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Molecular and morphological phylogenetics of the digitate-tubered clade within subtribe Orchidinae s.s. (Orchidaceae: Orchideae)

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Summary. The digitate-tubered clade (*Dactylorhiza* s.l. plus *Gymnadenia* s.l.) within subtribe Orchidinae is an important element of the North-temperate orchid flora and has become a model system for studying the genetic and epigenetic consequences of organism-wide ploidy change. Here, we integrate morphological phylogenetics with Sanger sequencing of nrITS and the plastid region *trnL-F* in order to explore phylogenetic relationships and phenotypic character evolution within the clade. The resulting morphological phylogenies are strongly incongruent with the molecular phylogenies, instead reconstructing through parsimony the genus-level boundaries recognised by traditional 20th Century taxonomy. They raise fresh doubts concerning whether *Pseudorchis* is sister to *Platanthera* or to *Dactylorhiza* plus *Gymnadenia*. Constraining the morphological matrix to the topology derived from ITS sequences increased tree length by 20%, adding considerably to the already exceptional level of phenotypic homoplasy. Both molecular and morphological trees agree that *D. viridis* and *D. iberica* are the earliest-diverging species within *Dactylorhiza* (emphasising the redundancy of the former genus *Coeloglossum*). Morphology and ITS both suggest that the former genus *Nigritella* is nested within (and thus part of) *Gymnadenia*, the Pyrenean endemic *'N.' gabasiana* apparently forming a molecular bridge between the two radically contrasting core phenotypes. Comparatively short subtending molecular branches plus widespread (though sporadic) hybridisation indicate that *Dactylorhiza* and *Gymnadenia* approximate the minimum level of molecular divergence acceptable in sister genera. They share similar tuber morphologies and base chromosome numbers, and both genera are unusually prone to polyploid speciation. Another prominent feature of multiple speciation events within *Gymnadenia* is floral paedomorphosis. The 'traditional' morphological and candidate-gene approaches to phylogeny reconstruction are critically appraised.

Key Words. character evolution, cladistics, *Dactylorhiza*, genus circumscription, *Gymnadenia*, Internal Transcribed Spacer, morphology, phylogeny, speciation, species circumscription, *trnL-F*.

Introduction

Subtribe Orchidinae s.s. (i.e. excluding the still poorly resolved subtribe Habenariinae) dominates the Eurasian orchid flora, encompassing a considerable range of phenotypes (Fig. 1) and evolutionary mechanisms (reviewed by Bateman 2009, 2012a). This clade has, through the last two decades, been subjected to several molecular phylogenetic studies utilising many samples that together spanned the subtribe (e.g. Bateman *et al.* 2003; Inda *et al.* 2012; Jin *et al.* 2014; Tang *et al.* 2015; Jin *et al.* 2017). Several genera within the subtribe have also been subjected individually to more detailed molecular phylogenetic examination; these include *Ophrys* (Soliva & Widmer 2003; Devey *et al.* 2008; Breitkopf *et al.* 2015; Bateman *et al.* 2018), *Serapias* (Bellusci *et al.* 2008), *Himantoglossum* s.l. (Sramkó *et al.* 2014), *Orchis* s.s. (Tyteca *et al.* 2012), *Platanthera* (Hapeman & Inoue 1997; Bateman *et al.* 2009), *Hemipilia* s.l. (Jin *et al.* 2014; Tang *et al.* 2015), *Dactylorhiza* (Devos *et al.* 2006a; Pillon *et al.* 2007; Hedrén *et al.* 2011) and *Gymnadenia* (Bateman *et al.* 2006; Stark *et al.* 2011; Efimov 2013; Sun *et al.* 2015; Hedrén *et al.* 2018).

This study is focused at an intermediate taxonomic level, seeking to clarify (a) genus-level relationships within the taper-tuber clade sensu Bateman *et al.* (2006) (i.e. those genera that do not rely entirely on roots emerging near-horizontally from the base of the stem, but also emit at least one large near-vertical root from the apex of the consequently tapered tuber:

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Fig. 1. **A** *Platanthera hyperborea* (Iceland); **B** *Galearis spectabilis* (Maryland, U.S.A.); **C** *Dactylorhiza incarnata* subsp. *pulchella* (England); **D** *D. viridis* (England); **E** *Gymnadenia* (*Nigritella*) *austriaca* (left), *G. conopsea* s.s. (centre), *G. odoratissima* (right) (co-occurring in the Italian Dolomites); **F** *D. romana* (yellow-flowered morph, left), *Orchis provincialis* (right) (Sicily); **G** *G. densiflora* (U.K.); **H** *G. borealis* (England); **J** *G. frivaldii* (N Greece); **K** *Pseudorchis albida* (Scotland). PHOTOS: RICHARD BATEMAN.

Platanthera, *Galearis*, *Neolindleya*, *Pseudorchis*, *Dactylorhiza*, *Gymnadenia*) and (b) species-level relationships (and the underlying causes of speciation) within *Dactylorhiza* s.l. (including the former genus *Coeloglossum*) and *Gymnadenia* s.l. (including the former genus *Nigritella*). These two genera have been consistently found to be sisters; they have been shown to share digitate tubers (i.e., those emitting more than one large root from the tuber apex) and a presumed chromosomal fusion event that converted $n = 21$ to $n = 20$ (Pridgeon *et al.* 1997; Bateman *et al.* 2003). These genera were segregated as subtribe Dactylorhizinae by Vermeulen (1977).

Trees based on Sanger sequencing of nuclear ribosomal ITS and the plastid region *trnL-F* are compared with trees generated via a morphological cladistic analysis spanning the subtribe, with the dual key objectives of (a) comparing these contrasting phylogenetic signals and (b) elucidating the sequence of acquisition of phenotypic characters within the taper-tubered clade in general and the digitate-tubered clade in particular. In addition to

Table 1. Details of accessions sequenced for molecular phylogenetic analysis.

Species	Locality	Publication	RB no.	Ribotype	Haplotype
Platanthera bifolia (L.) Rich.	Loch a Mhuilinn, Applecross, Wester Ross	Pridgeon *et al.* 1997	62		Present study
Platanthera micrantha Schltr. A	E+N Lagoa do Canario, SE Sete Cidades, Sao Miguel	Bateman *et al.* 2015	2694		
Pseudorchis straminea (Fernald) Soják		Bateman *et al.* 2003			Present study
Pseudorchis albida (L.) Á. Löve & D. Löve s.s.	Balvattan Hill, SE Aviemore, Rothiemurchus, U.K.	Pridgeon *et al.* 1997	63		Present study
Dactylorhiza iberica (M. Bieb. Ex Willd.) Soó	Cyprus	Pillon *et al.* 2006, 2007	Chase O-960	DQ022878	
Dactylorhiza euxina (Nevski) Czerep.	Turkey	Pillon *et al.* 2006, 2007		DQ022886	
Dactylorhiza cf. *hatagirea* (D. Don) Soó	Tibetan Plateau, China	Bateman *et al.* 2003	191		
Dactylorhiza incarnata (L.) Soó subsp. *pulchella* (Druce) Soó	Loch Kernsary (E), E Poolewe, Wester Ross, U.K.	Pridgeon *et al.* 1997	56		Present study
Dactylorhiza viridis (L.) R. M. Bateman, Pridgeon & M. W. Chase		Pridgeon *et al.* 1997	Chase O-576		Present study
Dactylorhiza viridis	Sichuan, China	Tang *et al.* 2015		KM651262	KM651588
Dactylorhiza viridis			???		GQ244815
Dactylorhiza aristata (Fisch. ex Lindl.) Soó		Pridgeon *et al.* 1997	366	DQ022872	
Dactylorhiza romana (Sebast.) Soó		Pridgeon *et al.* 1997			Present study
Dactylorhiza sambucina (L.) Soó s.s.	Col du Sarennes, French Alps	Bateman *et al.* 2003	112		Present study
Dactylorhiza foliosa (Soland. ex Lowe) Soó	Madeira	Pridgeon *et al.* 1997	Chase O-537	DQ022866	
Dactylorhiza fuchsii (Druce) Soó		Pridgeon *et al.* 1997	Chase O-1123		Present study
Dactylorhiza saccifera (Brongn.) Soó	Kastoria, N Greece	Bateman *et al.* 2003	172		Present study
Dactylorhiza majalis (Rchb.) P. F. Hunt & Summerh.	?Belgium	Devos *et al.* 2006b		DQ074217	
Dactylorhiza cordigera (Fr.) Soó	Hairpins imm. S Vitsi Ski, Kastoria-Florina rd, NW Greece	Present study	3761		Present study
Gymnadenia conopsea (L.) R. Br. s.s.	Solzan, Irkutsk, Russia	Efimov 2013		JQ768208	
Gymnadenia conopsea s.s.	Vayda Mt., Sakhlain Island, Russia	Efimov 2013		JQ768209	
Gymnadenia conopsea s.s.	Kilmovo, Leningrad, Russia	Efimov 2013		JQ768199	
Gymnadenia odoratissima (L.) Rich.)	Woods 3 km NE Scharnitz, NW Innsbruck, Austria	Bateman *et al.* 2003	138		
Gymnadenia odoratissima	Vallunga Reserve, NE Selva, SE Brixen, Italy	Present study	149		
Gymnadenia odoratissima	Near Schladming, S Dachstein, SE Salzburg, Austria	Present study	163		
Gymnadenia odoratissima	Gramais-Rosskarsee, Bavarian Alps, Germany	Present study	179		
Gymnadenia odoratissima A	Salvagny, Hautes Savoie, French Alps	Present study	174		
Gymnadenia odoratissima B	Salvagny, Hautes Savoie, French Alps	Present study	175		
Gymnadenia cf. *conopsea* s.s.	West Balkan Mts, Bulgaria	Bateman *et al.* 2006	868		
Gymnadenia conopsea s.l.	Pendelfos Pass, N Greece	Bateman *et al.* 2006	882		
Gymnadenia conopsea ?s.s.	Woods 3 km NE Scharnitz, NW Innsbruck, Austria	Present study	137		
Gymnadenia conopsea s.s.		Pridgeon *et al.* 1997	Chase O-574?		
Gymnadenia conopsea s.s.	Morgans Hill, SE Calne, Wilts, U.K.	Present study	1032		
Gymnadenia conopsea s.s.	Anga hay meadow, N Kraklingbo, EC Gotland, Sweden	Present study	3456		
Gymnadenia conopsea s.s.	Kallgateburgs reserve, ENE Hejnum, NE Visby, Gotland	Present study	3480		
Gymnadenia conopsea	Lone Robinia meadow, S Chamaloc, SE Vercors, France	Present study	1725		
Gymnadenia conopsea s.s.	Vallunga Reserve, NE Selva, SE Brixen, Italy	Present study	148		Present study
Gymnadenia conopsea s.s.	S Germany	Stark *et al.* 2011	Gc17	JF414033	
Gymnadenia conopsea s.s.	[N+E Germany]	Stark *et al.* 2011	Gc12	JF414028	
Gymnadenia conopsea s.s.	S Germany	Stark *et al.* 2011	Gc15	JF414031	
Gymnadenia cf. *densiflora* A. Dietr.	Vitosha Mountain, Bulgaria	Bateman *et al.* 2006	869		
Gymnadenia densiflora	Col del Canto, Pyrenees, Spain	Present study	3076		
Gymnadenia borealis (Druce) R. M. Bateman, Pridgeon & M. W. Chase A	Heath E Wych Cross, Ashdown Forest, Sussex, U.K.	Present study	3587		
Gymnadenia cf. *borealis* A	Lille Sandvatn, Glomfjellet, Meloy, Norway	Present study	2371		
Gymnadenia cf. *borealis*	Gangvatnet, Straumoya, Bodo, Norway	Present study	2373		

Gymnadenia orchidis Lindl.	(W?) China	Bateman *et al.* 2003	Luo 078		Present study
Gymnadenia orchidis	Sichuan, China	Tang *et al.* 2015	Tang 144	KM651267	KM651593
Gymnadenia crassinervis Finet	(W?) China	Pillon *et al.* 2006, 2007	Luo 705	DQ022890	
Gymnadenia 'conopsea'	(W?) China	Pillon *et al.* 2006, 2007		DQ022891	
Gymnadenia borealis	Loch a Mhuilinn, Applecross, Wester Ross, U.K.	Pridgeon *et al.* 1997	64		Present study
Gymnadenia borealis	Orton pastures, N Tebay, Cumbria, U.K.	Present study	3334		
Gymnadenia borealis	Dumbrock Loch, Mugdock Park, Milngavie, N Glasgow, U.K.	Present study	166		Present study
Gymnadenia borealis	N shore Loch Kernsary, E Poolewe, Wester Ross, U.K.	Present study	1866		
Gymnadenia borealis B	Heath E Wych Cross, Ashdown Forest, Sussex, U.K.	Present study	3588		
Gymnadenia frivaldii Hampe ex Griseb.	Pirin Mountain, Vihrenheit, Bulgaria	Bateman *et al.* 2006	867		
Gymnadenia frivaldii	Kaimaktealan, N Greece	Bateman *et al.* 2006	878		
Gymnadenia frivaldii	Smolikas, N Greece	Present study	879		
Gymnadenia frivaldii	Mount Vitsi, Kastoria, NC Greece	Present study	3589		
Gymnadenia densiflora	NE Blair Atholl, NW Pitlochry, Tayside, U.K.	Bateman *et al.* 2003	165		
Gymnadenia densiflora	Chippenham Fen, Cambs, U.K.	Present study	865		
Gymnadenia densiflora	Deegerty (Farrell) Fen, W Rathkeale, W Limerick, Eire	Present study	2315		
Gymnadenia densiflora	Outrediere, Hautes Savoie, French Alps	Present study	173		
Gymnadenia densiflora	Near Schladming, S Dachstein, SE Salzburg, Austria	Present study	162		
Gymnadenia cf. *densiflora* A	Ditchling Beacon, E Sussex, U.K.	Present study	3610		
Gymnadenia cf. *densiflora* A	Heyshott Down, SE Midhurst, W Sussex	Present study	3613		
Gymnadenia densiflora	Glyadino, Leningrad, Russia	Efimov 2013		JQ768211	
Gymnadenia densiflora	Lopushinka, Pskov, Russia	Efimov 2013		JQ768215	
Gymnadenia densiflora	E Germany	Stark *et al.* 2011	Gd4	JF414020	
Gymnadenia densiflora	[S+E Germany, Austria, Sweden]	Stark *et al.* 2011	Gd3	JF414019	
Gymnadenia densiflora friesica B	Kenfig Dunes, W Glamorgan, U.K.	Present study	3609		
Gymnadenia densiflora friesica A	Kenfig Dunes, W Glamorgan, U.K.	Present study	3608		
Gymnadenia gabasiana (Teppner & E. Klein) Teppner & E. Klein	Gabas, Pyrenees, France	Present study	3067		
Gymnadenia lithopolitanica (Ravnik) Teppner & E. Klein	Nr top of Mt Krvavec, N Ljubjana, Slovenia	Present study	1278		
Gymnadenia cf. *rhellicani* (Teppner & E. Klein) Teppner & E. Klein	Imm. E Groedner Joch, E Selva, SE Brixen, Italy	Present study	153		
Gymnadenia rubra Wettst.	Imm. E Groedner Joch, E Selva, SE Brixen, Italy	Bateman *et al.* 2003	154		
Gymnadenia rubra	NW slope Goldenknopf, S Compatsch, SE Brixen, Italy	Present study	158		
Gymnadenia nigra Rchb. f. s.s.	Sweden	Bateman *et al.* 2003	Hed 97-322		
Gymnadenia austriaca (Teppner & E. Klein) P. Delforge	Col du Menee, French Alps	Pridgeon *et al.* 1997	67		
Gymnadenia austriaca	Footpath, Hahntennjoch to Steinjoechl, Bavarian Alps	Present study	180		
Gymnadenia austriaca	Imm. NW junction A242–3, SE Selva, SE Brixen, Italy	Present study	150		
Gymnadenia austriaca	Imm. E Groedner Joch, E Selva, SE Brixen, Italy	Present study	152		
Gymnadenia austriaca	NW slope Goldenknopf, S Compatsch, SE Brixen, Italy	Present study	157		
Trnl–f only					
Gymnadenia nigra		Pornon *et al.* 2016			KU974016
Dactylorhiza viridis	N Norway	Soininen *et al.* 2009			GQ244815
Gymnadenia densiflora 1	St Moritz, Switzerland	Soliva & Widmer 1999			AF105324
Gymnadenia densiflora 2	St Moritz, Switzerland	Soliva & Widmer 1999			AF105325
Gymnadenia odoratissima 1	Davos, Switzerland	Soliva & Widmer 1999			AF105326
Gymnadenia odoratissima 2	Davos, Switzerland	Soliva & Widmer 1999			AF105327

further exploring pattern and process within this particular clade of orchids, this study constitutes an introspective re-examination of the strengths and weaknesses evident in what has become a traditional — arguably even passé — approach to phylogeny reconstruction.

Materials and Methods

Fieldwork directed toward this study occurred sporadically across the northern hemisphere from 1996 onward (RB, ID, PR and several associates), collecting samples in the now ubiquitous silica gel sachets. Laboratory work took place in three separate phases: in the late 1990s at RBG Edinburgh (RB, PH, MH), in the early 2000s at NHM London (RB, K. James: Bateman *et al.* 2006), and in 2017 at RBG Kew (RB, PR, AM); some details of the earlier phases of laboratory analyses have consequently been lost. Samples that yielded novel DNA sequences generated specifically for the present project are summarised in Table 1.

Data generation: nuclear ITS

The complete nuclear ribosomal ITS region was amplified using primers modified from White *et al.* (1990): "ITS 5p" (5'–GGAAGGAGAAGTCGTAACAAG) and "ITS 4p" (5'–TCCTCCGCTTATTGATATGC). PCR reactions of 50 µl contained 2 µl DNA template, 100 µM of each dNTP, 0.3 µM of each primer, 2 units Taq polymerase (Bioline), 2 µM $MgCl_2$ and 5 µl reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris HCl, 0.1% Tween 20, pH 8.8). The following PCR protocol was used: 1 cycle at 94°C for 3 minutes; 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 90 sec; 1 cycle at 72°C for 5 min. The resulting PCR products were purified using Qiagen PCR purification kits according to the manufacturer's instructions. Sequencing PCR was performed using Thermosequenase II TM dye terminator sequencing premix kit (Amersham Pharmacia, UK) according to the manufacturer's recommendations. Sequencing reactions were run on an ABI 377 automated sequencer and the output files were edited using Sequence Navigator (Applied Biosystems Inc.).

Data generation: plastid *trnL-F*

DNA was extracted from single bracts using a protocol modified from Doyle & Doyle (1990). A ground-glass rod attached to a domestic power drill was used to homogenise samples in 2× CTAB buffer (pre-heated to 65°C), with 0.2 % β-mercaptoethanol along with a pinch of polyvinylpolypyrrolidone (PVPP) and acid-washed sand. Samples were incubated at 65°C for 60 min. An equal volume of 24: 1 chloroform/isoamyl alcohol was then added and the samples were spun for 10 min at 13,000 rpm in a microfuge. The supernatant was removed and the chloroform/isoamyl alcohol step repeated. Following centrifugation, the supernatant was decanted, and the DNA precipitated by the addition of two-thirds volume freezer-cold isopropanol. The samples were then centrifuged at 13,000 rpm for 10 min to collect the resulting pellet. Finally, the isopropanol was decanted, and after air-drying for 20 min, the DNA was re-suspended in 300 µL of tris-ethylenediaminetetraacetic acid (TE) prior to sequencing, which encompassed the *trnL* intron, *trnL-F* intergenic spacer and the short intervening exon.

Data generation: Morphology

The morphological cladistic matrix was compiled from a wide range of sources with the primary aim of describing all aspects of the plants' phenotype that could realistically be summarised as qualitative (bistate or multistate) rather than quantitative (metric or meristic) characters. When selecting characters, no prior consideration was given to the likelihood of encountering extensive homoplasy, although character selection did attempt to minimise the risk of character duplication caused by underlying pleiotropy. The range of coded taxa was selected to broadly correspond with that — despite the near-typological sampling — represented in the corresponding ITS matrix. However, once it became evident that the outgroup genera selected initially (two species of *Platanthera* s.l., one each of *Galearis* and *Neolindleya*) did not resolve as monophyletic in the morphological tree, we then scored two additional, more phylogenetically distant outgroups (*Orchis*, *Hemipilia* s.l.).

Character states were obtained from the extensive literature (Vermeulen 1972; Luer 1975; Landwehr 1977; Strack *et al.* 1989; Luo & Chen 2000; Barone-Lumaga *et al.* 2006; Bateman *et al.* 2006; Delforge 2006; Box *et al.* 2008; Gamarra *et al.* 2008; Bateman *et al.* 2009; Bell *et al.* 2009; Chen *et al.* 2009; Efimov *et al.* 2009; Claessens & Kleynen 2011; Bateman *et al.* 2015; Gamarra *et al.* 2015; Tang *et al.* 2015; Bateman & Rudall 2018), liberally supplemented with our own observations based on the study of living plants, dried specimens held in the RBG Kew herbarium, and scanning electron microscopy performed in Kew's Jodrell Laboratory (Fig. 2).

Preparation for SEM involved selecting flowers from each preserved inflorescence for dehydration through an alcohol series to 100% ethanol. They were then stabilised using an Autosamdri 815B critical-point drier, mounted onto stubs using double-sided adhesive tape, coated with platinum using an Emtech K550X sputter-coater, and examined under a Hitachi cold-field emission SEM S-4700-II at 2 kV. The resulting images were recorded digitally for subsequent enhancement in Adobe Photoshop.

After considerable experimentation, 51 morphological characters were scored for 27 coded taxa, thereby generating a matrix of 1,377 cells. The characters (listed in Table 2) encompassed cytology, breeding system, and the morphology of every organ of the plant, the 13 vegetative

characters being numerically subordinate to 35 floral characters. A total of 47 cells (3.4%), distributed among 14 of the 51 characters, were coded as polymorphic (i.e. containing two or more character states). The most severely affected characters were labellum colour (C36: 10 of the 27 cells), leaf number (C10: 7 cells) and leaf arrangement along the stem (C13: 7 cells). No organ was represented by fewer than two characters. A further 133 cells (9.6%) were coded as missing, including a few cells in which the character was inapplicable for that species. In this case, the most severely affected characters were all micromorphological: spur papilla shape (C26: 18 cells), pollen exine surface sculpture (C51: 16 cells), spur interior striations (C24: 14 cells) and labellum adaxial epidermal cell morphology (C38: 14 cells). These figures are significant because the influence on tree building of any character containing more than approximately one-third polymorphic or missing values is considerably reduced.

Tree-building: ITS

Data for both the ITS and *trnL-F* were entered as Fasta files into MacClade v4.05 (Maddison & Maddison 2002), aligned by eye, and each of the respective resulting matrices was subjected to parsimony analyses in PAUP 4.0b10 (Swofford 2003), supported by GTR maximum likelihood (ML) analyses. Parsimony analyses were conducted as heuristic searches under TBR branch swapping and the amb–collapse criterion. Subsequent bootstrap analyses involved 1,000 replicates for *trnL-F* and 500 replicates for ITS for parsimony analyses, but was reduced to 100 replicates for both matrices in the more time-consuming ML analyses. Gaps were treated as missing, the less ambiguous among the apparent indels being coded as independent characters largely following the "simple coding" logic of Simmons & Ochoterena (2000).

Our initial ITS matrix combined 83 sequences generated for the present study or its direct predecessors (Pridgeon *et al.* 1997; Bateman *et al.* 2003; Bateman *et al.* 2006; Pillon *et al.* 2006; Pillon *et al.* 2007; Bateman *et al.* 2015; Tang *et al.* 2015) with a smaller number of sequences downloaded from studies published by several other research groups (Soliva & Widmer 1999; Stark *et al.* 2011; Jin *et al.* 2012; Efimov 2013; Sonkoly *et al.* 2016). The accumulated matrix of 129 sequences was then pared down to 46 representative ribotypes that exhibited non-polymorphic differences in nucleotides or indels, plus one sequence from Gotland showing several polymorphisms that clearly demonstrated the presence of two ribotypes that presumably reflect recent hybridisation between two *Gymnadenia* species. (Note: we are aware that the term "ribotype" was originally coined to describe the less complex ITS sequences of prokaryotes; nonetheless, we find it a useful shorthand across all the biotic kingdoms for "nrITS sequence variant".)

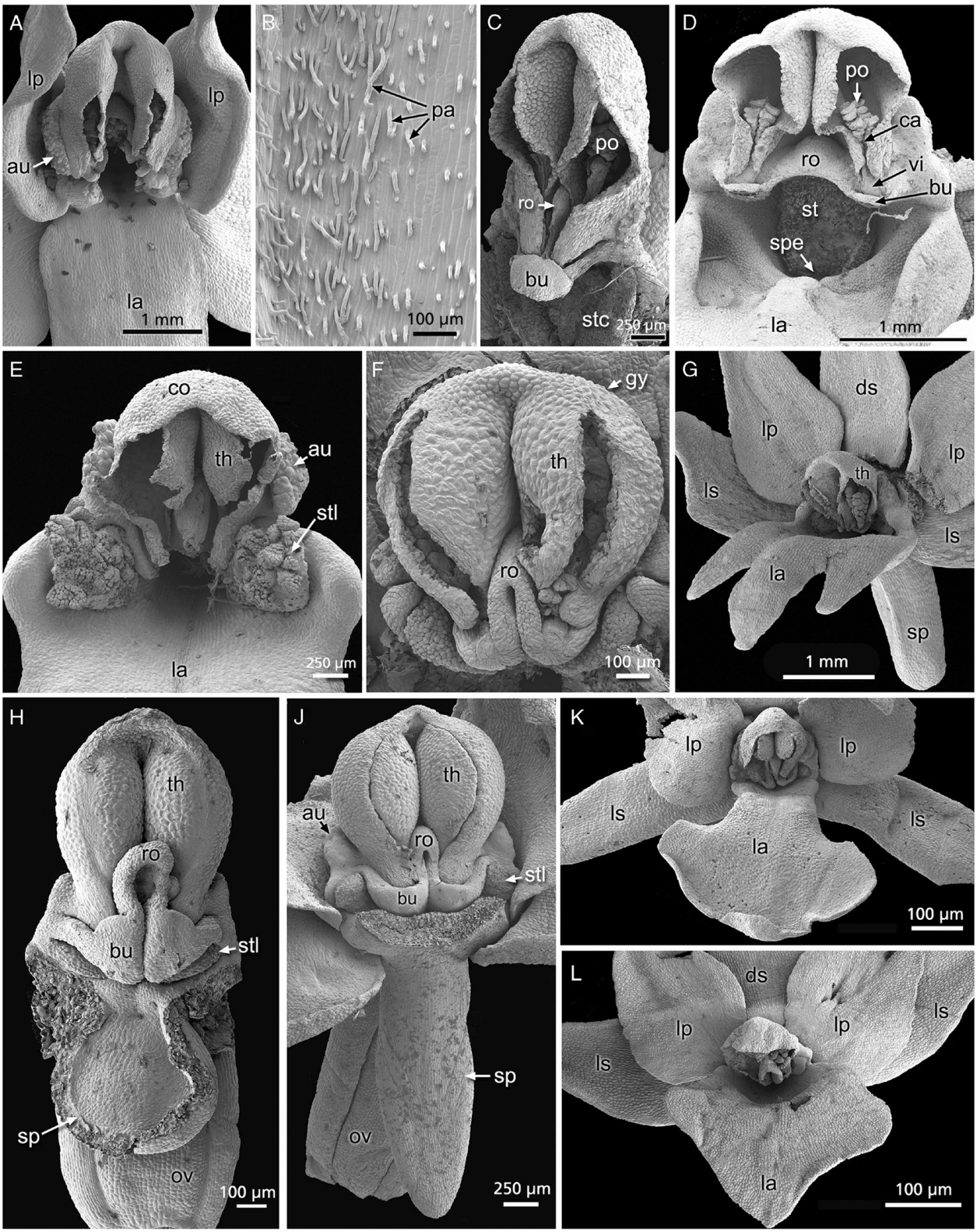
Fig. 2. Scanning electron micrographs of representative Orchidinae flowers (most are open flowers, although H and J are dissected flower buds). **A** *Platanthera bifolia*, gynostemium and proximal portion of labellum, featuring spur opening and lateral auricles; **B** *P. bifolia*, inside of spur, showing elongate papillae; **C** *Dactylorhiza iberica*, gynostemium, showing prominent bursicle, vertical rostellum and expansive thecae; **D** *D. viridis*, gynostemium and proximal portion of labellum, showing spur opening, suprajacent stigma, broad gynostemium and paired viscidia; **E** *Gymnadenia densiflora*, gynostemium and proximal portion of labellum, showing spur opening and lateral stigmatic surfaces encrusted in pollinium massulae; **F** *Pseudorchis albida*, showing compact gynostemium; **G** *Ps. straminea*, flower illustrating petals and sepals; **H** *Gymnadenia* (*Nigritella*) *austriaca*, immature compact gynostemium and longitudinally sectioned spur lacking papillae; **J** *G. densiflora*, immature gynostemium with labellum removed to reveal developing elongate spur; **K** *G. conopsea*, flower illustrating petals and sepals; **L** *G. frivaldii*, flower illustrating petals (including comparatively undifferentiated labellum) and sepals, paedomorphic relative to **K**; Labels: **au** auricle; **bu** bursicle; **ca** caudicle; **co** connective; **ds** dorsal sepal; **gy** gynostemium; **la** labellum; **ls** lateral sepal; **lp** lateral petal; **ov** ovary; **po** pollinium; **ro** rostellum; **sc** cordate stigma; **spe** spur entrance; **stl** lateral stigma lobe (bilobed stigma 'lappets'); **sp** labellar spur; **th** theca; **vi** viscidium. MICROGRAPHS: PAULA RUDALL.

Tree-building: *trnL-F*

Most of the 45 *trnL-F* sequences initially collated were generated specifically for the present study at Royal Botanic Garden Edinburgh, although eight were downloaded from GenBank (between one and four sequences each derived from the studies of Soliva & Widmer 1999; Soininen *et al.* 2009; Tang *et al.* 2015; Pornon *et al.* 2016). The overall alignment of the matrix required 712 base positions plus a further 20 indels. Two sequences lacked the initial 168 bp (Chinese samples of *Gymnadenia orchidis* and *Dactylorhiza viridis*) and one sequence lacked the terminal 138 bp (*Pseudorchis straminea*). One phylogenetically important species present in the ITS matrix, *G. gabasiana*, was absent from the *trnL-F* matrix, wherein the many controversial allotetraploid *Dactylorhiza* species were represented as a placeholder by *D. cordigera* rather than *D. majalis*. The 45 available sequences were pared down to 21 core sequences that differed in SNPs and/or indels; these 21 haplotypes formed the basis of subsequent tree-building, after setting aside a poly-A region located near the 5' end of the *trnL* intron (first reported by Soliva & Widmer 1999) that varied in length from 9 bp to 18 bp and failed to correlate with any credible taxonomy.

Tree building: morphology

The data presented as Table 3 — including polymorphic cells — were entered into MacClade v4.05 (Maddison & Maddison 2002) and analysed in PAUP 4.0b10 (Swofford 2003) using both parsimony and the widely used (if frequently criticised) phenetic algorithms neighbour joining (NJ) and unweighted pair group method with



arithmetic mean (UPGMA). Parsimony analyses were conducted as heuristic searches under the amb– collapse criterion and Acctran optimisation. The subsequent bootstrap analysis involved 1,000 replicates.

Table 2. Characters scored for morphological cladistic analysis.

1. Chromosome number (*n*) — 21 (0) : 20 (1) : 18 or 19 (2).
2. Ploidy base level (*x*) — 2 (0) : 3 (1) : 4 or 6 (2).
3. Breeding system — allogamous (0) : facultatively autogamous (1).
4. Tuber distal division — radially symmetrical (divided to base) (0) : bilaterally symmetrical, digitate (1).
5. Tuber diameter — wide, robust (0) : narrow, filiform (1).
6. Tuber distal root — absent (0) : present (1).
7. Stoloniferous growth — absent (0) : present (1).
8. Stem architecture — solid or narrow central cavity (0) : broad central cavity (1).
9. Stem sheath — absent (0) : present (1).
10. Leaf number (excluding bracteoidal leaves) — 1 – 2 (0) : 3 – 4 (1) : 5 – 7 (2) : >7 (3).
11. Leaf width — broad (0) : narrow (1).
12. Leaf arrangement viewed vertically — distichous (0) : more-or-less whorled (1).
13. Leaf arrangement viewed laterally — distributed along lower part of stem (0) : concentrated in basal rosette (1).
14. Leaf texture — robust and clearly keeled (0) : flexible and obscurely keeled (1) : flexible and multiply ribbed rather than keeled (2).
15. Leaf margin — straight (0) : regularly undulating (1).
16. Leaf purple spots — absent (0) : present (in most plants) (1).
17. Pedicel — long (>20% of ovary) (0) : short (<20% of ovary) (1).
18. Ovary torsion — 180° resupinate (0) : non-resupinate (1).
19. Basal bracts — foliose, shorter than flowers (0) : foliose, slightly exceeding flowers (1), foliose, greatly exceeding flowers (2) : membranous (3).
20. Bract cells — non-papillate (0) : moderately papillate (1) : strongly papillate (2) : microserrate (3).
21. Inflorescence density — loose (0) : moderate (1) : dense (2).
22. Fragrance — absent (0) : slight (1) : strong (2).
23. Nectar — absent (0) : trace (1) : reservoir (2).
24. Spur interior striations — absent (0) : weak or moderate (1) : strong (2).
25. Spur interior papillae size — absent (0) : short or medium (1) : long (2).
26. Spur internal papillae shape — absent/cylindrical (0) : clavate (1) : multicellular (2).
27. Spur length — long (0) : medium (1) : short (2).
28. Spur diameter — narrow (0) : broad (1).
29. Spur curvature — downward (0) : straight (1) : upward (2).
30. Labellum dissection — entire (sepaloid) (0) : shallowly three-lobed (1) : deeply three-lobed, central lobe rounded (2) : deeply three-lobed, central node invaginated (3).
31. Labellum dimensions — approximately equidimensional (0) : length approximately 1.5 times width (1) : length more than twice width (2).
32. Labellum three-dimensionality — strongly concave (0) : slightly concave (1) : more-or-less planar or slightly convex (2) : strongly convex (3).
33. Labellum marginal serrations — absent (0) : present (1).
34. Labellum lateral constriction — absent or weak (0) : pronounced (1).
35. Labellum median ridge — absent (0) : present (1).
36. Labellum colour — white tinged yellow-green (0) : yellow-green (1) : pale pink orchicyanins (2) : moderate orchicyanins and ophrysanthins (3) : intense red orchicyanins (4) : brown orchicyanins (5).
37. Labellum markings — absent (0) : discrete dashes and/or loops (1).
38. Labellum adaxial epidermis — planar cells (0) : domed cells (1) : densely packed papillate cells (2).
39. Lateral sepal position — connate with median sepal (0) : spreading laterally (1) : spreading vertically, erect (2) : spreading vertically, patent and recurved (3).
40. Lateral petal position — connate with median sepal (0) : spreading (1).
41. Gynostemial auricles — absent (0) : subdued (1) : prominent (2).
42. Bursicles — absent (0) : single (shared by pair of viscidia) (1) : paired (2).
43. Pollinaria placement on gynostemium — proximal, near-parallel (0) : distal. upwardly convergent (1).
44. Pollinarium shape — caudicle short (<30% of length of pollinium) (0) : caudicle long (>30% of length of pollinium) (1).
45. Stigma lateral lobes — absent or contiguous with mid-lobe (0) : lappets spreading laterally (1) : lappets projecting forward (2).
46. Viscidium size — absent (0) : small or medium (1) : large (2).
47. Viscidium outline shape – absent (0) : approximately circular (1) elliptical or oblong (2).
48. Rostellar median fold — subdued (0) : prominent (1).
49. Seed testa shape — fusiform (0) : clavate (1).
50. Seed testa external ornamentation — smooth (0) : trabeculate (1) : reticulate (2).
51. Pollen exine surface sculpture — micro-psilate (0) : psilate (1) : reticulate (2).

Many of the terms used to describe floral characters are illustrated in Fig. 2.

Results

TrnL-F phylogeny

Of the 732 positions plus indels analysed for 21 haplotypes, only 59 were variable and only 22 (including 11 indels) were parsimony informative. The resulting single most-parsimonious tree (shown under Acctran optimisation as Fig. 3) was 74 steps long, yielding a consistency index of 0.816 (0.611 excluding uninformative characters) and a retention index of 0.823. The node separating *Gymnadenia orchidis* from the remaining *Gymnadenia* species

Table 3. Matrix used for morphological cladistic analyses of the taper-tubered clade.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
H.chusua	0	0&1	0	0	0	0	0	0	0	0	0	?	1	1	0	0	0
O. mascula	0	0	0	0	0	0	0	1	1	2	0	1	1	0	0	0&1	0
Ps. albida	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	1
Ne. camtschatica	2	0	1	0	1	1	0	1	0	2&3	0	1	0	2	1	0	0
Ga. spectabilis	0	0	0	0	1	1	1	0	0	0	0	?	1	0	0	0	0
P. hyperborea	0	0	1	0	0&1	1	0	1	0	1&2	0	1	0	0	0	0	1
P. bifolia	0	0	0	0	0	1	0	0	0	0	0	?	1	1	0	0	0
D. iberica	1	0	?	0	1	1	1	0	0	1	1	0	0	0	0	0	1
D. viridis	1	0	0	1	0	1	0	0	0	1	0	1	0&1	1	0	0	1
D. incarnata s.s.	1	0	1	1	0	1	0	1	0	1	0	0	0	0	0	0	0
D. euxina	1	0	0	1	0	1	0	1	0	1&2	0	0	0	0	0	0&1	0
D. aristata	1	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0&1	0
D. sambucina	1	0	0	1	0	1	0	1	0	2	0	0	1	0	0	0	0
D. majalis s.l.	1	1	0	1	0	1	0	1	0	1&2	0	0	0	0	0	0&1	0
D. foliosa	1	0	0	1	0	1	0	1	0	2&3	0	0	0	1	0	0	0
D. fuchsii	1	0	0	1	0	1	0	0	0	2	0	0	0&1	1	0	1	0
G. conopsea s.s.	1	0&2	0	1	0	1	0	0	0	2	1	0	0&1	0	0	0	0
G. odoratissima	1	0	0	1	0	1	0	0	0	2	1	1	0&1	0	0	0	0
G. borealis	1	0	0	1	0	1	0	0	0	1&2	1	0	0&1	0	0	0	0
G. orchidis	1	0	0	1	0	1	0	0	0	2	0	1	0&1	0	0	0	0
G. crassinervis	?	?	?	1	0	1	0	0	0	1&2	1	0	0&1	0	0	0	0
G. frivaldii	1	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	1
G. densiflora	1	0&2	0	1	0	1	0	0	0	2	1	0	0	0	0	0	0
G rhellicani	1	0	0	1	0	1	0	0	0	3	1	1	1	0	0	0	1
G. nigra s.s.	1	1	1	1	0	1	0	0	0	3	1	1	1	0	0	0	1
G. austriaca	1	2	1	1	0	1	0	0	0	3	1	1	1	0	0	0	1
G. miniata	1	2	1	1	0	1	0	0	0	3	1	1	1	0	0	0	1

Species	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
H. chusua	0	2	?	0	0	0	?	2	2	1	1	1	2	0	2	0	0
O. mascula	0	3	?	0&1	0	0	?	?	?	1	1	2	3	0	3	0	0
Ps. albida	0	0	0	1	2	2	1	0&1	0	1	0	0	1&2	0	1	0	0
Ne. camtschatica	0	2	3	1	?	0	0	0	0	1	0	0	1	2	2	0	0
Ga. spectabilis	0	2	3	0	0	0	1	0	0	1	1	0	0	1	2	1	0
P. hyperborea	0	1	3	1	0	2	0	2	1	1	0	0	0	2	2	0	0
P. bifolia	0	0	0	0	2	2	0	2	0	2	0	1	0	2	2	0	0
D. iberica	0	0	0	0	0	0	0	0	0	1	0	0	1	1	2	0	0
D. viridis	0	1	0	1	1	1	1	0	0	0	1	0	1	2	2	0	0
D. incarnata s.s.	0	1	0	1	0	0	?	1	?	1	1	0	0&1	0	3	0	0
D. euxina	0	1	?	1	0	0	?	?	?	1	1	0	0&1	0	2&3	1	0
D. aristata	0	2	0	1	0	0	?	1	?	1	1	1	0	0	1&2	0	0
D. sambucina	0	1	0	1	0	0	2	1	0	1	1	2	1	0	2	0	0
D. majalis s.l.	0	1	0	1	0	0	?	1	?	1	1	0	1	0	2	0	0
D. foliosa	0	0&1	0	0&1	0	0	?	0	0	1	0	0	1	0	2	0	0
D. fuchsii	0	0	0	1	0	0	1	2	0	1	0	0	2	0	2	0	0
G. conopsea s.s.	0	0	0	1	2	2	1	2	0	2	0	0	1	0	2	0	0
G. odoratissima	0	0	1	1	2	2	0	1	0	1	0	0	0&1	0	2	0	0
G. borealis	0	0	0	1	2	2	?	1	?	2	0	0	1	0	2	0	0
G. orchidis	0	0	1	1	2	2	?	1	?	2	0	0	1	0	2	0	0
G. crassinervis	0	1	?	0&1	?	2	?	?	?	1	0	0	1	0	2	0	0
G. frivaldii	0	0	0	2	2	2	0	0	0	1	0	0	0&1	0	1	0	0
G. densiflora	0	0	0	1	2	2	?	?	?	2	0	0	1	0	2	0	0
G rhellicani	1	0	2	2	2	1	?	?	?	0	0	0	0	1	0	0	0
G. nigra s.s.	1	0	1	2	2	1	?	?	?	0	0	0	0	1	0	0	0
G. austriaca	1	0	1	2	2	1	2	1	0	0	0	0	0	1	0	0	0
G. miniata	1	0	1	2	2	1	?	?	?	0	0	0	0	1	0	0	1

Species	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
H. chusua	0	2	1	?	2	0	2	2	0	1	0	2	2	0	?	?	?
O. mascula	0	3	1	?	2	0	2	1	0	1	0	1	1	1	1	0	1
Ps. albida	0	0	0	0	0	0	2	0	0	1	1	1	2	1	0	0	2
Ne. camtschatica	0	2&3	0&1	?	1	0	0	1	0	0	1	0	0	1	?	?	0
Ga. spectabilis	0	2	0	?	0	0	0	1	0	0	0	1	1	0	0	1	?
P. hyperborea	0	1	0	0	3	0	0	0	0	0	0	1	1	1	1	0	0
P. bifolia	0	0	0	0	1	0	2	0	1	0	2	2	2	0	0	1	0
D. iberica	1	2	1	2	0	0	1	1	0	0	0	?	?	0	?	?	2
D. viridis	1	1&5	0	1	0	0	2	2	1	0	0	1	1	0	0&1	0	2

Table 3 (Continued)

Species	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
D. incarnata s.s.	0	2&3	1	?	2	0	2	1	0	1	0	?	?	1	0	0	?
D. euxina	0	3	1	?	2	0	?	1	0	1	?	?	?	1	?	?	?
D. aristata	0	2&3	1	?	2	0	?	1	0	1	0	?	?	1	?	?	?
D. sambucina	0	1&3	1	1	2	0	2	1	0	1	0	?	1	1	0	2	1
D. majalis s.l.	0	2&3	1	?	2	0	?	1	0	1	0	?	?	1	0	1	?
D. foliosa	0	2&3	1	?	1	1	?	1	0	?	0	?	?	?	0	1	?
D. fuchsii	0	2&3	1	1	1	0	2	1	0	1	0	1	1	1	0	1	1&2
G. conopsea s.s.	0	3	0	1	1	0	2	0	0	1	1	2	2	1	1	0	2
G. odoratissima	0	2	0	1	1	0	2	0	0	1	1	2	2	1	1	0	?
G. borealis	0	3	0	1	1	0	2	0	0	1	1	?	?	1	?	?	?
G. orchidis	0	2&3	0	?	1	0	2	0	0	0	1	2	2	1	?	?	?
G. crassinervis	0	1&3	0	?	1	0	?	0	0	?	?	?	2	?	?	?	?
G. frivaldii	0	2	0	0	1	0	1	0	0	0	1	?	?	1	?	?	?
G. densiflora	0	3	0	1	1	0	2	0	0	1	1	?	?	1	1	0	?
G rhellicani	0	4	0	?	1	1	2	0	0	0	1	2	2	1	1	0	1
G. nigra s.s.	0	4	0	?	1	1	2	0	0	?	1	2	2	1	1	0	?
G. austriaca	0	4	0	1	1	1	2	0	0	?	1	2	2	1	1	0	?
G. miniata	0	4	0	?	1	1	2	0	0	0	1	2	2	1	1	0	?

reflected only a single homoplastic SNP and hence collapsed in the strict consensus tree (it was also polytomous in the equivalent likelihood tree: not shown). The three ingroup genera all received at least 68% bootstrap support in the parsimony tree, but the node that identified *Dactylorhiza* and *Gymnadenia* s.l. as sister genera relative to *Pseudorchis* received only 50% (and less than 50% in the equivalent ML tree), reflecting the presence of just one non-homoplastic indel. The greatest separation was evident between the outgroup *Platanthera* and the ingroup, the observed disparity consisting of eight SNPs plus four indels.

Much of the phylogenetic signal in *trnL-F* was provided by a 9 bp minisatellite region (typical motif ATAATAGTA) located midway through *trnL-F*; this occurred once in *Dactylorhiza*, *Pseudorchis* and (in a modified motif) in the *Platanthera* outgroup, twice or thrice in 'classic' *Gymnadenia*, but was absent from the former genus *Nigritella*. The apparent deletion event of this minisatellite from the former genus *Nigritella* and the apparent insertion event in the remainder of *Gymnadenia* were the only characters separating the two groups, thereby inevitably leading to their poorly-supported representation as sister groups (Fig. 3).

Little variation (and thus little resolution) was evident within any of the genera analysed. All members of the former genus *Nigritella* yielded identical sequences once the poly-A region had been excluded. *Gymnadenia orchidis* deviated from the remainder primarily in four indels, although it also possessed a "rogue" SNP that otherwise characterised both *Dactylorhiza* and *'Nigritella'*. Remarkably, a single haplotype characterised the majority of individuals analysed of each of the other species of 'core' *Gymnadenia* analysed (60% of *conopsea*, 63% of *odoratissima*, 100% of *borealis*, 60% of *densiflora*). Other haplotypes observed in few individuals of each species deviated from the core haplotype by only one or two autapomorphies (the majority of them indels: Fig. 3).

ITS phylogeny

Of the 660 positions plus 12 indels analysed for 47 ribotypes, 166 proved variable and 117 were parsimony informative (including seven indels). The resulting 179 most-parsimonious trees of length 251 yielded a consistency index of 0.783 (0.729 excluding uninformative characters) and a retention index of 0.908. A representative tree is shown as Fig. 4. Of the three nodes that collapsed in the 50% majority rule consensus tree, two had only trivial implications: that supporting the pairings of *Dactylorhiza fuchsii* plus *D. majalis* and that supporting the rare ribotypes VII and VIII of *Gymnadenia conopsea*. However, the third ambiguous node was of greater significance: it was equally parsimonious to place *G. frivaldii* as sister to *G. densiflora* (as in Fig. 4) or as sister to *G. borealis*. The equivalent likelihood tree (not shown) differed only in collapsing the node shared by the two species of *Pseudorchis* in the parsimony tree.

Few branches attracted more than 50% bootstrap support under parsimony criteria, and even fewer under likelihood. *Platanthera* was clearly an appropriate outgroup, and the monophyly of each of the three ingroup genera is supported with at least 71% bootstrap support. *Pseudorchis* is placed as sister to the two remaining ingroup genera with 100% support (Fig. 4). Sampling of *Dactylorhiza* was largely typological, only the especially phylogenetically controversial *D. viridis* being represented by multiple accessions. In contrast, comparatively intensive sampling of

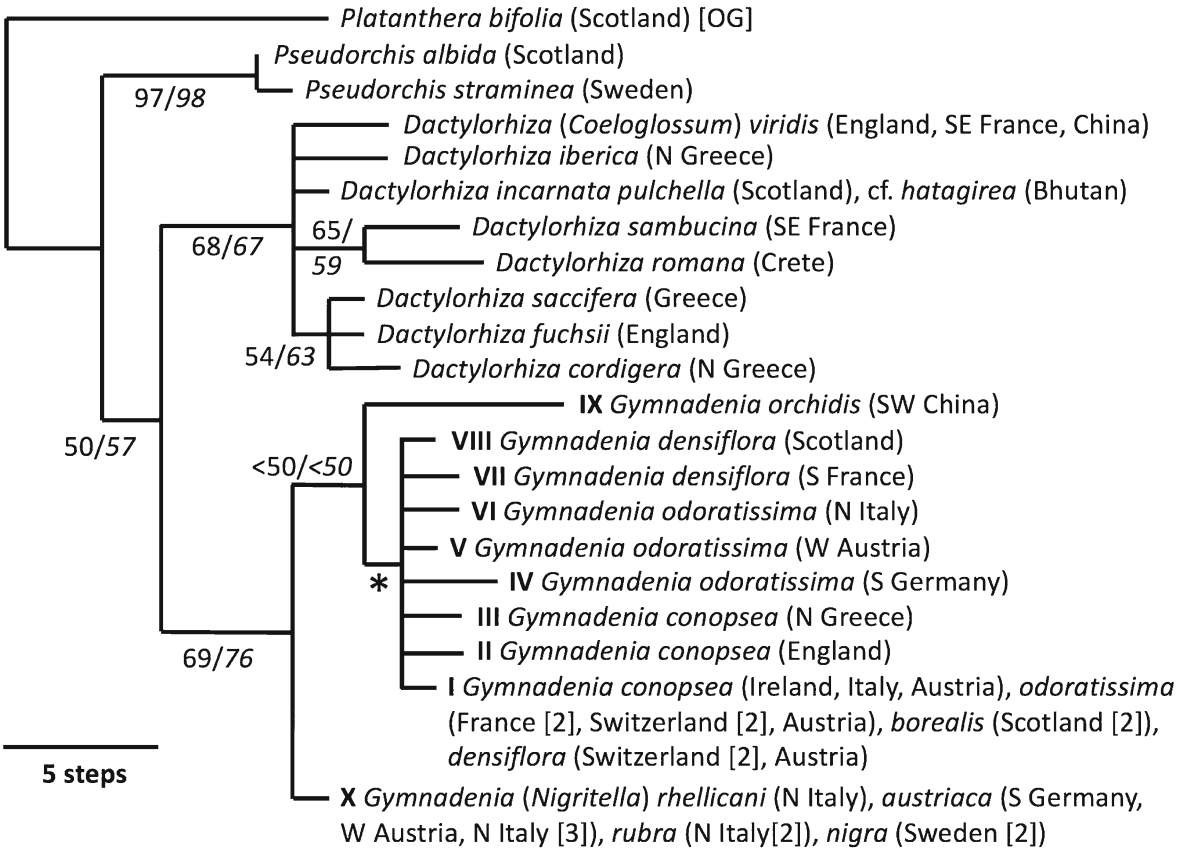


Fig. 3. Single most-parsimonious tree of the taper-tubered clade of subtribe Orchidinae, generated via maximum parsimony from the *trnL-F* (plastid) matrix. Acctran optimisation. Asterisk indicates the node that collapsed in the strict (and also majority rule) consensus tree. The six branches that achieved bootstrap support values exceeding 50% are indicated in roman script; equivalent bootstrap values obtained through ML analysis are italicised. Haplotypes within *Gymnadenia* s.l. are distinguished by roman numerals.

Gymnadenia s.l. identified a total of 30 ribotypes, representing 13 putative species. The six analysed species of the former genus *Nigritella* collectively yielded only two ribotypes, whereas nine putative ribotypes were found within *G. densiflora* alone and eight within *G. conopsea* s.s. Remarkably, three ribotypes were shared between the phenotypically distinct species *G. conopsea* and *G. odoratissima*.

Morphological phylogenies

Of the 51 coded characters analysed for 27 representative species of Orchidinae, all 51 were variable but only 46 were parsimony informative. The resulting 10 most-parsimonious trees of length 181 yielded a consistency index of 0.475 (0.457 excluding uninformative characters) and a retention index of 0.666. A representative tree is shown as Fig. 5. Only two comparatively unimportant nodes collapsed in the strict consensus tree: that linking *Dactylorhiza foliosa* with *D. fuchsii*, and that supporting a clade composed of *D. incarnata*, *D. euxina*, *D. aristata* and *D. majalis* (the allotetraploid derivative of *D. fuchsii* and *D. incarnata*). Despite the small number of most-parsimonious trees, the topological structure of the trees proved exceptionally fragile; only three nodes gained bootstrap values in excess of 50%, and only that underpinning the former genus *Nigritella* exceeded 75% (Fig. 5). The explanation for this topological weakness lies in the exceptionally low values for the retention index and especially the consistency index; only two individual character-state transitions were both non-homoplastic and non-autapomorphic (i.e. unambiguous synapomorphies): the absence of resupination throughout the former genus *Nigritella* (C18) and the shared presence of a central ridge on the labella of *D. iberica* and *D. viridis* (C35).

Neither the pair of *Platanthera* species nor the putative pairing of *Galearis* and *Neolindleya* species formed monophyletic groups (Fig. 5). Next to diverge was the pair of primitive *Dactylorhiza* species, *D. iberica* and *D. viridis*. The more derived species of *Dactylorhiza* emerged as sister to *Gymnadenia* s.l. (including the former genus *Nigritella*). However, *Orchis mascula* was embedded within *Dactylorhiza* as sister to *D. sambucina*, and *Ps. albida* was embedded within *Gymnadenia* as

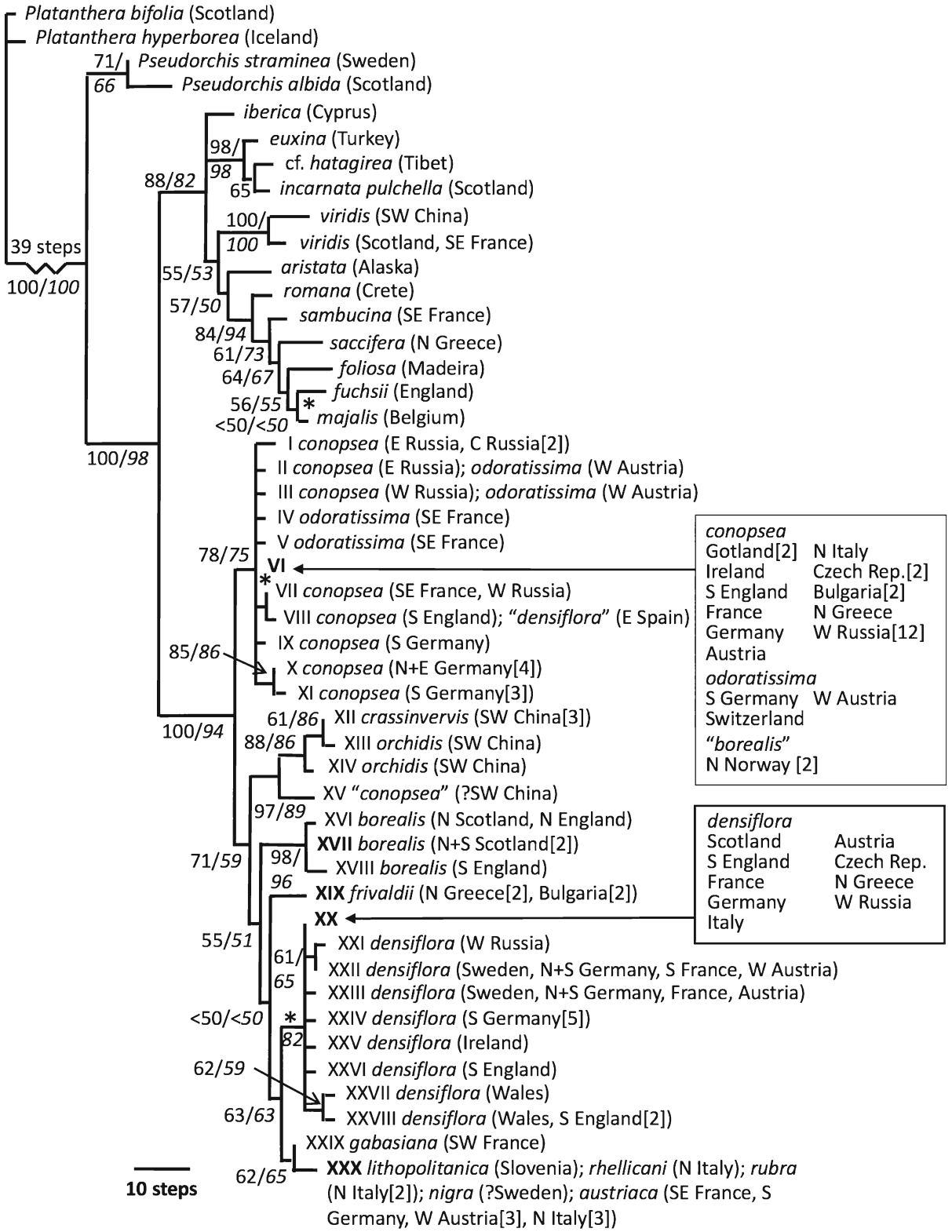


Fig. 4. Representative example of 179 maximum parsimony trees of the taper-tubered clade of subtribe Orchidinae, generated via maximum parsimony from the ITS (nuclear ribosomal) matrix. Acctran optimisation. Asterisks indicate nodes that collapsed in the strict (and also majority rule) consensus tree. The 26 branches that achieved bootstrap support values exceeding 50% are indicated in roman script; equivalent bootstrap values obtained through ML analysis are italicised. Ribotypes within *Gymnadenia* s.l. are distinguished by roman numerals (core ribotypes are emphasised in boldface), and the geographic origins of the relevant samples are also shown.

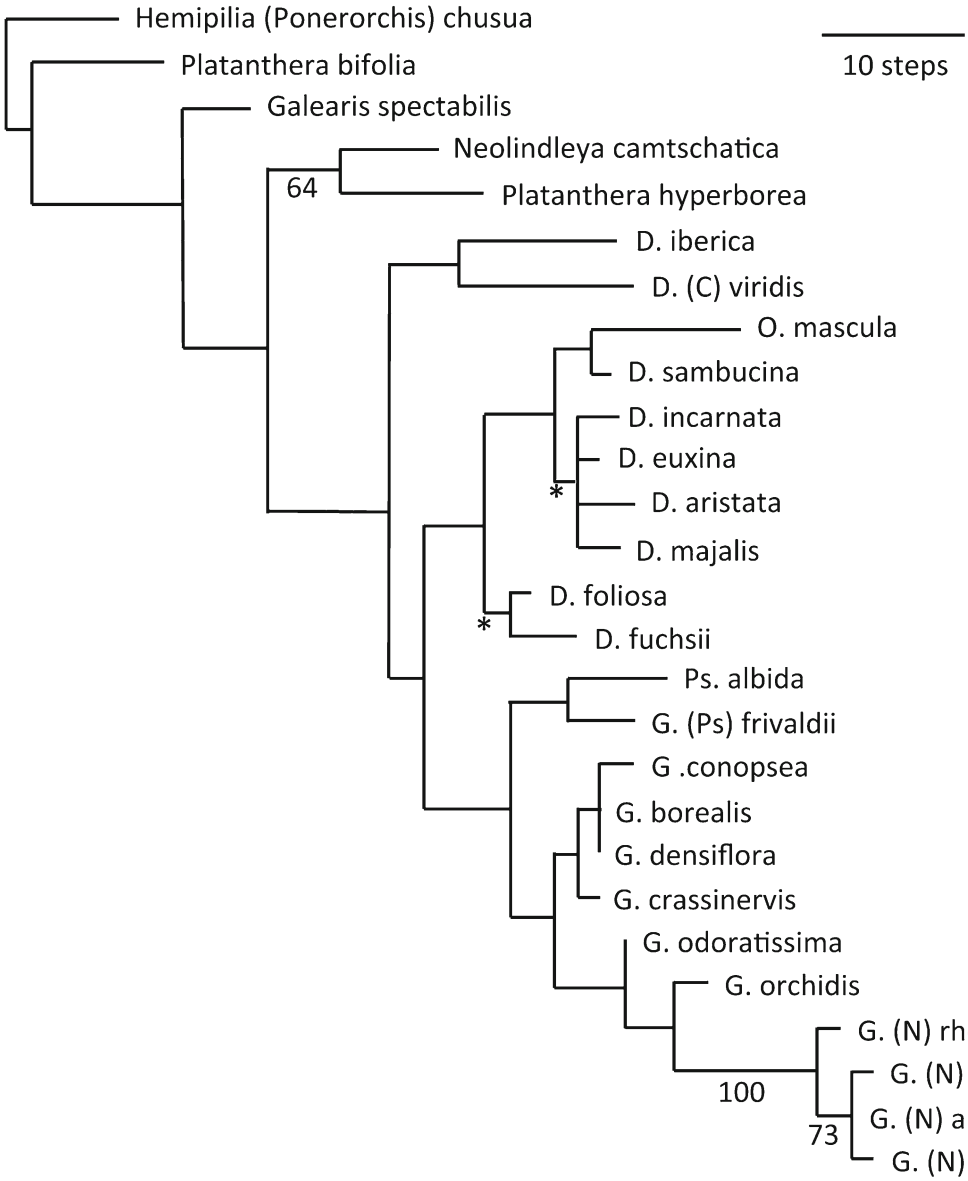


Fig. 5. Representative example of the 10 maximum parsimony trees of the taper-tubered clade of subtribe Orchidinae, generated from the morphological matrix given in Table 2. Acctran optimisation. Asterisks indicate nodes that collapsed in the strict (and also majority rule) consensus trees. The three branches that achieved bootstrap support values exceeding 50% are indicated.

sister to *G. frivaldii*. Within the more derived *Dactylorhiza* species, *D. fuchsii* and *D. foliosa* were separated from the remainder. Within *Gymnadenia*, *G. orchidis* was identified as sister to the deeply embedded former genus *Nigritella*.

Phenetic trees were also generated from the morphological matrix in PAUP using the NJ (Fig. 6A) and UPGMA (Fig. 6B) algorithms. Both deviated substantially from the most-parsimonious topologies. In the NJ tree, *Galearis spectabilis* was promoted to a position as sister to *Dactylorhiza iberica*, the pairing of *Platanthera hyperborea* and *Neolindleya camtschatica* diverged later than the core *Dactylorhiza* clade, *Pseudorchis* was placed as sister to *Gymnadenia*, and *G. frivaldii* replaced *G. orchidis* as sister to the former genus *Nigritella*. The UPGMA tree diverged most radically from the parsimony trees, yielding a highly improbable topology. Most notably, the former genus *Nigritella* diverged earliest, reflecting the large number of character-state transitions that occur on its subtending branch. The pairing of *Galearis spectabilis* and *D. iberica* was next to diverge, followed by *D. viridis* as an isolated lineage. *Orchis mascula* was shown as sister to the remainder of *Dactylorhiza*, and *Pseudorchis* was shown as sister to the non-nigritellan portion of *Gymnadenia* s.l.

Having obtained these diverse, weakly supported topologies, we decided to map the morphological cladistic matrix across the topology recovered from

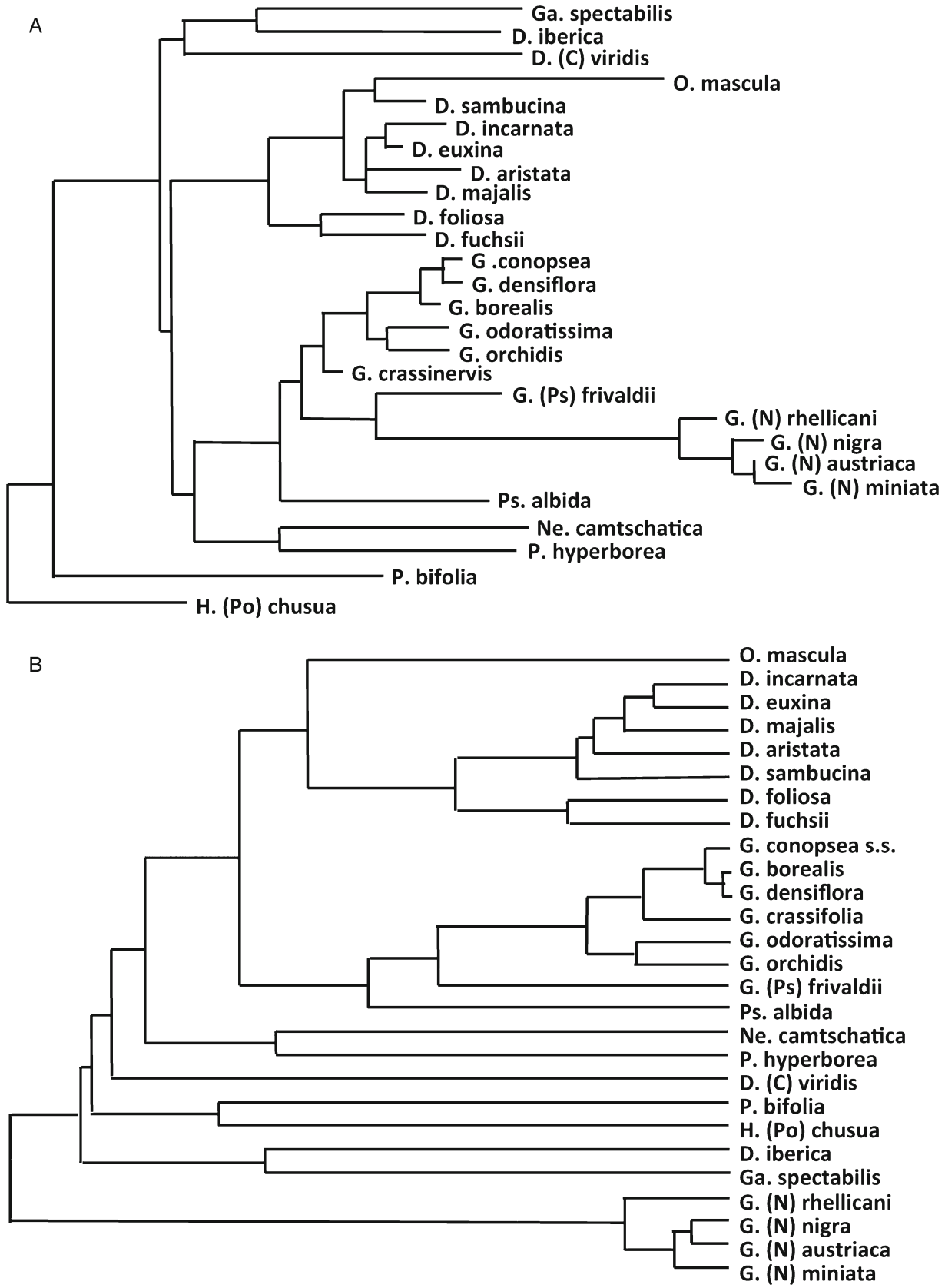


Fig. 6. Unique phenograms generated from the present morphological cladistic matrix using (A) Neighbour joining (NJ) and (B) unweighted pair-group method with arithmetic mean (UPGMA).

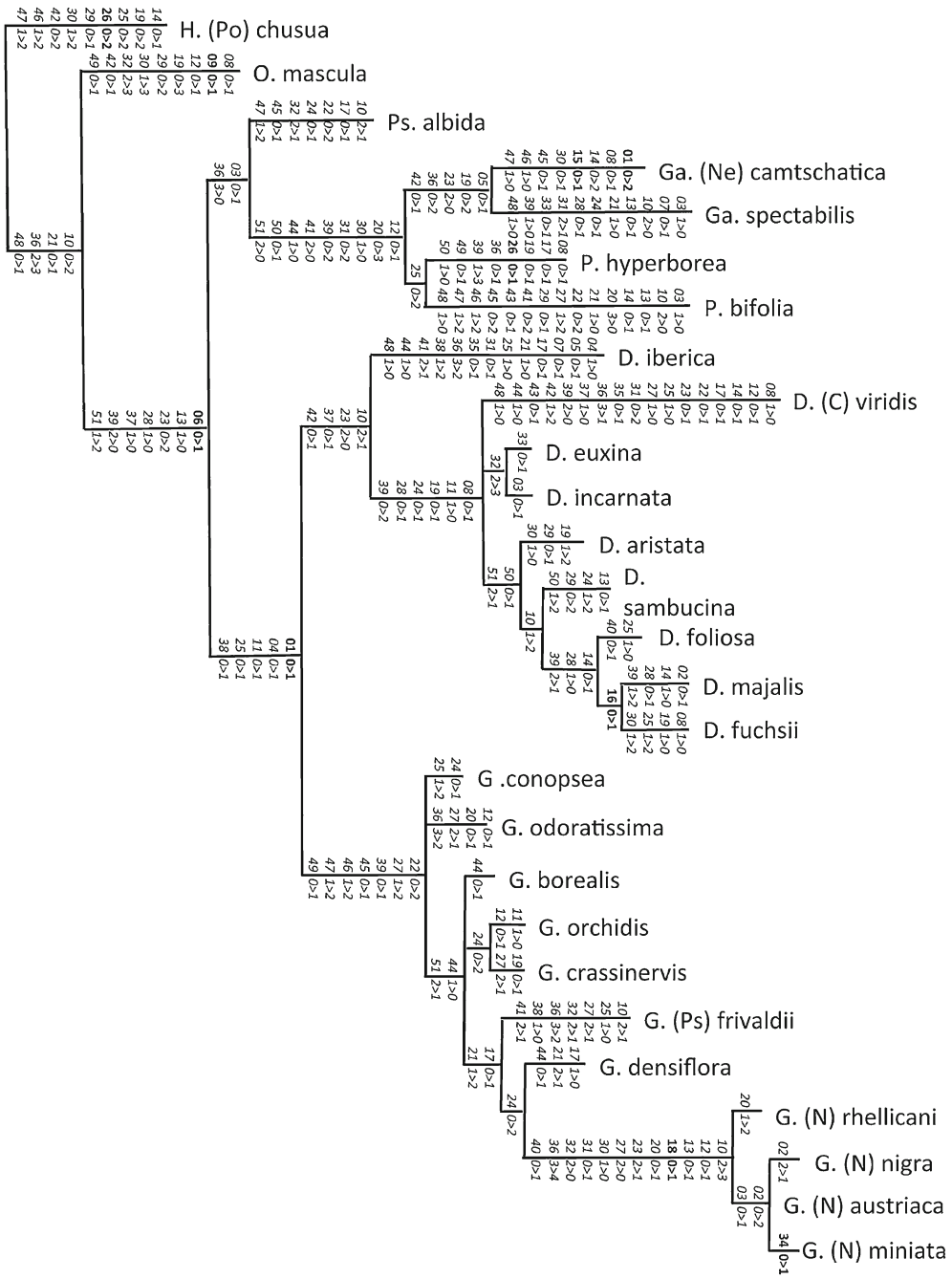


Fig. 7. Morphological character-state transitions (Acctran optimisation) dictated by constraining the morphological cladistic matrix to the preferred topology generated from the ITS matrix (presented here as Fig. 4) The initial number matches characters listed in Table 2, whereas the following numbers indicate the transition between ancestral and derived states of that character. Italicised characters are homoplastic, boldface characters are non-homoplastic.

the ITS matrix (Fig. 7). In order to generate the required backbone constraint prior to analysis in PAUP we combined four pairs of morphologically very similar species present in the ITS tree (*D. saccifera* into *D. fuchsii*, *D. romana* into *D. sambucina*, *D.* cf. *hatagirea* into *D. incarnata*, *G. gabasiana* into *G. rhellicani*), and added as more distant outgroups *Galearis*, the monotypic genus *Neolindleya*, *Orchis* s.s. and *Hemipilia* s.l. (the latter used in the original morphological cladistic analysis). Constraining the morphological characters to the molecular topology added 20% to the length of the most parsimonious tree(s), the increase to 218 steps further reducing the ensemble consistency index to 0.395 (0.377), the ensemble retention index to 0.535, and the total number of non-homoplastic synapomorphies to just four.

Consistency indices from the constraint tree were tabulated for each of the 51 morphological characters (Table 4) and mean values were calculated for each organ of the plant (including the five characters either fixed or optimised as autapomorphic). Mean consistency indices were also calculated for the three pigmentation values (C16, 36, 37: mean = 0.592) and the seven cellular-level micromorphological characters (C20, 24 – 26, 38, 50, 51: mean = 0.514). Homoplasy proved to be lowest in the character sets representing cytology and rootstocks, and greatest in those representing stem plus inflorescence, fragrance and nectar, labellar spurs, lateral petal/sepal positions, gynostemium morphology and (once adjusted for an unusually large number of ambiguous cells) seed/pollen morphology. Specific characters yielding consistency index values of less than 0.3 were breeding system (C3), stem architecture (C8), leaf arrangement (C12, C13), pedicel length (C17), spur papilla size (C25), spur length and diameter (C27, C28), pollinarium caudicle length (C44) and prominence of rostellar median fold (C48).

Discussion

Circumscription of, and relationships among, genera

Summary of previous molecular phylogenetic analyses — Fig. 8 compares topologies from four previous molecular phylogenetic studies of subtribe Orchidinae. Given the substantial proportion of branches lacking strong statistical support and thus incurring considerable uncertainty, it is perhaps unsurprising that considerable topological incongruence is evident between the various studies. Only two nodes persist across all five trees, but fortunately, they are the two nodes of greatest relevance to the present study: that linking *Dactylorhiza* and *Gymnadenia* as sister genera (usually with strong bootstrap support), and that linking this sister pairing to the remainder of the taper-tubered clade (i.e. *Platanthera* plus *Galearis* plus *Neolindleya* plus *Pseudorchis*). All five trees also agree, with strong bootstrap support, that both *Dactylorhiza* s.l. and *Gymnadenia* s.l. are monophyletic. Four of the trees agree that *Pseudorchis* is sister to *Platanthera* plus *Galearis* plus *Neolindleya*, and although the fifth (Tang *et al.* 2015) places *Pseudorchis* as sister to *Platanthera*, this inferred relationship lacks bootstrap support.

The apparent identity of the sister-group to the digitate-tubered clade differs greatly among trees, from *Traunsteinera* plus *Chamorchis* alone (Bateman *et al.* 2003) and *Neotinea* alone (Jin *et al.* 2017) through to a clade consisting of all of the remaining genera of Orchidinae other than *Hemipilia* s.l. (Inda *et al.* 2012). The morphologically mismatched Alpine pairing of *Traunsteinera* plus *Chamorchis* appears to be a particularly strong source of topological instability in these trees, as is *Neolindleya* within the *Platanthera–Galearis* clade (BS reliably <50%). Also noteworthy is the especially radical topological contrast between the two studies richest in "informative" bases: Inda *et al.* (2012) and Jin *et al.* (2017). Evidently, size is not everything.

Present molecular phylogenetic analysis — With the exception of the use of *Orchis* and *Hemipilia* as "outer outgroups" in the morphological cladistic analysis, the present study is confined to the six genera that constitute the taper-tubered clade (i.e. *Dactylorhiza* through to *Galearis* in Fig. 8). This narrower genus-level sampling inevitably weakens any conclusions

Table 4. Mean consistency indices and mean number of ambiguous cells for each of 11 organ-based suites of characters scored for the present morphological cladistic study, together with two thematic suites consisting of the three pigmentation characters and the seven cellular-level micromorphological characters.

Character set	Character nos.	Mean CI	Mean no. ambiguous cells/char.
Organ sets			
Chromosomes and breeding system	C1 – 3	0.622	2.0
Tubers	C4 – 7	0.625	0.3
Stem and inflorescence	C8, 21	0.250	1.5
Leaves	C9 – 16	0.551	2.6
Ovary and bracts	C17 – 20	0.511	1.0
Secretions (nectar and fragrance)	C22, 23	0.450	1.0
Spur	C24 – 29	0.407	6.7
Labellum	C30 – 38	0.541	3.6
Lateral petals and sepals	C39, 40	0.417	0
Gymnostemium	C41 – 48	0.396	4.1
Seeds and pollen	C49 – 51	0.444	11.7
Trans-organ sets			
Pigmentation	C16, 36, 37	0.592	5.0
Cellular micromorphology	C20, 24 – 26, 38, 50, 51	0.514	11.9

Original CI values per character were calculated on the basis of the characters as mapped across the present ITS phylogeny for the ingroup.

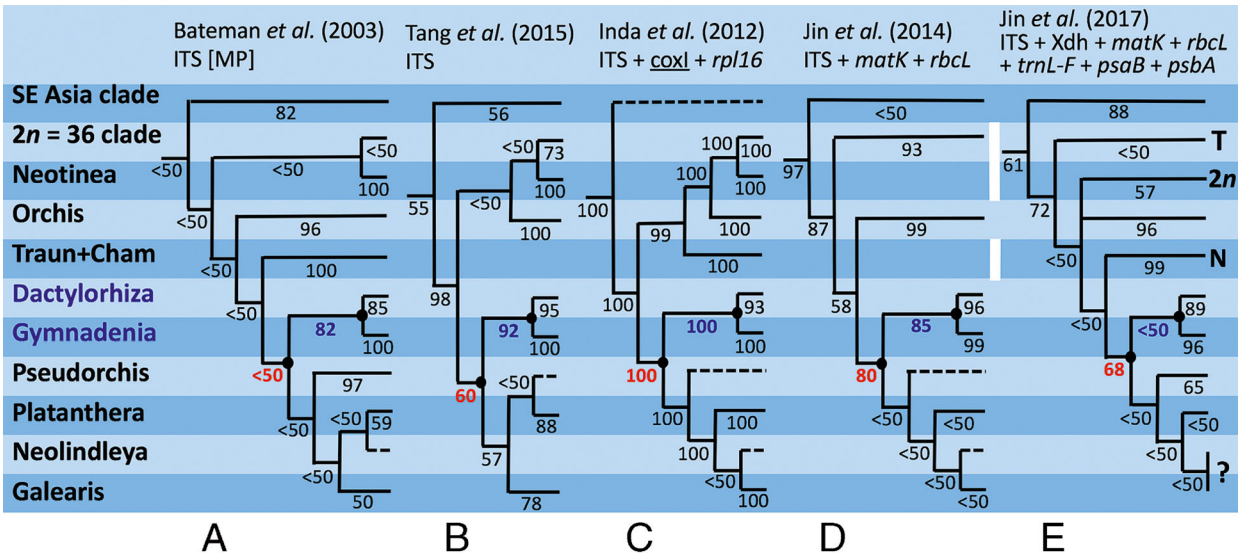


Fig. 8. Topological comparison of five previous molecular phylogenetic analyses of subtribe Orchidinae s.s. (A – E). All trees were generated using Bayesian inference except that of Bateman *et al.* (maximum parsimony). Nuclear regions are shown in roman font, plastid regions are italicised, and mitochondrial regions are underlined. Dashed lines were represented only by a single accession. Statistical support is uniformly given as bootstrap values calculated via maximum parsimony; that supporting the taper-tubered clade is given in red font and that supporting the digitate-tubered clade in blue font. 'Traun+Cham' is *Traunsteinera* plus *Chamorchis*, the SE Asia clade is *Hemipilia* (including the former genera *Neottianthe*, *Amitostigma*, *Ponerorchis* and *Chusua*: Tang *et al*. 2015), and the 2*n* = 36 clade consists of *Steveniella*, *Himantoglossum* s.l., *Anacamptis* s.l., *Serapias* and *Ophrys* (Bateman *et al*. 2003). Note that the lower half of the topology of Jin *et al.* (2017) provides substantially different placements for *Traunsteinera-Chamorchis* (T), *Neotinea* (N) and the 2*n* = 36 clade (2*n*); also, the upper half of their topology nests '*Neolindleya*' within *Galearis* s.s.

regarding relationships between the genera. Nonetheless, we note that our attempts to use *Pseudorchis* as an outgroup alongside *Platanthera* reliably failed. In both the nuclear (Fig. 4) and plastid (Fig. 3) trees, *Pseudorchis* is placed as sister to *Dactylorhiza* plus *Gymnadenia*, contradicting its placement in every previous molecular phylogenetic tree as sister to *Platanthera* or, more commonly, sister to *Platanthera* plus *Galearis/Neolindleya* (Fig. 8). Moreover, the branch separating *Pseudorchis* from *Dactylorhiza* plus *Gymnadenia* is significantly shorter than that separating *Pseudorchis* from *Platanthera* in both of our molecular trees. In the morphological tree (Fig. 5), *Pseudorchis* is placed in an even more derived position, as sister to the basalmost unequivocal *Gymnadenia* species. And when the morphological data were constrained to a typical molecular topology (Fig. 7), *Pseudorchis* fitted poorly, being linked with *Platanthera* plus *Galearis* by only two homoplastic characters (facultatively autogamous breeding system, C3; white flowers, C36) that are inadequate to circumscribe the putative aggregate clade.

Another variable placement is that of the former genus *Nigritella*, which is nested deeply within *Gymnadenia* s.s. in the nuclear and morphology trees but placed as sister to *Gymnadenia* s.s. in the plastid tree — a distinction based entirely on the basis of contrasting, group-specific copy numbers of a distinctive 9 bp minisatellite. A corresponding sister-group relationship between these genera was derived through UPGMA analysis of allozyme data by Stahlberg (1999) and Hedrén *et al.* (2000), and through a median-joining network generated from haplotypes based on five plastid regions by Hedrén *et al.* (2018). The most likely scenario for our data suggests that the minisatellite was lost from *'Nigritella'* but duplicated (and, in some cases, triplicated) in *Gymnadenia* s.s. On balance, the evidence remains strong for nesting of a monophyletic *'Nigritella'* within a paraphyletic *Gymnadenia* s.s.

With regard to other former genera, both the present nuclear and plastid trees place the former genus *Coeloglossum* firmly within *Dactylorhiza*. It occupies this position in all of the previous studies summarised in Fig. 8; thus far, only Devos *et al.* (2006b) have ever succeeded in placing *D. viridis* (marginally) below the remainder of the genus *Dactylorhiza* in a molecular phylogeny (reviewed by Bateman & Rudall 2018). The morphological phylogenetic tree does place *D. viridis* below the remainder of *Dactylorhiza* plus *Gymnadenia* plus *Pseudorchis*, but transferred alongside *D. viridis* as putative sister is *D. iberica*. This species was never challenged by previous authors as being anything other than a fully-fledged member of the genus *Dactylorhiza*, and we would strongly discourage any future attempt to establish it as a monotypic genus (Bateman & Rudall 2018).

'Pseudorchis' frivaldii was omitted from the plastid tree in the absence of relevant sequences, although a previous study utilising multiple plastid sequences (Bateman *et al.* 2006) placed this species firmly within *Gymnadenia*, as does the present ITS tree. In contrast, the morphology tree places *G. frivaldii* as sister to *Pseudorchis albida*, thereby recovering its traditional genus-level assignment.

Taken at face value, the molecular phylogenies generated during the present study (Figs 3, 4) merely add to the complexity and ambiguity of relationships suggested by the previous studies here summarised in Fig. 8. Moreover, the present morphological cladogram — the first to be generated for the taper-tubered clade (Fig. 5) — deviates strongly from any of the relevant molecular topologies. Given these strongly contrasting topologies, the only overall conclusion permitted by pragmatism is that, although molecular phylogenetics has undoubtedly proven effective for re-circumscribing genera within Orchidinae, it has been rather less successful at determining with confidence the relationships between those re-circumscribed genera. Admittedly, morphology performs even less well than candidate-gene molecular studies.

Morphological cladistic analysis — Interestingly, in the case of Orchidinae, morphological cladistics has achieved neither credibly circumscribed genera nor clarified relationships. Nonetheless, the results of this experiment are of considerable interest. Firstly, our study illustrates the great importance to phylogeny reconstruction of thorough taxon sampling. Multiple molecular phylogenetic studies (Hapeman & Inoue 1997; Bateman *et al.* 2003; Bateman *et al.* 2009; Jin *et al.* 2014; Tang *et al.* 2015) have all made convincing arguments that *Platanthera* s.l. is monophyletic, yet in the present morphological study the two species of *Platanthera* selected as outgroups (*P. chlorantha* from section *Platanthera*, *P. hyperborea* from section *Limnorchis*) are sufficiently morphologically divergent to be separated phylogenetically by interpolation of *Galearis spectabilis* (Fig. 5). Moreover, *Neolindleya* — recently assigned to *Galearis* on molecular phylogenetic evidence by Jin *et al.* (2014) — is here shown as sister to *P. hyperborea*, the two species being linked by four ambiguously optimised but homoplastic characters: autogamous breeding system, stem central cavity, leaves well-distributed along stem, and loss of spur interior striations.

Within the ingroup, with one notable exception, the morphological cladogram (Fig. 5) reconstructs the superseded genus-level circumscriptions that dominated taxonomy of the group immediately prior to early applications of molecular phylogenetics (Pridgeon *et al.* 1997, *et seq.*). The former genus *Coeloglossum* is separated from the majority of *Dactylorhiza* species, albeit carrying with it *D. iberica* on the basis of reduced leaf number, shortened pedicel, labellum median ridge (non-homoplastic), lateral sepal connate with median sepal, and subdued median fold of rostellum. More radically, the putative outgroup *Orchis mascula* is instead placed within the ingroup as sister to *D. sambucina*, supported by two characters: expanded leaves concentrated into a basal rosette, and upwardly curved spur. Comparison of the flowers of co-occurring plants of *D. romana* (a close relative of *D. sambucina*) and *O. provincialis* (a close relative of *O. mascula*) shows remarkable evidence of convergence in several floral features (Fig. 1F).

Above *Dactylorhiza*, the morphological tree places *Pseudorchis albida* as sister to *Gymnadenia*, but detaches *G. frivaldii* from *Gymnadenia* and instead supports its more traditional placement within *Pseudorchis*, even though this placement is conclusively rejected by molecular studies showing their floral similarities to reflect convergence (Bateman *et al.* 2006). Characters supporting the pairing are reduced leaf number, shortened pedicel, greater concavity of the labellum, loss of doming in adaxial labellar epidermal cells. The one issue where the morphological cladogram differs from immediately pre-molecular circumscription is the placement of the former genus *Nigritella*; as previously shown in a preliminary morphological cladistic analysis (Bateman & DiMichele 2002), it is embedded deeply within *Gymnadenia* s.s. despite its subtending long branch. Here, it is linked to *G. orchidis* as sister by only one ambiguously optimised character state: short (i.e. reduced) caudicle length.

Given an initial assumption that traditional taxonomists are more likely to use overall morphological similarity than parsimony as a cerebral classificatory tool, we predicted from first principles that the pre-molecular genus circumscriptions would more likely emerge from the phenetic tree-building techniques NJ and/or UPGMA, but this proved to be a false assumption (cf. Figs 5, 6). Instead, the NJ tree pushed *Dactylorhiza viridis* and *D. iberica* even further from the remainder of *Dactylorhiza* and elevated *Gymnadenia frivaldii* further up the tree, to a position deeply embedded within *Gymnadenia* s.s. as sister to *'Nigritella'* (Fig. 6A). In contrast, the UPGMA algorithm was seduced by the exceptionally long morphological branch subtending *'Nigritella'* into placing this former genus in a highly improbable position as the earliest divergent among all of the taxa analysed (Fig. 6B). (Note: We are aware of the philosophical impurity inherent in the phrase 'early divergent', but nonetheless find it a useful shorthand for identifying within a topology particular lineages that would otherwise require unnecessarily complex descriptions.)

After taking both past and present studies into account, and carefully weighing up the available spectrum of evidence, we conclude that the ITS

phylogeny (Fig. 4) is most likely to contain the greatest proportion of accurately reconstructed phylogenetic nodes. The plastid region *trnL-F* yielded undesirably few informative sites and thus gained poor statistical support across the tree. The morphological matrix incurred exceptionally high homoplasy, apparently encompassing both extensive convergence and occasional character reduction or loss; moreover, the combined total of 13% of cells coded as either polymorphic or unknown further reduced statistical confidence. A topology entirely congruent with our ITS tree between *Gymnadenia conopsea* s.s., *G. odoratissima*, *G. densiflora* and the former genus *Nigritella* (the latter paired as sisters) was generated by Sun *et al.* (2015) after adding to ITS sequences data from three low-copy nuclear genes.

Summary: Genus-level circumscription reviewed — Taxonomic literature published during the molecular era has led to the DNA-based classification being followed by some, questioned and selectively rejected by others on the basis of inadequate evidence (a "final solution" was rather inadvisedly demanded by Kropf in Kadereit *et al.* 2016: 43), or simply ignored without comment by perhaps the majority (reviewed by Bateman 2009, 2012a). In truth, the accumulated evidence supports the molecular re-circumscription of the taper-tubered genera that accommodates *'Nigritella'* and *'Pseudorchis' frivaldii* within *Gymnadenia* s.l. and *'Coeloglossum'* within *Dactylorhiza*.

Ongoing opponents of classifications that prioritise DNA data tend to rely on special pleading rather than explicitly proposing their own rule-based logic or directly challenging monophyly as the fundamental classificatory concept (e.g. Devos *et al.* 2006a; Tyteca *et al.* 2012; Delforge 2016; Kropf *in* Kadereit *et al.* 2016; Perazza 2016). The few attempts to "rescue" *Nigritella* as a full genus or occasionally to incorporate *Pseudorchis* into *Gymnadenia* (e.g. Delforge 2006 — a decision wisely reversed by Delforge 2016) can only be viewed as brave in the light of so much contradictory DNA-based evidence. Even if trees showing the non-nigritellan *Gymnadenia* species to be monophyletic were eventually to be generated by a future study (as is implied by the unrooted haplotype network recently published by Hedrén *et al.* 2018), following our explicit rules for deriving classifications from trees (e.g. Bateman & Rudall 2018), the inevitably comparatively short and poorly supported branch separating the two species groups would still require inclusion of '*Nigritella*' within *Gymnadenia*.

The one remaining uncertainty is the phylogenetic position of the monotypic southeast Asian genus *Neolindleya*, which has been included in at least four molecular studies (Bateman *et al.* 2003; Inda *et al.* 2012; Jin *et al.* 2014; Jin *et al.* 2017: Fig. 8) and one morphological study (Fig. 5) but has never been placed with >50% bootstrap support; irrespective of the data used, it appears to be intermediate between, and have approximately equal phylogenetic disparities from, *Platanthera* and *Galearis* (see also Efimov *et al.* 2009). On the basis of their multi-gene phylogeny, Jin *et al.* (2014) argued that *Neolindleya* should be incorporated into *Galearis*, but we believe that the genus should be retained despite its monotypic status, at least until multiple sources of evidence are found that together offer much stronger support for one sister-group relationship rather than the other.

Circumscription of, and relationships among, species within genera

Dactylorhiza — Although the most recent taxonomic treatment of the genus *Dactylorhiza* (Delforge 2016) recognised 60 species as native to Europe plus Asia Minor (though omitted *'Coeloglossum'* from the genus), 30 species would be a more scientifically justifiable estimate. Species-level relationships within western European *Dactylorhiza* s.l. have been explored in several molecular studies (e.g. Hedrén *et al.* 2001; Bateman *et al.* 2003; Devos *et al.* 2006a; Pillon *et al.* 2007; Hedrén *et al.* 2008; Hedrén *et al.* 2011), and those occurring further east in Eurasia have also received informed attention (Hedrén 2001; Hedrén *et al.* 2007). Given the limited and largely typological sampling of taxa and equally limited sampling of genic regions that characterise representation of *Dactylorhiza* in the present study, it was unlikely that additional major insights would emerge, and so it proved.

The plastid region *trnL-F* served only to link two pairs of species already grouped according to classical taxonomy: *Dactylorhiza romana* with *D. sambucina*, and *D. saccifera* with *D. fuchsii* and the allotetraploid complexes (Fig. 3). However, *trnL-F* is clearly useful for separating the main phylogenetic groups within the genus and indicates greater disparity among lineages shown as early divergent in the ITS tree. The morphological phylogenetic study similarly offered little resolution, although it did link the Canary Islands endemic *D. foliosa* with *D. fuchsii* on the basis of their comparatively thin leaves and trabeculate seed testa. The trabeculate testa is shared with *D. majalis* — the allotetraploid progeny of members of the *D. fuchsii* and *D. incarnata* groups — which is grouped with the latter (its paternal parent) in Fig. 5.

Most of the relationships indicated by the ITS tree receive only modest bootstrap support (Fig. 4). The topology agrees with those of previous studies in showing *Dactylorhiza iberica*, *D. viridis* and the *D. incarnata* group as competing to be the earliest diverging lineage within the genus (reviewed by Bateman & Rudall 2018). Relationships among the remaining species are already well-established, including inheritance of minor modifications of the

fuchsii group ribotype by *D. majalis* and most other allotetraploid taxa.

Gymnadenia — The present study was conceived in the late 1990s primarily to explore species relationships within *Gymnadenia* s.l. (using *Dactylorhiza* as a de facto "inner outgroup") and to provide a framework for a parallel population-level study that employed in situ morphometrics in the hope of better circumscribing those species (Bateman & Denholm 2019; R. Bateman, P. Rudall & I. Denholm, unpublished). The study therefore included representatives (typically several) of most species of the genus that have been at least adequately characterised (i.e. excluding several putative species of the former genus *Nigritella* that are near-identical in both morphology and DNA sequences: reviewed by Teppner & Klein 1985; Teppner 2004; Delforge 2016). Unlike *Dactylorhiza*, the genus *Gymnadenia* has not yet been the subject of 21st Century attempts at taxonomic revision, monographers perhaps having been intimidated by accumulating evidence for the presence of several comparatively cryptic species.

The *trnL-F* phylogeny served largely to distinguish the former genus *Nigritella* according to its lack of the 9 bp minisatellite, although it also separated the Chinese accession of *Gymnadenia orchidis* downloaded from the work of Tang *et al.* (2015) from the remaining species according to several unique SNPs (most of them occurring within the 9 bp minisatellite) plus four indels. Most accessions of all the remaining species shared a single core haplotype, although slightly deviant haplotypes were reported in two accessions of *G. conopsea*, two of *G. densiflora* and three of *G. odoratissima* (Fig. 3); in total, *Gymnadenia* yielded ten haplotypes (I – X).

As in previous ITS-based studies (Bateman *et al.* 2003; Gustafsson & Lönn 2003; Bateman *et al.* 2006; Stark *et al.* 2011; Efimov 2013; Sun *et al.* 2015), the resulting trees showed good resolution and taxonomic grouping but only modest bootstrap support in both parsimony and likelihood analyses (Fig. 4). Nonetheless, the improved taxonomic and geographic sampling relative to previous studies allowed some additional, long-standing conundra to be addressed.

In total, 30 ribotypes (I – XXX: Fig. 4) were recovered from the genus *Gymnadenia* s.l. Stark *et al.* (2011) reported 35 ribotypes, but unlike us they also included variants that showed ambiguous sites resolved via statistical reconstruction techniques. Our 30 ribotypes boil down to six clades: *conopsea* plus *odoratissima*, all available Chinese accessions, *borealis*, *frivaldii*, *densiflora*, and the former genus *Nigritella*. *Gymnadenia* encompasses a few widespread low altitude/mid-latitude species (*conopsea* s.s., *densiflora*) admixed with montane/high latitude specialists (the remainder). The main taxonomic instability persisting in the genus derives from the southeast European high-altitude specialist *G. frivaldii*, which has an ITS sequence approximately equally similar to those of *G. borealis* and *G. densiflora* plus *'Nigritella'*. Setting aside the under-sampled Sino-Himalayan clade, four of the five ITS groups each have a clearly dominant ribotype that is plesiomorphic within the group and hence is presumed to be ancestral (VI, XVII, XIX and XX, respectively, in Fig. 4). The exception is the former genus *Nigritella*; where by far the more common ribotype (XXX) is derived rather than primitive.

Perhaps the most surprising aspect of both this and previous candidate-gene trees exploring *Gymnadenia* is their consistent inability to distinguish between the dominantly lowland *G. conopsea* s.s. and the upland/boreal *G. odoratissima*. Ribotype VI is most common among individuals of both species, despite the fact that these species are readily distinguished morphologically — they are separated by a disparity of seven characters on our constrained morphology tree (Fig. 7). Most of the remaining ribotypes in the *conopsea–odoratissima* clade deviate by a single step, although two or three steps separate apparent regional genetic divergences in central and eastern Russia (ribotype I: Efimov 2013) and Germany (ribotypes X and XI: Stark *et al.* 2011). Three of the samples sequenced for ITS by us that were supposedly members of other species actually yielded ribotypes typical of *conopsea* and *odoratissima* and therefore appear to have been mis-identified when collected. One sample originally attributed to *"densiflora"*, collected on our behalf in the Pyrenees, yielded ribotype VIII; we have no knowledge of its morphology, but its flowering time suggests a more likely attribution to *conopsea* s.s. Two other samples collected on our behalf from the Norwegian coast had a morphology and habitat preference consistent with *G. borealis* but surprisingly yielded the classic *conopsea–odoratissima* ribotype VI.

Gustafsson & Sjögren-Gulve (2002) found similarly admixed microsatellites in populations of *Gymnadenia conopsea* and *G. odoratissima* in Sweden, although Sun *et al.* (2015) had more success in distinguishing the two species within 11 mixed-ploidy populations of these species in Switzerland, a result achieved by adding three low-copy nuclear regions to their ITS data. However, Sun *et al.* (2015) erroneously claimed that their tree showed the two species to be sisters. In fact, their molecular tree shows (admittedly with limited statistical support) *G. odoratissima* to be monophyletic but originating from within *G. conopsea* s.s., rendering *conopsea* paraphyletic. Nonetheless, at least one of their low-copy regions is clearly competent to reliably distinguish (albeit narrowly) between the two species, at least in Switzerland. Huber *et al.* (2005) reported different spectra of fragrance volatiles, and thus of pollinators, between Swiss populations of the two species.

The next clade of ribotypes to diverge in the ITS tree was confined to southwest China and

encompassed all of the Sino-Himalayan taxa analysed by us. Support for this clade also exists in the form of a consistent 1 bp insertion toward the 5' end of the *trnS-trnG* spacer in the plastid genome (Y. Pillon, pers. comm. 2004). Five putative species of *Gymnadenia* were recognised in the *Flora of China* (Chen *et al.* 2009): long-spurred *G. conopsea* s.s. and *G. orchidis*, together with three less well-known short-spurred taxa: *G. crassinervis*, *G. emeiensis* and *G. bicornis*. Traditional taxonomic accounts suggest that *G. emeiensis* and *G. bicornis* are typically vegetatively robust, morphologically distinct from the remaining taxa but perhaps not from each other. Unfortunately, we were unable to obtain DNA samples of either putative species, and within this group as a whole, our sampling is insufficient to identify a core ribotype. Our ITS study did, however, show that our two samples of the geographically widespread *G. orchidis*, which differs from *G. conopsea* s.s. primarily in its broader leaves, to be paraphyletic relative to three samples of *G. crassinervis* (Fig. 4). Most perturbing is a sample attributed to *G. conopsea* by Pillon *et al.* (2006) but placed here with 97% bootstrap support as the earliest divergent lineage within the Sino-Himalayan clade. It seems likely that plants growing in southeast Asia that closely resemble *G. conopsea* s.s. in morphology deviate more substantially in ITS sequences, as discussed below for *G. densiflora*. Viewed together, these results suggest that Sino-Himalayan samples of *Gymnadenia* have not been consistently identified. More fundamentally, they suggest that circumscription of *Gymnadenia* species in the region remains far from optimal and is in considerable need of a combined morphometric and population genetic survey.

The most appropriate taxonomic status for *Gymnadenia borealis* has long been debated (cf. Rose 1991; Lang 2001; Bateman *et al.* 2003; Bateman *et al.* 2006), as has its possible confinement to the British Isles (cf. Bateman *et al.* 2006; Harrap & Harrap 2009; Rankou 2011; Travnicek *et al.* 2012). Delforge (2006) elected to treat both *borealis* and *densiflora* as mere taxonomic varieties in the third edition of his monograph, later illogically elevating *borealis* but not *densiflora* to species status in the fourth edition (Delforge 2016). Our five bona fide samples of the species yielded three slightly divergent ribotypes (Fig. 4) and, predictably, a core ribotype that was concentrated in Scotland (XVII). Morphologically the plants resemble miniaturised versions of *G. conopsea* s.s., although the restriction of *G. borealis* populations to acid heaths spatially separates them from those of *G. conopsea* s.s. Thus, the southern English outlier population that we sampled in a Sussex heath yielded a ribotype that was only slightly divergent from the remainder and was clearly attributable to *G. borealis*. We continue to fail to find bona fide populations of this species in mainland Europe, despite the seemingly similar morphology and habitat preference of some populations in northern Norway (Bjerke & Strann 2009).

Gymnadenia (formerly *Pseudorchis*) *frivaldii*, an alpine specialist of wet acid heaths endemic to the Balkans, was the subject of a morphological and molecular study by Bateman *et al.* (2006) and so need not be discussed in detail here. All four of the northern Greek and Bulgarian samples at our disposal yielded the species-specific ribotype XIX, and this genetic uniformity apparently extends to the morphology of its populations; its distinctive small, pale pink flowers are borne in compact inflorescences on plants that show comparatively little variation in size.

The molecular phylogenetic disparity that separates *Gymnadenia densiflora* from *G. conopsea* s.s. (Fig. 4) continues to perplex observers despite their similar morphologies. Substantial differences are evident in both allozyme studies (cf. Scacchi & de Angelis 1989; Soliva & Widmer 1999; Gustafsson 2000) and sequencing data (Gustafsson & Lönn 2003; Bateman *et al.* 2006; Campbell *et al.* 2007; Jersáková *et al.* 2010; Stark *et al.* 2011; Meekers *et al.* 2012; Efimov 2013; Saenruen 2014). *Gymnadenia densiflora* inhabits calcareous to neutral marshes across Europe, and the biochemistry of its scent apparently differs from those of both *G. conopsea* s.s. and *G. odoratissima* (Gupta *et al.* 2014), although the difference in fragrances may nonetheless be insufficient to influence pollinator behaviour (Jersáková *et al.* 2010). Despite the large number of papers demonstrating a reliable and substantial molecular separation, in the eyes of a few authors, *G. densiflora* still remains an infraspecific taxon within *G. conopsea*, either a subspecies (e.g. Lang 2001; Gustafsson & Lönn 2003) or even a mere variety (Kreutz 2004; Bournérias & Prat 2005; Saenruen 2014; Delforge 2006, 2016).

A combination of our own accessions plus GenBank sequences provided us with extensive Europe-wide coverage of this species, yet the ITS clade for *densiflora* remained stubbornly robust. Most of the deviations from the core ribotype (XX) were slight, although a more interesting and unexpected deviation of four steps distinguished late-flowering plants that were assignable to *Gymnadenia densiflora* but occurred in both dune-slacks in southern Wales (Kreutz & Lewis 2015) and chalk downland in southern England (cf. Lang 2001; Campbell *et al.* 2007). Morphologically similar plants occupying dune slacks in the Dutch Frisian Islands were named in rapid succession *G. conopsea* var. *friesica* (Kreutz & Lewis 2015) and then *G. densiflora* var. *friesica* (Lewis 2015), but have not yet been subjected to either molecular or morphometric study to test whether they represent the same biological entity. However, the flow cytometry profiles of these plants show diploidy and are consistent with *G. densiflora* (P. Travnicek, pers. comm. 2012).

It is possible that this ITS-delimited taxon — broadly resembling typical marsh-dwelling *Gymnadenia densiflora* in both morphology (R. Bateman, P. Rudall and I. Denholm, unpublished) and ribotypes (Fig. 4) but tolerant of at least periodically drier soils — could at least partly explain the conundrum presented by a series of reports that *G. conopsea* s.s. maintains both early-flowering and late-flowering forms. The latter bloom contemporaneously with *G. densiflora* and reputedly maintain less variation, both morphological and molecular (cf. Gustafsson & Lönn 2003; Marhold *et al.* 2005; Lönn *et al.* 2006; Vöth & Sontag 2006; Jersáková *et al.* 2010; Stark *et al.* 2011; Saenruen 2014; Gross & Schiestl 2015). Several studies (Gustafsson & Lönn 2003; Stark *et al.* 2011; Saenruen 2014) have argued that the late-flowering populations of *G. conopsea* s.s. from Sweden group with *G. densiflora* in ITS trees, a conclusion that would be in accord with our results from *friesica*-like populations in England (Fig. 4). However, the converse situation was reported for Czech populations, where the late-flowering tetraploid populations assigned to *G. conopsea* s.s. have retained the ribotypes of the early-flowering diploid populations (Jersáková *et al.* 2010). The main weakness of these studies is that only Stark *et al.* (2011) seriously addressed the morphology of the plants described as late-flowering *G. conopsea*, and even they scored only nine morphological characters. Early investigations of ploidy suggested that *conopsea* was predominantly diploid and *densiflora* tetraploid (e.g. Groll 1965; Jongepierová & Jongepier 1989; Mrkvicka 1993), but more recently this viewpoint has become inverted, *densiflora* being regarded as predominantly diploid and *conopsea* as similarly predominantly diploid in western and northern Europe but often tetraploid (with subordinate triploids and pentaploids) further south and east, notably in the Alps and Pyrenees (Marhold *et al.* 2005; Jersáková *et al.* 2010; Stark *et al.* 2011; Trávnicek *et al.* 2012).

Despite being generally considered to contain approximately 12 species (Hedrén *et al.* 2000; Kreutz 2004; Teppner 2004; Delforge 2016), the former genus *Nigritella* yielded only two ribotypes and, in contrast with all other clades within *Gymnadenia*, by far the most common ribotype (XXX) proved to be derived (Fig. 4). The rarer ribotype (XXIX) appears to be confined to a single species — the diploid, allogamous Pyrenean endemic *G. gabasiana*. This species is particularly intriguing because its ribotype lacks four of the five synapomorphies possessed by the remaining *'Nigritella'* species (only the non-homoplastic C–T transition at position 378 was present in all species analysed). However, when *G. gabasiana* was assessed for possible inclusion in our morphometric matrix it proved to be identical to the widespread diploid *G. rhellicani* in all characters that could be scored (sadly, *G. gabasiana* was omitted from the recent morphometric study by Lorenz & Perazza 2012, which focused on Alpine rather than Pyrenean species). This strong similarity reduces the probability of a recent origin through hybridity analogous to that of the localised apomict *Gymnigritella runei* in Sweden (Stahlberg 1999; Hedrén *et al.* 2000) or pentaploid *G. buschmanniae* in the Italian Alps (Hedrén *et al.* 2018). It also increases the likelihood that *G. gabasiana* resembles — and could even be — the ancestor of the remaining *'Nigritella'* species.

Interestingly, *Gymnadenia gabasiana* was the only species that formed a cohesive group in the AFLP analysis of Stahlberg (1999), in which geographic origin influenced inferred relationships among samples more strongly than did taxonomic assignment. Also, Stahlberg placed *G. gabasiana* as the earliest-diverging species in his UPGMA tree (but see the criticisms of UPGMA clustering outlined below). In contrast, *gabasiana* failed to form a cohesive group when subjected to allozyme profiling, and it appeared derived rather than primitive in the resulting UPGMA phenograms (Hedrén *et al.* 2000). Similarly, the haplotype network of Hedrén *et al.* (2018) nested *G. gabasiana* (appearing monophyletic in this data-set) within *'Nigritella'*, their topology suggesting that it diverged later than the autogamous polyploids *G. widderi* and *G. archducis-joannis* — taxa that may be conspecific and are highly localised within the Alps and Apennines.

Our failure to detect any differentiation in either ITS or *trnL-F* among the remaining putative species of *'Nigritella'* suggests either over-splitting into species via traditional taxonomic practices, recent diversification, or (as inferred by Hedrén *et al.*) both. These species maintain a reservoir of morphological character variation (albeit subtle, as evidenced by our morphology trees: Figs 4, 7), often occupy contrasting mountain ranges, present a spectrum of reproductive modes from allogamy to autogamy (including apomixis) that correlate with a range of ploidies and karyotypes (cf. Teppner & Klein 1985; Stahlberg 1999; Hedrén *et al.* 2000; Lorenz & Perazza 2012; Hedrén *et al.* 2018). Building upon the excellent taxon molecular foundations recently laid by Hedrén *et al.* (2018) using haplotypes, ITS sequences and nuclear SSRs for a good spectrum of samples, the former genus *Nigritella* appears to us to be an ideal subject for a more detailed phylogeographic study of the influence of repeated glaciations on speciation, as discussed in the following section.

Likely causes of speciation within the digitate-tubered clade

The divergence in ITS and plastid regions between *Dactylorhiza* s.l. and *Gymnadenia* s.l. is less than that evident between any other genus-level sisters recognised by us within Orchidinae other than the specialised Alpine pairing of *Traunsteinera* versus *Chamorchis*. It is therefore unsurprising that in nature intergeneric hybrids have been recorded at F_1 level and sometimes

beyond between a large percentage of the combinations of species permitted by geographic distributions and ecological preferences (e.g. Oddone *et al.* 2016; Stace *et al.* 2015). Artificial breeding experiments (S. Malmgren, pers. comm. 2015; J. Haggar, pers. comm. 2017) have shown both genera to have at most weak post-zygotic isolation mechanisms, as have *G. conopsea* and *G. odoratissima* within the genus *Gymnadenia* (Sun *et al.* 2015; but see Sletvold *et al.* 2012b). Hybrids between either *Dactylorhiza* or *Gymnadenia* and *Pseudorchis* are considerably rarer, presumably reflecting the greater molecular divergence of the latter.

Dactylorhiza and *Gymnadenia* share a chromosomal fusion event that yielded $n = 20$, a character which is readily coded in a cladistic matrix (Table 3). They also show an unusually strong tendency toward both auto- and especially allopolyploidy, a trend rather than a discrete character and hence less readily scored. It has long been known that the *D. majalis* complex (whose species richness continues to be much debated) is the allopolyploid product of repeated polyploidisation between *D. fuchsii*-like and *D. incarnata*-like parents (Heslop-Harrison 1954; Heslop-Harrison 1968; Hedrén *et al.* 2008; Paun *et al.* 2010; Hedrén *et al.* 2011; Balao *et al.* 2016). More recently, similar events have been demonstrated in eastern European analogues that include *D. cordigera* (Hedrén *et al.* 2007) (Fig. 3). Limited evidence has now accrued for a possible third allopolyploid complex occurring in the Sino-Himalayan region. Autopolyploidy has long been known to characterise *D. maculata* (Hagerup 1944) and has now been inferred to occur in the earlier-divergent *D. viridis* (R. Bateman, P. Rudall & I. Denholm, unpublished). Both primary hybrids and allotetraploids reliably place closer to their ovule-parent than their pollen parent in morphological analyses (Fig. 5), suggesting a significant element of epigenetic inheritance in determining the phenotype of the next generation.

Recognition arose only later that similar karyological fluidity pertains within both *Gymnadenia* s.s. (Marhold *et al.* 2005; Trávnicek *et al.* 2012) and the former genus *Nigritella* (Teppner & Klein 1985; Hedrén *et al.* 2000; D'Emerico & Grünanger 2001). Extensive surveys across Continental Europe of base genome size in *G. conopsea* s.s. and *G. densiflora* by the late Jan Suda and colleagues have revealed a startling complexity of cytotypes that are dominated by diploids, triploids and tetraploids and can extend as far as hexaploids (Trávnicek *et al.* 2011; Trávnicek *et al.* 2012). Moreover, *Gymnadenia* species routinely exhibit the unusual phenomenon of progressively partial endoreplication, duplicating most but not all of the genetic material present in the nucleus in the absence of cell division (Hribova *et al.* 2016). In contrast, the apparently exclusively British and Irish *G. borealis* proved to be almost wholly diploid, as did — albeit on the basis of more limited sampling — the Carpathian endemic *G. frivaldii* and more widespread *G. odoratissima* (Trávnicek *et al.* 2012). These observations cast some doubt on recent arguments that polyploidy may confer selective advantage in mixed-ploidy populations of *G. conopsea* s.s. and *G. densiflora* (e.g. Jersáková *et al.* 2010; Gross & Schiestl 2015). Nevertheless, the remarkable lability of ploidy levels within several species of both genera, combined with weak post-zygotic isolation, leaves the clade subject to iterative allopolyploidy of the kind most effectively detailed in the *Dactylorhiza majalis* complex.

The patterns of morphological diversification contrast between *Dactylorhiza* s.l. and *Gymnadenia* s.l. That of *Dactylorhiza* is more typical of eukaryotic clades in general, being fractal: the early-divergent species *D. iberica* and *D. viridis* have accumulated much greater character change than have later-diverging species (Fig. 7), to the extent that *D. viridis* remains accused by some observers of being better treated as a separate genus (reviewed by Bateman & Rudall 2018). Although the more derived species of *Dactylorhiza* differ in relatively few characters both morphologically and molecularly, they are readily distinguishable by field botanists with the exception of the allopolyploid complexes. And natural hybridisation occurs readily throughout the genus, wherever biogeography, habitat preference and phenology permit (Eccarius 2016; Stace *et al.* 2015). Hybrids between allotetraploids and their diploid parents are particularly frequent and are problematic to identify with confidence due to their strong similarity in both phenotype and genotype.

Remarkably, the converse pattern of morphological diversification apparently pertains within *Gymnadenia* (Fig. 7). Here, it is the earlier divergent species that are more likely to differ only subtly in both morphological and molecular properties, whereas the long-branch clade — the former genus *Nigritella* — is nested well within *Gymnadenia* s.l. in our trees. We were surprised that *G. odoratissima* did not place as sister to *'Nigritella'* in our morphological cladistic tree (Fig. 5), as these taxa share the unusual character states of whorled leaves and papillate bract cells (Table 3). Instead, it is *G. orchidis* that is improbably shown as sister to *'Nigritella'*, despite the fact that the proximal margins of their present distributions are separated by c. 4,000 km. The ITS-based placement of *G. densiflora* as sister to *'Nigritella'* is more consistent with biogeography and phenology (both taxa flower comparatively late) but is less persuasive in terms of either habitat preference or morphology. Thus, the identity of the sister-species to *'Nigritella'* remains in doubt.

The origin of the *'Nigritella'* clade has long fascinated one of us as bearing all the likely hallmarks of a saltational morphological speciation event (e.g. Bateman & DiMichele 2002). Specifically, the reduced flower size, simplified labellum and spur, and loss of resupination are features commonly attributed to pseudopeloria (Bateman & Rudall 2006) and would be more easily achieved through genetic or epigenetic suppression of a key developmental gene rather than through gradual acquisition of several smaller changes

during the short time-span that has been available to evolution to so radically modify the basic *Gymnadenia* morphology. The recognition that the diploid *G. gabasiana* is almost intermediate in ITS sequences between the remainder of *'Nigritella'* and *G. densiflora* (Fig. 4), yet apparently possesses all of the phenotypic features typical of the *'Nigritella'* morphology (Figs 5, 7), further reduces the time available for these evolutionary steps to accrue sequentially, and so increases the credibility of the saltational hypothesis.

In addition, the short molecular branch subtending *'Nigritella'* raises the possibility that this remarkable phenotype arose in lowland areas during one of the Quaternary glaciations, retreating to montane refugia as the climate ameliorated to temperate conditions. An initially periglacial environment would have largely constrained potential pollinating insects to small-bodied guilds that would have been more likely to be compatible with these small-bodied, small-flowered orchids than with larger-flowered antecedents. We further note that most of the later-derived *'Nigritella'* species resulting from allopolyploidy (the majority) indulge in autogamy rather than allogamy (Stahlberg 1999; Hedrén *et al.* 2000; Claessens & Kleynen 2011; Hedrén *et al.* 2018).

Setting aside *'Nigritella'*, the most striking aspect of the relationships inferred within *Gymnadenia* s.s. is the phylogenetic alternation of species that bear flowers that are (a) medium-sized (*G. conopsea*, *G. orchidis*, *G. densiflora*) versus small and (b) those that bear spurs that are long (the above plus *G. borealis*) versus short; these phenotypic groups do not form clades (Fig. 7). Reduction in plant and flower size has been sufficiently extensive in *G. frivaldii* (syn. *'Pseudorchis' frivaldii*) to require changes in several characters, resulting in phenotypic convergence with bona fide *Pseudorchis* (Bateman *et al.* 2006). We suspect that the larger flowers were ancestral to the genus, which subsequently experienced iterative bouts of paedomorphic heterochrony to produce the smaller-flowered taxa, culminating in the more profound phenotypic shifts evident in *G. frivaldii* and the former genus *Nigritella* (Box *et al.* 2008; Bateman 2012b). If so, morphological convergence would be confined to the smaller-flowered species of *Gymnadenia*, the larger-flowered species more closely representing the common ancestor of the genus (Fig. 7). In order for this hypothesis of iterative paedomorphosis to be explored more effectively, and to tease out the significance of the contrasting habitat preferences of the controversial western European species *G. conopsea* s.s., *G. densiflora* and *G. borealis*, continuous morphological characters need to be added to the more discrete characters already scored. This approach would transfer the analytical emphasis to the realm of morphometric ordination rather than phylogeny reconstruction. Such a study is currently in preparation, building on the morphometric comparison of *G. conopsea* and *G. densiflora* presented by Stark *et al.* (2011) but based on a much wider range of characters and comparing all of the European species of *Gymnadenia* s.s. (Bateman and Denholm 2019; R. Bateman, P. Rudall & I. Denholm, unpublished).

Gymnadenia conopsea s.s. was determined to be a mycorrhizal generalist by Stark *et al.* (2009), whereas Tesitelová *et al.* (2013) detected divergence between the mycorrhizal associates of both seeds and adult plants of diploid versus tetraploid individuals collectively assigned by them to *G. conopsea* s.s. Numerous authors have stated that *G. conopsea* s.s., like most orchids that offer substantial nectar rewards, is a generalist with regard to pollination (e.g. Vöth 2000; Meyer *et al.* 2007; Meekers *et al.* 2012; Sletvold *et al.* 2012a). Claessens & Kleynen (2011) listed 40 species of Coleoptera, Diptera, Hymenoptera and especially Lepidoptera that are implicated as its pollinators (although it is unlikely that all of these observations actually pertain only to *G. conopsea* s.s.). However, despite this broad spectrum of pollinators, Sun *et al.* (2015) provided evidence that there is little if any gene flow affecting *G. conopsea* where it co-occurs with *G. odoratissima*, at least within their Swiss study populations. Interest will no doubt be maintained in the pronounced fragrances of these plants, not least because recent research showed that diurnal and nocturnal emissions differ in quantitative composition and that nocturnal divergence between populations is greater than diurnal divergence (Chapurlat *et al.* 2018).

As with *Dactylorhiza*, most species of *Gymnadenia* that come into regular contact have proven capable of producing occasional natural hybrids to at least F_1 generation (although these are difficult to identify morphologically when the parents are phenotypically similar, as in the case of *G. conopsea* s.s. × *G. densiflora*: reviewed by Bateman *et al.* 2006); moreover, *Gymnadenia* species cross readily in cultivation. For example, *G. conopsea* s.s. and *G. odoratissima* can produce natural hybrid swarms even in undisturbed habitats (R. Bateman, unpublished) and are easily artificially crossed as far as an F_3 generation (although the F_3 plants appear surprisingly unappealing to potential pollinating insects: S. Malmgren, pers. comm. 2015). Indeed, Sun *et al.* (2015) showed that the two species owe their restricted gene flow largely to pre-zygotic pollinator choice, which is assumed to be influenced primarily by demonstrated differences in the composition of their respective scent cocktails. On the other hand, frequent hybridisation between species of *Gymnadenia* and those of *Dactylorhiza* indicates that at least some pollinators remain blissfully ignorant of the behaviour that they are required by theory to exhibit (reviewed by Bateman *et al.* 2017).

Overall, a striking diversity of evolutionary mechanisms are implicated as having contributed appreciably to the diversity of both characters and species evident within the digitate-tubered clade (Bateman 2009; Bateman 2012b). They include allopolyploidy, autopolyploidy, mutationally driven lineage divergence ('dichotomous saltation' sensu

Bateman & DiMichele 2002), geographical isolation, diversification of ecotypes into contrasting habitats, autogamy, and perhaps even pollinator switching.

Conclusions

1) Several molecular phylogenetic studies based on Sanger sequencing have provided an optimal circumscription of genera within the taper-tubered clade of subtribe Orchidinae (although we remain unable to confidently incorporate the monotypic genus *Neolindleya* into either *Galearis* s.l. or *Platanthera* s.l.). However, the approach has been less successful at determining relationships among the genera. The best-supported phylogenetic nodes are the focus of this paper — those subtending the digitate-tubered clade (*Dactylorhiza* s.l. plus *Gymnadenia* s.l.) and the taper-tubered clade (the two digitate-tubered genera plus *Platanthera*, *Galearis* and *Neolindleya*). Ironically, it is the genus-level circumscriptions that continue to attract greatest criticism (reviewed by Bateman 2012a), despite the fact that they are the most conclusive result of two decades of molecular phylogenetic study pursued in a rigorous conceptual framework by multiple research groups.
2) Intriguingly, the application of parsimony to our morphological cladistic matrix reproduced a topology (albeit highly unstable) that closely resembled the most common genus-level classification of Orchidinae achieved through traditional authoritarian taxonomy, prior to the advent of molecular phylogenetics. The pre-molecular classification would, from first principles, have been predicted to have been more closely mirrored by phenetic analyses of our matrix, yet the equivalent NJ-based and especially UPGMA-based morphological topologies diverge more strongly from molecular topologies.
3) The contrasting phylogenetic topologies obtained here between nuclear ribosomal, plastid and morphological cladistic matrices challenge the wisdom of the now near-ubiquitous practice of routinely combining such data sets, given that they evolve within highly contrasting milieux. Similar conclusions have been reached following studies of other groups of orchids (e.g. van de Niet & Linder 2008; Sramkó *et al.* 2014; Tang *et al.* 2015; Pérez *et al.* 2016). Our results also emphasise the crucial importance of intensive sampling of species across the groups of interest. It remains to be seen whether topologies based on matrices obtained through next-generation sequencing technologies (reviewed by Olson *et al.* 2016) will offer the substantially greater stability that some commentators anticipate (e.g. Kropf *in* Kadereit *et al.* 2016; Pellegrino & Cozzolino 2016); our initial results suggest that only modest improvements are likely (Bateman *et al.* 2017; G. Sramkó, R. Bateman & O. Paun, unpublished; R. Bateman, P. Rudall & O. Pérez, unpublished). Unfortunately, morphological cladistic matrices — the foundation of the phylogenetics revolution and still a valuable component of any genuinely integrated comparative study — are rarely produced today, having become seriously under-valued by the research community.
4) We offer further support for earlier pre-molecular (e.g. Vermeulen 1947; Heslop-Harrison 1954, Vermeulen 1977) and molecular phylogenetic (e.g. Bateman *et al.* 1997; Pridgeon *et al.* 1997; Bateman *et al.* 2006) recognition that tapered tubers (those expanded into one or more distal roots) delimit the broader clade, and that apically divided digitate tubers plus a chromosomal fusion event ($n = 21 > 20$) plus a predisposition to polyploidy together delimit the digitate-tubered clade.
5) Mapping of 51 morphological cladistic characters across (i.e. constrained to) an ITS-based molecular topology increased levels of homoplasy in an already highly homoplastic morphological matrix by a further 20%, indicating an exceptionally high degree of evolutionary lability within the digitate-tubered clade that reflects convergence and so-called 'losses' of features, although in truth, paedomorphic reduction is a more frequent phenomenon than complete loss.
6) NrITS remains the best single region of choice for classic phylogenetic purposes approximating the genus level, due to its high mutation rate and coalescence properties (e.g. Hein *et al.* 2004; Bateman 2018). Ironically, these properties are often misrepresented as a negative feature, the term 'concerted evolution' often being applied in a pejorative context. Nonetheless, the failure of ITS to distinguish between the morphologically distinct species *Gymnadenia conopsea* s.s. and *G. odoratissima* demonstrates that at least a small minority of bona fide orchid species exist within the "genetic divergence lag phase" of Bateman (2016), thereby showing that even ITS is not a panacea for species delimitation. The present study will be used as a framework for a future paper comparing in detail the morphometric properties of all species of *Gymnadenia* other than those from southeast Asia (R. Bateman, P. Rudall & I. Denholm, unpublished), with the aim of better characterising these problematic species in a way more useful to typical field botanists.
7) One clear message to emerge from this study (yet again) is the great desirability, when circumscribing species and/or attempting to understand the underlying speciation mechanisms, of combining genetic studies with morphometric surveys of the same study populations. Most of the molecular studies cited in this paper (including many of those published by the present authors) have relied entirely on molecular data for their conclusions, yet it is phenotypes rather than genotypes that dictate any form of interaction between organisms. For example, the failure to collect morphometric data in most studies (the notable exception being Stark *et al.*

2011) when studying the relationship between *Gymnadenia conopsea* (diploid), *G. conopsea* (tetraploid) and *G. densiflora* (reputedly mostly diploid) has left the significance of studies of these three (or more?) biological entities virtually indecipherable — it is not clear exactly which phenotypes have been studied (and would not be even if herbarium vouchers were available for each plant studied — they are inevitably subject to post-mortem changes in morphometric parameters).

8) *Dactylorhiza* s.l. has become a model system for studying the adaptive and/or epigenetic consequences of ploidy change (Paun *et al.* 2010; Paun *et al.* 2011; Balao *et al.* 2016; Balao *et al.* 2017), while *Gymnadenia* s.s. has provided valuable information about the phylogeography of ploidy change (Trávnicek *et al.* 2011; Trávnicek *et al.* 2012) and the attraction of pollinators to orchid species that offer substantial nectar rewards (Huber *et al.* 2005; Lönn *et al.* 2006; Jersaková *et al.* 2010; Sletvold & Agren 2011; Sletvold *et al.* 2012a; Gupta *et al.* 2014; Gijbels *et al.* 2015; Gross & Schiestl 2015; Sun *et al.* 2015). Within *Gymnadenia* s.l., the radically morphologically divergent former genus *Nigritella* remains a strong candidate for having evolved from within *Gymnadenia* s.s. by saltational rather than gradual evolutionary mechanisms (Bateman & DiMichele 2002) and should be a prime target for future evolutionary-developmental genetic study. The ability of the genus to diversify into highly contrasting ecotypes (cf. *G. conopsea* s.s., *G. densiflora* and *G. borealis* in the British Isles) also merits investigation.

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