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Notes on Phylogeny of the Genus *Chiloschista* Lindl. (Orchidaceae) in Thailand

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Abstract: The genus *Chiloschista* Lindl. belongs to subtribe Aeridinae, family Orchidaceae. It is a leafless epiphytic orchid, which is one of the popular orchids, especially in Thailand. Due to floral variation, there is some doubt about species delimitation especially *C. usneoides*, *C. parishii*, *C. viridiflava* group. Thus, this study was conducted to employ the molecular evidence to clarify the species of *Chiloschista*. Sequences of *matK* and ITS regions of 8 species of *Chiloschista* collected from natural habitats in Thailand were used to construct the phylogenetic tree. Three endemic species from Thailand have been firstly sequenced *C. exuperei*, *C. extinatoriformis*, and *C. rodriguezii*. The result showed that *Chiloschista* is a monophyletic group as indicated in the previous study. The infrageneric relationship has not shown any subclade. The similar species based on morphology are well separated with higher statistic support. This molecular phylogenetic has supported the morphological species concept from previous study.

Keywords: Molecular phylogeny, *Chiloschista*, Orchidaceae, leafless epiphyte.

1. Introduction

The genus *Chiloschista* was established by Lindley [1] based on specimen collected from Nepal, previous named as *Epidendrum usneoides* D. Don. Some botanist such as Reichenbach.f. did accept as *Sarcochilus* and later he placed it in the genus *Thrixspermum*. Until Smith [2] re-introduced *Chiloschista*, it has since accepted this genus [3]. It distributed in tropical and subtropical Asia to NW Pacific, with 26 accepted species [4]. In Thailand, Seidenfaden [3] reported this genus 7 species, *C. exuperei* (Guillaumin) Garay, *C. lunifera* (Rchb.f.) J.J.Sm., *C. parishii* Seidenf., *C. ramifera* Seidenf., *C. trudelii* Seidenf., *C. usneoides* (D. Don) Lindl. and *C. viridiflava* Seidenf. Later, Casvestro and Ormerod described *C. rodriguezii* in 2005 [5] and Dalstrom and Kolan. described *C. lindstroemii* in 2020 [6]. Until now, there are 9 species recorded in Thailand.

The genus *Chiloschista* Lindl. is epiphytic or lithophytic orchids, usually leafless herbs, bearing flattened, or subcylindrical roots. Its roots are green and photosynthetic. Inflorescence is racemose, rarely panicle, erect or pendent, and comprises many flowers. Flowers are resupinate, fragrant yellow to white, without or with reddish or purplish spots. Sepals and petals separate, oblong-ovate or oblong-elliptic. The lateral

sepals usually adnate to column foot. Labellum is saccate or concave, articulate to apex of column foot, movable, trilobe at the apex. Column is semi-terete. Anther cap has two long-filiform appendages, sometime reduced to a small tooth. Pollinia has four masses, waxy and lay on the sublaminar stipe which having a small viscidium. Fruit is a capsule with numerous dust-like seeds (Fig. 1) [7].



Fig. 1. *Chiloschista* spp. A-B: *C. exuperei* (Guill.) Garay C-D: *C. viridiflava* Seidenf. E-F: *C. parishii* Seidenf.

The phylogenetic tree reflects the evolutionary relationship among taxa. The accepted taxonomic unit required as a monophyletic group. Moreover, this method can be used to identify the species by using the molecular information [8]. The result of phylogenetic analysis previously done by Topik [9],[10] and Carlswald et al. [11] revealed that the position of *Chiloschista* was different. This may be because *matK* sequences used are pseudogene [7]. The later reports agree that this genus is monophyletic based on molecular phylogenetic analysis [7],[12].

For the morphological features, they seem to be variable and not so clear for species delimitation. Indeed, some species similar on the floral morphology, except

the color pattern. Many species have not known the phylogenetic position, due to no sequence available. To update the phylogenetic relationship of the genus *Chiloschista*, we aimed to add more taxa from Thailand for phylogenetic analysis, based on *matK* and ITS sequences to illustrate the evolutionary relationship at infrageneric level and also clarify the species delimitation of similar species group.

2. Methods

2.1 Taxon selection

Eight Thai species of *Chiloschista* obtained from natural habitat in Thailand were available for DNA extraction, *C. exuperei*, *C. extincitoriformis*, *C. lunifera*, *C. parishii*, *C. rodriguezii*, *C. usneoides*, *C. viridiflava* and *C. sp.* Sequence data of *Hygrochilus parishii*, *Ornithochilus yingjiangensis*, *O. difformis*, *Taeniophyllum glandulosum*, *Thrixpernum annamense*, *T. centipeda* and *Vanda alpina* were obtained from GenBank to be used as outgroup. *C. yunnanensis* was also downloaded to compare with ingroup (Table 1).

Table 1. Species and DNA regions used in this study, downloaded from GenBank showing the accession numbers.

Species	nrITS	<i>matK</i>
<i>Chiloschista yunnanensis</i>	KJ021015	KJ021019
<i>Hygrochilus parishii</i>	KF545876	KF545887
<i>Ornithochilus difformis</i>	KF545878	KF545889
<i>Ornithochilus yingjiangensis</i>	KF545879	KF545894
<i>Taeniophyllum glandulosum</i>	KJ733455	KJ733612
<i>Thrixpernum annamense</i>	KF545883	KF545893
<i>Thrixpernum centipeda</i>	KJ733456	KJ733621
<i>Vanda alpina</i>	KC244656	KC244660

2.2 DNA extraction

The Genomic DNA Isolation Kit (Plant) was used to extract DNA from dried leaves (Bio-Helix, Taiwan). The *matK* region was amplified using a primer pair of 390F and 1326R [13]. The 50 μ L amplification reaction contained 25 μ L of OnePCR Ultra, 2.5 μ L of each primer (5 pmol/ μ L), 1 μ L of template DNA, and 19 μ L free water. An initial 5 minutes pre-melting stage at 95°C was followed by 30 cycles of 30 seconds at 95°C (denaturation), 1 minute at 55°C (annealing), 40 seconds at 72°C (extension), and a final 7 minutes extension at 72°C, for the polymerase chain reaction (PCR) [14]. For ITS sequences, amplification was performed using a primer pair, 1 7 SE and 2 6 SE [15]. The 50 μ L amplification reaction included 25 μ L OnePCR Ultra, 5.2 μ L each primer (2 pmol/ μ L), 1 μ L of template DNA, 1 μ L of DMSO, and 18 μ L of free water. The polymerase chain reaction (PCR) profile was performed as mentioned earlier. The obtained PCR products were used to perform direct PCR sequencing by U2Bio (Thailand) Co., ltd.

3. Analysis

A total of 23 nucleotide sequences of *matK*, ITS, and combined *matK* and ITS of ingroup and outgroup were aligned using Bioedit ver. 7.2.5 [16], followed by manual corrections. The evolution model was evaluated using

jModelTest2 on XSEDE in the CIPRES Gateway ver. 3.3 (<https://www.phylo.org/>) [17]. AIC selected the *matK*, ITS, and combined sequences with TPM1uf+G, GTT+ G, and TVM+ G, respectively. Phylogenetic analyses were done using RAxML BlackBox with 1,000 bootstrap replicates for maximum likelihood (ML) tree and MrBayes on XSEDE with a Markov chain Monte Carlo (MCMC) chain length of 1,000,000 was used for Bayesian analysis (BA) in the CIPRES Science Gateway v. 3.3 (<https://www.phylo.org/>) [18]. The obtained trees were inspected and altered using FigTree ver. 1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

4. Results and discussion

All sequences obtained in this study were compared to GenBank database sequences using NCBI nucleotide BLAST (blastn) (<http://blast.ncbi.nlm.nih.gov/>), and the results confirmed that all sequences are from orchids. The aligned *matK*, ITS, and combined *matK* and ITS matrices with gaps were respectively 1,634, 997, and 2,687 bp long.

The phylogenetic analysis of *matK* showed the highly statistic support of 2 clades between ingroup and outgroup. The genus *Chiloschista* form a monophyletic group with strong bootstrap percentage (BP) and Bayesian posterior probabilities (PP). The resolution infrageneric level is very poor (Fig. 2). Based on ITS, the resolution is quite better than of *matK* (Fig. 3). *Chiloschista* spp. form a clade with high BP and PP, separated from the outgroup as same as of *matK* result. While the combined genes showed the result congruent with of ITS result (Fig. 4). However, no subclade appears in the *Chiloschista* clade, but each species specimen forms a clade, indicating a monophyletic group at the species level.

Topik [9],[10] employed only *Chiloschista viridiflava* representative on his phylogenetic analysis of subtribe Aeridinae. His result showed that *Chiloschista* is a sister group with *Ornithochilus fifformis*. The later reported by Zou et al. [12] and our result indicated that both genera are still monophyletic group when more than one species were added and belonged to different clades. The last update on *Chiloschista* phylogenetic position involvement was reported by Zou et al. [12], showed that the *Chiloschista* clade is a sister group with the clade of *Phalaenopsis*, *Thrixpernum*, *Vanda*, *Aerides*, *Trichoglottis*, *Abdominea*, *Gastrochillus* and *Cleisostoma*. Another genus of leafless orchid, *Taeniophyllum* in this analysis is in the different clade with *Chiloschista* clade as same as the previous studies [12],[11], indicating the leafless character evolved more than one time in the subtribe Aeridinae. From previous studies and this study support the monophyly of the genus *Chiloschista*. However, the relationship in the genus based on combined ITS and *matK* showed that Thai *Chiloschista* does not reveal any subgroup in the genus level.

Some species, *C. parishii*, *C. viridiflava* and *C. usneoides*, are similar in morphological characteristics. They are different only color pattern, but it is difficult to recognize from herbarium and spirit specimens. However, the phylogenetic tree showed that each species from a monophyletic group. Thus, the molecular evident,

based on *matK* and ITS regions is congruent to Seidenfaden species delimitation [3].

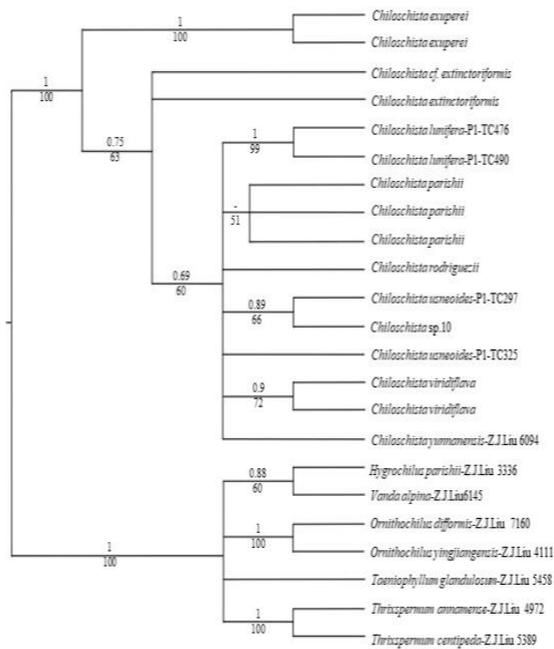


Fig. 2. Phylogenetic tree based on *matK*, showing bootstrap percentage (BP) and Bayesian posterior probabilities (PP).

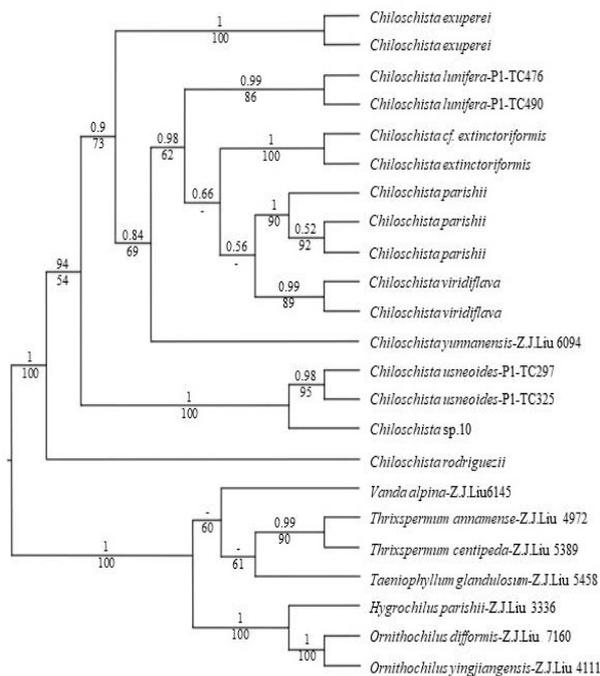


Fig. 3. Phylogenetic tree based on ITS, showing bootstrap percentage (BP) and Bayesian posterior probabilities (PP).

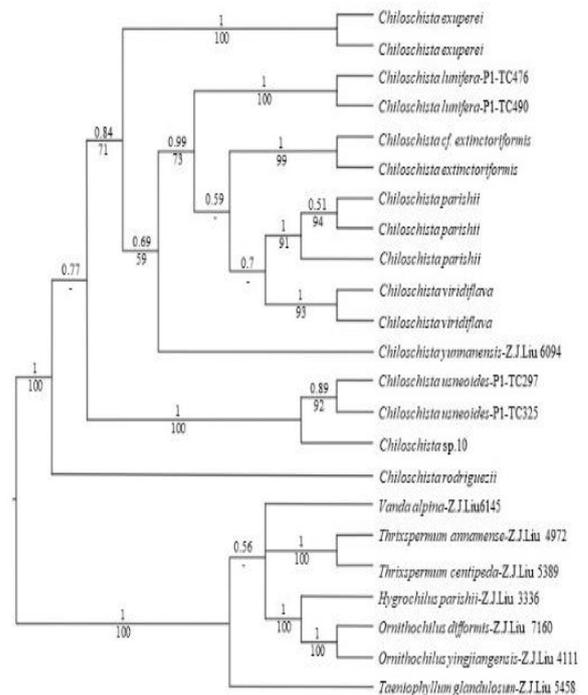


Fig. 4. Bootstrap consensus tree of *Chiloschista* based on the combined nuclear (ITS) and plastid (*matK*) markers, showing bootstrap percentage (BP) and Bayesian posterior probabilities (PP).

5. Conclusions

Three endemic species, *Chiloschista extinatoriformis*, *C. exuperei*, *C. rodriguezii* have been firstly sequenced. This study supported the monophyly of the genus *Chiloschista* based on *matK*, ITS and combined genes. All species of *Chiloschista* in this analysis are well separated from each other and not form any intrageneric group. *C. parishii*, *C. viridiflava*, and *C. usneoides* which are similar on morphological characteristic are the monophyletic species. It indicated that the recent species delimitation i.e. Seidenfaden [3] is accepted species.

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