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ORIGINAL RESEARCH

Phylogenetics and Biogeography of the *Phalaenopsis violacea* (Orchidaceae) Species Complex Based on Nuclear and Plastid DNA

Chi-Chu Tsai · Chiou-Rong Sheue · Chih-Hsiung Chen · Chang-Hung Chou

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Abstract The *Phalaenopsis violacea* complex includes two species: *P. violacea* Witte and *Phalaenopsis bellina* (Rchb.f.) E. A. Christ. However, three forms of *P. violacea* have been found in different areas, including Sumatra, the Malay Peninsula, and Mentawai Island. The phylogenetic tree inferred from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA), the *trn*L intron, and the *atp*B-*rbc*L spacer of plastid DNA were used to clarify the phylogenetics and biogeography of the *P. violacea* complex. Analyses of the *trn*L intron sequences and of the *atp*B-*rbc*L spacer did not allow for apparent discrimination among these three species of the *P. violacea* complex. Based on the phylogenetic tree inferred from the ITS sequence, *P. bellina* cannot be separated from populations of *P. violacea*, with the exception of the population distributed on Mentawai Is., Indonesia. Based on morphological characteristics, *P. violacea* distributed on Mentawai Is. has a long and roundish rachis and is separate from the other groups of the *P. violacea* complex described by Christenson (Timber, Portland, OR, 2001). Therefore, the results of this study show a trend that supports the conclusion that the population of the *P. violacea* complex on Mentawai Is. is a separate species from *P. violacea*. Based on the biogeography of the *P. violacea* complex, Mentawai plants of this complex might be descended from those on the Sumatra/Malay Peninsula.

Keywords *Phalaenopsis violacea* · Species complex · Phylogenetics · Biogeography

C.-C. Tsai
Crop Improvement Division,
Kaohsiung District Agricultural Research and Extension Station,
908, Pingtung, Taiwan

C.-C. Tsai (✉)
Graduate Institute of Biotechnology,
National Pingtung University of Science and Technology,
912, Pingtung, Taiwan
e-mail: tsaicc@mail.kdais.gov.tw

C.-R. Sheue
Department of Life Science, National Chung Hsing University,
402, Taichung, Taiwan

C.-H. Chen
Department of Botany, National Museum of Natural Science,
Taichung 404, Taiwan

C.-H. Chou (✉)
Research Center for Biodiversity, China Medical University,
Taichung 404, Taiwan
e-mail: choumasa@mail.cmu.edu.tw

The genus *Phalaenopsis* Blume (Orchidaceae), a group of beautiful and popular orchids, comprises approximately 66 species according to the latest classification of Christenson (2001), who divided this genus into five subgenera: *Proboscidioides*, *Aphyllae*, *Parishianae*, *Polychilos,* and *Phalaenopsis*. Of these, the subgenus *Polychilos* was subdivided into four sections: *Polychilos*, *Fuscatae*, *Amboinenses*, and *Zebrinae*. In addition, the subgenus *Phalaenopsis* was also subdivided into four sections: *Phalaenopsis*, *Deliciosae*, *Esmeralda*, and *Stauroglottis*.

The *Phalaenopsis violacea* Hort. ex H. Witte species complex was classified in the section *Amboinenses* of the subgenus *Polychilos*, in which 19 species are included according to the classification by Christenson (2001); based on molecular data, these species do not seem to be monophyletic (Tsai et al. 2006a, b, 2010). Within this complex, two species, *P. violacea* and *P. bellina*, were identified by the latest systematic study of the genus

Phalaenopsis. Formerly, plants of *P. bellina* (Rchb.f.) E. A. Christ. were traditionally classified as the "Borneo form" of *P. violacea* Witte based on their similar lip and calli (Kuhn and Kuhn 1965). Furthermore, plants of the *P. violacea* "Borneo form" are only distributed in Borneo and are separated from other plants of this complex, which are distributed in either the Sumatra (*P. violacea* "Sumatra form") or the Malay Peninsula (*P. violacea* "Malay form"). Until an examination of floral fragrance and a review of other morphological differences by Christenson and Whitten (1995), plants of the *P. violacea* "Borneo form" were treated as a separate species, *P. bellina*. The characteristics of *P. bellina* include a purplish inside at the base of the lateral sepals, in contrast to *P. violacea*, which has a rose-pink color over the entire surface of the sepals and petals. Furthermore, the leaf shape of *P. bellina* is broader (generally more than 10 cm wide) than that of *P. violacea* (generally less than 8 cm wide; Christenson and Whitten 1995). Furthermore, the lateral sepals in *P. bellina* are "bow-legged"-like and the apices of the three sepals form an isosceles triangle. In contrast, the lateral sepals of *P. violacea* are not "bow-legged"-like and the apices of the three sepals form an equilateral triangle (Christenson and Whitten 1995).

Plants of *P. violacea* are divided into two different groups, the "Sumatra form" and the "Malay form", based on the geographical distribution of the *P. violacea* species (Masaaki 2002). The author identified these two forms of *P. violacea* based on the difference in flower shape and floral color pattern. In addition, Masaaki (2002) also suggested that the "Sumatra form" of *P. violacea* comes from a natural hybridization between *P. bellina* and the "Malay form" of *P. violacea*. In addition, a distinct population of *P. violacea* can be found on Mentawai Is. (the west coast of Sumatra). Upon further study of the *P. violacea* plants from Mentawai Is., we found that these plants have much longer inflorescences and more roundish rachis than those of the Sumatra and Malay populations (Christenson 2001).

The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) has provided valuable information for determining phylogenetic relationships of *Phalaenopsis* at intra-generic levels (Padolina et al. 2005; Yukawa et al. 2005; Tsai et al. 2006a) and at the species complex level (Tsai et al. 2009). Plastid DNA has also been extensively applied to evolutionary and phylogenetic research (Palmer 1987). Compared to the gene regions of the plastid genome, the intron and intergenic spacer (IGS) region of plastid DNA evolved faster and thus may be more useful for resolving phylogeny at lower taxonomic levels, such as the levels of tribes and genera (Gielly and Taberlet 1994). Taberlet et al. (1991) developed a series of universal primers for several non-coding plastid regions. The *trn*L (UAA) intron, the *trn*L-F (GAA) spacer, and the *atp*B-*rbc*L spacer have been successfully used for the phylogenetic study of *Phalaenopsis* at an intra-generic level (Tsai et al. 2010) and to reveal the natural hybridization of *Phalaenopsis* (Tsai et al. 2006b).

The objective of this study was to elucidate the phylogenetics of the *P. violacea* complex using DNA sequence data, including the ITS region of nrDNA and the *trn*L intron and *atp*B-*rbc*L spacer of plastid DNA. Furthermore, the biogeographic and evolutionary trends of the species complex are discussed based on the molecular data, the geographical distribution, and the historical geology.

Materials and Methods

Plant Materials

The materials of the 13 specimens of the *P. violacea* complex, *P. violacea* and *P. bellina*, were used in this study (Table 1). Two other species of the section *Amboinenses*, *Phalaenopsis amboinensis* and *Phalaenopsis venosa*, were placed in the outgroup of the *P. violacea* complex, according to a previous study of molecular phylogeny in *Phalaenopsis* (Tsai et al. 2006a, b), to clarify the phylogenetics and evolutionary trends of this complex.

DNA Extraction

Total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). The approximate DNA yields were then determined using a spectrophotometer (Hitachi U-2001, Tokyo).

PCR Amplification and Electrophoresis

The primer sets designed to amplify the ITS of nrDNA and the *trn*L intron and *atp*B-*rbc*L spacer of plastid DNA from *Phalaenopsis* plants, as well as the PCR conditions used, can be found in Tsai et al. (2006a) and Tsai et al. (2010), respectively. These PCR products were detected by agarose gel electrophoresis (1.0%, *w*/*v* in TBE), stained by 0.5 μg/mL ethidium bromide, and finally, photographed under UV light exposure.

DNA Recovery and Sequencing

PCR products from the plant materials studied were recovered by glassmilk (BIO 101, California) to sequence directly by the dideoxy chain-termination method using an ABI377 automated sequencer with Bigdye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, California). The sequencing primers were the same as those

Table 1 A list of 13 accessions from two closely related *Phalaenopsis* species, *P. bellina* and *P. violacea*, to their different geographical distributions

Taxa and systematic classification[a]	Distribution	Source/voucher[b]	GenBank accession no.
P. violacea	Malay Peninsula	KDAIS KC-423/C.C. Tsai 1151	AY390227
P. violacea	Malay Peninsula	KDAIS KC-152/C.C. Tsai 1424	AY390228
P. violacea	Malay Peninsula	KDAIS KC-153/C.C. Tsai 1425	AY390229[c]
P. violacea	Sumatra	KDAIS KC-349/C.C. Tsai 1349	AY390230
P. violacea	Sumatra	KDAIS KC-365/C.C. Tsai 1467	AY390231
P. violacea	Sumatra	KDAIS KC-366/C.C. Tsai 1475	AY390232
P. violacea	Mentawai Island	KDAIS KC-367/C.C. Tsai 1053	AY390234
P. violacea	Mentawai Island	KDAIS KC-422/C.C. Tsai 1216	AY390235
P. violacea	Mentawai Island	KDAIS KC-439/C.C. Tsai 1360	AY390236
P. bellina	Borneo	KDAIS KC-67/C.C. Tsai 1067	AY390237
P. bellina	Borneo	KDAIS KC-351/C.C. Tsai 1377	AY390238
P. bellina	Borneo	KDAIS KC-106/C.C. Tsai 1408	AY900289
P. bellina	Borneo	KDAIS KC-107/C.C. Tsai 1409	AY900290[c]

The Genbank accession numbers are included

KDAIS Kaohsiung District Agricultural Improvement Station

[a] The classification of *Phalaenopsis* is based on Christenson (2001)

[b] Voucher specimens were deposited at the herbarium of the National Museum of Natural Science, Taiwan (TNM)

[c] Previously published sequences in Tsai et al. (2006a, b)

used for PCR. These reactions were performed as recommended by the manufacturers.

Data Analyses

The boundaries of the ITS regions (including ITS1, 5.8S rDNA, and ITS2) in the *P. violacea* complex samples were determined by comparison to several published sequences, as described by Tsai et al. (2006a). The sequences were aligned using the program Clustal W Multiple alignment in BioEdit (Hall 1999). Genetic relationships were performed using the program MEGA version 2.1 (Kumar et al. 2001). Genetic distance matrix was calculated by the two-parameter method of Kimura (1980) and was used to construct a tree using the neighbor-joining method (NJ; Saitou and Nei 1987) with interior branch tests of 1,000 replicates (Sitnikova et al. 1995). The sequence alignment was determined using the program Clustal W multiple alignment in BioEdit (Hall 1999). The alignment was then checked, and apparent alignment errors were corrected by hand. Genetic relationships were then determined using the program MEGA version 2.1 (Kumar et al. 2001). The genetic distance matrix was calculated using the two-parameter method of Kimura (1980), and it was then used to construct phylogenetic trees using the neighbor-joining (NJ) method (Saitou and Nei 1987). Maximum likelihood analyses were conducted using the program DNAml DNA Maximum Likelihood in BioEdit (Hall 1999), and the phylogenetic tree is shown using the TREEVIEW program (Page 1996). Maximum parsimony (MP) analyses (Fitch 1971) were performed using code modified from the close-neighbor-interchange (CNI) algorithm (Rzhetsky and Nei 1992) in MEGA version 2.1 (Kumar et al. 2001). Bootstrapping (1,000 replicates) was carried out to estimate the support for both NJ and MP topologies (Felsenstein 1985; Hillis and Bull 1993). The strict consensus parsimonious tree was then constructed using the program MEGA version 2.1 (Kumar et al. 2001).

Results and Discussion

Sequence Characteristics

The accession numbers of the 13 samples of the *P. violacea* complex are shown in Table 1. Sequences of the ITS regions of the 13 accessions of the *P. violacea* complex were aligned, resulting in 669 characters. No gap sites and two variable sites were found in the alignment sequence of the 13 accessions of the *P. violacea* complex (data not shown). The sequence lengths of the ITS regions were shown to be identical among all accessions of this complex. The sequence lengths of ITS1, 5.8S rDNA, and ITS2 were 243, 163, and 263 bp, respectively. Two substitutions were revealed in the ITS1 region among the 13 accessions of the *P. violacea* complex. The percentages of the G+C content across the ITS region of the *P. violacea* complex varied from 72.0% to 72.4%. In addition, the percentage of G+C

content across the ITS2 region from different accessions in the complex was 77.9% in all cases. The genetic distances of ITS1 and ITS2 among the 13 accessions of the *P. violacea* complex ranged between 0.000 and 0.008, with an average of 0.003 (data not shown). Among the accessions of *P. violacea*, the range of genetic distances was also between 0.000 and 0.008, with an average of 0.003. There were no genetic differences among any of the accessions of *P. bellina*. Excluding the accessions collected from Mentawai Is., the genetic difference between the accessions of *P. violacea* and those of *P. bellina* was shown to be 0.000. For accessions of the *P. violacea* complex, only accessions of *P. violacea* collected from Mentawai Is. showed a genetic distance of 0.007 compared to the other studied specimens of this complex (data not shown).

The sequence lengths of the *trn*L introns from the 13 accessions of the *P. violacea* complex ranged from 555 to 588 bp. These sequences were aligned, resulting in 588 characters. However, we did not find base substitutions in this region (data not shown). In contrast, a mutational hot spot of length variations was found in the sequences of the *trn*L introns from the *P. violacea* complex. This type of hot spot region can also be found in other *Phalaenopsis* species (Tsai et al. 2010). This hot spot region was highly enriched in A+. Several reports have indicated that AT-rich sequences were also found in hot spot regions of plastid DNA (Ogihara et al. 1991, 1992). In the study, length variations of the *trn*L intron mainly come from two types of long insertions/deletions (indels), TCTATTAATATTAT and TAATATTATATT (Fig. 1). In fact, the hot spot region as related to length mutations within plastid DNA has been described in several reports (Tassopulu and Kung 1984; Ogihara et al. 1991; Guo and Terachi 2005).

Within the hot spot region of the *trn*L intron of the 13 specimens of the *P. violacea* complex, four types of length variation were found. Accessions of both *P. bellina* and *P. violacea* from Mentawai Is. had the same DNA length variation, and all the accessions of *P. violacea* from Sumatra had the same DNA length variation, while the regions of *P. violacea* from Malay were highly variable. Each of the accessions had a different DNA length (Fig. 1). These results indicate that there is no constituent rule in the hot spot region of the sequences of the *trn*L intron of plastid DNA among species/forms of the *P. violacea* complex. In the sequences of the *atp*B-*rbc*L spacer, the sequence length and base pairs of the sequences of the *atp*B-*rbc*L spacer obtained from the 13 accessions of the *P. violacea* complex were shown to be identical (689 bp; data not shown). In the analysis of the sequences of the *trn*L intron, there was no genetic distance between the 13 accessions of the *P. violacea* complex due to the lack of any substitutions among those sequences. In the analysis of sequences of the *atp*B-*rbc*L spacer in the *P. violacea* complex, there was also no genetic distance among the 13 accessions of the *P. violacea* complex due to identical sequences among all samples (data not shown).

Phylogenetic Reconstructions

Molecular data for ITS1, 5.8S rDNA, ITS2 of nrDNA, the *trn*L intron, and the *atp*B-*rbc*L spacer were examined for the *P. violacea* complex. Only the ITS1 of nrDNA contains valuable information for identifying the accessions of the *P. violacea* complex. The phylogenetic tree inferred from the ITS of nrDNA to reconstructed following the NJ and ML method is shown in Figs. 2 and 3. Based on the MP method, the analysis yielded 210 equally parsimonious trees with a length of 23 steps, a consistency index (CI) of 1.0 and a retention index (RI) of 1.0. The strict consensus tree is shown in Fig. 4. Based on phylogenetic trees, only the Mentawai population of *P. violacea* was shown to be monophyletic, which suggests a single origin for the insular

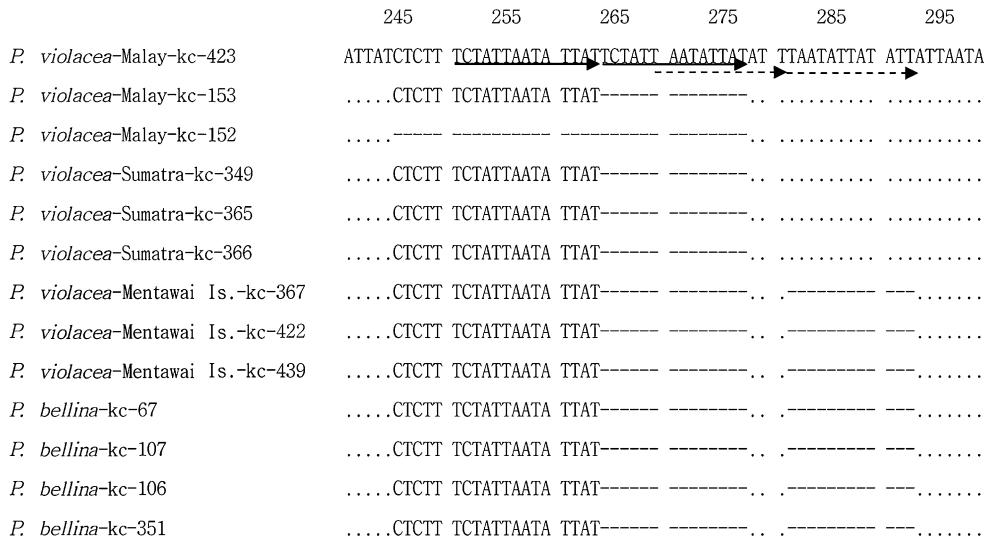

Fig. 1 The mutational hot spot of length variations within the *trn*L intron of chloroplast DNA from the *P. violacea* complex. *Arrows* represent repeat sequences within the hot spot region

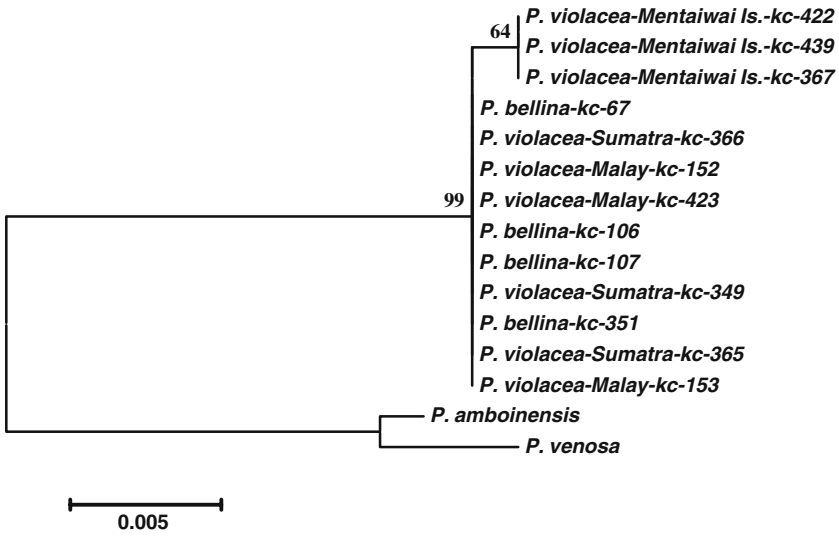

Fig. 2 The neighbor-joining tree of the 13 accessions of the *Phalaenopsis violacea* species complex plus two outgroups, *P. amboinensis* and *P. venosa*, obtained from sequence comparisons of the ITS region of rDNA. *Numbers above internodes* indicate values of a bootstrap test from 1,000 replicates. More than 50% of interior branch test is shown on each branch. Branch lengths are proportional to the number of base changes along each branch

population. This Mentawai population is nested within *P. bellina* and the Malay and Sumatra accessions of *P. violacea*, and thus, the insular plants may have derived from any of those populations.

All of the sequences of the *atp*B-*rbc*L spacer from the *P. violacea* complex were identical. Therefore, the sequences of the *atp*B-*rbc*L spacer do not aid in reconstructing the phylogenetics of the *P. violacea* complex. Although the mutational hot spot of length variations of the *trn*L introns from the *P. violacea* complex were highly variable, base substitution of the regions among accessions of the *P. violacea* complex cannot be found. In addition, these length variations cannot offer valuable information for identifying the accessions of the *P. violacea* complex, as they did not have consistent deletions/insertions among the populations/species of the *P. violacea* complex. In particular, each of the accessions of *P. violacea* distributed in the Malay Peninsula has different lengths of the deletions/insertions (Fig. 1). Therefore, information based on the *trn*L intron also does not contribute towards constructing the phylogenetic tree of the *P. violacea* complex.

Although the floral fragrance and the leaf shape have been shown to be different between *P. violacea* and *P. bellina* (Christenson and Whitten 1995), these two species still have a close relationship based on morphological characteristics, as described by Kuhn and Kuhn (1965). Therefore, *P. violacea* and *P. bellina* were suggested that they were of more recent origin (Kuhn and Kuhn 1965). The molecular data analyzed in this study are also unable to discriminate between these two species. The results support both species of *P. bellina* and *P. violacea* having close relationship. Among the specimens of *P. violacea*, plants of the Sumatra and Malay forms could not be separated based on the ITS sequences of nrDNA. This result did not support the separation of these two forms of *P. violacea* as described by Masaaki (2002). In fact, Christenson (2001)

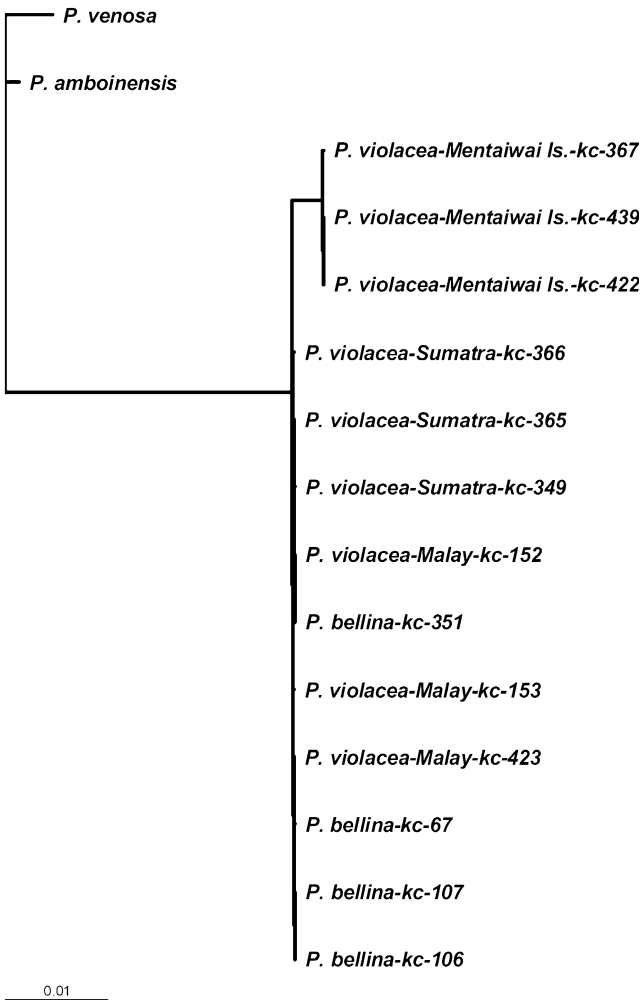

Fig. 3 Maximum likelihood tree of the 13 accessions of the *Phalaenopsis violacea* species complex plus two outgroups, *P. amboinensis* and *P. venosa*, obtained from sequence comparisons of the ITS region of rDNA

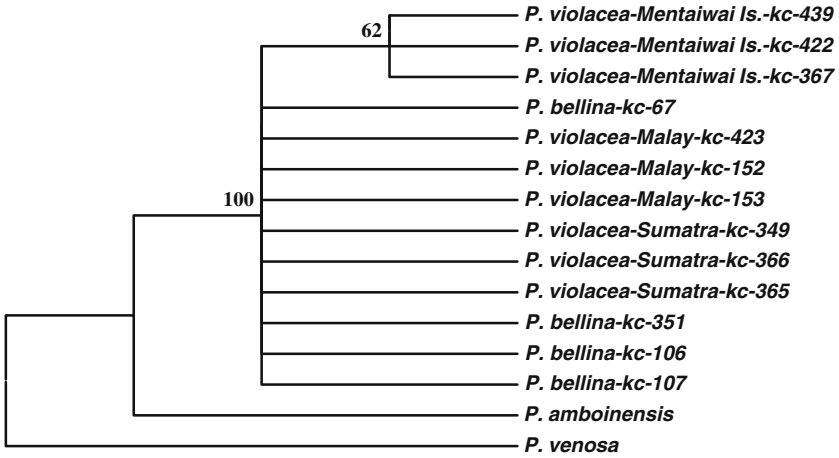

Fig. 4 The strict consensus tree of parsimony of the 13 accessions of the *Phalaenopsis violacea* species complex plus two outgroups, *P. amboinensis* and *P. venosa*, obtained from sequence comparisons of the ITS region of rDNA. *Numbers above internodes* indicate values of a bootstrap test from 1,000 replicates. More than 50% of interior branch test is shown on each branch

also did not accept the separation of these two forms of *P. violacea*.

Currently, the population of Mentawai Is. is still treated as *P. violacea* (Christenson 2001). However, this population is unique and separate from the other specimens of this complex based on the ITS sequence of nrDNA using NJ, MP, and ML trees. Furthermore, the Mentawai plants of the *P. violacea* complex have much longer inflorescences (to ca. 50 cm), and they lack a somewhat flattened instead of a roundish rachis. In contrast, both the "Sumatra form" and the "Malay form" of *P. violacea* as well as plants of *P. bellina* bear shorter (shorter than the leaves, usually less than 15 cm) and much more flattened inflorescences. In addition, the flowers of the Mentawai plants are larger, fuller in shape, and more brilliant in color than the other plants of *P. violacea* (Christenson 2001). Therefore, plants of the *P. violacea* group from Mentawai Is. showed characteristics that were unique in the *P. violacea* complex based on molecular and morphological data. Because the founder effect and the bottleneck effect can easily take place on small isolated islands, this could allow island plants to evolve uniquely (Tamarin and Leavitt 1991) and could thus explain why the *P. violacea* distributed on Mentawai Is. is unique among all *P. violacea*.

Our morphological and molecular evidence suggest that the population of the *P. violacea* complex that is found on Mentawai Is. should be treated as a separate species. Several morphological characteristics that are different between Mentawai plants and the other plants of the *P. violacea* complex have been described (Christenson 2001).

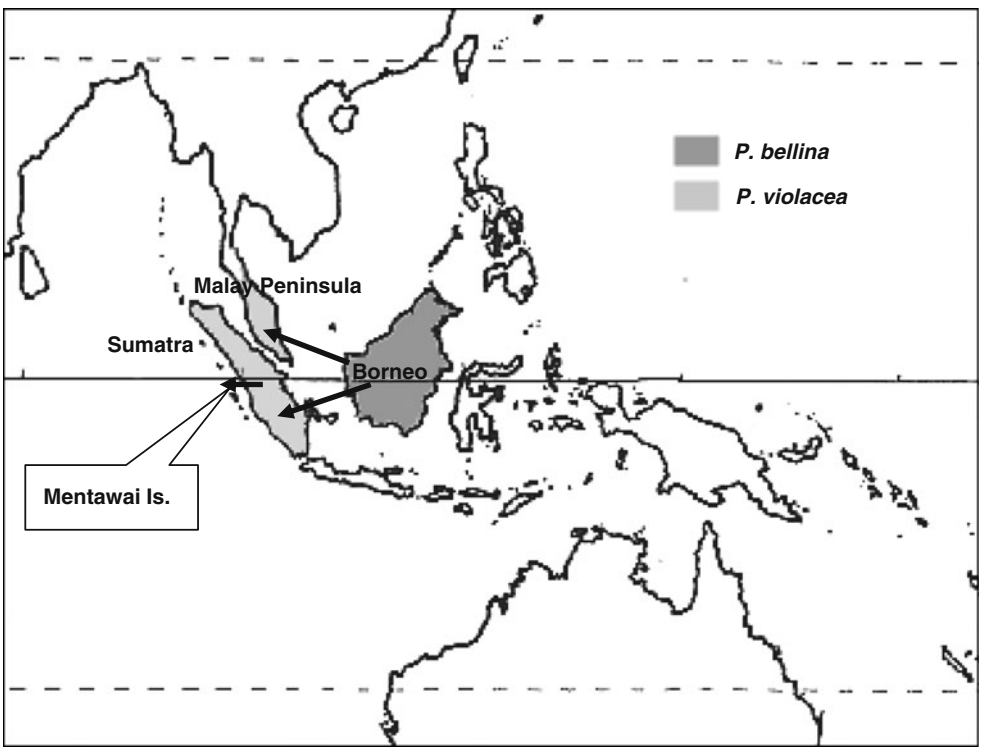

Fig. 5 Evolutionary trends of *P. violacea* complex based on the phylogenetic tree

The molecular data derived from this study are in agreement with that as well. Recognition of the Mentawai population as a distinct species is consistent with the taxonomy of the *P. violacea* complex in terms of recognition of *P. bellina*. *P. bellina* distributed in Borneo has been treated as a separate species based on floral fragrance and some morphological differences (Christenson and Whitten 1995), although our molecular data and RAPD markers (Niknejad et al. 2009) do not have high enough resolution to support this *P. bellina* classification. However, other RAPD markers (Goh et al. 2005; Taywiya et al. 2008) support the classification described by Christenson and Whitten (1995).

According to the geographical distribution of the *P. violacea* complex, Mentawai Is. is closer to Sumatra than to Malay Peninsula/Borneo. Thus, there is a high chance for dispersal of *P. violacea* plants between Mentawai Is. and Sumatra. Based on the phylogenetic tree of the *P. violacea* complex inferred from the ITS sequences of nrDNA, Mentawai *P. violacea* plants are a derived population. Therefore, Mentawai plants of the *P. violacea* complex are likely derived from Sumatra based on the phylogenetic tree to biogeography. In addition, plants of the *P. violacea* complex in Sumatra/Malay Peninsula/Borneo are relative origin regions, according to the phylogenetic trees (Figs. 2, 3, and 4). However, it is uncertain which region is the original distribution site of the *P. violacea* complex because differences among plants of the *P. violacea* complex, with the exception of the Mentawai population, cannot be further resolved in this study.

Biogeography and Evolutionary Trends

Evolutionary trends of the *P. violacea* complex were deduced based on the phylogenetic tree (Fig. 5). Apparently, these two species share a common ancestor. Based on the phylogenetic tree inferred from the ITS of nrDNA, the evolutionary trends of the *P. violacea* complex were deduced. Because specimens of *P. bellina/P. violacea* distributed in Borneo/Sumatra were located as the basal group within the *P. vioalacea* complex, *P. bellina/P. violacea* distributed in Borneo/Sumatra were suggested to be the relative ancestral group of the *P. violacea* complex. Except for species distributed in the Philippines, Borneo is the origin of species diversity for the section *Amboinenses* (Christenson 2001). Therefore, *P. bellina* distributed in Borneo is the relative ancestral group for the species complex. *P. violacea* distributed in Sumatra, the Malay Peninsula, or Mentawai Is. might have evolved from *P. bellina* during glacial periods because the land bridge between Mindoro, Palawan, and Borneo may have become the Sunda Shelf during the Pleistocene times (about 0.01 to 1.8 Mya; van Oosterzee 1997). According to the molecular data from the *trn*L intron (Fig. 1), sequences from the Mentawai populations are identical with those of *P. bellina* and separate from all others. Therefore, plants of the Mentawai population might be derived from plants of *P. bellina*. We thus suggest that plants of the *P. violacea* complex were distributed across the Sunda Shelf during the Pleistocene. Based on the molecular and morphological data, *P. violacea* from Mentawai Is. is clearly separated from *P. violacea* distributed in Sumatra/Malay Peninsula, to the dispersal event that occurred on Mentawai Is. Following the postglacial sea level rise, we would expect an isolation of different populations of the *P. violacea* complex. The small island population in Mentawai Is. began with only a few individuals. Thus, genetic drift occurred as a result of the founder effect (Templeton 1979). This makes *P. violacea* distributed on Mentawai Is. highly unique both morphologically and by DNA analysis. In fact, the high efficiency of endemics in Mentawai Is., approximately 65% of animals and 15% of plant species, has been demonstrated (Mitchell 1982). Therefore, species on the island are likely to have evolved quickly because of the dynamic evolutionary events that occurred on the island and the Sunda Shelf during the Pleistocene times.

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