept of a clade united by globose root tubers (*sensu* Stern, 1998), sheathed inflorescences and membranous bracts; rather, these characters are optimized as plesiomorphic across this particular topology.

At this point, it is useful to briefly consider the less densely sampled ITS trees of Orchidinae published by Aceto *et al.* (1999) and Cozzolino *et al.* (2001), which revealed similarly composed major clades but differed in the representation of the relative positions of some of those clades (admittedly, none of the incongruencies between their results and ours are supported by significant bootstrap values in either set of trees). Most notably, Cozzolino *et al.* (2001: fig. 1) depicted *Himantoglossum s.l.* as sister to *Ophrys* rather than to *Ophrys* plus *Serapias* plus *Anacamptis s.l.*, and *Neotinea s.l.* as sister to *Platanthera*, thus implying that this oval-tubered genus is nested within the fusiform-tubered clade. Neither of these placements is likely on morphological evidence, and we suspect that they reflect the use of very few 'placeholder' species for *Himantoglossum s.l.*, *Ophrys*, and the *Platanthera* group, compounded by the absence of representatives of the major clades that separate these relatively derived Orchidinae from the disiid and satyrinid outgroups (i.e. the *Amerorchis–Neottianthe* group, *Brachycorythis*, and the habenariids). In our analysis, the basal groups usefully reduced topological instability among major clades higher in the tree.

Perhaps the greatest novelty of the updated ITS tree presented here (Fig. 2b) is the inclusion of the Asian *Neottianthe–Hemipilia* clade and Asian–South African *Brachycorythis*, revealing their clear position as basally divergent to the remaining clades within the Orchidinae s.s. Given the great morphological and

karyotypic diversity evident among the habenariids, and the equivocal relationships obtained in this and other recent studies, our decision to include in the analysis these additional clades of Orchidinae significantly improved the chances of obtaining realistic polarities for morphological and cytological characters (Luo, 1999; Luo & Chen, in press). The few relevant karyotypes available, of $2n = 42$ for both *Amitostigma gracile* (Tanaka, 1965; D'Emérico, 2000), *Neottianthe cucullata* (Sundermann, 1980) and *Brachycorythis helfei/B. obcordata* (Larsen, 1966; Mehra & Kashyap, 1978), argue against primitiveness for the $2n = 36$ clade. Inflorescences are unsheathed, bracts non-membranous and tubers relatively small and globose – interestingly, these are plesiomorphic features that also characterize Northern Hemisphere temperate Habenariinae such as *Hermidium*. Relationships within the Habenariinae remain decidedly equivocal.

Brief scrutiny of the bootstrap tree (Fig. 3) reveals that the 'spine' of the cladogram lacks any moderately supported or well-supported branches, other than the unusually close positioning of the *Dactylorhiza s.l.* and *Gymnadenia s.l.* clades. Indels are few and mostly homoplastic; of the exceptions, one links *Dactylorhiza* with *Gymnadenia* and the other groups all clades above *Brachycorythis*.

EVOLUTIONARY IMPLICATIONS

NON-MOLECULAR HOMOPLASY AND EVOLUTIONARY TRENDS

The above brief discussion focuses on a few morphological and cytological characters – in other words, potential synapomorphies – as 'mapped' across the ITS trees. Ideally, such characters should themselves be subjected to parsimony analysis (e.g. Bateman, 1999a, 2001), but this has not yet been achieved for the Orchidinae, which is species-rich and thus especially time-consuming to code. Thus far, our discussion has focused on highly conserved non-molecular characters of low homoplasy and therefore of high phylogenetic information content, notably karyotype and tuber morphology. In this section, in contrast, we examine highly homoplastic characters that are more likely to play central roles in lower-level (and potentially coadaptive) speciation events.

PIGMENTATION

Our knowledge of the floral pigmentation of the Orchidinae owes much to the pioneering quantitative work of Uphoff (1979, 1980) and the subsequent, more detailed, survey of Strack, Busch &

Klein (1989). Both studies were weakened by the presence of substantial proportions of pigments that could not be precisely identified, by having sampled from concentrated regions within the phylogeny rather than more evenly, and by attempting to draw taxonomic conclusions from such highly homoplastic characters under the mistaken assumption that simplicity equates with plesiomorphy. Nonetheless, some intriguing trends are evident. Firstly, *Ophrys* and *Serapias*, distinctive long-branch genera demonstrating particularly close evolutionary relationships with pollinators, have developed their own specific pigments. In contrast, most species of *Orchis* s.s., *Neotinea* s.l., *Himantoglossum* p.p. and *Anacamptis* s.l. appear evolutionarily labile; many rely on various combinations of cyanins and especially orchicyanins, and sister-species within these genera repeatedly demonstrate the ability to switch readily from one dominant pigment to another. Most species express most pigments in trace amounts, suggesting that subtle switches allow the differential enhancement or suppression of biosynthetic pathways, in turn permitting rapid coevolutionary responses that replace suites of one to three dominant pigments.

Discrete anthocyanin markings on the vegetative parts of the orchids, restricted to the upper epidermis of the leaves in most species, originated at least three times in the Orchidinae. They characterize a wide but phylogenetically disparate range of *Dactylorhiza* species, both diploid and tetraploid, usually showing varying degrees of polymorphism within individual species and often forming transversely elongated patches. Leaf markings are more often longitudinally elongate in *Orchis* s.s., wherein mode of expression differs subtly in three phylogenetically disparate groups (*O. italica*, the *O. mascula* aggregate, and the *O. anatolica* aggregate), possibly reflecting independent origins of the character. In the *Neotinea* s.l. clade, leaf markings are confined to the basally divergent *Neotinea maculata*, where yet again they are polymorphic within the species, and occur as distinctive, strongly elongate dashes that decorate the stem as well as the leaves. In contrast, *Steveniella satyrioides* typically bears diffuse anthocyanins throughout its above-ground organs. The function of vegetative anthocyanins remains speculative; two contrasting but credible inferences are relatively long-distance attraction of pollinators or avoidance of herbivory.

OTHER POLLINATOR-RELATED CHARACTERS

The genus *Serapias* and the often sympatric species *Anacamptis papilionacea* seem to have converged on

another supplementary method of probable pollinator attraction, namely a greatly expanded bract that is prominently veined and pigmented. Also, characters summarizing contrasting degrees of lobing of the labellum incur predictably high levels of homoplasy (Aceto *et al.*, 1999: fig. 2), making them unsuitable for high-level classification despite a recent determined attempt to resurrect them (Buttler, 2001).

Within the labellum, various forms of pilose and papillose ornamentations have evolved in several distinct lineages. Large multicellular trichomes characterize the labellum of *Ophrys*, wherein they tend to be concentrated toward the margin, and the labellum of *Serapias*, wherein they tend to be concentrated toward the near-cylindrical throat. Most members of the former *Orchis* and of the 'Comperia'-*Himantoglossum* clade bear discrete dark spots of anthocyanin in the throat (again, presumably acting as pollinator attractants), but in the 'core' clade of anthropomorphic *Orchis* species (*punctulata*-*militaris*) and in the more derived species of *Himantoglossum* s.s. (*hircinum* s.s.-*caprinum*) these features are physically elevated as clusters of substantial epidermal papillae that presumably improve the orientation and/or traction of the pollinating insect upon landing. Less prominent but still brightly pigmented papillae characterize some species in other clades, including *Traunsteinera globosa*, *Orchis spitzelii*, *Neotinea tridentata* s.l., *Anacamptis papilionacea* and *Anacamptis coriophora*-*A. fragrans*.

Another character that strongly influences pollination syndromes in Orchideae is the labellar spur; specifically, its dimensions, posture and nectar-producing ability. A moderately large, downcurved spur is the plesiomorphic condition in the tribe, but this has been profoundly modified on many occasions (see also Cozzolino *et al.*, 2001). Unusually short spurs characterize *Gennaria*, *Neotinea maculata*, *Traunsteinera*, *Pseudorchis*, 'Coeloglossum', 'Nigritella' and the *Himantoglossum*-*Steveniella* clade. Spurs are even less developed in other groups, being rudimentary or non-existent in *Herminium*, 'Aceras', *Chamorchis*, *Serapias* and *Ophrys*. In contrast, long, downcurved spurs evolved independently in *Platanthera* (found in most species, nectariferous), *Gymnadenia* s.s. (found in most species, nectariferous) and *Anacamptis pyramidalis* (not nectariferous), presumably to facilitate lepidopteran pollination. Wider, upcurved and elongate spurs lacking nectar evolved independently in the *Dactylorhiza sambucina* group, the *Orchis mascula*-*anatolica* clade, the *Anacamptis laxiflora*-*robusta* clade and the *A. boryi*-*longicornu* clade (Fig. 2), whereas 'Habenaria' *purpureopunctata* has innovated an apical sac (Luo & Chen, 2000, in press).

Fusion (typically congenital fusion) of floral structures is a frequent evolutionary trend in orchids

(Rudall & Bateman, 2002, 2003). The presence of a galea (hood formed of the sepals and lateral petals) is also highly homoplastic, although it may prove more phylogenetically informative if dissected into more precise characters describing (a) relative degrees of connivence and fusion, and (b) whether these phenomena affect only three perianth segments (median sepal plus lateral petals) or all five (including the two lateral sepals). Certainly, preliminary study suggests that the basal fusion of the three sepals is a useful (and novel) character for distinguishing in the field *Anacamptis s.l.* (most species of which were formerly assigned to *Orchis s.l.*) from *Orchis s.s.* (Bateman, 1999b).

Among the gymnostemium-based characters that have long been favoured by most orchid taxonomists, the presence of two viscidia (adhesive discs = retinacles) and absence of an enclosing bursicle (pollinarium sac) are primitive characters within tribe Orchideae (cf. Dressler, 1993). Fusion of the pair of pollinarium bases into a single viscidium occurred three times within subtribe Orchidinae: in *Anacamptis pyramidalis*, in the genus *Serapias*, and in the *Himantoglossum s.s.*–*Barlia* clade. Fused viscidia were foreshadowed by juxtaposed but rarely fused paired viscidia in the sister group, the former '*Comperia*', and a similar condition has been observed in the former '*Aceras*' (e.g. see the well-informed downgrading of gynostemium characters by Delforge, 1999).

There is considerable disagreement in the literature regarding whether some genera (e.g. *Herminium*, *Chamorchis*, *Traunsteinera*, *Galearis*, *Amerorchis*, '*Nigritella*', '*Coeloglossum*') lack bursicles or possess rudimentary bursicles; consequently, several of these genera, plus *Dactylorhiza*, were the focal points of arguments over the boundaries of two putative subtribes within the tribe Orchideae, the *Gymnadeniinae* and the *Serapiadinae* (cf. Delforge, 1995). Several other, relatively derived genera unequivocally possess well-developed bursicles, apparently reflecting at least three separate origins of the feature: the $2n = 36$ clade, *Neotinea s.l.*–*Orchis s.s.*, and *Dactylorhiza s.s.* In addition, *Dactylorhiza* apparently experienced (a) partial loss of the bursicle in *D. viridis* (the former '*Coeloglossum*'), and (b) reputed division into two bursicles in a few other species (Nelson, 1976). Among these genera there is a reliable positive correlation between juxtaposed or fused viscidia and simple rather than bilobed bursicles. A bursicle wholly divided into two segments is a genus-level apomorphy of *Ophrys*, and a strongly elongate gynostemium is a genus-level apomorphy of *Serapias*. Deeply divided pollinia characterize *Platanthera* section *Piperia* (fig. 1 of Ackerman, 1977) and have also been observed in *Neotinea maculata* (plate 13 of Ross-Craig, 1971). Lastly, the grouping of *Platanthera* and *Gymnadenia* with *Habenaria* that

was inferred using rostellar structure by Rasmussen (1985) is clearly refuted by the DNA data.

Many orchid specialists continue to give pre-eminence to gynostemium characters in typological classifications of the Orchideae and/or in phylogenetic hypotheses that are not rooted in numerical data-matrices (e.g. Vöth, 1999; Wucherpfennig, 1999; Buttler, 2001). For example, Vöth's (1999) insightful ecological interpretations of similarities in the floral morphologies of *Neottia*, *Listera* (both Epidendroideae: Neottieae), *Chamorchis*, '*Coeloglossum*' and '*Aceras*' were converted by him into a phylogenetic hypothesis of these taxa, mistakenly indicating a close relationship between the last three taxa (cf. figs 2 and 3 of Vöth). Although less homoplastic than spur dimensions and labellum shape, it appears that gynostemium characters are also modifiable (and reversible) under the influence of evolutionary interactions between flowers and pollinators. Cozzolino *et al.* (2001) presented a more detailed exploration of the presumed phylogenetic implications of pollination biology, notably the apparently recent separation of sister-species of the former *Orchis s.l.*, which were attributed to switching among specialized pollinators.

Such observations offer a salutary warning regarding the dangers of a priori weighting of taxonomic characters, and further emphasize the importance of applying to systematic data the congruence text of homology through explicit, quantified cladistic analysis. However, they are *not* an acceptable justification for omitting potentially adaptive morphological characters from phylogenetic analyses (Bateman, 1999a, 2001).

OTHER EVOLUTIONARY TRENDS WITHIN THE MAJOR CLADES

Although more detailed quantitative exploration is desirable, some potential evolutionary trends are evident within specific clades as one traces the divergence of species distally from the base. Firstly, there is a tendency for increased concentration of leaves at the base of the flowering stem; leaves are relatively widely spaced up the stems of the *Anacamptis laxiflora*–*robusta*, *Ophrys insectifera*, *Pseudorchis* and *Traunsteinera*. Interestingly, the first three examples are basally divergent members of clades that more typically possess leaves that are few in number and concentrated as basal rosettes (there is also a continued trend of reduction in leaf number in the *Platanthera s.s.* and *Galearis*–*Amerorchis* clades). Also, flowers tend to become larger and more striking in appearance within major clades (exceptions being *Anacamptis s.l.* and *Gymnadenia s.l.*).

Moving on to more cryptic characters, increasing chromosomal asymmetries were reported within all three clades formerly attributed to *Orchis* (Yokata, 1987; D'Emérico *et al.*, 1996; D'Emérico, 2000), whereas microanatomical characters such as seed morphology and developmental embryogeny appear to have remained resolutely constant; all reputedly possess 'Orchis' group 4b seeds (*sensu* Wildhaber, 1972), and the 'Orchideae type' embryological model extends across the Orchidinae and Habenariinae (Clements, 1999). Pollen grains vary subtly in surface sculpture among species (Schill & Pfeiffer, 1977), but no clear phylogenetic patterns are evident. The intriguing suggestion by Aceto *et al.* (1999: 74) that basally divergent genera are more species-rich than more derived genera is not upheld by Figure 2, and is of course dependent on partly subjective choices of generic circumscription by the observer.

Not all of the evolutionary transitions implied by the cladogram are likely to reflect subtle adaptative 'tweaking'; punctuationist interpretations of the initial subfamilial radiation of Orchidaceae were offered by both Bateman (1999a; see also Bateman, 2001; Bateman & DiMichele, 2002; Rudall & Bateman, 2002) and Aceto *et al.* (1999). As noted by Bateman (1999a), the strong contrast in degrees of molecular and morphological divergence implies that '*Nigritella*' probably represents a relatively recent but successful establishment, in Europe's uplands, of a mutant of *Gymnadenia*, most likely originating by a single-gene mutation that radically simplified the perianth segments and rendered the flower both non-resupinate and pseudopeloric (Bateman, 1985; Rudall & Bateman, 2002). If so, this evolutionary event qualifies as non-adaptive dichotomous saltation *sensu* Bateman & DiMichele (1994, 2002). A similar mode of origin, probably from within *Galearis*, is likely for the pseudopeloric '*Aceratorchis*' *tshiliensis* (cf. Chen *et al.*, 1999: 4). Such evolutionary-developmental events could also have initiated the highly distinct floral morphologies of, for example, *Ophrys* and *Serapias*.

HYBRIDIZATION

The frequent records of hybrids in the Orchidinae require considerable critical appraisal when, as in the vast majority of cases, they are based entirely on qualitative morphological comparison (e.g. Averyanov, 1990). Infrageneric hybrids are often under-recorded due to preclusion of identification by morphological overlap between the putative parents (Bateman *et al.*, 1997; Bateman, 2001; Bateman & Hollingsworth, 2003). In contrast, past records of intergeneric hybrids are probably vastly inflated, due to (a) recognition of phylogenetically spurious genera, and (b) erroneous

identification of morphological extremes of single species, including teratological mutants, as hybrids (e.g. Rudall & Bateman, 2002).

Those few genuine bigeneric hybrids – in other words, those that survived our past and present taxonomic rearrangements of the Orchidinae – are generally sister-genera. The most frequent hybrids between the 12 major clades of Orchidinae discussed above also occur between sisters, such as *Gymnadenia* × *Dactylorhiza*, *Pseudorchis* × *Dactylorhiza*, *Anacamptis* s.l. × *Serapias* (e.g. Sundermann, 1980; Ettlinger, 1999), and *Traunsteinera* with members of the fusiform/digitate-tubered clade (Peitz, 1972), supporting their revised ITS placements as sister-clades. Convincing records of hybrids are very rare between members of any pair of the six most distinct groups on Figure 2 (Habenariinae, *Brachycorythis*, *Neottianthe* group, *Neotinea* plus *Orchis* s.s. (paraphyletic), *Platanthera*–*Gymnadenia*, *Himantoglossum*–*Anacamptis*). One recent example involved two natural specimens of *Orchis mascula* × *Anacamptis morio* found in Cumbria, UK (Halliday, 1997: 579). The plants were short-lived and the flowers imperfectly formed, implying a predictably high degree of infragenomic incompatibility upon combining these two highly divergent lineages. Similar genetic instability is evident in a photograph of a hybrid generated artificially between *Orchis mascula* and *Dactylorhiza fuchsii* (Ettlinger, 1999: 202), suggesting that the long-term evolutionary prognosis is generally poor for orchid lineages blending two such disparate genomes. Other recent experiments in artificial hybridization have shown that the maternal parent has a far greater influence over the resulting hybrid morphology than does the paternal parent (e.g. R. Manuel, pers. comm., 2002; see also Bateman & Hollingsworth, 2003).

BIOGEOGRAPHY

Renz (1980: fig. 6; see also Chen, 1982) presented a simple evolutionary–biogeographical scenario for the Orchideae that involved an origin in south-east Asia followed by two migrations: of the Disperideae (since shown using ITS sequences to be sister to the remainder of Diseae by Douzery *et al.*, 1999) plus Diseae plus Satyriineae (presumably also plus Habenariinae, though this was not explicitly stated) across south-western Asia and into a centre of radiation in southern Africa, and of the Orchidinae across western Asia into a centre of radiation in Europe.

Comparing the topology of Figure 2 with the current distributions of the analysed species yields an equivocal result, due to the relative instability of the sparsely sampled *Brachycorythis* and the various habenariid genera. In other trees (e.g. Bateman, 2001) the north-western African *Gennaria* lies phylogeneti-

cally outside the other habenariids, but here it groups tentatively (and morphologically improbably) with the co-occurring *H. tridactylites*. *Bonatea*, *Cynorkis* and *Pecteilis* each cluster with contrasting species of *Habenaria s.l.* (Fig. 2b), though many of these supposed relationships collapse in the consensus tree (Fig. 3b). The Asian '*Peristylus*' is firmly nested within the similarly Asian *Herminium s.l.* Crucially from a biogeographical viewpoint, the southern African *Stenoglottis* and Afro-Asiatic *Brachycorythis* are interpolated between the aforementioned unequivocal Habenariinae genera and the basal-most bona fide clade of Orchidinae, *Neottianthe-Hemipilia*; indeed, these two genera occupy similar positions in the maximum-parsimony and maximum-likelihood trees generated from ITS data by Douzery *et al.* (1999). Having the Afro-Asiatic *Brachycorythis* tentatively basal within the Orchidinae and the southern African *Stenoglottis* tentatively basal within the Habenariinae raises the possibility of an 'out of Africa' (and into central Asia) hypothesis for the origin of both subtribes.

Overall, the difficulty of resolving the relationships among the major clades within Orchidinae suggests a relatively early and rapid evolutionary radiation (*sensu* Bateman, 1999a). Above *Brachycorythis*, the next divergent clade, *Neottianthe-Hemipilia*, is exclusively Asian. The *Pseudorchis-Platanthera* clade exhibits the most complex biogeography – one that implies a migration following phylogenetic divergence into *Pseudorchis*, *Galearis-Amerorchis*, *Neolindleya* and *Platanthera* – that tracked across north-east Asia and into North America before eventually (and somewhat improbably) colonizing Europe (Bateman *et al.*, 1997). The present centre of diversity of *Dactylorhiza* is Europe, but the more basally divergent species extend into central/eastern Asia and the basal-most species analysed (*D. cf. hatagirea*, excluded from this analysis pending confirmation of its ITS sequence, but see Bateman, 2001) is exclusively central/eastern Asian, suggesting a western migration of the clade.

In contrast, the remaining major clades (*Neotinea*, *Orchis s.s.*, *Traunsteinera-Chamorchis*, and the four clades collectively delimited by $2n = 36$) are exclusively European, the intriguing exceptions being *Stenieniella* and '*Comperia*'. These two taxa, localized within Asia Minor, are the two basal-most members of the *Stenieniella-Himantoglossum s.l.* clade, which is in turn the basal-most group in the $2n = 36$ clade. In terms of overall biogeographical patterns, the phylogenetic interpolations in Figure 2 of (a) *Neotinea s.l.* beneath the $2n = 36$ clade, and (b) *Orchis s.s.* and *Traunsteinera-Chamorchis* below the more geographically widespread *Platanthera-Dactylorhiza-Gymnadenia* clade, are not optimally parsimonious.

TAXONOMIC IMPLICATIONS

GENERIC RE-DELIMITATION

The original ITS analysis of Pridgeon *et al.* (1997) and the subsequent, monophyly driven taxonomic rearrangements formalized by Bateman *et al.* (1997) caused predictable controversy (cf. Delforge, 1998, 1999, 2001; Gerbaud, 1998, 1999; Hughes, 1998; Teppner & Klein, 1998; Bateman, 1999b, 2001; Ettlinger, 1999; Marren, 1999: 86–88; Quentin, 1999; Wucherpennig, 1999; Breiner & Breiner, 2000; Hardwick, 2000; Hedrén *et al.*, 2000; Vöth, 2000; Bateman *et al.*, 2001; Buttler, 2001; Grünanger, 2001; Kocyan & Widmer, 2001; Kretzschmar, *et al.*, 2001, 2002; Temple, 2001; Dusak & Pernot, 2002). Nonetheless, given subsequent confirmation by independent ITS analysis of a similar range of species (Aceto *et al.*, 1999), these taxonomic revisions have begun to permeate the secondary literature; for example, they have been used in recent detailed plant atlases for counties within the UK (e.g. French, Murphy & Atkinson, 1999), France (Dusak & Pernot, 2002) and various Mediterranean islands (Kretzschmar, Kretzschmar & Eccarius, 2001, 2002). They have also been wholly adopted in *Genera Orchidacearum* (Pridgeon *et al.*, 2001), which in turn provides the nomenclature recommended by two key British societies, the Royal Horticultural Society and the Hardy Orchid Society. Generic transfers implemented by Bateman *et al.* (1997) involved the incorporation of *Nigritella* into *Gymnadenia*, *Coeloglossum* into the conserved *Dactylorhiza*, *Aceras* into *Orchis s.s.*, and the expansion of the formerly monotypic *Neotinea* and *Anacamptis* to encompass tranches of the formerly triphyletic *Orchis s.l.*

Publication of the previous phylogenies (Bateman *et al.*, 1997; Pridgeon *et al.*, 1997; later Bateman, 1999a, 2001; Bateman *et al.*, 2001) prompted further generic transfers of '*Nigritella*' species by other workers: firstly the widespread Scandinavian species '*N. nigra s.s.*' by Delforge (1998; a species since sequenced for the present study), and then a series of more finely divided and geographically localized Alpine species (examples of which were only recently sequenced by us) by Teppner & Klein (1998) and Fölsche *et al.* (1999). Gerbaud (1999) since resurrected *Nigritella*, this time as a monophyletic subgenus of *Gymnadenia s.l.*, but he did not attempt the recognition of several corresponding subgenera within *Gymnadenia s.s.* necessary to erect a bona fide phylogenetic classification of the group. In contrast, Hedrén *et al.* (2000) used a substantial body of allozyme data to argue that *Nigritella* and *Gymnadenia s.s.* are sister-taxa (albeit close sisters) and should therefore be maintained as separate genera.

More controversially, Delforge (1999) used Pridgeon *et al.*'s (1997) evidence of sister-group relationships to

sink 'Barlia' into *Himantoglossum* s.s., also sinking 'Comperia' on suspicion of a sister-group relationship with 'Barlia' that has been conclusively disproved by the present study (Fig. 2). Nonetheless, as discussed in detail above, the overall balance of evidence supports Delforge's decision to incorporate both 'Barlia' and 'Comperia' into a revised concept of *Himantoglossum* s.l. that encompasses a substantially increased range of morphological variation. The less certain phylogenetic placement of *Steveniella* (Figs 2, 3) currently precludes its incorporation into an even further expanded *Himantoglossum*, even if it was considered sufficiently morphologically similar to the other species in the clade.

Synonymization of 'Piperia' into *Platanthera* is clearly required by the tenets of monophyly, as continued recognition of 'Piperia' as a genus would render *Platanthera* paraphyletic. Floral and vegetative similarities linking the two former genera are reflected in the fact that the first species of 'Piperia' to be described was assigned to *Platanthera* by its author, Lindley (1835). However, this placement of 'Piperia' increases perceived levels of homoplasy in two suites of morphological characters routinely prioritized for high-level orchid classification: pollinarium structure (many authors) and tuber morphology (Dressler, 1993; Pridgeon *et al.*, 1997), given that the very short caudicles and globose tubers ostensibly are characters shared with other species outside, but not within, *Platanthera* s.l.

On balance, *Chamorchis* and *Traunsteinera* merit continued generic recognition, unless a taxon is subsequently found that bridges the morphological and molecular gaps that currently readily distinguish them.

Decisions regarding other apparent taxonomic requirements of the present tree (Fig. 2) within the Orchidinae require additional data. The more likely changes concern the *Chusua*-*Neottianthe* clade: they include (a) confirming the distinction made here between *Ponerorchis* s.s. and *Chusua* s.s., and carefully examining the more narrowly delimited *Chusua* s.s. to determine whether it is monophyletic or paraphyletic, and (b) either incorporating 'Habenaria' *purpureopunctata* into an expanded *Hemipilia* s.l. or establishing this species as a new monotypic genus that is sister to *Hemipilia* (see also Luo & Chen, in press; Luo *et al.*, in press). 'Gymnadenia' *camtschatica* should be assigned to the pre-existing but rarely used genus name, *Neolindleya*.

Other options considered for taxonomic changes but ultimately rejected included erecting *Dactylorhiza hatagirea* as a new sister-genus to the other more derived dactylorchids, and redelimiting the *Anacamptis laxiflora*-*robusta* clade as a new genus. Even if all these suggested transfers, past, present and future,

were implemented and accepted they would affect only an estimated 12% of the total number of species widely recognized within the Orchidinae s.s.

INFRAGENERIC CLASSIFICATION

There are also clear implications for potential hierarchical classifications *within* the more species-rich genera. Bateman *et al.* (1997) and Pridgeon *et al.* (1997) discussed at length the severe taxonomic problems that had developed when over two centuries of taxonomic research attempted to shoe-horn the triphyletic *Orchis* s.l. into various infrageneric classifications. Indeed, new results presented here require further dismantling of Vermeulen's (1972, 1977) classification of *Orchis* s.l., since his subsections *Punctulatae*, *Provincialae* and *Masculacae* have all proved to be non-monophyletic (see Bateman *et al.*, 1997; note that all of the 'placeholder' species listed in their fig. 10 have now been sequenced). We are currently preparing a revised infrageneric classification of *Orchis* s.s. and have completed that of *Anacamptis* s.l. (R. Bateman and P. Hollingsworth, unpubl. obs.). Infrageneric classification is also now feasible for the relatively well-studied *Platanthera*-*Piperia* clade, as has already been achieved informally for *Platanthera* s.s. by Hapeman & Inoue (1997).

SPECIES DELIMITATION

The issue explicitly raised by Bateman *et al.* (1997: see also Cafasso *et al.*, 2000; Bateman, 2001) of the relevance of degrees of ITS sequence divergence to species delimitation can be further discussed in the light of the present results. Several disparate species within the Orchideae have now yielded multiple sequences, often acquired from conspecific individuals sampled in different countries: these include *Habenaria socotrana*, *Neottianthe cucullata*, several members of the *Gymnadenia*-*Dactylorhiza* clade (R. Bateman *et al.*, unpubl. obs.; M. Chase *et al.*, unpubl. obs.), *Neotinea tridentata*, *Orchis pauciflora* and *Anacamptis laxiflora*. All but the supposed *N. tridentata* (see above) yielded sequences that were identical or apparently differed by only a single substitution, indicating both the high repeatability of the laboratory analyses and the high conservation of ITS sequences within species. Admittedly, considerably higher levels of ITS divergence were evident between some of the 25 pairs or triplets of conspecific individuals included in the recent densely sampled study of tribe Epidendroideae subtribe Laeliinae by van den Berg *et al.* (2000). Also, a single individual of the allotetraploid *D. praetermissa* was inferred by Pridgeon *et al.* (1997) to contain three ITS variants diverging by up to 13 bases, but re-examination of the data suggests that these putative levels of divergence were greatly over-estimated. Overall,

significant ITS divergence appears a strong indicator (though not proof) of species-level differences.

However, the converse argument is not upheld; some pairs of orchid species that are clearly separable using morphological criteria reliably yield identical ITS sequences. Examples include *Platanthera bifolia* and *P. chlorantha*, *Gymnadenia conopsea* s.s. and *G. odoratissima*, and among the neottiid orchids several *Epipactis* species, both allogamous and autogamous (e.g. *E. helleborine*, *E. purpurata*, *E. phyllanthos*; P. Hollingsworth *et al.*, unpubl. obs.). Thus, genera that have recently been highly (and controversially) split on morphological grounds, notably *Ophrys*, *Serapias*, *Nigritella* and the more derived, iteratively polyploid species of *Dactylorhiza*, will require other more effective molecular (and morphometric) tools for delimiting bona fide species and determining their relationships. For example, Hedrén *et al.* (2001) used AFLP data to argue controversially that all of the tetraploid *Dactylorhiza* species should be amalgamated into a single species, *D. elata* (but see Appendix), and that the geographically widespread diploid *D. fuchsii* should be made conspecific with the tetraploid *D. maculata* (contra Bateman & Denholm, 2003). More such studies are needed in order to distinguish genuine but recent infrageneric radiations from pseudo-speciation events perpetrated by orchid taxonomists adopting an extreme 'splitters' approach (cf. Bateman, 2001; Delforge, 2001).

SUPRAGENERIC CLASSIFICATION

Moving to higher phylogenetic levels, it is also tempting to generate classifications that group combinations of the 12 well-founded major clades discussed individually above. However, the low bootstrap values associated with most of the internal nodes along the 'spine' of the cladogram suggest that further formal classification would be premature and that a simultaneous analysis of multiple data sources (e.g. adding morphological and plastid sequence data to the ITS matrix: Bateman, 1999a; R. Bateman *et al.*, unpubl. obs.) is required. Also, although the taxonomic history of the Orchidinae was well reviewed by Klinge (1898), Vermeulen (1947) and Bateman *et al.* (1997), there nonetheless remains an opportunity to compare in greater detail the suprageneric classifications of such orchidological luminaries as (in chronological order) Linnaeus, Haller, Swartz, Brown, Richard, Lindley, Reichenbachs p. and f., Klinge, Schlechter, Camus, Vermeulen, Dressler and Delforge. All unknowingly (or, in the case of Delforge, 2001, knowingly) deviated considerably from the dictates of monophyly due to typological reliance on a relatively small number of often highly homoplastic morphological characters.

For example, Dressler (1993) classified the Orchidinae primarily according to tuber morphology into four

tentative 'alliances' (tabulated with distributional data as table 1 of Pridgeon *et al.*, 1997). Of these, Alliance 4, consisting of the African genera *Holothrix* and *Bartholina*, certainly does not belong in the Orchidinae, nor is its ITS placement in the Habenariinae by Douzery *et al.* (1999) convincing, as the single *Holothrix* species is subtended by an improbably long-terminal branch; we believe that the group may even lie outside the tribe Orchideae. Figure 2 demonstrates that Dressler's Alliance 1, incorporating genera with minimal morphological expression of tubers such as *Galearis* and *Amerorchis*, is a subgroup of the more typically fusiform-tubered *Platanthera* clade, which occurs in Dressler's Alliance 2 alongside digitate-tubered genera of the *Dactylorhiza* and *Gymnadenia* clades. Also erroneously placed in Alliance 2 are two far more primitive groups of Orchidinae, the Asian *Ponerorchis* s.l. and the dominantly African *Brachycorythis* (see also Douzery *et al.*, 1999). Dressler's globose-tubered Alliance 3 is the most genus-rich and morphologically heterogeneous assembly, mixing primitive Asian genera such as *Amitostigma* and *Hemipilia* with the derived $2n = 36$ clade of *Himantoglossum*–*Anacamptis* and the globose-tubered sister-groups (*Neotinea* s.l.–*Orchis* s.s. and *Traunsteinera*–*Chamorchis*) of the fusiform-digitate tubered clade. Moreover, this study suggests that the globose tubers of *Platanthera* subgenus *Piperia*, included as '*Piperia*' by Dressler in his Alliance 3, represent a reversal from the fusiform tubers that characterize the remaining subgenera of *Platanthera* (see above). Also included in Alliance 3 is the as-yet unsequenced South African genus *Schizochilus*, which in our view is more likely to be a habenariid.

Thus, Dressler's (1993) morphologically based classification contains valuable insights into the phylogeny of the Orchidinae but has required considerable modification following DNA sequencing. The fairly radical generic re-delimitation accompanying our earlier ITS phylogeny (Pridgeon *et al.*, 1997; see also Bateman, 2001) has been followed by finer tuning to reflect the present ITS phylogeny (Figs 2, 3). Current evidence suggests that the ITS phylogeny is now stabilizing for the Orchidinae if not the Habenariinae, presumably due to the much-improved taxonomic sampling of the Orchidinae. Phylogenetic knowledge of the tribe would now benefit most from confirmation by sequencing other regions of the plant genome, particularly to increase resolution along the spine of the tree. Such work is now underway (R. Bateman *et al.*, unpubl. obs.).

FUTURE RESEARCH

Much of the additional sampling advocated by Bateman *et al.* (1997) and Pridgeon *et al.* (1997) has since

been achieved. Consequently, the Orchidinae is now among the most thoroughly sampled tribes/subtribes of the Orchidaceae, along with the Cypripedioideae (Cox *et al.*, 1997, 1998; Pridgeon *et al.*, 1999) and the Epidendroideae subtribe Laeliinae (van den Berg *et al.*, 2000). Moreover, the Orchidaceae is in turn one of the most thoroughly sampled and actively researched large families of plants.

Nonetheless, we will continue to gather additional ITS sequences with the aim of covering all species widely recognized within the group. The new data can be integrated with sequences for other taxa, either gathered by us recently but too late for inclusion in the present analysis (*Himantoglossum metlesicsianum*, *Neotinea ustulata* var. *aestivalis*, *Dactylorhiza iberica*, the diploid species of '*Nigritella*'), or requiring re-analysis due to possible misidentification of original samples (*Orchis provincialis*, *Dactylorhiza hatagirea*, *Habenaria repens*). Additional taxa are also required for a complete ITS-based phylogenetic analysis. Remaining priorities for further ITS sequencing within the Orchidinae include five supposed monotypic genera from Asia: *Aorchis*, *Aceratorchis*, *Chondradenia*, *Symphyosepalum* and *Pseudodiphryllum* (e.g. Vermeulen, 1972; Dressler, 1993; Chen *et al.*, 1999). The need for further species-level sampling within genera already sequenced by us also focuses on (Eur)asia. '*Pseudorchis*' *frivaldii* and, arguably, *Himantoglossum formosum* remain generically ambivalent, the former with *Gymnadenia* (Delforge, 1995) and the latter with the former genus *Barlia* (Bateman *et al.*, 1997). Also, resolution is required of ambiguities in the *Neottianthe*-*Hemipilia* clade, notably the apparent paraphyly of *Ponerorchis* as provisionally re-delimited here.

Further molecular phylogenetic research is also desirable on putatively related southern African orchids, as their phylogenetic positions are crucial to interpretations of both the putative monophyly and respective relationships of the Orchidinae s.s. and Habenariinae (cf. Douzery *et al.*, 1999; Linder & Kurzweil, 1999), which were controversially synonymised in *Genera Orchidacearum* (Pridgeon *et al.*, 2001). *Brachycorythis* may encompass at least parts of *Neobolusia*, the monotypic *Dracomonticola* and *Schizochilus*. Moreover, *Holothrix* and *Bartholina* may even lie outside the Habenariinae. The erroneous identification of '*H.*' *repens* is noteworthy, as this species was used as representative of the habenariids in early molecular phylogenetic studies of the Orchideae (e.g. Neyland & Urbatsch, 1995, 1996a,b). Similarly, the use of *Peristylus coeloceras* in both this and earlier studies (Douzery *et al.*, 1999) to exemplify the genus was misleading, since morphological (Luo *et al.*, in press) and sequence (this study) evidence shows that *P. coeloceras* and other high-

altitude temperate species are distinct from *Peristylus* s.s. and should be transferred to *Herminium*, thereby rendering the latter genus monophyletic. Certainly, the habenariids require far more intensive (and preferably internationally collaborative) sampling for both sequencing and karyotypic studies (cf. D'Emerico, 2000). Current lists of orchids native to Africa, the centre of distribution of *Habenaria*, are fraught with taxonomic hazards (cf. Minasiewicz & Olszewski, 1999).

Tree rooting also remains ambiguous. We previously believed that rooting of the Habenariinae in this study would be improved by adding to our ITS matrix at least one *Satyrium* sequence, since the genus was phylogenetically interpolated between the Orchidinae-Habenariinae and Diseae in the phylogenetic studies of Linder & Kurzweil (1994, 1999; see also Kurzweil & Linder, 1999) and Douzery *et al.* (1999). However, the more detailed sampling of Bellstedt, Linder & Harley (2001) led to a near-polytomy among *Disa*, *Satyrium* and *Habenaria*, and in some most-parsimonious trees obtained during this study the single *Satyrium* sequence analysed by us nested within the habenariids. We also believe that ITS sequence alignment ambiguities, which are most problematic among the Habenariinae in our study (ultimately precluding satisfactory incorporation of *Holothrix*) and proved even more troubling among the Diseae in the analysis of Bellstedt *et al.* (2001), could be improved by reciprocal illumination with computer-generated secondary structures (cf. Hershkovitz *et al.*, 1999; admittedly, this procedure requires sequence similarities in excess of 60%).

Indeed, reciprocal illumination has already greatly aided our understanding of the phylogeny of the Orchidinae in other ways. For example, our original ITS sequences revealed the strongly conserved phylogenetic signal of cytological characters (cf. Brandham, 1999; D'Emerico, 2001); these observations in turn allowed accurate predictions of the broad-brush phylogenetic positions of additional taxa characterized by $2n = 36, 40$ or, to a lesser degree, 42. Reappraisal of morphological characters then reveals additional synapomorphies, such as the basally fused sepals of *Anacamptis* s.l. that apparently separate it from *Orchis* s.s. with unfused sepals (Bateman, 1999b; R. Bateman and P. Hollingsworth, unpubl. obs.). Interesting micro-morphological distinctions have been observed between the pollinaria of *Serapias* plus *Orchis* s.s., *Platanthera*, and *Dactylorhiza* plus *Gymnadenia* (Barone Lumaga *et al.*, 2000). Ongoing construction of matrices for the plastid regions *trnL* and *matK*, and for morphology (R. Bateman and P. Hollingsworth, unpubl. obs.) plus anatomy and ontogeny (Kurzweil, 1987, 1990, 1999, 2000; Kurzweil & Weber, 1991, 1992; Stern, 1998; Luo & Chen, in press; Luo *et al.*, in press), will aid the more distant goal of inter-matrix compar-

ison followed by simultaneous analyses of all three matrices (cf. Bateman, 1999a, 2001).

At lower taxonomic levels, the weaknesses elucidated by Bateman (2001) of the current trend towards 'literative speciation' – upgrading infraspecific taxa to full species without gathering additional scientific data to test direct or indirect evidence of gene flow among populations – are clear; contrast for example the species-rich classifications of *Dactylorhiza* by Averyanov (1990) and of all European orchids by Delforge (1995, 2001) with earlier, more conservative comparable publications. As noted above and by Bateman *et al.* (1997), there are considerable dangers in delimiting species using degrees of ITS divergence as the only molecular evidence. Rather, combining morphometrics with the rapidly improving range of molecular fingerprinting techniques (cf. Qamaruz-Zaman *et al.*, 1998; Soltis *et al.*, 1998; Hollingsworth *et al.*, 1999; Qamaruz-Zaman, 2000; Soliva *et al.*, 2000; Tyteca, 2001) offers a more powerful approach to delimiting species, identifying putative hybrids, and placing phylogenetically troublesome polyploids, especially those within the *Dactylorhiza*–*Gymnadenia* clade (Hedrén *et al.*, 2001; Bateman & Denholm, 2003; R. Bateman *et al.*, unpubl. obs., M. Chase *et al.*, unpubl. obs.).

Lastly, we urgently need more rigorous ecological observations to generate less speculative coevolutionary studies, not only with pollinators of Orchideae (cf. Dafni, 1987; Paulus & Gack, 1990; Nilsson, 1992; van der Cingel, 1995; Neiland & Wilcock, 1998; Johnson & Steiner, 2000; Gumbert & Kunze, 2001) but also with their perennially under-researched mycorrhizal symbionts (Rasmussen, 2000; J. Leake and S. McKendrick, pers. comm., 2001).

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APPENDIX: NOMENCLATURAL NOTE

Terrestrial orchids continue to ‘benefit’ more than most plant groups from concerted attempts to find and establish older specific and infraspecific epithets by judicious application of the laws of nomenclatural priority. Of the many taxa analysed here, the most recent controversy has surrounded the replacement of the well-established *D. majalis* (Rchb. f. 1828) P.F. Hunt and Summerh. with *D. comosa* (Scop. 1772) P.D. Sell that was advocated by Sell in Sell & Murrell (1996; 364). Pedersen (2000) attributed the lectotype of *D. comosa* to the diploid *D. incarnata*, but the arguments of Baumann *et al.* (2002) that the lectotype is attributable to *D. praetermissa* are more convincing. However, Baumann *et al.* (2002) then proceeded to reopen the long-running nomenclatural debate concerning whether *Platanthera montana* (F.W. Schmidt 1793) Rchb. f. has priority over the widely recognized *P. chlorantha* (Custer 1827) Rchb. p. Given that in both cases holotypes are absent and the identity of the lectotype is ambiguous, the consequent nomenclatural complexity of the suggested changes is difficult to justify. In contrast, the generic transfers made in this paper are rooted in unambiguous phylogenetic relationships.