

Phylogenetics of the slipper orchids (*Cypripedioideae*, *Orchidaceae*): nuclear rDNA ITS sequences

ANTONY V. COX, ALEC M. PRIDGEON, VICTOR A. ALBERT, and MARK W. CHASE

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Abstract: *Cypripedioideae* (*Orchidaceae*) have been the subject of numerous taxonomic treatments with conflicting interpretations of relationships among the five genera and the 150–170 species. We have produced nuclear ribosomal ITS nucleotide sequences for nearly 100 slipper orchid species and used parsimony analysis to investigate their relationships. Our results demonstrate that each genus, as currently circumscribed, is monophyletic (*Mexipedium* and *Selenipedium* being represented by a single taxon). Like *rbcL* data, ITS sequences place *Mexipedium* sister to *Phragmipedium*. Relationships at the sectional level in *Paphiopedilum* are largely as described by CRIBB. However, the division of *Paphiopedilum* into subgg. *Brachypetalum* and *Paphiopedilum* is not supported; subg. *Brachypetalum* is paraphyletic to subg. *Paphiopedilum*. *Phragmipedium* species are divided into the same three major clades as in the taxonomic scheme of McCook. The plicate-leaved genera, *Cypripedium* and *Selenipedium*, are successive sister groups to the rest of the subfamily, confirming generally held opinions that they display plesiomorphic characters compared to the conduplicate-leaved genera. A survey of karyotypes in the context of the ITS tree reveals a general trend toward increased chromosome number, probably brought about by centric fission. These data also accord with a previously suggested biogeographic hypothesis of a widespread Northern Hemisphere distribution, followed by range fragmentation due to Miocene cooling.

The orchid subfamily *Cypripedioideae* LINDLEY has been considered a distinct lineage since LINDLEY (1840) separated them by virtue of their possession of two fertile anthers and the mistaken belief that they all possessed unilocular ovaries. Despite this oversight, the subfamily is clearly circumscribed by a number of synapomorphies which include a deeply saccate labellum, two fertile stamens, a shield-like staminode and a synsepal composed of the fused lateral sepals.

The distinct morphological characteristics of slipper orchid flowers have fascinated botanists and horticulturists for well over a hundred years. Their flowers are highly modified to attract pollinating bees and flies by deceit (DRESSLER 1993). Those insects enticed into the inflated median petal, or labellum, may escape from the flower only by passing the stigma and anther where pollen masses are removed

and/or deposited. Reliance upon such a complex pollination mechanism has no doubt been a significant constraint on the evolution of floral diversity and makes *Cypripedioideae* an instantly recognisable orchid group.

Slipper orchids have a wide geographical distribution and occupy diverse habitats. They range from trunk epiphytes in wet tropical forests and tall bamboo-like shrubs in lowland tropical understories to terrestrial herbs in Arctic zones. The five genera of slipper orchids occupy individual geographical ranges that are much more restricted. *Selenipedium* RCHB. f. and *Phragmipedium* ROLFE are restricted to northern South America and Mesoamerica, whereas *Cypripedium* L. is widely distributed throughout most of the temperate Northern Hemisphere. *Mexipedium* V. A. ALBERT & M. W. CHASE is a narrow endemic, found only in the state of Oaxaca in Mexico, whereas *Paphiopedilum* PFITZER is distributed from the Himalayas and southern China through Malaysia to Guadalcanal. *Paphiopedilum druryi* (BEDD.) STEIN occurs only in southern India. No slipper orchids are known from Africa or Australia.

Some taxonomists take the view that, in addition to the apostasioids, the slipper orchids form an “ancestral” group of orchids insofar as they are likely to approximate the hypothetical orchid ancestor (DRESSLER & DODSON 1960). However, anatomical studies by STERN & al. (1993) found no evidence to support the view that apostasioids are “primitive” orchids, a view that could be carried over to the cypripedioids as well. Until outgroup relationships are better known, these hypotheses are highly speculative. Recent studies of *rbcL* have identified several potential outgroups, but these require further work because they themselves are poorly known (DRESSLER & CHASE 1995, CHASE & al. 1995). RASMUSSEN in DAHLGREN & al. (1985) elevated the *Cypripedioideae* to family rank and treated it as the sister group to *Orchidaceae* s. str. Despite this, the precise relationships between diandrous and monandrous orchids remain to be clearly defined. Recent molecular studies (CHASE & al. 1994; CAMERON & al., unpubl.) have largely supported the position of *Cypripedioideae* as a distinct and potentially ancestral group (i.e. one that is sister to the rest of *Orchidaceae* excluding *Apostasioideae*).

The cypripedioids have received much attention from taxonomists in the last two centuries (LINNAEUS 1753; RAFINESQUE 1836; LINDLEY 1840; REICHENBACH 1854; PFITZER 1888, 1894, 1903; ROLFE 1896; HALLIER 1897; KRAENZLIN 1897; BRIEGER 1971; ATWOOD 1984). Great interest in the plants has prompted a large number of studies and revisions that often conflict with each other (Table 1). Following descriptions of the first species over two hundred years ago, there was a long period of instability in generic nomenclature. LINNAEUS (1753) recognised only one species and a number of varieties. LINDLEY (1840) recognised 22 species, all of which he placed in *Cypripedium*. REICHENBACH (1854: 1) recognised ovary condition as an important character and created *Selenipedium* which initially comprised all taxa with unilocular ovaries. BENTHAM & HOOKER (1883) recognised both genera (sensu REICHENBACH). PFITZER (1886) segregated the conduplicate-leaved taxa into *Paphiopedilum*, and ROLFE (1896) followed this by transferring all Neotropical conduplicate taxa to *Phragmipedium*. Following a period of confusion over this set of nomenclatural changes, most authors now recognise four genera. Recently a fifth genus, *Mexipedium*, was created to resolve conflicting taxonomic characters in *Phragmipedium* and *Paphiopedilum* (ALBERT & CHASE 1992). ALBERT

Table 1. Taxonomy of the *Cyripedioideae*

LINDLEY (1840)	PFITZER (1888)	PFITZER (1894)	ROLFE (1896)	KRAENZLIN (1897)	BRIEGER (1971)	ALBERT & CHASE (1992)	ALBERT & PETTERSSON (1994)
	<i>Selenipedium</i> <i>Cyripedium</i>	<i>Selenipedium</i> <i>Cyripedium</i>	<i>Selenipedium</i> <i>Cyripedium</i>	sect. <i>Selenipedium</i> sect. <i>Calceolaria</i> <i>Calceolus</i> group <i>Arietina</i> group	tribe <i>Selenipedieae</i> tribe <i>Cyripedioideae</i>	<i>Selenipedium</i> <i>Cyripedium</i>	<i>Selenipedium</i> <i>Cyripedium</i>
<i>Arietinium</i> group	<i>Arietina</i> group	<i>Arietinum</i> group					
<i>Foliosa</i> group	<i>Foliosa</i> group	<i>Eucyripedium</i> group <i>Trigonopedium</i> group		<i>Macrantha</i> group <i>Obtusifolia</i> group			
<i>Bifolia</i> group <i>Acaulia</i>	<i>Diphylla</i> group <i>Paphiopedilum</i> (excl. <i>C. acaule</i>) <i>Genuina</i> group	<i>Paphiopedilum</i> <i>Coelopedium</i> group <i>Eremantha</i> <i>Polyantha</i>	<i>Paphiopedilum</i>	<i>Bifolia</i> group sect. <i>Barbata</i> sect. <i>Concoloria</i> sect. <i>Insignia</i>	tribe <i>Paphiopedileae</i>	<i>Paphiopedilum</i> subg. <i>Paphiopedilum</i>	<i>Paphiopedilum</i> subg. <i>Paphiopedilum</i>
<i>C. caudatum</i>	<i>C. caudata</i> group	<i>Phragmopedium</i> group	<i>Phragmipedium</i>	sect. <i>Lortifolia</i> sect. <i>Caudata</i>	tribe <i>Phragmipedioideae</i>	<i>Phragmipedium</i> subg. <i>Phragmipedium</i>	<i>Paphiopedilum</i> subg. <i>Phragmipedium</i>
						<i>Mexipedium</i>	<i>Paphiopedilum</i> subg. <i>Mexipedium</i>

& PETTERSSON (1994) recently combined all conduplicate genera (*Paphiopedilum*, *Mexipedium* and *Phragmipedium*) under *Paphiopedilum*.

Generic boundaries in *Cypripedioideae* have traditionally been defined using a combination of four characters: leaf type, vernation, locule number and placentation. Unfortunately, all are individually inconsistent. Reliance upon these "critical" characters has been questioned (ATWOOD 1984), although no more obvious ones have yet been found (a number of correlated but cryptic characters exist, e.g., perianth aestivation and persistence after flowering). *Paphiopedilum*, *Phragmipedium* and *Mexipedium* have conduplicate leaves, and *Selenipedium* and *Cypripedium* have plicate leaves. *Paphiopedilum* and *Cypripedium* are unilocular with parietal placentation, whereas *Selenipedium* and *Phragmipedium* are trilocular with axile placentation. The recent description of conduplicate-leaved *Phragmipedium xerophyticum* SOTO, SALAZAR & HAGSATER (SOTO & al. 1990) served only to blur the distinctions among existing genera because it possesses a mixture of *Paphiopedilum*-like and *Phragmipedium*-like features. Its New World distribution (Mexico) is like that of *Phragmipedium*, but it possesses unilocular ovaries like the Old World genus *Paphiopedilum*. *Phragmipedium xerophyticum* may be distinguished from *Paphiopedilum* by having branched racemes and valvate sepal aestivation. Because of its mixed suite of characters ALBERT & CHASE (1992) transferred *Phragmipedium xerophyticum* to a new monotypic genus, *Mexipedium*. Although ALBERT & PETTERSSON'S (1994) combination of all conduplicate-leaved genera under *Paphiopedilum* has not been widely accepted, and the three conduplicate genera are still generally recognised, this union certainly solved many of the apparent inconsistencies in slipper orchid taxonomy.

The boundaries of most genera have been supported by crossing experiments. Extensive horticultural interest in the slipper orchids has resulted in many interspecific and complex hybrids being produced. As yet no verified intergeneric hybrids have been registered, although *Mexipedium xerophyticum* has been crossed with many *Phragmipedium* species (H. KOPOWITZ, pers. comm.). Hybridisation has never been used as a generic character in orchids, and thus neither accords with, nor refutes, either the generic status of *Mexipedium* or the broad concept of *Paphiopedilum* (sensu ALBERT & PETTERSSON 1994).

To investigate further the nature of these generic and subgeneric relationships we have obtained DNA sequence data for nearly 100 of the 122 slipper orchid species (DRESSLER 1993) and here demonstrate that the division of *Cypripedioideae* into five genera is entirely appropriate from the perspective of monophyly. Although botanists have always been able to suggest taxon groupings, few hypotheses of relationships among these groups have been advanced (but see ALBERT 1994). We have conducted the most comprehensive molecular study of an orchid subfamily to date and interpret our findings with reference to existing morphological, cytological and biogeographic data.

Materials and methods

Plant material. Details of plant materials, voucher information, chromosome numbers and geographical distributions are listed in Table 2. Vouchers are located in the Royal Botanic Gardens, Kew Herbarium (K) and/or the Kew spirit collection. Karyotype data are from

Table 2. Plant materials used in this study. ¹ chromosome numbers not available indicated by question mark, number of telocentric chromosomes in complement indicated in parentheses, where known; ² character states used in cladistic biogeography: 1 USA/Canada/N Mexico, 2 S Mexico/Central America, 3 South America, 4 temperate Asia, 5 subtropical Asia, 6 tropical Asia

Taxon	Taxonomic placement	Voucher	Chromosome number (2n) ¹	Zone ²	EMBL acc. no.	Geographical distribution
<i>Selenipedium</i>	(sensu PRITZER 1888)	ALBERT 166	?	3	Z78534	Panama
<i>S. chica</i> RCHB.						
<i>Cypripedium</i>	(sensu PRITZER 1888)	TC3	?	1	Z78525	USA, Canada
<i>C. acaule</i> AITON		TCGB1	20	4	Z78521	Europe, Russian Fed., China
<i>C. calceolus</i> L.		ALBERT 68	?	1	Z78532	USA (Oregon)
<i>C. californicum</i> A. GRAY		ALBERT 176	20	1	Z78531	USA (Colorado)
<i>C. fasciculatum</i> KELL. ex S. WATTS.		TC6	?	4	Z78517	China, Tibet
<i>C. flavum</i> WARD		ALBERT 91	20	4	Z78524	Taiwan
<i>C. formosanum</i> HAY		(C.J. SHEVIAK 2845, NYS)				
<i>C. guttatum</i> Sw.		ALBERT 72	20	1, 4	Z78526	USA, Russian Fed., China, Japan, India, Korea, Nepal, Bhutan
<i>C. himalaicum</i> ROLFE		ALBERT70	20	4	Z78523	Nepal, Bhutan
<i>C. irapeanum</i> LLAVE & LEX.		ALBERT47	?	2	Z78533	Nepal, Bhutan
<i>C. lichiangense</i> CRIBB		TC1	?	4	Z78529	Guatemala, Honduras, Mexico
<i>C. macranthum</i> Sw.		ALBERT 123	20	4	Z78522	China
<i>C. margaritaceum</i> FRANCH.		ALBERT 113	?	4	Z78530	China, Japan, Russian Fed., Korea, Taiwan
<i>C. passerinum</i> RICHARDS		ALBERT 48	20	1	Z78516	China
<i>C. plectrochilum</i> FRANCH.		ALBERT 124	?	4	Z78528	USA, Canada
<i>C. pubescens</i> WILLD.		ALBERT 80	20	1	Z78519	Russian Fed., China
<i>C. reginae</i> WALT.		ALBERT 83	20	1	Z78518	USA (Michigan)
<i>C. segawai</i> MASAMUME		ALBERT 79	20	4	Z78520	Russian Fed., China
<i>C. yatabeanum</i> MAKINO		(C.J. SHEVIAK 2952, NYS)	20	4	Z78527	Russian Fed., China

(Contd.)

Table 2 (continued)

Taxon	Taxonomic placement	Voucher	Chromosome number (2n) ¹	Zone ²	EMBL acc. no.	Geographical distribution
<i>Phragmipedium</i>						
	(sensu McCook 1989)					
<i>P. besseae</i> DODSON & J. KUHN	sect. <i>Micropetalum</i>	ALBERT 8	24,28 ³	3	Z78513	Ecuador, Peru
<i>P. schlimii</i> LINDEN & RCHB. f.		ALBERT 128	30 (24)	3	Z78514	Colombia
<i>P. caricinum</i> (LINDL. & PAXTON) ROLFE	sect. <i>Phragmipedium</i>	1988-1360	20,22	3	Z78510	Bolivia
<i>P. caudatum</i> (LINDL.) ROLFE		ALBERT 170	28 (20)	2,3	Z78501	Costa Rica, Guatemala, Panama, Bolivia, Ecuador, Peru
<i>P. exstaminodium</i> (LINDL.) ROLFE		1986-2484	?	1	Z78511	Mexico
<i>P. lindleyi</i> (LINDL.) DRESSLER & N. H. WILLIAMS		ALBERT 17	28 (20)	3	Z78507	Colombia, Ecuador
<i>P. pearcei</i> (RCHB. f.) RAUH & SENGHAS		ALBERT 131	20 (4), 21,22	3	Z78509	Ecuador, Peru
<i>P. wallisii</i> (RCHB. f.) GARAY		1983-344	28 (20)	3	Z78512	Colombia, Ecuador
<i>P. warszewiczianum</i> (RCHB. f.) GARAY		ALBERT 130	28	3	Z78500	Panama
<i>P. boissierianum</i> (RCHB. f.) ROLFE	sect. <i>Lorifolia</i>	ALBERT 129	18	3	Z78502	Ecuador, Peru
<i>P. czerwiakowianum</i> (RCHB. f.) ROLFE		ALBERT 121	?	3	Z78503	Peru
<i>P. longifolium</i> (RCHB. f. & WARSZ.) ROLFE		ALBERT 174	21 (5) 23 (7)	2,3	Z78508	Costa Rica, Panama, Colombia, Ecuador
<i>P. kaeteurum</i> (N. E. BR.) GARAY	sect. <i>Platypetalum</i>	ALBERT 7	?	3	Z78504	Venezuela
<i>P. lindleyanum</i> (LINDL.) ROLFE		ALBERT 108	22 (10)	3	Z78506	Brazil, French Guiana, Guyana, Suriname, Venezuela
<i>P. sargentianum</i> ROLFE		1984-1461	22 (10)	3	Z78505	Brazil
<i>Mexipedium</i>						
<i>M. xerophyticum</i> (SOTO ARENAS, SALAZAR & HAGSATER) V. A. ALBERT & M. W. CHASE	(ALBERT & CHASE 1992)	ALBERT 148	?	2	Z78515	Mexico
<i>Paphiopedilum</i>						
	(sensu CRIBB 1987)					
<i>P. bellatulum</i> (RCHB. f.) STEIN	subg. <i>Brachypetalum</i>	ALBERT 140	26	5	Z78492	China, Burma, Thailand, Laos
<i>P. concolor</i> (LINDL.) PFTIZER	sect. <i>Concoloria</i>	ALBERT 11	26	5	Z78491	China, Laos, Burma, Thailand, Vietnam
<i>P. godefroyae</i> (GODEFR.-LEBEUF) STEIN		ALBERT 162	26	5	Z78493	Thailand
<i>P. niveum</i> (RCHB. f.) STEIN		1987-3331	26	5,6	Z78494	Thailand, Malaysia
<i>P. armeniacum</i> CHEN & LIU	sect. <i>Parvisepalum</i>	648	26	5	Z78496	China

<i>P. delenatii</i> GULL.	ALBERT 141	26	5	Z78497	Vietnam
<i>P. emersonii</i> KOOP. & P. J. CRIBB	ALBERT 158	26	5	Z78495	China
<i>P. malipoense</i> CHEN & TSI	ALBERT 12	26	5	Z78498	China, Vietnam
<i>P. micranthum</i> T. TANG & F. T. WANG	629	26	5	Z78499	China, Vietnam
subg. <i>Paphiopedilum</i>					
sect. <i>Coryopeditum</i>					
<i>P. adductum</i> ASHER	ALBERT 96	?	5	Z78468	Philippines
<i>P. glanduliferum</i> (BLUME) STEIN "A"	1953-3850	26	6	Z78463	Indonesia, Papua New Guinea
<i>P. glanduliferum</i> (BLUME) STEIN "B"	1986-02309	26	6	Z78464	Indonesia, Papua New Guinea
<i>P. kolopakingii</i> FOWLE	633	?	6	Z78474	Indonesia
<i>P. philippinense</i> (RCHB. f.) STEIN	ALBERT 25	26	5,6	Z78466	Malaysia, Philippines
<i>P. rothschildianum</i> (RCHB. f.) STEIN	ALBERT 133	26	6	Z78465	Malaysia
<i>P. sanderianum</i> (RCHB. f.) STEIN	ALBERT 34	?	6	Z78473	Malaysia
<i>P. stonei</i> (HOOK.) STEIN	ALBERT 13	26	6	Z78467	Malaysia
<i>P. supardii</i> BRAEM & LOEB	ALBERT 118	26	6	Z78475	Malaysia
<i>P. dianthum</i> T. TANG & F. T. WANG	GREL	?	5	Z78471	China
<i>P. haynaldianum</i> (RCHB. f.) STEIN	ALBERT 109	26	5	Z78469	Philippines
<i>P. lowii</i> (LINDL.) STEIN	ALBERT 30	26	6	Z78472	Indonesia, Malaysia
<i>P. parishii</i> (RCHB. f.) STEIN	ALBERT 2	26	5	Z78470	China, Burma, Thailand
<i>P. glaucophyllum</i> J. J. SMITH	ALBERT 28	36 (22), 37	6	Z78476	Indonesia
sect. <i>Cochlopetalum</i>					
<i>P. primulum</i> M. WOOD & TAYLOR	634	32 (14)	6	Z78479	Indonesia
<i>P. victoria-mariae</i> (ROLFE) ROLFE	ALBERT 116	?	6	Z78477	Indonesia
<i>P. victoria-regina</i> (SANDER) M. WOOD	630	32,33,34, 35,36	6	Z78478	Indonesia
sect. <i>Paphiopedilum</i>					
<i>P. barbigerum</i> T. TANG & F. T. WANG	ALBERT 106	26	5	Z78486	China
<i>P. charlesworthii</i> (ROLFE) PFITZER	632	26	5	Z78484	Burma, Thailand
<i>P. druryi</i> (BEDD.) STEIN	Albert117	30 (8)	5	Z78489	India
<i>P. exul</i> (RIDLEY) ROLFE		26	5	Z78482	Thailand
<i>P. fairieanum</i> (LINDL.) STEIN	ALBERT 107				
<i>P. gratixianum</i> (MASTERS) GULL.	ALBERT 185	26	4	Z78490	Bhutan, India
<i>P. henryanum</i> BRAEM	ALBERT 112	?	5	Z78480	Laos, Vietnam
<i>P. hirsutissimum</i> (LINDL. ex HOOK.) STEIN	O645	?	5	Z78485	China, Vietnam
<i>P. tigrinum</i> KOOP. & N. HASEG.	ALBERT 105	26	5	Z78487	China, India, Bhutan
<i>P. insigne</i> (WALL. ex LINDL.) PFITZER	713	?	4	Z78488	China
<i>P. villosum</i> (LINDL.) STEIN	ALBERT 110	26	4	Z78481	India, Nepal
<i>P. acmodontium</i> SCHOSER ex M. WOOD	ALBERT 111	26	5	Z78483	India, Burma, Thailand
<i>P. appletonianum</i> (GOWER) ROLFE	ALBERT 135	36 (20)	5	Z78446	Philippines
	ALBERT 102	38 (24)	5	Z78444	China, Cambodia, Laos, Thailand, Vietnam

Table 2 (continued)

Taxon	Taxonomic placement	Voucher	Chromosome number (2n) ¹	Zone ²	EMBL acc. no.	Geographical distribution
<i>P. argus</i> (RCHB. f.) STEIN		ALBERT 36	38 (24)	5	Z78448	Philippines
<i>P. barbatum</i> (LINDL.) PFITZER		ALBERT 145	38 (28)	6	Z78439	Malaysia
<i>P. bullenianum</i> (RCHB. f.) PFITZER		ALBERT 182	40 (28)	6	Z78442	Borneo
			40 (28) ⁴			
			42 (32) ⁵			
<i>P. bougainvilleanum</i> FOWLIE		ALBERT 97	40 (28)	6	Z78452	Bougainville
<i>P. callosum</i> (RCHB. f.) STEIN		ALBERT 3	32 (12)	5	Z78457	Cambodia, Laos, Thailand, Vietnam
<i>P. ciliolare</i> (RCHB. f.) STEIN		ALBERT 33	32 (12)	5	Z78460	Philippines
<i>P. dayanum</i> (LINDL.) STEIN		ALBERT 136	36 (20)	6	Z78459	Malaysia
<i>P. fowleii</i> BIRK		1984-1266	36 (20)	5	Z78454	Philippines
<i>P. hennisianum</i> (M. WOOD) FOWLIE		ALBERT 38	36 (18)	5	Z78458	Philippines
<i>P. hookerae</i> (RCHB. f.) STEIN		ALBERT 142	28 (0)	6	Z78451	Indonesia, Malaysia
<i>P. javanicum</i> (REINW. ex LINDL.) PFITZER		635	38 (24)	6	Z78455	Indonesia, Malaysia
<i>P. lawrenceanum</i> (RCHB. f.) PFITZER		ALBERT 115	36 (20)	6	Z78433	Malaysia
<i>P. masterianum</i> (RCHB. f.) STEIN		ALBERT 19	36 (20)	6	Z78449	Indonesia
<i>P. papuanum</i> (RIDL.) RIDL.		O823	?	6	Z78450	Indonesia, Papua New Guinea
<i>P. purpuratum</i> (LINDL.) STEIN		ALBERT 180	40 (28)	5	Z78440	China, Vietnam
<i>P. schoseri</i> BRAEM & H. MOHR		ALBERT 138	?	6	Z78453	Indonesia, Malaysia
<i>P. sukhakulii</i> SCHOSER & SENGHAS		ALBERT 35	40 (28)	6	Z78462	Indonesia
<i>P. superbiens</i> (RCHB. f.) PFITZER		ALBERT 155	38 (24)	6	Z78441	Indonesia
			36 (20) ⁶			
<i>P. tonsum</i> (RCHB. f.) STEIN		ALBERT 98	32 (12)	6	Z78456	Indonesia
<i>P. urbanianum</i> FOWLIE		ALBERT 39	?	5	Z78445	Philippines
<i>P. venustum</i> (WALL.) PFITZER ex STEIN		ALBERT 101	40 (28)	4	Z78447	Bhutan, India, Nepal
<i>P. wardii</i> SUMMERH.		ALBERT 134	41 (29)	5	Z78461	Burma

³ WIMBER (1993) and pers. comm.⁴ *Paphiopedilum johrense* FOWLIE & YAP = *P. bullenianum* (CRIBB 1987)⁵ *Paphiopedilum celebensis* FOWLIE & BIRK = *P. bullenianum* (CRIBB 1987)⁶ *Paphiopedilum curtisii* (RCHB. f.) STEIN = *P. superbiens* (CRIBB 1987)

KARASAWA (1979), KARASAWA & Aoyama (1986, 1988), KARASAWA & TANAKA (1981) and ATWOOD (1984). Within *Cypripedioideae* generic delimitations are controversial: AVC, AMP, and MWC prefer to recognize five genera whereas VAA prefers to recognize three; the former circumscription is used throughout this paper.

Molecular techniques. DNA was extracted from either fresh or silica-gel dried material (CHASE & HILLS 1991) according to the methods described by DOYLE & DOYLE (1987). Nuclear ribosomal internal transcribed spacers were amplified using the methods and primers described by BALDWIN (1992). Amplified double stranded DNA fragments (~750bp) were purified using the "Wizard" DNA cleanup system (Promega) and directly sequenced on an ABI 373A automated sequencer using standard dye-terminator chemistry following manufacturer's protocols (Applied Biosystems Inc.). For sequence editing and assembly of the two complimentary strands, "Sequence Navigator" and "AutoAssembler" (Applied Biosystems Inc.) were used. Generally two sequencing reactions per taxon were required and in most cases greater than 80% strand overlap was achieved.

DNA sequences were aligned by eye and using ClustalW for Power Macintosh (THOMPSON & al. 1995). Gaps were coded as missing values. Although regions of insertion/deletion ("indel") activity were found, no sequence data were excluded from the analysis. Six species of *Vanilla* MILL. were designated as outgroup taxa. All cladistic analyses were performed using PAUP version 3.1.1 (SWOFFORD 1993). The data matrix was analysed in the first place using 150 replicates of random taxon addition order, tree bisection-reconnection (TBR) branch swapping, MULPARS, and with all character transformations weighted equally and unordered (Fitch parsimony; FITCH 1971). Only ten trees were saved from each replicate, which minimises the large amounts of time searching on sub-optimal "islands" with thousands of trees (MADDISON 1991). All trees thus collected were used as starting trees, with MULPARS on, in a heuristic search with the same search and weighting criteria mentioned above until the number of trees exceeded memory capacity (approximately 8000 trees were swapped). Successive weighting was used to improve the data matrix (FARRIS 1969) rather than as a means of selecting a subset of most parsimonious trees (CARPENTER 1988). A random subset of 2000 most-parsimonious trees retained during branch swapping was selected for successive weighting. Different random subsets were tested without influence on resulting weighted trees. Tree and character manipulations were performed using MacClade version 3.05 (MADDISON & MADDISON 1992). Internal character support for various branching diagrams was assessed using 1000 bootstrap replicates and weights derived from successive weighting (FELSENSTEIN 1985) and by 10000 parsimony jackknife replicates with equal weights ("Parsimony Jackknifer" version 4.22; FARRIS & al. 1996).

Results

The cladistic analysis of 102 taxa yielded more than 8000 most-parsimonious trees (the maximum permitted by available memory) of length 3249 steps with a consistency index (CI)=0.431 and a retention index (RI)=0.784. Successive weighting identified 14 most-parsimonious trees of Fitch length 3250 steps (CI=0.458, RI=0.784). A generalised diagram of the strict consensus of all 14 trees indicating generic relationships is shown in Fig. 1. Numbers above branches indicate nucleotide substitutions along each branch. Clades weakly supported by the jackknife procedure are indicated on all Figures with open circles, whereas those that receive strong support are indicated by filled circles. Arrows indicate branches that collapse in the strict consensus of all 14 trees. Numbers in

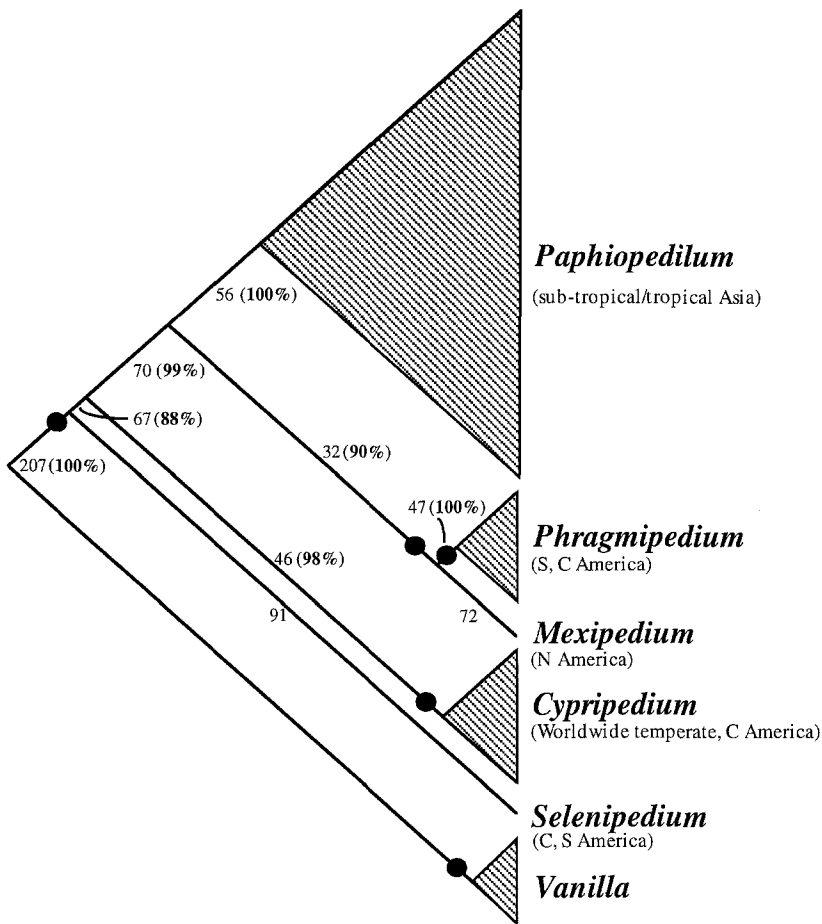


Fig. 1. Strict consensus of all 14 successively weighted, most-parsimonious trees indicating general cladistic relationships among the five slipper orchid genera, using *Vanilla* as an outgroup

parentheses indicate percent support for 1000 bootstrap replicates using values determined during successive weighting.

Insertion/deletion (“indel”) activities in the ITS region are useful evolutionary events because they provide clear synapomorphies that can be used to group taxa with a degree of confidence. These characters may be analysed independently of the nucleotide sequence data if sufficient numbers exist in the data set to make such an analysis valuable. Within subg. *Brachypetalum*, *P. niveum* (RCHB. f.) STEIN and *P. godefroyae* (GODEFR.-LEBEUF) STEIN share a 13bp deletion (at nucleotide positions 742–756) in the aligned matrix. In addition, all species comprising section *Cochlopetalum* share a 25bp deletion (at nucleotide position 173–196). Although these deletions are not coded as additional characters and are not included in the present analysis, they may be interpreted as additional evidence for the monophyly of these groups. No coincident indels occur across generic boundaries. All currently described genera are monophyletic (*Selenipedium* and *Mexipedium* are represented by a single taxon).

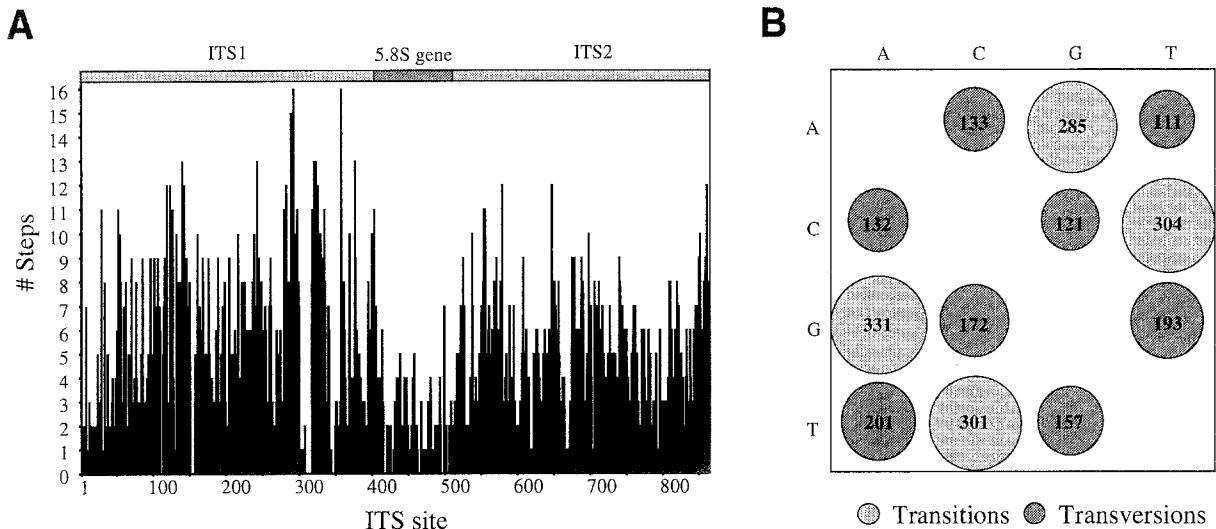


Fig. 2. Nucleotide substitution rates in Cyripedioid nuclear ITS sequences. *A* Number of fixed substitutions inferred at each base along the ITS sequence; a notable reduction in substitution rate occurs in the 5.8S rRNA coding region. *B* Transition/transversion ratios

As expected, the rate at which substitutions are fixed varies along the length of the ITS region (see Fig. 2A). A mean of approximately five and three substitutions per nucleotide position occurs for ITS1 for ITS2 respectively. The substitution rate within the 5.8S ribosomal gene coding region is noticeably lower. The transition to transversion ratio (Fig. 2B) varies from approximately 1.5:1 to 3:1; these are typical of non-coding DNA sequences.

Despite a large number of most-parsimonious trees, a highly resolved consensus topology is recovered that describes species relationships within slipper orchid genera (Figs. 3–5). Poor resolution in *Paphiopedilum* sect. *Barbata* appears to be responsible for the high number of equally most-parsimonious trees. Species groupings in *Paphiopedilum* are largely as in CRIBB (1987; Table 3 and Fig. 3) with one important difference; section *Concoloria* [*Paphiopedilum* subg. *Brachypetalum* sect. *Brachypetalum* was used by CRIBB (1987) without citing the basionym; *Paphiopedilum* subg. *Brachypetalum* sect. *Concoloria* (KRAENZLIN) ALBERT & PETTERSSON (ALBERT & PETTERSSON 1994) therefore has priority and will be used here] alone is sister to subg. *Paphiopedilum*. Subgenus *Brachypetalum* is therefore paraphyletic (Fig. 3).

Taxon sampling in *Phragmipedium* is less complete than in *Paphiopedilum*, and this may contribute to the less clearly resolved relationships seen in this portion of the tree (Fig. 4). However, all subgeneric sections (sensu McCook 1989) are clearly identifiable (Table 4). *Mexipedium* is sister to *Phragmipedium*, a position that receives significant support from the jackknife procedure. This agrees with the topology obtained from *rbcl* sequence data (ALBERT & CHASE 1992).

The 18 species of *Cyripedium* included in this study form a monophyletic group which is sister to all conduplicate taxa (Figs. 1, 5). *Cyripedium irapeanum*

Table 3. Infrageneric treatments of *Paphiopedilum* (revised from CRUBB 1987)

PRITZER (1894)	HALLIER (1897)	PFITZER (1903)	BRIEGER (1971)
a. <i>Eremantha Tessellata</i> (in part)	<i>Aphanoneura Brachypetalum</i>	<i>Brachypetalum</i>	<i>Brachypetalum</i>
b. <i>Polyantha</i>	<i>Chromatoneura Viridia Polyantha</i>	<i>Anotopedilum</i>	<i>Polyantha</i>
	XI <i>Sireptopetalum</i> (in part)	sect. <i>Corypedium</i>	sect. <i>Streptopetalum</i>
	XII <i>Mastigopetalum</i>	sect. <i>Gonatopetalum</i>	sect. <i>Mastigopetalum</i>
		sect. <i>Prenipedilum</i>	
		<i>Otopedilum</i>	
	XI <i>Sireptopetalum</i> (in part)	sect. <i>Mystriopetalum</i>	sect. <i>Polyantha</i>
	X <i>Pardalopetalum</i>	sect. <i>Pardalopetalum</i>	
	XIII <i>Cochlopetalum</i>	sect. <i>Cochlopetalum</i>	sect. <i>Cochlopetalum</i>
a. <i>Eremantha Viridia</i>	<i>Chromatoneura Viridia Eremantha</i>		<i>Paphiopedilum</i>
	VIII <i>Stictopetalum</i>	sect. <i>Stictopetalum</i>	sect. <i>Stictopetalum</i>
	IX <i>Neuropetalum</i>	sect. <i>Neuropetalum</i>	sect. <i>Paphiopedilum</i>
	V <i>Thiopetalum</i>	sect. <i>Thiopetalum</i>	sect. <i>Paphiopedilum</i>
	VII <i>Cymatopetalum</i>	sect. <i>Cymatopetalum</i>	
	V <i>Ceratopetalum</i>	sect. <i>Ceratopetalum</i>	<i>Barbata</i>
	<i>Chromatoneura Tessellata</i>		
a. <i>Eremantha Tessellata</i> (in part)	II <i>Sigmatopetalum</i>	sect. <i>Spathopetalum</i>	sect. <i>Sigmatopetalum</i>
	IV <i>Drepanopetalum</i>	sect. <i>Blepharopetalum</i>	sect. <i>Blepharopetalum</i>
		sect. <i>Phacopetalum</i>	sect. <i>Barbata</i>

KARASAWA & SAITO (1982)	ATWOOD (1984)	BRAEM (1988)	CRIBB (1987)	COX & al., this paper
<i>Brachypetalum</i> <i>Parvisepalum</i>	<i>Brachypetalum</i> <i>Parvisepalum</i>	<i>Brachypetalum</i> <i>Parvisepalum</i>	<i>Brachypetalum</i> sect. <i>Brachypetalum</i> sect. <i>Parvisepalum</i> <i>Paphiopedilum</i>	<i>Parvisepalum</i> <i>Paphiopedilum</i>
<i>Polyantha</i>	<i>Paphiopedilum</i>	<i>Polyantha</i>		
sect. <i>Mastigopetalum</i>	sect. <i>Coryopedilum</i>	sect. <i>Mastigopetalum</i>	sect. <i>Coryopedilum</i>	sect. <i>Pardalopetalum</i>
sect. <i>Mystropetalum</i> sect. <i>Polyantha</i>	sect. <i>Pardalopetalum</i>	sect. <i>Mystropetalum</i>	sect. <i>Pardalopetalum</i>	
<i>Cochlopetalum</i> <i>Paphiopedilum</i>	sect. <i>Cochlopetalum</i> sect. <i>Paphiopedilum</i>	<i>Cochlopetalum</i> <i>Paphiopedilum</i>	sect. <i>Cochlopetalum</i> sect. <i>Paphiopedilum</i>	sect. <i>Cochlopetalum</i> sect. <i>Paphiopedilum</i>
sect. <i>Stictopetalum</i> sect. <i>Paphiopedilum</i> sect. <i>Thiopetalum</i>	sect. <i>Stictopetalum</i> sect. <i>Paphiopedilum</i> sect. <i>Thiopetalum</i>	sect. <i>Stictopetalum</i> sect. <i>Paphiopedilum</i> sect. <i>Thiopetalum</i>		
sect. <i>Ceratopetalum</i> <i>Sigmatipetalum</i> sect. <i>Spathopetalum</i> sect. <i>Sigmatopetalum</i> sect. <i>Blepharopetalum</i> sect. <i>Punctatum</i> sect. <i>Planipetalum</i> sect. <i>Barbata</i>	sect. <i>Barbata</i>	sect. <i>Ceratopetalum</i> <i>Sigmatopetalum</i> sect. <i>Spathopetalum</i> sect. <i>Blepharopetalum</i> sect. <i>Punctatum</i> sect. <i>Planipetalum</i> sect. <i>Barbata</i>	sect. <i>Barbata</i>	sect. <i>Barbata</i> <i>Brachypetalum</i>

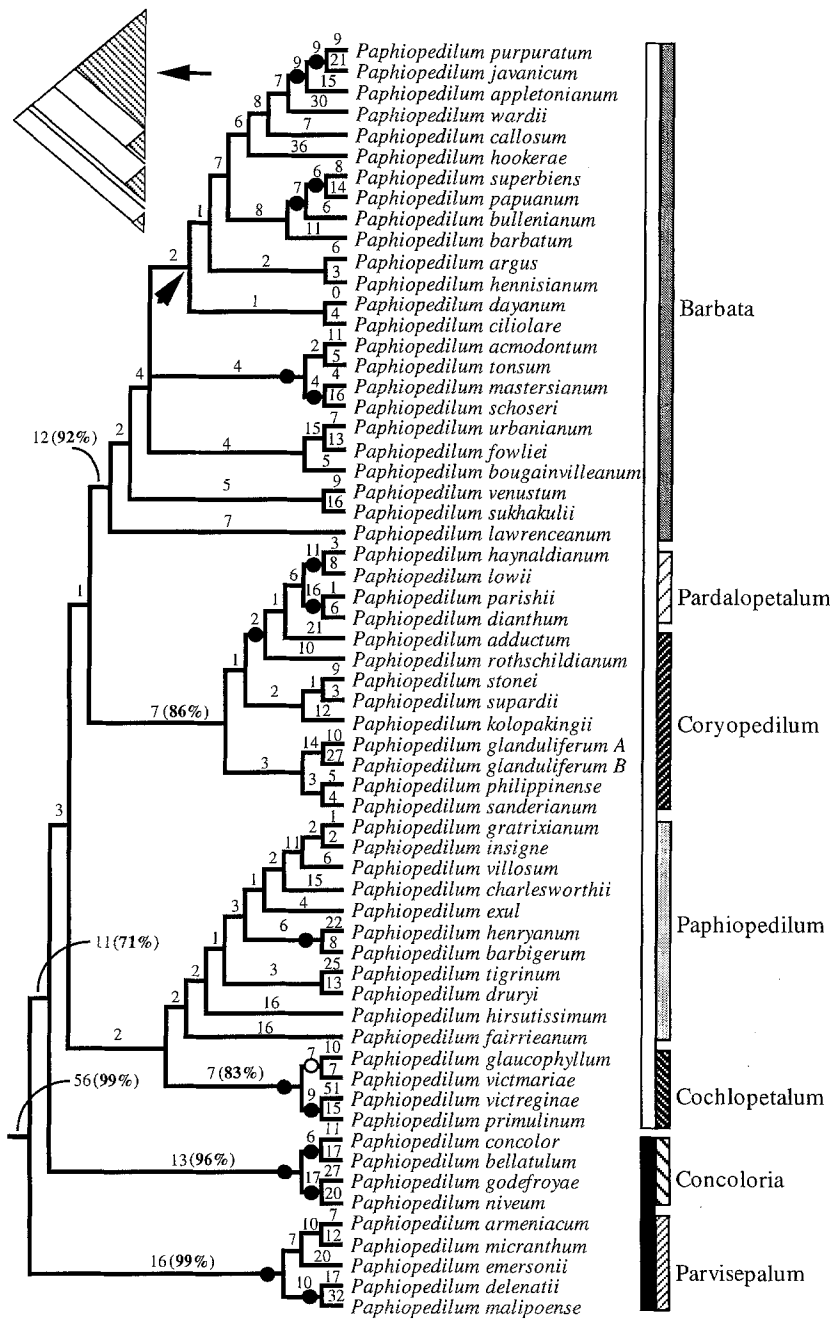


Fig. 3. One of the most parsimonious weighted trees showing cladistic relationships in the genus *Paphiopedilum*. The open bar indicates subg. *Paphiopedilum* and the solid black bar subg. *Brachypetalum* (both sensu CRIBB 1987). Sectional limits (sensu CRIBB 1987) are indicated by various shaded bars. Solid circles indicate clades strongly supported by the jackknife procedure (node score > 0.63); open circles indicate those that are weakly supported (node score > 0.5 but < 0.63). An arrow indicates the node that collapses in the strict consensus of all most-parsimonious weighted trees. ITS sequences from two accessions of *P. glanduliferum* (BLUME) STEIN were determined, and are labelled "A" and "B" respectively

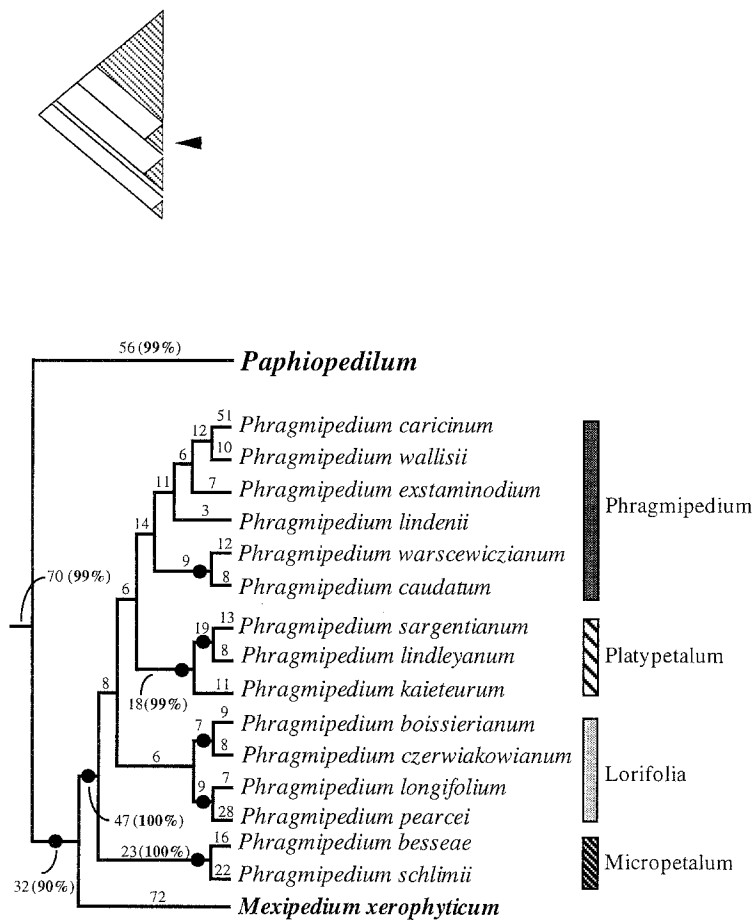


Fig. 4. One of the most-parsimonious trees showing cladistic relationships in the genus *Phragmipedium*. Sectional limits (sensu McCook 1989) are indicated by various shaded bars. Solid circles indicate clades strongly supported by the jackknife procedure (node score > 0.63)

LLAVE & LEX., from Mexico and Guatemala, is sister to all other cyripediums which are distributed throughout the temperate Northern Hemisphere. Excluding *C. irapeanum*, the genus is divided into three clades which do not reflect relationships that may be inferred from their geographical distribution. Since taxon sampling is less complete in *Cyripedium* we refrain from making confident statements about species relationships. A more comprehensive study of the genus is nearing completion (COX & CHASE, unpubl.).

Support for relationships among the currently accepted slipper orchid genera is robust. The jackknife test (FARRIS & al. 1996) gave strong support (node score > 0.63) for the monophyly of *Cyripedioideae*, *Cyripedium* and the *Mexipedium-Phragmipedium* clade as well as for *Phragmipedium* alone. There was no support for the monophyly of either the conduplicate genera or *Paphiopedilum* (Fig. 1).

Table 4. Infrageneric treatments of *Phragmipedium* (revised from McCook 1989)

PFITZER (1903)	BRIEGER (1973)	GARAY (1979)	ATWOOD (1984)	MCCOOK (1989)	COX & al., this paper
sect. <i>Micropetalum</i> <i>P. schlimii</i>	sect. <i>Micropetalum</i> <i>P. schlimii</i>	sect. <i>Micropetalum</i> <i>P. schlimii</i>	sect. <i>Micropetalum</i> <i>P. schlimii</i>	sect. <i>Micropetalum</i> <i>P. schlimii</i>	sect. <i>Micropetalum</i> <i>P. schlimii</i>
sect. <i>Platypetalum</i> <i>P. lindleyanum</i> <i>P. sargentianum</i>	sect. <i>Platypetalum</i> <i>P. lindleyanum</i> <i>P. sargentianum</i>	sect. <i>Platypetalum</i> <i>P. lindleyanum</i> <i>P. sargentianum</i> <i>P. kaeteurum</i>	sect. <i>Platypetalum</i> <i>P. lindleyanum</i> <i>P. sargentianum</i> <i>P. kaeteurum</i>	sect. <i>Platypetalum</i> <i>P. lindleyanum</i>	sect. <i>Platypetalum</i> <i>P. lindleyanum</i>
sect. <i>Lorifolia</i> <i>P. boissierianum</i> <i>P. czerwiakowianum</i>	sect. <i>Lorifolia</i> <i>P. boissierianum</i> <i>P. czerwiakowianum</i>	sect. <i>Lorifolia</i> <i>P. boissierianum</i> <i>P. czerwiakowianum</i>	<i>P. boissierianum</i>	sect. <i>Lorifolia</i> <i>P. boissierianum</i>	<i>P. kaeteurum</i> sect. <i>Lorifolia</i> <i>P. boissierianum</i>
<i>P. longifolium</i>	<i>P. longifolium</i>	<i>P. longifolium</i> <i>P. reticulatum</i>	<i>P. longifolium</i>	<i>P. longifolium</i>	<i>P. longifolium</i>
<i>P. hartwegii</i>	<i>P. hartwegii</i>	<i>P. dartense</i> <i>P. hartwegii</i> <i>P. hinksonianum</i> <i>P. roezlii</i>			
<i>P. vittatum</i>	<i>P. vittatum</i>	<i>P. vittatum</i>	<i>P. vittatum</i>	<i>P. vittatum</i>	<i>P. vittatum</i> ¹ <i>P. pearcei</i>
sect. <i>Phragmipedium</i> <i>P. caricinum</i>	<i>P. caricinum</i>	sect. <i>Himantopetalum</i> <i>P. caricinum</i> <i>P. ecuadorensis</i>	<i>P. caricinum</i> <i>P. ecuadorensis</i>	sect. <i>Phragmipedium</i> <i>P. caricinum</i>	sect. <i>Phragmipedium</i> <i>P. caricinum</i>
<i>P. klotzschianum</i>	<i>P. klotzschianum</i>	<i>P. klotzschianum</i> <i>P. pearcei</i>	<i>P. klotzschianum</i> <i>P. pearcei</i>	<i>P. klotzschianum</i> <i>P. pearcei</i>	<i>P. klotzschianum</i> ¹ <i>P. pearcei</i>
sect. <i>Caudatum</i> <i>P. caudatum</i>	<i>P. caudatum</i>	sect. <i>Phragmipedium</i> <i>P. caudatum</i>	sect. <i>Caudatum</i> <i>P. caudatum</i>	<i>P. caudatum</i> <i>P. exstaminodum</i> <i>P. lindenbergii</i> <i>P. wallisii</i>	<i>P. caudatum</i> <i>P. exstaminodum</i> <i>P. lindenbergii</i> <i>P. wallisii</i>
		<i>P. lindenbergii</i> <i>P. wallisii</i> <i>P. warszewiczianum</i>	<i>P. lindenbergii</i> <i>P. wallisii</i> <i>P. warszewiczianum</i>	<i>P. caudatum</i> <i>P. exstaminodum</i> <i>P. lindenbergii</i> <i>P. wallisii</i>	<i>P. caudatum</i> <i>P. exstaminodum</i> <i>P. lindenbergii</i> <i>P. wallisii</i>

¹ Material not available for the present study

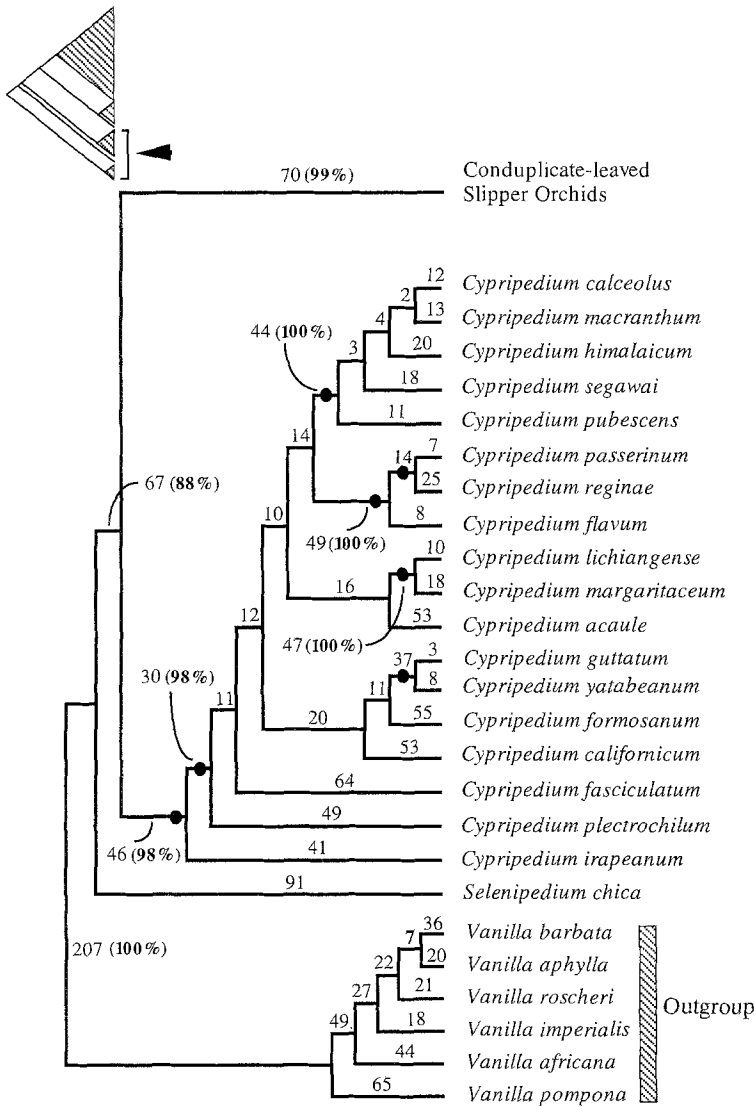


Fig. 5. One of the most-parsimonious weighted trees showing cladistic relationships in the genus *Cypripedium*. Solid circles indicate clades strongly supported by the jackknife procedure (node score > 0.63)

Discussion

The systematic inferences that can be drawn from ITS nucleotide sequences are in general agreement with previous studies of the slipper orchids. Although overall internal support for the ITS tree is not high, confidence in the topology is gained from congruence with previous taxonomies as well as many morphological, anatomical and cytological data. Such congruence establishes ITS as a reliable indicator of phylogenetic relationships at higher levels within and among genera. It is unlikely that such a comprehensive agreement with the ITS tree should occur by chance, and thus many of the weakly supported groupings appear more reasonable.

It is within the context of the basic congruence between molecular and non-molecular data that we discuss several aspects of the overall topology that are weakly supported by the ITS data alone. The lack of support for some groups (e.g. *Paphiopedilum*) may be due to problems with DNA alignment which could inject a degree of randomness into the data. For this reason use of ITS sequences for phylogenetic analyses at the subfamily level or above may be problematic in the orchids.

***Selenipedium/Cypripedium*.** The conduplicate-leaved genera *Selenipedium* and *Cypripedium* form the first two successively diverging branches. This agrees with general views that these two genera possess what have been described as plesiomorphic character states, e.g. plicate leaves, crustose seeds (in the case of *Selenipedium*) and multiple-flowered inflorescences. However, the precise status of these characters in *Orchidaceae* is uncertain, and more detailed investigations are needed to determine their general distribution. Until such characters are better understood it is too speculative to assume that these are “primitive”; instead they could be autapomorphies. Many of these assessments depend upon knowing more about outgroup characters, and this topic is presently poorly examined.

Cypripedium irapeanum is sister to the rest of the genus (Fig. 5). This taxon resembles the most “primitive” slipper orchid genus, *Selenipedium*, in general plant habit, flower lip morphology, possession of two purple spots on the involute lip margin and multiple-flowered inflorescences. *Selenipedium* has been considered by some (e.g. Rosso 1966) to grade into *Cypripedium* via *C. irapeanum*. Relationships within *Cypripedium* (Fig. 5) are well resolved, relatively well supported and the subject of a separate study (Cox 1995) that will be described elsewhere (Cox & al., unpubl.).

A notable feature of the most parsimonious trees recovered from our analysis is the unusual distribution of terminal branch lengths. Figure 3 (genus *Paphiopedilum*) shows instances in which morphologically similar taxon pairs have either long or short branch lengths. For example, the morphologically similar *P. insigne* (WALL. ex LINDL.) PFITZER and *P. gratixianum* (MASTERS) GUILL are separated by just three substitutions; contrasted with this are two accessions of *P. glanduliferum* (BLUME) STEIN (“A” and “B”) that are separated by 37 substitutions. Additionally, morphologically similar species, such as *P. wardii* SUMMERH. and *P. sukhakulii* SCHOSER & SENGHAS are widely separated in sect. *Barbata*.

The unusual distributions of branch lengths and taxa suggest that the results of this large analysis may be distorted by inadvertent analysis of paralogous ITS sequences (McDADE 1990, 1992; RIESEBERG 1990). Paralogous ITS copies may arise by several routes. Duplication of chromosomal segments bearing nucleolar organisers may occur; multiple secondary constrictions have been reported in slipper orchids (KARASAWA 1979). Amplification in vitro of duplicated but subsequently diverged DNAs may lead to random recovery of paralogous copies. Alternatively, some species may have undergone non-observed hybridisation. Introgression through successive back-crossing to one parent may lead to offspring that genetically resemble one parent but morphologically resemble the other. Use of characters displaying such reticulating histories will inevitably lead to recovery of a phylogeny that is a mixture of both gene and species trees; the relative contributions of each tree type to the overall phylogeny is difficult to estimate. The

likelihood of recovering trees that are distorted by such factors will increase as the size of the data set increases. Thus the misleading effects that paralogous sequences introduce must be considered carefully when interpreting phylogenies from DNA sequences that are obviously susceptible to reticulating patterns of inheritance (i.e. like ITS rDNA sequences).

Paphiopedilum. Subgenus *Brachypetalum* was erected to circumscribe a group of “primitive” or “basal” taxa. Originally comprised of only the species of sect. *Concoloria*, the subgenus has been divided and expanded to include sect. *Parvisepalum* following their relatively recent discovery. Although most species in sect. *Concoloria* have been known for 100 years, those in sect. *Parvisepalum* have only been discovered since 1950 and most after 1980. The two sections were grouped because they share flowers with similar involute lip margins (P. CRIBB, pers. comm.), although detailed examination of floral structures does not reveal evidence of other similarities. This type of labellum morphology would appear to be plesiomorphic for the *Cypripedioideae*. CRIBB (1987) suggested that the resemblance in lip morphology may be the result of parallel pollinator pressure rather than being the truly plesiomorphic condition; this is not a parsimonious explanation from the perspective of our trees. Our results indicate (albeit weakly) that subg. *Brachypetalum* is not monophyletic; all species of a monophyletic section *Parvisepalum* are together sister to a clade containing the remaining *Paphiopedilum* species.

Section *Parvisepalum* has been considered by CHEN & TSI (1984) to be the “link” group between *Paphiopedilum* and *Cypripedium*. This is again based on observations that these species have flowers that bear a strong resemblance to those in both *Cypripedium* and *Selenipedium*. The *Parvisepalum* group may legitimately be considered a “link” because it occupies a position that is sister to the rest of the genus and has also retained *Cypripedium*-like flower characteristics. However, to view the group as “primitive” simply on the basis of gross floral morphology would be questionable; sect. *Parvisepalum* has undergone considerable vegetative and molecular divergence from both *Cypripedium* and *Selenipedium* (Fig. 1).

Although weakly supported, all other sections of subg. *Paphiopedilum* are as described by CRIBB (1987) with the exception of sects. *Pardalopetalum* and *Coryopedilum* (Fig. 3). Taxa of these two sections form a single clade with species in sect. *Coryopedilum* forming a series paraphyletic to those of sect. *Pardalopetalum*. Both *P. rothschildianum* (RCHB. f.) STEIN and *P. adductum* ASHER are successive sister taxa to sect. *Coryopedilum* (sensu CRIBB 1987).

As suggested by ATWOOD (1984), sect. *Barbata* is the most derived section in *Paphiopedilum* and mostly comprises species that are narrow endemics often found only on single Malaysian islands. Section *Barbata* has probably undergone a recent, rapid radiation which likely accounts for the short branch lengths in this clade.

To reconcile intrageneric nomenclature with the monophyletic taxa identified here, we would consider the following changes to genus *Paphiopedilum* (but are not making these without further study): (i) elevation of sect. *Concoloria* to subg. *Brachypetalum*; (ii) elevation of sect. *Parvisepalum* to subgeneric rank; (iii) combination of sect. *Coryopedilum* with sect. *Pardalopetalum*; and (iv) elimination of the subsectional groupings proposed by BRAEM (1988). These numerous

subsections containing only one or two species contributed little to systematic or phylogenetic understanding of the group. Few of the groups identified are supported, thus refuting this narrow approach based on trivial characters and intuition.

The rate of change of ITS sequences in the *Cypripedioideae* is sufficient to ensure that nearly all accessions examined possess a unique sequence, although some, e.g. *P. insigne* and *P. gratixianum* (MASTERS) GULL, are little diverged. It is difficult to distinguish an amount of sequence divergence that is significant when trying to differentiate species or populations. An arbitrarily chosen number of substitutions is unlikely to be correlated with morphological differentiation. Addition of taxa to investigate if they are “good species” would not a sensible way to investigate species boundaries. For example, examination of the branch lengths in *Paphiopedilum* sect. *Pardalopetalum* reveals as many substitutions inferred between the synonymized species *P. parishii* (RCHB. f.) STEIN and *P. dianthum* T. TANG & F. T. WANG (CRIBB 1987) as have been inferred between the accepted species *P. haynaldianum* (RCHB. f.) STEIN and *P. lowii* (LINDL.) STEIN.

The question of whether it is justifiable to assign synonymy or specific rank in such cases remains unresolved. In other cases a more definite decision can be reached; the synonymy of *P. fowliei* BIRK and *P. hennisianum* (M. WOOD) FOWLIE (CRIBB 1987) is clearly refuted; the two are not even sister taxa. One possible explanation for the position of *P. fowliei* is that it is an introgressed individual with an ITS sequence resembling that of one parent and morphological characters resembling those of the other.

Phragmipedium. Most taxonomic schemes place sect. *Micropetalum* sister to the rest of *Phragmipedium*, and our analysis supports these conclusions (Fig. 4). *Phragmipedium besseae* DODSON & J. KUHN and *P. schlimii* LINDEN & RCHB. f., both of sect. *Micropetalum*, have inflated labella with involute lip margins, resembling sectt. *Parvisepalum* and *Concoloria* of *Paphiopedilum*, *Mexipedium* and most species of *Cypripedium* and *Selenipedium*. CRIBB (1987) has suggested that this is a parallel adaptation to exploit similar pollinators; our results cannot confirm that this flower form is a plesiomorphic type, but they do not refute this or similar interpretations drawn by ALBERT (1994). This flower stereotype may be useful in attracting certain pollinators (mostly bees) and has subsequently been modified for attracting flies in both *Paphiopedilum* and *Phragmipedium*.

Phragmipedium species relationships inferred from this study closely parallel those in the monograph of the genus by McCOOK (1989), which was based primarily on morphological and isozyme data. McCOOK synonymized *P. kaieteurum* (N. E. BR.) GARAY and *P. sargentianum* ROLFE under *P. lindleyanum* (LINDL.) ROLFE, and our data can do little to resolve this issue. The same is true for *P. czerwiakowianum* (RCHB. f.) ROLFE, which McCOOK treats as a synonym of *P. boissierianum* (RCHB. f.) ROLFE, and for *P. warscewiczianum* (RCHB. f.) GARAY, which she treats as a synonym of *P. caudatum* (LINDL.) ROLFE. We have been unable to obtain tissue of *P. vittatum* (VELL.) ROLFE and *P. klotzchianum* (RCHB. f.) ROLFE. The only discrepancy between our findings and McCOOK'S scheme concerns the relationship of *P. pearcei* (RCHB. f.) RAUH & SENGHAS. McCOOK included this species in sect. *Phragmipedium*, but our data place it in sect. *Lorifolia* as sister to *Phragmipedium longifolium* (RCHB. & WARSCEWICZ) ROLFE. With a diploid chromosome

number of 20–22, *P. pearcei* appears better placed in sect. *Lorifolia* in which the range in chromosome number is 18–22, rather than in sect. *Phragmipedium* which generally have 28 chromosomes (see Table 2).

Mexipedium. The description of *Mexipedium* highlighted a number of internal conflicts in generic delimitation of the conduplicate genera. ALBERT & PETERSSON (1994) attempted to resolve these conflicts by combining all three conduplicate genera under *Paphiopedilum*. Our data place *Mexipedium xerophyticum* as the sister taxon to *Phragmipedium*. Such an arrangement receives significant support from the jackknife test (Fig. 1), and a similar topology has been recovered from an analysis of chloroplast *rbcL* sequences (ALBERT & CHASE 1992).

Although similarities between the two genera may indeed be as significant as their differences, our results demonstrate a degree of divergence between *Mexipedium* and *Phragmipedium* that is comparable to that between conduplicate- and plicate-leaved genera (Fig. 1). AVC, AMP and MWC therefore suggest that division of the *Cypripedioideae* into five genera is most appropriate, although a strong supporting argument based on sequence divergence alone is insufficient to justify generic status. This finding makes *Mexipedium* one of the most interesting slipper orchids in many ways, for it may be pivotal in trying to understand biogeography and evolution in the subfamily.

Biogeography. The ITS phylogenetic hypothesis for slipper orchids is difficult to reconcile with their present-day geographical distribution. It is tempting to hypothesise southern North America/Mesoamerica as the cradle of slipper orchid evolution since it supports today a significant number of the taxa that display plesiomorphic characters (*Selenipedium*, *C. irapeanum*, *M. xerophyticum*). One can confirm this idea by overlaying the ITS tree on geographical provinces (Fig. 6); generic branching events appear to be centred in this region. An interesting test of this hypothesis will occur when material of *Cypripedium subtropicum* CHEN & LANG becomes available for DNA analysis. This enigmatic species is known from a single collection in Tibet and bears a striking resemblance to several species of *Selenipedium*.

The evidence favours a hypothesis that slipper orchids were once much more widespread and that their distributions are now largely relictual. ATWOOD (1984) proposed that the slipper orchids were once widespread in North America/Asia, a hypothesis also favoured by ALBERT (1994). Separation of the continents fragmented their original distribution; subsequent cooling and glaciation combined with range restriction has resulted in the complicated, discontinuous distribution pattern seen today. A boreotropical explanation is a likely scenario (WOLFE 1975; TIFFNEY 1985a, b; LAVIN & LUCKOW 1993).

Cytology. The cytology of the slipper orchids has been studied extensively. Comprehensive accounts have been published for *Paphiopedilum* (KARASAWA 1979, 1986; KARASAWA & TANAKA 1981; ATWOOD 1984; KARASAWA & AOYAMA 1988) and *Phragmipedium* (KARASAWA 1980, ATWOOD 1984). Less is known about the chromosomes of *Cypripedium*, although counts for a number of species have been published (KARASAWA & AOYAMA 1986); neither *Mexipedium* nor *Selenipedium* has been examined.

Figure 7 shows chromosome numbers (Table 2) plotted onto the strict consensus of all most-parsimonious trees with a “pseudo-regression” line

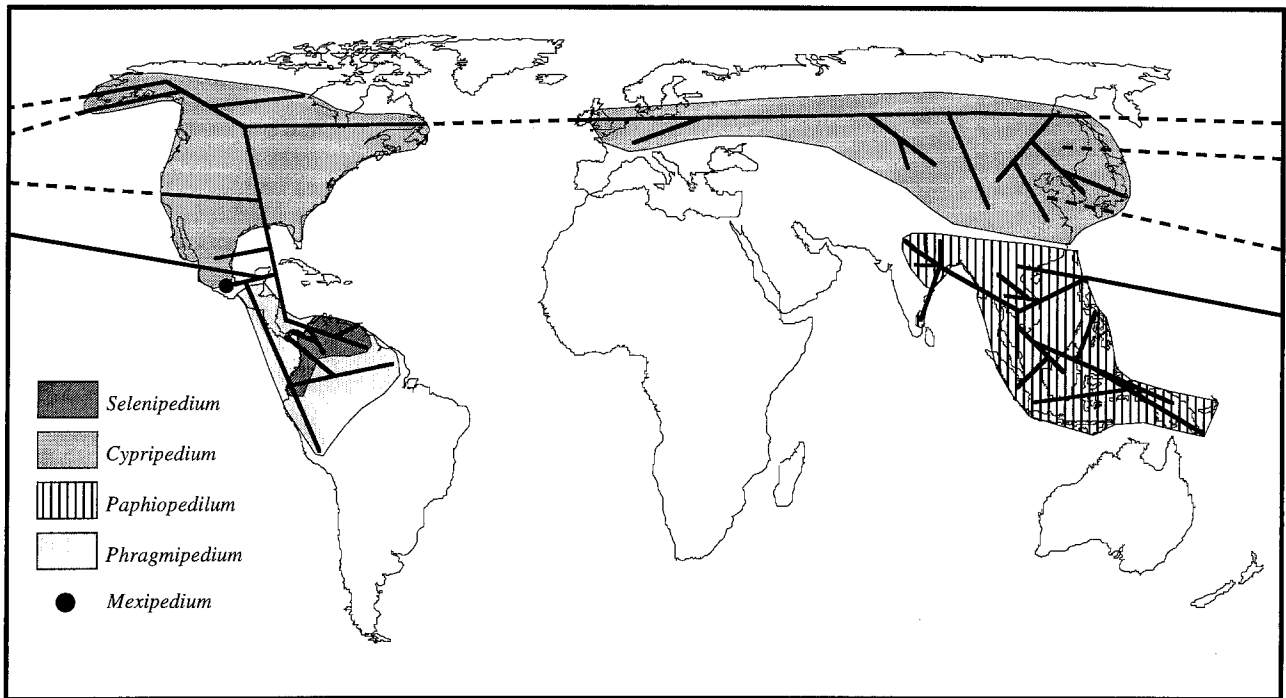


Fig. 6. Distribution map for the slipper orchids overlaid with the ITS phylogeny. This arrangement highlights the concentration of “deep” branching points in southern North America/Mesoamerica indicating a possible origin for the subfamily in this region. Since the exact migration route for *Cyripedium* is unclear from these trees, trans-oceanic branches are shown as dotted lines

indicating the gradual trend toward increased chromosome number in more derived clades. The base chromosome number for *Cyripedium* is $x = 10$, which has been observed in nearly all species examined to date. Counts other than $2n = 20$ are attributed to either polyploidy (rare in slipper orchids) or error.

Chromosome numbers in species of the conduplicate-leaved genera display a wide range of variation. In most species of *Phragmipedium* the complement consists of variable numbers of metacentric or sub-metacentric chromosomes. The remainder of the complement comprises, almost invariably, telocentric chromosomes (KARASAWA 1980). If all telocentrics are paired to form metacentrics, a nearly uniform number of 18 is recovered in *Phragmipedium*.

In *Paphiopedilum*, most sections comprise species possessing 26 meta- or sub-metacentrics, often displaying a degree of bimodality; four large chromosomes are present with the remaining chromosomes gradually decreasing in size (KARASAWA 1979). In two sections (*Cochlopetalum* and *Barbata*), chromosome numbers are as high as 37 and 41, respectively. In cases for which accurate karyotype data are known, telocentrics may be arranged so as to form 26 metacentric chromosomes. Studies of some species in sect. *Cochlopetalum* reveal karyotypes that equate to 25 metacentric chromosomes. This is thought to be due to the loss of a pair of

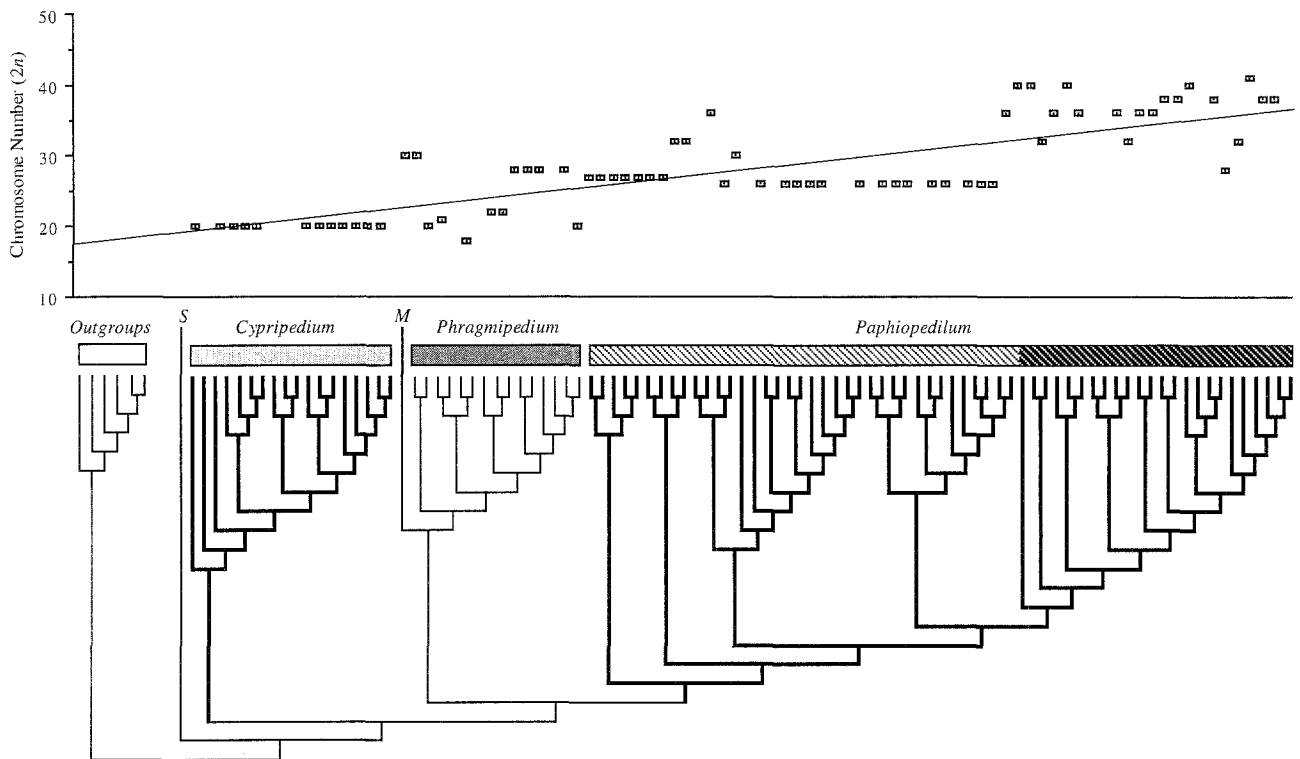


Fig. 7. Diploid chromosome number variation in the slipper orchids. Chromosome number on the vertical axis is plotted against position in the cladogram (horizontal “axis”). If sect. *Barbata* is considered the most derived clade (Arwood 1984), a clear trend (indicated by a “pseudo-regression” line) toward increased chromosome number can be identified. Generic limits are indicated by shaded bars. Section *Barbata* in the genus *Paphiopedilum*, which has the highest chromosome numbers in the subfamily, is indicated by reversed shading. M *Mexipedium*, S *Selenipedium*

telocentric chromosomes (KARASAWA 1979). It would therefore appear that 26 is the ancestral number in *Paphiopedilum* just as 18 is in *Phragmipedium*.

Since nuclear DNA amount is known for only a single slipper orchid species (*Paphiopedilum insigne*: $4C = 82.4$ pg; Cox & al. 1993) we are unable to determine whether there has been any general tendency to changes in genome size during phyletic diversification. Similarly, it is also unknown whether slipper orchids possessing more chromosomes than the base number for their genus have the same genome size.

The first application of these chromosome data to a phylogenetic hypothesis for the slipper orchids clearly demonstrates several conspicuous changes from symmetric to asymmetric karyotypes. These observations raise the question of why such a marked difference in karyotype symmetry should occur. Centric fission has clearly played a major role in karyotype evolution in some slipper orchid groups (KARASAWA 1979, KARASAWA & TANAKA 1980). The latent karyotype numbers of 26 metacentrics in *Paphiopedilum* and 18 metacentrics in *Phragmi-*

pedium are compelling evidence for fragmentation of the nuclear genome in these genera. Such fragmentation may be attained in different ways. Centromeres may have become unstable in some groups, leading to chromosome breakage. Alternatively, division of the nuclear chromatin among more chromosomes may confer a selective advantage to the plants. More chromosomes may permit an increased level of recombination at meiosis (STEBBINS 1971). Slipper orchids have not been observed to undergo apomixis, appear unable to harness polyploidy (possibly due to their already large genome size) and produce flowers that are highly modified to prevent self-fertilisation; changes that favour selection of asymmetric karyotypes may be a means of generating elevated levels of recombination.

There are two exceptions to the generally consistent pattern of chromosome numbers in slipper orchid genera (apart from those mentioned above for some species in sect. *Cochlopetalum* and *Barbata*).

The first example of chromosome change is displayed by *Paphiopedilum hookerae* (RCHB. f.) STEIN; whereas nearly all members of *Paphiopedilum* sect. *Barbata* exhibit greatly elevated chromosome numbers, this species possesses 28 chromosomes, none of which is telocentric. Its karyotype does, however, include two sub-telocentric chromosomes. These may have been derived from telocentrics with the centromeres being repositioned through the gain of terminal chromatin or by a pericentric inversion.

A counter example is provided by *Paphiopedilum druryi* which has 30 chromosomes (including 8 telocentrics), whereas the other species comprising *Paphiopedilum* sect. *Paphiopedilum* have 26 metacentric or sub-metacentric chromosomes. *Paphiopedilum druryi* is the only member of the section that appears to have undergone centric fission. This may be correlated with its narrow endemism; it is the only slipper orchid to occur in southern India, restricted to the Travencore Hills, near Quilon.

These observations can be correlated with the fact that slipper orchid species displaying highly asymmetric karyotypes are nearly all narrow endemics either far removed from the main centres of generic distribution (i.e. *P. druryi*) or on Malaysian islands such as Borneo, Java and Sumatra (CRIBB 1987). Some species are known only in single locations. The colonisation of such islands may require increased levels of generic variation to cope with the rigorously selective environments to which island floras may be subjected (STEBBINS 1971). An alternative but not mutually exclusive explanation is that island habitats may simply permit a greater fixation rate of chromosomal (and other) variation following from founder effect and effective inbreeding occurring within small populations occupying novel ecological niches.

This study establishes the ITS rDNA region as a reliable indicator of phylogenetic relationships at higher levels within and among orchid genera. Investigations into species relationships may become complicated by atypically long branch lengths. This may indicate that ITS sequences are undergoing episodic evolutionary change or else phylogenetic analyses are misleading through recovery of paralogous copies of rDNA regions. However, in groups in which few other data are available ITS should be a good tool for exploratory examinations of taxonomic delimitations.

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Addresses of the authors: ANTONY V. COX (correspondence), ALEC M. PRIDGEON, MARK W. CHASE, Department of Molecular Systematics, Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, United Kingdom. Fax: (+44) - (0)181-332-5310; email: a.cox@rbgkew.org.uk. – VICTOR A. ALBERT, The Lewis B. & Dorothy Cullman Program for Molecular Systematics Studies, The New York Botanical Garden, Bronx, New York 10458-5126, USA. Email: valbert@nybg.org.