2006

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Recommended Citation

Cameron, Kenneth M. (2006) "A Comparison and Combination of Plastid *atpB* and *rbcL* Gene Sequences for Inferring Phylogenetic Relationships within Orchidaceae," *Aliso: A Journal of Systematic and Floristic Botany*. Vol. 22: Iss. 1, Article 36.

Available at: <https://scholarship.claremont.edu/aliso/vol22/iss1/36>

MONOCOTS

Comparative Biology and Evolution
Excluding Poales

Asparagales
Orchidaceae

A COMPARISON AND COMBINATION OF PLASTID *atpB* AND *rbcL* GENE SEQUENCES FOR INFERRING PHYLOGENETIC RELATIONSHIPS WITHIN ORCHIDACEAE

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ABSTRACT

Parsimony analyses of DNA sequences from the plastid genes *atpB* and *rbcL* were completed for 173 species of Orchidaceae (representing 150 different genera) and nine genera from outgroup families in Asparagales. The *atpB* tree topology is similar to the *rbcL* tree, although the *atpB* data contain less homoplasy and provide greater jackknife support than *rbcL* alone. In combination, the two-gene tree recovers five monophyletic clades corresponding to subfamilies within Orchidaceae, and fully resolves them with moderate to high jackknife support as follows: Epidendroideae are sister to Orchidoideae, followed by Cypripedioideae, then Vanilloideae, and with Apostasioideae sister to the entire family. Although this two-gene hypothesis of orchid phylogeny is an improvement over all single-gene studies published to date, there is still no consensus as to how all the tribes of Epidendroideae are related to one another. Nevertheless, these new topologies help to clarify some of the anomalous results recovered when *rbcL* was previously analyzed alone, and demonstrate the value of continued plastid gene sequencing within Orchidaceae.

Key words: *atpB*, DNA, evolution, molecular, Orchidaceae, phylogeny, plastid, *rbcL*, systematics.

INTRODUCTION

Orchidaceae are distributed throughout the world, and are by far the largest family of monocotyledons, with more than 750 genera and 30,000 species currently recognized. This immense diversity of taxa coupled with the complexity of orchid flowers presents a great challenge to plant systematists concerned with phylogeny reconstruction and classification (Atwood 1986). For this reason, molecular sequence data have been a boon to orchidologists, since it allows for large amounts of data to be generated for many taxa, and with relatively minimal cost, time expenditure, and difficulty.

The first published molecular phylogeny for Orchidaceae employed plastid *rbcL* sequences from 33 orchids plus 62 other lilioid monocots (Chase et al. 1994). Although limited in taxon sampling, that study showed that the neottiid orchids are polyphyletic, and thus implied that the orchids might be divided best into five subfamilial lineages: the apostasioid and cypripediooid orchids (each sometimes treated as distinct families), orchidoids (including the diurid and spiranthoid orchids), and epidendroids (including the neottiid and vandoid orchids). Only *Vanilla* Plum. ex Mill. and *Pogonia* Andrews were sampled to represent the vanilloid orchids, but this lineage showed itself to most likely represent a distinct subfamilial clade (Vanilloideae) as well.

The *rbcL* matrix used by Chase et al. (1994) was expanded substantially by Cameron et al. (1999) to include 158 ingroup and 13 outgroup taxa. Since that study contains the greatest genus and species level sampling to date, it has remained a standard to which most subsequent molecular phylogenetic studies of orchids have been compared (e.g., van den Berg et al. 2005). A handful of additional phylogenetic studies focused on the entire Orchidaceae have been published using genes other than *rbcL* (e.g., *ndhF* by Neyland and Urbatsch 1995, 1996; 18S by Cameron and Chase 2000;

matK by Freudenstein et al. 2004), but only the mitochondrial *nad1b-c* intron published by Freudenstein et al. (2000) and Freudenstein and Chase (2001) have considered enough taxa (ca. 100) to adequately depict higher level orchid relationships. Unfortunately, the level of sequence variation exhibited in the mitochondrial *nad1b-c* intron was also insufficient for addressing relationships much below the rank of subfamily or tribe.

Since no clear consensus has been reached regarding relationships among the subfamilies, tribes, or subtribes of Orchidaceae, a return to the plastid genome as a source of new phylogenetic information is warranted and justified given the well-known advantages of working with chloroplast DNA. These have been discussed previously by Palmer et al. (1988), Clegg and Zurawski (1992), Olmstead and Palmer (1994), among others, and include unilateral inheritance, numerous copies per cell, ease of amplification and sequencing, absence in animals and fungi, etc. Although there are a number of plastid loci that could have been chosen, *atpB* was sequenced to complement the previously published *rbcL* data of Cameron et al. (1999) since its utility for family level systematics studies has been documented for a number of other plant groups (Hoot et al. 1995; Savolainen et al. 2000). This gene is located downstream from *rbcL* in the large single copy region, and encodes one of the subunits of ATP synthase, an enzyme that couples proton translocation across membranes during the synthesis of ATP. Furthermore, a coding region was desired for sequencing so that issues of alignment (i.e., character homology assessment) would be minimized for this diverse assemblage of taxa.

MATERIALS AND METHODS

Taxon Sampling and Gene Sequencing

Nearly complete DNA sequences for *atpB* (ca. 1500 base-pairs [bp]) were obtained from 173 species of Orchidaceae

(representing 150 different genera) and nine genera from Asteliaceae, Blandfordiaceae, Boryaceae, Hypoxidaceae, and Lanariaceae to serve as outgroup taxa. In order to fill in taxonomic gaps and to compare these data with the published *rbcL* phylogeny of Orchidaceae (Cameron et al. 1999), an additional 30 new sequences of *rbcL* were also completed; the remaining *rbcL* sequences were downloaded from GenBank as indicated in Table 1. This resulted in completely congruent matrices for all 182 taxa at the generic level. An effort was made to sequence *atpB* from the same species as previously done for *rbcL* (in many cases the same DNA aliquot was used), but this was not always possible (see Table 1). The entire data matrix is available from the author upon request or can be downloaded from The New York Botanical Garden website at <http://www.nybg.org/bsci/res/cullb/dna.html> (May 2005).

All of the newly generated sequences were produced by automated methods, briefly described as follows. Most of the total DNA was extracted using the FastPrep[®] (Qbiogene, Inc., Carlsbad, California, USA) and glassmilk method from approximately 0.5 cm² dried leaf tissue, as described by Struwe et al. (1998). In some cases, DNA aliquots were obtained from the Royal Botanic Gardens, Kew DNA bank (see Table 1). Target loci were amplified in 50 μ L volumes using standard polymerase chain reaction (PCR) protocols that typically included the addition of bovine serum albumin (BSA) and betaine, which may or may not have been necessary. The annealing temperature used to amplify both genes was 55°C. For *atpB*, primers originally published by Hoot et al. (1995), herein designated "ny172": TATGA-GAATCAATCTACTACTTCT and "ny173": TCAGTA-CACAAAGATTAAAGGTCAT, were used for amplification. Both of these together with "ny174": AACGTACTCGTGA-AGGAAATGATCT and "ny175": TAACATCTCGGAAA-TATTCCGCCAT were used for sequencing. For *rbcL*, primers "ny35": CTTCAAGCAGCAGCTAGTTC and "ny149": ATGTCACCACAAACAGAAAC were used for amplification together with "ny23": GCGTTGGAGAGAT-CGTTTCT and "ny28": TCGCATGTACCYGCAGTTGC for sequencing. In all cases, resulting PCR products were purified using QIAquick[®] spin columns (QIAGEN, Inc., Valencia, California, USA) according to the manufacturer's protocols. Cycle sequencing reactions were performed using a combination of purified PCR template, primer, and BigDye Reaction mix (Applied Biosystems, Inc., Foster City, California, USA) for 20 cycles. These reactions resulted in complete forward and reverse strands of the genes for nearly all sequences. Centri-Sep[®] sephadex columns (Princeton Separations, Inc., Adelphia, New Jersey, USA) were used according to the manufacturer's instructions to remove excess dye terminators and primer from the cycle sequencing products. These were subsequently dehydrated, resuspended in a mixture of formamide and loading dye, and loaded onto a 5% denaturing polyacrylamide gel. Samples were analyzed on an Applied Biosystems ABI 377XL automated DNA sequencer, and resulting electropherograms were edited using Sequencher vers. 3.0 software (Gene Codes Corporation, Ann Arbor, Michigan, USA).

Phylogenetic Analyses

The individual *atpB* and *rbcL* matrices, as well as the combined two-gene matrix, were analyzed using the parsimony criterion in PAUP* vers. 4.0b10 (Swofford 2002) with

gaps treated as missing data, characters weighted equally, and with DELTRAN optimization of characters onto resulting trees. The sequenced genera from Asteliaceae, Blandfordiaceae, Boryaceae, Hypoxidaceae, and Lanariaceae were specified as a monophyletic outgroup based on topologies uncovered in broader phylogenetic studies of monocots (Chase et al. 1995, 2000). Equally parsimonious trees were found by executing a heuristic search of 1000 random addition replicates using tree bisection and reconnection (TBR) branch swapping, but saving only five trees per replicate in order to discover possible "islands" of maximum parsimony (MP) (Maddison 1991). All trees obtained in the first round of searching were then used as starting trees for a second heuristic search using the same parameters, but this time saving all shortest trees (MULTREES option in effect) until a MAXTREE limit of 10,000 trees was reached. Support values for the relationships discovered by analysis of each matrix were calculated by performing jackknife (jck) analyses of 5000 heuristic search replicates using the TBR branching swapping algorithm and the following settings: 37% deletion, emulate "jac" resampling, one random addition per replicate, holding one tree, and saving two trees per replicate. Finally, a partition homogeneity test (= incongruence length difference [ILD] test) was conducted in PAUP* to test for incongruence between the *atpB* and *rbcL* matrices.

RESULTS

Analysis of *atpB*

The strict consensus of all equally parsimonious trees discovered by independent analysis of the *atpB* data is presented as Fig. 1–2 with jackknife values >50% indicated. The *atpB* matrix contains 1504 characters of which 668 (44%) are variable and 459 (31% of total) parsimony informative. Analysis of these data resulted in more than 10,000 trees of maximum parsimony (length of 2499 steps, CI of 0.389, and RI of 0.730). A single *atpB* tree is presented as a phylogram in Fig. 3 to highlight variation in branch lengths. Overall, the strict consensus of these trees is similar to published phylogenetic reconstructions for Orchidaceae (e.g., Kores et al. 1997; Cameron et al. 1999; Freudenstein and Chase 2001). The five subfamilies recognized by Pridgeon et al. (1999) are monophyletic and each is supported by high jackknife support ranging from 87–100%. Epidendroideae are supported as sister to Orchidoideae s.l. (94% jck), and Cypridioideae are sister to this pair (84% jck). The positions of Apostasioideae and Vanilloideae among the subfamilies are unresolved. In total, 80 clades receive jackknife support >50% (64 of these $\geq$ 75%).

Analysis of *rbcL*

A strict consensus topology similar to that obtained with *atpB* and with comparable resolution and jackknife support resulted from independent analysis of the *rbcL* matrix (tree not shown). In this case, the matrix consists of 1330 characters; 605 (46%) of these are variable and 373 (28% of total) are parsimony informative. Again, the analysis yielded more than 10,000 equally parsimonious trees with a length of 2217 steps, CI of 0.368, and RI of 0.687. The trees are

Table 1. Species analyzed. Arranged by family, subfamily, tribe, subtribe, and genus where applicable (Orchidaceae sensu Dressler 1993).

Family, subfamily, tribe, subtribe, genus, and species	Voucher— <i>rbcL</i>	GenBank accession— <i>rbcL</i> ^a	Species— <i>atpB</i>	Voucher— <i>atpB</i>
ASTELIACEAE				
<i>Astelia alpina</i> R. Br.	Chase 1103 (K) ^b	Z77261	<i>Astelia menziesiana</i> Sm.	Cameron 1129 (NY)
<i>Milligania stylosa</i> F. Muell. ex Benth.	Chase 511 (K)	Z73693	same DNA	
BLANDFORDIACEAE				
<i>Blandfordia punicea</i> Sweet	Chase 519 (K)	Z73694	same DNA	
BORYACEAE				
<i>Borya septentrionalis</i> F. Muell.	Chase 2205 (K)	Y14985	same DNA	
<i>Curculigo capitulata</i> Kuntze	Chase 205 (NCU)	Z73701	same species	Cameron 1118 (NY)
<i>Hypoxis hirsuta</i> L.	Chase 108 (NCU)	Z73702	same species	Cameron 2132 (NY)
<i>Pauridia longituba</i> M. E. Thompson	Snijman s.n. (WBG)	Y14991	same DNA	
<i>Rhodohypoxis milloides</i> (Baker) Hilliard & B. L. Burt	Chase 479 (K)	Z77280	same species	Cameron 1120 (NY)
LANARIACEAE				
<i>Lanaria lanata</i> Druce	Goldblatt 9410 (MO)	Z77313	same species	Chase 458 (K)
ORCHIDACEAE				
APOSTASIOIDEAE				
<i>Apostasia stylioides</i> Rchb. f.	Clements 4823 (CBG)	Z73705	same species	Cameron 2229 (NY)
<i>Neuwiedia veratrifolia</i> Blume	Clements 5910 (CBG)	AF074200	same species	Cameron 1100 (NY)
CYPRIPEDIOIDEAE				
<i>Cypripedium passerinum</i> Richards	Albert 48 (NCU)	AF074142	<i>Cypripedium pubescens</i> Willd.	NYBG—living 512-96
<i>Mexipedium xerophyticum</i> V. A. Albert & M. W. Chase	Hegedus s.n. (AMO)	AF074193	same species	Cameron 2141 (NY)
<i>Paphiopedilum sukhakulii</i> Schoser & Senghas	Albert 100 (NCU)	AF074209	<i>Paphiopedilum philippinense</i>	Cameron 1134 (NY)
<i>Phragmipedium longifolium</i> (Rchb. f. & Warsc.) Rolfe	Albert 18 (NCU)	AF074212	same species	Cameron 1107 (NY)
<i>Selenipedium chica</i> Rchb. f.	Albert 167 (NCU)	AF074227	<i>Selenipedium aequinoctiale</i> Garay	NYBG—living s.n.
SPIRANTHOIDEAE				
CRANICHIDEAE				
CRANICHIDINAE				
<i>Cranichis fertilis</i> Schltr.	Chase O-401 (K)	AF074137	<i>Cranichis</i> Sw. sp.	
<i>Ponthieva racemosa</i> (Walter) Mohr	Chase O-398 (K)	AF074223	<i>Ponthieva</i> R. Br. sp.	ML 3041 (NY)
<i>Pterichis</i> Lindl. sp.	Weigend 2000-448 (NY)	AY381132	same DNA	Weigend 2000-448 (NY)
GOODYERINAE				
<i>Gonatostylis veillardii</i> (Reich. f.) Schltr.	Cameron 1174 (NY)	AY381122	same DNA	
<i>Goodyera pubescens</i> (Willd.) R. Br.	Chase O-212 (K)	AF074174	same species	Cameron 1064 (NY)
<i>Platythelys querceticola</i> (Lindl.) Garay	Chase O-378 (K)	AF074216	same DNA	
<i>Pristiglotis montana</i> (Schltr.) Cretz. & J. J. Sm.	Cameron 2151 (NY)	AY381129	same DNA	
PACHYPLECTRONINAE				
<i>Pachyplectron arifolium</i> Schltr.	Ziesing 22 (CBG)	AF074205	same species	Cameron 1171 (NY)
PRESCOTTIINAE				
<i>Aa paleacea</i> Kunth	Chase O-535 (K)	AF074105	same DNA	
<i>Prescottia</i> Lindl. sp.	Christenson s.n.	AY381128	same DNA	
SPIRANTHINAE				
<i>Spiranthes cernua</i> (L.) Richard	Chase O-402 (K)	AF074229	<i>Spiranthes romanoffiana</i> Cham.	Grant 3757 (NEU)
<i>Stenorrhynchos speciosum</i> (Jacq.) Rich. ex Spreng.	NYBG—living 293-82b	AY381135	same DNA	

Table 1. Continued.

Family, subfamily, tribe, subtribe, genus, and species	Voucher— <i>rbcL</i>	GenBank accession— <i>rbcL</i> ^a	Species— <i>atpB</i>	Voucher— <i>atpB</i>
TROPIDIEAE				
<i>Corymborkis</i> Thouars sp.	Chase O-542 (K)	AF074136	<i>Corymborkis veratrifolia</i> (Reinw.) Bl.	Motley 2231 (NY)
<i>Tropidia</i> Lindl. sp.	Chase O-211 (K)	AF074237	<i>Tropidia effusa</i> Rchb. f.	Motley 2234 (NY)
DISEAE				
DISINAE				
<i>Disa tripetaloides</i> (L. F.) N. E. Br.	Cameron 1047 (NCU)	AF074151	same species	Cameron 2143 (NY)
SATYRIINAE				
<i>Satyrium nepalense</i> D. Don	Chase O-539 (K)	AF074226	same DNA	
DIURIDEAE				
ACIANTHINAE				
<i>Acianthus exsertus</i> R. Br.	Chase O-565 (K)	AF074101	same DNA	
<i>Corybas diemenicus</i> (Lindl.) Rupp	Chase O-564 (K)	AF074135	<i>Corybas trilobus</i> Rchb. f.	Cameron 1101 (NY)
CALADENIINAE				
<i>Adenochilus nortonii</i> Fitzg.	Chase O-5670 (K)	AY381108	same DNA	
<i>Aporostylis bifolia</i> (Hook. f.) Rupp & Hatch	Cameron 1144 (NY)	AY381109	same DNA	
<i>Caladenia</i> cf. <i>caerulea</i> R. Br.	Chase O-487 (K)	AF074116	<i>Caladenia lyallii</i> Hook. f.	Cameron 1103 (NY)
<i>Cyanicula gemmata</i> (Lindl.) S. D. Hopper & A. P. Brown	Chase O-830 (K)	AY381116	same DNA	
<i>Eriochilus cucullatus</i> (Labill.) Rchb.	Chase O-566 (K)	AF074166	same DNA	
<i>Glossodia major</i> R. Br.	Chase O-568 (K)	AF074173	same DNA	
<i>Leporella fimbriata</i> (Lindl.) George	Chase O-835 (K)	AY381124	same DNA	
<i>Lyperanthus nigricans</i> R. Br.	Chase O-836 (K)	AF074187	same DNA	Chase O-567 (K)
<i>Rimacola elliptica</i> (R. Br.) Rupp	Chase O-5672 (K)	AY381133	same DNA	
CHLORAEINAE				
<i>Chloraea</i> Rchb. f. sp.	Chase O-551 (K)	AF074125		Weigend 2000-361 (NY)
<i>Codonorchis lessonii</i> Lindl.	Sobel & Strudwick 2626 (NY)	AY381113	same DNA	
<i>Megastylis glandulosus</i> Schltr.	Ziesing 29 (CBG)	AF074191	same species	Cameron 9814 (NY)
<i>Megastylis latissima</i> (Schltr.) Schltr.	Motley 2149 (NY)	AY381125	same DNA	
<i>Megastylis rara</i> (Schltr.) Schltr.	Cameron 2003 (NY)	AY381126	same DNA	
CRYPTOSTYLIDINAE				
<i>Coilochilus neocaledonicus</i> Schltr.	Cameron 2117 (NY)	AY381114	same DNA	
<i>Cryptostylis subulata</i> (Labill.) Rchb.	Chase O-332 (K)	AF074140	<i>Cryptostylis arachnites</i> Bl.	Cameron 1105 (NY)
DIURIDINAE				
<i>Diuris sulphurea</i> R. Br.	Chase O-554 (K)	AF074152	same DNA	
<i>Orthoceras strictum</i> R. Br.	Chase O-571 (K)	AF074204	same species	Cameron 1161 (NY)
DRACAENINAE				
<i>Chiloglottis trapeziformis</i> Fitzg.	Chase O-569 (K)	AF074124	<i>Chiloglottis cornuta</i> Hook. f.	Cameron 1104 (NY)
PRASOPHYLLINAE				
<i>Microtis parviflora</i> R. Br.	Chase O-553 (K)	AF074194	<i>Microtis unifolia</i> Rchb. f.	Cameron 1152 (NY)
PTEROSTYLIDINAE				
<i>Pterostylis nutans</i> R. Br.	Chase O-533 (K)	AF074224	<i>Pterostylis oliveri</i> Petrie	Cameron 1102 (NY)
THELYMITRINAE				
<i>Calochilus robertsonii</i> Benth.	Chase O-570 (K)	AF074118	<i>Calochilus</i> R. Br. sp.	Chase O-488 (K)

Table 1. Continued.

Family, subfamily, tribe, subtribe, genus, and species	Voucher— <i>rbcL</i>	GenBank accession— <i>rbcL</i> ^a	Species— <i>atpB</i>	Voucher— <i>atpB</i>
<i>Thelymitra</i> J. R. Forst. & G. Forst. sp.	Chase O-489 (K)	AF074232	<i>Thelymitra longifolia</i> J. R. Forst. & G. Forst.	Cameron 989 (NY)
ORCHIDEAE				
HABENARIINAE				
<i>Cynorkis fastigiata</i> Thou.	Motley 2273 (NY)	AY381117	same DNA	
<i>Habenaria repens</i> Nutt.	Chase O-381 (K)	AF074177	<i>Habenaria</i> Willd. sp.	Alves 2165
CORYCINIINAE				
<i>Corycium carnosum</i> Rolfe	Chase O-692 (K)	AY381115	same DNA	
<i>Disperis capensis</i> Sw.	Chase O-1203 (K)	AY381120	same DNA	
ORCHIDINAE				
<i>Galearis spectabilis</i> (L.) Raf.	Cameron 1096 (NY)	AY381121	same DNA	
<i>Ophrys apifera</i> Hudson	Chase O-536 (K)	AF074202	same DNA	
<i>Orchis quadripunctata</i> Cyrillo ex Tenore	Chase O-911 (K)	AF074203	same DNA	
<i>Platanthera ciliaris</i> (L.) Lindl.	Albert 54 (NCU)	AF074215	<i>Platanthera obtusata</i> Lindl.	Grant 3758 (NEU)
EPIDENDROIDEAE				
NEOTTIEAE				
LIMODORINAE				
<i>Cephalanthera damasonianum</i> (Miller) Druce	Chase O-575 (K)	AF074123	<i>Cephalanthera longifolia</i> Fritsch	Cameron s.n.
<i>Epipactis helleborine</i> (L.) Crantz	Chase O-199 (K)	Z73707		Cameron 1097 (NY)
LISTERINAE				
<i>Listera smallii</i> Wiegand	Cameron 1001 (NCU)	AF074184	<i>Listera ovata</i> R. Br.	Paris—living s.n.
NERVILLEAE				
<i>Nervilia bicarinata</i> Schltr.	Chase O-580 (K)	AF074199	same DNA	
PALMORCHIDEAE				
<i>Palmorchis trilobulata</i> L. O. Williams	Chase O-462 (K)	AF074206	same DNA	
TRIPHOREAE				
<i>Monophyllorchis</i> Schltr. sp.	Chase O-435 (K)	AF074195	<i>Monophyllorchis maculata</i> Garay	Cameron 2142 (NY)
<i>Triphora trianthophora</i> (Swartz) Rydb.	Chase O-379 (K)	AF074236	same DNA	
VANILLEAE				
GALEOLINAE				
<i>Cyrtosia septentrionalis</i> (Rchb. f.) L. A. Garay	Chase O-793 (K)	AY381118	same DNA	
<i>Erythrorchis altissima</i> (Bl.) Bl.	Cameron 1029 (NCU)	AF074168	same DNA	
<i>Erythrorchis cassythoides</i> (Cunn. ex Lindl.) Garay	Weston 1831 (NCU)	AF074169	same DNA	
<i>Pseudovanilla foliata</i> (F. Muell.) Garay	Chase O-790	AY381130	same DNA	
<i>Pseudovanilla ponapensis</i> (Kaneh. & Yamam.) L. A. Garay	Cameron 1114 (NY)	AY381131	same DNA	
VANILLINAE				
<i>Clematepistephium smilacifolium</i> Hallé	Ziesing 33 (CBG)	AF074131	same DNA	
<i>Epistephium</i> Humbert sp.	Chase O-432 (K)	AF074159	same DNA	
<i>Epistephium</i> sp.	Chase O-433 (K)	AF074160	same DNA	
<i>Epistephium</i> cf. <i>lucidum</i> Cogn.	Chase O-795 (K)	AF074161	same DNA	
<i>Epistephium parviflorum</i> Lindl.	Chase O-794 (K)	AF074162	same DNA	

Table 1. Continued.

Family, subfamily, tribe, subtribe, genus, and species	Voucher— <i>rbcl</i>	GenBank accession— <i>rbcl</i> ^a	Species— <i>atpB</i>	Voucher— <i>atpB</i>
<i>Epistephium subrepens</i> Hoehne	Chase O-815 (K)	AF074163	same DNA	
<i>Eriaxis rigida</i> Rchb. f.	Ziesing 5 (CBG)	AF074165	same species	
<i>Vanilla africana</i> Lindl.	Chase O-584 (K)	AF074239	same DNA	
<i>Vanilla aphylla</i> Bl.	Chase O-578 (K)	AF074238	same DNA	
<i>Vanilla</i> cf. <i>barbellata</i> Rchb. f.	Chase O-591 (K)	AF074240	same DNA	
<i>Vanilla imperialis</i> Kraenzlin	Chase O-587 (K)	AF074241	same DNA	
<i>Vanilla inodora</i> Schiede	McCartney s.n.	AY381136	same DNA	
<i>Vanilla palmarum</i> Lindl.	Santo s.n.	AY381137	same DNA	
<i>Vanilla</i> cf. <i>planifolia</i> Andrews	Chase O-170 (K)	AF074242	same DNA	
<i>Vanilla roscheri</i> Rchb. f.	Chase O-540 (K)	AF074243	same DNA	
CYMBIDIOD PHYLLAD				
CALYPSOEAE				
<i>Aplectrum hyemale</i> Nutt.	Chase O-104 (K)	AF074108	same species	Cameron 2144 (NY)
<i>Calypso bulbosa</i> (L.) Oakes	Grant 92-02165 (US)	AF074120	same species	Grant 3763 (NEU)
<i>Tipularia discolor</i> (Pursh) Nutt.	Freudenstein s.n.	AF074234	same species	Cameron 2145 (NY)
CYMBIDIEAE				
CATASETINAE				
<i>Catasetum expansum</i> Rchb. f.	Chase O-224 (K)	AF074121	<i>Catasetum</i> Rich. ex Kunth sp.	NYBG—living 4255-95c
<i>Dressleria eburnea</i> (Rolfe) Dodson	Chase O-313 (K)	AF074153	same DNA	
CYRTOPODIINAE				
<i>Ansellia gigantea</i> Rchb. f.	Chase O-429 (K)	AF074107	<i>Ansellia africana</i> Lindl.	NYBG—living 3199
<i>Cymbidium ensifolium</i> (L.) Sw.	Chase O-290 (K)	AF074141	<i>Cymbidium goeringii</i> (Rchb. f.) Rchb. f.	Cameron 1098 (NY)
<i>Cyrtopodium andersonii</i> (Lamb. ex Andrews) R. Br.	Chase O-341 (K)	AF074143		NYBG—living 246
<i>Galeandra devoniana</i> Schomb. ex. Lindl.	Chase O-382 (K)	AF074171	same DNA	
<i>Grammatophyllum speciosum</i> Blume	Chase O-890 (K)	AF074176	<i>Grammatophyllum scriptum</i> Bl.	NYBG—living s.n.
EULOPHIINAE				
<i>Eulophia nuda</i> Lindl.	Chase O-292 (K)	AF074170	<i>Eulophia petersii</i> Rchb. f.	NYBG—living 1042
GOVENIINAE				
<i>Govenia</i> Lindl. sp.	Chase O-146 (K)	AF074175	<i>Govenia superba</i> Lindl. ex Lodd.	Cameron 2153 (NY)
MALAXIDEAE				
<i>Liparis liliifolia</i> (L.) L. C. M. Rich. ex Lindl.	Chase O-214 (K)	AF074183	<i>Liparis viridiflora</i> Lindl.	NYBG—living 2025
<i>Malaxis spicata</i> Sw.	Chase O-377 (K)	AF074188	same species	McCartney s.n.
MAXILLARIEAE				
CRYPTARRHENINAE				
<i>Cryptarrhena</i> Lindl. sp.	Chase O-307 (K)	AF074138	<i>Cryptarrhena lunata</i> R. Br.	FLAS—living 98000
LYCASTINAE				
<i>Lycaste cruenta</i> Lindl.	unknown	AF074185	<i>Lycaste aromatica</i> Lindl.	NYBG—living 79449
MAXILLARIINAE				
<i>Bifrenaria harrissoniae</i> (Hook.) Rchb. f.	Chase O-95 (K)	AF074112	same species	Chase 86086 (K)
<i>Cryptocentrum peruvianum</i> (Cogn.) C. Schweinf.	Chase O-115 (K)	AF074139	<i>Cryptocentrum Benth</i> sp.	FLAS—living s.n.
<i>Maxillaria cucullata</i> Lindl.	Chase O-85 (K)	AF074190	<i>Maxillaria nasuta</i> Rchb. f.	Cameron 1071 (NY)

Table 1. Continued.

Family, subfamily, tribe, subtribe, genus, and species	Voucher— <i>rbcL</i>	GenBank accession— <i>rbcL</i> ^a	Species— <i>atpB</i>	Voucher— <i>atpB</i>
<i>Xylobium</i> Lindl. sp.	unknown	AF074245	<i>Xylobium variegatum</i> (Ruiz & Pav.) Garay & Dunst.	NYBG—living 3104
ONCIDIINAE				
<i>Oncidium excavatum</i> (Rchb. f.) Lindl.	Chase O-86 (K)	AF074201	<i>Oncidium ornithorhynchum</i> H. B. & K.	Cameron 1124 (NY)
STANHOPEINAE				
<i>Acineta chrysanthia</i> Lindl. & Paxt.	Chase O-251 (K)	AF074102	same species	NYBG—living 2291
<i>Coryanthes verrucolineata</i> G. Gerlach	Chase O-510 (K)	AF074134	<i>Coryanthes mastersiana</i> F. C. Lehm.	NYBG—living s.n.
<i>Houlletia sanderi</i> Rolfe	Chase O-500 (K)	AF074178		FLAS—living 93079
<i>Kegeliella kupperi</i> Mansf.	Chase O-495 (K)	AF074181	<i>Kegeliella atropilosa</i> L. O. Williams & A. H. Heller	Chase 89142 (K)
<i>Lycomormium squalidum</i> Rchb. f.	Chase O-273 (K)	AF074186	<i>Lycomormium</i> Rchb. f. sp.	FLAS—living 87056
<i>Stanhopea ecorruta</i> Lemaire	Chase O-255 (K)	AF074230	<i>Stanhopea</i> Frost ex Hook. sp.	Cameron 1126 (NY)
TELIPOGONINAE				
<i>Stellilabium pogonostalex</i> (Rchb. f.) Garay & Dunst.	Chase O-123 (K)	AF074231	same DNA	
ZYGOPETALINAE				
<i>Dichaea riopalenquensis</i> Dodson	Chase O-114 (K)	AF074149	<i>Dichaea</i> Lindl. sp.	Cameron 1127 (NY)
<i>Huntleya heteroclita</i> (Poepp. & Endl.) Garay	Whitten 88023 (FLAS)	AF074179	same DNA	
<i>Zygopetalum intermedium</i> Hort. Petrop. ex Regel	Chase O-160 (K)	AF074246	<i>Zygopetalum mackaii</i> Hook.	NYBG—living 232
EPIDENDROID PHYLAD				
ARETHUSEAE				
ARETHUSINAE				
<i>Arethusa bulbosa</i> L.	Chase O-880 (K)	AF074109	same DNA	
BLETIINAE				
<i>Acanthephippium mantinianum</i> Lindl. & Cogn.	Chase O-397 (K)	AF074100	same DNA	
<i>Bletia</i> cf. <i>purpurea</i> (Lam.) DC.	Chase O-581 (K)	AF074113	<i>Bletia</i> Ruiz & Pav. sp.	FLAS—living s.n.
<i>Bletilla striata</i> (Thunb.) Rchb. f.	Chase O-556 (K)	AF074114	same species	NYBG—living s.n.
<i>Calanthe triplicata</i> (Willemet) Ames	Chase O-207 (K)	AF074117	same species	Motley 2291 (NY)
<i>Calopogon tuberosus</i> (L.) Britton, Sterns & Poggenb.	Chase O-876 (K)	AF074119	<i>Calopogon pallidus</i> Chapm.	Cameron 1061 (NY)
<i>Phaius minor</i> Blume	Chase O-325 (K)	AF074210	<i>Phaius tankervilleae</i> (Aiton) Blume	NYBG—living 4406-95
<i>Spathoglottis pacifica</i> Rchb. f.	Motley 2277 (NY)	AY381134	same DNA	
CHYSIINAE				
<i>Chysis bractescens</i> Lindl.	Chase O-436 (K)	AF074126	same species	NYBG—living 1129
COELOGYNEAE				
COELOGYNINAE				
<i>Coelogyne cristata</i> Lindl.	Chase O-491 (K)	AF074133	same species	NYBG—living 3025
<i>Dendrochilum cobbianum</i> Rchb. f.	Cameron 1116 (NY)	AY381119	same DNA	
THUNIINAE				
<i>Thunia alba</i> Rchb. f.	Chase O-589 (K)	AF074233	<i>Thunia marshalliana</i> Rchb. f.	NYBG—living 3481
EPIDENDREAE I				
ARPOPHYLLINAE				
<i>Arpophyllum giganteum</i> Hartweg ex Lindl.	Chase O-586 (K)	AF074110	same species	NYBG—living 3023

Table 1. Continued.

Family, subfamily, tribe, subtribe, genus, and species	Voucher— <i>rbcL</i>	GenBank accession— <i>rbcL</i> ^a	Species— <i>atpB</i>	Voucher— <i>atpB</i>
COELIINAE				
<i>Coelia triptera</i> (Smith) G. Don ex Steud.	Chase O-324 (K)	AF074132	<i>Coelia bella</i> Rchb. f.	FLAS—living s.n.
LAELIINAE				
<i>Cattleya dowiana</i> Batem. & Rchb. f.	Chase O-282 (K)	AF074122	<i>Cattleya percivaliana</i> Hort.	NYBG—living 3052
<i>Dilomilis montana</i> (Sw.) Summerh.	Chase O-206 (K)	AF07415	same DNA	
<i>Encyclia</i> Hook. sp.	unknown	AF074157	<i>Encyclia cochleata</i> (L.) Dressler	Cameron 1115 (NY)
<i>Epidendrum</i> L. sp.	unknown	AF074158	<i>Epidendrum ciliare</i> Linn.	NYBG—living 2017
MEIRACYLLIINAE				
<i>Meiracyllium trinasutum</i> Rchb. f.	Chase O-202 (K)	AF074192	same species	NYBG—living 5178
PLEUROTHALLIDINAE				
<i>Masdevallia infracta</i> Lindl.	Chase O-294 (K)	AF074189	same species	NYBG—living 5377
<i>Pleurothallis endotrachys</i> Rchb. f.	Chase O-306 (K)	AF074217	<i>Pleurothallis restrepioides</i> Lindl.	Cameron 1128 (NY)
<i>Restrepia</i> Kunth sp.	unknown	AF074225	<i>Restrepia sanguinea</i> Rolfe	NYBG—living 2892-95a
SOBRALIINAE				
<i>Elleanthus</i> Presl sp.	Chase O-374 (K)	AF074156	<i>Elleanthus caravata</i> Rchb. f.	NYBG—living 1437
<i>Sobralia macrantha</i> Lindl.	Chase O-200 (K)	AF074228	same species	NYBG—living 3557
EPIDENDREAE II				
GLOMERINAE				
<i>Earina autumnalis</i> Hook. f.	Chase O-298 (K)	AF074155	<i>Earina deplanchei</i> Rchb. f.	Cameron 9810 (NY)
<i>Glomera</i> Blume sp.	Chase O-555 (NCU)	AF074172	<i>Glomera macdonaldii</i> (Schltr.) Ames	Motley 2200 (NY)
POLYSTACHYINAE				
<i>Polystachya pubescens</i> (Lindl.) Rchb. f.	Chase O-152 (K)	AF074222	<i>Polystachya</i> Hook. sp.	NYBG—living 4470-95a
PODOCHILEAE				
BULBOPHYLLINAE				
<i>Bulbophyllum lobbii</i> Lindl.	Chase O-474 (K)	AF074115	same DNA	
DENDROBIINAE				
<i>Cadetia taylori</i> (F Muell.) Schltr.	Shiraishi 132 c	D58406	same species	NYBG—living 191-75a
<i>Dendrobium kingianum</i> Bidw.	Chase O-164 (K)	AF074146	same species	NYBG—living 4527
<i>Epigeneium acuminatum</i> (Rolfe) Summerh.	Shiraishi 804c	D58410	<i>Epigeneium cymbidioides</i> (Bl.) Summerh.	NYBG—living 4549
ERIINAE				
<i>Eria ferruginea</i> Teijsm. & Binn.	Chase O-590 (K)	AF074164	<i>Eria javanica</i> (Swartz) Bl.	NYBG—living 28106
<i>Trichotosia ferox</i> Blume	Chase O-396 (K)	AF074235	same DNA	
PODOCHILINAE				
<i>Podochilus cultratus</i> Lindl.	Chase O-559 (K)	AF074218	same DNA	
THELASIINAE				
<i>Phreatia</i> Lindl. sp.	Chase O-203 (K)	AF074214	<i>Phreatia</i> sp.	Cameron 2048 (NY)
VANDEAE				
AERIDINAE				
<i>Cleisostoma rolfeanum</i> (King & Pantling) Garay	Jarrell s.n.	AF074130	<i>Cleisostoma arietinum</i> (Rchb. f.) Garay	NYBG—living 4592
<i>Neofinetia falcata</i> (Thunb.) S. Y. Hu	Jarrell s.n.	AF074197	same species	NYBG—living 5097

Table 1. Continued.

Family, subfamily, tribe, subtribe, genus, and species	Voucher— <i>rbcL</i>	GenBank accession— <i>rbcL</i> ^a	Species— <i>atpB</i>	Voucher— <i>atpB</i>
<i>Phalaenopsis equestris</i> (Schauer) Rchb. f.	Jarrell s.n.	AF074211	same species	NYBG—living s.n.
ANGRAECINAE				
<i>Aeranthus ramosa</i> Rolfe	Jarrell s.n.	AF074104	<i>Aeranthus grandiflorus</i> Lindl.	FLAS—living s.n.
<i>Angraecum sesquipedale</i> Thouars	Jarrell s.n.	AF074106	<i>Angraecum comorense</i> Finet	Cameron 2129 (NY)
AERANGIDINAE				
<i>Aerangis calligera</i> (Rchb. f.) Garay	Jarrell s.n.	AF074103	<i>Aerangis ugandensis</i> Summerh.	NYBG—living 720-75
<i>Diaphananthe rutila</i> (Rchb. f.) Summerh.	Jarrell s.n.	AF074147	same species	NYBG—living 543
ANOMALOUS	Chase O-395 (K)	AF074111	same species	Motley 2289 (NY)
EPIDENDROIDEAE				
ARUNDINAE				
<i>Arundina graminifolia</i> (D. Don) Hochr.				
POGONIINAE				
<i>Cleistes</i> L. C. Richard sp. 1	Chase O-430 (K)	AF074129	same DNA	
<i>Cleistes</i> sp. 2	Jardim 2579 (NY)	AY381110	same DNA	
<i>Cleistes</i> sp. 3	Thomas 12975 (NY)	AY381111	same DNA	
<i>Cleistes cipoana</i> Hoehne	Thomas 12976 (NY)	AY381112	same DNA	
<i>Cleistes divaricata</i> (L.) Ames	Chase O-376 (K)	AF074127	same species	Cameron 1062 (NY)
<i>Cleistes rosea</i> Lindl.	Cameron 1038 (NCU)	AF074128	same DNA	
<i>Duckeella adolphii</i> Porto & Brade	Romero 3013 (AMES)	AF074154	same DNA	
<i>Isotria medeoloides</i> Rafin.	Keenan s.n.	AY381123	same DNA	
<i>Isotria verticillata</i> (Muhl. ex Willd.) Raf.	Cameron 1030 (NCU)	AF074180	same DNA	
<i>Pogonia japonica</i> Rchb. f.	Cameron 1034 (NCU)	AF074219	same DNA	
<i>Pogonia minor</i> (Makino) Makino	Cameron 1033 (NCU)	AF074220	same DNA	
<i>Pogonia ophioglossoides</i> (L.) Jussieu	Chase O-437 (K)	AF074221	same DNA	
ANOMALOUS				
<i>Eriopsis biloba</i> Lindl.	Chase O-502 (K)	AF074167	same species	FLAS—living s.n.
<i>Neomoorea irrorata</i> (Rolfe) Rolfe	Chase O-503 (K)	AF074198	<i>Neomoorea wallisii</i> Schltr.	FLAS—living 90010
<i>Nephelaphyllum pulchrum</i> Bl.	Cameron 1106 (NY)	AY381127	same DNA	
<i>Xerorchis amazonica</i> Schltr.	Romero 3014 (AMES)	AF074244	same DNA	

^a Accession numbers prefixed by AF, Y, or Z are from GenBank and those with D are DNA Data Bank of Japan (DDBJ).

^b Chase vouchers represent orchid DNA collection numbers from the Kew (K) DNA bank. New *atpB* sequences for this study will be made available in GenBank at a future time.

Fig. 2.—Genera from Epidendroideae (including neottiid and vandoid orchids) representing half of the strict consensus tree resulting from analysis of *atpB* sequences for Orchidaceae. Continued from Fig. 1. Tribes (in boldface) sensu Chase, Freudenstein, and Cameron (2003) are indicated where applicable, and jackknife values >50% are given for supported clades.

Table 2. Matrix and tree statistics for the separate and combined gene analyses of Orchidaceae *rbcL* and *atpB*.

No. taxa	182	182	182
No. characters	1330	1504	2834
No. variable characters (% of total)	605 (45.49%)	668 (44.1%)	1273 (44.92%)
No. informative characters (% of total)	373 (28.05%)	459 (30.52%)	832 (29.36%)
No. trees	>10,000	>10,000	>10,000
Tree length steps	2217	2499	4773
CI/CI excluding uninformative	0.368/0.285	0.389/0.324	0.374/0.302
RI	0.687	0.730	0.705
No. clades with >50%/>75% bootstrap support	66/47	80/64	112/75

based on ca. 10% greater taxon sampling than the *rbcL* consensus tree for Orchidaceae published by Cameron et al. (1999), but differ only slightly. Although there is no jackknife support for the relationships among the five subfamilies, they are fully resolved as follows: Epidendroideae are sister to Orchidoideae, Vanilloideae are sister to this pair, followed by Cypridioideae, and with Apostasioideae sister to all the remaining orchids. Overall support within the family (66 clades >50%; only 47 of these $\geq 75\%$) is considerably less than found with *atpB* alone.

Combined Two-Gene Analysis

Using $P < 0.01$ as a significance threshold for the partition homogeneity test (Cunningham 1997), the *rbcL* and *atpB* data sets cannot be considered incongruent ($P = 0.02$). Moreover, there are no strongly supported clades in conflict between the *rbcL* and *atpB* trees. For these reasons, the two matrices were combined to create a matrix of 2834 total characters. Once again, the MAXTREE limit of 10,000 trees was reached, at which point the analysis was aborted. The CI and RI values (0.374 and 0.705, respectively) are comparable and intermediate to those obtained by the individual gene analyses, but overall resolution and the number of clades supported by the jackknife (especially $\geq 75\%$) increased considerably when the data were combined. The two-gene tree is based on 1273 variable characters, of which 832 are phylogenetically informative. Table 2 shows a comparison of all data matrix and tree statistics. The *atpB* + *rbcL* strict consensus tree with jackknife values is presented as Fig. 4–5 and shows strong support for each of the five subfamilies. Epidendroideae are sister to Orchidoideae, Cypridioideae are sister to this pair, followed by Vanilloideae, and with Apostasioideae sister to the entire Orchidaceae. These intersubfamilial relationships are supported by the jackknife analysis, but the placements of Cypridioideae and Vanilloideae are only weakly so (67–68% jck).

DISCUSSION

Chase, Freudenstein, and Cameron (2003) recently proposed a new classification system for Orchidaceae based on a variety of molecular phylogenetic studies focused at the

subtribe, tribe, and family level (including the data presented here). No fewer than five subfamilies and 17 tribes were recognized (2 in Vanilloideae, 4 in Orchidoideae, and 11 in Epidendroideae). Four tribes of the latter (Cymbidieae, Epidendreae, Podochileae, and Vandaeae) were subdivided further into 26 subtribes. Within Orchidoideae, there were at least 15 subtribes recognized. For the purpose of this discussion, their new classification system will be followed with only slight modifications where noted.

In many ways, the combined *atpB* + *rbcL* tree presented here is not greatly different from the *rbcL* tree published by Cameron et al. (1999). The two studies are difficult to compare, however, in that successive weighting was used to reduce the total number of equally parsimonious trees and increase resolution in the *rbcL* consensus tree. Nevertheless, it is obvious that the addition of these new *atpB* data to the *rbcL* matrix gives a much clearer picture of orchid phylogeny. The *atpB* sequence is 174 bp longer than *rbcL* and consequently provides 86 more phylogenetically informative characters. The *atpB* data also contains less homoplasy than the *rbcL* (CI of 0.324 vs. 0.285 when uninformative characters are excluded), and the tree receives greater jackknife support (80 vs. 66 clades >50%). The possibility exists that part of the inequity between these two matrices is due to the fact that these *rbcL* sequences were completed nearly 10 years ago by less precise manual methods that were more prone to human error than the automated methods of today.

atpB Sequence Anomalies

Length differences were encountered for *atpB* sequences in select orchids. The particular insertions/deletions (indels) were few and not coded separately since most appear to be isolated events of no phylogenetic significance. Species of *Vanilla* are an exception as they all share a six bp insertion at the 3'-end of the gene. The most dramatic cases of sequence anomaly are seen in *Earina* Lindl., which has a divergent *atpB* sequence ca. 12 bp shorter than in other taxa. Near the 5'-end there is a gap of 16 bp, but this is followed downstream by a seven bp insertion, then a nine bp deletion. The gaps resulting from these indels (treated as missing data) may explain the instability of *Earina* in the resulting trees.

Fig. 3.—One of >10,000 equally parsimonious *atpB* trees chosen at random and presented as a phylogram to highlight relative branch lengths (DELTRAN optimization) among subfamilies and genera of Orchidaceae. Note that whereas Cypridioideae and especially most of Epidendroideae have relatively low levels of sequence divergence, all members of Vanilloideae have accelerated rates of substitution compared to other orchids.

Fig. 4.—Strict consensus tree obtained when *atpB* and *rbcL* sequence data for Orchidaceae are analyzed together. Genera from outgroup families and orchid subfamilies Apostasioideae, Vanilloideae, Cyripedioideae, and Orchidoideae are shown; the tree continues in Fig. 5. Subfamilies, tribes, and subtribes sensu Chase, Freudenstein, and Cameron (2003) are indicated in boldface where applicable, and jackknife values >50% are given for supported clades.

The sequence of *Spathoglottis* Blume is also an extreme in that it is ca. 12 bp longer than other taxa; it contains one insertion of five bp and another of seven bp.

In contrast, full length *atpB* sequences were amplified from the mycoheterotrophic and mostly achlorophyllous vanilloid genera *Cyrtosia* Blume, *Erythrorchis* Blume, and *Pseudovanilla* Garay. A sequence from the genus *Galeola* Lour. was not included in this analysis because of missing data for *rbcL*, but even that taxon yielded an intact *atpB* sequence that placed it sister to *Cyrtosia*, as expected based on morphology. Such has not been the case for *rbcL* or *psaB* in which pseudogenes have been documented for *Cyrtosia* (Cameron 2004), and for which *Galeola* has resisted amplification. Whether or not ATP synthase is functional in these nonphotosynthetic orchids is uncertain. Overall, *atpB* sequence divergence is predictably low among genera of Epidendroideae, but extremely high among genera of Vanilloideae, as documented for nuclear (Cameron and Chase 2000), mitochondrial (Freudenstein and Chase 2001), and other plastid genes (Cameron et al. 1999; Cameron 2004) as well.

Subfamily Relationships

One significant difference between the *rbcL* tree and the *atpB* tree is the position of Cyprripedioideae relative to the other subfamilies. In the case of *rbcL*, this monophyletic subfamily of diandrous orchids is sister to all other orchids except Apostasioideae. Both the *atpB* tree and the combined tree (Fig. 2) place Cyprripedioideae as sister to the Epidendroideae + Orchidoideae clade. Cameron and Chase (2000) also recovered this arrangement with 18S data. Most orchid systematists have considered Cyprripedioideae to be only slightly less “primitive” than Apostasioideae on account of their terrestrial habit, two fertile anthers, abscission layer between perianth and ovary, pollen monads, and crustose seeds enclosed within fleshy trilocular fruits in *Selenipedium* Rchb. f., a supposedly relictual genus. However, Atwood (1984) argued against this view of their “primitiveness” and Dressler (1986) at one time felt very strongly that Cyprripedioideae was the sister group to what was then treated as subfamily Neottioideae (i.e., *Epipactis* Zinn, *Listera* Adans., and their relatives, which are now considered “lower” Epidendroideae). He cited evidence in the form of shared seed structure, cytology, and habit between these groups. It is worth pointing out (Fig. 1) that *Cyprripedium* L., not *Selenipedium*, is sister to all other taxa of Cyprripedioideae (94% jck) according to the *atpB* and combined data (its position is unresolved with *rbcL* alone). Some of the presumably plesiomorphic characters of *Selenipedium* may in fact be secondary gains. Moreover, members of Vanilloideae exhibit just as many, or even more, plesiomorphic characters as Cyprripedioideae, and their single anther is developmentally not homologous with that observed in Epidendroideae/Orchidoideae (Freudenstein et al. 2002). Hence, the reversed positions of Cyprripedioideae and Vanilloideae may not be so surprising as they seem at first glance.

Relationships of Problematic Taxa

Within each subfamily there are one or more genera whose position in the orchid phylogeny continues to be un-

stable. For example, the relationships among the four major lineages of Vanilloideae are fully resolved and supported by *atpB*, but not in the two-gene tree. Moreover, the relationships of *Cleistes divaricata* and *Isotria* Raf. to the other genera of Pogonieae in the *atpB* tree are unlike any other topology seen before (e.g., Cameron and Chase 1999). Sequence divergence among all these vanilloid orchids is very high, and the possibility is real that long-branch attraction may be a factor in this clade. The positions of *Chloraea*, *Codonorchis* Lindl., *Megastylis glandulosa*, and *Pterostylis* R. Br. of Orchidoideae are ambiguous in these trees as well. These genera exhibit a number of plesiomorphic characters, and are possible descendents of an ancient ancestor(s) distributed across Gondwana. Today they are isolated relicts of disjunct lineages distributed in Chile, New Caledonia, and Australia. The other species of *Megastylis* Schltr. are firmly embedded within Diurideae, making the genus polyphyletic, but *M. glandulosa* shows an unexpected affinity with *Pachyplectron* Schltr. (both endemic to New Caledonia) according to the *atpB* data (Fig. 4). With *rbcL* (Cameron et al. 1999) and *psaB* (Cameron 2004) *Megastylis glandulosa* is sister to *Chloraea*, whereas *Pachyplectron* is allied to Goodyerinae—a more logical arrangement based on column morphology, pollen structure, and other floral features. The *atpB* tree places *Chloraea* sister to all other genera of Orchidoideae, whereas the combined tree relocates the genus to the base of Cranichideae (Fig. 4). Neither position is supported by the jackknife. Likewise, *Codonorchis* is either sister to all Orchidoideae or unresolved among the tribal branches of Orchidoideae. Recognition of Codonorchideae, in either case, is probably justified since this monotypic genus with whorled leaves is morphologically unique in the family. Greater character and taxon sampling may help to settle these mobile taxa in future analyses.

It is difficult to make firm conclusions regarding relationships among the major lineages of Epidendroideae since resolution and jackknife support is poor in this group. Nevertheless, a few sister relationships in the subfamily are worth pointing out (Fig. 2). An alliance between Malaxideae and Dendrobieae (both with naked pollinia) continues to hold in these trees, just as it did with *rbcL* (Cameron et al. 1999). Podochileae may also be closely related to them. *Polystachya* seems firmly positioned now as sister to Vandaeae (72% jck). Cameron et al. (1999) expressed concern for the placement of *Polystachya* near Laeliinae in their *rbcL* tree, and felt that it might represent a spurious result of incomplete taxon sampling. The *rbcL* sequence of *Polystachya* contains some 30 bp of missing data, and is one of the most divergent in Epidendroideae. It is probably of dubious quality and should be resequenced. Chase, Freudenstein, and Cameron (2003) proposed Collabiinae as a subtribe to encompass several genera typically classified as part of Arethuseae (Fig. 5). The two-gene tree shows that this subtribe is monophyletic and clearly unrelated to the core Arethuseae. It may or may not be part of Epidendreae, but the monophyly of that tribe is not resolved here. More sampling is needed in the form of genera such as *Isochilus* R. Br. or *Ponera* Lindl., which may help to bring these groups together, since van den Berg et al. (2000) identified them as basal members of the Laeliinae clade. Likewise, it may be wise to sequence *Cremastra* Lindl. and/or other members of Calypsoeae for

future studies, because that tribe continues to be polyphyletic.

Conclusions and Future Directions

The addition of these new *atpB* characters to the *rbcL* matrix of Cameron et al. (1999) gives a much clearer picture of phylogenetic relationships within Orchidaceae since the overall two-gene tree's resolution and jackknife support levels are increased substantially over either individual gene tree. Perhaps the greatest value of these new data is in documenting that although they are relatively conserved, collecting additional plastid gene sequences for Orchidaceae is worth the effort. They are easy to sequence and avoid many of the pitfalls encountered with sequencing nuclear, mitochondrial, or more variable plastid regions (especially issues of alignment). Certainly, other plant systematists have found the combination of several plastid genes to be a profitable strategy for improving hypotheses of phylogeny (Graham and Olmstead 2000; Reeves et al. 2001; Sytsma et al. 2002), and so the next step in this program of research will be to combine the *rbcL* and *atpB* data with a third plastid gene (*psaB*) for the same set of taxa. Ultimately, the fundamental issues of orchid origins, speciation, and coevolution with animals, fungi, and other plants can be addressed more objectively when a robust phylogeny for the family is in hand.

ACKNOWLEDGMENTS

Special thanks are extended to Ken Wurdack and Susan Pell for their assistance with DNA extraction and gene sequencing. Mark Chase, Harold Koopowitz, Barry Glick, Mark Whitten, Tomohisa Yukawa, Eric Christenson, and others who generously shared plant material and DNA samples. The Lewis B. and Dorothy Cullman Foundation as well as the USA National Science Foundation (grant DEB-0108100) are acknowledged for their financial support.

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Fig. 5.—Strict consensus tree obtained when *atpB* and *rbcL* sequence data for Orchidaceae are analyzed together. Genera from Epidendroideae are shown as a continuation from the previous figure. Tribes and subtribes sensu Chase, Freudenstein, and Cameron (2003) are indicated in boldface where applicable, and jackknife values >50% are given for supported clades.

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