Phylogeny and Historical Biogeography of *Paphiopedilum* Pfitzer (Orchidaceae) Based on Nuclear and Plastid DNA

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The phylogeny and biogeography of the genus *Paphiopedilum* were evaluated by using phylogenetic trees derived from analysis of nuclear ribosomal internal transcribed spacer (ITS) sequences, the plastid *trnL* intron, the *trnL*-F spacer, and the *atpB*-rbcL spacer. This genus was divided into three subgenera: *Parvisepalum*, *Brachypetalum*, and *Paphiopedilum*. Each of them is monophyletic with high bootstrap supports according to the highly resolved phylogenetic tree reconstructed by combined sequences. There are five sections within the subgenus *Paphiopedilum*, including *Coryopedilum*, *Pardalopetalum*, *Cochlopetalum*, *Paphiopedilum*, and *Barbata*. The subgenus *Parvisepalum* is phylogenetic basal, which suggesting that *Parvisepalum* is comprising more ancestral characters than other subgenera. The evolutionary trend of genus *Paphiopedilum* was deduced based on the maximum likelihood (ML) tree and Bayesian Evolutionary Analysis Sampling Trees (BEAST). Reconstruct Ancestral State in Phylogenies (RASP) analyses based on the combined sequence data. The biogeographic analysis indicates that *Paphiopedilum* species were firstly derived in Southern China and Southeast Asia, subsequently dispersed into the Southeast Asian archipelagoes. The subgenera *Paphiopedilum* was likely derived after these historical dispersals and vicariance events. Our research reveals the relevance of the differentiation of *Paphiopedilum* in Southeast Asia and geological history. Moreover, the biogeographic analysis explains that the significant evolutionary hotspots of these orchids in the Sundaland and Wallacea might be attributed to repeated migration and isolation events between the south-eastern Asia mainland and the Sunda Super Islands.

**Keywords:** *Paphiopedilum*, molecular phylogeny, biogeography, evolutionary trend, dispersal events
INTRODUCTION

The orchid genus *Paphiopedilum* Pfitzer belongs to the subfamily Cypripedioideae Lindley. This subfamily has been considered a distinct lineage since Lindley (1840) separated them from other orchids based on the characteristic of having two separated fertile anthers [see (Cribb, 1998)]. This subfamily includes only five genera: *Cypripedium*, *Mexipedium*, *Paphiopedilum*, *Phragmipedium*, and *Selenipedium*. *Mexipedium* and *Selenipedium* are monotypic genera (Albert and Chase, 1992), which was a finding supported by ITS sequence analysis (Cox et al., 1997). These five genera are distributed in separate and restricted geographical ranges (Cribb, 1998).

*Paphiopedilum* is distinguished from genera *Cypripedium* and *Selenipedium* by its conduplicate coriaceous leaves, as opposed to the plicate persistent leaves of the latter two genera. Furthermore, *Paphiopedilum* differs from *Phragmipedium* and *Mexipedium*, as they display imbricate sepal vernation, different chromosome base numbers and a unilocular ovary (Albert and Chase, 1992; Albert, 1994).

The systematics of the genus *Paphiopedilum* proposed by Cribb (1997b) are largely consistent with Atwood (1984), except that Cribb placed the *Parvisepalum* group within subgenus *Brachypetalum*. Cribb (1997b) accepted the suggestion of Karasawa (1982) and Karasawa and Saito (1982) to promote the *Parvisepalum* group (e.g., *Parvisepalum delenatii*, *Parvisepalum armeniacum*, *Parvisepalum malipoense*, and *Parvisepalum emersonii*) to the subgeneric rank, since the two relatively new species (i.e., *P. malipoense* and *P. emersonii*) found in this group have been described. According to the classification of Cribb (1997b), the genus *Paphiopedilum* comprised of approximately 69 species worldwide. Cribb divided this genus into three subgenera, *Parvisepalum*, *Brachypetalum*, and *Paphiopedilum*, which are mainly based on the morphological characteristics of flower inflorescence, leaf type, floral morphology, and molecular data on ITS sequences (Cox et al., 1997). Recently, several new species and treatment have been described for this genus. The genus *Paphiopedilum* was described as containing approximately 98 species worldwide by the year 2000 (Koopowitz, 2000). In this genus, *Paphiopedilum* was divided into five sections: *Coryopedilum*, *Pardalopetalum*, *Cochlopetalum*, *Paphiopedilum*, and *Barbata*. Subgenera of the genus *Paphiopedilum* distribute in distinct geographic regions (Cribb, 1997b). The subgenera *Parvisepalum* and *Brachypetalum*, as well as the section *Paphiopedilum* of the subgenus *Paphiopedilum*, are found only in mainland Asia. The *Parvisepalum* subgenus is concentrated in southern China and Vietnam, the subgenus *Brachypetalum* is mostly found in Thailand (*Figure 1*). Among the subgenus *Paphiopedilum*, *Paphiopedilum* ranges from India to southern China, Thailand and Indo-China, and the species diversity found in southern China was the most concentrated. The section *Cochlopetalum* is restricted to the islands of Sumatra and Java. The section *Pardalopetalum* is widespread in Southeast Asia, the Malay Archipelago, as far east as Sulawesi, and Luzon in the Philippines. The enormous species diversity of the section *Coryopedilum* locates in Borneo, and this section range from the Philippines to Sulawesi in New Guinea. The section *Barbata* is widespread from eastern Nepal, across to Hong Kong and the Philippines, south to the Malay Archipelago, New Guinea, and the Solomon Islands (Cribb, 1998).

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**FIGURE 1** | Map of the geographical distribution of *Paphiopedilum* based on the phylogeny of Cribb (1998). Comparison of Southeast Asian landmasses between the Pleistocene era and the present. During the Pleistocene, Indochina, Malaya, Sumatra, Java, Borneo, and the Philippines were interconnected and were separated from Sulawesi by the Makassar Strait.
Plant Materials

Seventy-eight taxa of *Paphiopedilum* and two outgroups from genus *Phragmipedium* were used in this study (Table 1). All leaf materials were taken from living plants in the greenhouse of the Kaohsiung District Agricultural Improvement Station (KDAIS) in Taiwan.

DNA Extraction, PCR Amplification, and Sequencing

Total DNA was extracted from fresh etiolated leaves by using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). Approximate DNA yields were determined by using the spectrophotometer (model U-2001, Hitachi).

The PCR reaction was used to amplify nuclear ribosomal ITS sequence and chloroplast (cp) DNA fragments *trnL* intron and the *trnL*-*trnF* spacer, *atpB*-rbcL spacer. ITS primers were designed from conserved regions of the 3’ end of the 18S rRNA gene and the 5’end of the 26S rRNA gene using sequences from different species present in GenBank. Universal primers for *trnL* intron and the *trnL*-*trnF* spacer were referenced from Taberlet et al. (1991). Primer sequences for amplifying of the *atpB*-rbcL spacer were designed from the conserved regions of the 3’end of the *atpB* gene and the 5’end of the rbcL gene of chloroplast DNA using sequences of different species obtained from GenBank. Detailed amplification conditions and primer sequences are given in Supplementary Table S1. All PCR products were separated by agarose gel electrophoresis (1.0%, w/v in TBE) and were recovered using glassmilk (BIO 101, California).

PCR products were directly sequenced using the dideoxy chain-termination method on an ABI377 automated sequencer with the Ready Reaction Kit (PE Biosystems, California) of the BigDye™ Terminator Cycle Sequencing. The PCR reaction primer sequences were used as sequencing primers. Each sample was sequenced two or three times to confirm the sequences. Reactions were performed as recommended by the product manufacturers.

Sequence Alignment and Phylogenetic Reconstruction

The sequence alignment was determined using the MUSCLE multiple alignment program in BioEdit (Hall, 1999), and four regions were combined for the following analysis. The alignment was checked, and apparent alignment errors were corrected by hand. Indels (insertion/deletions) were treated as missing data. For phylogenetic reconstruction, two *Phragmipedium* taxa treating as outgroups were sequenced to resolve whether all ingroup taxa formed a monophyletic lineage. The best-fitting substitution model was selected (Supplementary Table S2) by a model test using the model testing tool MEGA 6.0 (Tamura et al., 2013). Tamura 3-parameter model (T92) using a discrete Gamma distribution (+G) was selected for following neighbor-joining (NJ) phylogenetic reconstruction. The general time reversible (GTR) using a discrete gamma distribution (+G) and considering the proportion of invariable sites (+I) were chosen for following divergence time estimation using the Yule model methods in BEAST 1.8.0 (Drummond and Rambaut, 2007; Drummond et al., 2012). The phylogenetic tree for the combined multiple sequence datasets used equally weighted characters. Moreover, because the sequence data of the four genera (*Mexipedium*, *Selenipedium*, *Cypripedium*, and *Goodyera*) in NCBI is limited, only two sets of fragment data (ITS: *Mexipedium xerophyticum*-MK161260.1; *Selenipedium aequinoctiale*-JF825977.1; *Cypripedium macranthos*-KT338684.1; *Goodyera procera*-MK451741.1and *trnL*-*trnF* spacer: *Mexipedium xerophyticum*-FR851215.1; *Selenipedium aequinoctiale*-JF825975.1; *Cypripedium macranthos*-JF797026.1; *Goodyera procera*-MK451782.1) are used as an additional analysis and compared with the data using only genus *Phragmipedium* as outgroup. The results of six outgroups are showed in Supplementary Data.

**MATERIALS AND METHODS**

**Plant Materials**

*Paphiopedilum* is a genus of tropical Asiatic origin, and its range extends eastward, reaching the Philippines, Southeast Asia, Borneo, and the Malay Archipelago, crossing Wallace's Line into Sulawesi, the Moluccas, New Guinea, and the Solomon Islands (Cribb, 1998). Tracking back to the geological history of Southeast Asian, the Palawan, Mindoro, Zamboanga, and the adjacent small islands are the older islands of the Southern Philippines. These regions are located on the border of the Eurasian Plate and have been shifting away from the mainland mass by tectonic collision since the early Miocene (~30 Mya) and the shell of the older plate was merged to Borneo until 5~10 Mya (Karig et al., 1986; Stephan et al., 1986; Hall, 1996). In contrast, most of the Philippine islands formed less than 5 Mya (Aurelio et al., 1991; Quebral et al., 1994). In addition, the Sundaland was comprised of the Malay Peninsula, Sumatra, Java, Sulawesi, and Borneo and merged with Bali, the Philippines, and even New Guinea/Australia into Sunda Superland interconnecting by land bridge during the last glacial period (0.01~1.8 Mya) (van Oosterzee, 1997). Since the last glacial period, species migrated forward and backwards between these regions and isolated after the last glacial maximum (LMG), causing the broken of Sunda Superland (Tsai et al., 2015).

The chloroplast primers for the *atpB*-rbcL, *trnL*-*trnF* spacer, and *trnL* intron are useful for phylogenetic studies at the intrageneric level. The primers for the *trnL*-*trnF* spacer and *trnL* intron developed by Taberlet et al. (1991) have been applied for inferring phylogenies at the intrageneric level (Goldblatt et al., 2002; Hodkinson et al., 2002; Mogensen, 1996; Van Raamsdonk et al., 2003), and have also been used successfully on Orchidaceae (Tsai et al., 2012). The *atpB*-rbcL regions are high length differences due to frequent occurrence of indels and are often used in combination with other primers to provide more information (Yoshinaga et al., 1992; Chiang et al., 1998; Von Konrat et al., 2010). Therefore, this study aims to further elucidate the phylogeny of *Paphiopedilum* through analysis ITS (internal transcribed spacer) sequences and three non-coding plastid DNA sequences (*trnL* intron, *trnL*-F, and *atpB*-rbcL spacers). In addition, the biogeography of this genus is clarified based on the phylogenetic tree derived from the molecular evidence.
## TABLE 1 | Names of specimens, geographical distribution, source, and GenBank accession numbers for sequences of the internal transcribed spacer (ITS) of ribosomal DNA (rDNA), the plastid trnL intron, the trnL-F spacer, and the atpB-rbcL spacer.

| Taxa and systematic classification | Geographical distribution | Voucher a | GenBank accession no. |
|-----------------------------------|---------------------------|-----------|-----------------------|
|                                   |                           |           | ITS                   |
|                                   |                           |           | trnL intron           |
|                                   |                           |           | trnL-F spacer         |
|                                   |                           |           | atpB-rbcL spacer      |
| **Genus Paphiopedilum**           |                           |           |                       |
| **Subgenus Parvisepalum**         |                           |           |                       |
| Paphiopedilum armeniacum S.C. Chen & F.Y. Liu | Southwest China | C. C. Tsai 2021 | EF156086 EF156001 EF156171 GQ850803 |
| Paphiopedilum delenatii Guill.    | Vietnam                   | C. C. Tsai 2073 | EF156096 EF156011 EF156181 GQ850813 |
| Paphiopedilum emersonii Koop. & P.J. Cribb | China                  | C. C. Tsai 2351 | EF156099 EF156104 EF156184 GQ850816 |
| Paphiopedilum hangianum Perner & Gruss | China                   | C. C. Tsai 2201 | EF156109 EF156024 EF156194 GQ850826 |
| Paphiopedilum jackii H.S. Hua     | China, Vietnam            | C. C. Tsai 2330 | EF156118 EF156033 EF156203 GQ850832 |
| Paphiopedilum malipoense S.C. Chen & Z.H. Tsi | China, Vietnam         | C. C. Tsai 2024 | EF156125 EF156040 EF156210 GQ850839 |
| Paphiopedilum micranthum var. eburneum Fowlie | China                   | No voucher | EF156127 EF156042 EF156212 GQ850841 |
| Paphiopedilum niveum (Rchb.f.) Stein | Southern Thailand, Malay peninsula | C. C. Tsai 2039 | EF156130 EF156045 EF156215 GQ850844 |
| **Subgenus Brachypetalum**        |                           |           |                       |
| Paphiopedilum concolor (Bateman) Pfitzer | China, Burma, Thailand, Laos, Vietnam | C. C. Tsai 2307 | EF156093 EF156008 EF156178 GQ850810 |
| Paphiopedilum godefroyae (God.-Leb.) Stein | Thailand               | C. C. Tsai 2321 | EF156107 EF156022 EF156192 GQ850824 |
| Paphiopedilum godefroyae var. leucochilum (Masters) Hallier | Thailand | C. C. Tsai 2031 | EF156106 EF156021 EF156191 GQ850823 |
| Paphiopedilum niveum (Rchb.f.) Stein | Northern Borneo, Philippines | C. C. Tsai 2007 | EF156142 EF156050 EF156220 GQ850848 |
| **Subgenus Paphiopedilum**        |                           |           |                       |
| **Section Coryopedilum**          |                           |           |                       |
| Paphiopedilum adductum Asher      | Philippines              | C. C. Tsai 2025 | EF156082 EF155997 EF156167 GQ850799 |
| Paphiopedilum anitum Golamco      | Philippines              | C. C. Tsai 2295 | EF156083 EF155998 EF156168 GQ850800 |
| Paphiopedilum gigantifolium Braem, M.L. Baker & C.O. Baker | Sulawesi | No voucher | EF156103 EF156018 EF156188 GQ850821 |
| Paphiopedilum klopatingsi Fowlie  | Borneo                   | C. C. Tsai 2057 | EF156121 EF156036 EF156206 GQ850835 |
| Paphiopedilum ooil Koopowitz      | Borneo                   | No voucher | EF156138 EF156046 EF156216 GQ850845 |
| Paphiopedilum philippinense (Rchb.f.) Stein | Northeast Borneo, Philippines | C. C. Tsai 2007 | EF156142 EF156050 EF156220 GQ850848 |
| Paphiopedilum glanduliferum (Blume) Stein | New Guinea              | C. C. Tsai 2040 | EF156104 EF156019 EF156189 GQ850822 |
| Paphiopedilum randaii fowlie      | Mindanao, Philippines    | C. C. Tsai 2297 | EF156132 EF156053 EF156223 GQ850851 |
| Paphiopedilum rothschildianum (Rchb.f.) Stein | Borneo                  | C. C. Tsai 2249 | EF156135 EF156056 EF156226 GQ850853 |
| Paphiopedilum sandermanianum (Rchb.f.) Stein | Borneo                  | C. C. Tsai 2309 | EF156136 EF156057 EF156227 GQ850854 |
| Paphiopedilum stonei (Hook.) Stein | Borneo                   | C. C. Tsai 2310 | EF156146 EF156061 EF156231 GQ850858 |
| Paphiopedilum suparidae Braem & Loeb | Borneo                  | C. C. Tsai 2189 | GQ505309 GQ505312 GQ505315 GQ505316 GQ850860 |
| Paphiopedilum wilhelminae L.O. Williams | New Guinea              | C. C. Tsai 2205 | GQ505310 GQ505313 GQ505316 GQ850875 |
| **Section Pardalopetalum**        |                           |           |                       |
| Paphiopedilum dianthum T. Tang & F.T. Wang | China                  | C. C. Tsai 2085 | EF156097 EF156012 EF156182 GQ850814 |
| Paphiopedilum haynaldianum (Rchb.f.) Stein | Philippines            | No voucher | EF156110 EF156025 EF156195 GQ850827 |
| Paphiopedilum lowii (Lindl.) Stein | Peninsular Malaysia, Sumatra, Borneo, Sulawesi | C. C. Tsai 2285 | EF156124 EF156039 EF156209 GQ850838 |
| Paphiopedilum parishii (Rchb.f.) Stein | Southwest China, Burma, Thailand | C. C. Tsai 2276 | EF156140 EF156048 EF156218 GQ850847 |
| Paphiopedilum richardianum Asher & Beaman | Sulawesi               | C. C. Tsai 2068 | EF156133 EF156054 EF156224 GQ850852 |
| **Section Cochlopetalum**         |                           |           |                       |
| Paphiopedilum victoria-regina (Sander) M.W. Wood | Sumatra             | C. C. Tsai 2045 | EF156157 EF156072 EF156242 GQ850870 |

(Continued)
| Taxa and systematic classification a | Geographical distribution | Voucher b | GenBank accession no. |
|-----------------------------------|---------------------------|-----------|----------------------|
|                                   |                           |           | ITS                  |
|                                   |                           |           | trnL intron          |
|                                   |                           |           | trnL-F spacer        |
|                                   |                           |           | atpB-rbcL spacer     |

### Paphiopedilum

**Section Paphiopedilum**

- **Paphiopedilum barbigerum** Tang & Wang
  - China, northern Vietnam
  - C. C. Tsai 2023
  - EF156088
  - EF156003
  - EF156173
  - GQ850805

- **Paphiopedilum charlesworthii** (Rolfe) Pfitzer
  - Burma, northern Thailand, southwest China
  - C. C. Tsai 2192
  - EF156091
  - EF156006
  - EF156178
  - GQ850808

- **Paphiopedilum druryi** (Bedd.) Stein
  - Southern India
  - C. C. Tsai 2079
  - EF156102
  - EF156017
  - EF156187
  - GQ850819

- **Paphiopedilum exul** (Ridl.) Rolfe
  - Peninsular Thailand
  - C. C. Tsai 2155
  - EF156108
  - EF156023
  - EF156193
  - GQ850825

- **Paphiopedilum esquirolei** Schlr. China, India, Bhutan (Southeast Asia)
  - Laos, Vietnam
  - C. C. Tsai 2229
  - EF156145
  - EF156060
  - EF156230
  - GQ850857

- **Paphiopedilum fairrieanum** (Lindl.) Stein
  - India, Burma, Thailand (Southeast Asia)
  - C. C. Tsai 2216
  - EF156159
  - EF156074
  - EF156244
  - GQ850872

### Section Barbata

- **Paphiopedilum acmodontum** Schoser ex M.W. Wood
  - Philippines
  - C. C. Tsai 2094
  - EF156081
  - EF155996
  - EF156166
  - GQ850879

- **Paphiopedilum argentatum** (Gower) Rolfe
  - China, Thailand, Cambodia, Laos, Vietnam (Southeast Asia)
  - C. C. Tsai 2153
  - EF156084
  - EF155999
  - EF156169
  - GQ850801

- **Paphiopedilum braecki** (Rchb.f.) Stein
  - Northern Sumatra, Indonesia
  - C. C. Tsai 2151
  - EF156089
  - EF156004
  - EF156174
  - GQ850806

- **Paphiopedilum barbatum** (Lindl.) Pfitzer
  - Southern Thailand, peninsular Malaysia, Sumatra
  - C. C. Tsai 2227
  - EF156087
  - EF156002
  - EF156172
  - GQ850804

- **Paphiopedilum calliophyllum** (Rchb.f.) Stein
  - Thailand, Cambodia, Laos, Vietnam (south-east Asia)
  - C. C. Tsai 2267
  - EF156090
  - EF156005
  - EF156175
  - GQ850807

- **Paphiopedilum ciliolare** (Rchb.f.) Stein
  - Philippines
  - C. C. Tsai 2078
  - EF156092
  - EF156007
  - EF156177
  - GQ850809

- **Paphiopedilum curtisii** (Rchb.f.) Stein
  - Sumatra
  - C. C. Tsai 2107
  - EF156094
  - EF156009
  - EF156179
  - GQ850811

- **Paphiopedilum dayanum** (Lindl.) Stein
  - Borneo
  - C. C. Tsai 2280
  - EF156095
  - EF156010
  - EF156180
  - GQ850812

- **Paphiopedilum foetidum** Birk
  - No voucher
  - GQ505311
  - GQ505314
  - GQ505317
  - GQ850820

- **Paphiopedilum hookeri** (Rchb.f.) Stein
  - Borneo
  - C. C. Tsai 2089
  - EF156116
  - EF156031
  - EF156201
  - GQ850831

- **Paphiopedilum volutatum** (Sander) Stein
  - No voucher
  - EF156115
  - EF156030
  - EF156200
  - GQ850873

- **Paphiopedilum javanicum** (Reinw. ex Lindl.) Pfitzer
  - Borneo, southeast Sumatra, Java
  - C. C. Tsai 2326
  - EF156120
  - EF156035
  - EF156205
  - GQ850834

- **Paphiopedilum javanicum var. viridiflorum** (Rchb.f.) Stein
  - North Borneo
  - No voucher
  - EF156119
  - EF156034
  - EF156204
  - GQ850833

- **Paphiopedilum lawrenceanum** (Rchb.f.) Stein
  - Borneo
  - C. C. Tsai 2013
  - EF156122
  - EF156037
  - EF156207
  - GQ850836

- **Paphiopedilum mastersianum** (Rchb.f.) Stein
  - Moluccas
  - C. C. Tsai 2341
  - EF156126
  - EF156041
  - EF156211
  - GQ850840

- **Paphiopedilum papuanum** (Ridl.) Ridl.
  - New Guinea
  - No voucher
  - EF156139
  - EF156047
  - EF156217
  - GQ850846

- **Paphiopedilum purpuratum** (Lindl.) Stein
  - China, Vietnam
  - C. C. Tsai 2049
  - EF156131
  - EF156052
  - EF156222
  - GQ850850

- **Paphiopedilum sangii** Braem
  - Sulawesi
  - C. C. Tsai 2088
  - EF156137
  - EF156058
  - EF156228
  - GQ850855

(Continued)
Genetic relationships were determined using NJ in the MEGA 6.0 (Tamura et al., 2013), maximum parsimony (MP) in PHYLIP 3.68 (Felsenstein, 2004), and maximum likelihood (ML) in MEGA 6.0 (Tamura et al., 2013). Bootstrapping (1,000 replicates) was carried out to estimate the support for NJ, MP, and ML topologies (Felsenstein, 1985; Hillis and Bull, 1993). The strict consensus parsimonious tree was then constructed by using the MEGA 6.0 (Tamura et al., 2013).

**Divergence Time Estimation**

The combined chloroplast DNA (cpDNA) dataset was used to estimate the divergence times using the Bayesian Yule model methods (BEAST version 1.7.5). The characteristic of uniparental inheritance in cpDNA prevents the inference of recombination introgression on phylogenetic reconstruction (Drummond et al., 2012). The general-time reversible (GTR) model with estimates of invariant sites (+I) and gamma-distributed among site rate variation (+G) in all matrices without partitions model was determined by the nucleotide substitution model test, conducted in MEGA 6.0 (Tamura et al., 2013). The general-time reversible (GTR) model with estimates of invariant sites (+I) and gamma-distributed among site rate variation (+G) in all matrices without partitions model was determined by the nucleotide substitution model test, conducted in MEGA 6.0 (Tamura et al., 2013). To estimate the divergence time, two strategies, the strict and relaxed clock models, were adopted. For the relaxed clock, the calibration point at the most recent common ancestor (MRCA) of *Paphiopedilum* and *Phragmipedium* for 22 Ma (Gustafsson et al., 2010) were used to calculate the divergence times of each node. However, since there is no suitable fossil record to correct the calibration of divergence times for the ingroup, we re-calculated the divergence time by strict clock model for consistency. For the strict clock, the mean substitution rate of 1.82 × 10^{-9} subs/site/year with the lower and upper limits 1.11 × 10^{-9} subs/site/year and 2.53 × 10^{-9} subs/site/year, respectively, were used for the cpDNA spacers in *Phalaenopsis amabilis* complex (Tsai et al., 2015).

We conducted four independent runs of a Yule prior and four Markov Chain Monte Carlo (MCMC) chains with a different starting seed. The first 10% simulations were discarded (burn-in) in a total of 10^8 generations. For thinning, one tree was reserved every 10,000 trees, and finally, 10,000 trees were left to calculate the posterior probability of each node. The effective sample size (ESS) > 200 was used as a criterion to check whether the sampling (simulations) is proper and is reaching a stationary distribution by Tracer v1.6 (Rambaut et al., 2018). Four independent-runs results, including the log file and tree file, were combined with the assistance of LogCombiner 1.6.1 (Drummond and Rambaut, 2007). TreeAnnotator 1.6.1 (Drummond and Rambaut, 2007) was used to summarize a consensus tree with a criterion of the maximum clade reliability using the mean heights option. The final consensus tree was drawn by FigTree 1.3.1 (Rambaut, 2009).

**Biogeographic Inference Using Reconstruct Ancestral State in Phylogenies**

The Statistical dispersal–vicariance analysis was used to assess the biogeographic patterns of *Paphiopedilum* species [Statistical Dispersal-Vicariance Analysis (S-DIVA), (Yu et al., 2010)]. Bayes–Lagrange Statistical dispersal–extinction–cladogenesis (S-DEC) model (Ree and Smith, 2008) was performed in Reconstruct Ancestral State in Phylogenies (RASP) 3.2 (Yu et al., 2015) to distinguish the events of vicariance, dispersal, and extinction. Five geographic areas were determined mainly according to Myers et al. (2000) with a little modification to illuminate the vicariance, dispersal and extinction events of *Paphiopedilum* species. The hotspot areas in South-Central China and Indo-Burma with the Malay Peninsula were combined as area A, consisting of China, Nepal, India, Bhutan, Burma, Thailand, Malaysia, Cambodia, Vietnam, and Laos. The hotspot “Sundaland” including Borneo, Java, and Sumatra were set as the area B. We move the Malay Peninsula from the area “Sundaland” to area A due to the integrity of the current landmass. The hotspots “Wallacea” (include Sulawesi and Moluccas) and “Philippines” were set as area C and area E.

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**TABLE 1 | Continued**

| Taxa and systematic classification a | Geographical distribution | Voucher b | GenBank accession no. |
|-----------------------------------|---------------------------|-----------|----------------------|
|                                   |                           |           | ITS                  |
|                                   |                           |           | trnL intron          |
|                                   |                           |           | trnL-F spacer        |
|                                   |                           |           | atpB-rbcL spacer     |

aThe systematics of Phalaenopsis are based on Christenson (2001).

bVoucher specimens were deposited at the herbarium of the National Museum of Natural Science, Taiwan (TNM).
RESPECTIVELY, ISLANDS OF NEW GUINEA EASTERN FROM THE WALLACEA ARE DEFINED AS AREA D. SPECIES OF OUTGROUP WERE ALL DEFINED AS THE AREA I. THESE TWO OUTGROUP SPECIES ARE DISTRIBUTED IN ECUADOR, PERU, COSTA RICA, PANAMA, AND COLOMBIA. THE REAL DISTRIBUTION OF OUTGROUPS IS TOO FAR FROM THE AREAS OF THE SPECIES IN THIS STUDY. THEREFORE, THE RANGES OF OUTGROUPS ARE ASSIGNED TO A NEW AREA IN WHICH NONE OF THE INGROUP SPECIES OCCURS (YU ET AL., 2014). THE ML TREE TOPOLOGIES WERE USED IN S-DIVA ANALYSIS.

RESULTS

Sequence Alignment and Characteristics

The lengths of the ITS sequences obtained from the Paphiopedilum and outgroup samples were similar to those reported for a broad example of angiosperms (Baldwin, 1992; Baldwin et al., 1995). The alignment length of the ITS sequence is 735 nucleotides, of which 343 were identified as variable sites with 235 potentially parsimony informative sites. The average genetic distance between the 78 Paphiopedilum samples was 0.039 in ITS, and the average genetic distance between the 78 Paphiopedilum species was 0.01 in cpDNA. The alignment of combined plastid DNA fragments contained a total of 2,409 characters, of which 872 were identified as variable sites with 588 potentially parsimony informative sites. Since the sequences of three samples of every species are the same within species, only one sequence per species was used for the analyses and deposited in NCBI GenBank. The accession numbers of the nuclear ribosomal ITS sequences and the three fragments of plastid DNA from the 78 Paphiopedilum taxa and the two outgroup samples (from the genus Phragmipedium) are shown in Table 1.

Phylogeny Reconstruction

Both NJ and MP trees revealed a monophyletic relationship of 78 Paphiopedilum taxa with high bootstrap supports (Figure 2). Moreover, the use of six outgroups for phylogeny reconstruction showed similar bootstrap supports (Supplementary Figure S1). In the Paphiopedilum monophyletic clade, three subgenera Parvisepalum, Brachypetalum, and Paphiopedilum formed independent monophyletic clades with 100/100/100, 98/91/84, and 61/59/86% bootstrap supporting values in NJ/MP/ML trees, in which subgenus Parvisepalum was diverged firstly from other lineages (Figure 2, Supplementary Figure S1). In subgenus Paphiopedilum, sections Barbata, Cochlopetalum, Pardalopetalum, and Coryopedilum are monophyletic with 94/96/100, 79/81/94, 100/100/100, and 100/100/100% bootstrap supporting values in NJ, MP, and ML trees. Additionally, the use of six outgroups for phylogeny reconstruction showed similar patterns (Supplementary Figure S1). However, section Paphiopedilum showed a low support [56/51/54% bootstrap values (Supplementary Figure S2); 59% bootstrap values (Supplementary Figure S1)] for the monophyly. In addition, the molecular data demonstrates that a newly described variety, P. micranthum var. eburneum, is closely related to P. malipoense based on the plastid DNA within subgenus Parvisepalum, which is inconsistent with the inference by nuclear ITS and combined data. In ITS tree, P. micranthum var. eburneum is sister with P. micranthum var. micranthum (Figures 2 and 3). Therefore, we infer a hybridization event between the ancestor of P. micranthum and P. malipoense that lead to a plastid capture in P. micranthum var. eburneum.

Divergence Time Estimates

The coalescence time of the genus Paphiopedilum was estimated to be 7.09 Mya with 95% confidence intervals (95% CI) of 5.88–8.41 Mya (Figure 4) according to the substitution rate referenced from Tsai et al. (2015). If calibrating by relaxed clock referring to Gustafsson et al. (2010), the estimated coalescence time of the genus Paphiopedilum was 5.72 Mya (Figure 4). In the genus Paphiopedilum, the coalescence times estimated by strict clock were 4.30 Mya (95% CI: 3.50–5.18 Mya), 2.47 Mya (95% CI: 1.73–3.33 Mya), and 4.08 Mya (95% CI: 3.39–4.86 Mya) for subgenera Parvisepalum, Brachypetalum, and Paphiopedilum, respectively (Figure 4). After re-calibrating by a relaxed clock, the coalescence time was estimated to be 3.3 Mya, 2.24, and 3.38 Mya for subgenera Parvisepalum, Brachypetalum, and Paphiopedilum, respectively (Figure 4). In addition, the strict clock suggested that the coalescence times were tracked back to 2.19 Mya (95% CI: 0.17–2.77 Mya), 1.54 Mya (95% CI: 0.93–2.27 Mya), 3.12 Mya (95% CI: 2.49–3.81 Mya), 2.48 (95% CI: 1.86–3.17 Mya), and 1.60 Mya (95% CI: 1.10–2.18 Mya) for clades of subgenera Barbata, Cochlopetalum, Paphiopedilum, Coryopedilum, and Pardalopetalum of subgenus Paphiopedilum, respectively. By relaxed clock, the coalescence time was estimated to be 1.94, 1.3, 2.59, 1.81, and 1.08 Mya for clades of subgenera Barbata, Cochlopetalum, Paphiopedilum, Coryopedilum, and Pardalopetalum of subgenus Paphiopedilum, respectively. Additionally, the use of six outgroups for divergence time estimation also showed similar supports (Supplementary Figure S2). In short, the estimates of the coalescence times by strict and relaxed clocks are similar, but the time calculated is slightly shorter by relaxed clocks. Regardless, the coalescence time of the genus Paphiopedilum will not be earlier than Upper Miocene.

Demographic History and Historical Biogeography Inference

Complicated evolutionary processes of continuous and episodic dispersal, vicariance, and extinctions determined the current geographic distribution of genus Paphiopedilum. Since the most probable ancestral areas located on continental Asia (area A in Figure 5), dispersal events seem to determine the extant distributions of subgenera largely. The results supported vicariance events on nodes 159, 146, and 109 shown in Table 2 and Figure 5, and on nodes 163, and 132 (Supplementary Table S3 and Figure S3). The node 147 revealed dispersal events among section Coryopedilum/Pardalopetalum of Paphiopedilum and other sections of Paphiopedilum causing by migration route from area A (China, Nepal, India, Bhutan, Burma, Thailand, Malaysia, Cambodia, Vietnam, and Laos) to B area (Sumatra, Borneo, and Java). Meanwhile, the nodes 145, 129, 114, and 102 also revealed dispersal events from north to south, according to Sundaland and Sunda Super Islands.

Furthermore, the use of six outgroups for dynamic historical inference showed similar patterns (Supplementary Table S3 and
Figure S3). Only the nodes 146 and 109 were detected vicariance event causing by the geological separation between Indochina and Sumatra/Borneo/Java. In addition, in subgenus *Paphiopedilum*, 2 vicariance and 10 dispersal events were detected, which suggesting a significant dispersal process affected biogeographical patterns in shaping the current distribution in the subgenus *Paphiopedilum*. Areas A and B might be the two possible ancestral areas and likely shaped by several complicated dispersal events in the subgenus *Paphiopedilum*.

**DISCUSSION**

**Systematics Revision of Genus *Paphiopedilum***

In general, our phylogenetic inference is mostly congruent with that of Cox et al. (1997), Cribb (1997b), and Guo et al. (2015). In the genus *Paphiopedilum*, tessellated leaves, single flowers with broad elliptic to subcircular petals, and a sizeable thin-textured lip characterize subgenus *Parvisepalum* in southwest China and Vietnam (Cribb, 1998). Within this subgenus, the phylogenetic topography and the divergence time of at least 4.30 Mya rejected the previous hypothesis of the sister-species relationship between *P. armeniacum* and *P. delenatii* (Cribb, 1983) (Figure 4). The geographical distribution of these two species is also separated (Yunnan, China for *P. armeniacum*, and Vietnam for *P. delenatii*) (Cribb, 1998).

Furthermore, a newly described variety, *P. micranthum* var. *eburneum*, is phylogenetically close to *P. malipoense* in maternal-inherited plastid DNA but close to *P. micranthum* in biparental-inherited nuclear ITS sequences (Figure 5), suggesting that *P. micranthum* var. *eburneum* is a natural hybrid between the maternal parent *P. malipoense* and the paternal parent *P. micranthum* and experienced the event of chloroplast
capture. The overlap of the geographical distribution of these three taxa also supports this hypothesis (Cribb, 1998). In addition, ITS sequences are usually concertedly evolved via unequal crossing-over (Schlotterer and Tautz, 1994) and biased gene conversion (Hillis et al., 1991), which results in sequence homogeneity between paralogs (Maynard, 1989).

The monophyly of subgenus Brachypetalum inferred in this study is congruent with the inference by Cox et al. (1997). The subgenus Brachypetalum is geographically confined to Southeast Asia (Cribb, 1998). Albeit overlapping distribution with subgenus Parvisepalum (Cribb, 1998), subgenus Brachypetalum is phylogenetically separated, consistent with the distinguishable leaf anatomy between these two subgenera (Cribb, 1998). Both molecular and morphological evidences support the independent taxonomic treatment between subgenera Brachypetalum and Parvisepalum (Karasawa, 1982; Karasawa and Saito, 1982; Cribb, 1997b), but object with Atwood’s (1984) opinion of taking the subgenus Parvisepalum as a synonym of Brachypetalum.
The monophyletic subgenus *Paphiopedilum* can be morphologically and phylogenetically subdivided into five sections: *Coryopedilum*, *Pardalopetalum*, *Cochlopetalum*, *Paphiopedilum*, and *Barbata* (Cox et al., 1997; Cribb, 1997b). Section *Coryopedilum* is characterized by its plain green, strap-like leaves, and multi-flowered inflorescences, which flowers are blooming simultaneously (Cribb, 1998). This section distributes throughout Borneo, Sulawesi, New Guinea, and the Philippines (Cribb, 1998). Except placing *Paphiopedilum parishii* and *Paphiopedilum dianthum* into section *Pardalopetalum* from section *Coryopedilum*, Cribb (1997b) agreed with Atwood (1984) that section *Pardalopetalum* is independent from section *Coryopedilum* taxonomically, according to the ITS analysis (Cox et al., 1997) and similar green strap-like leaves and staminodes (Cribb, 1997b), which is consistent with our phylogenetic inference. However, the only character that separates sections of *Pardalopetalum* and *Coryopedilum* is the morphology of staminode. Whether this single character is sufficient to characterize them as separating sections should be re-evaluated with more evidence.

Unlike the simultaneous bloom of section *Coryopedilum*, section *Cochlopetalum* flower in succession, and their flowers bear elliptic bracts, linear, spirally twisted, spreading, ciliate petals, and a pot-shaped spotted lip (Cribb, 1998).
Cochlopetalum distributes in Sumatra and Java only (Cribb, 1998). The extensive section Barbata is the sister of section Cochlopetalum, also characterized by a solitary flower with a lip and prominent incurved side-lobes, but the leaf tessellated (Cribb, 1998). The morphological dissimilarity and reciprocally monophyletic relationship indicate that, despite recently diverged, these two sections should be independent taxonomically.

**Biogeography and Evolutionary Trends**

The clade of genus Paphiopedilum is coalesced to 7.09 or 5.72 Mya, similar to the estimate of 7.62 Mya by Guo et al. (2015). The flower morphology of subgenus Parvisepalum is intermediate between other subgenera of Paphiopedilum and Cypripedium (Chen and Tsi, 1984), which could be explained by the earlier divergence of subgenus Parvisepalum in genus Paphiopedilum (Guo et al., 2012). Presently, the genus Cypripedium is distributed throughout worldwide temperate zones (Cox et al., 1997), with China as a center for species diversity (Cribb, 1997a). Therefore, genera Paphiopedilum and Cypripedium have most likely diverged in mainland Asia (Chen and Tsi, 1984).

However, genus Paphiopedilum was suggested as the sister with two American genera Phragmipedium and Mexipedium according to morphology, plastid rbcL (Albert, 1994), ITS (Cox et al., 1997), and both nuclear and plastid genes (Guo et al., 2012). These inferences are conflict to the hypothesis of the divergence between Paphiopedilum and Cypripedium in China, but implied the divergence of Paphiopedilum from the group of Phragmipedium + Mexipedium, by which the slipper orchids (Cypripedioideae) were hypothesized widespread throughout North America and Asia in the past (Atwood, 1984; Albert, 1994; Cox et al., 1997).

Subgenus Parvisepalum in southwest China and Vietnam diverged earlier from the other subgenera of genus Paphiopedilum. The coalescence time of subgenera Parvisepalum, Brachypetalum, and Paphiopedilum were tracking back to the Upper Miocene (Guo et al., 2015). Subgenus Brachypetalum in mainland Southeast Asia was descended from the subgenus Parvisepalum inferred by S-DIVA (Figure 5), which agrees with other disjunctions at the Southern China and Indochina (Guo et al., 2015) or Sunda Shelf and New Guinea/Australia (Tougard, 2001; Lohman et al., 2011; Tsai et al., 2015).

The subgenus Paphiopedilum is further descended and evolved quickly in the Sunda Shelf. A land bridge might connect Mindoro, Palawan, Borneo, the Malay Peninsula, Borneo, Sumatra, Java, Bali, and various parts of the Philippines when sea levels falling during Pleistocene (about 0.01~1.8 Mya) (van Oosterzee, 1997) (Figure 1). Surfaced land bridge connected these regions and was beneficial to the interisland and continent-island dispersal in Southeast and South Asia (van Oosterzee, 1997). The clade of Coryopedilum + Pardalopetalum was the first derived in subgenus Paphiopedilum based on the phylogenetic tree, which reflects in the sympatric distribution of subgenus Brachypetalum and section Pardalopetalum (Figure 6). Following this clade formation, sections Paphiopedilum and Barbata were subdivided and dispersed throughout Southeast Asian archipelagoes across the land bridge during the glacial. The southward expansion from continental Asia into the Greater Sunda Islands through the Indochina and Malay Peninsulas were also reported in other taxa, e.g., Lithocarpus (Fagaceae) (Yang et al., 2018). Such colonization events between continental Asia and the Greater Sunda Islands mostly occurred during Miocene and Plio-Pleistocene (de Bruyn et al., 1997).
et al., 2014). As a "corridor," Indochina reveals high flora diversity and the high species richness, which facilitates the in situ speciation (de Bruyn et al., 2014).

Another flora diversity hotspot is Borneo (de Bruyn et al., 2014), which is also important for the genus *Paphiopedilum*. The section *Cochlopetalum*, which is found only in Sumatra and Java, might represent a group derived from Borneo. The S-DIVA inferred multiple times dispersal events sourced from Borneo with two vicariances to illustrate the current distribution of the species. The repeated submergence and emergence of land bridges could promote repeated genetic isolation and gene flow between closed related taxa. During the Plio-Pleistocene glacial oscillations, this process accelerates the diversification rates in the Sunda Super Islands.

Dispersal and vicariance events that exposed geographic isolation among taxa might be due to the land bridge submergence (Chiang et al., 2009; Chiang et al., 2013; Ge et al., 2012; Ge et al., 2015; Hsu et al., 2013) in Sunda Shelf and Sunda Super Island during the Pleistocene (Figure 5 and Table 2). The Borneo is the second original center of *Paphiopedilum*. The tropical forests and rugged topography harbor diversified niches opened to the speciation of organisms. The repeated submergence and emergence of land bridges could promote repeated genetic isolation and gene flow between closed related taxa. During the Plio-Pleistocene glacial oscillations, this process accelerates the diversification rates in the Sunda Super Islands.

Conclusions

In summary, the origin and coalescence time of genus *Paphiopedilum* tracked back to Southern China/Eastern Indochina since late Miocene and early Pliocene, while the range expansion and species divergence were related to sea-level fluctuations during the Plio-Pleistocene glacial cycles. Historical geological barriers shaped a pattern of vicariance among disjunct distributed subgenera after isolated ancestral populations. The ancestral taxa of subgenus *Paphiopedilum* migrated from Southern China/Eastern Indochina to south which developed quickly in the Sunda Shelf. Due to the submergence of the Sunda Shelf and Sunda Super Island, species of subgenus *Paphiopedilum* dispersed with isolation between islands as well as subsequent in situ speciation within islands from other taxa within section or subgenus, which accelerated species divergence in subgenus *Paphiopedilum*. *Paphiopedilum* distributes in four of 25 biodiversity hotspots (Myers et al., 2000), the Indo-Burma, Sundaland, Wallacea, and Philippines, where are also the major evolutionary hotspots” (de Bruyn et al., 2014). It suggests that rich and fascinating historical biogeographic events have created rich species diversity there, such as the case of *Paphiopedilum*. However, deforestation has caused the so-called “empty forest syndrome” (de Bruyn et al., 2014). We hope that these areas will not become extinction hotspots, even though they are almost now.

Data Availability Statement

The datasets generated for this study are available on request to the corresponding author.

Author Contributions

Conceived and designed the experiments: C-CT and Y-CC. Performed the experiments: C-CT, P-CL, Y-ZK, C-HC, and Y-CC. Analyzed the data: C-CT, P-CL, Y-ZK, C-HC, and Y-CC. Contributed reagents/materials/analysis tools: C-CT, P-CL, Y-ZK, C-HC, and Y-CC. Wrote the paper: C-CT, P-CL, and Y-CC. Conceived of the study, edited the manuscript, and approved the final manuscript: C-CT, P-CL, Y-ZK, C-HC, and Y-CC.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00126/full#supplementary-material

SUPPLEMENTARY FIGURE S1 | Phylogenetic relationships using Maximum Likelihood resulting from analysis of the combined data matrix (nuclear ribosomal ITS, and trnL-F spacer) from 78 Paphiopedilum and 6 outgroup species.

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