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MOLECULAR IDENTIFICATION OF CYPRIPEDIOID ORCHIDS IN INTERNATIONAL TRADE

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ABSTRACT. Two cypripedioid orchid genera, *Paphiopedilum* and *Phragmipedium,* are listed in Appendix I of CITES and are restricted from international trade. Because of their morphological similarity to other genera, however, they may be disguised as belonging to one of the other cypripedioids listed along with other orchids in Appendix II of CITES. Sequence analysis was performed on the internal transcribed spacer region (ITS) of ribosomal DNA of cypripedioid orchids to develop a molecular marker system capable of discriminating among rare species in trade. Molecular analyses concentrated on rare cypripedioid orchids from the genera *Paphiopedilum* and *Phragmipedium,* which are known to be poached from the wild and smuggled across international borders disguised as common species. A total of 48 taxa representing two genera (*Paphiopedilum,* N = 43; *Phragmipedium,* N = 5) have been sequenced and compared for distinctiveness. Phylogenetic analyses clearly distinguish between these two genera and among other cypripedioid genera, with 5–10 fixed nucleotide differences reported between genera. Within a genus, sections of closely related taxa are recoverable in phylogenetic analyses, in most cases, with low sequence divergence within sections. ITS sequences available in GenBank have been aligned with data generated for this project, resulting in a comprehensive sequence library of 151 sequences representing all genera of cypripedioid orchids: 70 *Paphiopedilum* taxa, 16 *Phragmipedium* taxa, and 14 *Cypripedium* taxa, as well as representatives from *Selenipedium* and the monotypic genus *Mexipedium (Phragmipedium) xerophyticum.* Additionally, several organelle intron regions have been screened for variation among genera and species. Both the chloroplast *trn*S-M and the mitochondrial NAD1 intron regions, which varied between genera in nucleotide substitutions and indels, hold promise for increasing ability to distinguish between these orchids. The set of DNA markers examined for this project are diagnostic of these genera, appear to be robust, and are suitable for rapid assay to avoid unnecessary complication in the legitimate trade of orchids listed in CITES Appendix II.

Key words: cypripedioid orchid genera, *Paphiopedilum, Phragmipedium,* DNA markers, ITS sequences, chloroplast *trn*S-*trn*fM, mitochondrial *nad*1 intron

INTRODUCTION

The Orchidaceae is the largest family of flowering plants, with more than 25,000 species belonging to 800 genera. Import and export of all orchids are regulated by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), regardless of specific conservation status. The U.S. Department of the Interior, Fish and Wildlife Service, Division of Scientific Authority is responsible for determining the impacts of U.S. trade on orchid species, and for developing proposals to amend the CITES appendices. Although ca. 20,000 orchid species are not considered at risk, all species are regulated because many closely resemble rare species of concern. Some rare orchids, particularly in the cypripedioid (slipper orchids) genera *Paphiopedilum* from Southeast Asia and *Phragmipedium* from South America are known to be poached from the wild and smuggled across international borders disguised as more common species. This smuggling is facilitated by the fact that the cypripedioid genera share many morphological traits through either common descent or convergence (Atwood 1984).

The slipper orchids are a distinct group with many primitive features relative to other mem-

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bers of the family Orchidaceae (Dressler 1993), yet they also have been described as advanced with specialized structures and on-going speciation, suggesting that the possession of primitive features indicates early divergence during the evolution of orchids (Atwood 1984). The five genera comprising the subfamily Cypripedioideae (*Cypripedium* L., *Mexipedium* V.A. Albert & M.W. Chase, *Paphiopedilum* Pfitzer, *Phragmipedium* Rolfe, and *Selenipedium* Rchb.f) are united by features such as two fertile anthers and a saccate lip (pouch) (Averyanov et al. 2003). Although several floral differences exist between genera, the main vegetative difference is the growth pattern. The plicate-leaved genera (*Cypripedium* and *Selenipedium*) have leaves arranged spirally on an elongate stem, where the conduplicate-leaved slipper orchids (*Mexipedium, Paphiopedilum, Phragmipedium*) have leaves arranged in a distichous rosette (Atwood 1984). Without appreciable vegetative differences between species that can be recognized by customs officials, it is difficult to accurately identify unknown plants that are not producing flowers.

Molecular genetic techniques have been gaining widespread use in wildlife forensics and can potentially be used in U.S. ports to determine whether a particular trade specimen is actually a *Paphiopedilum* or *Phragmipedium* species listed in CITES Appendix I (commercial trade banned), or is simply a species that resembles these taxa yet is listed in CITES Appendix II and can be legally traded with proof of proper cultivation. For genetic techniques to be applied to orchid trade, specific genetic characteristics of these genera must first be identified, and then the appropriate techniques can be disseminated to points of entry for the final monitoring of specimens being transported.

A number of DNA markers have been used to distinguish between, or determine historical relationships between, related orchid species. Nuclear ribosomal DNA, mitochondrial DNA, and chloroplast DNA, have all been used in past studies. One of the most popular gene regions used for drawing phylogenetic inferences at the generic level and above in plants has been the internally transcribed spacer regions (ITS) of nuclear ribosomal cistron (Álvarez & Wendel 2003). In particular, the ITS region has been useful in discriminating genera and species of slipper orchids (Cox et al. 1997). Although this gene region has contributed to our phylogenetic understanding of plants, a review of the molecular genetic processes has suggested that the sole use of ITS sequences may be misleading with regard to phylogenetic inference (Álvarez & Wendel 2003). Because of a relatively high rate of substitution and maternal inheritance in plants, non-coding organelle gene regions have recently been used to determine relationships both at higher taxonomic levels (e.g., Zhang et al. 2003) and among closely related orchid species (e.g., Szalanski et al. 2001). Among chloroplast genes, the *rbcL* gene has shed new light on relationships within the Orchidaceae (Cameron et al. 1999) and at the genus level among slipper orchids (Albert 1994). Detecting population-level polymorphism has been more challenging using chloroplast genes, but many are now turning to non-coding intron regions that separate coding genes throughout the chloroplast genome. Universal primers now exist allowing amplification of intron regions in a range of higher plants, and these regions have been found to be highly polymorphic in mononucleotide repeats (Provan et al. 2001). Because of similar modes of inheritance and utility, the chloroplast genome in plants and the mitochondrial genome of animals have been considered as natural counterparts for use in phylogenetic studies of their respective groups (Olmstead & Palmer 1994). The mitochondrial genome of plants, however, is large in size and poorly known relative to the animal mitochondrial genome, with structure and evolutionary dynamics that are dramatically different as well (Palmer et al. 1992). Many aspects of the structure of the genome change rapidly, yet primary sequence changes very slowly (Palmer et al. 1992), and as a result, the genome has not been utilized extensively in plant molecular systematics. Like the chloroplast genome, however, non-coding intron regions are now being utilized with universal primers (Demesure et al. 1995), and the utility of such regions, especially length-change characters, is now being realized in the Orchidaceae (Freudenstein & Chase 2001).

The availability of robust molecular markers to discriminate among orchid specimens could facilitate commercial trade in species that are not an endangered species concern to resource managers (i.e., those not listed in CITES Appendix I). To reach this end, an examination was made of variability and identification of discriminating characters in multiple DNA regions within and between the five genera belonging to the orchid subfamily Cypripedioideae. This study describes the utility of molecular tools that may aid in the taxonomic identification of orchids imported to and exported from the United States and to assist in effectively enforcing the provisions of CITES.

Materials and Methods

Sample Collection

With the help of collaborators, we were able to collect tissue from many cypripedioid orchid

species belonging to the genera *Paphiopedilum* and *Phragmipedium* (APPENDIX 1). The specimens were assigned collection numbers using the prefixes: Cp = *Cypripedium,* Pp = *Paphiopedilum,* and Pg = *Phragmipedium,* which accompany the GenBank submissions. These ID numbers are useful in distinguishing data unique to this study versus the data from GenBank in FIGURES 1–4. Two collections of leaf tissue were made from the United States Botanic Garden, Washington D.C., by the authors and by P. Ford, U.S. Fish and Wildlife Service, Arlington, Virginia. Additionally, plant tissue collections were obtained from P. Simpson, an orchid enthusiast and professor at Shepherd College, Shepherdstown West Virginia. Several methods of collecting tissue were attempted, including storing leaf punches on ice and flash-freezing leaf-punches in liquid nitrogen. The best results for long-term sample stability were obtained when leaf tissue was crushed into an FTA card (Whatman Inc., Newton, Massachusetts) using a pestle; the FTA card then was air-dried and stored at room temperature in individual envelopes.

Molecular Methods

Total DNA was isolated from leaf tissue using the plant protocol from the PureGene DNA extraction kit (Gentra Systems Inc., Minneapolis, Minnesota), in quantity and quality suitable for subsequent laboratory use. The internal transcribed spacer regions (ITS-1 and ITS-2), separating structural ribosomal DNA genes, were amplified from genomic DNA using the polymerase chain reaction (PCR) and universal primers (Baldwin 1992). PCR reactions were carried out using 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 20 mM KCl), 2 mM $MgCl_2$, 0.2 mM of dNTPs, 0.375 μM of each primer, 0.5 mg/ml bovine serum albumin, 2.5 U of Taq polymerase, and ca. 100 ng of template DNA in a 20 ul reaction volume. Thermal cycling used an MJ-Research (Watertown, Massachusetts) PTC-200 thermocycler using the following cycle parameters: initial denaturing at 94°C for 2 min.; followed by 35 cycles of 94°C denaturing (1 min.), 52–56°C annealing (1 min.), 72°C extension (1 min), ending with a 5 min. extension at 72°C. PCR products were purified with Microcon spin columns (Millipore Corp., Bedford, Massachusetts). Purified PCR products were sequenced directly using amplification primers and conditions suggested for Big Dye Cycle Sequencing (Applied Biosystems, Foster City, California). Both heavy and light strands were sequenced for confirmation.

An intron in the chloroplast genome that lies between transfer RNA genes for Serine (*trn*S) and Methionine (*trn*fM) was amplified using the universal primers "*trn*S" and "*trn*fM" (Demesure et al. 1995). Since this region is too long to obtain overlapping sequence data using only the amplification primers (~1700 bp in *Quercus robur,* Demesure et al. 1995), internal sequencing primers were developed based upon sequences obtained and aligned with *Spiranthes romanzoffiana* (Forrest et al. 2004, accession number AY363054). The 3′ end of the amplicon can be confirmed using the primer Cp2-R1 5′-TCCATAATGATGTACCAGAA-3′, and the 5′ end can be confirmed using either Cp2-F1 5′-TTCTGGTACATCATTATGGA-3′ or Cp2-F2 5′-TTGGATTAGTCTTTCTG-3′.

The mitochondrial *nad*1 intron separating exons B and C was amplified and sequenced using the primers "*nad*1 exon B" and "*nad*1 exon C" from Demesure et al. (1995). Again, the length of the amplicon required designing internal amplification primers (~1550 bp in *Quercus robur,* Demesure et al. 1995). Internal sequencing primers were designed for the 3′ end of the amplicon: *nad*1b-F1 5′-GGGATATACACCAGGGCAAC-3′ [20-mer, forward] and *nad*1b-R1 5′-GTTGCCCTGGTGTATATCCC-3′ [20-mer, reverse]; and for the 5′ end: *nad*1b-F2 5′-CACCACTTGGGATGGGAAT-3′ [19-mer, forward], and *nad*1b-R2 5′-ATTCCCATCCCAAGTGGTG-3′ [19-mer, reverse].

PCR products were sequenced directly using the ABI Prism Big Dye Terminator Cycle Sequencing reaction kit utilizing AmpliTaq DNA Polymerase, FS (Applied Biosystems, Foster City, California). Sequencing reactions were analyzed by capillary electrophoresis using the ABI PRISM-3100 Genetic Analyzer and DNA Sequencing Analysis Software (Applied Biosystems). Forward and reverse sequences were assembled using Sequencher 4.0 (Gene Codes Corporation, Ann Arbor, Michigan). Multiple sequence alignment was carried out using Clustal X ver. 1.4b (Thompson et al. 1994), with gap opening/extension penalties of 20/5, respectively. The resulting alignment was checked by eye using MacClade 4.0 (Maddison & Maddison 2002).

Molecular Analyses

Phylogenetic analyses of DNA sequences were carried out using PAUP* 4.0b10 (Swofford 2002). Both distance- and parsimony-based tree reconstruction methods were used. Pair-wise Kimura two-parameter genetic distances (Kimura 1980) were calculated from the sequence alignment, and the neighbor-joining method was used to determine phylogenetic relationships. For parsimony analyses, heuristic searches were run

TABLE 1. Eighteen single nucleotide polymorphisms in the nuclear internal transcribed spacer region (ITS) that are diagnostic of cypripedioid genera.

Base position in alignment	87	102	190	197	235	252	383	543	549	573	616	635	647	676	720	749	751	789
Cypripedium	C	C	T	T	A	T	G	G	T/C	T	C/T	G	G/T	T	G/T	C	T	A
Selenipedium	C	C	T	T	A	G	G	G	C	C	T	G	G	T	A	G	T	G
Mexipedium	C	A	C	T	G	G	G	G	T	T	T	A	A	T	G	T	T	G
Phragmipedium	C	G	T	C	G	G	G	G	T	A	A	A	A	C	G	T	C/T	T
Paphiopedilum	T	G	C	C/-	C/T	G	A	A	A	A	T	G	G	T	A	C/A	G	A

using unweighted, parsimony-informative characters with the following settings: starting trees for branch swapping were obtained via stepwise addition, 10 random addition sequences per run, and tree bisection-reconnection (TBR) branch swapping on best trees. Alignment gaps were treated as missing data and as a 5th base in separate runs, and results differed little in topology. Bootstrap resampling (Felsenstein 1985) was used to assess the support of relationships recovered in phylogenetic analyses.

RESULTS

The multiple sequence alignment of 151 ITS sequences from cypripedioid orchids both from the present study and from GenBank (Cox et al. 1997) was 909 base pairs (bp) in length. This sequence library contains representative samples from 57 out of 69 species of *Paphiopedilum* recognized by Cribb (1998), several more recently described species (5), and several hybrids, for a total of 70 *Paphiopedilum* species (see APPENDIX). Included in the data set are 30 taxa that have replicate samples, including both *Paphiopedilum* and *Phragmipedium* taxa, allowing analysis of intraspecific genetic variation for the first time in these genera. The nuclear ITS region was highly polymorphic, including 699 polymorphic sites and 526 sites that were phylogenetically informative. Sequence divergence among genera was generally high (ca. 16–29%), with comparisons to *Selenipedium* being highest (28–41%). Within a genus, genetic distances were intermediate (2–8%), except between species of *Cypripedium,* where genetic distances between most species were more similar to distances between genera (15–22%, data not shown). There were 18 diagnostic fixed differences (nucleotides) between genera and/or between CITES categories (TABLE 1). Also several fixed differences were found for sections within *Paphiopedilum* (Parvisepalum, Brachypetalum, and Barbata, data not shown). Many fixed differences occurred in alignment gaps, or insertion and deletion mutations (indels) between genera. For example, a 55 bp deletion was present in most *Paphiopedilum* relative to the other cypripedioid orchids, with the exception of the section Parvisepalum species, the most basal section of *Paphiopedilum,* which retained this stretch of bases.

We analyzed a subset of the aligned sequences, (51 from the present study and four from the genera *Cypripedium, Selenipedium,* and *Mexipedium* downloaded from GenBank as outgroups). *Selenipedium chica* was used as the outgroup for phylogenetic analyses because of the high sequence divergence estimates relative to other cypripedioid genera and because of the basal placement of this genus in a previous phylogenetic analysis of relationships within the cypripedioids (Albert 1994, Cox et al. 1997). Both neighbor-joining and parsimony analyses yielded similar tree topologies (see parsimony results, FIGURE 1). Although including gaps as characters decreased the number of most parsimonious trees (TABLE 2), the general topology remained very similar, so the analysis with higher consistency index (gaps = missing) is shown (FIGURE 1). Monophyly of each genus was strongly supported in these analyses, as seen in the high bootstrap values for these clades (100%, FIGURE 1), signifying the ability to distinguish between genera using ITS sequence data. Generally, taxa belonging to sections within a genus clustered together and were supported by bootstrap analysis (FIGURE 1; sections of *Paphiopedilum* following Cribb 1987, 1998). Within sections of *Paphiopedilum* and *Phragmipedium,* distances were <2%, with the exceptions of Parvisepalum and Brachypetalum, which were slightly higher (2.4–4.6%, FIGURE 1). The section Parvisepalum is the most genetically distinct *Paphiopedilum* section, contained the greatest genetic diversity within a section (4.6% divergence between species, FIGURE 1), and was the most basal (i.e., primitive) group of *Paphiopedilum* orchids based on the ITS sequence data. Another interesting result from this analysis is the lack of variation within the section Barbata. Note the low sequence divergence among species within this section (0.5%, FIGURE 1), which is less than the differentiation in the *Paphiope-*

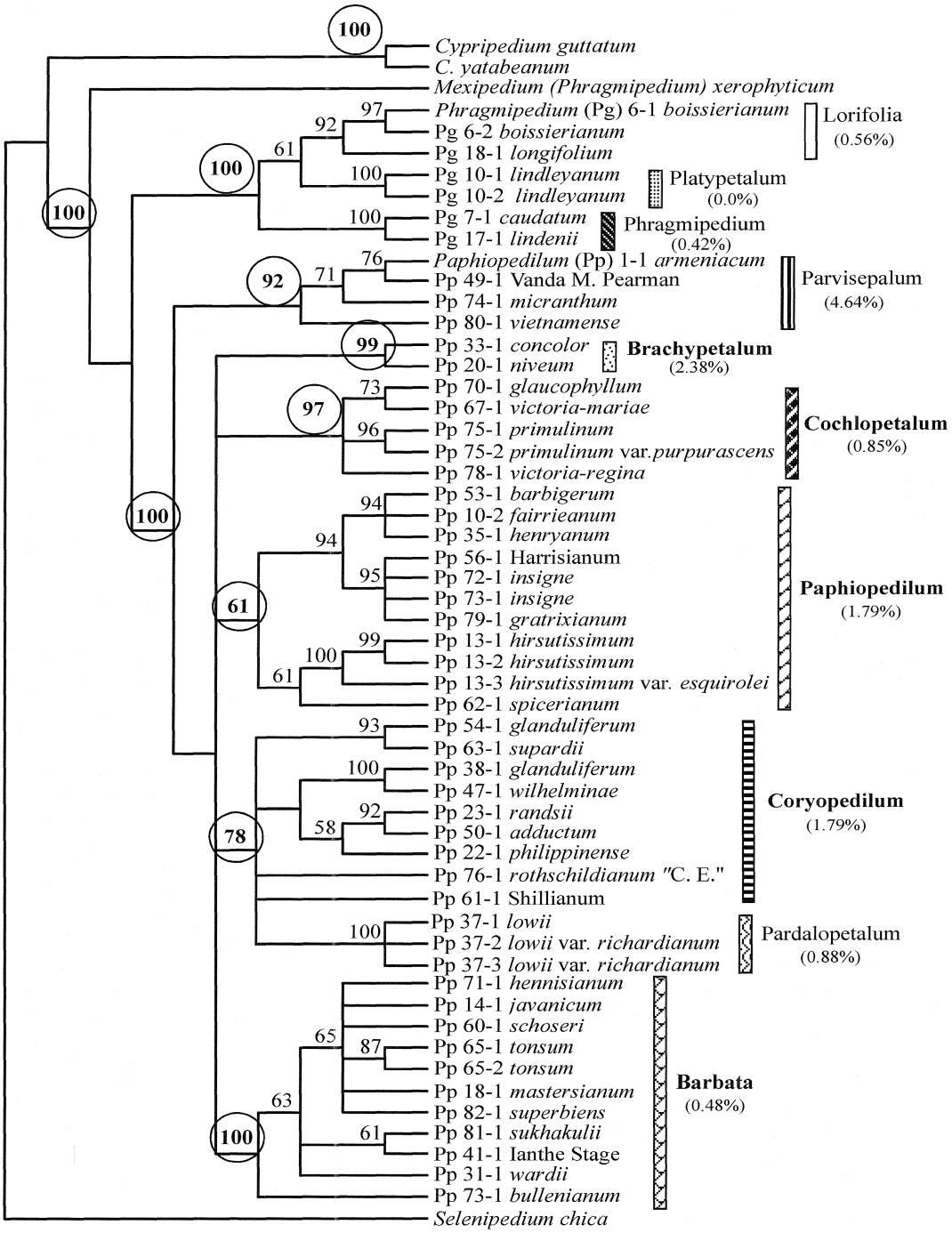

FIGURE 1. Strict consensus of 19,498 most parsimonious trees based upon 296 phylogenetically informative sites of nuclear ITS sequence data. Numbers above branches refer to bootstrap support values, with circled values for generic and sectional support. Bars to the left of clades indicate sections within genera according to Cribb (1987), and numbers under section names are percent sequence divergence within sections.

TABLE 2. Variability of gene regions examined and supporting statistics from parsimony analyses.

Gene region analysis	Variable characters/P.I. characters	Taxa no.	Trees no.	Steps no.	C.I.
ITS, new data, 925 bp, no gaps	431/296	56	19,498	608	0.7188
ITS, new data, gaps=5th	592/440	56	84	1108	0.6916
ITS, new + GenBank, no gaps	709/360	126	39,790	1192	0.5126
ITS, new + GenBank, gaps=5th	831/419	126	1323	1542	0.3946
CP *trn*S-*trn*fM, 1100 bp, no gaps	97/46	20	2	48	0.9583
CP *trn*S-*trn*fM, gaps=5th	316/101	20	3	123	0.9024
Mt *nad*1, 1559 bp, no gaps	37/18	20	10	23	0.7826
Mt *nad*1, gaps=5th	217/141	20	2	173	0.8555

Note: P.I. = phylogenetically informative; C.I. = consistency index.

dilum section Pardalopetalum or the *Phragmipedium* section Lorifolia, both of which contained either individuals from one species or varieties of the same species. Several species within section Barbata have been described as a "natural hybrid swarm" (*P. schoseri, P. javanicum, P. acmodontum*; Koopowitz 2000), which may help to explain low levels of divergence between species. The section Paphiopedilum, which received the lowest overall bootstrap support among *Paphiopedilum* sections, appears to contain two distinct groups, each of which receive higher support: one containing *P. hirsutissimum* and *P. spicerianum,* and the other group containing the remaining taxa sampled. Although most sections of *Paphiopedilum* could be distinguished in phylogenetic analysis of ITS data, relationships among taxa belonging to the sections Coryopedilum and Pardalopetalum were an exception. Taxa belonging to the Pardalopetalum group cluster together, but this clade is contained within the section Coryopedilum clade. These sections also appeared closely related in a previous phylogenetic analysis (Cox et al. 1997). Few, if any, nucleotide substitutions were observed in comparisons of individuals belonging to the same taxon, and monophyly of these sequences received high bootstrap support (FIGURE 1).

Branch lengths between sections were very short (See FIGURE 2C for a comparison of branch lengths within *Paphiopedilum*), signifying few differences between sections, yet species within sections share polymorphisms (or are genetically similar), as they are recovered in both distance-based (not shown) and parsimony-based (FIGURE 1) analyses. Probably as a result of these short branches (i.e., little genetic variation distinguishing between sections), relationships between most *Paphiopedilum* sections were not recovered in analysis of this ITS data set (note the collapsed nodes between *Paphiopedilum* sections except Parvisepalum in FIGURE 1).

A separate phylogenetic analysis combined data from this study with that available in GenBank (Cox et al. 1997) with the intent of maximizing the number of taxa included belonging to the genera *Paphiopedilum* and *Phragmipedium.* This analysis included 126 sequences representing 15 *Phragmipedium* and 69 *Paphiopedilum* taxa, with multiple individuals and/or different varieties of some taxa. *Mexipedium xerophyticum* (*Phragmipedium xerophyticum*) was used as the outgroup, as this species clearly lies outside of the two genera in question based on our analysis (FIGURE 1), and the analysis presented by Cox et al. (1997). FIGURE 2A shows the general structure of the phylogram resulting from parsimony analysis using 360 parsimony-informative characters (TABLE 2), and note the long branch lengths separating genera. All *Phragmipedium* taxa included in this analysis were monophyletic (FIGURE 2B, 100% bootstrap value). The monophyly of sections Micropetalum and Platypetalum was highly supported, with Micropetalum being the most primitive group of *Phragmipedium,* which agrees with results from Cox et al. (1997). The section Phragmipedium received poorer bootstrap support (67%, FIGURE 2B), and the Lorifolia taxa clustered together, but the monophyly of the group was not supported by bootstrap analysis. Where multiple individuals of a taxon were included, they generally clustered together in this analysis, including cases where species formerly considered separate are now synonymized, such as *P. sargentianum* Albert & Borge Pett, which is now accepted as *P. lindleyanum* (Rolfe) Rolfe.

Sections of *Paphiopedilum* were also recovered in the combined analysis (FIGURE 2C, 2D). Again, Parvisepalum was the most primitive *Paphiopedilum* section, and branch lengths leading to taxa were long, or in other words, there were many substitutions between species. The section most closely related to Parvisepalum was Brachypetalum, which would be expected following the designation of these sections in a separate subgenus (Brachypetalum) following Cribb

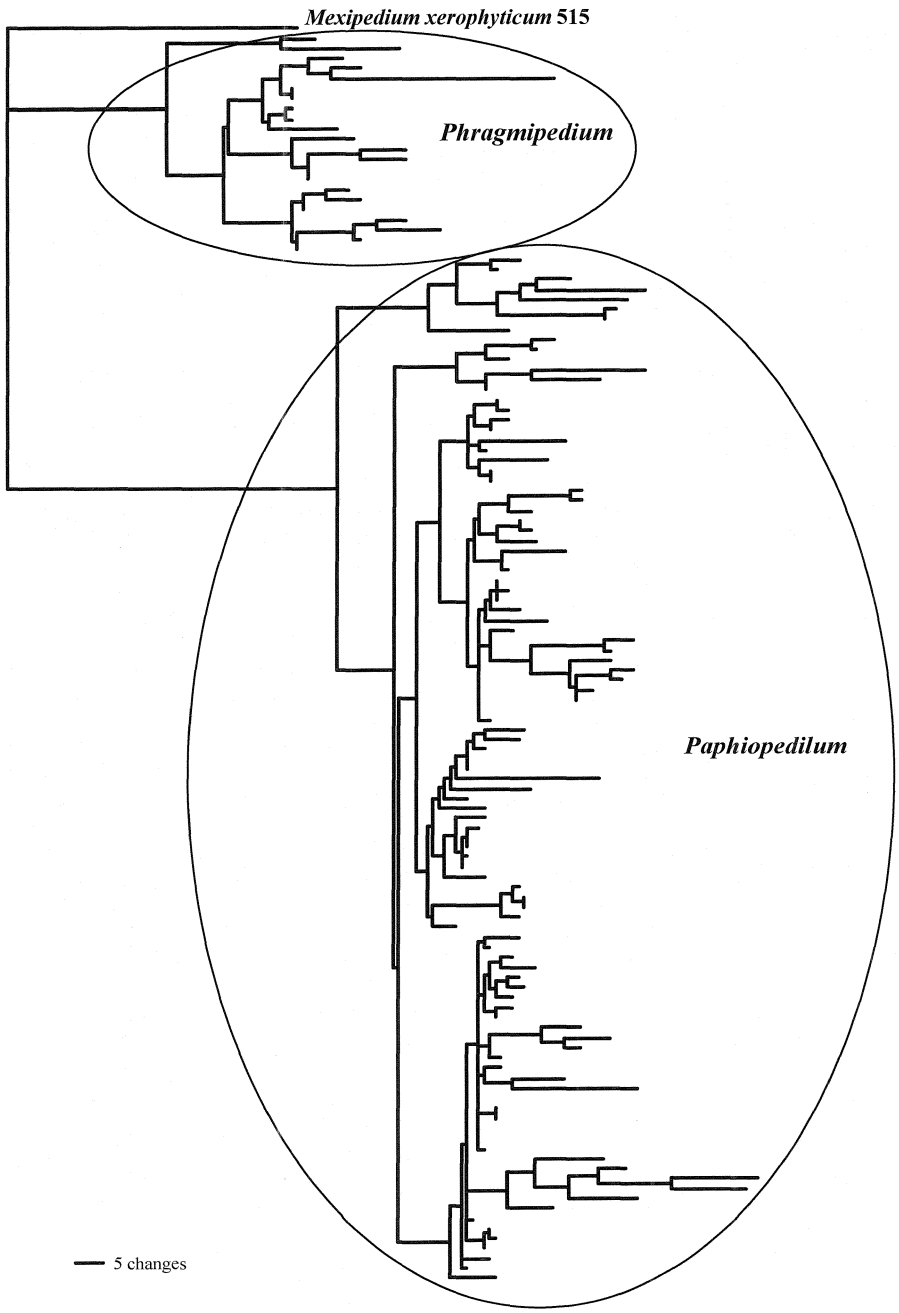

FIGURE 2. Results of parsimony analysis on nuclear ITS sequence data for 126 taxa. FIGURE 2A. One of 39,790 most parsimonious trees based upon 360 phylogenetically informative sites of nuclear ITS sequence data. Branch lengths are proportional to amount of change (see scale bar).

(1987), yet the unity of these sections is not supported by bootstrap analysis. Support for the Cochlopetalum section was high, and individuals from the same species clustered together. As in the first analysis, the Pardalopetalum section was well supported and nested within the Coryopedilum. Included in section Pardalopetalum are two sister species, *P. haynaldianum* and *P. lowii,* which form a well-supported clade within the section (95% bootstrap value, FIGURE 2C). In

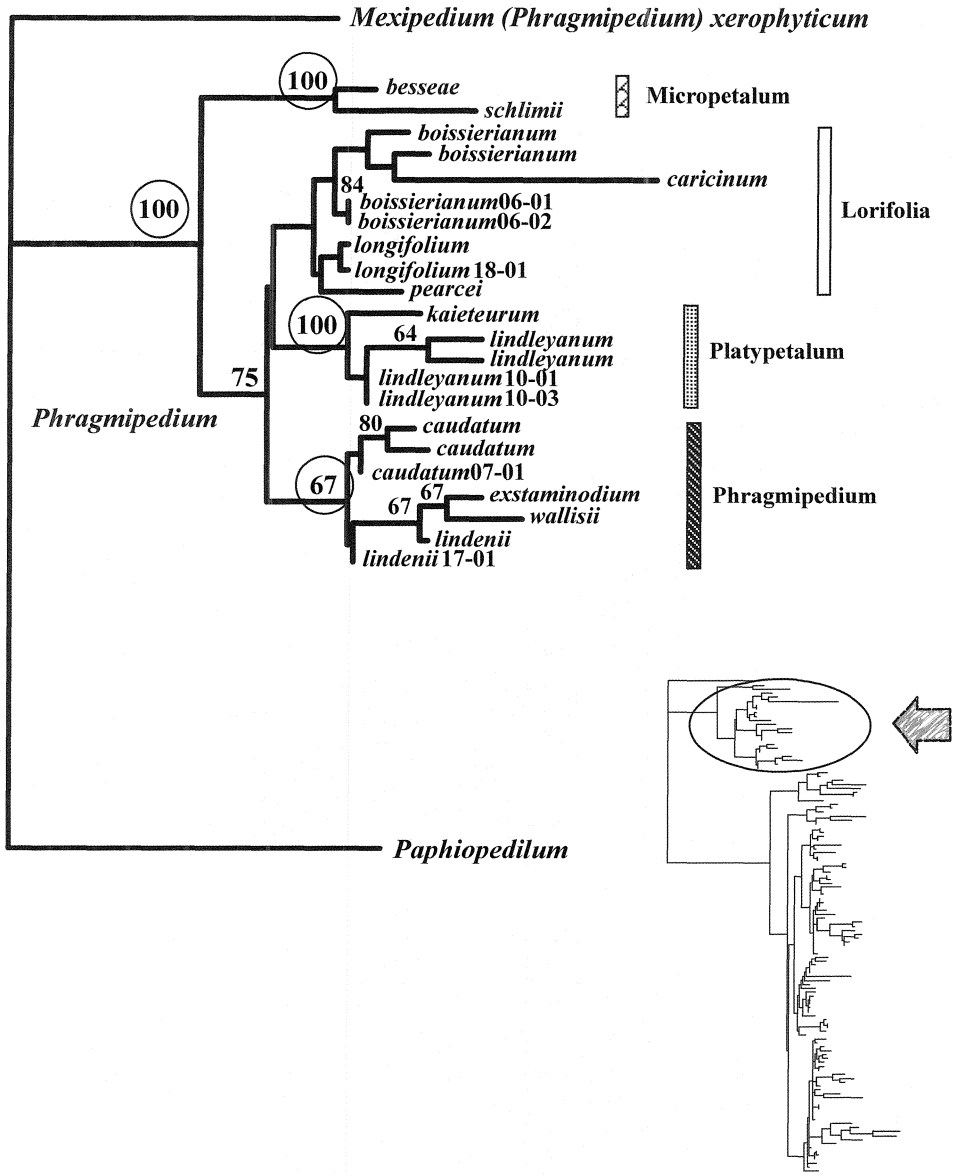

FIGURE 2B. Subset of phylogeny showing phlogenetic relationships among *Phragmipedium* species, with bootstrap support values above branches and circled values for generic and sectional support.

section Coryopedilum, controversy exists regarding the status of *P. praestans* (see Koopowitz 2000). *Phaphiopedilum praestans* (Rchb.f.) Pfitzer has been synonymized with *P. glanduliferum* (Blume) Stein (Cribb 1998, Govaerts 2003), and has been associated with *P. wilhelminae,* L.O. Williams, all of which appear closely related in this analysis. The section Paphiopedilum species again formed two clusters (*P. hirsutissimum* and *P. spicerianum* versus all others), yet in this analysis there is no bootstrap support for these clades or for the section. The addition of taxa to the Barbata clade in this analysis relative to the previous one resulted in a substantial decrease in bootstrap support (100% in FIGURE 1 versus 65% in FIGURE 2D). Most of the intraspecific duplicates in this section did not cluster together (e.g., individuals of *P. superbiens* and *P. javanicum,* FIGURE 2D). Many of the Barbata species sequences from GenBank had very long branches compared to those included in the first analysis as well (e.g., those in

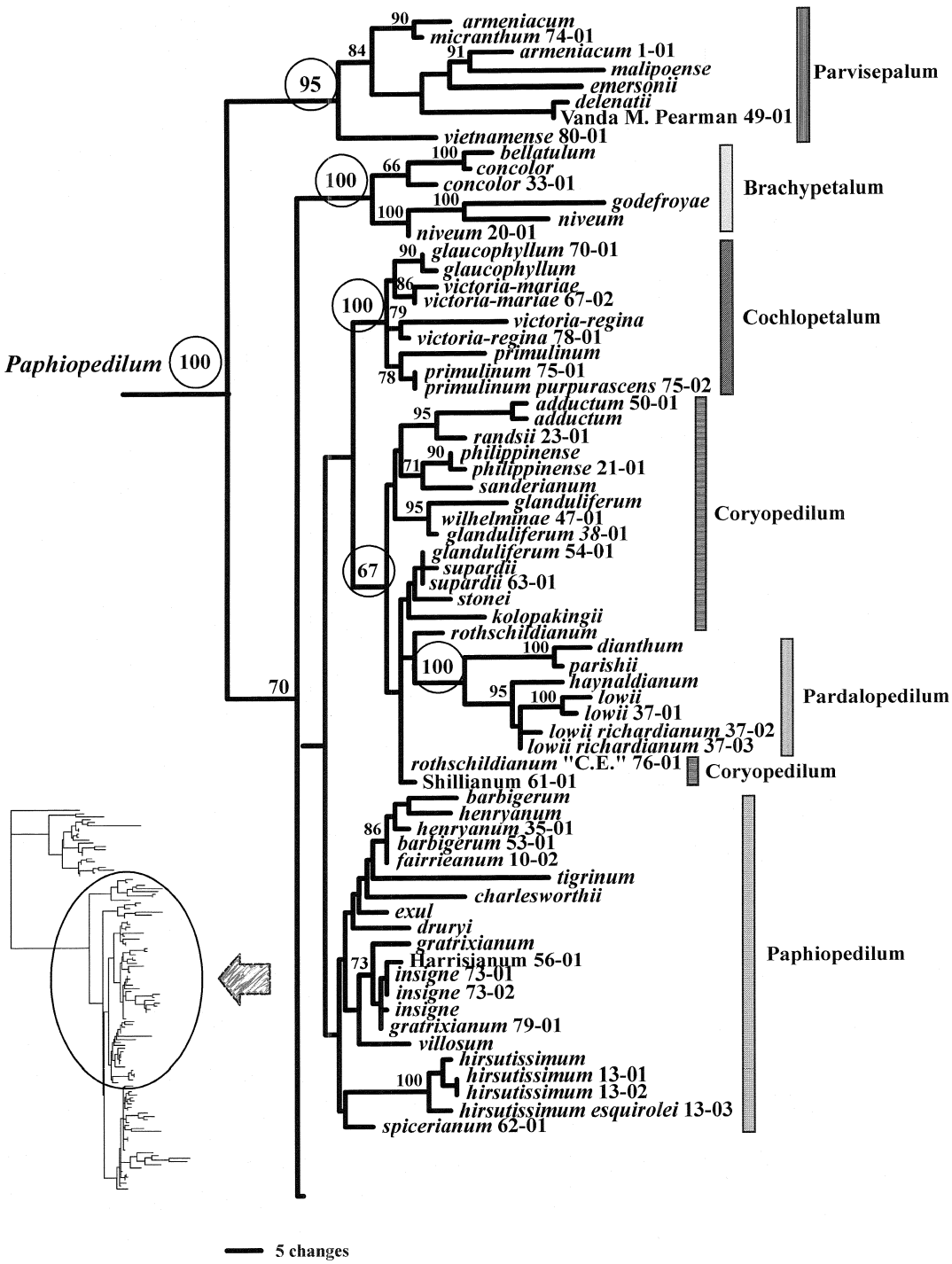

FIGURE 2C. Subset of phylogeny showing phylogenetic relationships among *Paphiopedilum* species, with bootstrap support values as above.

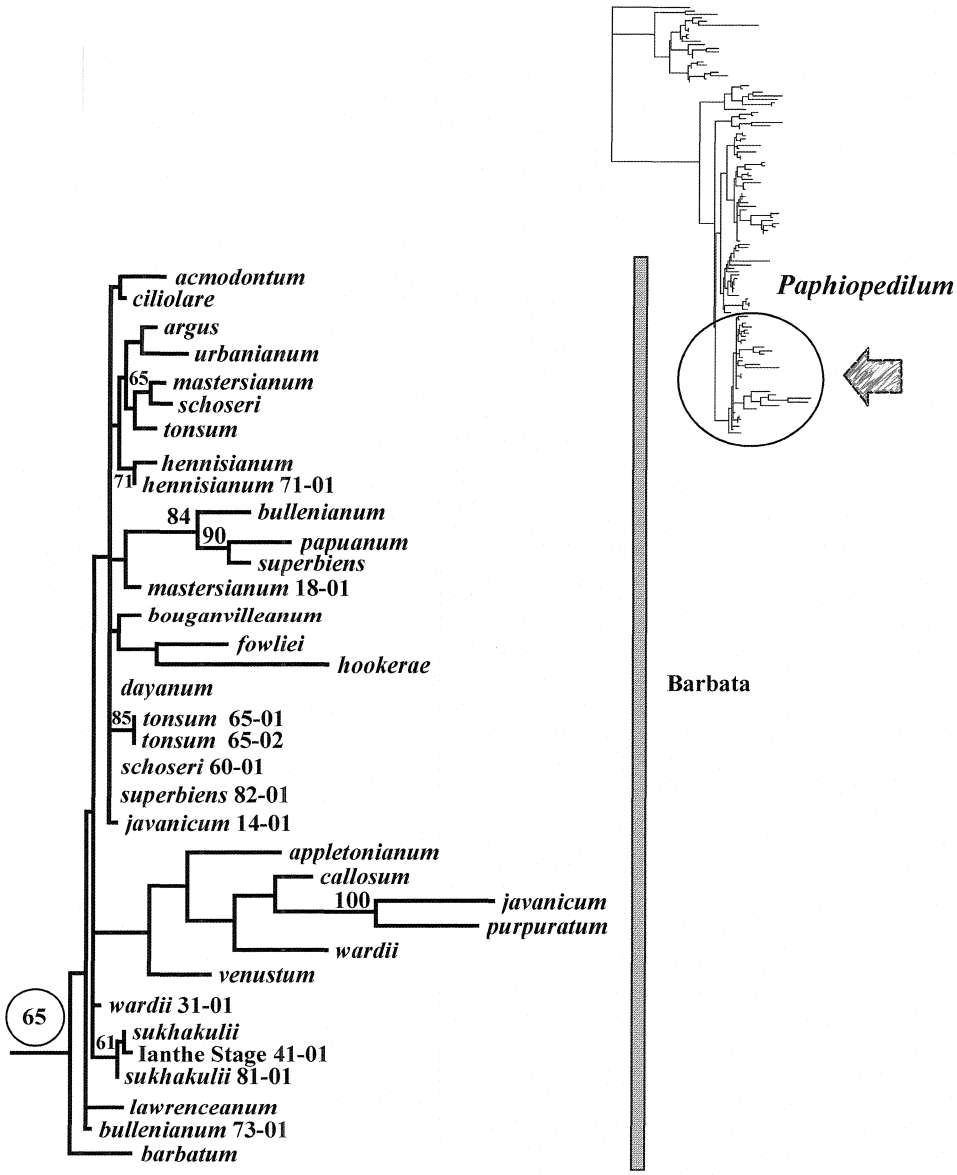

FIGURE 2D. Subset of phylogeny showing phylogenetic relationships among *Paphiopedilum* section Barbata species, with bootstrap support values as above.

the clade containing *P. venustum* and *P. appletonianum,* FIGURE 2D). Samples of hybrid origin (*P.* Vanda M. Pearmen, *P.* Ianthe Stage, *P.* Shillianum, *P.* Harrisianum) clustered with one of the parent species (*P. delenatii, P. sukhakulii, P. rothschildianum,* and *P. villosum,* respectively) and not with the other parent. The hybrids did not cluster as an intermediate between both parent species, which were from different *Paphiopedilum* sections (APPENDIX 1 and FIGURE 2C, 2D).

In a preliminary search for novel gene regions that can be used to verify results based upon ITS sequences, we have screened several *Phragmipedium* and *Paphiopedilum* taxa for variation in intron (i.e., non-coding) regions of organelle genomes. In the chloroplast genome, we sequenced a 1100 bp intron region (*trn*S-*trn*fM) for two *Cypripedium,* five *Phragmipedium,* and 13 *Paphiopedilum* species that, for the latter two genera, were chosen to represent sections, allowing for maximum sampling of potential genetic di-

TABLE 3. Twenty-eight single nucleotide polymorphisms in the chloroplast *trn*S-*trn*fM intron region that are diagnostic of cypripedioid orchid genera.

Base position in alignment	77	94	109	110	115	161	165	186	192	207	513	540	546	584
Cypripedium	A	T	T	A	A	G	G	T	G	T	T	G	A	A
Phragmipedium	C	C	G	G	C	G	G	G	A	C	G	A	G	G
Paphiopedilum	C	T	T	G	C	A	A	G	A	T	T	G	G	G

Base position in alignment	596	605	611	632	644	706	711	724	729	752	765	766	783
Cypripedium	A	T	C	T	T	G	T	A	T	G	T	C	T
Phragmipedium	G	C	C	T	T	T	A	G	C	A	C	C	T
Paphiopedilum	G	C	A	G	A	G	T	G	C	G	C	A	G

versity. The region contained 97 variable sites, of which 46 were phylogenetically informative (TABLE 2). Of these informative characters, there were a surprising 28 fixed nucleotide differences between genera (TABLE 3). These differences included indels, or gaps in the alignment of sequences, which greatly increased the number of variable and informative sites to 316 and 101, respectively (TABLE 2). Four indel regions (24 bp) represented fixed differences between these genera (TABLE 4). Separate parsimony analyses were preformed with gaps treated as missing data and as a fifth character. Resulting topologies were very similar (see FIGURE 3, from analysis with gaps treated as missing). *Paphiopedilum* and *Phragmipedium* species were easily distinguished using this data set, as can be seen by the high bootstrap values and long branch lengths between these genera (FIGURE 3). Unlike results based upon ITS sequence data (e.g., FIGURE 2B), there was little genetic variation among species of *Phragmipedium* at this chloroplast locus (FIGURE 3). Similar to results from the ITS analyses, *Paphiopedilum micranthum,* from section Parvisepalum, was basal to the genus (FIGURE 3). Species from section Brachypetalum were intermediate between *Paphiopedilum micranthum* (section Parvisepalum) and other *Paphiopedilum* species included in the analysis.

In a preliminary screening of the mitochondrial *nad*1 intron, sampling of taxa followed that of the chloroplast gene region, where individuals were selected to represent sections within *Phragmipedium* and *Paphiopedilum,* including two *Cypripedium* species, four *Phragmipedium,* and 13 *Paphiopedilum* species. The multiple sequence alignment also contained *Cypripedium passerinum* and *Paphiopedilum delenatii* from GenBank (Freudenstein & Chase 2001) and was 1559 bp in length. There were fewer fixed nucleotide differences between genera at this gene region (seven sites, TABLE 5), yet indel regions were numerous, including 61 positions that were fixed between genera (TABLE 6). A parsimony analysis of the *nad*1 data set, with gaps coded as a 5th base, included 114 phylogenetically informative sites and yielded twelve trees (TABLE 2, FIGURE 4). This *nad*1 gene intron is clearly useful for distinguishing between the three genera represented, with high bootstrap values at nodes defining genera (FIGURE 4). Also, there was structuring among *Paphiopedilum* species, with the basal species belonging to section Parvisepalum and closely related species clustered together (e.g., *P. insigne,* FIGURE 4). The hybrid *P.* Vanda M. Pearman clustered with one of its parent species, *P. delenatii* (FIGURE 4), which agrees with results from phylogenetic analysis of ITS data (FIGURE 2C).

DISCUSSION

The data presented here clearly demonstrates the utility of the assayed gene regions for determining the genus of unknown slipper orchids. Gene regions in the nuclear, chloroplast, and mi-

TABLE 4. Twenty-four indel positions in the chloroplast *trn*S-*trn*fM intron region that are diagnostic of cypripedioid orchid genera.

Base position in alignment	262	263	264	265	694	695	696	697	698	699	700	701	798	799	800	801	802	803	804	1002	1003	1004
Cypripedium	T	T	T	T	A	A	T	T	T	A	G	A	C	A	T	A	T	A	T	T	A	G
Phragmipedium	—	—	—	—	A	A	T	T	T	A	G	A	C	A	T	A	T	A	T	T	A	G
Paphiopedilum	T	T	T	T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

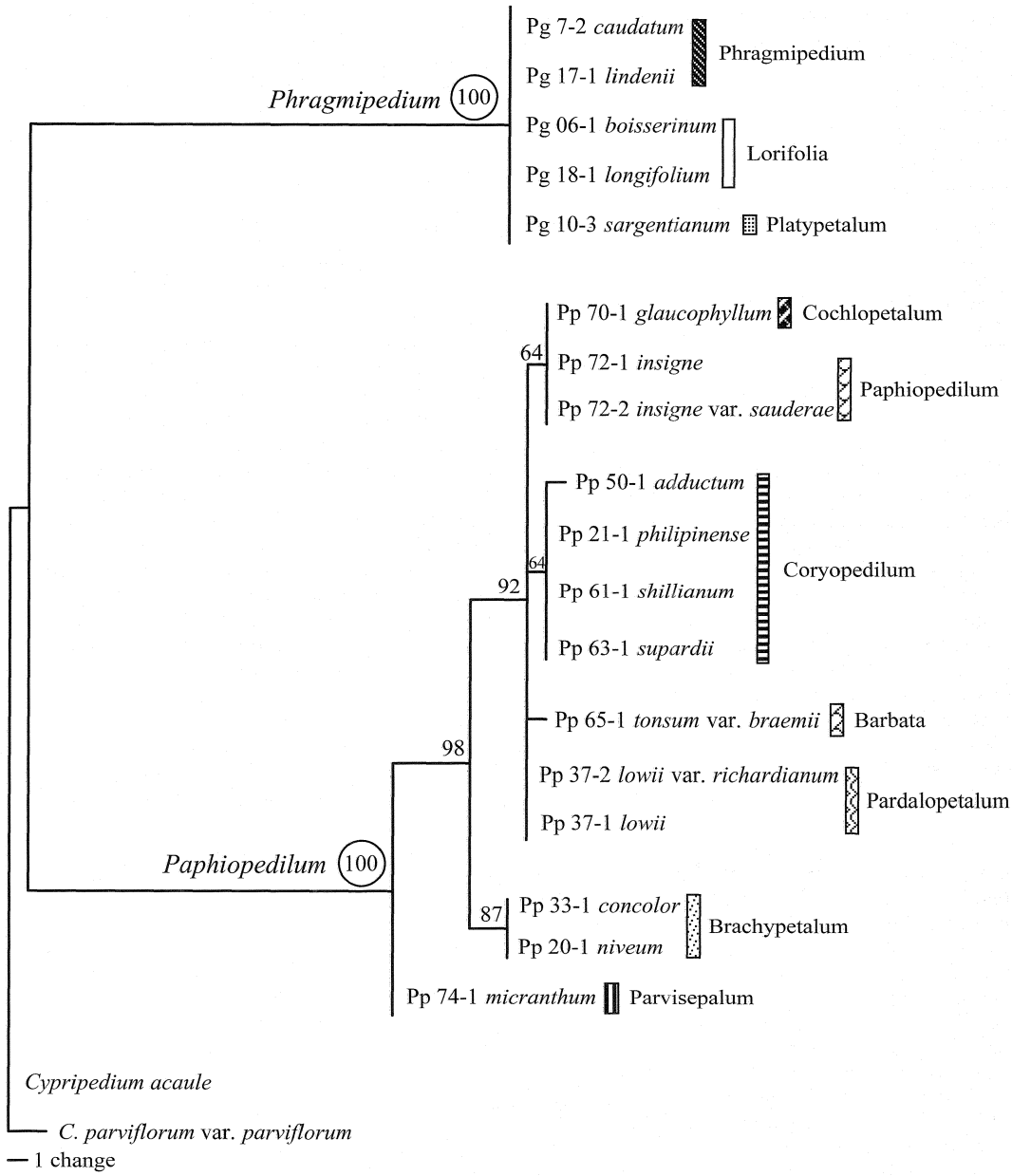

FIGURE 3. One of two most parsimonious trees based upon 46 phylogenetically informative sites in an analysis of chloroplast *trn*S-*trn*fM intron DNA sequences. Bootstrap support values are shown above branches with circled values for generic and sectional support.

tochondrial genomes contained many informative substitutions. Data collected from all three regions would allow for high accuracy and cross-checking of results from any one region, although each region had strengths that could be drawn upon for certain types of assays. For example, the nuclear ITS region contained the highest level of variability and was able to best recover relationships among closely related taxa, such as sections among species of *Paphiopedilum*. The best method for assay for the ITS region may be DNA sequencing, which would yield a high percentage of informative characters. The chloroplast *trn*S-*trn*fM region had a

TABLE 5. Seven single nucleotide polymorphisms in the mitochondrial *nad*1 intron region that are diagnostic of genera.

Base position in alignment	398	424	425	426	427	568	630
Cypripedium	T	C	T	C	C	C	A
Phragmipedium	T	C	T	C	C	C	C
Paphiopedilum	A	A	A	A	A	G	C

large number of fixed nucleotide substitutions and would likely be assayed effectively using a method such as single nucleotide polymorphism (SNPs) detection through real-time PCR or perhaps a simple fluorometric reaction visible on a typical microplate reader. The mitochondrial *nad*1 gene intron had the fewest number of nucleotide substitutions, yet it had a high number of indels that appear to be informative between slipper orchid genera. In previous studies, indel variation has been observed at low levels, such as among species of *Spiranthes* (Chen & Sun 1998), and at higher taxonomic levels, such as within the Orchidaceae (Freudenstein et al. 1998). To assay these substitutions, DNA sequencing or restriction fragment length polymorphism (RFLP) analysis will likely be required. Once sequence data is gathered, there are methods of analysis to consider that treat strings of gaps as one evolutionary event, each of which can be coded as a separate binary character (see Freudenstein & Chase 2001). For example, in the *nad*1 data set presented here, the 61 bp with fixed indels between taxa likely represent 10 evolutionary events, or are made up of strings of bases 3–18 bp in length (TABLE 6). In this way, presence or absence of sequence in one of these regions becomes the necessary phylogenetic information.

The ITS data set with the new data presented here combined with that from GenBank contains some of the only sequence data where more than one individual of a species is represented, and several cases point to the importance of including such replicates. Scattered throughout the ITS *Paphiopedilum* subtree are examples of both well-supported clusters of individuals from one taxon (e.g., *P. lowii, P. glaucophylum, P. hirsutissimum*) and cases where individuals of the same taxon do not cluster together (e.g., *P. schoseri, P. javanicum, P. glanduliferum,* FIGURE 2C). There are several reasons for why this may have occurred. For one, all described *Pa-*

TABLE 6. Twenty-four indel positions in the mitochondrial *nad*1 intron region that are diagnostic of cypripedioid orchid genera.

Base position in alignment	433	610	611	612	613	614	615	616	617	677	678	679	680	681
Cypripedium	—	G	C	T	T	C	T	T	A	G	G	G	C	C
Phragmipedium	G	C	C	T	T	C	T	T	A	G	G	G	C	C
Paphiopedilum	G	—	—	—	—	—	—	—	—	—	—	—	—	—

Base position in alignment	682	683	684	685	686	687	688	689	690	691	692	693	694	718
Cypripedium	—	—	—	—	G	C	—	—	—	—	—	—	—	—
Phragmipedium	G	T	T	T	G	C	G	—	—	—	—	—	T	—
Paphiopedilum	—	—	—	—	—	—	—	T	A	T	A	G	T	A

Base position in alignment	719	881	882	883	884	885	886	925	926	927	1245	1246	1247	1248
Cypripedium	—	A	G	C	C	C	G	A	T	A	—	—	—	—
Phragmipedium	—	A	G	C	C	C	G	A	T	A	C	A	T	C
Paphiopedilum	A	—	—	—	—	—	—	—	—	—	—	—	—	—

Base position in alignment	1249	1250	1251	1265	1266	1267	1268	1273	1274	1275	1276	1420	1421
Cypripedium	—	—	—	G	C	C	G	—	A	A/T	A/G	C	C
Phragmipedium	T	A	A	—	—	—	—	—	—	—	—	C	C
Paphiopedilum	—	—	—	G	C	C	—	A	A	A	A	—	—

Base position in alignment	1422	1423	1424	425	1426	1427
Cypripedium	T	C	G	T	A	A
Phragmipedium	T	C	G	T	A	A
Paphiopedilum	—	—	—	—	—	—

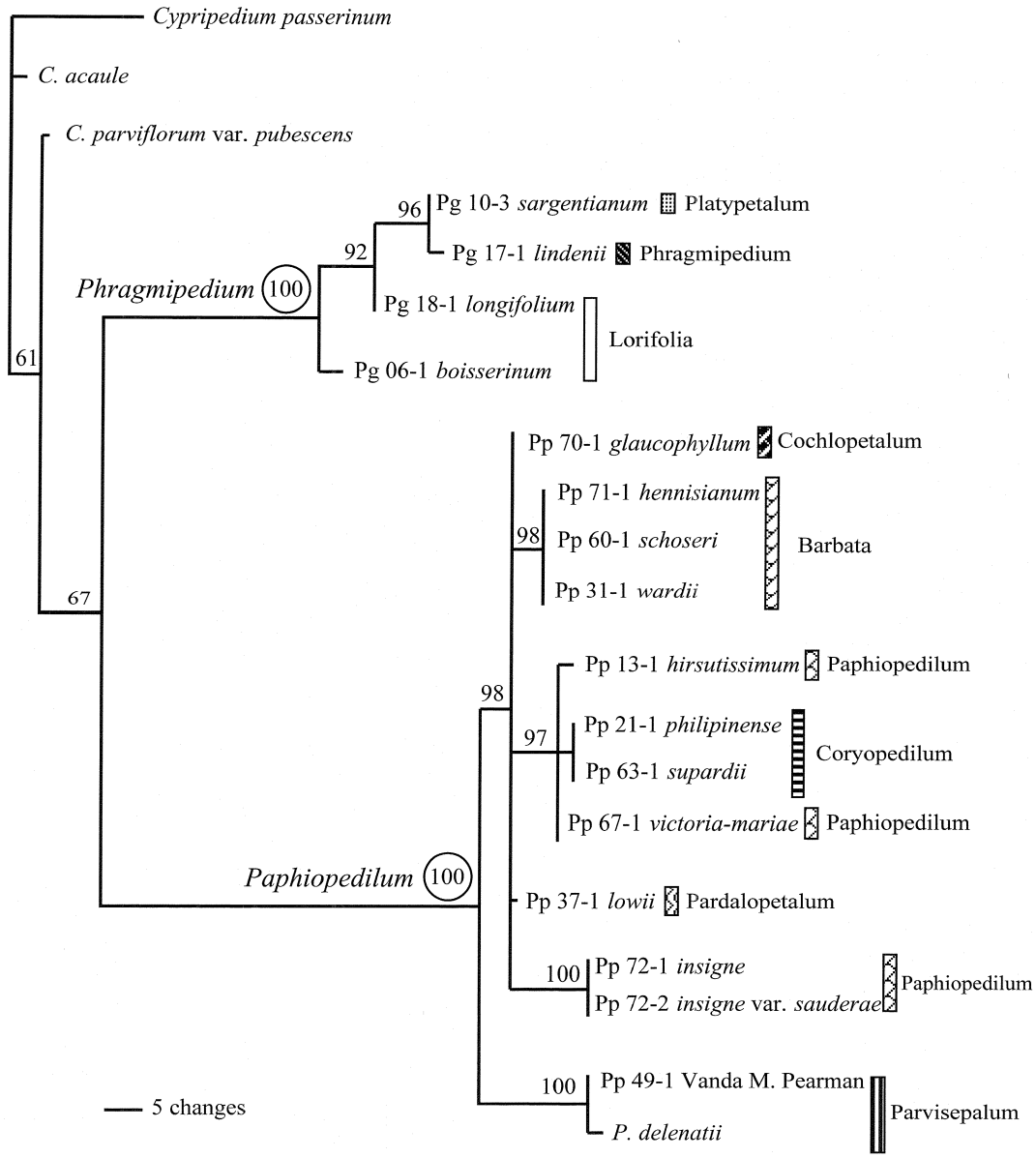

FIGURE 4. One of two most parsimonious trees based upon 141 phylogenetically informative sites in an analysis of mitochondrial *nad*1 intron DNA sequences. Bootstrap support values are shown above branches with circled values for generic and sectional support.

phiopedilum species may not be good phylogenetic species, instead some designated "species" may represent closely related varieties of the same species; or in other words, the speciation process is probably still ongoing in many cases, and the species boundaries seen in DNA after speciation may not yet have formed. Little information is available regarding morphological variation among individuals in the field (Koopowitz 2000), and information regarding natural intraspecific genetic variation is even less known. Morphological intraspecific variation may have lead to overzealous "splitting" of some genera into more species than may be necessary based on genetic differences between species (e.g., section Barbata, see Koopowitz 2000). Given the rarity of plants remaining in the wild for many of these species, it becomes

complicated to estimate possible gene flow among populations. The possibility of other errors exists also. Some plants may have been misidentified because of convergence of a particular diagnostic character or coloration. Since our methods have been non-invasive (leaf tissue was taken from live plants), voucher specimens are not available to go back to for verifying species identity. The ideal situation would be to begin with tissue from herbarium plants with preserved flowers and vegetative material. This, however, becomes increasingly difficult when working with rare or endangered species. Good digital photographs of the plants also may serve as a reference in case of future identification questions. Sequencing techniques used by different labs may introduce errors that may be enough to cause individuals belonging to the same taxon to cluster separately. This would be problematic in sections where genetic differences between species are few. Identification of these types of problems may need to be conducted on a species by species case. Clearly, additional samples of all taxa need to be sought to develop the most extensive baseline data set to facilitate the verification of results. These examples point to the need for multiple gene regions to be used along with ITS to distinguish between errors in taxonomy, poor resolution of molecular data, and to verify results from analyses based upon a single gene region. Nonetheless, this nuclear gene region clearly allows differentiation between genera and between most sections within the genera *Paphiopedilum* and *Phragmipedium.*

Additional tissue samples from positively identified species belonging to any of the five genera of cypripedioid orchids are needed to have a comprehensive representation of as many taxa as possible. Especially important will be representative samples from species in the genera *Cypripedium, Selenipedium,* and *Mexipedium* for comparisons to be made with *Paphiopedilum* and *Phragmipedium* for organelle gene regions. We request assistance from all interested parties in obtaining tissue from additional species of interest. The sequencing protocol we have followed to this point appears to allow rapid and accurate identification to genus and often to section of the CITES Appendix I genera of slipper orchids.

Acknowledgments

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APPENDIX. Collection information for slipper orchid samples/sequences included in phylogenetic analyses.

Genus	Species	Subspecies, variety, or cross	Authority*	Sample source**	ID No.	ITS GenBank	CP *trn*S-fM GenBank	Mt *nad*1 GenBank
Cypripedium	*acaule*		Aiton	2	Ca-01			AY642483
Cypripedium	*guttatum*		Sw.	6		Z78526		
Cypripedium	*parviflorum*	*pubescens*	(Willd.) O.W. Knight	3	Cp-01		AY642767	
Cypripedium	*parviflorum*	*parviflorum*	Salisb.	3	Cp-02			AY642484
Cypripedium	*passerinum*		Richardson	7				AF314830
Cypripedium	*yatabeanum*		Makino	6		Z78527		
Mexipedium	*xerophyticum*		(Soto Arenas, Salazar & Hágsater) V.A. Albert & M.W. Chase	6		Z78515		
Paphiopedilum	*acmodontum*		M.W. Wood	6		Z78446		
Paphiopedilum	*adductum*		Asher	5	Pp50-1	AY643459	AY642472	
Paphiopedilum	*adductum*		Asher	6		Z78468		
Paphiopedilum	*appletonianum*		(Gower) Rolfe	6		Z78444		
Paphiopedilum	*argus*		(Rchb.f.) Stein	6		Z78448		
Paphiopedilum	*armeniacum*		S.C. Chen & F.Y. Liu	1	Pp01-1	AY643431		
Paphiopedilum	*armeniacum*		S.C. Chen & F.Y. Liu	6		Z78496		
Paphiopedilum	*barbatum*		(Lindl.) Pfitzer	6		Z78439		
Paphiopedilum	*barbigerum*		Tang & F.T. Wang	6		Z78486		
Paphiopedilum	*barbigerum*		Tang & F.T. Wang	5	Pp53-1	AY643442		
Paphiopedilum	*bellatulum*		(Rchb.f.) Stein	6		Z78492		
Paphiopedilum	*bougainvilleanum*		Fowlie	6		Z78452		
Paphiopedilum	*bullenianum*		(Rchb.f.) Pfitzer	4	Pp73-1	AY643465		
Paphiopedilum	*bullenianum*		(Rchb.f.) Pfitzer	6		Z78442		
Paphiopedilum	*callosum*		(Rchb.f.) Stein	6		Z78457		
Paphiopedilum	*charlesworthii*		(Rolfe) Pfitzer	6		Z78484		
Paphiopedilum	*ciliolare*		(Rchb.f.) Stein	6		Z78460		
Paphiopedilum	*concolor*		(Lindl. ex. Bateman) Pfitzer	1	Pp33-1	AY643435	AY642478	
Paphiopedilum	*concolor*		(Lindl. ex. Bateman) Pfitzer	6		Z78491		
Paphiopedilum	*dayanum*		(Lindl.) Stein	6		Z78459		
Paphiopedilum	*delenatii*		Guillaumin	6		Z78497		
Paphiopedilum	*delenatii*		Guillaumin	7				AF314831
Paphiopedilum	*dianthum*		Tang & F.T. Wang	6		Z78471		
Paphiopedilum	*druryi*		(Bedd.) Stein	6		Z78489		
Paphiopedilum	*emersonii*		Koop. & P.J. Cribb	6		Z78495		
Paphiopedilum	*exul*		(Ridl.) Rolfe	6		Z78482		

APPENDIX. Continued.

Genus	Species	Subspecies, variety, or cross	Authority*	Sample source**	ID No.	ITS GenBank	CP *trn*S-fM GenBank	Mt *nad*1 GenBank
Paphiopedilum	*fairrieanum*		(Lindl.) Stein	5	Pp10-2	AY643443		
Paphiopedilum	*fairrieanum*		(Lindl.) Stein	6		Z78490		
Paphiopedilum	*fowliei*		Birk	6		Z78454		
Paphiopedilum	*glanduliferum*		(Blume) Stein	5	Pp54-1	AY643451		
Paphiopedilum	*glanduliferum*		(Blume) Stein	6		Z78463		
Paphiopedilum	*glanduliferum*		(Blume) Stein	1	Pp38-1	AY643452		
Paphiopedilum	*glaucophyllum*		J.J. Sm.	4	Pp70-1	AY643437	AY642470	AY642772
Paphiopedilum	*glaucophyllum*		J.J. Sm.	6		Z78476		
Paphiopedilum	*godefroyae*		(God.-Leb.) Stein	6		Z78493		
Paphiopedilum	*gratrixianum*		Rolfe	4	Pp79-1	AY643472		
Paphiopedilum	*gratrixianum*		Rolfe	6		Z78480		
Paphiopedilum	Harrisianum	*barbatum* × *villosum*		5	Pp56-1	AY643444		
Paphiopedilum	*haynaldianum*		(Rchb.f.) Stein	6		Z78469		
Paphiopedilum	*hennisianum*		(M.W. Wood) Fowlie	4	Pp71-1	AY643460		AY642773
Paphiopedilum	*hennisianum*		(M.W. Wood) Fowlie	6		Z78458		
Paphiopedilum	*henryanum*		Braem	1	Pp35-1	AY643445		
Paphiopedilum	*henryanum*		Braem	6		Z78485		
Paphiopedilum	*hirsutissimum*		(Lindl. ex Hook.) Stein	1	Pp13-1	AY643446		AY642774
Paphiopedilum	*hirsutissimum*		(Lindl. ex Hook.) Stein	5	Pp13-2	AY643447		
Paphiopedilum	*hirsutissimum*		(Lindl. ex Hook.) Stein	6		Z78487		
Paphiopedilum	*hirsutissimum*	*esquirolei*	(Schltr.) K. Karas. & K. Saito	4	Pp13-3	AY643473		
Paphiopedilum	*hookerae*		(Rchb.f.) Stein	6		Z78451		
Paphiopedilum	*insigne*		(Wall. ex Lindl.) Pfitzer	4	Pp72-1	AY643448	AY642471	AY642778
Paphiopedilum	*insigne*		(Wall. ex Lindl.) Pfitzer	6		Z78481		
Paphiopedilum	*insigne*		(Wall. ex Lindl.) Pfitzer	4	Pp72-2	AY643449	AY642473	AY642780
Paphiopedilum	Ianthe Stage	*rothschildianum* × *sukhakulii*		1	Pp41-1	AY643470		
Paphiopedilum	*javanicum*		(Reinw. ex Lindl.) Pfitzer	5	Pp14-1	AY643461		
Paphiopedilum	*javanicum*		(Reinw. ex Lindl.) Pfitzer	6		Z78455		
Paphiopedilum	*kolopackingii*		Fowlie	6		Z78474		
Paphiopedilum	*lawrenceanum*		(Rchb.f.) Pfitzer	6		Z78443		
Paphiopedilum	*lowii*		(Lindl.) Stein	1	Pp37-1	AY643456	AY642480	AY642777
Paphiopedilum	*lowii*	*richardianum*	(Asher & Beaman) O. Gruss	5	Pp37-2	AY643457	AY642475	

APPENDIX. Continued.

Genus	Species	Subspecies, variety, or cross	Authority*	Sample source**	ID No.	ITS GenBank	CP *trn*S-fM GenBank	Mt *nad*1 GenBank
Paphiopedilum	*lowii*	*richardianum*	(Asher & Beaman) O. Gruss	4	Pp37-3	AY643474		
Paphiopedilum	*lowii*		(Lindl.) Stein	6		Z78472		
Paphiopedilum	*malipoense*		S.C. Chen & Z.H. Tsi	6		Z78498		
Paphiopedilum	*mastersianum*		(Rchb.f.) Stein	1	Pp18-1	AY643466		
Paphiopedilum	*mastersianum*		(Rchb.f.) Stein	6		Z78449		
Paphiopedilum	*micranthum*		Tang & F.T. Wang	4	Pp74-1	AY643432	AY642481	
Paphiopedilum	*niveum*		(Rchb.f.) Stein	1	Pp20-1	AY643436	AY642482	
Paphiopedilum	*niveum*		(Rchb.f.) Stein	6		Z78494		
Paphiopedilum	*papuanum*		(Ridl ex Rendle) L.O. Williams	6		Z78450		
Paphiopedilum	*parishii*		(Rchb.f.) Stein	6		Z78470		
Paphiopedilum	*philippinense*		(Rchb.f.) Stein	1	Pp21-1	AY643475	AY642476	AY642775
Paphiopedilum	*philippinense*		(Rchb.f.) Stein	6		Z78466		
Paphiopedilum	*primulinum*		M.W. Wood & P. Taylor	4	Pp75-1	AY643438		
Paphiopedilum	*primulinum*		M.W. Wood & P. Taylor	6		Z78479		
Paphiopedilum	*primulinum*	*purpurascens*	(M.W. Wood) & P.J. Cribb	4	Pp75-2	AY643439		
Paphiopedilum	*purpuratum*		(Lindl.) Stein	6		Z78440		
Paphiopedilum	*randsii*		Fowlie	1	Pp23-1	AY643458		
Paphiopedilum	*rothschildianum*		(Rchb.f.) Stein	6		Z78465		
Paphiopedilum	*rothschildianum*	'Charles Edward'		4	Pp76-1	AY643453		
Paphiopedilum	*sanderianum*			6		Z78473		
Paphiopedilum	Shillianum	(curtisii × lawrenceanum) × rothschildianum		5	Pp61-1	AY643471	AY642477	
Paphiopedilum	*stonei*		(Hook.) Stein	6		Z78467		
Paphiopedilum	*supardii*		Braem & Löb	5	Pp63-1	AY643454	AY642479	AY642779
Paphiopedilum	*supardii*		Braem & Löb	6		Z78475		
Paphiopedilum	*tonsum*		(Rchb.f.) Stein	6		Z78456		
Paphiopedilum	*tonsum*	*braemii*	(Rchb.f.) Stein	5	Pp65-1	AY643463	AY642474	
Paphiopedilum	*tonsum*	*braemii*	(Rchb.f.) Stein	4	Pp65-2	AY643464		
Paphiopedilum	*schoseri*		Braem & H. Mohr	5	Pp60-1	AY643462		AY642776
Paphiopedilum	*schoseri*		Braem & H. Mohr	6		Z78453		
Paphiopedilum	*spicerianum*		(Rchb.f) Pfitzer	5	Pp62-1	AY643450		
Paphiopedilum	*superbiens*		(Rchb.f.) Stein	1	Pp82-1	AY643467		
Paphiopedilum	*superbiens*		(Rchb.f.) Stein	6		Z78441		

APPENDIX. Continued.

Genus	Species	Subspecies, variety, or cross	Authority*	Sample source**	ID No.	ITS GenBank	CP *trn*S-fM GenBank	Mt *nad*1 GenBank
Paphiopedilum	*sukhakulii*		Schoser & Senghas	4	Pp81-1	AY643468		
Paphiopedilum	*sukhakulii*		Schoser & Senghas	6		Z78462		
Paphiopedilum	*tigrinum*		Koop. & N. Haseg.	6		Z78488		
Paphiopedilum	*urbanianum*		Fowlie	6		Z78445		
Paphiopedilum	Vanda M. Pearman	*bellatulum* × *delenatii*		1	Pp49-1	AY643434		AY642781
Paphiopedilum	*venustum*		(Wall. ex Sims) Pfitzer	6		Z78447		
Paphiopedilum	*victoria-mariae*		(Sander ex Mast.) Rolfe	6		Z78477		
Paphiopedilum	*victoria-mariae*		(Sander ex Mast.) Rolfe	5	Pp67-2	AY643440		AY642782
Paphiopedilum	*victoria-regina*		(Sander) M.W. Wood	6		Z78478		
Paphiopedilum	*victoria-regina*		(Sander) M.W. Wood	4	Pp78-1	AY643441		
Paphiopedilum	*vietnamense*		O. Gruss & Perner	4	Pp80-1	AY643433		
Paphiopedilum	*villosum*		(Lindl.) Stein	6		Z78483		
Paphiopedilum	*wardii*		Summerh.	1	Pp31-1	AY643469		AY642783
Paphiopedilum	*wardii*		Summerh.	6		Z78461		
Paphiopedilum	*wilhelminae*		L.O. Williams	1	Pp47-1	AY643455		
Phragmipedium	*besseae*		Dodson & J. Kuhn	6		Z78513		
Phragmipedium	*boissierianum*		(Rchb.f.) Rolfe	1	Pg06-1	AY643424	AY642467	AY642766
Phragmipedium	*boissierianum*		(Rchb.f.) Rolfe	1	Pg06-2	AY643425		
Phragmipedium	*boissierianum*		(Rchb.f.) Rolfe	6		Z78502		
Phragmipedium	*boissierianum*	*czerwiakowianum*	(Rchb.f.) Rolfe	6		Z78503		
Phragmipedium	*caricinum*		(Lindl. & Paxton) Rolfe	6		Z78510		
Phragmipedium	*caudatum*		(Lindl.) Rolfe	1	Pg07-1	AY643429		
Phragmipedium	*caudatum*		(Lindl.) Rolfe	5	Pg07-2		AY642465	
Phragmipedium	*caudatum*		(Lindl.) Rolfe	6		Z78501		
Phragmipedium	*caudatum*	*(warscewiczianum)*		6		Z78500		
Phragmipedium	*exstaminodium*		Castaño, Hagsater & E. Aguirre	6		Z78511		
Phragmipedium	*lindenii*		(Lindl.) Dressler & N.H. Williams	5	Pg17-1	AY643430	AY642469	AY642770
Phragmipedium	*lindenii*		(Lindl.) Dressler & N.H. Williams	6		Z78507		
Phragmipedium	*lindleyanum*	*(sargentianum)*	(R.H. Schomb. ex Lindl.) Rolfe	6		Z78506		
Phragmipedium	*lindleyanum*		(Lindl.) Rolfe	1	Pg10-1	AY643427		

APPENDIX. Continued.

Genus	Species	Subspecies, variety, or cross	Authority*	Sample source**	ID No.	ITS GenBank	CP *trn*S-fM GenBank	Mt *nad*1 GenBank
Phragmipedium	*lindleyanum*		(Lindl.) Rolfe	5	Pg10-2			
Phragmipedium	*lindleyanum*		(Lindl.) Rolfe	5	Pg10-3	AY643428	AY642468	AY642768
Phragmipedium	*lindleyanum*	*(sargentianum)*	(Lindl.) Rolfe	6		Z78505		
Phragmipedium	*lindleyanum*	*(kaieteurum)*		6		Z78503		
Phragmipedium	*longifolium*		(Warsz ex Rchb.f.) Rolfe	5	Pg18-1	AY643426	AY642466	AY642769
Phragmipedium	*longifolium*		(Warsz ex Rchb.f.) Rolfe	6		Z78508		
Phragmipedium	*pearcei*		Rauh & Senghas	6		Z78509		
Phragmipedium	*schlimii*		(Linden & Rchb.f.) Rolfe	6		Z78514		
Phragmipedium	*wallisii*		(Rchb.f.) Garay	6		Z78512		
Selenipedium	*chica*		Rchb.f.	8				

* Species description authorities and synonyms following Cribb 1998, Govaerts 2003.

** Sample sources: (1) Shepherd College by P. Simpson; (2) Greenville Co., SC, and (3) Linville, NC, by Lucy Dueck; U.S. Botanic Garden (4) by P. Ford or (5) by the authors. Samples from GenBank: (6) nuclear ITS sequences by Cox et al. 1997, with species names used in submission but now synonymized in parentheses; (7) mitochondrial NAD1 by Freudenstein & Chase 2001; (8) ITS sequence for *Selenipedium chica* by M. Chase pers. comm.

Note: ID No. = collection numbers listed in the figures.