Postprint: Confirmation of the Systematic Position of Biondia and Merrillanthus (Apocynaceae) Based on Molecular Evidence

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Abstract

Biondia Schltr. is a Chinese endemic genus comprising approximately 13 species, whereas Merrillanthus Chun & Tsiang is a monotypic genus distributed exclusively in China and Cambodia. Currently, both genera have been subsumed into Vincetoxicum Wolf; however, deficiencies remain in sampling and phylogenetic analysis, necessitating further verification of their systematic positions and taxonomic placements. Phylogenetic trees of Tylophorinae were reconstructed using data from two ribosomal gene sequence regions (ITS, ETS), five chloroplast gene sequence regions (psbA-trnH, trnG, trnL, trnL-F, trnT-L), and combined datasets thereof (including the type species of the genus, Biondia chinensis Schltr. = V. shaanxiense (Schltr.) Meve & Liede, and Merrillanthus hainanensis Chun & Tsiang = V. hainanense (Chun & Tsiang) Meve, H.H.Kong & Liede). The results demonstrate that both Biondia and Merrillanthus are nested within Vincetoxicum. Biondia chinensis and Biondia henryi (Warb.) Tsiang & Li = V. henryi (Warb.) Meve & Liede form a sister group and cluster with V. kawaroense Meve & Liede, while Biondia insignis Tsiang = V. insigne (Tsiang) Meve, H.H.Kong & Liede is placed in a separate clade (Subtropical Clade). Merrillanthus forms a clade with V. cissoides (Blume) Kuntze and V. philippicum Meve, Omlor & Liede. Both ribosomal and chloroplast gene data support the merger of Biondia and Merrillanthus into Vincetoxicum; however, Biondia is polyphyletic. Additional sampling and combined data analysis are required to thoroughly investigate interspecific phylogenetic relationships and positions within the merged Vincetoxicum.

Full Text

Confirmation of the Systematic Position of *Biondia* and *Merrillanthus* (Apocynaceae) Based on Molecular Evidence

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Abstract

Biondia Schltr. is a genus endemic to China comprising approximately 13 species, while *Merrillanthus* Chun & Tsiang is a monotypic genus distributed only in China and Cambodia. Both genera have been subsumed into *Vincetoxicum* Wolf, but their phylogenetic positions and taxonomic status require further investigation due to limited sampling and incomplete phylogenetic analysis. We reconstructed phylogenetic trees of subtribe Tylophorinae using two nuclear ribosomal DNA regions (ITS, ETS) and five plastid DNA regions (psbA-trnH, trnG, trnL, trnL-F, trnT-L), analyzed both separately and in combination. Our sampling included the type species of each genus: *Biondia chinensis* Schltr. (= *V. shaanxiense* (Schltr.) Meve & Liede) and *Merrillanthus hainanensis* Chun & Tsiang (= *V. hainanense* (Chun & Tsiang) Meve, H.H. Kong & Liede). The results demonstrate that both *Biondia* and *Merrillanthus* are nested within *Vincetoxicum*. *Biondia chinensis* is sister to *B. henryi* (Warb.) Tsiang & Li (= *V. henryi* (Warb.) Meve & Liede), and this clade groups with *V. kawaroense* Meve & Liede, whereas *B. insignis* Tsiang (= *V. insigne* (Tsiang) Meve, H.H. Kong & Liede) falls within a separate lineage (the Subtropical Clade). *Merrillanthus* forms a clade with *V. cissoides* (Blume) Kuntze and *V. philippicum* Meve, Omlor & Liede. Both nuclear and plastid datasets support the inclusion of *Biondia* and *Merrillanthus* within *Vincetoxicum*, but *Biondia* is polyphyletic. Additional species sampling and combined data analysis are needed to further elucidate interspecific relationships within the expanded *Vincetoxicum*.

Keywords: Apocynaceae, *Vincetoxicum*, *Biondia*, *Merrillanthus*, phylogeny, China

Introduction

Apocynaceae, as circumscribed in the Angiosperm Phylogeny Group IV (APG IV) system, belongs to Gentianales along with Gelsemiaceae, Gentianaceae, Loganiaceae, and Rubiaceae (Chase et al., 2016). The family encompasses the traditionally recognized Apocynaceae s.s. and Asclepiadaceae, comprising approximately 5,350 species primarily distributed in tropical and subtropical regions (Endress et al., 2018). A relatively stable molecular phylogenetic framework for Apocynaceae has been established (Fishbein et al., 2018), leading to the consolidation or repositioning of several revised genera and reducing the total number from 422 (Endress & Bruyns 2000) to 378 (Endress et al., 2018).

Vincetoxicum Wolf belongs to tribe Asclepiadeae, subtribe Tylophorinae, and has undergone substantial taxonomic revision (Endress et al., 2018). Traditionally, *Vincetoxicum* is characterized by erect stems (occasionally twining at the apex), fascicled fibrous roots, rotate corollas, five fleshy corona lobes, and the presence of translucent latex (Qiu et al., 1989; Liede, 1996). The genus shares similar corona, gynostegium, and pollinarium structures with *Cynanchum* L., leading some researchers to advocate for its inclusion within *Cynanchum* (Jiang and Li, 1977; Forster, 1991; Li et al., 1995). However, others have argued for its recognition as a distinct genus (Markgraf, 1972; Ali & Khatoon, 1982). Qiu et al. (1989) proposed the independence of *Vincetoxicum* from *Cynanchum* based on evidence from chemical constituents, morphology, chromosome numbers, and distribution patterns, a view supported by Liede (1996) using morphological and chemical data, who further suggested its closest relationship with *Tylophora* R. Br.

Molecular phylogenetic studies have revealed that *Vincetoxicum* is distantly related to *Cynanchum* (Liede, 2001; Rapini et al., 2007), instead showing closer affinity with *Tylophora*, although both genera are non-monophyletic. Six genera —*Biondia* Schltr., *Blyttia* Arn., *Diplostigma* K. Schum., *Goydera* Liede, *Pleurostelma* Baill., and *Rhyncharrhena* F. Muell.—are nested within the *Vincetoxicum*–*Tylophora* clade (Liede-Schumann et al., 2012, 2016). Consequently, *Tylophora* and these six morphologically similar genera have been subsumed into *Vincetoxicum*, resulting in numerous new combinations and names (Kidyoo & Kidyoo, 2018; Liede-Schumann & Meve, 2018; Hsu et al., 2021; Shah et al., 2021). Three genera originally distributed primarily in China—*Merrillanthus* Chun & Tsiang, *Pentastelma* Tsiang & Li, and *Belostemma* Wall. ex Wight—have also been incorporated into *Vincetoxicum*. The newly defined *Vincetoxicum* comprises over 150 species, typically with translucent latex, small flowers with rotate corollas (occasionally campanulate, urceolate, or long-conical), coronas composed of fleshy, separate lobes on the stamens or forming rings from fused stamens and interstaminal parts, erect, horizontal, or ascending caudicles, and a distribution spanning tropical and subtropical Africa, Asia, and Eurasia (Endress et al., 2018).

Biondia comprises approximately 13 species endemic to eastern and southwestern China, while *Merrillanthus* is monotypic, containing only *M. hainanensis*, distributed in Guangdong, Hainan (China), and Cambodia (Jiang and Li, 1977; Li et al., 1995). In the phylogenetic analyses by Liede-Schumann et al. (2012, 2016), *Biondia* was represented by only two species—*B. insignis* and *B. henryi*—lacking the type species *B. chinensis*, and no *Merrillanthus* samples were included. Some researchers have reported chloroplast genome data for *B. chinensis* and *M. hainanensis* with preliminary phylogenetic analyses: *B. chinensis* appeared sister to *V. rossicum*, but the tree included only a single *Vincetoxicum* species (Rao et al., 2018), while *M. hainanensis* appeared sister to *B. insignis*, again with limited sampling (Xiong et al., 2019). Thus, the placement of these two species within *Vincetoxicum* and their relationships to specific lineages remain unclear.

In this study, we incorporated data for *B. chinensis* and *M. hainanensis*, with newly generated sequences for *M. hainanensis* and published shallow-sequencing data for *B. chinensis* from Rao et al. (2018), combined with existing molecular data for other *Vincetoxicum* species (Liede-Schumann et al., 2016), to conduct phylogenetic analyses aimed at clarifying the systematic positions and taxonomic 归属 of these two genera.

1.1 Taxon Sampling and Molecular Data Sources

Data for *Biondia chinensis* (voucher: ZJB-2017-152-1, deposited at Shaanxi Normal University) were obtained from shallow-sequencing genomic data in Rao et al. (2018). For *Merrillanthus hainanensis* (voucher: LHB-AP17, deposited at South China Agricultural University), fresh leaves were sampled, dried in silica gel, and stored on dry ice before being shipped to Novogene Bioinformatics Technology Co., Ltd. (Wuhan) for total genomic DNA extraction, library construction, and sequencing. Following quality control of the DNA, random fragmentation produced ~350 bp libraries for paired-end 150 bp sequencing, yielding 10 Gb of clean data after quality filtering. Both the existing shallow-sequencing data for *B. chinensis* and the newly generated data for *M. hainanensis* were assembled using GetOrganelle v1.7 (Jin et al., 2020) with default parameters. The resulting fastg files were visualized and extracted using Bandage 0.8 (Wick et al., 2015) and Geneious Prime 2019 (https://www.geneious.com/) to obtain the final ribosomal genome sequence for *B. chinensis* and both chloroplast and ribosomal genome sequences for *M. hainanensis*. The chloroplast genome of *M. hainanensis* was annotated using Plastid Genome Annotator (PGA) (Qu et al., 2019) with *Amborella trichopoda* Baill. (AJ506156) and *Apocynum venetum* L. (MT313688) as references, followed by manual correction in Geneious Prime 2019 based on log files. Ribosomal genomes for both species were annotated in Geneious Prime 2019 using the nrDNA continuous fragment (18S+ITS1+5.8S+ITS2+26S) from *Asclepias coulteri* A.Gray (JN665084) and the ETS sequence from *V. biglandulosum* (Endl.) Kuntze (LN880610) as references. Required ribosomal and chloroplast gene fragments were extracted using the Extract function, and all data were submitted to GenBank (https://www.ncbi.nlm.nih.gov/).

Based on previously published molecular phylogenetic data for subtribe Tylophorinae (Liede-Schumann et al., 2016), we supplemented DNA data for *M. hainanensis* and *B. chinensis*, selecting three species from subtribe Cynanchinae as outgroups. Phylogenetic trees were constructed using: (1) five chloroplast gene regions (psbA-trnH, trnG, trnL, trnL-F, trnT-L) for 139 species; (2) two ribosomal gene regions (ETS, ITS) for 136 species; and (3) combined datasets for 139 species. Sequence details are provided in Table 1, and voucher specimen information is available in Liede-Schumann et al. (2016). Missing sequence data for some samples were coded as gaps in the alignment matrices.

1.2 Sequence Alignment and Concatenation

Individual gene regions were aligned using MAFFT (Katoh & Standley, 2013). Multiple alignments were concatenated in Geneious Prime 2019 to produce combined matrices.

1.3 Phylogenetic Analysis

Maximum likelihood (ML) phylogenetic analyses were performed using IQ-TREE (Nguyen et al., 2015). ModelFinder within IQ-TREE was used to automatically test and select the best-fit substitution models according to the Bayesian Information Criterion (BIC): TVM+F+R3 for the combined ribosomal dataset, K3Pu+F+R3 for the combined plastid dataset, and TVM+F+R3 for the combined nuclear and plastid dataset. Branch support was assessed using 1,000 SH-aLRT replicates (Guindon et al., 2010) and ultrafast bootstrap (UFBoot) analyses (Minh et al., 2013). Branches with SH-aLRT $\geq$ 80% and UFBoot $\geq$ 95% were considered well-supported and reliable. Resulting trees were visualized in FigTree 1.4.2 (Rambaut, 2012).

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2 Results and Analysis

Phylogenetic trees constructed from the ribosomal gene regions (ETS, ITS) revealed that *Vincetoxicum* is non-monophyletic within subtribe Tylophorinae, with *Pentatropis* R. Br. ex Wight & Arn. nested within it and forming a sister clade to *V. apiculatum* + *V. sylvaticum* + *V. tenuipedunculatum* (SH-aLRT = 82.8%, UFBoot = 82%) (Fig. 1). Trees based on plastid gene regions and the combined dataset both resolved Tylophorinae into two major clades, with *Pentatropis* as sister to *Vincetoxicum* (support values: 99.8/100 and 99.9/100, respectively).

All three datasets strongly supported *Merrillanthus* grouping with *V. cissoides* (T. *cissoides*) and *V. philippicum* (T. *parviflora*) (support values: 100/100, 97.9/100, and 100/100 in Figs. 1, 2, and 3, respectively). The three *Biondia* species were nested within the *Vincetoxicum* clade but were non-monophyletic. In the ribosomal and combined datasets, *B. chinensis* was sister to *B. henryi* (= *V. henryi*) with strong support (97.7/100 and 97.8/100, respectively; Figs.

1 and 3), and this clade grouped with *V. kawaroense* (T. *japonica*) (97.1/100 and 97.8/100). In the plastid dataset tree, *B. chinensis* and *B. henryi* formed a clade but with low support (31.3/67) (Fig. 2). *Biondia insignis* (= *V. insigne*) consistently fell within the Subtropical Clade across all three trees (support values: 100/100, 93.8/100, and 100/100 in Figs. 1, 2, and 3, respectively), where it grouped with *V. villosum* (T. *villosa*) (support values: 74.3/100, 0/96, and 72.6/100).

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3 Discussion

3.1 Phylogenetic Position and Generic Status of *Biondia* When Schlechter (1905) established *Biondia*, he distinguished it from *Marsdenia* R. Br. by its pendulous pollinia (vs. erect) and from *Tylophora* by its campanulate or sub-campanulate corolla (vs. deeply 5-lobed rotate or broadly rotate corollas), placing it in subtribe Asclepiadinae. Gilbert et al. (1995) noted that *Biondia* is characterized by narrow leaves with minute pale spots on the adaxial surface, small flowers with reduced, often annular corollas and well-developed corolla tubes, and observed similar pale spots in some *Tylophora* species, indicating morphological overlap between the genera. Since *Tylophora* has been subsumed into *Vincetoxicum* (Liede-Schumann et al., 2012; Liede-Schumann & Meve, 2018; Endress et al., 2018), our molecular results support the inclusion of *Biondia* within *Vincetoxicum*.

Phylogenetic analyses show that all three *Biondia* species are nested within *Vincetoxicum*. *Biondia chinensis* and *B. henryi* form one clade, while *B. insignis* groups with *V. villosum* (T. *villosa*) within the Subtropical Clade. However, these species differ markedly in morphology: *B. insignis* has branches and petioles covered with uniseriate short hairs, linear-lanceolate leaves that are glabrous except for the midvein, and a sub-campanulate, glabrous corolla, whereas *V. villosum* has densely pubescent branches and petioles, ovate or ovate-oblong leaves that are hairy on both surfaces, and a rotate corolla with long soft hairs. Geographically, *B. insignis* is distributed in Guizhou, Hunan, Sichuan, Tibet, and Yunnan, China (Li et al., 1995), while *V. villosum* occurs on Java (Backer & Bakhuizen, 1965). The close phylogenetic relationship between *B. insignis* and *V. villosum* despite their substantial morphological and geographic differences warrants further investigation with expanded sampling.

3.2 Systematic Position and Generic Status of *Merrillanthus* When *Merrillanthus* was described, it was considered most similar to *Pycnostelma* Bunge ex Decne. (now subsumed in *Vincetoxicum*; Liede-Schumann & Meve, 2018). Both genera have coronas adnate to the stamens but differ in growth habit, corona lobe shape, corolla lobe fusion patterns, and fruit morphology. *Merrillanthus* is a woody liana with dorsally raised, basally thickened corona lobes, connate corolla lobes that are fused at the apex before anthesis, and solitary, fusiform, large follicles (9–12 cm long, 3.5–4 cm in diameter). In contrast, *Pycnostelma* is an erect herb with coronas thickened toward the apex, deeply lobed corollas, and smaller, lanceolate fruits (Chun & Tsiang, 1941). The corolla lobe fusion in *Merrillanthus* resembles that of *Heterostemma* Wight & Arn. and *Hoya* R. Br., while its membranous, cordate leaves are similar to *Telosma* Coville. However, *Merrillanthus* can be distinguished by its pendulous pollinia (vs. erect in the other three genera) (Chun & Tsiang, 1941). Jiang and Li (1977) noted similarities between *Merrillanthus* and the Indian genus *Iphisia* Wight & Arn. (= *Vincetoxicum* Wolf), differing in corolla lobe adhesion at the bud stage and pollinium orientation. The floral structure of *Merrillanthus* is also similar to *Tylophora augustiniana* (Hemsl.) Craib (= *V. augustinianum* (Hemsl.) Meve & Liede), though with larger flowers. The large follicles with thick fibrous mesocarp in *Merrillanthus* contrast with the thin-walled, papery follicles typical of *Tylophora*, supporting its recognition as a distinct genus (Gilbert et al., 1995).

Although we did not include *V. augustinianum* in our analysis, our results show *Merrillanthus* grouping with two former *Tylophora* species, *V. cissoides* (T. *cissoides*) and *V. philippicum* (T. *parviflora*). These species share large fruits (10–12 cm × 3–5 cm and 13–15 cm × 3–4 cm, respectively) and similar leaf sizes and shapes. Geographically, *V. cissoides* is widespread in eastern Indonesia and New Guinea (Forster, 1994), *V. philippicum* is restricted to the Philippines (Meve et al., 2002), and *Merrillanthus* occurs in Guangdong, Hainan (China), and Cambodia (Li et al., 1995). These findings suggest that fruit size, caudicle orientation, and leaf morphology may be insufficient characters for generic delimitation within Tylophorinae but could serve as useful species-level diagnostic traits within *Vincetoxicum.* Initially placed in subtribe Asclepiadinae based on its pendulous pollinia (Chun & Tsiang, 1941), *Merrillanthus* was subsequently assigned to subtribe Cynanchinae (Endress et al., 2007) and then to Tylophorinae (Endress et al., 2014, 2018) following the merger of Apocynaceae and Asclepiadaceae (Endress & Bruyns, 2000). Our study supports the current classification of *Merrillanthus* within *Vincetoxicum* and its placement in subtribe Tylophorinae.

3.3 Phylogeny of *Vincetoxicum* Although the combined nuclear and plastid dataset resolves *Vincetoxicum* as monophyletic, the dataset contains relatively few informative sites, resulting in low support for many interspecific relationships within the genus. High-throughput sequencing-based phylogenetic analyses will be necessary to reconstruct robust relationships within *Vincetoxicum.* The genus has experienced considerable taxonomic changes, making a comprehensive revision based on genomic data and morphological character analysis essential.

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Note: Figure translations are in progress. See original paper for figures.