A New Molecular Phylogeny and a New Genus, Pendulorchis, of the Aerides–Vanda Alliance (Orchidaceae: Epidendroideae)

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Abstract

Background: The Aerides–Vanda alliance is a complex group in the subtribe Aeridinae (subfamily Epidendroideae, Orchidaceae). Some phylogenetic systems of this alliance have been previously proposed based on molecular and morphological analyses. However, several taxonomic problems within this alliance as well as between it and its allies remain unsolved.

Methodology/Principal Findings: We utilized ITS and five plastid DNA regions in this phylogenetic analysis. Consensus trees strongly indicate that the Aerides–Vanda alliance is monophyletic, and the 14 genera of this alliance can be grouped into the following clades with 14 subclades: 1. Aerides, comprising two subclades: Rhynchostylis and Aerides; 2. Ascocentropis; 3. Papilionanthe; 4. Vanda, comprising five subclades: Neofinetia, Christensonaria, Seidenfadenia, Ascocentrum, and Vanda–Trudelia, in which Vanda and Trudelia form a subclade; 5. Tsiorchis, comprising three subclades: Chenorchis, Tsiorchis, and two species of Ascocentrum; 6. Paraholcoglossum; and 7. Holcoglossum. Among the 14 genera, only Ascocentrum is triphyletic: two species of the Ascocentrum subclade, an independent subclade Ascocentrum subclade in the Tsiorchis clade; the Ascocentrum subclade in the Vanda clade; and one species in the Holcoglossum clade. The Vanda and Trudelia species belong to the same subclade. The molecular conclusion is consistent with their morphological characteristics.

Conclusions: We elucidate the relationship among the 14 genera of the Aerides–Vanda alliance. Our phylogenetic results reveal that the Aerides–Vanda alliance is monophyletic, but it can be divided into 14 genera. The data prove that Ascocentrum is triphyletic. Plants with elongate-terete leaves and small flowers should be treated as a new genus, Pendulorchis. Saccolabium himalaicum (Ascocentrum himalaicum) should be transferred to Pendulorchis. Ascocentrum pumilum, endemic to Taiwan, should be transferred to Holcoglossum. A new combination, Holcoglossum pumilum, was also established. Trudelia should not be recognized as an independent genus. Two new species, Pendulorchis gaoligongensis and Holcoglossum singchianum, were described as well.

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Introduction

Orchidaceae is possibly the largest family of angiosperms with almost 25,000 species. The traditional typological classification divides Orchidaceae into Apostasioideae, Cypripedioideae, Spiranthoideae, Orchidoideae, and Epidendroideae [1]. Recently, Spiranthoideae has been integrated into Orchidoideae, where a new subfamily Vanilloideae was established [2] based on the affinity analysis of internal transcribed spacer (ITS), trnL-F, and matK sequences. Epidendroideae is a subfamily that accounts for more than 80% of the orchid species. The Aerides–Vanda alliance...
described in this study is a member of the subtribe Aeridineae and an advanced but complex group in Epidendroideae.

Tsi [3] and Christenson [4–7] identified the Aerides–Vanda alliance by a comparative analysis of Holcoglossum and its allied genera, such as Vanda, Papilionanthe, Asocentrum, Aerides, Rynchostylis, Seidenfadenia, Trudelia [9], and Neofinetia. Subsequently, the following genera were established within the alliance: Christensonia [9], Chenorchis [10], Paraholcoglossum [11], Tsiorchis [11], and Asocentropsis [12]. Consequently, 14 genera were included in the alliance. However, the Aerides–Vanda alliance is somewhat ambiguous in taxonomic literature, i.e., the same species may be classified under different genera [2,13]. This confusion arises from using partly overlapping morphological characteristics to distinguish one genus from another. Similarly, Seidenfaden [14] pointed out that “The difficulties arise because we again and again meet with species that can be accommodated in a genus only by widening such a generic circumscription until the situation becomes completely blurred.” Christension [4] conducted a branch analysis of this alliance (excluding Neofinetia and genera subsequently established) using 11 features, and divided the Aerides–Vanda alliance into two branches. One branch comprises Vanda and Asocentrum, and the other branch includes Holcoglossum, Papilionanthe, Aerides, Rynchostylis, and Seidenfadenia. The latter branch is further divided into the three sub-branches, namely, Aerides, Papilionanthe, and Holcoglossum-Rynchostylis-Seidenfadenia sub-clades.

In the Aerides–Vanda alliance, Garay [15] placed Papilionanthe between Vanda and Aerides, but it is more closely linked to Aerides. By contrast, Jin [16] considered Vanda as a relatively primal genus of this alliance.

The members of Asocentrum considerably differ in the shapes of their leaves, which can be divided into two types: subterete and nearly lorate leaves. Based on the morphological analysis of the mid-lobe of the lip, stipe, and spur of this genus, Jin [16] stated that Asocentrum may be grouped between Aerides and Seidenfadenia because the bilobed uplifted rostellum is unique to Asocentrum and Seidenfadenia in the Aerides–Vanda alliance, and the leaves of some Asocentrum species are similar to those of Seidenfadenia. However, Christension [4] classified Asocentrum under the same branch where Vanda belongs because of its notch-tipped leaves. This finding indicates that the Asocentrum species with terete leaves do not belong to the Vanda-Asocentrum branch. Christension [4] inferred that Holcoglossum and Seidenfadenia are two parallel evolutive branches, whereas Jin [16] considered Seidenfadenia to be more evolutive because of the special structure of its spur and rostellum. Most Seidenfadenia, Holcoglossum, and Asocentrum species have closely similar vegetative organs, specifically, a very short stem and subterete leaves with a ventrally longitudinal groove. Their distribution areas usually overlap with one another, and they have very similar habits. However, the floral structures of these three genera are distinct, particularly their rostellum, pollinia, and stipe. The vegetative comparison in these three genera can be considered as their adaptation to similar habitats [16]. Given that this finding is only a speculation, molecular confirmation is still necessary.

Rynchostylis is relatively close to Vanda in terms of its morphological structures, including its robust habit, entirely or slightly trilobed labellum, bilaterally compressed spur with its apex pointing backward, and two cleft pollinia. This species is a relatively primitive genus in the Aerides–Vanda alliance. However, its stipe is long and narrows downwards, which make it appear specialized.

Christension [4] performed the initial branch analysis of the Aerides–Vanda alliance and stated that the generic relation of this alliance may have undergone considerable changes after thoroughly researching each genus. The character status of some genera has changed, but some rather ambiguous genera are reclassified. For example, although Holcoglossum is polymorphic, it has been treated as a single genus until recently [17–19]. However, Liu et al. [11] divided the Holcoglossum alliance into three genera, namely, Holcoglossum, Tsiorchis, and Paraholcoglossum, based on further molecular and morphological analyses of more taxa under this alliance and its allied groups. The two new genera were treated by Jin [18] and Fan et al. [19] as either subgeneric or sectional rank.

Although some molecular and morphological systems of this alliance have been proposed in previous studies [2,20], the relationships among the members of this alliance are unclear. Two species of Papilionanthe have been placed in the section Nupangensia of Holcoglossum after molecular analysis [11]. Thus, to seek clarification of the Aerides–Vanda alliance phylogenetically, molecular and morphological analyses of more species are necessary.

The two recently published genera, Asocentropsis and Chenorchis, which genetically belong to the Aerides–Vanda alliance, are both monotypic. Asocentropsis has been established based on Asocentrum pusillum [12], whereas Chenorchis is perceived to be genetically related to Holcoglossum and Asocentrum [10].

In this study, we focused on improving the sampling of the Aerides–Vanda alliance to facilitate a more accurate reconstruction of the phylogenetic relationships. We collected specimens of 68 species under the 14 genera of the Aerides–Vanda alliance and its three allied genera, with emphasis on Holcoglossum, Paraholcoglossum, Tsiorchis, Chenorchis, Asocentrum, Neofinetia, Seidenfadenia, Christensonia, Trudelia, and Asocentropsis. Based on the molecular and morphological analyses, we provided a well-supported phylogenetic resolution for the placement of the 14 genera in the Aerides–Vanda alliance.

Results

The DNA sequences of 70 taxa, including 68 species of 17 allied genera and two species of Cymbidium as outgroup, were obtained and analyzed. The DNA sequences of all species of Holcoglossum, three species of Paraholcoglossum, two species of Tsiorchis, six species of Asocentrum and one new species similar to Asocentrum himalacum, one species of Chenorchis, three species of Neofinetia, six species of Vanda, two species of Papilionanthe, and three species of Aerides were newly obtained, and other species were accessed from GenBank. Tables 1 and 2 provide the detailed sequence information, aligned length, numbers of variable sites, parsimony informative sites, tree statistics for maximum parsimony (MP) analysis, and the best fit model selected by Modeltest.

ITS Analysis

A total of 67 taxa were analyzed. Most genera form independent clades or subclades. The generic relationships are mostly well resolved. However, the phylogenetic positions of some species are unclear, such as those of Paraholcoglossum auriculatum, Seidenfadenia mitrata, Rynchostylis gigantea, Rynchostylis retusa, and Aerides odorata. Figs. S1, S2, and S3 provide the detailed results.

Chloroplast DNA Analysis

In this analysis, chloroplast DNA (cpDNA), including trnL-F, matK, psbA-trnH, atpB-atpH, and trnS-trnF regions were combined as a dataset for analysis. A total of 68 taxa were analyzed. Most genera form independent clades or subclades. The generic
relationships are mostly well resolved. However, the position of Papilionanthe hookeriana and the phylogenetic relationships of Ascocentrum, Christensonia and Seidenfadenia mirata are unclear. Figs. S4, S5, and S6 provide the detailed results.

Combined Dataset Analysis

We also combined all datasets into a single dataset for the phylogenetic analysis of the Aerides–Vanda alliance. The strict consensus trees strongly suggest that the Aerides–Vanda alliance is monophyletic, and the 14 genera under this alliance can be divided into the following 7 clades with 14 subclades: 1. Aerides, comprising two subclades: Rhynchostylis and Aerides; 2. Ascocentrum; 3. Papilionanthe; 4. Vanda, comprising five subclades: Neofinetia, Christensonia, Seidenfadenia, Ascocentrum, and Vanda; 5. Tsiorchis, comprising three subclades: Chenorchis, Tsiorchis, as well as one species of Ascocentrum and one new species, Pendulorchis gaoligongensis; 6. Paraholcoglossum; and 7. Holcoglossum. Among these clades, only Ascocentrum is triphyletic and comprises two subclades. One is the Ascocentrum subclade that is related to the Seidenfadenia subclade; the other is the Ascocentrum himalacium and Pendulorchis gaoligongensis form an independent subclade that is much more closely related to Tsiorchis than to Ascocentrum subclade. Another species, Ascocentrum piumulum, should be transferred to the Holcoglossum clade. Trudelia species do not form an independent subclade but belong to the Vanda subclade. The molecular conclusion is consistent with their morphological characteristics. Fig. 1 provide the detailed results.

Discussion

Data Analysis

In this study, six DNA regions were utilized, including one nuclear (ITS) and five plastid (trnL-F, matK, psbA-trnH, atpA-atpH, and trnS-trnM) regions. The results show that most phylogenetic relationships based on ITS agree with the plastid datasets and their combination, but some genera such as Rhynchostylis, Siedenfadenia and Paraholcoglossum appear to have some different phylogenetic relationships between ITS and plastid data. Such differences may result from intergeneric hybridization or introgression at some point during the evolution of these genera, which should need further study to testify. We performed an incongruence length difference test between ITS with cpDNA, and the result shows incongruence to a certain extent between the ITS and plastid regions ($P=0.01$), but it did not affect the whole phylogenetic relationship. In fact, different genes (including ITS and cpDNA) are incongruent in many cases. Different genes are known to have different evolutionary rates and can provide different evolutionary information. Thus, we need to use more than one gene to assess their phylogenetic relationship because of their incongruence. Based on our experience and those of other researchers, the obtained phylogenetic relationship is better when more genes are used. The combination dataset still produced the best trees. Among them (Fig. 1), most species belong to their phylogenetic clades or subclades and most nodes have good support. Therefore, we believe that combining a nuclear ITS and plastid regions to solve the phylogenetic relationship is appropriate.

Table 2. Best-fit model and parameter for the analysis datasets.

| Region      | AIC select model | Base frequencies | substitution model(rate matrix) | I | G |
|-------------|------------------|------------------|--------------------------------|---|---|
|             |                  |                  | A-C                           | A-G | A-T | C-G | C-T | G-T |
| ITS         | GTR+H+G          | 0.1717            | 0.3255                        | 0.3616 | 0.1411 | 1.0095 | 3.4540 | 0.9949 | 0.5939 | 4.4265 | 1.0000 | 0.2185 | 0.9418 |
| matK        | GTR+H+G          | 0.3229            | 0.1532                        | 0.1424 | 0.3814 | 1.0576 | 1.2520 | 0.1481 | 0.4903 | 1.0083 | 1.0000 | 0.4153 | 0.9585 |
| trnL-F      | TVM+H+G          | 0.3683            | 0.1222                        | 0.1292 | 0.3802 | 1.2574 | 1.1553 | 0.7785 | 1.362 | 1.1553 | 1.0000 | 0.3645 | 0.3229 |
| psbA-trnH   | GTR+H+G          | 0.3282            | 0.1548                        | 0.1606 | 0.3563 | 1.0275 | 0.9166 | 0.8045 | 0.1412 | 0.2959 | 1.0000 | 0.7543 | 0.7690 |
| atpA-atpH   | K81uf+G          | 0.3534            | 0.1267                        | 0.1812 | 0.3387 | 1.0000 | 1.0952 | 0.2983 | 0.2983 | 1.0952 | 1.0000 | 0.0000 | 1.4207 |
| trnS-trnM   | K81uf+G          | 0.3409            | 0.1666                        | 0.1457 | 0.3469 | 1.0000 | 1.6369 | 0.4514 | 0.4514 | 1.6369 | 1.0000 | 0.0000 | 0.2414 |
| cpDNA       | GTR+H+G          | 0.3388            | 0.1508                        | 0.1518 | 0.3587 | 0.9560 | 1.1765 | 0.5484 | 0.2459 | 0.9456 | 1.0000 | 0.5416 | 0.7061 |
| Combined    | TIM+H+G          | 0.3157            | 0.1757                        | 0.1786 | 0.3301 | 1.0000 | 1.7855 | 0.5976 | 0.5976 | 1.5632 | 1.0000 | 0.4865 | 0.5983 |

Table 1. Statistics from the analysis datasets.

| Information | No. of taxa | Aligned length | No. variable characters | No. informative characters (%) | Tree length | Consistency index | Retention index |
|-------------|-------------|----------------|-------------------------|-----------------------------|-------------|------------------|-----------------|
| ITS         | 67          | 736            | 379                     | 236(32.1%)                 | 991         | 0.5550           | 0.7442          |
| matK        | 68          | 1640           | 359                     | 186(11.3%)                 | 589         | 0.7029           | 0.7528          |
| trnL-F      | 62          | 1541           | 395                     | 221(14.3%)                 | 643         | 0.7372           | 0.8126          |
| psbA-trnH   | 42          | 1025           | 106                     | 53(5.2%)                   | 127         | 0.8740           | 0.8621          |
| atpA-atpH   | 39          | 886            | 223                     | 176(19.9%)                 | 268         | 0.9067           | 0.9351          |
| trnS-trnM   | 39          | 1122           | 110                     | 655(5.8%)                  | 134         | 0.8881           | 0.9198          |
| cpDNA       | 68          | 6214           | 1193                    | 701(11.3%)                 | 1885        | 0.7220           | 0.7720          |
| Combined    | 70          | 6950           | 1572                    | 937(13.5%)                 | 2965        | 0.6445           | 0.7379          |
Overall Tree and Effect of Taxon Sampling

The phylogenetic analyses identified the following seven major clades: 1. *Aerides*, comprising two subclades: *Rhynchostylis* and *Aerides*; 2. *Ascoentropis*, monotypic; 3. *Papilionanthe*, comprising four species; 4. *Vanda*, comprising five subclades, *Neofinetia*, *Chenorchis*, *Seidenfadenia*, four species of *Ascoentrum*, and *Vanda*; 5. *Tsiorchis*, comprising three subclades: *Chenorchis*, *Tsiorchis*, as well as two species of *Ascoentrum* and *Pendulorchis*, *gaoligongensis* (PP 1.00, BS ML 100 and BS MP 97); 6. *Paraholcoglossum*, and 7. *Holcoglossum*, comprising all species of *Holcoglossum* and one species of *Ascoentrum* (PP 1.00, BS ML 95 and BS MP 63). Among the 14 genera, only *Ascoentrum* is monotypic and comprises three subclades. First is the *Ascoentrum* subclade that is related to the *Seidenfadenia* subclade; second is the *Ascoentrum* *himalaticum* and *Pendulorchis gaoligongensis* form an independent subclade that is much more closely related to *Tsiorchis* than to *Ascoentrum*; and third is a species, *Ascoentrum pulinum*, that should be transferred to the *Holcoglossum* clade. The parsimony, maximum likelihood, and Bayesian approaches for the combined dataset result in similar tree topologies, with the identification of seven well-supported major clades (Fig. 2). This topology partially agrees with the proposal of Liu et al. [11].

The seven clades mostly receive moderate to high support (over 50). Species of the *Holcoglossum* clade are related to the species of the *Paraholcoglossum* clade (PP 1.00, BS ML 97 and BS MP 68). *Ascoentrum himalaicum*, often placed in *Holcoglossum*, is related to the *Tsiorchis* subclade (PP 0.99, BS ML 88 and BS MP 78). The *Ascoentrum* species are divided into three groups, belonging to the *Tsiorchis*, *Vanda*, and *Holcoglossum* clades respectively.

Morphological Characteristics and Distribution Analyses

The morphological characteristics of the *Aerides–Vanda* alliance support its division into the following 14 genera. (1) *Rhynchostylis*, which includes two species that are both large plants with flat leaves, laterally compressed and backward-pointing spur, cleft pollinia with narrow stipe much longer than the pollinia, and a small viscidium. This genus is widespread in tropical Asia north to south China. (2) *Aerides* includes 17 species with flat leaves, elongated column, appendiculate spur, bifid rostellum, cleft pollinia, and semicircular viscidium. This genus is widespread in tropical Asia from north China to the Himalayas. (3) *Ascoentropis* is a monotypic genus similar to *Ascoentrum*, from which it differs by having more or less cross-shaped pollinarium with sulcate or split pollinia, visible caudicle, narrowly linear stipe much longer than either the pollinia or viscidium, and strongly incurved side-lobes of the lip. This genus is found in Thailand. (4) *Papilionanthe* includes four species with terete leaves, large flowers, trifoliated lip attached to the column foot, elongated rostellum, and cleft pollinia attached to a common stipe, and purple-marked lip. This genus is found in China (Yunnan, Hainan), Laos, Myanmar, Thailand, and North Vietnam. (12) *Ascoentrum himalaicum* and new species (*Pendulorchis gaoligongensis*), pendulous plant with terete leaves 30 cm to 60 cm long, very small flowers, white lip with very long spur, and very short sepals and petals. These plants are distributed in China (Yunnan), Bhutan, NE India, and Myanmar. (13) *Paraholcoglossum*, consisting of *P. amesianum*, *P. subaflatium*, and *P. auricalatum*, is characterized by a lip shallowly saccate at the base, a ridged callus at the sac entrance, a mid-lobe clawed at the base, and oblong stipe. These plants are found in China (Yunnan, Hainan), India, Laos, Myanmar, Thailand, and Vietnam. (14) *Holcoglossum*, which has 13 species including one new species and one new combination, is characterized by horn-shaped spur, porate pollinia directly attached to a common stipe, and white lip. This genus is found in China (Taiwan, Fujian, Sichuan, Guangxi, and Yunnan) and North Vietnam.

*Holcoglossum* Clade

This clade comprises all species of *Holcoglossum*, including one species transferred from *Ascoentrum*. Our data strongly suggest (PP 1.00) that the *Holcoglossum* clade is related to the *Paraholcoglossum* clade. However, *Tsiorchis* should be placed distantly outside the *Holcoglossum* clade. *Papilionanthe* is treated as an independent clade outside the *Holcoglossum* clade. The aforementioned four genera have marked differences in morphology (Fig. 1). The phylogenetic relationships between *Holcoglossum* and its allied genera are well resolved, and all data sets supported the recognition of *Papilionanthe*. The *Holcoglossum* alliance is divided into the *Tsiorchis*, *Paraholcoglossum*, and *Holcoglossum* clades (PP 1.00).

*Holcoglossum* is treated as a genus of 13 species, including a new species, *H. singchianum*, and a new combination. They all combine to form a homologous subclade. The new species is related to *Holcoglossum quasipumilum*, but distinguishable from it by some marked characteristics and should be treated as a new species.

*Paraholcoglossum* Clade

From the tree of the combined dataset, the *Paraholcoglossum* clade is related to the *Holcoglossum* clade. *Paraholcoglossum* comprises three species: *P. subaflatium*, *P. auricalatum*, and *P. amesianum*. Morphonologically, this genus differs from *Holcoglossum* by a lip that is saccate (not spurred) at the base, a ridged (not crested or fleshy) callus at

Figure 1. Phylogram obtained from Bayesian inference analysis of the combined nrDNA ITS and cpDNA data. Numbers near the nodes are Bayesian posterior probabilities (×100) and bootstrap percentages (PP left, BS ML middle, BS MP right), respectively. “——” indicates that the node was not supported in ML and MP analysis.
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the sac entrance (not mid-lobe base), and an oblong (not tapering) stipe [11].

Three distinct pollination systems are observed in the Holcoglossum and Paraholcoglossum alliance, specifically, the autogamy in Paraholcoglossum amesianum [21], beetle pollination in Holcoglossum rupestre [22], and bee pollination in Holcoglossum nujangense [23].

**Rhynchostylis–Aerides Clade**

This clade consists of the two subclades *Rhynchostylis* and *Aerides*. *Rhynchostylis* has three or four species, and its two Chinese species, which combine to form a subclade, were analyzed in this study. This study further suggests that *Rhynchostylis* is an independent genus. *Aerides* has approximately 20 species, among which 16 species were analyzed in this study. They combine to form a basal subclade of the *Aerides–Vanda* alliance and another basal subclade.
related to the *Rhyynchostylis* subclade. The topology of *Aerides* partially agrees with that described by Kocyan et al. [2].

**Papilionanthe Clade**

This clade is composed of all 12 *Papilionanthe* species. The four species selected in this study combine to form a group distantly related to *Holcoglossum*, which contradicts previous findings [11]. *Papilionanthe* has an elongated stem, terete leaves, two- or three-lobed mid-lobe of the lip, cylindrical or horn-shaped spur, and short cleft pollinia. These characteristics relatively leave an evolutionary trace in *Tsiorchis*, *Paraholcoglossum*, and *Holcoglossum*.

**Ascocentropsis Clade**

The *Ascocentropsis* clade includes a monotypic genus formerly established based on *Ascocentropsis pumila* [12]. The molecular data from this study strongly support the independent position of this clade at the generic level, and prove that the *Ascocentropsis* clade is a basal clade compared with the *Holcoglossum* clade.

**Vanda (Trudelia)–Ascocentrum–Neofinetia Clade**

This group is a multifarious clade composed of the following subclades: (1) *Neofinetia* comprising the monotypic genus *Neofinetia* with three species; (2) *Christensonia*, a monotypic genus that is considered independent at the generic level and related to the *Seidenfadenia* and *Ascocentrum* subclades; (3) *Seidenfadenia*, also a monotypic genus and a subclade related to the *Ascocentrum* subclade; (4) *Ascocentrum*, comprising four species with big flowers and flat leaves, i.e., a subclade related to the *Seidenfadenia* subclade. One other species and one new species (*Pendulorchis gaoligongensis*) with small flowers and terete leaves are considered independent genera placed in the *Pendulorchis–Tsiorchis–Chenorchis* clade. *Pendulorchis gaoligongensis* has unique characteristics and should be treated as a new species. *Ascocentrum pumilum* should be transferred to *Holcoglossum*; and (5) *Vanda*, comprising approximately 40 species, seven of which were selected in this study and combined to form a subclade. *Trudelia alpina* and *Trudelia pumila* should be transferred back to *Vanda*.

**Pendulorchis–Tsiorchis–Chenorchis Clade**

This clade is composed of the following subclades. (1) *Chenorchis* comprising a monotypic genus described in 2008 in China [10]. This species was previously believed [13] to be identical with the species selected in this study; (2) *Tsiorchis*, comprising a monotypic genus described in 2008 in China [10]. The sample in our study is from the type specimen of *Z. J. Liu*, sp. nov. Fig. 2, Fig. S7. [urn:lsid:ipni.org:names: 77125664-1].

**Molecular Phylogeny of the Aerides-Vanda Alliance**

The *Aerides–Vanda* alliance is confirmed to be monophyletic, but it can be divided into 14 genera, including the recently established *Chenorchis*, *Ascocentropsis*, *Christensonia*, *Seidenfadenia*, *Paraholcoglossum*, and *Tsiorchis*. We elucidate the relationship among the 14 genera of the *Aerides–Vanda* alliance, which comprises 7 main clades with 14 subclades. Its basal groups are *Rhyynchostylis* and *Aerides*. Our molecular data prove that *Ascocentrum* is triphyletic. *Ascocentrum pumilum* is closely related genetically to *Holcoglossum* and should be transferred to this genus. One species of *Ascocentrum* and one new species with terete leaves and tiny flowers are treated in this study as a new genus, *Pendulorchis*, which is more closely related to *Tsiorchis* than to *Ascocentrum*. This study suggests that *Vanda alpina* and *Vanda pumila* should not be separated from *Vanda* to form an independent genus, *Trudelia*. A new genus, *Pendulorchis*, and two new species, *Holcoglossum singchanum*, are also described. Three new combinations, *Pendulorchis gaoligongensis*, *Pendulorchis himalaica*, and *Holcoglossum pumilum*, are established as well.

**Taxonomic Treatment**

**Pendulorchis.** Z. J. Liu, Ke Wei Liu et G. Q. Zhang, gen. nov. [urn:lsid:ipni.org:names: 77125664-1].

**Diagnosis.** Genus novum *Pendulorchis* Schlechter ex J. J. Smith et Tsiorchide Z. J. Liu, S. C. Chen et L. J. Chen similis, a quibus plantis pendulis, caulibus 60 cm longis, foliis 5–10 cm latis, lobi mid-lobe spreading forward, obovate, adaxially with three longitudinal midveins; spur cylindric; column stout and short; pollinia two globose, cleft, attached by a common stipe to a large suborbicular viscidium.

**Type.** *Pendulorchis gaoligongensis* G. Q. Zhang, Ke Wei Liu et Z. J. Liu.

**Pendulorchis gaoligongensis.** G. Q. Zhang, Ke Wei Liu et Z. J. Liu, sp. nov. Fig. 2, Fig. S7. [urn:lsid:ipni.org:names: 77125664-1].

**Type.** China, Yunnan, Gaoligongshan, Lushui, 2010 m, growing on the branch of a big tree, 2011. 10. 10, Z. J. Liu 5871 (NOCC).

**Diagnosis.** Species nova *Ascocentrum himalaica* (Deb, Sengupta & Malick) Christenson similis, a quo caulibus 14–25 cm longis foliis 6–16 praeditis, inflorescentiis 5–18 floribus 18 ad 41 praebentibus, viscidio diametro pollinium fere aequanti bene differt.
labello rubello colora sepalasque acuamanti, viscidio diametro pollinium ferre acuamanti bene differit.

**Description.** Epiphytic plants, pendent, with many flattened roots. Stem 14–25 cm long, 4–5 mm in diameter, enclosed by leaf sheaths, often branched. Leaves 6–16, fleshy, deep green, subteere, 40–60 cm long, 4–5 mm in diameter, channelled adaxially; acute at apex, joined and sheathing at base; sheaths 4–5 cm long. Inflorescences 5–16, racemose, arising from the axis of the lower leaves, 7–15 cm long, with 18–41 flowers; floral bracts broadly ovate, 2.2–5 mm long; flowers 6.9 mm in diameter, reddish; pedicel and ovary 1.2–1.5 mm long; dorsal sepal oblong, 4.5–5 mm long; 1.8–2.2 mm wide, rounded at apex; lateral sepal elliptic, 4.5–5 mm long, 2.5–2.8 mm wide, obtuse at apex; petals obovate-elliptic, 4.5–5 mm long, 2.2–4.2 mm wide, obtuse at apex; lip 3-lobed; side-lobe erect, oblone, 2.2–2.5 mm long, 1–1.2 mm wide, obtuse, toward abaxial base strongly concave forming a callus-like structure; mid-lobe spreading forward, obovate, 2.5–3 mm long, 2.5–5 mm wide, adaxially with 3 longitudinal midveins; spur cylindric, 1.2–1.5 cm long, 1.2–1.5 mm thick, obtuse-tipped; column stout and short, 1.8–2 mm long; anther cap purple; pollinia 2, globose, clef, attached by a common stipe to a large suborbicular viscidium.

**Flowering period.** October–November.

**Distribution.** China, SW Yunnan (Lushui County).

**Habitat.** Epiphytic, on branches of tall trees in evergreen forest at an altitude of 1800–2100 m.

**Pendulorchis himalaica.** (Deb, Sengupta & Malick) Z. J. Liu, Ke Wei Liu et X. J. Xiao, comb. nov. [urn:lsid:ipni.org:names: 77125664-1].

**Basionym.** Saccolabium himalaicum (Deb, Sengupta, & Malick) Averyanov in Bot. J. (Leningrad) 73 (1) 101–107, 1988; H. numeicum Z. H. Tsi in Acta Phytotax. Sin. 20 (4): 442. Fig. 1. 1982; Ascocentrum hiiialaicum (Deb, Sengupta, & Malick) Christenson in Notes Bot. Gard. Edinb. 44:256. 1987.

**Distribution.** China and W Yunnan; Bhutan, NE India, and Myanmar.

**Holocoglossum singchianum.** G. Q. Zhang, L.J. Chen, & Z. J. Liu sp. nov. Fig. 3, Fig. S8. [urn:lsid:ipni.org:names: 77125663-1].

**Type.** Yunnan, Xichou, in forest, on tree trunk, alt. 1300 m. 2009. 04. 30. Z. J. Liu 4532 (NOCC).

**Diagnosis.** Species nova Holocoglossum linearifolium similis, a quo differit foliiis 3–4 mm in diam., inflorescentia 12–16-flora, lobo intermedio labelli subobovato-rhambico, ejus lobis lateralibus flavis et bruno-maculatis.

**Description.** Epiphytic plant. Stem nearly ascending, 5–6 cm long, enclosed in persistent leaf sheaths, 7- to 8-leaved. Leaves fleshy, cylindric, 19–37 cm long, 3–4 mm thick, adaxially channelled, acumenate at apex, base dilated into amplexicaul sheaths. Inflorescence 12–16-flowered; peduncle 8–9 cm long, with 2 to 3 tubular sheaths; rachis 14–20 cm long; floral bracts broadly ovate, 3–4.5 cm long, obtuse at apex; pedicel and ovary 2.4–3.5 cm long; flowers 3.5–3.8 cm across; sepalas and petals white with a purple midvein; mid-lobe of lip white, purple-spotted on 3 mm long; flowers 2–3 mm long; column stout and short, 1.8–2 mm long; anther cap purple; pollinia 2, globose, clef, attached by a common stipe to a large suborbicular viscidium.

The new species is similar to H. linearifolium, differing by its nearly ascending stem, leaves 3–4 mm thick, inflorescence 12- to 16-flowered, lip with subobovate-rhombic mid-lobe, and yellow side-lobes spotted with brown.

**Flowering period.** November to December.

**Distribution.** China, SE Yunnan (Malipo County).

**Habitat.** Epiphytic, on tree trunks in broad-leaved forests, 1300–1500 m.

**Holocoglossum pumilum.** (Hayata) L. J. Chen, J. Xiao et G. Q. Zhang comb. nov. [urn:lsid:ipni.org:names: 77125664-1].

**Basionym.** Saccolabium pumilum Hayata in Bot. Mag. (Tokyo) 20:77, 1906.

**Synonym.** Ascocentrum pumilum (Hayata) Schlechter in Repert. Spec. Nov. Regni Veg. Br. 4:285. 1919; Ascolabium pumilum (Hayata) S. S. Ying in Col. Ill. Inding. Orch. Taiwan 1:54, 1977.

**Distribution.** Taiwan, China.

**Materials and Methods**

**Total DNA was extracted from fresh material or silica gel-dried plant tissue using a MultiSource Genomic DNA Miniprep Kit (Axygen Biosciences) following the manufacturer's instructions. The amplification reaction included total DNA, primers, Ex-Taq buffer, and Ex-Taq DNA polymerase (Takara Bio). The polymerase chain reaction (PCR) profile consisted of an initial 5 min pre-melting stage at 95°C, followed by 30 cycles of 30 s at 95°C (denaturation), 30 s at 50°C to 55°C (annealing temperature was determined based on the primer requirement), and 1 min to 3 min at 72°C (extension time was determined based on the length of the target DNA region), and a final 10 min extension at 72°C.

The amplification of the ITS region was performed using the primer pairs ITS A and ITS B [26]. The trnL-F region was amplified with primers c and f [27] or the two sets of primers developed by Liu et al. [11]. The trnK-F sequence was amplified using the primer pairs trnK-19F and trnK-2R [26], and several fragments were amplified using the three sets of primers developed by Liu et al. [11]. The psbA-trnH region was amplified and sequenced by the primer pairs psbAF and trnHR [28]. The atpF-atpH region was amplified and sequenced using the primer pairs atpF and atpH [29]. The trnS-trnM region was amplified and sequenced using the primer pair trnS-trnM [30]. Table 3 contains the detailed information.

The PCR products were run on 1.5% agarose gels to assess the quality of the amplified DNA. The gels with the target products were excised, purified using DNA Gel Extraction Kits (Axygen Biologicals), and then sequenced at Macrogen (South Korea).
Sequence Editing and Assembling

Both forward and reverse sequences, as well as electropherograms were edited and assembled using DNASTAR (http://www.dnastar.com/). DNA sequences were aligned to the muscle model and manually adjusted using MEGA5.05 [31]. Aligned sequences are available from the corresponding authors upon request.

Data Analyses

The datasets included a nuclear ITS, plastid DNA (cpDNA; including the trnL-F, matK, psbA-trnH, atpH-atpI, trnS-trnG) and their combination. Insertions, deletions, and some unavailable sequences were treated as missing. Phylogenetic analyses were performed under ML, MP, and Bayesian inference (BI) methods. The best-fit model for each dataset was selected by Modeltest 3.7 [32] under the Akaike Information Criterion (Table 2). The homogeneities between nrDNA ITS data and the combined cpDNA dataset were tested using the incongruence length difference (ILD) test [33], as implemented in PAUP* version 4.0b10 [34]. The ILD test was conducted with 1000 replicates, each with 10 random addition sequence replicates, TBR branch swapping, and keeping no more than 100 trees per random addition replicate. Following Cunningham [35], a significance level of P = 0.01 was adopted for this test.

MP analyses were performed using the PAUP* version 4.0b10 [34]. All characters were equally weighed and unordered. Test settings included 1000 replications of random addition sequence and heuristic search with tree bisection-reconnection branch swapping. Table 1 lists the tree length, consistency index (CI), and
Table 3. Primers used in this study.

| Primer | Sequence(5’→3’) | Origin |
|--------|-----------------|--------|
| ITS A  | GGAAGGGAGAACGTCAACAGG | Mike et al. [26] |
| ITS B  | CTTTCTCCCGCTTATGAGT | Mike et al. [26] |
| trnL-C | CGAATCGGTAAGGCCTAGG | Taberlet et al. [27] |
| trnL-F | ATGGAAGATCCGAGCAGG | Taberlet et al. [27] |
| trnL-MF | TAAAGGGAAGTTGCTCATTTTAC | Liu et al. [11] |
| trnL-MR | GGAGGAAATGAAATGCTG | Liu et al. [11] |
| trnK-2R | AACCTAGGCGGCCGAGTAG | Mike et al. [26] |
| matK-19F | CGTCCTCATATGGCCACTAG | Mike et al. [26] |
| matK-969R | CTTTCTCTGATATCGAAGATC | Liu et al. [11] |
| matK-731F | AAGAAAAGATCTTGGTCCCT | Liu et al. [11] |
| psbAF | GTTATGCGATACGTTGGTCTG | Sang et al. [28] |
| mmHR | CGGCGATGAGCAATCAGCAT | Sang et al. [28] |
| atpI | TATTTAAGGATCGTTGACCCTAAGCT | Shaw et al. [29] |
| atpH | CCAAYCAGCCAGCATAAAC | Shaw et al. [29] |
| trnS | GAGAGAGGGATATTGCTG | Demesure [30] |
| trnM | CATACCCTTGAGCTCCGGG | Demesure [30] |

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Supporting Information

Figure S1 Bayesian consensus trees based on ITS data. The Bayesian posterior probability (×100) is given above the branches. (TIF)

Figure S2 Maximum likelihood (ML) trees of ITS computed by RAxML with 100 bootstrap replicates. The bootstrap values are given above the branches. (TIF)

Figure S3 Strict consensus tree of most parsimonious trees based on ITS data. Tree length = 991 steps, CI = 0.5550, and RI = 0.7442. The bootstrap values of the maximum parsimony analysis are given above the branches. (TIF)

Figure S4 Bayesian trees based on cpDNA combined dataset. The Bayesian posterior probability (×100) is given above the branches. (TIF)

Figure S5 Maximum likelihood (ML) trees of cpDNA combined dataset computed by RAxML with 100 bootstrap replicates. The bootstrap values are given above the branches. (TIF)

Figure S6 Strict consensus tree of most parsimonious trees based on cpDNA combined dataset. Tree length = 1885 steps, CI = 0.7220, and RI = 0.7720. The bootstrap values of the maximum parsimony analysis are given above the branches. (TIF)

Figure S7 Pendulorchis gaoligongensis G. Q. Zhang, Ke Wei Liu et Z. J. Liu. a. Plant on tree trunk, b. Flowering plant; c. Inflorescence; d. Flower, front view; e. Pollinariaium, front view; f. Pollinariium, back view; g. Flower, side view. (TIF)

Figure S8 Holcoglossum singchianum G. Q. Zhang, L. J. Chen et Z. J. Liu. a. Flowering in cultivation; b. Inflorescence; c and d. Flower, front view and side view; e and f. Pollinariaium, front and back views. (TIF)

Table S1 Species and gene regions sequenced for analysis, as well as GenBank accession numbers. (DOC)

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Author Contributions

Conceived and designed the experiments: ZJL GQZ WCT KWL XJX. Performed the experiments: ZJL GQZ WCT LJC JWZ. Analyzed the data: ZJL JC YHY GQZ LJC XYM KWL. Contributed reagents/materials/analysis tools: ZJL WHR JH SWC LQL. Wrote the paper: ZJL GQZ LQH KWL XJX WCT LJC.
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