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A PHYLOGENY OF *PHALAENOPSIS* USING MULTIPLE CHLOROPLAST MARKERS

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ABSTRACT. A phylogeny of *Phalaenopsis* was reconstructed using three chloroplast markers, *mat*K, *atp*H-*atp*F, and *trn*D-*trn*E, totaling 2177 base pairs. Results of Bayesian and Maximum Parsimony analyses support the placement of the species of *Doritis* and *Kingidium* into a more broadly defined *Phalaenopsis,* as proposed in a revision of the genus by Christenson. While some of Christenson's subgeneric groups appear to be monophyletic, several species may need to be reclassified, if a natural classification is desired. Taxon sampling, however, must be completed, and the identities of several plants confirmed, before definitive conclusions can be made. Another marker of approximately 1300 base pairs will be added to the analysis. It is expected to provide more support and to improve the resolution of the tree, especially at the basal nodes and in the subgenus *Polychilos.*

Key words: *Phalaenopsis,* phylogeny, taxonomy, Orchidaceae

INTRODUCTION

Phalaenopsis is a genus of orchids containing 63 species that occur throughout Southeast Asia and the Pacific Islands. Christenson (2000) recently revised the genus and included within it the species that were considered *Doritis* and *Kingidium* in the earlier work of Sweet (1980). Christenson divided the genus into five subgenera, *Proboscidioides, Aphyllae, Parishianae, Phalaenopsis,* and *Polychilos,* based on flower color and pigmentation patterns, lip and callus structure, number of pollinia, and deciduousness. Subgenus *Phalaenopsis* was further divided into four sections, *Phalaenopsis, Stauroglottis, Deliciosae,* and *Esmeralda.* Subgenus *Polychilos* was also divided into four sections, *Polychilos, Fuscatae, Amboinenses,* and *Zebrinae.*

Here we present a molecular phylogenetic analysis of the genus *Phalaenopsis,* including the species formerly in *Doritis* and *Kingidium,* using DNA sequences from three regions of the chloroplast genome: the maturase K gene (*mat*K), the ATP synthase genes *atp*H, *atp*F, and their intergenic spacer (*atp*HF), and the *trn*D–*trn*E intergenic region (*trn*DE). Relationships between the species are inferred and the monophyly of Christenson's subgeneric groups are tested.

MATERIALS AND METHODS

Sampling

Sixty-five individuals are included in the current data set, 47 of which are *Phalaenopsis* species (one variety and 10 replicates), and four are outgroup taxa from three genera (*Paraphalaenopsis, Sarcoglyphis,* and *Amesiella*) shown to be closely related to *Phalaenopsis* (Whitten pers. comm.). Two of the three species that were previously placed in *Doritis* and seven species that were *Kingidium* have been sampled. All individuals included were obtained through horticultural sources, either private or commercial growers, or the collections of the Marie Selby Botanical Gardens, the New York Botanical Garden, or the University of Florida. Individuals obtained through private or commercial growers were identified as they bloomed, and vouchers have been placed in The University of Texas Herbarium (TEX).

DNA Extraction, PCR Amplification, and Sequencing

Total DNA was extracted either from fresh or silica-dried tissue using the CTAB protocol of Doyle and Doyle (1987). Extracts were purified with the QIAGEN QIAEX II Suspension kit. Standard PCR protocols were used (Mullis & Faloona 1987), and dimethyl sulfoxide (DMSO) was added to reactions when necessary for successful amplification. Primers for *mat*K and *trn*DE were taken from Whitten et al. (2000) and Demesure (1995), respectively. Primers for *atp*HF were designed from the complete Maize chloroplast genome available in GenBank (Maier 1995). Amplifications were visualized on agarose gels and purified with QIAGEN QIAquick PCR purification kits. Cycle sequencing reactions were performed using BigDye Terminator 3.0 and visualized on an MJ BaseStation.

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Analyses

Sequences were assembled and edited in Sequencher 4.2. Initial alignments were carried out using ClustalX (Thompson et al. 1997), and then modified in MacClade 4.0 (Madison & Madison 2000). All three markers were combined for all analyses. Parsimony analyses were run in PAUP* 4.0 (Swofford 2002) using a heuristic search with ten random-addition-replicates and TBR branch swapping. A bootstrap analysis was performed to determine branch support with 1000 replicates and 1000 trees saved from each replicate.

Bayesian analyses were performed using MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001). Appropriate models of evolution were determined separately for each marker using the likelihood ratio test implemented in ModelTest 3.06 (Posada & Crandall 1998). Model Test determined that the Felsenstein 81 (F81) + gamma distributed rate variation model was appropriate for *trn*DE and *atp*HF, and the general time reversible model (GTR) + invariant sites + gamma distributed rate variation was appropriate for *mat*K. Data were partitioned in the Bayesian analysis, and the appropriate model was applied to each partition. Four MCMC chains were run for 3 million generations, with one tree saved every 100 generations. The first 3000 generations prior to stationarity were discarded as the burn-in period.

Results

The *mat*K region used in these analyses consisted of 1301 base pairs, the *trn*DE region of 493 base pairs, and the *atp*HF region of 383 base pairs after alignment. Combined, these regions produced a total of 2177 aligned base pairs, 179 of which were parsimony informative. Parsimony analyses resulted in 100,000 most-parsimonious trees with a score of 470, consistency index (CI) of 0.679, and retention index (RI) of 0.823.

Results of the Bayesian analysis are summarized as a majority rule consensus tree of 27,000 trees sampled after stationarity (Figure 1). Parsimony bootstrap values and Bayesian posterior probabilities are indicated above the branches. Individuals that have not yet bloomed (and therefore not positively identified) are marked with an asterisk (Figure 1). The Bayesian majority-rule tree was very similar to the majority rule consensus tree from the parsimony analysis.

The Bayesian analysis recovers two major sister clades within *Phalaenopsis.* Subgenus *Polychilos* is represented by Clade 1, with strong Bayesian support and moderate bootstrap support (100 and 62, respectively). The second major clade contains a polytomy of three groups, Clades 2, 3, and 4. It has, however, very low Bayesian support (62) and bootstrap support below 50%. This group collapses in the maximum parsimony majority rule consensus tree, with Clade 2 falling into a polytomy with Clade 1 and a third group containing Clades 3 and 4. This group is only recovered in 61% of the 100,000 best trees; and in the bootstrap consensus, it collapses, resulting in a polytomy of Clades 1, 2, 3, and 4.

Clade 2 contains the species in subgenera *Aphyllae* (previously *Kingidium*) and *Proboscidioides.* It has very strong support with a posterior probability of 100 and a bootstrap value of 99. Clade 3 contains species in subgenus *Phalaenopsis* sections *Esmeralda* (previously *Doritis*) and *Deliciosae* (*Kingidium*) and subgenus *Parishianae* (as well as *P. minus,* which was previously *Kingidium*). This clade has moderate to low support with a posterior probability of 93 and bootstrap support of 53. Clade 4 contains the species in subgenus *Phalaenopsis* sections *Phalaenopsis* and *Stauroglottis.* This clade is also well supported with a posterior probability of 100 and a bootstrap value of 85.

Discussion

Doritis and *Kingidium*

Christenson's treatment of *Phalaenopsis* as a more broadly defined genus that includes species of the genus *Doritis* and *Kingidium* is supported by the chloroplast phylogeny presented here. Subgenus *Aphyllae* and the other species that were previously placed in *Kingidium* and subgenus *Phalaenopsis* section *Esmeralda,* which was *Doritis,* are well supported by both the Bayesian analysis (100) and the parsimony analysis (87) to be included within *Phalaenopsis.* Although they may have morphological differences that distinguish them from the traditional *Phalaenopsis,* these differences are apparently adaptations to a terrestrial habit, in the case of *Doritis,* and to colder, drier climates associated with high altitudes in the case of *Kingidium.*

Subgenus *Polychilos*

Results strongly support the monophyly of subgenus *Polychilos,* although sectional relationships within the subgenus are still unclear because of a lack of resolution and low support for some clades. Branch lengths within the group are extremely short (data not shown), and more variable markers will be needed to discern the relationship between these species. Section

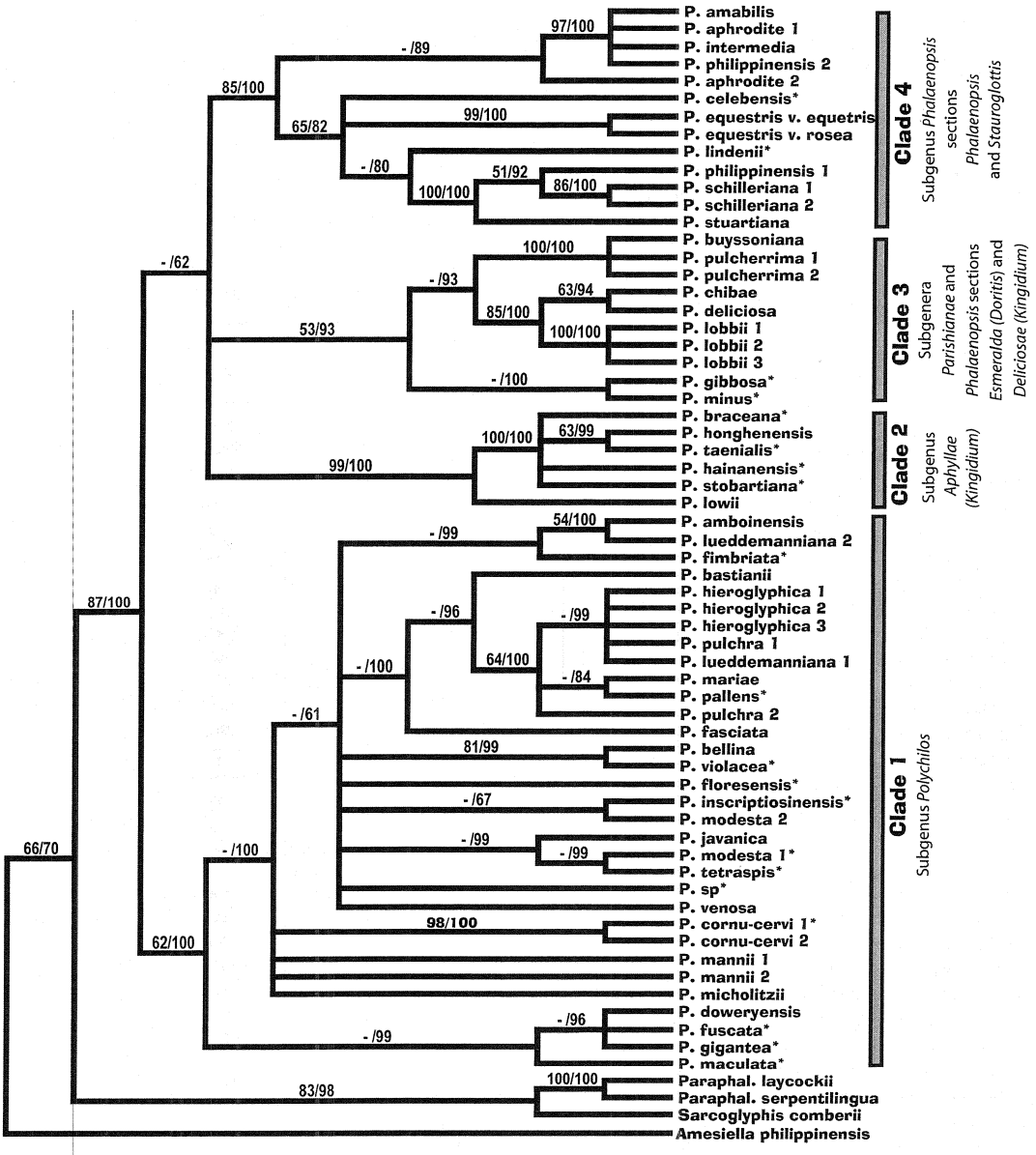

FIGURE 1. Phylogenetic tree of *Phalaenopsis* reconstructed with Bayesian analysis. Numbers above branches indicate parsimony bootstrap value/posterior probability. Taxa for which species identity has not been confirmed are marked with an asterisk (*).

Amboinenses, the most species-rich section of subgenus *Polychilos,* is well sampled. There is support for a close relationship between *Phalaenopsis hieroglyphica, P. pulchra,* and *P. lueddemanniana* (posterior probability of 99), which is not surprising, since *P. hieroglyphica* and *P. pulchra* were originally described as varieties of *P. lueddemanniana. Phalaenopsis mariae* and *P. pallens* are sister taxa with *P. bastianii* occurring basally within the group. Sweet (1980) pointed out that *P. mariae* and *P. pallens* have similar lip morphology, and Christenson (2000) noted the probable, close relationship between *P. mariae* and the relatively recently described *P. bastianii.*

Unfortunately, taxon sampling in the sections other than *Amboinenses* is not thorough. Section *Polychilos* is represented by two of the four spe-

cies in that group, *Phalaenopsis mannii* and *P. cornu-cervi,* which occur basal to most of the species in *Amboinenses.* Sections *Fuscatae* is represented by only one of the four species, *P. fuscata,* which occurs in a clade with *P. doweryensis, P. gigantea,* and *P. maculata* that is basal to the rest of subgenus *Polychilos.* Section *Zebrinae* is represented by two of its five species, *P. tetraspis* and *P. inscriptiosenensis,* which are well nested with the species in section *Amboinenses.*

Subgenera *Aphyllae* and *Proboscidioides*

Most of the species in subgenus *Aphyllae* occur as a monophyletic group (Clade 2), except for *Phalaenopsis minus.* In this analysis, *P. minus* occurs, with moderate support (posterior probability 93, bootstrap 53), basal in a group composed of members of subgenus *Parishianae* and subgenus *Phalaenopsis* sections *Esmeralda* and *Deliciosae* (Clade 3). In some trees it falls out in a paraphyletic grade between Clades 3 and 4 (basal to Clade 4). Placement by Christenson (2000) of *P. minus* in subgenus *Aphyllae* was tenuous, and he noted that it "is an oddball of sorts and does not appear to be closely related to the other species in the subgenus." The position of *Phalaenopsis lowii,* of the monotypic subgenus *Proboscidioides,* sister to subgenus *Aphyllae,* is strongly supported in Bayesian and bootstrap analysis (100 and 99, respectively).

Subgenus *Parishianae*

Subgenus *Parishianae* is represented by two of the four species in the group, *Phalaenopsis lobbii* and *P. gibbosa.* It was thought that three species were included, but an individual tentatively identified as *Phalaenopsis parishii* was identified as *P. lobbii.* The fourth species of subgenus *Parishianae, P. appendiculata,* is extremely rare in cultivation and has not been available for sampling. Results strongly support *P. lobbii* as sister to subgenus *Phalaenopsis* section *Deliciosae* and closely related to section *Esmeralda.* Although it occurs in the same clade, these analyses do not support *P. gibbosa* as most closely related to *P. lobbii*; however, the sequences representing *P. gibbosa* are from an individual that has not yet flowered and may be misidentified.

Subgenus *Phalaenopsis*

Subgenus *Phalaenopsis* sections *Phalaenopsis* and *Stauroglottis* form a well-supported monophyletic group and perhaps should be combined to form a subgenus with no sectional delimitations. The species in section *Stauroglottis* (*Phalaenopsis equestris, P. lindenii,* and *P. celebensis*) do not form a monophyletic group; rather they occur as a paraphyletic grade (in the majority rule consensus tree) between the basal species of section *Phalaenopsis* and the more derived species. Support at this branch, however, is low (posterior probability 82 and bootstrap 65), and more data may recover different relationships.

Sections *Esmeralda* and *Deliciosae* do not appear to belong in subgenus *Phalaenopsis.* They occur in a clade with subgenus *Parishianae* and *Phalaenopsis minus.* The relationship of this clade to the other clades in the genus is unclear, but it appears to be more closely related to subgenera *Phalaenopsis* and *Aphyllae* than to *Polychilos.* Support at these nodes is very weak, and more data are necessary to make conclusions as to the relative relationships between any of the clades.

Conclusions

Phalaenopsis is a phylogenetic challenge. The preliminary results presented here provide an initial understanding of the evolutionary relationships in *Phalaenopsis. Phalaenopsis* seems to have diversified relatively recently, especially within the subgenus *Polychilos.* Genetic variation is low, especially in the chloroplast. To improve resolution and branch support, an additional chloroplast marker, *pet*D, consisting of approximately 1400 base pairs, is being added to the data set. Inclusion of a nuclear DNA marker is also desirable. The internal transcribed spacer (ITS) of nuclear ribosomal DNA is the most commonly used nuclear marker for reconstructing infrageneric phylogeny. It is problematic, however, in *Phalaenopsis,* as individuals exhibit multiple, paralogous types that may be phylogenetically misleading or may in some cases indicate hybridization between species. In our search for nuclear markers, we have found several other regions of the nuclear genome that also occur in multiple copies. Additional plants are being sought to complete taxon sampling of *Phalaenopsis* as thoroughly as possible.

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