A preliminary phylogenetic revision and temporal build-up of *Aeschynanthus* (Gesneriaceae) with insights into the evolution of bird pollination systems

Jing-Yi Lu

Introduction:

Gesneriaceae possess exceptional diversity in flowers. Research on pollination ecology and floral morphology has revealed the members of families interact with almost all major functional groups of pollinators, namely bees, butterflies, flies, birds, and bats (Sanmartin-Gajardo and Sazima 2005; Martén-Rodríguez et al. 2009). Among the diverse pollination systems, the interactions with hummingbirds have been demonstrated to trigger the diversification of the neotropical clades (Serrano-Serrano et al. 2017). On the other hand, the overall ornithophily in the family is thought to reflect the globally uneven distribution of specialist birds (Cronk and Ojeda 2008). That is, bird pollination is less common and important as the diversification diver in the hummingbird-absent Old World. However, detailed research on the evolution of pollination systems and diversification in Southeast Asia, where sunbirds may take over the roles of hummingbirds, are scarce. In Gesneriaceae, *Aeschynanthus* and *Agalmyla* make up a mainly ornithophilous group with 240 species and their diversification patterns await exploration.

The genus *Aeschynanthus* is particular of-interest since the floral morphology varies among its 160 species and pollination syndromes of a few species indicate putative pollinator shifts or reversal to insect pollination. For example, a pollinator shift was inferred in *A. acuminatus* since it possesses a floral morphology deviated from most of its typical ornithophilous congeneric species. A recent study has confirmed that the species is pollinated by a group of uncommon pollinators, generalist birds, in Taiwan, where sunbirds are absent (Chen et al. 2019). Furthermore, the widespread distribution of the species provides an opportunity to investigate the process of speciation in ornithophilous clades. However, the lack of knowledge about its phylogenetic position in the genus blurs the interpretation of its evolutionary history.

The pioneering work on *Aeschynanthus* evolution reconstructed the phylogeny using a single nuclear marker, internal transcribed spacer region (*ITS*) (Denduangboripant et al. 2001). Two

major clades mostly congruent with the geographic distribution led to the inference of its biogeographic patterns: the origination in Southeast Asia followed by a vicariance event into mainland Southeast Asia (Clade I, India-Indochina clade) and Malasia (Clade II, Philippines, Borneo, Indonesia, and New Guinea). However, the single genetic region and incomplete taxa sampling, 50 out of over 160 species, in this study only provided a partial understanding of the evolutionary history. In the case of generalist pollinated *A. acuminatus*, the sister species remained unknown in the phylogeny. The section *Haplotichium s.s.*, a six species clade defined by seed morphology (Mendum et al. 2001), where the species belongs have only two species samples. Whether the species in this section are monophyletic and closest relatives of *A. acuminatus* still need further examination.

The increased numbers of sequences of different genetic regions from more *Aeschynanthus* species deposited in GenBank since the work can help to advance the phylogeny. Also, a series of taxonomy revisions in the Indochinese region (Middleton 2007, 2009, 2016), covering most species from Clade I, provides a more solid taxonomic background for updates on sample identification. Conversely, the taxonomic revision including several combinations can also be validated by the updated phylogeny. Furthermore, a recent comprehensive analysis of the diversification patterns of Gesneriaceae (Roalson and Roberts 2016) can act as a time framework to build up the temporal patterns of *Aeschynanthus* evolution. The inferred geological times of key events, such as the origination of *Aeschynanthus* and the speciation event of *A. acuminatus*, then can be compared with ornithological studies to test the proposed hypotheses of coevolution.

In this study, I aim to gather current available sequences data of *Aeschynanthus* on GenBank to reconstruct its evolutionary history. Firstly, update and revise the phylogeny to compare with the update taxonomic framework. Secondly, focusing on the historical event related to *A. acuminatus*, reveal its sister species and examine the monophyly of section *Haplotrichium.* Lastly, I will estimate the divergence times in the use of the molecular clock. Temporally build up the key events in the evolutionary history of *Aeschynanthus* and compare them with the evolutionary timing of interacting birds and geological events.

Methods:

Taxon sampling and sequences alignment

On GenBank, 219 currently available nucleotide accession records belong to the genus *Aeschynanthus* [accessed 2019-11-26] (see supplementary S1 for the entire list of sequences on GenBank). Among them, over half of the sequences are from the early phylogenetic studies of the genus using only *ITS*: 158 sequences of *ITS1* and *ITS2* from 50 individual samples (Denduangboripant et al. 2001). Other available genetic regions include nuclear external transcribed spacer (*ETS*), plastid *trnL-trnF* and *psbA-trnH* intergenic spacer, maturase K (*MatK*) gene, etc., from individual submissions, studies of closely related genus *Cyrtandra* (Johnson et al. 2017) and intrageneric studies of Old World Gesneriaceae (Möller et al. 2009, 2011). The commonly used, especially for the New World Gesneriaceae, *CYCLOIDEA*-like genes (*GCYC*) were not included since multiple duplication events and possibly species-specific selective signals found in previous studies (see Hsin et al. 2019 for example).

After considering the overall coverage of taxa and the inclusion of novel species, four genetic regions were selected for further analyses: *ITS*, *ETS*, *trnL-trnF* and *psbA-trnH*. Four unpublished *trnL-trnF* sequences of *A. acuminatus*, *A. albidus*, and *A. chiritoides* were also included (see S2). To allow a secondary calibration points to be implemented (described below), outgroup sequences were chosen to represent the newly revised subtribe 'Didymocarpinae' (includes *Aeschynanthus*) and its closely related 'Streptocarpinae' (Weber et al. 2013).

Sequences for each genetic region were automated aligned separately using MUSCLE 3.8 (Edgar 2004) and then manually examined and adjusted in Mesquite 1.12 (Maddison and Maddison 2018). In contrast to Denduangboripant et al. (2001), the cloned sequences of ITS2 were not combined into consensuses as heterozygosity but treated as variants (alleles) sampled from a single individual. Sequences from different publications or individual vouchers were also pooled in several cases and treated as *samples* in the analyses. These treatments might confound the phylogenetic signals if species were misidentified but may be justified if the results are carefully interpreted, especially for non-monophyletic relationships of *samples* from a single species.

Phylogeny reconstruction

For each genetic region, the most probable substitution model was chosen by jModelTest 2 (Darriba et al. 2012), comparing 88 models and choosing based on corrected Akaike Information Criteria (AICc) (Hurvich and Tsai 1989). The four data sets were then concatenated into a single matrix for further analyses. The phylogenetic analyses for each region were not conducted because of the huge unbalance of taxa numbers, e.g. 108 for *ITS* vs. 24 for *trnL-trnF*.

The Maximum Likelihood (ML) analyses were conducted using RAxML v.8.1.12 (Stamatakis 2014). Each region was designated a separate partition with the substitution model as GTRCAT. The ML search algorithm was the option of rapid Bootstrap analysis and searching for besting scoring ML tree with 1000 bootstraps.

Bayesian inference of phylogenetic reconstruction was performed by MrBayes v.3.2.7 (Ronquist et al. 2012). Each region was also designated a separate partition. The substitution was not specified but estimated by Reversible jump MCMC (RJMCMC). Two runs of four Markov chain Monte Carlo (MCMC) chains were run in parallel for 5 million generations, with a burn-in of 10% and a sampling frequency of every 1000 generation. Convergence was assessed using the potential scale reduction factor (PSRF = 1.0) and the average standard deviation of split frequencies (<0.01). Additionally, the effective sampling size (ESS > 200) for all parameters and the traces of likelihoods were examined in Tracer v.1.7.1 (Rambaut et al. 2018). A consensus tree was reconstructed from trees of a single run and posterior probabilities determined by TreeAnnotator included in BEAST v.1.10.4 (Suchard et al. 2018).

Estimation of divergence time

The secondary time calibration point was chosen to be the crown age of the subtribe 'Didymocarpinae.' A lognormal distribution with mean = 42.0 and standard deviation = 7.0 was set to approximate the mean of 41.26 mya and 95% confidence interval of [21.96, 51.00] of the crown age inferred in the previous study of Gesneriaceae (Roalson and Roberts 2016). The four-region concatenated sequence matrix was analyzed using BEAST v.1.10.4 while the substitution models were unlinked and set to the closest approximates of the results of jModelTest 2. Two independent MCMC chains were run for 10 million generations with a sampling frequency of every 1000 generation. The two runs were also inspected in Tracer v.1.7.1 for ESS and traces of

likelihood. Then, the trees of two runs was combined using LogCombiner and the consensus tree were constructed with maximum clade credibility by TreeAnnotator.

Results:

Taxon sampling and sequences alignment

Each genetic region was compared after alignment to examine the unequal lengths resulting from various markers used by different studies. Two ends were trimmed to include only the portion that at least about half of the samples contains nucleotides (see S3 for sequence alignment). The resulted lengths of each genetic markers were as follows: ITS – 800 bps; *trnL-trnF* – 904 bps; *psbA-trnH* – 422 bps; and *ETS* – 441 bps.

The concatenated four-region matrix contained a total of 2567 bps and was composed of 96 *samples* from 55 unique taxa of *Aeschynanthus* (52 currently recognized species, list in S4, see discussion) with 14 *samples* from 12 taxa of outgroups.

Phylogeny reconstruction

The best substitution models based on the AICc criterion were as following: *ITS – TrNef*+G; *trnL-trnF – TVM*+I; *psbA-trnH – TPM1uf*+G; and *ETS – TrNef*+G. However, as the GTRCAT model and the RJMCMC were implemented in ML and BI analyses, respectively, the model selected was specified only in the further analyses of divergence time in BEAST.

The reconstructed phylogenies from ML (S5) and BI (Figure 1 and S6) were generally congruent. The monophyly of Didymocarpinae and the genus *Aeschynanthus* are highly supported by ML bootstrap values (95/100) and BI posterior probabilities (0.99/1). The sister genus of *Aeschynanthus* remains unclear with *Agalmyla* and *Oreocharis* as candidates, consistent with the results of Möller et al. 2011 but different from Roalson and Roberts (2016).

Within the genus, the two clades which were discovered in Denduangboripant et al. (2001), Clade I and Clade II, were also revealed in the current study with supports (ML: 100/67; BI: 1/1). In Clade I, a grade of three monophyletic clades were recognized, including the only monophyletic traditionally recognized taxonomic section, *Haplotrichium* (ML: 100/75/63; BI:

1/0.97/0.81). The last-diverging and the most specious clade have two major lineages (ML: 30/66; BI: 0.78/0.96). On the other hand, Clade II consisted of two major groups (ML: 73/76; BI: 0.93/0.99) with two early-diverging species *A. rhododendron* and *A. philippinensis* having uncertain positions. Different *samples* of the same species usually formed monophyletic clades with only a few exceptions, However, the interspecific relationships close to tips were generally unresolved. but most of the exceptions are the mix of sister species, e.g. *A. buxifolius* and *A. garretii*. The only truly contradicted position of *samples* from a single species is *A. burtii*, which are unknown species in the original article (Denduangboripant et al. 2001) with the identities coming from updated identification of voucher specimens.

Estimation of divergence time

The estimated divergence times generally have high variance (Figure 2, 95% confidence interval as node bars.) The posterior estimate of the crown age of Didymocarpinae was 33.45 mya with a 95% confidence interval = [22.10, 44.62], younger than the prior estimate with smaller 95% confidence interval. The crown age of the genus *Aeschynanthus* was estimated with a mean of 22.31 mya with a 95% confidence interval of [13.91, 31.78], older than the estimates of Roalson and Roberts (2016). Section of-interest, *Haplotrichium*, had a stem age of 14.64 mya [8.76, 21.55] and a crown age of 9.35 mya [3.57,15.96]. The speciation event of *A. acuminatus* from its sister species *A. moningeriae* in the current study was estimated with a mean of 2.73 mya with a 95% confidence interval of [0.68, 5.44].

Discussion:

Phylogenetic revision of Aeschynanthus: updates from increased sampling & taxonomy revision

The taxonomic revision of *Aeschynanthus* has affected several species *samples* included in the phylogeny. The cases of species combination include 1) *A. macranthus* in Thailand was included in *A. fulgens* and *A. pachytrichus* was regarded as synonyms of *A. parasiticus* (Middleton 2007, 2009). The four samples now recognized as *A. fulgens* form a highly supported clade (Figure 1). After the taxonomic revision, the relationships between *A. fulgens* and *A. parasiticus* can also be examined in the phylogeny. Despite their high morphological similarity, the two taxa each form a distinct clade respectively in a group of species with similar floral colors (Figure 1), supporting the treatments by Middleton. 2) *A. parvifolius* and *A. javanicus* were now synonymized as *A.*

pulcher (Middleton 2007, 2016). The *samples* of these two taxa, however, did not form an exclusive clade but group with *samples* from *A. radicans* and *A. chrysanthus* (Figure 1). According to Middleton (2016), *A. pulcher* is a variable species but different from *A. radicans* by the pubescence on the ovary. Natural hybridization was also suggested possible based on specimen records. This complex including *A. pulcher*, *A. radicans*, and *A. chrysanthus* can be a single species or close relatives with introgression and hybridization but need further investigation. 3) The inclusion of *A. hildebrandii* in *A. andersonii* (Middleton 2007). The *samples* of the former taxon were retained its name in this study (Asterisk in Figure 1 and Figure 2.) *A. andersonii* formed a well-supported clade with *A. humilis A. hildebrandii* was highly supported to be sister to these two taxa. Therefore, synonymized *A. hildebrandii* needs further validation. 4) The inclusion of *A. austroyunnanensis* into *A. micranthus* (Middleton 2009) cannot be validated as only *samples* from the former species are available in the phylogeny.

There are also a few novel species included in the updated phylogeny, providing new information on the species relationship and clade distribution. For example, the south and east most species, *A. solomonensis*, was placed in the Malasian Clade II and had the New Guinean *A. guttatus* as sister species (Figure 1). The putatively only insect-pollinated species, *A. chiritoides*, was also included and placed in the most specious subclade within Clade I. However, its sister species remained unknown. Multiple samples in Denduangboripant et al. (2001) with unknown identities were also updated with the further identification of the voucher specimens, including *A. burtii* and *A. lobaticalyx*. The two samples of *A. burtii* being placed in the different major clades (Daggers marked *samples* in Figure 1 and Figure 2) may be the results of misassignment of voucher number or misidentification though.

The monophyly of the section Haplotrichium and the sister species of A. acuminatus

The monophyly of the section *Haplotrichium* was supported by both ML and BI reconstructed phylogenies (Green arrow in Figure 1). The monophyletic group including three species in the current phylogeny differs from a grade-like two species relationship between *A. bracteatus* and *A. acuminatus* in the previous study. The change may result from the inclusion of the species *A. moningeriae* and more genetic regions (three species shared at least both *ITS* and *trnL-trnF* regions). In current sampling, the Hainan endemic species, *A. moningeriae*, was the sister species

of *A. acuminatus*. It is also the most morphologically similar species in the section with longer and redder corolla tubes. A recently published new species, *A. pedunculatus*, shares morphological features with *A. acuminatus* and *A. moningeriae* and is likely to be the closest relative of the two (Middleton 2009). Further sampling is in need to disentangle their relationship.

Temporal patterns of the evolution of Aeschynanthus

The origination time of *Aeschynanthus* was estimated to date back to 22.31 mya (Red arrow in Figure 2). Although large confidence interval hints the uncertainty, the time was plausibly older than several Asian bird pollinated clades, e.g. *Erythrina* (Fabaceae): 7.4 mya (Li et al. 2013). If *Agalmyla* was the sister genus as Möeller et al. (2011) suggested, the origination of ornithophily of this clade will extend further back. To compare the major pollinator group of *Aeschynanthus*, the estimates of the divergence time (stem age) of sunbirds (Nectariniidae) from its close relative flowerpeckers (Dicaeidae) was slightly earlier than 20 mya in the mid-Miocene (Oliveros et al. 2019). The appearance of ornithophily in *Aeschynanthus* and *Agalmyla* can be interpreted to coincide with or earlier than the origination of nectar specialist passerines, which can be either sunbirds alone or the ancestors of sunbirds and flowerpeckers.

Time comparison of the speciation event of A. acuminatus

The speciation time of *A. acuminatus* from its sister species in this study was estimated to be 2.73 mya. This time, even with uncertainty, could be used in the preliminary examination of speciation and coevolutionary hypotheses to rule out unlikely explanations.

One of the possible scenarios of the speciation process is the *shift on island and recolonization*: The speciation of *A. acuminatus* occurred on Taiwan island with strong selective pressure in the absent of sunbirds. The current distribution of this species was the consequence of recolonization after the species adopted the generalist bird pollination system. To allow the scenario to be possible, the speciation time must be no earlier than the formation of Taiwan island. The formation of the island dated back to 9 mya and attained its current form around 5-6 mya (Sibuet and Hsu 1997, 2004; summarized for plant biogeography in Chiang and Schaal 2006). The estimated speciation time was far later. Even considering the crown age of *Haplotrichium*, in the

situation that *A. moningeriae* speciate after their common ancestor embraced novel pollinator on Taiwan island, 9.35 mya is still not able to rule out this *shift on island* scenario.

Chen et al. (2019) identified three species of babblers, Taiwan yuhina (*Yuhina brunneiceps*), White-eared sibia (*Heterophasia auricularis*), and Grey-cheeked fulvetta (*Alcippe morrisonia*) from different bird families are the main pollinators for the species in Taiwan. The first two species are endemic to Taiwan. Thus, comparing their speciation time with the speciation could also explore the potential simultaneous or sequential movement of *A. acuminatus* and its interacting partners to Taiwan island. The recent divergence time investigation of all babblers (Cai et al. 2019) estimated the speciation of these two species from their close relatives to be 5 mya or slightly younger. Therefore, the appearance of the two endemic species in Taiwan was inferred to be right after the final formation of the island (5-6 mya), following by the speciation of *A. acuminatus* around 2.7 mya. However, if the oldest possible pollinator shift mentioned in the previous paragraph was considered, the age of 9.35 mya would predate the colonization of these generalist birds and coincide with the earliest appearance of "proto Taiwan."

These inferences were preliminary, and the interpretations need cautions. Besides the uncertainty of divergence time estimates, the incomplete sampling of the widespread *A. acuminatus* (Irimote, Taiwan, and the geographic adjacent mainland province, Guangdong) may lead to biased estimates of its relationships and divergence time with *A. moningeriae*. For example, in the discussion in previous paragraphs, the timing of the actual pollinator shifts could range from the stem age of 9.35 mya to the crown age of 2.73 mya in different possible scenarios. The inclusion of the information from molecular samples and pollinator assemblages of different populations can potentially resolve the timing to finer scales.

Reference:

Cai, T., A. Cibois, P. Alström, R. G. Moyle, J. D. Kennedy, S. Shao, R. Zhang, M. Irestedt, P. G. P. Ericson, M. Gelang, Y. Qu, F. Lei, and J. Fjeldså. 2019. Near-complete phylogeny and taxonomic revision of the world's babblers (Aves: Passeriformes). Mol. Phylogenet. Evol. 130:346–356.

Chen, K.-H., J.-Y. Lu, and C.-N. Wang. 2019. Effective pollination of *Aeschynanthus*

acuminatus (Gesneriaceae) by generalist passerines, in sunbird-absent East Asia. Sci. Rep. 9:17552.

Chiang, T.-Y., and B. A. Schaal. 2006. Phylogeography of plants in Taiwan and the Ryukyu Archipelago. Taxon 55:31–41.

Cronk, Q., and I. Ojeda. 2008. Bird-pollinated flowers in an evolutionary and molecular context. J. Exp. Bot. 59:715–727.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9:772–772. Nature Publishing Group.

Denduangboripant, J., M. Mendum, and Q. C. B. Cronk. 2001. Evolution in *Aeschynanthus* (Gesneriaceae) inferred from ITS sequences. Plant Syst. Evol. 228:181–197.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with improved accuracy and speed. Pp. 689–690 *in* Proceedings. 2004 IEEE Computational Systems Bioinformatics Conference, 2004. CSB 2004. IEEE.

Hsin, K.-T., J.-Y. Lu, M. Möller, and C.-N. Wang. 2019. Gene duplication and relaxation from selective constraints of *GCYC* genes correlated with various floral symmetry patterns in Asiatic Gesneriaceae tribe Trichosporeae. PLoS One 14:e0210054.

Hurvich, C. M., and C.-L. Tsai. 1989. Regression and time series model selection in small samples. Biometrika 76:297–307.

Johnson, M. A., J. R. Clark, W. L. Wagner, and L. A. McDade. 2017. A molecular phylogeny of the Pacific clade of *Cyrtandra* (Gesneriaceae) reveals a Fijian origin, recent diversification, and the importance of founder events. Mol. Phylogenet. Evol. 116:30–48. Elsevier Inc.

Li, H., W. Wang, L. Lin, X. Zhu, J. Li, X. Zhu, and Z. Chen. 2013. Diversification of the phaseoloid legumes: effects of climate change, range expansion and habit shift. Front. Plant Sci. 4:1–8.

Maddison, W. P. and D.R. Maddison. 2018. Mesquite: a modular system for evolutionary analysis. Version 3.51 http://www.mesquiteproject.org

Martén-Rodríguez, S., A. Almarales-Castro, and C. B. Fenster. 2009. Evaluation of pollination syndromes in Antillean Gesneriaceae: Evidence for bat, hummingbird and generalized flowers. J. Ecol. 97:348–359.

Mendum, M., P. Lassnig, A. Weber, and F. Christie. 2001. Testa and seed appendage morphology in *Aeschynanthus* (Gesneriaceae): phytogeographical patterns and taxonomic

implications. Bot. J. Linn. Soc. 135:195–213.

Middleton, D. J. 2009. A revision of *Aeschynanthus* (Gesneriaceae) in Cambodia, Laos and Vietnam. Edinburgh J. Bot. 66:391–446.

Middleton, D. J. 2016. A revision of *Aeschynanthus* (Gesneriaceae) in Singapore and Peninsular Malaysia. Gard. Bull. Singapore 68:1.

Middleton, D. J. 2007. A revision of *Aeschynanthus* (Gesneriaceae) in Thailand. Edinburgh J. Bot. 64:363–429.

Möller, M., A. Forrest, Y.-G. Wei, and A. Weber. 2011. A molecular phylogenetic assessment of the advanced Asiatic and Malesian didymocarpoid Gesneriaceae with focus on non-monophyletic and monotypic genera. Plant Syst. Evol. 292:223–248.

Möller, M., M. Pfosser, C.-G. Jang, V. Mayer, A. Clark, M. L. Hollingsworth, M. H. J. Barfuss, Y.-Z. Wang, M. Kiehn, and A. Weber. 2009. A preliminary phylogeny of the 'didymocarpoid Gesneriaceae' based on three molecular data sets: Incongruence with available tribal classifications. Am. J. Bot. 96:989–1010.

Oliveros, C. H., D. J. Field, D. T. Ksepka, F. K. Barker, A. Aleixo, M. J. Andersen, P. Alström, B. W. Benz, E. L. Braun, M. J. Braun, G. A. Bravo, R. T. Brumfield, R. T. Chesser, S. Claramunt, J. Cracraft, A. M. Cuervo, E. P. Derryberry, T. C. Glenn, M. G. Harvey, P. A. Hosner, L. Joseph, R. T. Kimball, A. L. Mack, C. M. Miskelly, A. T. Peterson, M. B. Robbins, F. H. Sheldon, L. F. Silveira, B. T. Smith, N. D. White, R. G. Moyle, and B. C. Faircloth. 2019. Earth history and the passerine superradiation. Proc. Natl. Acad. Sci. 116:7916–7925.

Rambaut, A., A. J. Drummond, D. Xie, G. Baele, and M. A. Suchard. 2018. Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. Syst. Biol. 67:901–904.

Roalson, E. H., and W. R. Roberts. 2016. Distinct Processes Drive Diversification in Different Clades of Gesneriaceae. Syst. Biol. 65:662–684.

Ronquist, F., M. Teslenko, P. van der Mark, D. L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard, and J. P. Huelsenbeck. 2012. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Syst. Biol. 61:539–542.

Sanmartin-Gajardo, I., and M. Sazima. 2005. Chiropterophily in sinningieae (Gesneriaceae): *Sinningia brasiliensis* and *Paliavana prasinata* are bat-pollinated, but *P. sericiflora* is not.

Not yet? Ann. Bot. 95:1097–1103.

Serrano-Serrano, M. L., J. Rolland, J. L. Clark, N. Salamin, and M. Perret. 2017. Hummingbird pollination and the diversification of angiosperms: an old and successful association in Gesneriaceae. Proc. R. Soc. B Biol. Sci. 284:20162816.

Sibuet, J.-C., and S.-K. Hsu. 2004. How was Taiwan created? Tectonophysics 379:159–181.

Sibuet, J. C., and S. K. Hsu. 1997. Geodynamics of the Taiwan arc-arc collision. Tectonophysics 274:221–251.

Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313.

Suchard, M. A., P. Lemey, G. Baele, D. L. Ayres, A. J. Drummond, and A. Rambaut. 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. Virus Evol. 4:1–5.

Weber, A., J. Clark, and M. Möller. 2013. A new formal classification of Gesneriaceae. Selbyana 31:68–94.

Figures

Figure 1. Reconstructed phylogeny of *Aeschynanthus* with selected samples from Didymocarpinae and Streptocarpinae. The topology is the consensus Bayesian Inference phylogeny from the first chain of 5 million MCMC search. The labels above branches are the posterior probabilities and the labels below are the bootstrap values from Maximum Likelihood analyses (Topology see S5). The statistical supports under 50% are not shown. The red arrow indicates the branch led to the genus while the green arrow points to the branch led to the section *Haplotrichium s.s*. The asterisk marks the retained taxon name of *A. hildebrandii* and the daggers label the two positions of *A. burtii* in the phylogeny.

Figure 2. Divergence time estimates of *Aeschynanthus*. The scale bar represents the geological time scale in million-year (my) units. The node labels are the estimated mean of every node (bifurcating event). The blue bars demonstrate the 95 % confidence interval of the estimates. The arrows, asterisk, and daggers indicate the same contents as in Figure 1.

Supplementary Data

The supplementary data are available on https://github.com/jingyilu/rtol_final_project.

S1 spreadsheet. The entire list of all the sequences available on GenBank of *Aeschynanthus* and selected outgroups with reference of each accession.

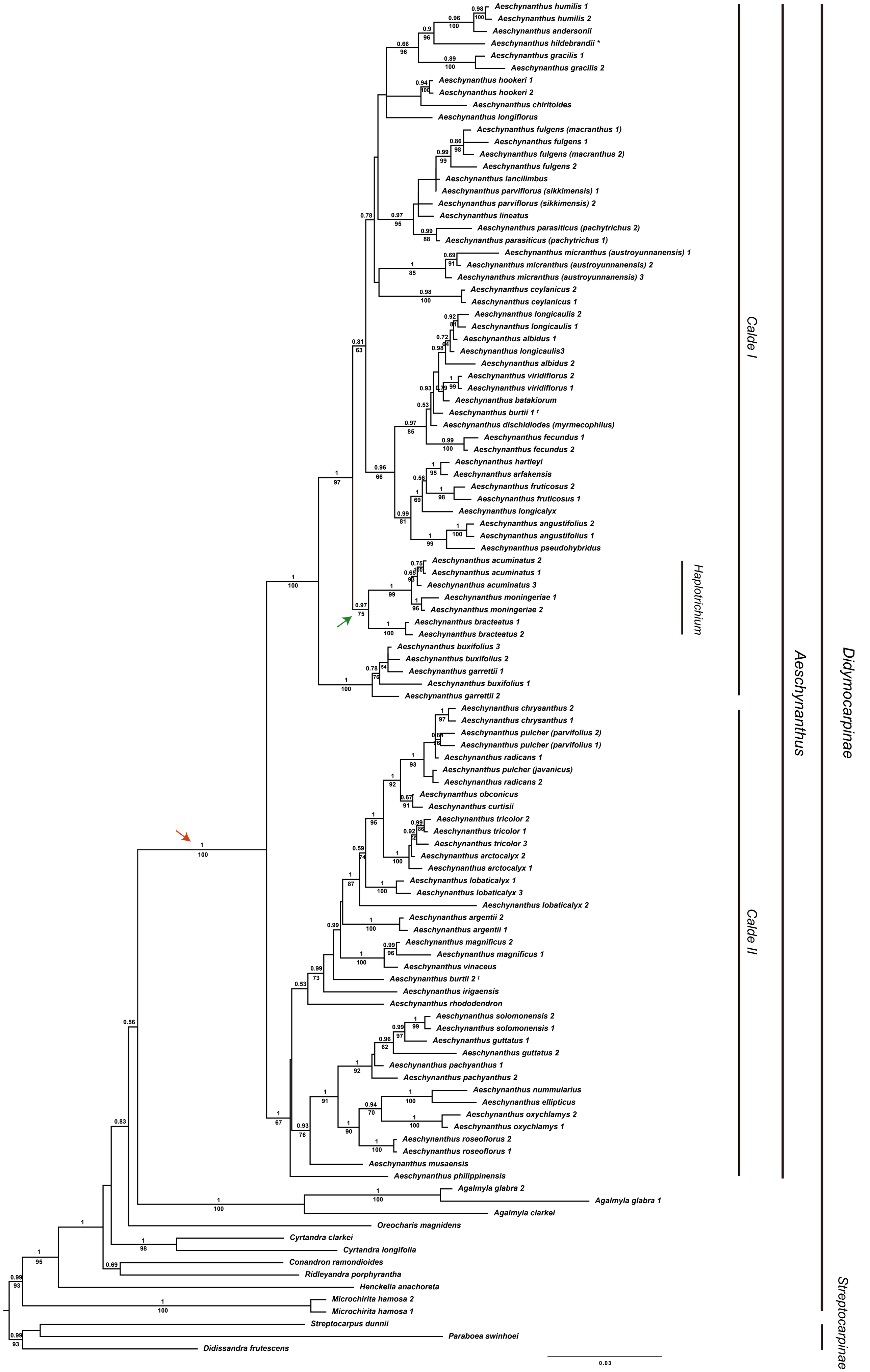
S2 spreadsheet. The species and voucher information of the newly included unpublished sequences.

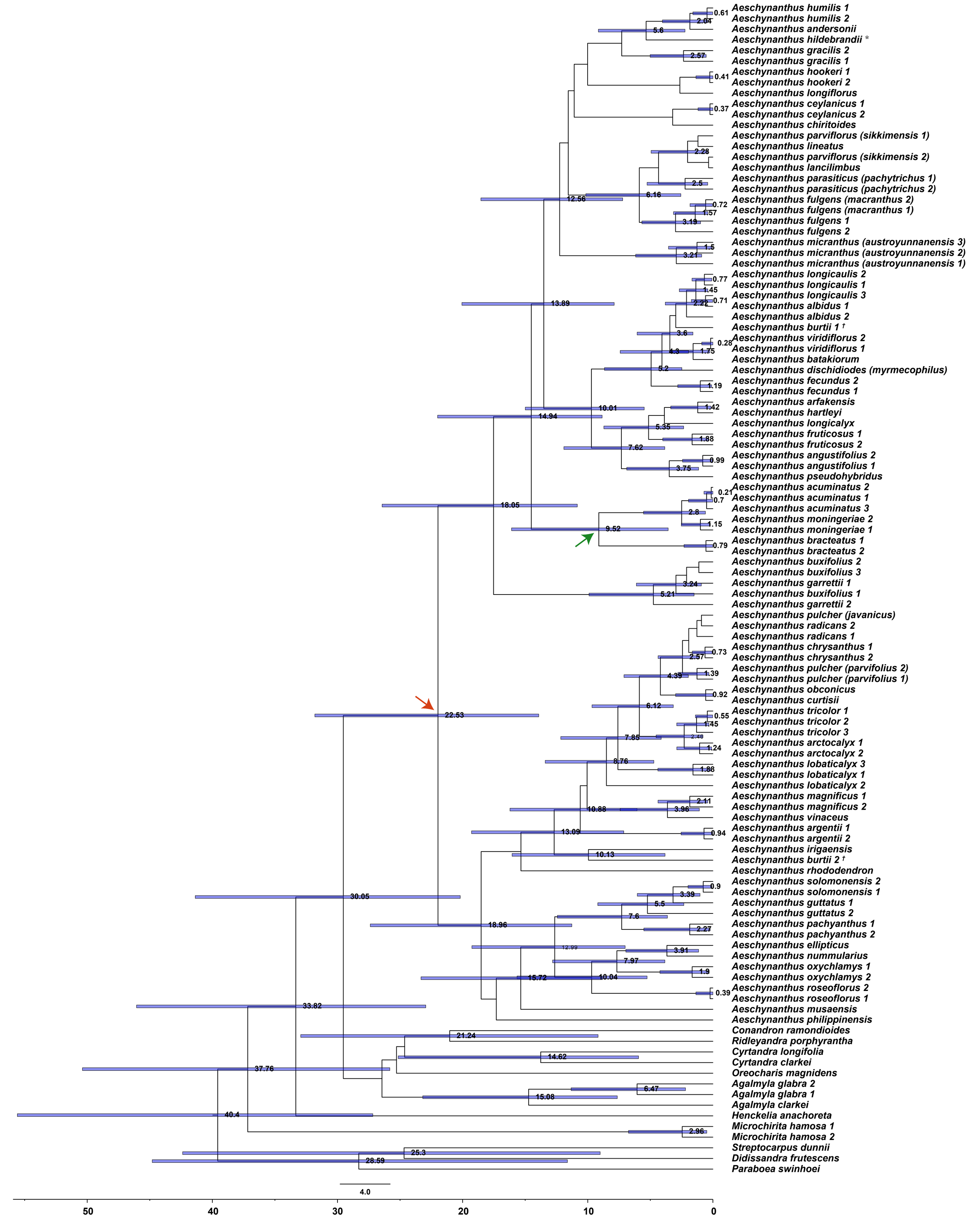
S3 alignment in NEXUS format. The concatenated alignment of four genetic regions: *ITS*, *trnL-trnF*, *psbA-trnH*, and *ETS*. This was also the input sequence file for MrBayes.

S4 spreadsheet. The list of all currently recognized species included in this study. The original taxa names which are now synonyms used in this study were noted.

S5 consensus ML phylogeny. The resulting phylogeny of ML analysis with 1000 rapid bootstraps by RAxML.

S6 consensus BI phylogeny. The forced bifurcated consensus phylogeny of the second chain of 5 million MCMC search by MrBayes.


Aeschynanthus humilis 1
Aeschynanthus humilis 2
Aeschynanthus andersonii
Aeschynanthus hildebrandii *
Aeschynanthus gracilis 1
Aeschynanthus gracilis 2
Aeschynanthus hookeri 1
Aeschynanthus hookeri 2
Aeschynanthus chiritoides
Aeschynanthus longiflorus
Aeschynanthus fulgens (macranthus 1)
Aeschynanthus fulgens 1
Aeschynanthus fulgens (macranthus 2)
Aeschynanthus fulgens 2
Aeschynanthus lancilimbus
Aeschynanthus parviflorus (sikkimensis) 1
Aeschynanthus parviflorus (sikkimensis) 2
Aeschynanthus lineatus
Aeschynanthus parasiticus (pachytrichus 2)
Aeschynanthus parasiticus (pachytrichus 1)
Aeschynanthus micranthus (austroyunnanensis) 1
Aeschynanthus micranthus (austroyunnanensis) 2
Aeschynanthus micranthus (austroyunnanensis) 3
Aeschynanthus ceylanicus 2
Aeschynanthus ceylanicus 1
Aeschynanthus longicaulis 2
Aeschynanthus longicaulis 1
Aeschynanthus albidus 1
Aeschynanthus longicaulis3
Aeschynanthus albidus 2
Aeschynanthus viridiflorus 2
Aeschynanthus viridiflorus 1
Aeschynanthus batakiorum
Aeschynanthus burtii 1 †
Aeschynanthus dischidiodes (myrmecophilus)
Aeschynanthus fecundus 1
Aeschynanthus fecundus 2
Aeschynanthus hartleyi
Aeschynanthus arfakensis
Aeschynanthus fruticosus 2
Aeschynanthus fruticosus 1
Aeschynanthus longicalyx
Aeschynanthus angustifolius 2
Aeschynanthus angustifolius 1
Aeschynanthus pseudohybridus
Aeschynanthus acuminatus 2
Aeschynanthus acuminatus 1
Aeschynanthus acuminatus 3
Aeschynanthus moningeriae 1
Aeschynanthus moningeriae 2
Aeschynanthus bracteatus 1
Aeschynanthus bracteatus 2
Aeschynanthus buxifolius 3
Aeschynanthus buxifolius 2
Aeschynanthus garrettii 1
Aeschynanthus buxifolius 1
Aeschynanthus garrettii 2
Aeschynanthus chrysanthus 2
Aeschynanthus chrysanthus 1
Aeschynanthus pulcher (parvifolius 2)
Aeschynanthus pulcher (parvifolius 1)
Aeschynanthus radicans 1
Aeschynanthus pulcher (javanicus)
Aeschynanthus radicans 2
Aeschynanthus obconicus
Aeschynanthus curtisii
Aeschynanthus tricolor 2
Aeschynanthus tricolor 1
Aeschynanthus tricolor 3
Aeschynanthus arctocalyx 2
Aeschynanthus arctocalyx 1
Aeschynanthus lobaticalyx 1
Aeschynanthus lobaticalyx 3
Aeschynanthus lobaticalyx 2
Aeschynanthus argentii 2
Aeschynanthus argentii 1
Aeschynanthus magnificus 2
Aeschynanthus magnificus 1
Aeschynanthus vinaceus
Aeschynanthus burtii 2 †
Aeschynanthus irigaensis
Aeschynanthus rhododendron
Aeschynanthus solomonensis 2
Aeschynanthus solomonensis 1
Aeschynanthus guttatus 1
Aeschynanthus guttatus 2
Aeschynanthus pachyanthus 1
Aeschynanthus pachyanthus 2
Aeschynanthus nummularius
Aeschynanthus ellipticus
Aeschynanthus oxychlamys 2
Aeschynanthus oxychlamys 1
Aeschynanthus roseoflorus 2
Aeschynanthus roseoflorus 1
Aeschynanthus musaensis
Aeschynanthus philippinensis
Agalmyla glabra 2
Agalmyla glabra 1
Agalmyla clarkei
Oreocharis magnidens
Cyrtandra clarkei
Cyrtandra longifolia
Conandron ramondioides
Ridleyandra porphyrantha
Henckelia anachoreta
Microchirita hamosa 2
Microchirita hamosa 1
Streptocarpus dunnii
Paraboea swinhoei
Didissandra frutescens
0.03
Calde I
Haplotrichium
Aeschynanthus
Calde II
Didymocarpinae
Streptocarpinae


Aeschynanthus humilis 1
Aeschynanthus humilis 2
Aeschynanthus andersonii
Aeschynanthus hildebrandii *
Aeschynanthus gracilis 2
Aeschynanthus gracilis 1
Aeschynanthus hookeri 1
Aeschynanthus hookeri 2
Aeschynanthus longiflorus
Aeschynanthus ceylanicus 1
Aeschynanthus ceylanicus 2
Aeschynanthus chiritoides
Aeschynanthus parviflorus (sikkimensis 1)
Aeschynanthus lineatus
Aeschynanthus parviflorus (sikkimensis 2)
Aeschynanthus lancilimbus
Aeschynanthus parasiticus (pachytrichus 1)
Aeschynanthus parasiticus (pachytrichus 2)
Aeschynanthus fulgens (macranthus 2)
Aeschynanthus fulgens (macranthus 1)
Aeschynanthus fulgens 1
Aeschynanthus fulgens 2
Aeschynanthus micranthus (austroyunnanensis 3)
Aeschynanthus micranthus (austroyunnanensis 2)
Aeschynanthus micranthus (austroyunnanensis 1)
Aeschynanthus longicaulis 2
Aeschynanthus longicaulis 1
Aeschynanthus longicaulis 3
Aeschynanthus albidus 1
Aeschynanthus albidus 2
Aeschynanthus burtii 1 †
Aeschynanthus viridiflorus 2
Aeschynanthus viridiflorus 1
Aeschynanthus batakiorum
Aeschynanthus dischidiodes (myrmecophilus)
Aeschynanthus fecundus 2
Aeschynanthus fecundus 1
Aeschynanthus arfakensis
Aeschynanthus hartleyi
Aeschynanthus longicalyx
Aeschynanthus fruticosus 1
Aeschynanthus fruticosus 2
Aeschynanthus angustifolius 2
Aeschynanthus angustifolius 1
Aeschynanthus pseudohybridus
Aeschynanthus acuminatus 2
Aeschynanthus acuminatus 1
Aeschynanthus acuminatus 3
Aeschynanthus moningeriae 2
Aeschynanthus moningeriae 1
Aeschynanthus bracteatus 1
Aeschynanthus bracteatus 2
Aeschynanthus buxifolius 2
Aeschynanthus buxifolius 3
Aeschynanthus garrettii 1
Aeschynanthus buxifolius 1
Aeschynanthus garrettii 2
Aeschynanthus pulcher (javanicus)
Aeschynanthus radicans 2
Aeschynanthus radicans 1
Aeschynanthus chrysanthus 1
Aeschynanthus chrysanthus 2
Aeschynanthus pulcher (parvifolius 2)
Aeschynanthus pulcher (parvifolius 1)
Aeschynanthus obconicus
Aeschynanthus curtisii
Aeschynanthus tricolor 1
Aeschynanthus tricolor 2
Aeschynanthus tricolor 3
Aeschynanthus arctocalyx 1
Aeschynanthus arctocalyx 2
Aeschynanthus lobaticalyx 3
Aeschynanthus lobaticalyx 1
Aeschynanthus lobaticalyx 2
Aeschynanthus magnificus 1
Aeschynanthus magnificus 2
Aeschynanthus vinaceus
Aeschynanthus argentii 1
Aeschynanthus argentii 2
Aeschynanthus irigaensis
Aeschynanthus burtii 2 †
Aeschynanthus rhododendron
Aeschynanthus solomonensis 2
Aeschynanthus solomonensis 1
Aeschynanthus guttatus 1
Aeschynanthus guttatus 2
Aeschynanthus pachyanthus 1
Aeschynanthus pachyanthus 2
Aeschynanthus ellipticus
Aeschynanthus nummularius
Aeschynanthus oxychlamys 1
Aeschynanthus oxychlamys 2
Aeschynanthus roseoflorus 2
Aeschynanthus roseoflorus 1
Aeschynanthus musaensis
Aeschynanthus philippinensis
Conandron ramondioides
Ridleyandra porphyrantha
Cyrtandra longifolia
Cyrtandra clarkei
Oreocharis magnidens
Agalmyla glabra 2
Agalmyla glabra 1
Agalmyla clarkei
Henckelia anachoreta
Microchirita hamosa 1
Microchirita hamosa 2
Streptocarpus dunnii
Didissandra frutescens
Paraboea swinhoei
0.61
2.04
5.6
2.57
0.41
0.37
2.28
2.5
6.16
12.56
0.72
1.57
3.19
1.5
3.21
0.77
1.45
13.89
0.71
2.22
3.6
0.28
4.3
1.75
5.2
1.19
10.01
1.42
14.94
5.35
1.88
7.62
0.99
3.75
0.21
0.7
18.05
2.8
1.15
9.52
0.79
3.24
5.21
0.73
2.57
4.39
1.39
0.92
22.53
6.12
0.55
1.45
7.85
2.46
1.24
8.76
1.88
2.11
10.88
3.96
13.09
0.94
10.13
0.9
3.39
30.05
5.5
7.6
18.96
2.27
12.99
3.91
7.97
1.9
15.72
10.04
0.39
33.82
21.24
14.62
37.76
6.47
15.08
40.4
2.96
25.3
28.59
4.0
50
40
30
20
10
0