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On the value of nuclear and mitochondrial gene sequences for reconstructing the phylogeny of vanilloid orchids (Vanilloideae, Orchidaceae)

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• *Background and Aims* Most molecular phylogenetic studies of Orchidaceae have relied heavily on DNA sequences from the plastid genome. Nuclear and mitochondrial loci have only been superficially examined for their systematic value. Since 40% of the genera within Vanilloideae are achlorophyllous mycoheterotrophs, this is an ideal group of orchids in which to evaluate non-plastid gene sequences.
• *Methods* Phylogenetic reconstructions for Vanilloideae were produced using independent and combined data from the nuclear 18S, 5·8S and 26S rDNA genes and the mitochondrial *atpA* gene and *nad1b-c* intron.
• *Key Results* These new data indicate placements for genera such as *Lecanorchis* and *Galeola*, for which plastid gene sequences have been mostly unavailable. Nuclear and mitochondrial parsimony jackknife trees are congruent with each other and previously published trees based solely on plastid data. Because of high rates of sequence divergence among vanilloid orchids, even the short 5·8S rDNA gene provides impressive levels of resolution and support.
• *Conclusions* Orchid systematists are encouraged to sequence nuclear and mitochondrial gene regions along with the growing number of plastid loci available.

Key words: 26S rDNA, 18S rDNA, 5·8S rDNA, *atpA*, *nad1*, orchids, plastid, *Vanilla*, vanilloid orchids, Vanilloideae.

INTRODUCTION

With few exceptions, molecular systematic studies of Orchidaceae above the species level have relied on sequences of plastid genes or intergenic spacers (ITSs)/introns. This has also been true for the majority of angiosperm phylogenetic studies. Among the plastid loci most commonly used by orchidologists are *ndhF* (Neyland and Urbatsch, 1996), *rbcL* (Cameron *et al.*, 1999), *atpB* (Cameron, 2006), *matK* (Goldman *et al.*, 2001) and *trnL-F* (Kores *et al.*, 2001). Cameron (2004) introduced *psaB* as an alternative plastid gene for intergeneric studies of Orchidaceae, and the plastid genes *psbB* and *psbC* were shown by Cameron and Molina (2006) to be of value in a study to determine the sister taxon of *Vanilla*. Others have explored the value of additional plastid regions such as *rpoC1*, *ycf1*, *trnS-G* and *trnH-psbA* for systematic studies of orchids (Dueck and Cameron, 2007; W. M. Whitten, University of Florida, pers. comm.). There is little doubt that the plastid genome holds a wealth of information for the orchid systematist. Now that the entire plastid genome sequence of *Phalaenopsis* is known (Chang *et al.*, 2005), and the plastid genomes of additional orchid genera have been sequenced (N. H. Williams, University of Florida, pers. comm.), even more plastid regions are likely to be introduced.

During this same time, there has been little effort to incorporate nuclear and/or mitochondrial gene sequences into orchid phylogenetic studies. The notable exception to this observation is the use of the two highly variable nuclear rDNA ITSs (ITS1 and 2), which are nearly ubiquitous among interspecific studies of orchids (e.g. Cox *et al.*, 1997; Gravendeel *et al.*, 2001; Pridgeon and Chase, 2001). Only a few other published studies have looked beyond the plastid genome or nuclear ITS region. Cameron and Chase (2000) sequenced a limited number of taxa for the nuclear 18S rDNA gene in an effort to place *Rhizanthella*, *Cyrtosia* and other achlorophyllous genera of uncertain affinity within appropriate subfamilies of Orchidaceae. Likewise, Molvray *et al.* (2000) used 18S rDNA sequences to gain a better understanding of phylogenetic relationships among genera of Gastrodieae and Epipogieae (both Epidendroideae), all species of which are non-photosynthetic mycoheterotrophs. The only study of Orchidaceae that has sampled the mitochondrial genome has been that of Freudenstein and Chase (2001), who provided a reconstruction of the family based on *nad1b-c* intron sequences. Again, this study proved itself to be especially valuable in providing data for those achlorophyllous taxa of Epidendroideae for which plastid gene sequencing has proved difficult or impossible.

Subfamily Vanilloideae also contain a number of non-photosynthetic genera, and many of these have been absent in phylogenetic studies using plastid DNA sequences alone. In fact, 40% of the 15 genera of vanilloid orchids *sensu* Cameron (2003) are mycoheterotrophic. These are *Cyrtosia*, *Erythrorchis*, *Galeola*, *Lecanorchis*, *Pogoniopsis* and, to a lesser extent, *Pseudovanilla*. Despite this high percentage, some of these taxa retain partial or even full-length copies of some plastid genes. Cameron and co-authors in a number of studies (e.g. Cameron *et al.*, 1999; Cameron, 2004, 2006; Cameron and Molina, 2006) have been able to place *Pseudovanilla*, *Cyrtosia* and *Erythrorchis* within Vanilloideae

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using partial sequences of *rbcL*, *psaB*, *psbB*, *psbC* and/or *atpB*. No plastid DNA sequences have been published for *Lecanorchis*, and no DNA sequences of any kind have been published for *Pogoniopsis* or *Galeola*, although Cameron (2006) stated that he had been able to amplify an intact copy of *atpB* from *Galeola*. DNA of *Pogoniopsis* has not been available.

Considering that two of the three plant genomes have been insufficiently sampled within Orchidaceae and that the phylogenetic relationships of Vanilloideae are still incompletely known due to missing data from achlorophyllous genera, this study was initiated to evaluate a few loci from the nuclear and mitochondrial genomes for these orchids. The loci under consideration are 18S, 5·8S and 26S rDNA, representing the tandemly repeated nuclear rDNA cistron, and the mitochondrial *atpA* gene and *nad1b-c* intron region.

MATERIALS AND METHODS

Taxon sampling and gene sequencing

Incomplete gene sequences from the nuclear ribosomal genes 26S, 18S and 5·8S rDNA were obtained for 35 taxa, representing 13 of the 15 genera of Vanilloideae (Table 1). Most of these taxa were also sampled for the mitochondrial loci *atpA* and *nad1b-c*. DNA was unavailable for two rare genera of Vanilloideae: *Pogoniopsis* and *Dictyophyllaria*. Most of the sampled taxa were field collected and vouchered, details of which are given in Table 1. Considering that tribe Pogonieae is known to be monophyletic and sister to tribe Vanilleae based on more broadly sampled studies of Orchidaceae (e.g. Cameron *et al*, 1999; Cameron, 2004), it was specified as the outgroup in all analyses presented here. The data matrices are available from the author upon request. All sequences have been submitted to GenBank (Table 1).

All newly generated sequences were produced by automated methods, briefly described as follows. Total DNA was extracted according to the manufacturer's protocols using the DNEasy™ (Qiagen, Valencia, CA, USA) method from approx. 0·5 cm^2 of dried leaf tissue. Target loci were amplified in 25 μL volumes using standard amplification protocols that typically included the addition of bovine serum albumin. Sometimes betaine was added to relax the secondary DNA structure. Amplification and double-stranded sequencing reactions were completed using primers previously published by various authors. The 18S rDNA primers are mostly based on those published by Bult *et al.* (1992) and modified later by others. Kuzoff *et al.* (1998) gave details concerning the primers for 26S rDNA sequencing. The 5·8S rDNA gene was amplified with the flanking ITS1 and 2 regions using primers that can be traced back to various publications, including Nickrent *et al.* (1994). The *nad1b-c* primers were synthesized based on information originally provided by Demesure *et al.* (1995). Primer sequences for *atpA* sequencing were taken from Davis *et al.* (1998). Many of these primers have been in use for over a decade, and their exact primary origins are difficult to trace. For this reason, the complete primer sequences are listed here 5'–3' as follows: for 18S, gtagtcatatgcttgtctc*, gcccttccgtcaattcctttaagtttcagc, tcctattgtgt tggcctt and cgacttctccttcctcta*; for 26S, agggaagcggatgg gggc*, gctatcctgagggaaacttc, cgtgcaaatcgttcgtct and acccatgtgcaagtgccgtt*; for 5·8S, tatgcttaaaytcagcgggt* and aacaaggtttccgtaggtga*; for *nad1b-c*, catcacctacagccctttc, gaaagggctgtaggtgatggyg, gcattacgatctgcagctca* and ggagctcgattagtttctgc*; for *atpA*, aagtggatgagatcggtcgag*, ttccgcgataatggaatgca, agcggctctttctaagagac and ggcattcgatcacaga*. Amplification primers are indicated with an asterisk (*). The nuclear rDNA ITS1 and 2 were amplified (with 5·8S), but the spacers could not be aligned among these genera. They were not included in these analyses. In all cases, resulting PCR products were purified using QIAquick™ spin columns (Qiagen). Cycle sequencing reactions were performed using a combination of purified PCR template, primer and BigDye™ reaction mix (Applied Biosystems, Foster City, CA, USA) for 20 cycles. To remove excess dye terminators and primer from the cycle sequencing products, Centri-Sep™ Sephadex columns (Princeton Separations, Adelphia, NJ, USA) were employed. Final purified samples were subsequently dehydrated, re-suspended in a mixture of formamide and loading dye, and pipetted onto a 5% denaturing polyacrylamide gel. Samples were analysed on an Applied Biosystems ABI 377XL automated DNA sequencer. Resulting electropherograms were edited and sequences aligned by eye using Sequencher 3·0 software (GeneCode, Ann Arbor, MI, USA).

Phylogenetic analyses

In this study the aim was to to determine whether selected nuclear and/or mitochondrial gene sequences would provide an appropriate level of variation to reconstruct phylogenetic relationships within Vanilloideae and whether they would result in parallel or conflicting topologies for intergeneric relationships within Vanilloideae when compared with previously published plastid DNA studies. Abbreviated heuristic searches were executed to calculate relative consistency index (CI) and retention index (RI) scores for each analysis, but not to find all equally parsimonious trees. Instead, parsimony jackknife consensus trees were calculated and used to address comparative issues of tree topology, resolution and support. In a few cases, a single tree was generated to highlight the variability of branch lengths and sequence divergence among taxa. In all cases, gaps were treated as missing data, and there was no attempt made to code indels. Jackknife support was calculated by performing 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 and saving two trees per replicate. All analyses were performed using PAUP* v. 4.0b10 (Swofford, 2002).

RESULTS

Trees for individual loci

The aligned nuclear 5·8S matrix contains 191 characters of which 45 (24%) are variable and 30 (16%) potentially informative. The CI is 0·78 and the RI is 0·90. Sequences were available for 24 taxa. Only eight nodes of the tree (not shown) were supported (>50% jackknife), including clades

TABLE 1. *Species of Vanilloideae (Orchidaceae) sequenced for this study; voucher information and GenBank accession numbers are provided*

Taxon	Voucher	GenBank, 18S	GenBank, 5·8S	GenBank, 26S	GenBank, *atpA*	GenBank, *nad1b-c*
Tribe Vanilleae						
Clematepistephium smilacifolium (Rchb.f.) N.Hallé	*Ziesing 33* (CBG)	FJ425740	FJ425838	FJ425773	–	FJ425846
Cyrtosia lindleyana Hook.f. & Thomson	*Cameron 2182* (WIS)	–	–	FJ425775	FJ425807	FJ425847
Cyrtosia septentrionalis (Rchb.f.) Garay	*Cameron 1048* (WIS)	FJ425742	FJ425826	FJ425774	FJ425808	FJ425848
Epistephium lucidum Cogn.	*Cameron 1039* (WIS)	FJ425744	FJ425836	FJ425780	FJ425810	FJ425853
Epistephium parviflorum Lindl.	*Cameron 1040* (WIS)	FJ425745	FJ425828	FJ425777	–	FJ425850
Epistephium sp. Kunth	*Chase O-432* (MICH)	FJ425746	FJ425839	FJ425778	FJ425811	FJ425851
Epistephium sp. Kunth	*Chase O-433* (MICH)	FJ425747	FJ425827	FJ425779	FJ425812	FJ425854
Epistephium subrepens Hoehne	*Cameron 1037* (WIS)	FJ425748	FJ425837	FJ425781	FJ425813	FJ425852
Eriaxis rigida Rchb.f.	*Ziesing 5* (CBG)	FJ425749	FJ425833	FJ425782	FJ425814	–
Erythrorchis altissima Blume	*Cameron 1029* (WIS)	FJ425750	–	FJ425784	FJ425815	FJ425855
Erythrorchis cassythoides (A.Cunn. ex Lindl.) Garay	*Weston 1831* (WIS)	FJ425751	FJ425841	FJ425783	FJ425816	AH010950
Galeola nudifolia Lour.	*Cameron 1045* (WIS)	FJ425752	–	FJ425785	–	–
Lecanorchis multiflora J.J.Sm.	*Cameron 1015* (WIS)	FJ425755	FJ425831	FJ425788	FJ425819	FJ425858
Lecanorchis nigricans Honda	*Yukawa* s.n. (WIS)	FJ425756	FJ425829	FJ425789	FJ425820	FJ425859
Pseudovanilla foliata (F.Muell.) Garay	*Cameron 1046* (WIS)	–	–	FJ425793	FJ425823	FJ425863
Pseudovanilla ponapensis (Kaneh. & Yamam.) Garay	*Cameron* s.n. (WIS)	FJ425760	–	FJ425794	FJ425824	FJ425864
Vanilla africana Lindl.	*Chase O-584* (K)	FJ425762	FJ425834	FJ425798	–	FJ425865
Vanilla aphylla Blume	*Cameron 1041* (WIS)	FJ425763	AF151006	FJ425795	–	FJ425871
Vanilla barbellata Rchb.f.	*Chase O-591* (K)	FJ425764	FJ425835	FJ425797	–	FJ425866
Vanilla cf. *planifolia* Andrews	*Chase O-170* (MICH)	FJ425765	FJ425832	FJ425800	–	FJ425869
Vanilla imperialis Kraenzl.	*Chase O-587* (K)	–	FJ425830	FJ425799	FJ425825	FJ425867
Vanilla mexicana Mill.	*McCartney* s.n.	FJ425761	–	FJ425796	–	FJ425868
Vanilla roscheri Rchb.f.	*Chase O-540* (K)	FJ425766	FJ425840	FJ425801	–	FJ425870
Tribe Pogonieae						
Cleistes cipoana Hoehne	*Thomas 12976* (NY)	–	–	FJ425771	FJ425802	FJ425842
Cleistes divaricata (L.) Ames	*Cameron 1062* (WIS)	FJ425738	AF151009	FJ425767	FJ425803	FJ425843
Cleistes rosea Lindl.	*Cameron 1038* (WIS)	FJ425739	–	FJ425769	FJ425804	–
Cleistes sp. 1	*Chase O-430* (MICH)	FJ425741	AF151013	FJ425772	FJ425805	FJ425845
Cleistes sp. 2	*Jardim 2579* (NY)	–	–	FJ425768	FJ425806	FJ425844
Cleistes sp. 3	*Thomas 12975* (NY)	–	–	FJ425770	–	–
Duckeella adolphii Porto & Brade	*Romero 3013* (AMES)	FJ425743	AF151007	FJ425776	FJ425809	FJ425849
Isotria medeoloides Raf.	*Keenan* s.n.	FJ425753	–	FJ425786	FJ425817	FJ425856
Isotria verticillata (Muhl. ex Willd.) Raf.	*Cameron 1030* (WIS)	FJ425754	AF151008	FJ425787	FJ425818	FJ425857
Pogonia japonica Rchb.f.	*Cameron 1034* (WIS)	FJ425757	AF151011	FJ425790	FJ425821	FJ425861
Pogonia minor Makino	*Cameron 1033* (WIS)	FJ425758	AF151010	FJ425791	FJ425822	FJ425860
Pogonia ophioglossoides (L.) Ker Gawl.	*Chase O-437* (MICH)	FJ425759	AF151012	FJ425792	–	FJ425862

comprised of *Vanilla* spp. (87%), *Epistephium* spp. (96%), *Lecanorchis* spp. (99%), *Pogonia* spp. (62%) and temperate Pogonieae (72%).

The aligned nuclear 18S matrix contains 1699 characters of which 207 (12%) are variable and 176 (10%) potentially informative. The CI for these data is 0·66 and RI 0·88. Sequences were available for 29 taxa. The parsimony jackknife tree is not shown, but see Fig. 1 for the results of the combined nuclear gene analysis.

The aligned nuclear 26S matrix contains 1098 characters of which 349 (32%) are variable and 291 (27%) potentially informative. The CI is 0·59 and RI 0·84. Sequences were available for 35 taxa. The parsimony jackknife tree is not shown, but see Fig. 1.

The aligned mitochondrial *atpA* matrix contains 1217 characters of which 76 (6%) are variable and 53 (4%) potentially informative. The CI is 0·77 and RI 0·92. Sequences were available for 24 taxa. The parsimony jackknife tree is presented (Fig. 2).

The aligned mitochondrial *nad1b-c* matrix contains 2412 characters of which 550 (23%) are variable and 407 (17%) potentially informative. The CI for these data is 0·84 and RI 0·92. Sequences, which ranged in size from 951 bp in the case of *Clematepistephium smilacifolium* to 2001 bp for *Erythrorchis altissima*, were available for 31 taxa. The average sequence length was approx. 1600 bp. A parsimony jackknife tree is presented as Fig. 3. Sequences from *Epistephium* spp. are highly divergent from all other taxa due to indels and possible inversions. To highlight this phenomenon, a single tree is depicted to show relative branch lengths (Fig. 4).

Trees for combined loci

The three nuclear genes, 26S, 5·8S and 18S rDNA, were combined into a single matrix containing 2988 characters, of which 497 are potentially informative. The CI is 0·63 and RI 0·84. A parsimony jackknife tree is presented (Fig. 1).

Likewise, the two mitochondrial loci, *atpA* and *nad1b-c*, were combined into a single matrix containing 3629

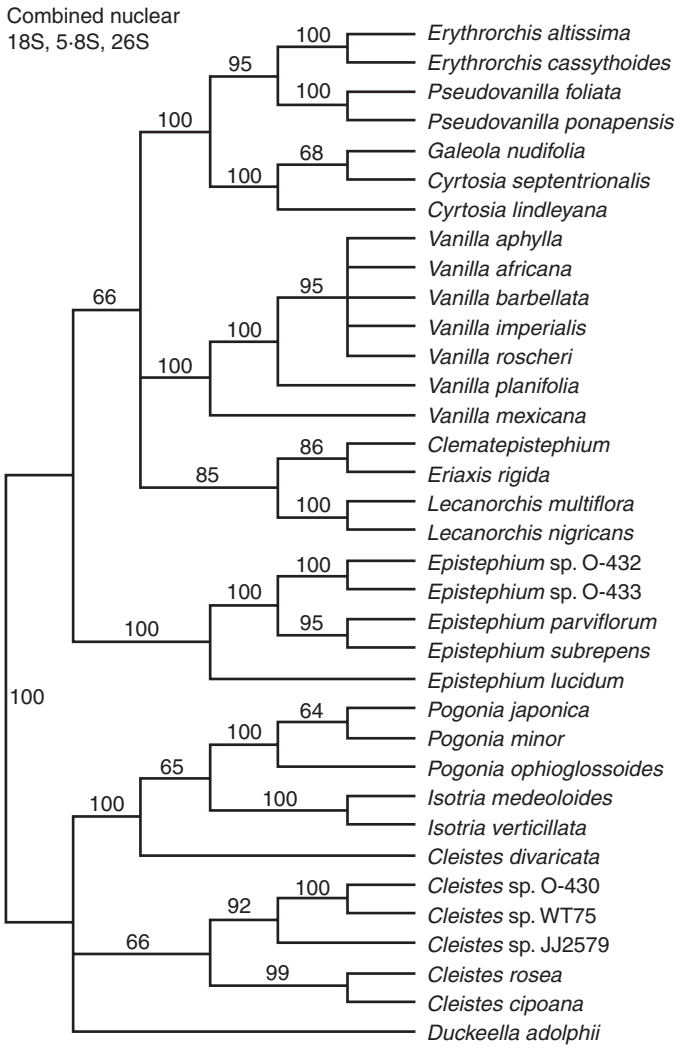

FIG. 1. Parsimony jackknife tree for Vanilloideae based on combined nuclear ribosomal gene sequences. Support percentages >50 are indicated above the branches.

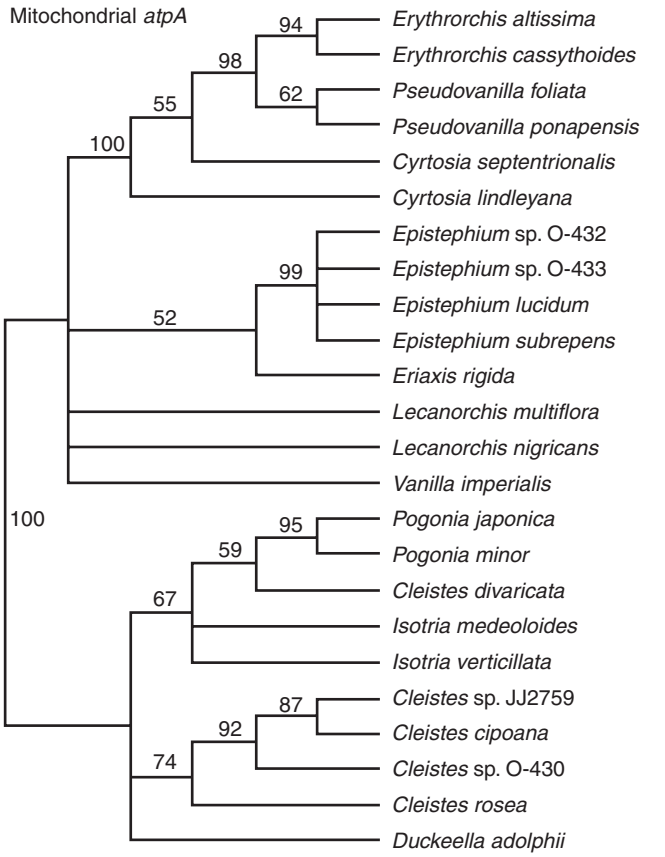

FIG. 2. Parsimony jackknife tree for Vanilloideae based on mitochondrial *atpA* gene sequences. Support percentages >50 are indicated above the branches.

characters, of which 459 are potentially informative. The CI is 0·83 and RI 0·92 (tree not shown).

The three nuclear and two mitochondrial loci were combined into a five-locus matrix containing 6617 characters. The matrix contains 35 taxa. The CI is 0·71 and RI 0·88. A parsimony jackknife tree is presented (Fig. 5). From this it can be seen that every node of the tree is supported by jackknife percentages >50%, and all but three are supported by >70%. *Vanilla* is monophyletic and sister to a clade containing mostly mycoheterotrophic vines. A second lineage of mycoheterotrophs, *Lecanorchis* spp., is sister to the pair of monotypic genera endemic to New Caledonia, *Eriaxis* and *Clematepistephium*. Sister to all of the rest of Vanilleae is *Epistephium*. Relationships within Pogonieae are highly supported. *Pogonia* is sister to *Cleistes divaricata*, and this pair is sister to *Isotria*. These orchids form a clade of temperate Pogonieae. Sister to the temperate clade are tropical Pogonieae, represented by five accessions of *Cleistes*, with *Duckeella* as their sister.

DISCUSSION

The value of non-plastid gene sequences within Vanilloideae

The greatest value of the data presented here lies in the positioning of those achlorophyllous taxa of Vanilloideae for which only partial, functionless and/or divergent plastid sequences have been obtained; in some cases no plastid gene sequences have been available. For all five loci considered in this rerport, complete sequences of *Lecanorchis multiflora* and *L. nigricans* were successfully amplified and sequenced. To date, no sequences from any species of this mycoheterotrophic genus have been obtained from the plastid genome, despite repeated efforts to do so. Similarly, there are no published sequences of the genus *Galeola*. This may, in part, be due to the fact that only a degraded DNA sample has been available. Nevertheless, quality sequences of nuclear 18S and 26S rDNA were obtained from this sample. The other non-photosynthetic members of the subfamily for which samples are available were also successfully sequenced for both mitochondrial loci and most of the nuclear loci. These included two species each of *Cyrtosia*, *Erythrorchis* and *Pseudovanilla*. As stated in the Introduction, plastid gene or pseudogene sequences have been recovered from these plants in the past, and so there are existing phylogenetic hypotheses with which to compare these nuclear and mitochondrial results.

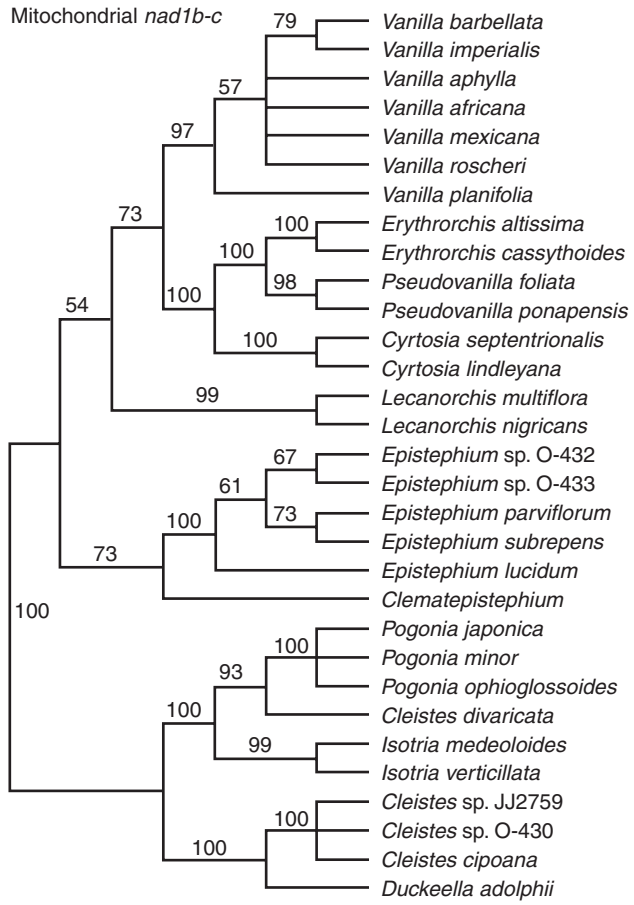

FIG. 3. Parsimony jackknife tree for Vanilloideae based on mitochondrial *nad1b-c* intron sequences. Support percentages >50 are indicated above the branches.

It is also worth commenting on the unexpected utility of the nuclear 5·8S rDNA gene for reconstructing relationships within Vanilloideae. This short gene (<200 bp) is flanked by the ITS1 and ITS2 regions, and is generally considered to be so highly conserved as to be of little value for systematic studies. However, Vanilloideae are known for their exceptional rates of molecular evolution in all three genomic compartments, and hypervariable regions such as nuclear ITS rDNA or the plastid *trnL-F* intron/spacer are almost impossible to align among them. On the other hand, genes typically considered useful primarily for interfamilial studies (i.e. many of those considered herein) are of value within Vanilloideae. Thus, it is not surprising that there is a relatively large amount of phylogenetic information in the 5·8S rDNA sequences for this set of taxa.

In general, the resulting trees presented here are congruent with one another and with previously published cladograms. Both nuclear and mitochondrial sequences, in this case, are worthy of further consideration as more taxa are collected and sampled. At the same time, however, caution must be exercised when using these regions, particularly those derived from the mitochondrial genome. Others have commented on the perils and pitfalls of analysing mitochondrial sequences in plants (Palmer *et al.*, 2000; Adams *et al.*, 2002; Peterson *et al.*, 2006), and some of the same anomalies of molecular evolution seen in other plant lineages are evident in these data as well. For example, among these *nad1* intron data are seen extremes in length variation ranging as much as 1500 bp between *Clematepistephium* and *Erythrorchis*. Numerous indels are present, making alignment a challenge, and putative inversions and/or highly divergent stretches of nucleotides relative to the other genera are evident within *Epistephium*, thereby placing the genus on an especially long branch (Fig. 4).

Relationships among Pogonieae

These data corroborate relationships among taxa of Pogonieae uncovered by earlier studies (e.g. Cameron *et al.*, 1999; Cameron, 2004; Cameron and Molina, 2006). In all cases, the three species of *Pogonia* form a clade, with the two Asian species sister to each other in most trees. The two species of *Isotria* are always sister to each other, and *Cleistes divaricata* is always part of a clade of temperate taxa. This renders the genus polyphyletic since the tropical species sampled almost always form an unrelated clade. *Cleistes divaricata* should be treated as a species of *Pogonia* or as a distinct, possibly monotypic genus [Smith *et al.* (2004) showed there to be two genetic lineages in North American *Cleistes*, but the division did not follow the current division into *C. bifaria* and *C. divaricata*, with some populations of *C. bifaria* being genetically closer to *C. divaricata* than to the other populations of *C. bifaria*]. Identifying morphological characters to support the generic status for *C. divaricata s.l.* separate from *Pogonia* or *Cleistes* has proved difficult.

The only taxa that vary in their placement within Pogonieae among the individual trees are *Duckeella* and *C. divaricata*. In the plastid and ITS analyses published to date, *Duckeella* is sister to all Pogonieae, but that position is not supported here. Instead, *Duckeella* is either sister to temperate Pogonieae (26S, 69% jackknife support; 5·8S, 50% jackknife support), sister to tropical *Cleistes* species (18S, 60% jackknife support; *nad1*, 100% jackknife support) or unresolved (*atpA*, <50% jackknife support). The conflicting positions are mostly poorly supported and may simply be due to sampling error since there are no taxa included in these analyses from other orchid subfamilies. In any case, combination of data from all three genomic compartments positions *Duckeella* sister to all Pogonieae with 100% jackknife support (tree not shown), and this would seem to be its proper placement. It is morphologically distinct from all other taxa in the tribe (see Cameron and Chase, 1999).

The position of *C. divaricata* from North America also varies among trees. In some cases it is sister to *Pogonia*; in other cases it is sister to *Pogonia* plus *Isotria*, and in other cases its position is unresolved within temperate Pogonieae. Among nuclear and mitochondrial gene trees, only *nad1b-c* provides high jackknife support (93%) for the position of *C. divaricata*, and this is sister to *Pogonia*. That same relationship is recovered when all data are combined, including plastid and morphological data (e.g. Cameron and Chase, 1999; Cameron *et al.*, 1999; Cameron, 2006).

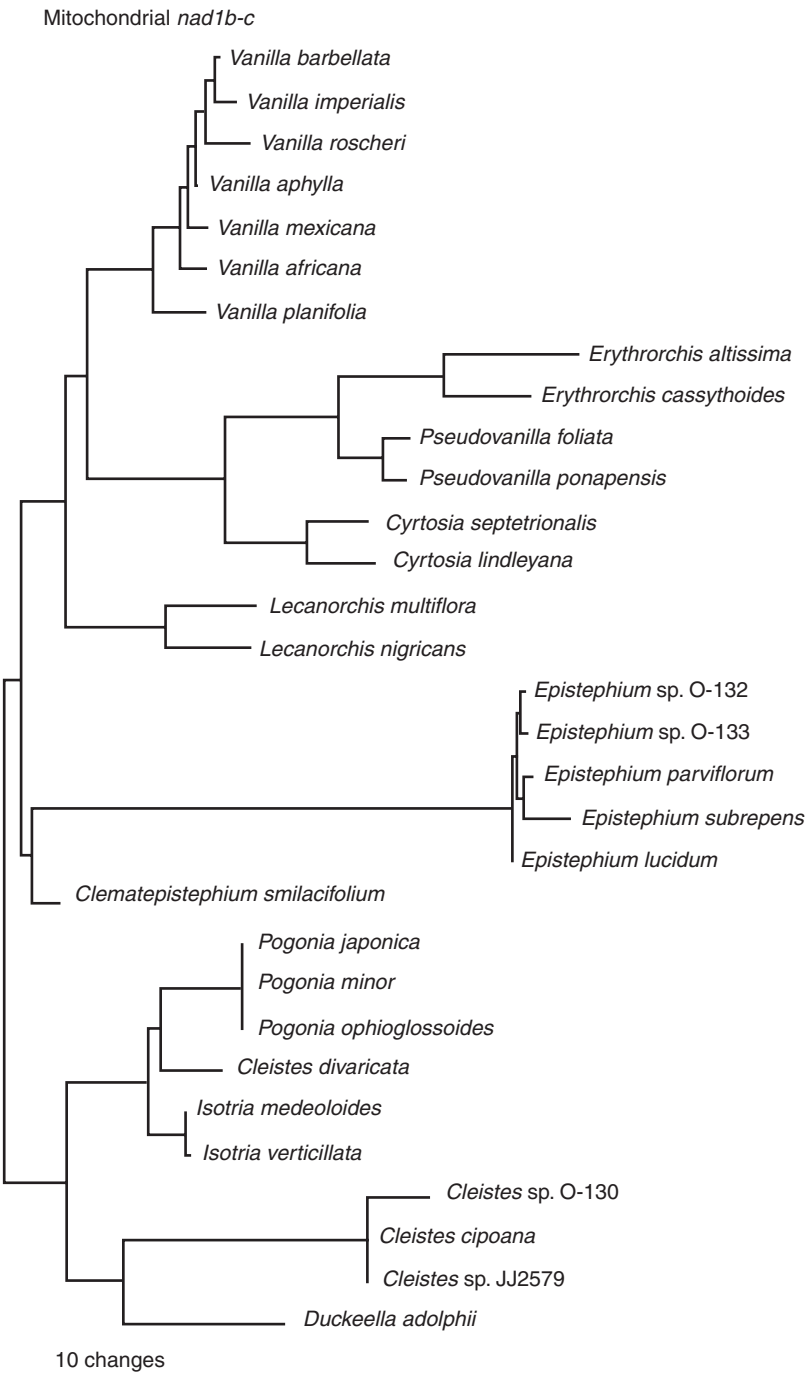

FIG. 4. A single tree depicting relative branch lengths for one of the most equally parsimonious trees for Vanilloideae based on mitochondrial *nad1b-c* intron sequences.

Relationships among Vanilleae

Unlike its sister clade, relationships among the major lineages of Vanilleae have been less certain in previous studies. Clear molecular and morphological evidence has been accumulating over the past decade to support the monophyly of *Epistephium*, *Vanilla*, the pair of New Caledonian endemics (*Clematepistephium* and *Eriaxis*) and the clade of mostly achlorophyllous mycoheterotrophic genera related to *Pseudovanilla*. These new data confirm those findings. Also, Cameron and Molina (2006) presented well-supported evidence for a sister relationship between *Vanilla* and the mycoheterotrophic *Pseudovanilla* clade based on plastid gene sequences. That sister relationship is also supported by the nuclear and mitochondrial gene trees depicted here.

What remains unclear is the exact relationship among *Epistephium*, the New Caledonian sister genera and *Lecanorchis*. Each individual jackknife consensus tree

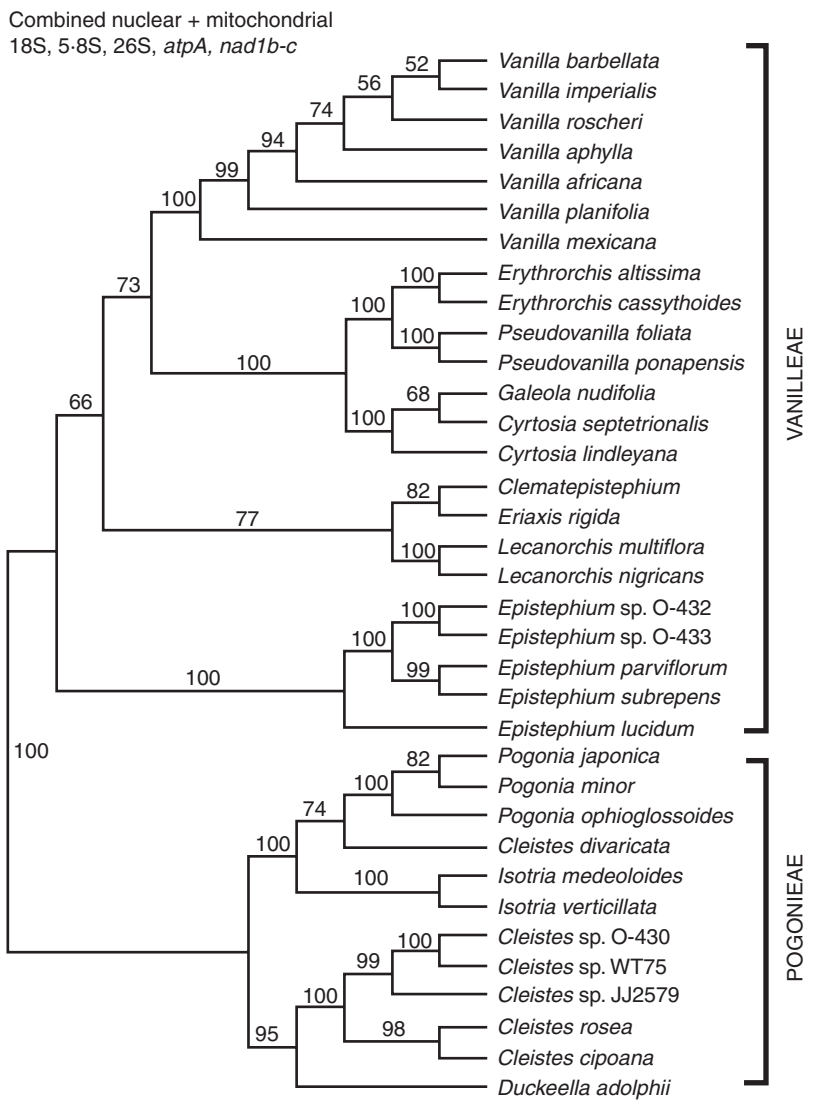

FIG. 5. Parsimony jackknife tree for Vanilloideae based on combined nuclear 26S, 5·8S and 18S rDNA gene sequences plus the mitochondrial *atpA* gene and *nad1b-c* intron sequences. Support percentages >50 are indicated above the branches.

calculated for the separate nuclear and mitochondrial gene matrices places *Lecanorchis* in an unresolved (or at least unsupported) position among Vanilleae. In combination, however, the three nuclear genes place *Lecanorchis* sister to the New Caledonian genera with 85% jackknife support (see Fig. 1). That relationship is not supported by the mitochondrial data, but holds up with 77% jackknife support in the combined nuclear + mitochondrial gene trees (Fig. 5). This is a surprising relationship. *Lecanorchis* has seeds unlike those of the New Caledonian genera (or any other orchid, for that matter), *Lecanorchis* spp. are distributed throughout Southeast Asia but not in Australia or the Pacific Islands, and the floral morphology of *Lecanorchis* is more similar to that of some *Vanilla* spp. than to anything else. However, *Lecanorchis* does share with *Eriaxis* and *Clematepistephium* the phenomenon of placental intrusion into the ovary resulting in a pseudo-trilocular condition. *Lecanorchis* is such an enigmatic genus of orchids, and with such reduced vegetative morphology, that its placement as sister to the New Caledonian taxa is just as believeable as a position anywhere else in Vanilleae. However, all three genera are relatively phylogenetically isolated, and undoubtedly there have been numerous extinction events of perhaps entire lineages throughout Vanilloideae that could confound reconstruction of phylogenetic relationships of the extant relictual members. More systematic data for *Lecanorchis* are needed before we can have confidence in the result uncovered in this study, but there is now a hypothesis in hand to be further evaluated. No plastid data from *Lecanorchis* exist for making further comparisons.

The position of *Galeola* in these trees must be addressed because it renders *Cyrtosia* paraphyletic. This is probably an anomaly related to taxon and gene sampling. Sequences for all three species in question, *Galeola nudifolia*, *Cyrtosia septentrionalis* and *Cyrtosia lindleyana*, are available only for 26S rDNA. That gene provides just 67% support for

Galeola being sister to *C. septentrionalis*; the same relationship is seen in the combined tree. For all other single locus matrices, *Galeola* and/or *C. lindleyana* are missing. There is little doubt that the two genera are closely related since they have similar floral morphology, but their seeds, fruits and habit are different. *Galeola* species are climbing vines with dry dehiscent fruits and winged seeds; *Cyrtosia* species are erect herbs with fleshy indehiscent fruits and crustose seeds. Further sampling of data and taxa may resolve each as monophyletic.

Future molecular systematics studies among Vanilloideae

This study demonstrates the potential value of sampling nuclear and mitochondrial gene regions (not only plastid) for reconstructing the phylogeny of Orchidaceae, especially for lineages that include mycoheterotrophic species. Most relationships supported by these new data confirm and even strengthen those uncovered with plastid gene data alone (results not shown). At the same time they remind us that issues related to incomplete sampling (of data and/or taxa) can cloud our interpretation of phylogeny. There are still no molecular data for *Pogoniopsis*, and this genus could be important for understanding the evolutionary history of vanilloid orchids, assuming that it even belongs within the subfamily. This achlorophyllous genus from Brazil shares reproductive characters with some Pogonieae and Vanilleae. Placing it among these taxa could affect current tree topologies in a manner that we have no way to predict. Even more enigmatic is *Dictyophyllaria*, an orchid that is known only from the type specimen and a line drawing. It shares morphological features with both *Vanilla* and *Epistephium*, but may well be extinct. If this is the case, then it serves to remind us that extant Vanilloideae, a line of orchids perhaps >76 million years old (Ramirez *et al.*, 2007), are relicts of what may have been an even more diverse clade of Orchidaceae with a complex history of evolution.

Our understanding of the fundamental biology of *Pogonia ophioglossoides*, *Cleistes divaricata* and *Vanilla mexicana* has come a long way since Linnaeus (1753) described them in *Species plantarum*. It has advanced most noticeably during the past decade, thanks in part to early molecular systematics studies, but, like all scientific knowledge, these are incomplete. Such studies of Vanilloideae must be expanded and refined.

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