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Photosystem II gene sequences of *psbB* and *psbC* clarify the phylogenetic position of *Vanilla* (Vanilloideae, Orchidaceae)

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Abstract

Nucleotide sequences of the plastid genes *psbB* and *psbC* were obtained for 34 taxa to represent most genera currently classified within Vanilloideae (Orchidaceae). These genes code for two of the subunits that make up the Photosystem II protein P680, and have only rarely been used for reconstructing phylogenetic relationships among plants. We failed to amplify *psbB* from the achlorophyllous genera *Cyrtosia* and *Lecanorchis*, but were able to align full length copies of *psbC* sequences from two species of *Cyrtosia* with the other taxa. This was not the case for an anomalous *psbC* sequence obtained for *Lecanorchis*. Nucleotide variation within each of these genes is sufficient to resolve the major relationships among Vanilloideae, and the combined two-gene tree is fully resolved at the genus level and highly supported. These gene trees demonstrate with a high degree of confidence (95% jackknife support) that a clade of mostly achlorophyllous tropical vines including *Pseudovanilla* and *Erythrorchis* are the sister group to *Vanilla*. The two New Caledonian endemic genera of Vanilloideae are sister to this pair, and *Epistephium* is sister to all remaining Vanillieae.

The genus *Vanilla* is one of the most familiar of all orchids. It contains as many as 100 different species distributed throughout the New and Old World tropics, and one of these—*V. planifolia* Andrews—is the primary source of natural vanilla flavoring. Despite the economic value of these climbing plants and their ubiquity in living orchid collections, there is still a great deal of uncertainty regarding the biology and natural history of *Vanilla*. For example, natural pollinators are generally unknown for the species, their fungal requirements for seed germination and growth are poorly studied, and the question of why some species produce aromatic fleshy berries is unclear since fruit and seed dispersal mechanisms have been mostly speculatively explained. Furthermore, the taxonomy and systematics of the genus are in a state of uncertainty. This is probably due to the fact that fertile specimens within the world's herbaria are few, and the plants are shy to flower in cultivation or even in their native habitats.

The phylogenetic position of *Vanilla* among Orchidaceae is also not clear. Until the use of DNA sequence data for reconstructing evolutionary relationships became routine, most orchid systematists (e.g., Dressler, 1981) classified *Vanilla* within a subtribe, Vanillinae, that contained such genera as *Epistephium*, *Eriaxis* and *Clematepistephium*. This subtribe was further classified within the tribe Vanilleae, together with subtribes Galeolinae, Lechanorchidinae, Pogoniinae and/or Palmorchidinae depending on the taxonomic system. Since all of these terrestrial orchids possess only a single, fertile, incumbent anther (an "advanced" character in orchids), but because they mostly shed their pollen as monads (a "primitive" character), they were traditionally considered basal members of the large subfamily Epidendroideae (e.g., *sensu* Dressler, 1993), or Neottioideae in systems where that subfamily was recognized (e.g., Garay, 1972).

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Molecular phylogenetic studies have changed substantially how *Vanilla* and its allies are now treated by orchidologists. All DNA-based cladograms published to date (e.g., Cameron et al., 1999; Cameron, 2004; Freudenstein et al., 2004) demonstrate with strong support that this group represent a subfamily all of their own, Vanilloideae, that contains approximately 15 genera (Pridgeon et al., 2003). Vanilloid orchids are remarkable not only for exhibiting transcontinental distributions and a mixture of plesiomorphic and apomorphic character states, but also because their position in the phylogeny of Orchidaceae as sister to the entire family, save Apostasioideae, is a pivotal one for understanding evolutionary trends and processes within what may be the largest family of plants on Earth.

Despite the advances made during the past decade regarding the systematics of the vanilloid orchids, there are still a number of outstanding questions that remain to be addressed. Among these are the exact position of *Vanilla* within Vanilloideae, and the sister group of this economically valuable genus. Fortunately, molecular analyses by Cameron (2004), Cameron and Chase (2000), and Cameron et al. (1999) have narrowed the candidates to a handful of monophyletic taxa and/or lineages within Vanilleae including: (1) *Lecanorchis*, (2) *Epistephium*, (3) *Eriaxis* plus *Clematepistephium* (the New Caledonian endemic vanilloid genera), or (4) *Pseudovanilla* plus its achlorophyllous cousins *Erythrorchis*, *Galeola* and *Cyrtosia* (together recognized in the past as subtribe Galeolinae). Unfortunately, there has been no well sampled phylogenetic study to date that has fully resolved the relationships among these four lineages with strong topological support.

The published molecular phylogenetic hypotheses for Orchidaceae that include *Vanilla* have been based on combinations or individual gene matrices of plastid *rbcL* (Cameron et al., 1999), *atpB* (Cameron, in press), and *psaB* (Cameron, 2004) gene sequences mostly with good sampling of Vanilloideae; or *nad1b-c* mitochondrial gene sequences with limited sampling of Vanilloideae (Freudenstein et al., 2004); or nuclear 18S gene sequences with moderate sampling of Vanilloideae (Cameron and Chase, 2000). In order to provide more data that could be applied to the construction of a hypothesis of phylogenetic relationships within Vanilloideae, and also to investigate the nature of photosynthetic genes among the achlorophyllous vanilloid orchids, we present here phylogenetic trees based on *psbB* and *psbC* sequences. This pair of plastid genes encode for two subunits of the P680 protein (Photosystem II), which serves a vital role in the process of harnessing photons of light during photosynthesis. The *psbB* and *psbC* genes themselves are located within the large single copy region (LSC) of the plastid genome, and the products derived from their translation are the CP43 and CP47 proteins, respectively. Both genes have been employed only rarely for phylogenetic reconstruction among plants (Graham and Olmstead, 2000; Nozaki et al., 2000; Rai et al., 2003), but showed potential during preliminary screening for alternative plastid genes of sufficient length and variation for use in molecular phylogenetic studies of Vanilloideae.

Methods

Taxon sampling and gene sequencing

Sequences of *psbB* and *psbC* were obtained for 34 taxa, representing 12 of the 15 genera of Vanilloideae. DNA was unavailable for the other three genera. Most of these taxa were field collected and vouchered. The 12 sampled species of Pogonieae were specified as a monophyletic outgroup, since broader family level analyses of Orchidaceae (Cameron et al., 1999; Cameron, 2004) indicated strongly that Vanilloideae is divided into two monophyletic tribes—Pogonieae and Vanilleae *sensu* Chase et al., 2003)—that are sister to each other. Complete voucher information and GenBank accession numbers are given in Table 1. The data matrices are available from the first author upon request or can be downloaded from The New York Botanical Garden website at http://www.nybg.org/bsci/res/cullb/dna.html.

All of the sequences were produced by automated methods, briefly described as follows. Total DNA was extracted according to the manufacturer's protocols using the DNEasy™ (Qiagen Inc., Valencia, California, USA) method from approximately 0.5 cm^2 of dried leaf tissue. Target loci were amplified in 25 μL volumes using standard polymerase chain reaction (PCR) protocols that typically included the addition of BSA and betaine. Initial gene amplifications were carried out using the primers described by Graham and Olmstead (2000), but new internal primers specific to Vanilloideae were designed when necessary (Figs 1 and 2). Specifically, the primers VAN1F (= "ny250") and VAN2R (= "ny249") were designed for the general sequencing of *psbB*. A primer specific to *Cyrtosia* (VAN5F = "ny357") was designed to sequence *psbC* for that genus more efficiently. Likewise, two primers (VAN4F = "ny356" and VAN3R = "ny355") were specifically designed in an attempt to obtain a sequence of the achlorophyllous species *Lecanorchis multiflora* (Figs 1 and 2). An annealing temperature of 45 °C was used to produce the greatest quality amplification products. In all cases, the resulting PCR products were purified using QIAquick™ spin columns (Qiagen Inc., Valencia, CA) according to the manufacturer's protocols. Cycle sequencing reactions were done using a combination of purified PCR template, primer, and BigDye™ reaction mix (Applied Biosystems Inc., Foster

Table 1
Species of Vanilloideae (Orchidaceae) sequenced for this study. Voucher information and GenBank accession numbers are provided

Taxon	Voucher	GenBank *psbB*	GenBank *psbC*
Tribe Vanilleae			
Clematepistephium smilacifolium Halle	*P. Ziesing 33* (CBG)	AY705158	AY705190
Cyrtosia lindleyana Hook.f. & Thomson	*K. Cameron 2182* (NY)	–	AY705208/AY705209
Cyrtosia septentrionalis (Rchb.f) L.A.Garay	*M. Chase O-793* (K)	–	AY705210
Epistephium lucidum Cogn.	*M. Chase O-795* (K)	AY705161	AY705193
Epistephium parviflorum Lindl.	*M. Chase O-794* (K)	AY705162	AY705194
Epistephium sp.	*M. Chase O-432* (K)	AY705159	AY705191
Epistephium sp.	*M. Chase O-433* (K)	AY705160	AY705192
Epistephium subrepens Hoehne	*M. Chase O-815* (K)	AY705163	AY705195
Eriaxis rigida Rchb.f.	*P. Ziesing 5* (CBG)	AY705157	AY705189
Erythrorchis altissima (Bl.) Bl.	*K. Cameron 1029* (NCU)	AY705148	AY705178
Erythrorchis cassythoides (Cunn. Ex Lindl.) L.A.Garay	*P. Weston 1831* (NCU)	AY705147	AY705177
Pseudovanilla foliata (F.Muell.) Garay	*M. Chase O-790* (K)	–	AY705187
Pseudovanilla ponapensis (Kaneh. & Yamam.) L.A.Garay		AY705156	AY705188
Vanilla africana Lindl.	*M. Chase O-584* (K)	AY705151	AY705181
Vanilla aphylla Bl.	*M. Chase O-578* (K)	AY705150	AY705180
Vanilla barbellata Rchb.f.	*M. Chase O-591* (K)	AY705153	AY705183
Vanilla planifolia Andrews	*M. Chase O-170* (K)	AY705152	AY705182
Vanilla imperialis Kraenzl.	*M. Chase O-587* (K)	AY705149	AY705179
Vanilla mexicana (L.) Miller	*C. McCartney* s.n.	AY705155	AY705186
Vanilla palmarum Lindl.	*M. Alves* s.n.	–	AY705185
Vanilla roscheri Rchb.f.	*M. Chase O-540* (K)	AY705154	AY705183
Tribe Pogonieae			
Cleistes cipoana Hoehne	*W. Thomas 12976* (NY)	AY705167	AY705199
Cleistes divaricata (L.) Ames	*K. Cameron 1062* (NY)	AY705166	AY705198
Cleistes rosea Lindl.	*K. Cameron 1038* (NCU)	AY705171	AY705203
Cleistes sp. 1	*M. Chase O-430* (K)	AY705169	AY705201
Cleistes sp. 2	*J. Jardim 2579* (NY)	AY705168	AY705200
Cleistes sp. 3	*W. Thomas 12975* (NY)	AY705170	AY705202
Duckeella adolphii Porto & Brade	*G. Romero 3013* (AMES)	AY705175	AY705207
Isotria medeoloides Raf.	*P. Keenan* s.n.	AY705164	AY705196
Isotria verticillata (Muhl. Ex Willd.) Raf.	*K. Cameron 1030* (NCU)	AY705165	AY705197
Pogonia japonica Rchb.f.	*K. Cameron 1034* (NCU)	AY705173	AY705205
Pogonia minor (Makino) Makino	*K. Cameron 1033* (NCU)	AY705172	AY705204
Pogonia ophioglossoides (L.) Juss.	*M. Chase O-437* (K)	AY705174	AY705206

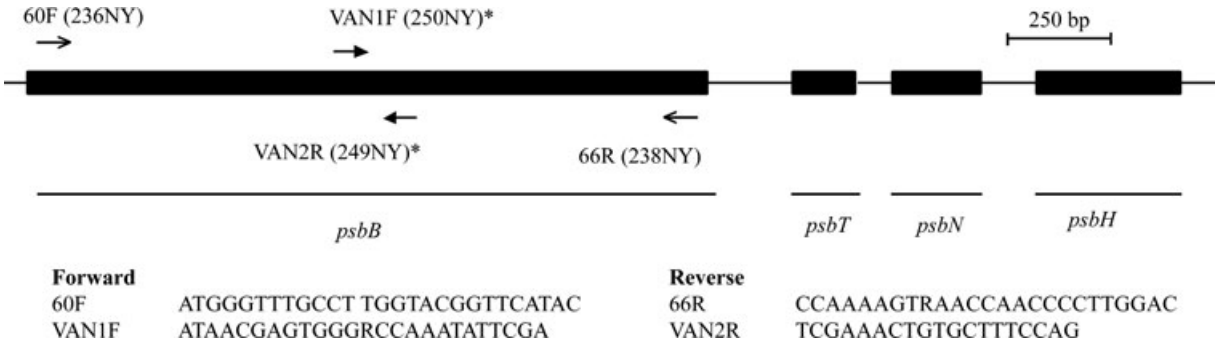
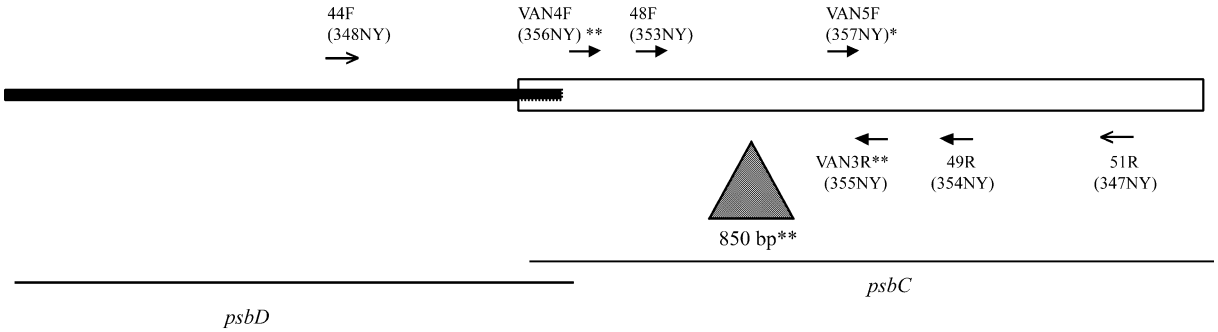


Fig. 1. Map of the primers used to amplify (open arrow) and sequence (all arrows) the chloroplast gene *psbB*. Primers 60F and 66R published by Graham and Olmstead (2000) were used for PCR amplification, whereas VAN1F* and VAN2R* were designed as internal primers specific to Vanilloideae.

City, CA) for 20 cycles. To remove excess dye terminators and primer from the cycle sequencing products, Centri-Sep^{TM} sephadex columns (Princeton Separations Inc., Adelphia, NJ) were employed. Final purified samples were subsequently dehydrated, resuspended in a mixture of formamide and loading dye, and pipetted onto a 5% denaturing polyacrylamide gel. Samples were analyzed on a Applied Biosystems ABI 377XL automated DNA sequencer, and the resulting electropherograms were edited using Sequencher 3.0 software (GeneCode Corp., Ann Arbor, MI), and data were aligned manually.



Forward		Reverse	
44F	GGKGTTGCTTTTTCCAATAAACG	VAN3R**	ATCCATCCTTCTCCCCCAA
VAN4F**	GAAGGTCTTCGTGCTTGGATGG	49R	GTATTATTGAACCAGACAAAACAACAAGC
48F	TTAGGTATTCACYTAATYTTGTTAG	51R	GCTAACCAACTTCTAGGRGARACAT
VAN5F*	GCTTTATCTATCTTTGGTTTCAT		

Fig. 2. Map of the primers used to amplify (open arrows) and sequence (all arrows) the chloroplast gene *psbC*. Primers 44F and 51R (Graham and Olmstead, 2000) were used for PCR amplification of the region. The primer VAN5F* was designed specifically for *Cyrtosia*, which is quite divergent from the other members of Vanilloideae. VAN4F** and VANrF* were designed to obtain a highly divergent sequence from *Lecanorchis multiflora*, which contains an insertion of nearly 850 bp, as indicated by the gray triangle.

Phylogenetic analyses

The independent *psbB* (30 taxa) and *psbC* (34 taxa) matrices, as well as the combined two-gene matrix (34 taxa) were analyzed using the parsimony criterion in PAUP* version 4.0b10 (Swofford, 2002) with gaps treated as missing data, characters weighted equally, collapsing branches if minimum length equals zero, and with DELTRAN optimization of characters onto the resulting trees. Equally parsimonious trees were found by executing a heuristic search of 1000 random addition replicates using TBR branch swapping, but keeping only five trees per replicate in order to discover possible "islands" of maximum parsimony (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 the shortest trees (MULTREES option in effect). Support values for the relationships discovered by an 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. Character optimizations onto the gene trees were performed using MacClade 4.0 (Maddison and Maddison, 2000).

Results

The *psbB* matrix contains 1306 characters of which 285 (21.8%) are variable and 205 (15.7%) are parsimony informative. Analysis of these data resulted in two trees of maximum parsimony (length of 440 steps, CI of 0.746, and RI of 0.893). A single *psbB* gene tree is presented as a phylogram in Fig. 3 to highlight the variation in branch lengths, but arrows are used to show the nodes that collapse in the strict consensus of all equally parsimonious *psbB* trees.

The *psbC* matrix contains 2012 characters of which 391 (19.4%) are variable and 197 (9.8%) are parsimony informative. Analysis of these data resulted in six trees of maximum parsimony (length of 556 steps, CI of 0.793, and RI of 0.895). A randomly chosen single *psbC* tree is presented as a phylogram in Fig. 4 to highlight the variation in branch lengths for this gene. Arrows mark the nodes that collapse in the strict consensus, and it is evident that there are no strongly supported clades in conflict between this *psbC* tree and the *psbB* tree.

Figure 5 shows the strict consensus of six parsimonious trees (length of 998 steps, CI of 0.771, and RI of 0.893) obtained after analysis of the combined two-gene matrix. Jackknife values > 50% are indicated. This matrix contains 3318 characters of which 676 (20.4%) are variable and 402 (12.1%) are parsimony informative. The two-gene tree, which is very similar in overall topology to the separate *psbB* and *psbC* trees, is well resolved, with members of the *Pseudovanilla* clade strongly supported (95% jck) as sister to *Vanilla*. The New Caledonian endemic genera *Eriaxis* and *Clematepistephium* are sister to each other (99% jck), but only moderately supported (73% jck) to the *Pseudovanilla/Vanilla* clade. *Epistephium* is sister to the remainder of Vanilleae.

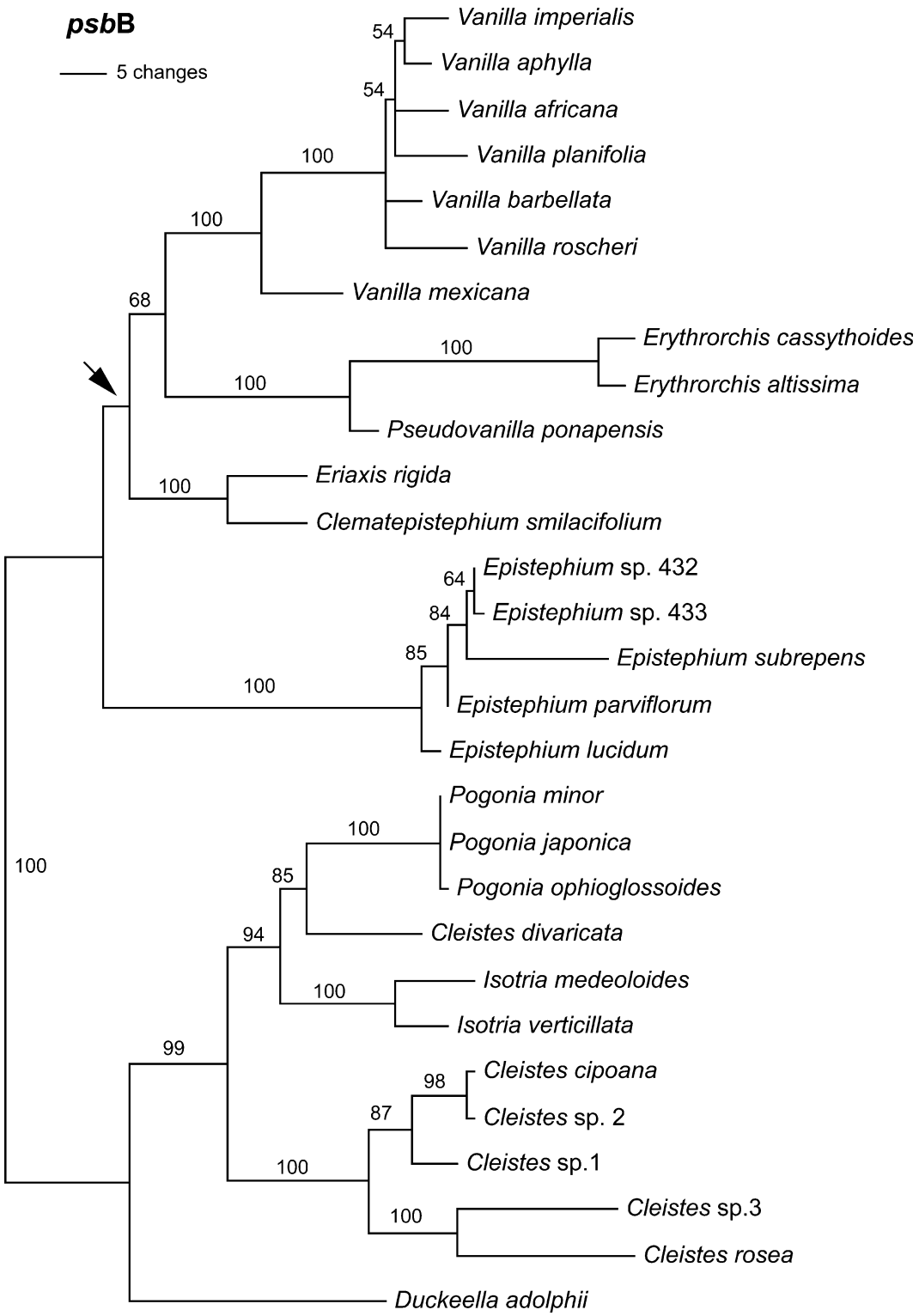


Fig. 3. One of two equally parsimonious trees resulting from analysis of *psbB* for Vanilloideae (Orchidaceae) depicted as a phylogram (DELTRAN optimization) to highlight the relative branch lengths. The arrow indicates the only node that collapses in the strict consensus, and jackknife values > 50% are presented above the branches.

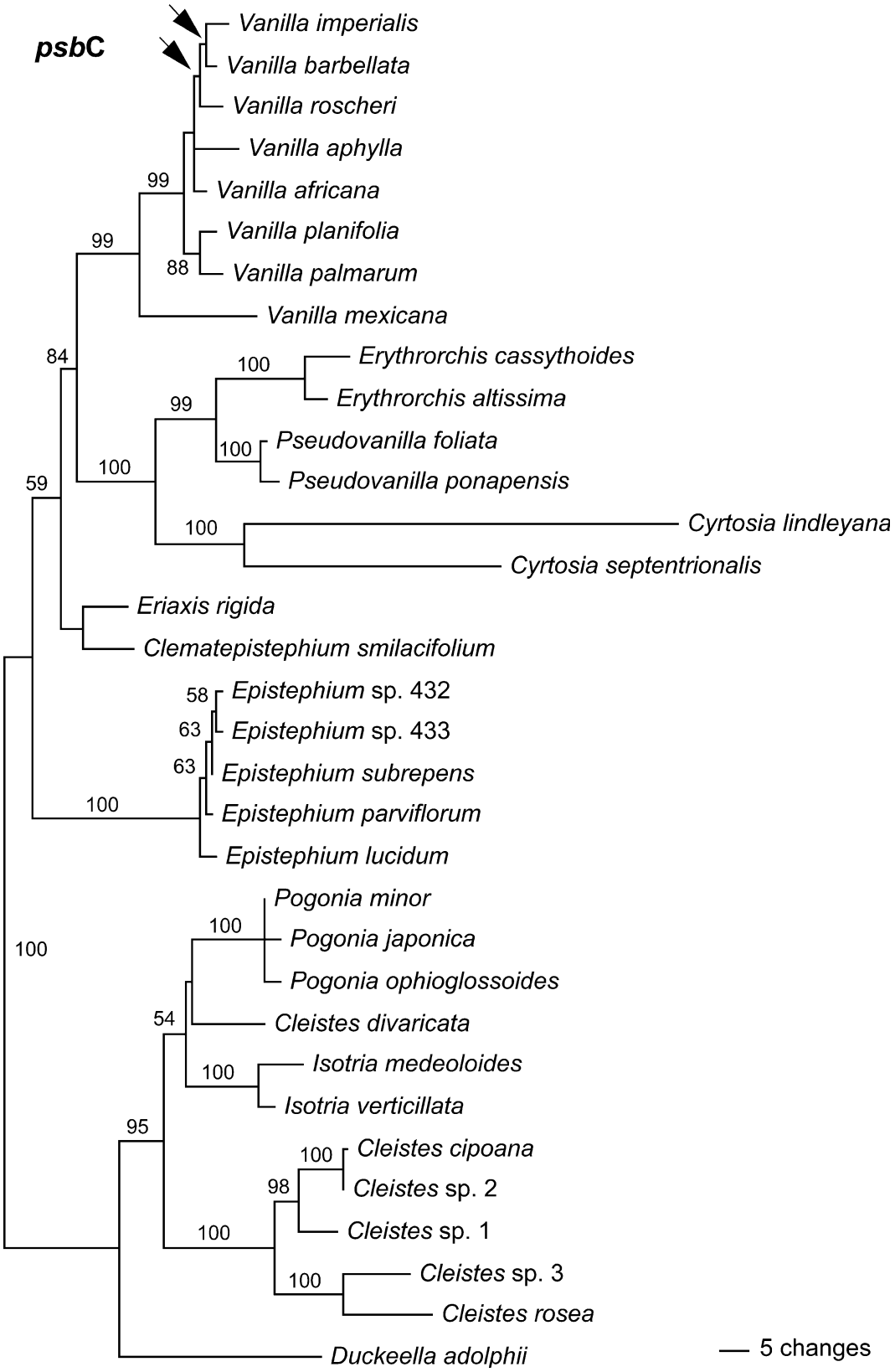


Fig. 4. One of the six equally parsimonious *psbC* trees chosen at random and presented as a phylogram to highlight relative branch lengths (DELTRAN optimization) among species of Vanilloideae (Orchidaceae). The two species of *Cyrtosia* are achlorophyllous mycoheterotrophs, and exhibit highly divergent *psbC* sequences relative to the other taxa.

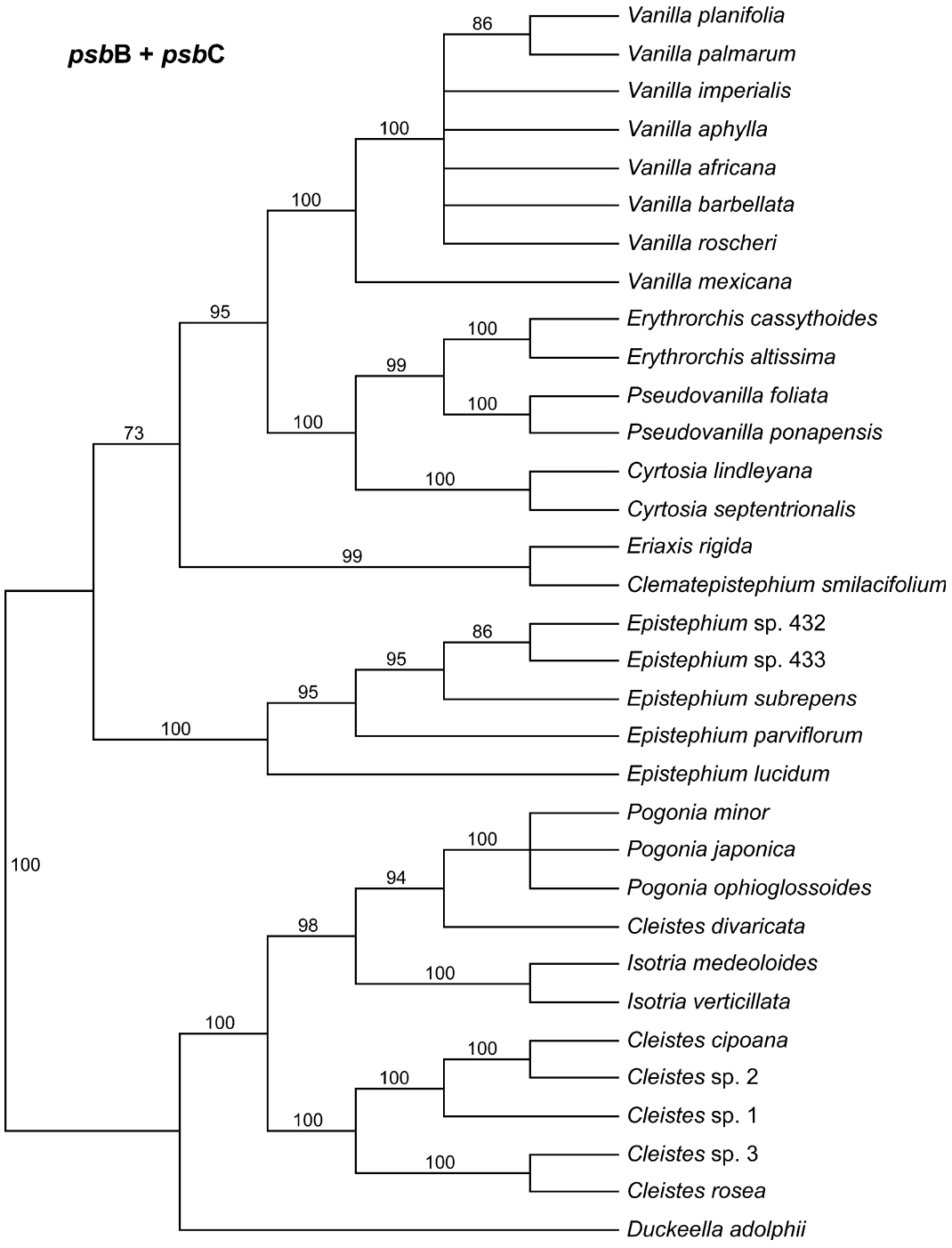


Fig. 5. The strict consensus of six equally parsimonious trees resulting from analysis of combined *psbB* and *psbC* sequence data for Vanilloideae (Orchidaceae). Jackknife values > 50% are indicated.

Discussion

The performance of photosystem II genes

Protein-coding genes of the plastid genome have been used extensively in higher order phylogenetic studies of photoautotrophic organisms because they are mostly easy to amplify, single copy loci with slow evolutionary rates. They also offer the advantage of being absent in fungi, which may contaminate DNA samples derived from organisms known to live in association with mycorrhizae (such as orchids). These plastid genes

generally lack introns, and therefore provide DNA sequences that are aligned with relative ease. Specifically for the case of *psbB* and *psbC*, it is worth noting that photosystem II genes have been characterized as having some of the lowest synonymous substitution rates among single-copy plastid genes (Olmstead and Palmer, 1994). They also have a correspondingly low level of multiple changes per site, which may provide for a good fit to differing models of phylogenetic reconstruction (Graham and Olmstead, 2000), when criteria other than strict parsimony are employed.

To our knowledge, *psbC* has been used for evolutionary studies in only three cases. First, in an attempt to deduce robust phylogenetic relationships among colonial green algae of the order Volvocales (Nozaki et al., 2000). Second, *psbC* was sequenced together with *psbB* and other genes to establish phylogenetic relationships among gymnosperms (Rai et al., 2003); and third, both genes proved useful for reconstructing a phylogenetic relationship among deep branches of the basal angiosperm lineage (Graham and Olmstead, 2000) when included in a large multigene analysis. Neither gene has been used to examine intergeneric relationships within a single family of plants.

The *psbB* + *psbC* tree presented here is the first published hypothesis of a phylogenetic relationship for Orchidaceae, or any other plant lineage for that matter, to consider photosystem II genes. In this analysis *psbC* and *psbB* revealed themselves to be relatively effective molecular markers for phylogenetic reconstruction among Vanilloideae. The total percentage of parsimony informative characters provided by these two loci (15.7% for *psbB*, and only 9.8% for *psbC*) can be considered low in comparison with other plastid genes such as *rbcL*, *psaB* and *atpB* which generally provide something closer to 30% informative characters for Orchidaceae (Cameron, 2004, 2005; Cameron, 2004). However, homoplasy is very low within the matrix (CI = 0.771), and a high quality phylogenetic signal is clearly present, as evidenced by the retention index value of 0.893, coupled with high jackknife support values on the well resolved cladograms.

The achlorophyllous vanilloid orchids

The leafless vanilloid genera *Cyrtosia* and *Lecanorchis* are of significant biological interest since they have lost their ability to produce chlorophyll and perform photosynthesis. Instead, both are mycoheterotrophs, exclusively feeding as parasites on soil fungi. To date, the first author has failed to amplify plastid genes from *Lecanorchis*, but has published rather divergent plastid gene/pseudogene sequences of *Cyrtosia* for *rbcL*, *atpB* and *psaB* (Cameron, 2004). Conversely, nuclear and mitochondrial gene sequences have been readily obtained for both of these taxa from the same DNA extractions (e.g., Cameron and Chase, 2000; Freudenstein et al., 2004). Repeated attempts to amplify *psbB* from two different species of *Cyrtosia* and two species of *Lecanorchis* were unsuccessful in this study. It may be speculated that these genes are no longer present within their plastid genomes, since other holoparasitic plants, such as *Epifagus virginiana,* are known to have completely lost their photosynthetic genes (dePamphilis and Palmer, 1990). However, the lack of a PCR product cannot be taken as definitive proof of gene loss in these taxa, and further study is warranted to confirm this hypothesis.

In the case of *psbC*, however, *Cyrtosia lindleyana* presents a large deletion of approximately 160 base pairs (bp) located near the 3′ end of the gene. Its sister species, *Cystosia septentrionalis*, shows this same large deletion, but also a small deletion of 3 bp, and an insertion of 4 bp located near the 5′ end. Except for these isolated indels, the two species of *Cyrtosia* were easily aligned to the other vanilloid genera. A *psbC* sequence obtained from *Lecanorchis*, on the other hand, was found to be highly divergent, and was not included in the analysis because of extreme difficulty in its alignment and uncertainty as to its homology. It shows an internal insertion of more than 800 bp (see Fig. 2), and must surely represent a vestigial gene remnant or pseudogene. The unusually long branches of such taxa as *Pseudovanilla*, *Cyrtosia* and *Erythrochis* in the cladogram support the hypothesis that elevated rates of mutation in plastid genes represent an advanced stage in the evolution towards a parasitic way of life (Haberhausen et al., 1992), ultimately leading to achlorophylly and complete gene loss. As such, these vanilloid orchids present a snapshot in time of the continuum of photosynthesis devolution in a unique lineage of plants.

Orchid evolution and the position of Vanilla

The monophyletic genus *Vanilla* is well supported (95% jac) as sister to the clade containing *Pseudovanilla* and its achlorophyllous relatives (i.e., the former subtribe Galeolinae). This is the same result obtained for *psaB* (Cameron, 2004) and for *atpB* (Cameron, 2005), but jackknife/bootstrap support was lacking in those cases. This result is ironic because Cameron (1996) recovered two islands of parsimony when Vanilloideae were analyzed first using only *rbcL* gene sequences. One island placed *Vanilla* sister to the entire Vanilleae clade, followed by *Epistephium*, the New Caledonian endemics, then Galeolinae (i.e., *Vanilla* was basal in the tribe, Galeolinae was derived). The alternative island reversed the positions of *Vanilla* and Galeolinae as derived and basal in the tree. In retrospect, it seems evident now that those *rbcL* trees may have been exhibiting a phenomenon related to poor sampling within lineages characterized by long branches, and that this has been

remedied by sequencing additional species of *Pseudovanilla*, *Cyrtosia*, *Erythrorchis* and *Vanilla mexicana*, all of which serve to shorten the long branches leading to the Galeolinae and *Vanilla* clades.

Finally, the topologies presented here have significance for interpreting trends in character evolution within Orchidaceae. For example, Cameron (2002) proposed that the climbing habit had evolved independently three times in the family: once in *Vanilla* which uses aerial roots at each node for climbing, once in Galeolinae which also uses aerial roots plus reduced hook-like leaves, and once in *Clematepistephium,* which simply twines around support trees. It is now evident, based on the trees presented here that a single origin for the climbing habit—with two separate reversals to the non-climbing habit (in both *Cyrtosia* and *Eriaxis*)—is an equally parsimonious hypothesis since it also requires three steps. The climbing habit by means of adventitious roots, however, clearly represents a synapomorphy for *Vanilla* and the *Pseudovanilla* clade.

It seems that the phylogenetic position of *Vanilla* within Orchidaceae is now firmly established, and that shared morphological features such as climbing habit, aerial roots, crustose seeds and unilocular ovaries with arrested placental development (Cameron, 2003a,b) are indicative of a common ancestry between *Vanilla* and the *Pseudovanilla* clade. Is this the final answer to the *Vanilla* enigma? Perhaps not. There are two vanilloid orchids so rare that their DNA has been unavailable for study. The first is *Dictyophyllaria dietschiana* (= *Vanilla dietschiana*), a non-climbing sympodial orchid with reticulate leaf venation and crustose seeds that lack a wing. The type specimen was collected nearly a century ago in Brazil, but not since then. Its relationship to *Vanilla*, with which is shares some morphological features, may forever be a mystery. Equally mysterious is the poorly known genus *Pogoniopsis*. This diminutive achlorophyllous orchid from Brazil is devoid of leaves, and would appear to be a member of Pogonieae (perhaps even a segregate of tropical *Cleistes*), based on its floral morphology. However, a fruiting specimen was collected recently, and SEM micrographs of the plant's seed sent to the first author by Mr Emerson R. Pansarin of São Paulo, Brazil, show that it has an ovoid shape and crustose seed coat reminiscent of *Vanilla* or *Cyrtosia*. Until more material (especially DNA) is available for careful examination, the phylogenetic position of *Pogoniopsis* and its possible relationship to *Vanilla* remain perplexing.

The scientific study of evolution and systematics, in general, and phylogenetic reconstruction, specifically, are dynamic ones. Interpretations of character homology, character state change and classification are ever changing as new data becomes available. Surely this study will not be the final word on *Vanilla*'s origin and evolution, but it does represent a step in the process of filling in gaps in our understanding of these remarkable plants. There is nothing plain about *Vanilla*!

Acknowledgments

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