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# *Encyonopsis indonesica* sp. nov. (Bacillariophyceae, Cymbellales), a new diatom from the ancient lake Matano (Sulawesi, Indonesia)

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## Abstract

A new species, *Encyonopsis indonesica*, is described from the ancient lake Matano, Sulawesi island, Indonesia. The morphology of this species was studied by means of light and scanning electron microscopy. *E. indonesica* has a remarkable valve ultrastructure. The valve surface is ornamented with numerous longitudinal siliceous ribs and siliceous verrucae. Valve face delineated from the mantle by a thickened marginal ridge. Raised sterna border the raphe branches. Raphe is distinctly undulate with distal ends hooked strongly to the ventral side. The only similar species to *E. indonesica* is *Amphora dissimilis* described from New Caledonia. Comparison of both taxa is given and *A. dissimilis* is transferred to *Encyonopsis*. The taxonomic placement of both taxa is evaluated, and the phenomenon of external siliceous ornamentation is discussed.

## Keywords

ancient lake, diatoms, Indonesia, morphology, SEM, taxonomy

## Introduction

The genus *Encyonopsis* Krammer was established during a comprehensive revision of cymbelloid diatoms by Krammer (1997a). It differs from morphologically related genus *Encyonema* Kützing mainly by its slightly dorsiventral valve outline and by terminal raphe fissures only slightly bent to the ventral margin (Krammer 1997a, b; Potapova 2014). Though described less than a quarter of a century ago, over 170 taxa have been assigned to *Encyonopsis* (Kociolek et al. 2020). Many taxa were transferred by Krammer (1997b) from other genera in his initial circumscription of the genus, but since then new species of the genus have been described from western North America (Bahls 2013; Graeff and Kociolek 2013; Kociolek et al. 2014; Mark et al. 2019), Central America (Wydrzycka and Lange-Bertalot 2001), South America (Metzeltin and Lange-Bertalot 2007; Wengrat et al. 2015), Europe (Van de Vijver et al. 2012; Kennedy et al. 2019), Asia (Krammer 2003; Potapova et al. 2014), Madagascar (Metzeltin and Lange-Bertalot 2002), and Reunion Island (Le Cohu et al. 2014).

Relatively recently the genus *Kurtkrammeria* Bahls (2015) was proposed, with most species of the genus having been transferred from *Encyonopsis*. This genus differs from *Encyonopsis* by having convergent striae at the apices, slit-like apically-oriented or crescent-shaped areolae, the internal proximal raphe ends hooked strongly towards the dorsal side of the valve and the presence (or absence) of stigmata (Bahls 2015; Marquardt et al. 2016; Zhang et al. 2020). Despite the rather weak differences, *Kurtkrammeria* is an accepted genus and several new species have been described since its description (Marquardt et al. 2016; Zhang et al. 2020). However, sometimes taxa of *Gomphonema* are misidentified as *Encyonopsis* or *Kurtkrammeria* (e.g., Bahls et al. 2018; Almeida et al. 2020), suggesting a review of the distinguishing features of these genera may be warranted.

The Malili lakes are located in the mountains of Central Sulawesi and are composed of five tectonic lakes, namely, Matano, Mahalona, Towuti, Lontoa (also known as Wawontoa) and Masapi (Brooks 1950; Vaillant et al. 2011; von Rintelen et al. 2012). Lake Matano is the oldest lake among them, and its estimated age is 2 to 4 million years old (Brooks 1950; Vaillant et al. 2011). The most extensive treatment on diatoms from the Malili lakes was performed by Hustedt (1942). Subsequently, many new taxa were described from these lakes (Bramburger et al. 2006; Kociolek et al. 2018; Kapustin et al. 2019) and some taxa were re-investigated (Kapustin et al. 2017; Kapustin and Kulikovskiy 2018; Kapustin et al. 2020; Kulikovskiy et al. 2020).

The aim of this paper is to describe a new *Encyonopsis* species based on light and scanning electron microscopy, detail its unusual morphology, and discuss its generic placement.

## Material and methods

An epilithic sample containing *Encyonopsis indonesica* was collected from Lake Matano in 2010 (02°28.433'S, 121°15.710'E). With a Hanna multiparameter probe meter

(HANNA HI98128), the temperature was recorded as 28.5 °C, pH as 8.53, and conductivity as 177 $\mu S\cdot cm^{-1}$.

The sample was heated in concentrated hydrogen peroxide (~37%) to dissolve the organic matter. It was then rinsed with deionized water four times at 12 h intervals. After decanting and filling with deionized water up to 100 ml, the suspension was spread on to coverslips and left to dry at room temperature. Permanent diatom slides were mounted in Naphrax. Light microscopic (LM) observations were performed with a Zeiss Scope A1 microscope equipped with an oil immersion objective (100×/n.a.1.4, differential interference contrast [DIC]) and Zeiss Axio-Cam ERc 5s camera. Valve ultrastructure was examined by means of a JSM-6510LV scanning electron microscope (Papanin Institute for Biology of Inland Waters RAS, Borok, Russia). For scanning electron microscopy (SEM), parts of the suspensions were fixed on aluminum stubs after air-drying. The stubs were sputter coated with 50 nm of gold.

## Results

**Class Bacillariophyceae Haeckel**
**Order Cymbellales D.G. Mann**
**Family Cymbellaceae Kützing**
**Genus *Encyonopsis* Krammer**

***Encyonopsis indonesica* Kapustin, Kulikovskiy & Kociolek, sp. nov.**
Figs 1–4

**Holotype** (here designated): MHA 01105. Fig. 1B illustrates the holotype.

**Type locality.** Indonesia, Island of Sulawesi, Lake Matano, 02°28.433'S, 121°15.710'E, *leg*. I.I. Ivanov, 14.XI.2010.

**Etymology.** The specific epithet refers to the type locality from Indonesia.

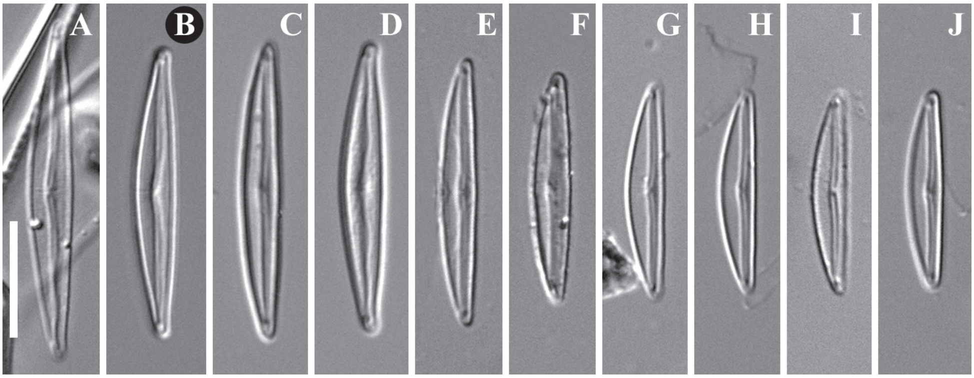

**Figure 1. A–J** *Encyonopsis indonesica* sp. nov. (LM). Size diminution series showing variation in valve outline **B** holotype specimen. Scale bar: 10 µm.

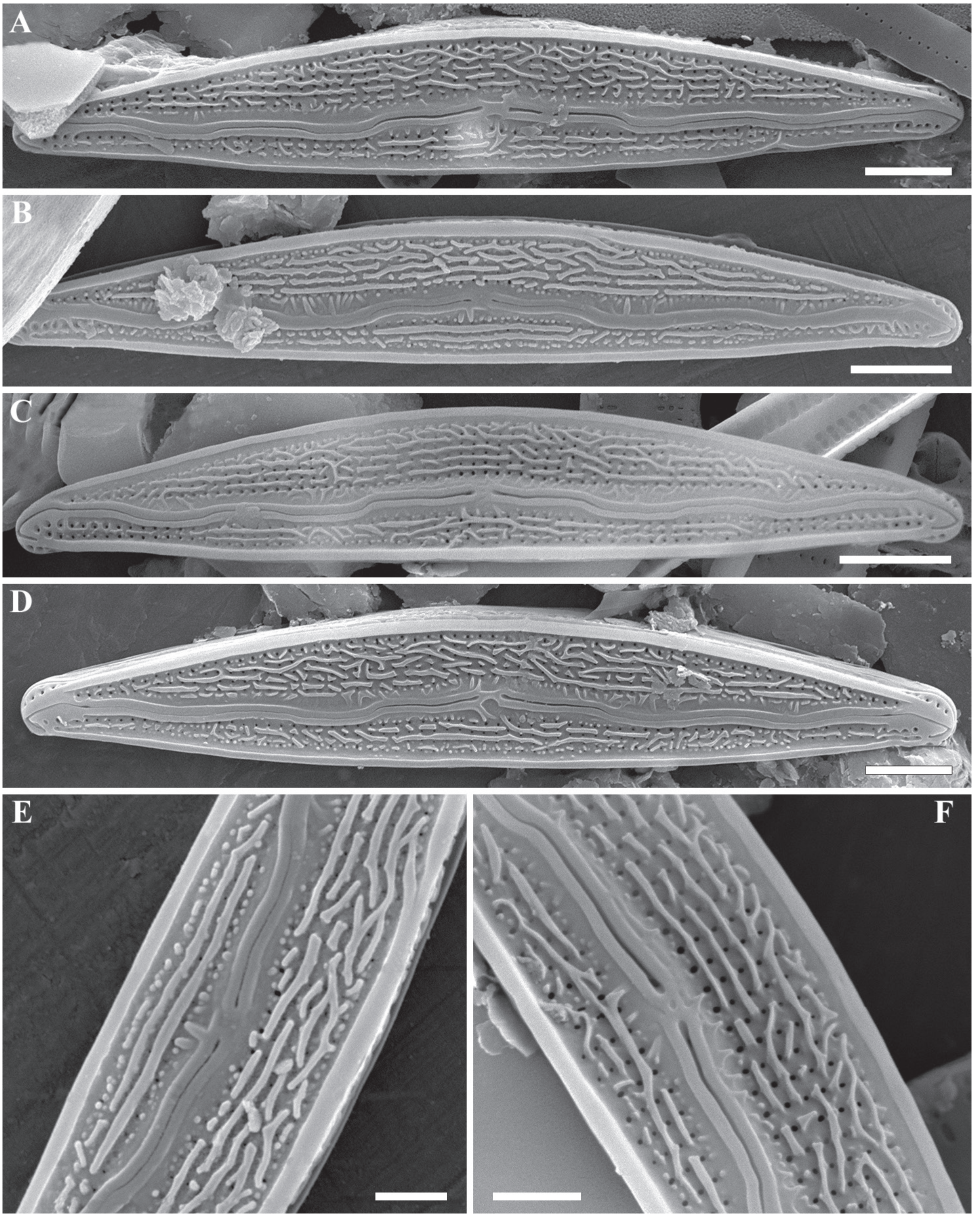

**Figure 2. A–F** *Encyonopsis indonesica* sp. nov. (SEM). External view **A–D** whole valves showing morphological variability in external ornamentation **E, F** central area with dorsally deflected proximal raphe ends. Note the thickened marginal ridge, longitudinal ribs and verrucae on the valve surface. Scale bars: 2 μm (**A, D**), 2.5 μm (**B, C**), 1 μm (**E, F**).

**Description. LM (Fig. 1A–J).** Valves dorsi-ventral, semi-lanceolate, with a slightly convex dorsal margin and a nearly straight ventral margin. Apices slightly protracted, cuneate to slightly rostrate. Axial area very narrow, following the course of the raphe

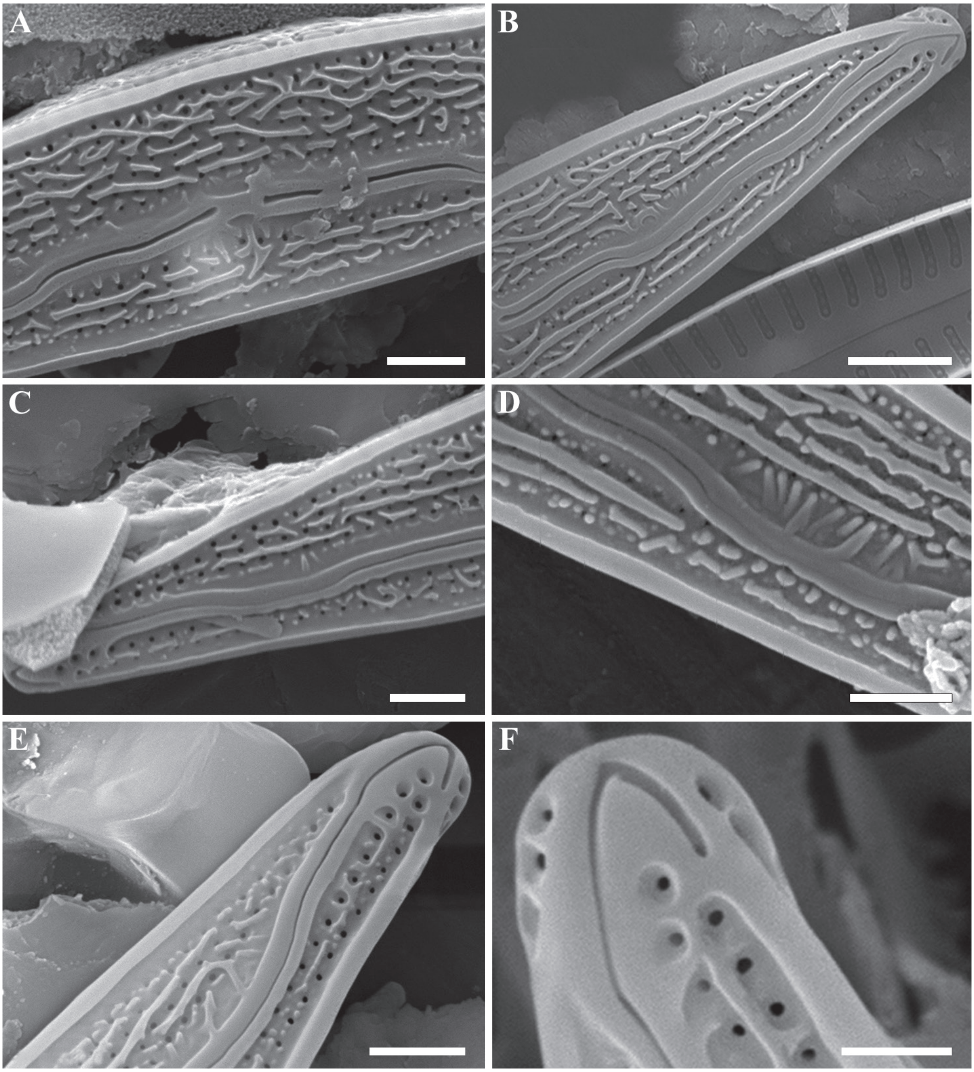

**Figure 3. A–F** *Encyonopsis indonesica* sp. nov. (SEM). External view **A** central area **B, C** valve ends. Note a hyaline area along the raised sternum **D** a part of a raphe with transapical ribs on the valve surface **E, F** valve ends with strongly hooked distal raphe fissures. Scale bars: 1 µm (**A, C, D, E**), 2 µm (**B**), 0.5 µm (**F**).

and central area not expressed. Raphe undulate. Striae indistinct in LM. Length 17–29 µm (21.9 ± 3.3; n = 16), breadth 3–4 µm (3.4 ± 0.3; n = 16), length/width ratio 5.7–7.4 (6.5 ± 0.6; n = 16).

**SEM (Figs 2–4).** External valve face occasionally covered with siliceous verrucae and numerous siliceous ribs of different lengths mostly aligned along the apical axis (Fig. 2A–F). Valve face delineated from the mantle by a thickened marginal ridge. Raphe distinctly undulate, with the proximal raphe ends deflected slightly towards the dorsal margin and the distal ends hooked strongly to the ventral side (Fig. 3E,

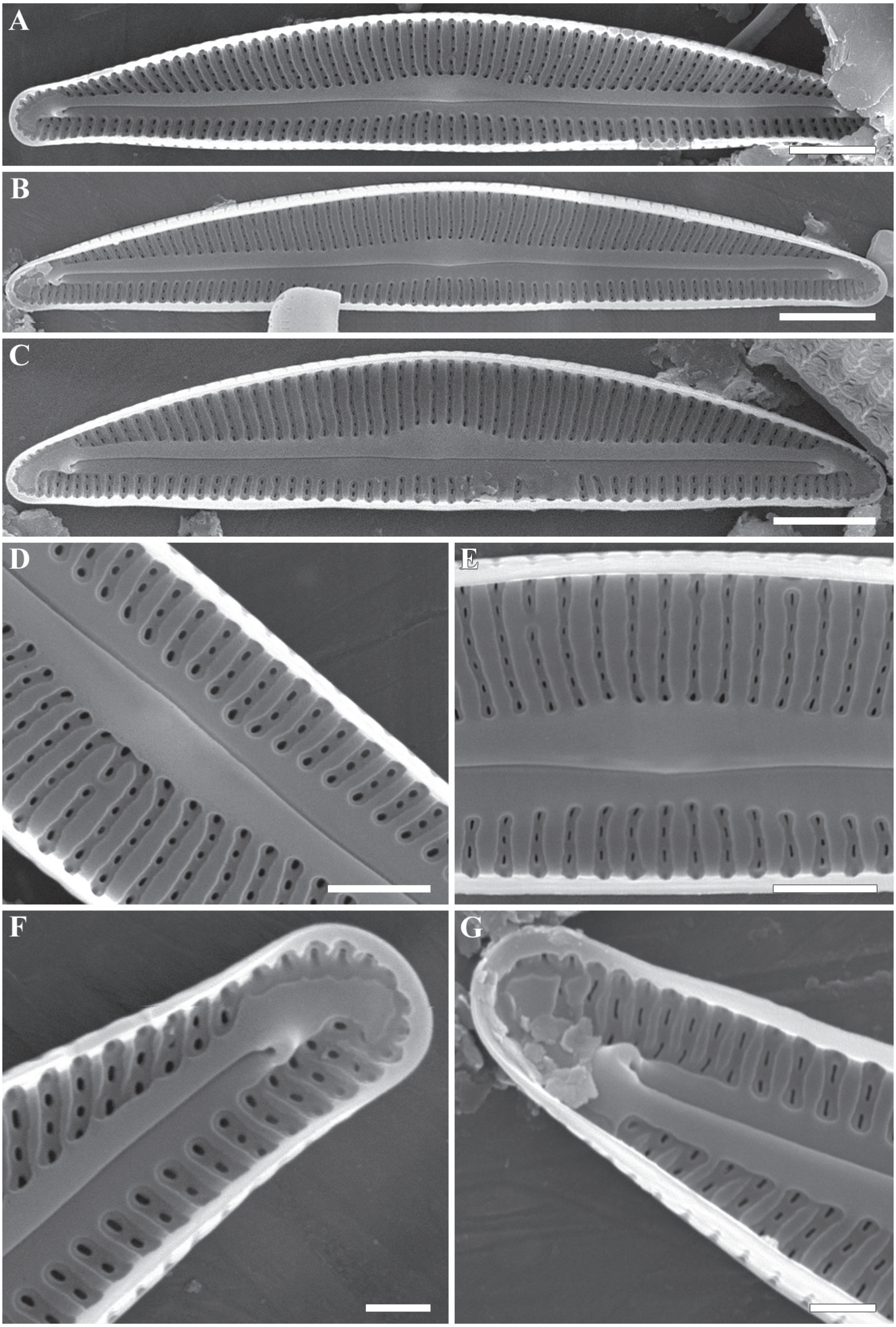

**Figure 4. A–G** *Encyonopsis indonesica* sp. nov. (SEM). Internal view **A–C** whole valve **D, E** central area with rounded or elongated areolae located in a transapically elongated grooves **F, G** valve ends with typical helictoglossa. Note a thin, plate-like silica thickening present between helictoglossa and apex. Scale bars: 2 µm (**A, C**), 2.5 µm (**B**), 1 µm (**D, E**), 0.5 µm (**F, G**).

F). Raised sterna border the raphe branches. Striae almost parallel at the valve center becoming weakly radiate towards the apices, 34–36 in 10 µm. Striae composed of 2–4 areolae on the ventral side and 5–6 areolae on the dorsal side. Areolae rounded and unoccluded, being occasionally slightly smaller near the valve margins, 50 in 10 µm. Internally, areolae rounded or transapically elongated located in a shallow groove. Internal proximal raphe ends obscured; distal raphe ends terminate in well-developed helictoglossae (Fig. 4A–E). A thin, plate-like silica thickening present between helictoglossa and apices (Fig. 4F, G).

## Discussion

*Encyonopsis indonesica* is very similar to *Amphora dissimilis* described from New Caledonia (Moser et al. 1998), however the latter is slightly larger (length 29–40 µm; breadth 4.6–5.4 µm) and has a more arched dorsal margin and more attenuated apices. Additionally, *E. indonesica* is more finely striated and has 34–36 striae in 10 µm whereas *A. dissimilis* has 18–20 striae in 10 µm, which are discernible under LM. External valve structure of both species looks very similar as well. The valve face is delineated from the mantle by a thickened marginal ridge. In *A. dissimilis* the valve face is more heavily silicified, the longitudinal siliceous ribs are longer and thicker (they are even discernible in LM) and they are present in smaller numbers than in *E. indonesica*. In *A. dissimilis* the raphe is almost straight and filiform. It is located close to the ventral side. At the valve center the raphe branches are abruptly curved dorsally to form an arc. Whereas in *E. indonesica* the raphe is distinctly undulate and does not form an arc at the valve center. Unfortunately, the internal valve structure of *A. dissimilis* remains unknown. Morphological and morphometric features of both species are summarized in Table 1.

The authors initially had some doubts when placing *Amphora dissimilis* in *Amphora* (Moser et al. 1998, pp. 90, 91). Levkov (2009) included this species in his monograph on *Amphora* but placed it with other species with uncertain systematic positions. He pointed out that the raphe system of *Amphora dissimilis* significantly differs from that of *Amphora* sensu stricto, however, he decided to keep this species in *Amphora* pending additional observations (Levkov 2009).

We agree that *Amphora dissimilis* does not belong to this genus. Taking into account the remarkably similar morphology between *Encyonopsis indonesica* and *Amphora dissimilis* we propose to transfer the latter species to *Encyonopsis*:

### ***Encyonopsis dissimilis*** **(Metzeltin & Krammer) Kapustin, Kulikovskiy & Kociolek, comb. nov.**

Basionym: *Amphora dissimilis* Metzeltin & Krammer in Moser et al. 1998. Biblioth. Diatomol. 38: 90, pl. 43: figs 1–8.

In terms of valve outline, the degree of asymmetry about the apical axis might suggest we assign *Encyonopsis indonesica* and *E. dissimilis* to *Cymbellopsis* Krammer rather

**Table 1.** Morphological and morphometric comparisons among *Encyonopsis indonesica* and *E. dissimilis*.

| Taxon | Valve shape | Valve / ends | Raphe | Valve length, µm | Valve width, µm | Striae in 10 µm | Areolae in 10 µm |
|---|---|---|---|---|---|---|---|
| *Encyonopsis indonesica* | semi-lanceolate | cuneate to rostrate | undulate | 17–29 | 3–4 | 34–36 | 50 |
| *Encyonopsis dissimilis* | semi-lanceolate | cuneate | filiform, proximal ends are abruptly curved dorsally to form an arc at the valve center | 29–40 | 4.6–5.4 | 18–20 | no data |

than to *Encyonopsis*. All species of the genus *Cymbellopsis* have distinctly dorsiventral valve outlines whereas the species of the genus *Encyonopsis* have only slightly dorsiventral valve outlines. However, in *Cymbellopsis* taxa possess an intermissio of Type 1, similar to what is seen in *Encyonema silesiacum* (Krammer 1997a). *Encyonopsis indonesica*, however, lacks an intermissio. Additionally, in *Cymbellopsis* the areolae are internally occluded with hymens whereas in *Encyonopsis indonesica* areolae are unoccluded. Moreover, internally *Encyonopsis indonesica* possesses typical *Encyonopsis* morphology. All described *Cymbellopsis* taxa are restricted to South America and Africa (Kociolek 2018).

Remarkably, many endemic diatoms from Lake Matano, e.g. *Celebesia distinguenda* (Hustedt) Kapustin, Kulikovskiy & Kociolek, *Cymbella eunotioformis* Kapustin, Kociolek & Kulikovskiy, *C. densigranulata* Kapustin, Kociolek & Kulikovskiy, *Gomphonema matanense* Kapustin, Kociolek & Kulikovskiy and *Encyonopsis indonesica* are additionally ornamented with siliceous outgrowths, ridges, granules, or spines (Kapustin et al. 2017, 2019; Kociolek et al. 2018). This situation was also shown for another cymbelloid taxon, in a species of *Delicatophycus* Wynne (Le Cohu et al. 2020) described from New Caledonia. Since silicification in diatoms is genetically encoded (e.g. Kröger 2007) and the ornamentation is rather stable and apparently does not vary significantly, it is possible this feature can be used for species delimitation.

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## References

Almeida FF, Ector L, Silva ES, Wetzel CE (2020) *Gomphonema frequentiformis* (Metzeltin & Krammer) comb. nov. (Bacillariophyta): Ecology and taxonomy of a Neotropical diatom. Phytotaxa 439: 265–275. https://doi.org/10.11646/phytotaxa.439.3.8

Bahls LL (2013) *Encyonopsis* (Bacillariophyta, Cymbellaceae) from western North America: 31 species from Alberta, Idaho, Montana, Oregon, South Dakota and Washington, incl. 17 species described as new. Northwestern Diatoms 5: 1–44.

Bahls LL (2015) *Kurtkrammeria*, a new genus of freshwater diatoms (Bacillariophyta, Cymbellaceae) separated from *Encyonopsis*. Nova Hedwigia 101(1–2): 165–190. https://doi.org/10.1127/nova_hedwigia/2015/0263

Bahls LL, Wetzel CE, Ector L (2018) Transfer of *Kurtkrammeria eileencoxiae* to the genus *Gomphonema* (Bacillariophyta, Gomphonemataceae). Notulae algarum 50: 1–4.

Bramburger AJ, Haffner GD, Hamilton PB, Hinz F, Hehanussa PE (2006) An examination of species within the genus *Surirella* from the Malili lakes, Sulawesi Island, Indonesia, with descriptions of 11 new taxa. Diatom Research 21(1): 1–56. https://doi.org/10.1080/0269249X.2006.9705650

Brooks JL (1950) Speciation in ancient lakes (concluded). The Quarterly Review of Biology 25(2): 131–176. https://doi.org/10.1086/397539

Graeff CL, Kociolek JP (2013) New or rare species of cymbelloid diatoms (Bacillariophyceae) from Colorado (USA). Nova Hedwigia 97(1): 87–116. https://doi.org/10.1127/0029-5035/2013/0106

Hustedt F (1942) Süßwasser-Diatomeen des indomalayischen Archipels und der Hawaii-Inseln. Internationale Revue der Gesamten Hydrobiologie und Hydrographie 42(1–3): 1–252. https://doi.org/10.1002/iroh.19420420102

Kapustin DA, Kulikovskiy MS (2018) Transfer of *Stenopterobia* and *Surirella* taxa (Bacillariophyceae) described from the insular Southeast Asia to the genus *Iconella*. Nova Hedwigia. Beiheft 147: 237–245. https://doi.org/10.1127/nova-suppl/2018/019

Kapustin DA, Kulikovskiy MS, Kociolek JP (2017) *Celebesia* gen. nov., a new cymbelloid diatom genus from the ancient Lake Matano (Sulawesi Island, Indonesia). Nova Hedwigia. Beiheft 146: 147–155. https://doi.org/10.1127/1438-9134/2017/147

Kapustin DA, Kociolek JP, Glushchenko AM, Kulikovskiy MS (2019) Four new species of *Cymbella* (Bacillariophyta) from the ancient Malili Lakes (Sulawesi Island, Indonesia). Botanicheskii Zhurnal 104(5): 766–780. https://doi.org/10.1134/S0006813619050065

Kapustin DA, Kociolek JP, Glushchenko AM, Kulikovskiy MS (2020) A rediscovery of *Cymbella mirabilis* Hustedt, a rare endemic diatom, and description of *Alveocymba* gen. nov. Diatom Research 35(3): 281–287. https://doi.org/10.1080/0269249X.2020.1772888

Kennedy B, Buckley Y, Allott N (2019) Taxonomy, ecology and analysis of type material of some small *Encyonopsis* with description of new species in Ireland. Phytotaxa 395(2): 89–128. https://doi.org/10.11646/phytotaxa.395.2.5

Kociolek JP (2018) A worldwide listing and biogeography of freshwater diatom genera: A phylogenetic perspective. Diatom Research 33(4): 509–534. https://doi.org/10.1080/0269249X.2019.1574243

Kociolek JP, Laslandes B, Bennett D, Thomas E, Brady M, Graeff C (2014) Diatoms of the United States 1. Taxonomy, ultrastructure and descriptions of new species and other rarely reported taxa from lake sediments in the Western U.S.A. Bibliotheca Diatomologica 61: 1–188.

Kociolek JP, Kapustin DA, Kulikovskiy MS (2018) A new, large species of *Gomphonema* Ehrenberg from ancient Lake Matano, Indonesia. Diatom Research 33(2): 241–250. https://doi.org/10.1080/0269249X.2018.1513868

Kociolek JP, Blanco S, Coste M, Ector L, Liu Y, Karthick B, Kulikovskiy M, Lundholm N, Ludwig T, Potapova M, Rimet F, Sabbe K, Sala S, Sar E, Taylor J, Van de Vijver B, Wetzel CE, Williams DM, Witkowski A, Witkowski J (2020) DiatomBase. http://www.diatombase.org/aphia.php?p=taxdetails&id=149565

Krammer K (1997a) Die cymbelloiden Diatomeen. Eine Monographie der weltweit bekannten Taxa. Teil 1. Allgemeines und *Encyonema* part. Biblioteca Diatomologica 36: 1–382.

Krammer K (1997b) Die cymbelloiden Diatomeen. Ein Monographie der weltweit bekannten Taxa. Teil 2. *Encyonema* part., *Encyonopsis* and *Cymbellopsis.* Bibliotheca Diatomologica 37: 1–463.

Krammer K (2003) *Cymbopleura*, *Delicata*, *Navicymbula*, *Gomphocymbellopsis*, *Afrocymbella*. In: Lange-Bertalot H (Ed.) Diatoms of Europe, Diatoms of the European Inland waters and comparable habitats. A.R.G. Gantner Verlag, Ruggell, 529 pp.

Kröger N (2007) Prescribing diatom morphology: Toward genetic engineering of biological nanomaterials. Current Opinion in Chemical Biology 11(6): 662–669. https://doi.org/10.1016/j.cbpa.2007.10.009

Kulikovskiy M, Kapustin D, Glushchenko A, Sidelev S, Maltsev Y, Gusev E, Kezlya E, Shkurina N, Kuznetsova I, Kociolek P (2020) Morphological and molecular investigation of *Gomphonema longissimum* and related taxa from Malili lakes (Indonesia) with comments on diatom evolution in ancient lakes. European Journal of Phycology 55(2): 147–161. https://doi.org/10.1080/09670262.2019.1664771

Le Cohu R, Gassiole G, Coste M (2014) Three new species of Cymbellales (Bacillariophyceae) from Réunion Island. Phytotaxa 156(3): 117–132. https://doi.org/10.11646/phytotaxa.156.3.3

Le Cohu R, Lange-Bertalot H, Van de Viver B, Tudesque L (2020) Analysis and critical evaluation of structural features in four Cymbellaceae taxa from New Caledonia. Fottea, Olomouc 20(1): 75–85. https://doi.org/10.5507/fot.2019.017

Levkov Z (2009) *Amphora* sensu lato. In: Lange-Bertalot H (Ed.) Diatoms of Europe. Diatoms of the European inland waters and comparable habitats (Vol. 5). A. R.G. Gantner Verlag, Ruggell, 916 pp.

Mark A, McKim S, Lowe RL, Kociolek JP (2019) Diatom community composition within *Ophrydium* colonies in northern Michigan, and the description of a new *Encyonopsis* Krammer species. Great Lakes Botanist 58: 210–221.

Marquardt GC, Da Rocha ACR, Wetzel CE, Ector L, Bicudo CEDM (2016) *Encyonema aquasedis* sp. nov. and *Kurtkrammeria salesopolensis* sp. nov.: two new freshwater diatom species (Cymbellales, Bacillariophyceae) from an oligotrophic reservoir in southeastern Brazil. Phytotaxa 247(1): 062–074. https://doi.org/10.11646/phytotaxa.247.1.4

Metzeltin D, Lange-Bertalot H (2002) Diatoms from the “Island Continent” Madagascar. In: Lange-Bertalot H (Ed.) Iconographia Diatomologica. Annotated Diatom Micrographs (Vol. 11). Taxonomy-Biogeography-Diversity. A.R.G. Gantner Verlag, Ruggell, 286 pp.

Metzeltin D, Lange-Bertalot H (2007) Tropical diatoms of South America II. Special remarks on biogeography disjunction. In: Lange-Bertalot H (Ed.) Iconographia Diatomologica. Annotated Diatom Micrographs (Vol. 18). Diversity-Taxonomy-Biogeography. A.R.G. Gantner Verlag K.G., Ruggell, 877 pp.

Moser G, Lange-Bertalot H, Metzeltin D (1998) Insel der Endemiten: Geobotanisches phänomen Neukaledonien. Bibliotheca Diatomologica 38: 1–464.

Potapova M (2014) *Encyonema appalachianum* (Bacillariophyta, Cymbellaceae), a new species from Western Pennsylvania, USA. Phytotaxa 184(2): 115–120. https://doi.org/10.11646/phytotaxa.184.2.4

Potapova M, Hamilton P, Kopyrina L, Sosina N (2014) New and rare diatom (Bacillariophyta) species from a mountain lake in Eastern Siberia. Phytotaxa 156(3): 100–116. https://doi.org/10.11646/phytotaxa.156.3.2

Vaillant J, Haffner GD, Cristescu ME (2011) The Ancient Lakes of Indonesia: Towards Integrated Research on Speciation. Integrative and Comparative Biology 51(4): 634–643. https://doi.org/10.1093/icb/icr101

Van de Vijver B, Verweij GL, Van der Wal J, Mertens A (2012) *Encyonopsis neerlandica*, a new freshwater diatom species (Bacillariophyta) from moorland pools in The Netherlands. Phytotaxa 66(1): 43–48. https://doi.org/10.11646/phytotaxa.66.1.7

von Rintelen T, von Rintelen K, Glaubrecht M, Schubart CD, Herder F (2012) Aquatic biodiversity hotspots in Wallacea: the species flocks in the ancient lakes of Sulawesi, Indonesia. In: Gower DJ (Ed.) Biotic Evolution and Environmental Change in Southeast Asia. Cambridge University Press, Cambridge, 290–315. https://doi.org/10.1017/CBO9780511735882.014

Wengrat S, Marquardt GC, Bicudo DDC, Bicudo CEDM, Wetzel CE, Ector L (2015) Type analysis of *Cymbella schubartii* and two new *Encyonopsis* species (Bacillariophyceae) from southeastern Brazil. Phytotaxa 221(3): 247–264. https://doi.org/10.11646/phytotaxa.221.3.3

Wydrzycka U, Lange-Bertalot H (2001) Las diatomeas (Bacillariophyceae) acidófilas del río Agrio y sitios vinculados con su cuenca, volcán Poás, Costa Rica. Brenesia 55–56: 1–68.

Zhang W, Gu X-W, Bao M-Y, Kociolek JP, Blanco S, Li Y-L ( (2020) *Kurtkrammeria tiancaiensis* sp. nov. a new cymbelloid species (Bacillariophyceae) from Lijiang Laojunshan National Park in Yunnan Province, China. Phytotaxa 451(3): 223–230. https://doi.org/10.11646/phytotaxa.451.3.5

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# New insights into the phylogeny of *Sinocarum* (Apiaceae, Apioideae) based on morphological and molecular data

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## Abstract

*Sinocarum* is a Sino-Himalayan endemic genus of Apiaceae and distributed in high-elevations from Nepal to SW China. In this study, morphological characteristics were combined with nuclear internal transcribed spacer (ITS) and two chloroplast DNA (cpDNA) intron sequences (*rpl16* and *rps16*) to determine the phylogenetic placement of *Sinocarum* and the infrageneric relationships between five *Sinocarum* species. The results confirmed that *Sinocarum* was a polyphyletic group separated into two clades, *Acronema* and East Asia clades. *S. coloratum*, the generic type of *Sinocarum*, *S. cruciatum*, *S. vaginatum* and *S. filicinum* are in the *Acronema* clade. Among them, the first three species are clustered into a subclade and are closely related to the genus *Acronema*. While *S. filicinum* has a close affinity with *Meeboldia*. *S. schizopetalum* did not ally with its congeners we collected and is allied closely with members of the distantly related East Asia clade. In addition, the fruit of the *Acronema* clade *Sinocarum* species is usually oblong-ovoid or ovoid, and the pollen is super-rectangular, while the *Sinocarum* species in the East Asia clade have broad-ovoid fruit and sub-rhomboidal pollen. This study has furnished cumulative evidence to reduce phylogenetic uncertainty and provide a more comprehensive description of the plant morphology, fruit morphology and anatomy, and pollen morphology of these five Chinese *Sinocarum* species.

## Keywords

Apiaceae, morphology, phylogeny, *Sinocarum*

## Introduction

*Sinocarum* H. Wolff ex R. H. Shan & F. T. Pu (1980: 374) was transferred from the genus *Carum* L. (1753: 263) by Wolff (1927), but formally described by Shan and Pu (1980). *Sinocarum* encompasses about 20 species, with eight species (four endemic) in China and is distributed at high-elevation in the Sino-Himalayan region from Nepal to SW China (Pu et al. 2005). It is usually classified by a suite of characteristics: elongate rhizome, expanded petiole sheaths, obtuse at apex and clawed at base petals and oblong-ovoid fruit (Shan et al. 1980; Pu et al. 2005). Despite several easily recognizable characteristics, there remains morphological and taxonomic confusion in the genus, including the lack of morphological description and specimens of mature fruit, unclear intergeneric boundary and excessive use of synonyms. *Sinocarum* is a taxonomically complex genus that is closely related to *Acronema* Falcon. ex Edgew. (1846: 51) and sometimes difficult to distinguish (Pu et al. 2005). Thus, further work and more extensive specimen collections are needed to clarify the situation.

Palynological study of *Sinocarum* mainly focused on seven species, these being *S. coloratum* (Diels) H. Wolff ex R. H. Shan & F. T. Pu (1985: 33), *S. cruciatum* (Franch.) H. Wolff ex R. H. Shan & F. T. Pu (1985: 33), *S. dolichopodum* (Diels) H. Wolff ex R. H. Shan & F. T. Pu (1985: 38), *S. filicinum* H. Wolff (1929: 182), *S. pauciradiatum* R. H. Shan & F. T. Pu (1980: 374), *S. schizopetalum* (Franch.) H. Wolff ex R. H. Shan & F. T. Pu (1985: 33) and *S. vaginatum* H. Wolff (1929: 183). The seven species were observed through light microscope (LM) and scanning electron microscope (SEM) (Wang 1998; Shu and She 2001). The study indicated that the equatorial view of *Sinocarum* pollen was usually broad-ellipsoidal, and the equatorial exine was rugulate (Shu and She 2001).

Previous studies have shown that fruit characteristics play a key role in the classification of subfamily Apioideae (Kljuykov et al. 2004; Lyskov et al. 2017; Guo et al. 2018; Jia et al. 2019). The fruit characteristics of *Sinocarum* species are described in *Flora Reipublicae Popularis Sinicae* and *Flora of China*. The fruit of *Sinocarum* is oblong-ovoid with 5 filiform ribs, but only young fruit is involved, and the mature fruit is unknown (Shan and Pu 1985; Pu et al. 2005). Hence, the definition of the fruit's morphological and anatomical characteristics needs to be supplemented to allow for better identification.

Similarly, previous molecular studies have been limited and results ambiguous. *Sinocarum* was found to be polyphyletic based on ITS, cpDNA sequences and limited specimen materials (*S. coloratum*, *S. cruciatum* and *S. dolichopodum*) and there has been no consensus on its phylogenetic placement (Valiejo-Roman et al. 2002; Zhou et al. 2008; Zhou et al. 2009; Downie et al. 2010). Consequently, there is a gap in our understanding of *Sinocarum's* phylogeny and infrageneric classification due to insufficient specimen sampling. Together with limited definitions of morphological characteristics, there is a need to study the phylogeny and morphology of this genus based on new, comprehensive materials.

Therefore, the objective of this study was to estimate the phylogenetic placement of *Sinocarum* and the infrageneric relationships of the five *Sinocarum* species we collected. This is the first comprehensive phylogenetic analysis of *Sinocarum* using morphology and three DNA regions data (i.e. ITS, *rpl16* and *rps16*). Given this more comprehensive analysis, we also discuss the significance of using morphology in phylogenic analyses. In addition, we provide more comprehensive descriptions for the plant morphology, fruit morphology and anatomy, pollen morphology and identification of herbarium specimens of five accepted *Sinocarum* species. We believed that this study will contribute to a better understanding of the phylogenetic status, infrageneric relationships and morphological identification of *Sinocarum*.

## Materials and methods

### Field investigation, morphology study and specimen examination

Samples were obtained from type localities and adjacent areas of *S. coloratum* (Mt. Yulong, Yunnan), *S. cruciatum* (Mt. Jizu, Yunnan), *S. filicinum* (Mt. Cang; Mt. Jizu, Yunnan), *S. schizopetalum* (Mt. Cang, Yunnan) and *S. vaginatum* (Mt. Cang, Yunnan). The fruit of *Meeboldia yunnanensis* (H. Wolff) Constance & F. T. Pu (1998: 70) was obtained from Kunming, Yunnan. Photographs of specimens were made using a Nikon D5600 camera. Fruits were observed and photographed using a stereomicroscope, Nikon SMZ 25 (Japan), and five representative fruit samples were selected to observe characters and measure their size, and then calculate the average value. Pollen grains from the anthers of specimens were directly mounted on copper stubs with conductive carbon adhesive tabs using a needle, sputtercoated with gold, and observed with a Hitachi-SX-450 SEM (Japan). The continuous section of the middle transection of the mericarp was made by the normal paraffin section method. And the section was observed and photographed using stereomicroscope Nikon SMZ25 (Japan). A total of ten pollen grains were selected to measure their length of polar axis (P) and equatorial axis (E), and calculate their average value, ratio of polar axis to equatorial axis (P/E) and size index ($\sqrt{P\times E}$). The micromorphological characteristics of pollen were described according to Shu and She (2001). Morphological characteristics were measured using Kayotype (Altınordu et al. 2016). Voucher specimens were deposited in the herbarium of Natural History Museum of Sichuan University (SZ) (Table 1).

The related specimens in A, BM, CDBI, E, GB, GH, HNWP, IBSC, JAY, K, KATH, KUN, NAS, NWFC, NY, P, PE, SZ, USP and W were studied and presented in Table 2. Information and photographs of type specimens were gathered from Tropicos (http://www.tropicos.org), the International Plant Names Index (http://www. ipni.org) and JSTOR Global Plants (http://plants.jstor.org).

### DNA extraction, amplification and sequencing

Total genomic DNA was extracted from silica gel-dried leaves and herbarium materials according to the protocols of plant genomic DNA kit (Tiangen Biotech, Beijing, China). Nuclear ribosomal DNA (nrDNA) ITS sequences and two chloroplast DNA (cpDNA)

**Table 1.** Voucher details and GenBank accession numbers of taxa used in this study.

| Taxa | Voucher | Locality | ITS | *rpl16* | *rps16* |
|---|---|---|---|---|---|
| *Acronema astrantiifolium* H. Wolff | T2010093003(SZ) | Muli, Sichuan, China | KP940757 | KP940829 | KP940901 |
| *A. muscicola* (Hand.-Mazz.) Hand.-Mazz. | XZ2011081741(SZ) | Xizang, China | KP940756 | KP940828 | KP940900 |
| *A. paniculatum* (Franch.) H. Wolff | T2010100602(SZ) | Xiangcheng, Sichuan, China | KP940758 | KP940830 | KP940902 |
| *A. schneideri* H. Wolff | ZJ810826(KUN) | Shangri-La, Yunnan, China | EU236156 | FJ385070 | – |
| *Anthriscus sylvestris* (L.) Hoffm. | ZJ0566(KUN) | Daocheng-Litang, Sichuan, China | EU236159 | FJ385078 | FJ385176 |
| *Chaerophyllum prescottii* DC. | ZJ0744(KUN) | Habahe, Xinjiang, China | FJ385039 | FJ385084 | FJ385183 |
| *Changium smyrnioides* H. Wolff | J101(KUN) | Jiangsu Institute of Botany, China | DQ517340 | FJ385088 | FJ385187 |
| *Chuanminshen violaceum* M. L. Sheh & R. H. Shan | J105(KUN) | Xinlong, Sichuan, China | FJ385040 | FJ385089 | FJ385188 |
| *Cyclorhiza peucedanifolia* (Franch.) Constance | J034(KUN) | Lijiang, Yunnan, China | FJ385042 | FJ385092 | FJ385191 |
| *C. waltonii* (H. Wolff) M. L. Sheh & R. H. Shan | ZJ0536(KUN) | Derong, Sichuan, China | EU236165 | FJ385093 | FJ385192 |
| *Ferula kingdon-wardii* H. Wolff | ZJ810846(KUN) | Shangri-La, Yunnan, China | EU236166 | FJ385094 | FJ385193 |
| *Halosciastrum melanotilingia* (H. Boissieu) Pimenov & V. N. Tikhom. | Pimenov & Kljuykov 200 (MW) | Khasan distr., Primorsk Terr., Russia | AY328937,<br>AY330503 | – | – |
| *Hansenia forbesii* (H. Boissieu) Pimenov et Kljuykov | 666939(SZ) | – | GU390407 | – | – |
| *H. weberbaueriana* (Fedde ex H. Wolff) Pimenov et Kljuykov | ZJ0697(KUN) | KIB nursery, Yunnan, China | EU236180 | FJ385115 | FJ385212 |
| *Harrysmithia franchetii* (M. Hiroe) M. L. Sheh | ZJ0748(KUN) | Luquan, Yunnan, China | FJ385044 | FJ385097 | FJ385195 |
| *H. heterophylla* H. Wolff | T2012052603 (SZ) | Baoxing, Sichuan, China | KP940763 | – | – |
| *Haplosphaera phaea* Hand.-Mazz. | ZJ0521(KUN) | Shangri-La, Yunnan, China | EU236167 | FJ385096 | FJ385194 |
| *Heptaptera anisoptera* (DC.) Tutin | Pimenov & Kljuykov 438 (MW) | Lorestan, Iran | AY941273,<br>AY941301 | – | – |
| *Komarovia anisosperma* Korovin | – | – | AF077897 | AF094434 | AF110555 |
| *Ligusticum delavayi* Franch. | ZJ810841(KUN) | Shangri-La, Yunnan, China | EU236174 | FJ385106 | FJ385204 |
| *Meeboldia achilleifolia* (DC.) P. K. Mukh. & Constance | Pimenov & Kljuykov 28 (MW) | Langtang National Park, Nepal | AY038206,<br>AY038220 | – | – |
| *M. yunnanensis* (H. Wolff) Constance & F. T. Pu | ZJ0673(KUN) | Fumin, Yunnan, China | EU236178 | FJ385110 | FJ385208 |
| *Oenanthe hookeri* C. B. Clarke | ZJ0519(KUN) | Shangri-La, Yunnan, China | EU236182 | – | – |
| *Oreocomopsis stelliphora* (Cauwet & Farille) Pimenov & Kljuykov | Farille 81-421 (G) | N Annapurna, Nepal | GQ379322 | – | – |
| *Oreomyrrhis involucrata* Hayata | J111(KUN) | Taiwan, China | FJ385052 | – | FJ385218 |
| *Ostericum scaberulum* (Franch.) C. Q. Yuan & R. H. Shan | YL757(KUN) | Lijiang, Yunnan, China | FJ385053 | FJ385121 | FJ385219 |
| *Pachypleurum xizangense* H. T. Chang & R. H. Shan | Watson & Gilbert 1580 (E, EBH) | Madoi, Qinghai, China, | KJ660841 | – | KJ660442 |
| *Physospermopsis cuneata* H. Wolff | J066(KUN) | Lijiang, Yunnan, China | FJ385055 | FJ385125 | FJ385221 |
| *P. kingdon-wardii* (H. Wolff) C. Norman | ZJ810822(KUN) | Sichuan, China | EU236190 | FJ385127 | FJ385223 |
| *P. muliensis* R. H. Shan & S. L. Liou | ZJ0686(KUN) | Ninglang, Yunnan, China | EU236191 | FJ385128 | FJ385224 |
| *P. rubrinervis* (Franch.) C. Norman | FED 378 (E) | Shangri-La, Yunnan, China | AF164836,<br>AF164861 | – | – |
| *P. shaniana* C. Y. Wu & F. T. Pu | ZJ0678(KUN) | Ninglang, Yunnan, China | EU236192 | FJ385129 | FJ385225 |
| *Pimpinella acuminata* (Edgew.) C. B. Clarke | ZJ0503(KUN) | Lijiang, Yunnan, China | EU236193 | FJ385130 | FJ385226 |
| *P. henryi* Diels | ZJ0524(KUN) | Shangri-La, Yunnan, China | EU236195 | FJ385132 | FJ385228 |
| *P. purpurea* (Franch.) H. Boissieu | ZJ0527(KUN) | Shangri-La, Yunnan, China | EU236197 | FJ385133 | FJ385229 |
| *Pleurospermum franchetianum* Hemsl. | ZJ0573(KUN) | Yajiang-Kangding, Sichuan, China | EU236198 | FJ385137 | FJ385232 |
| *P. hookeri* var. *thomsonii* C. B. Clarke | ZJ0545(KUN) | Sichuan, China | EU236199 | FJ385138 | FJ385233 |
| *P. wrightianum* H. Boissieu | ZJ0669(KUN) | Shangri-La, Yunnan, China | EU236201 | FJ385140 | FJ385235 |
| *P. yunnanense* Franch. | ZJ091033(KUN) | Shangri-La, Yunnan, China | EU236202 | FJ385141 | FJ385236 |
| *Pternopetalum botrychioides* (Dunn) Hand.-Mazz. | ZJ04(KUN) | Suijiang, Yunnan, China | EU236203 | FJ385142 | FJ385237 |
| *P. cardiocarpum* (Franch.) Hand.-Mazz. | ZJ0581(KUN) | Luding-Mianning, Sichuan, China | EU236204 | FJ385143 | FJ385238 |
| *P. davidii* Franch. | ZJ06(KUN) | Suijiang, Yunnan, China | EU236205 | FJ385144 | FJ385239 |

| Taxa | Voucher | Locality | ITS | *rpl16* | *rps16* |
|---|---|---|---|---|---|
| *Pterygopleurum neurophyllum* (Maxim.) Kitag. | – | – | AY509127 | – | – |
| *Rupiphila tachiroei* (Franch. & Sav.) Pimenov & Lavrova | Pimenov & Kljuykov 169 (MW) | Primorsk Terr., Russia | AY328952,<br>AY330518 | – | – |
| *Sinocarum bellum* (C. B. Clarke) Pimenov & Kljuykov | Skvortzov & Proskurjakova (MHA) | West Bengal, India | MK309872 | – | – |
| *S. coloratum* (Diels) H. Wolff ex R. H. Shan & F. T. Pu | XYP19071901(SZ) | Lijiang, Yunnan, China | MN846685* | MN852960* | MN852964* |
| *S. coloratum* (Diels) H. Wolff ex R. H. Shan & F. T. Pu | YL561(KUN) | Lijiang, Yunnan, China | FJ385063 | FJ385154 | FJ385248 |
| *S. coloratum* (Diels) H. Wolff ex R. H. Shan & F. T. Pu | – | – | AY328927 | – | – |
| *S. cruciatum* (Franch.) H. Wolff ex R. H. Shan & F. T. Pu | XYP19080301(SZ) | Dali, Yunnan, China | MN846686* | MN852961* | MN852965* |
| *S. cruciatum* (Franch.) H. Wolff ex R. H. Shan & F. T. Pu | ZJ0672(KUN) | Shangri-La, Yunnan, China | EU236209 | FJ385155 | FJ385249 |
| *S. cruciatum* (Franch.) H. Wolff ex R. H. Shan & F. T. Pu | – | – | AY038199,<br>AY038213 | – | – |
| *S. dolichopodum* (Diels) H. Wolff ex R. H. Shan & F. T. Pu | ZJ0548(KUN) | Sichuan, China | EU236208 | FJ385156 | FJ385250 |
| *S. filicinum* H. Wolff (JZS) | XYP19080302(SZ) | Dali, Yunnan, China | MT586806* | MT588116* | MT588118* |
| *S. filicinum* H. Wolff (CS) | XYP19091803(SZ) | Dali, Yunnan, China | MT586807* | MT588117* | MT588119* |
| *S. schizopetalum* (Franch.) H. Wolff ex R. H. Shan & F. T. Pu | XYP19080401(SZ) | Dali, Yunnan, China | MN846687* | MN852962* | MN852966* |
| *S. vaginatum* H. Wolff | XYP19080402(SZ) | Dali, Yunnan, China | MN846688* | MN852963* | MN852967* |
| *S. wolffianum* (Fedde ex H. Wolff) P. K. Mukh. & Constance | Pimenov & Kljuykov 62 (MW) | Yumthang, Sikkim, India | MK309871 | – | – |
| *Sinolimprichtia alpina* H. Wolff | 0465919(KUN) | Xizang, China | FJ385064 | FJ385157 | FJ385251 |
| *Sium frigidum* Hand.-Mazz. | ZJ0520(KUN) | Shangri-La, Yunnan, China | EU236210 | – | – |
| *S. ventricosum* (H. Boissieu) Li S. Wang & M. F. Watson | – | – | AY038200,<br>AY038214 | – | – |
| *Spuriopimpinella arguta* (Diels) X. J. He & Z. X. Wang | T2012091505 (SZ) | Songxian, Henan, China | KP940760 | – | – |
| *S. brachycarpa* (Kom.) Kitag. | T2012093001 (SZ) | Anshan, Liaoning, China | KP940761 | – | – |
| *Tilingia ajanensis* Regel & Til. | Pimenov & Kljuykov 139 (MW) | Saghalien, Russia | AY328939,<br>AY330505 | – | – |
| *Torilis japonica* (Houtt.) DC. | ZJ0623(KUN) | Hongyuan, Sichuan, China | EU236214 | FJ385163 | AF123741 |
| *Tongoloa elata* H. Wolff | Pimenov et al. 180 (MW) | Hongyuan-Barkam, Sichuan, China | AY038207,<br>AY038221 | – | – |
| *T. gracilis* H. Wolff | ZJ0554 (KUN) | Daocheng, Sichuan, China | EU236211 | – | – |
| *T. loloensis* (Franch.) H. Wolff | ZJ0501(KUN) | Lijiang, Yunnan, China | EU236212 | FJ385160 | FJ385254 |
| *T. silaifolia* (H. Boissieu) H. Wolff | ZJ810821(KUN) | Sichuan, China | EU236213 | FJ385161 | FJ385255 |
| *T. tenuifolia* H. Wolff | J075(KUN) | Lijiang, Yunnan, China | FJ385066 | FJ385162 | FJ385256 |
| *Trachydium simplicifolium* W. W. Sm. | J091(KUN) | Lijaing, Yunnan, China | FJ385067 | FJ385164 | FJ385257 |
| *Vicatia bipinnata* R. H. Shan & F. T. Pu | ZJ0564(KUN) | Daocheng-Litang, Sichuan, China | EU236217 | FJ385167 | FJ385260 |

– unavailable sequences. * Newly generated sequences; otherwise, sequences were obtained from GenBank.

intron sequences (*rpl16* and *rps16*) were applied to phylogenetic analyses. The primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3'; White et al. 1990) were used for PCR-amplification of a complete ITS fragment. The *rpl16* intron region was amplified with primers F71(5'-GCT ATG CTT AGT GTG TGA CTC GTT G-3') and R1516 (5'-CCC TTC ATT CTT CTA TGT TG-3'; Jordan et al. 1996; Kelchner and Clark 1997). The *rps16* intron was amplified using primers *rps16* 5'exon (5'-AAA CGA TGT GGN AGN AAR CA-3') and *rps16* 3'exon (5'-CCT GTA GGY TGN GCN CCY TT-3'; Downie and Katz-Downie 1999). Amplification was undertaken in a 30 μL mixture of 2 μL plant total DNA,

**Table 2.** *Sinocarum* specimens examined in this study.

| Species | Type specimens | Additional specimens examined |
|---|---|---|
| *S. coloratum* | **China. Yunnan**: Grassy ledges of cliffs on the eastern flank of the Lichiang Range, 11–12000 ft, Lat. 27°25'N, September 1906, *G. Forrest 3060* (lectotype: E!, designated by Watson, 1998: 382; isolectotype: BM0000574892). | **China**. Without specific locality, *Lianda expedition 21341* (KUN); without specific locality, *Lianda expedition 21612* (KUN). **Sichuan Province**: Konkaling, Tsungu, 3850 m, 30 August 1937, *T. T. Yu 13026* (PE); Daocheng, Mt. Gongga, 3200 m, 1 September 1981, *Qinghai-Xizang expedition 6025* (KUN); Daocheng, Mt. Gongga, 4500 m, 29 August 1981, *Qinghai-Xizang expedition 5581* (KUN). **Yunnan Province**: Shangri-La, 3500 m, 16 August 1962, *Zhongdian expedition 957* (PE); Lijiang, 3 September 1939, *Z. G. Zhao 30577* (PE); Lijiang, 3000 m, 11 August 1937, *T. T. Yu 15416A* (PE); Binchuan, Ki-chan, 2900 m, 18 September 1929, *R. C. Ching 24714* (PE); Mekong-Yangtze divide, 4000 m, August 1914, *G. Forrest 12970* (PE); Likiang Snow Range, 3 September 1939, *R. C. Ching 30577* (KUN); Fugong, Mt. Biluo, 4300 m, 12 September 1964, *S. G. Wu 8780* (KUN); Fugong, Mt. Biluo, 12 September 1964, *S. G. Wu 8807* (KUN); Shangri-La, Mt. Haba, 31 August 1962, *Zhongdian expedition 1800* (KUN); Shangri-La, Mt. Haba, 8 September 1962, *Zhongdian expedition 1923* (KUN); Lijiang, Mt. Yulong, 3800 m, 19 July 2019, *Y. P. Xiao & Q. P. Jiang XYP19071901* (SZ); Lijiang, Mt. Yulong, 3800 m, 27 September 2019, *Y. P. Xiao & Q. P. Jiang XYP19092701* (SZ). |
| *S. cruciatum* | **China. Yunnan**: Mt. Ki-chan, 2800 m, 10 September 1884, *Delavay 182* (lectotype: P03224861!, designated by Pimenov, 2017: 219; isolectotypes: P02284823, P03224868). | **China. Yunnan Province**: Binchuan, Mt. Jizu, 3000 m, 3 August 2019, *Y. P. Xiao & Q. P. Jiang XYP19080301* (SZ). |
| *S. filicinum* | **China. Yunnan**: Eastern flank of the Tali Range, 2540 m, *G. Forrest 6963* (lectotype: E!, designated by M. Farille; isolectotype: K000685663!). | **China**. Without specific locality, *Lianda expedition 12092* (KUN). **Yunnan Province**: Dali, 22 June 1945, *H. C. Wang 4412* (PE); Dali-Hejiang, September 1941, *H. C. Wang 1396* (PE); Binchuan, Mt. Jizu, 3200 m, *R. C. Ching 24920* (PE); Binchuan, Mt. Jizu, *Anonymous 2447* (PE); Binchuan, Mt. Jizu, 3000 m, 3 August 2019, *Y. P. Xiao & Q. P. Jiang XYP19080302* (SZ); Dali, Mt. Cang, 3200 m, 18 September 2019, *Y. P. Xiao & Q. P. Jiang XYP19091803* (SZ). |
| *S. schizopetalum* | **China. Yunnan**: Mt. Tsang-chan, 4000 m, 25 July 1884, *Delavay 196* (lectotype P!, designated by Pimenov, 2017: 221; isolectotypes: K000685665!, PE). | Without specific locality, *C. Y. Wu & D. Y. Liu 20581* (PE, PEY). **Yunnan Province**: Dali, Mt. Cang, 3800 m, 4 August 2019, *Y. P. Xiao & Q. P. Jiang XYP19080401* (SZ). |
| *S. vaginatum* | **China. Yunnan**: Mt. Ghi Shan, 11000 ft. (About 3350 m), open pasture, August 1917, *G. Forrest 15484* (lectotype: E!, designated by Pimenov, 2017: 221; isolectotype: K000685664). | **China. Sichuan Province**: Yanyuan, Mt. Xiaogao, 2150 m, October 1986, *Z. H. Pan & Y. J. Li & F. T. Pu 964* (CDBI). **Yunnan Province**: Dali, Mt. Cang, 21 August 1944, *H. C. Wang 4511* (PE); Dali, Mt. Cang, 4 August 2019, *Y. P. Xiao & Q. P. Jiang XYP19080402* (SZ). |

10 μL $ddH_2O$, 1.5 μL forward primer, 1.5 μL reverse primer and 15 μL 2 × Taq MasterMix (cwbio, Beijing, China). The amplification of the ITS region was obtained by initial denaturation for 3 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 60 s at 54 °C, and 90 s at 72 °C, and then a final extension of 10 min at 72 °C. The amplification of the *rpl16* region was obtained by initial denaturation for 3 min at 94 °C, followed by 36 cycles of 45 s at 94 °C, 70 s at 58.5 °C, and 90 s at 72 °C, and then a final extension of 10 min at 72 °C. Whereas amplification of *rps16* region was obtained by initial denaturation for 3 min at 94 °C, followed by 36 cycles of 45 s at 94 °C,70 s at 54 °C, and 90 s at 72 °C, and then a final extension of 10 min at 72 °C. All PCR products were separated using a 1.5% (w/v) agarose TAE gel and sent to Sangon (Shanghai, China) for sequencing. New sequences generated for this study have been deposited in GenBank (Table 1).

## Sequence alignment and phylogenetic analysis

We used 53 nrDNA ITS sequences obtained from GenBank, and six sequences newly sequenced for this study (Table 1), to infer the phylogenetic placement of *Sinocarum*. Seventy-four accessions obtained from GenBank for the nrDNA (ITS) and cpDNA (*rpl16* and *rps16*), and 15 accessions newly sequenced (Table 1) represented 35 species from 21 genera of Apiaceae and were used to reconstruct the phylogenetic tree of the *Acronema* clade. Tribe Scandiceae was selected as the outgroup (Downie et al. 2000a; Zhou et al. 2008; Zhou et al. 2009). Eighty-three accessions obtained from GenBank for the nrDNA (ITS) and cpDNA (*rpl16* and *rps16*), and three accessions newly sequenced (Table 1) represented 31 species from 15 genera of Apiaceae and were used to reconstruct the phylogenetic tree of the East Asia clade. Tribe Pleurospermeae was selected as the outgroup (Downie et al. 2000b; Zhou et al. 2008; Zhou et al. 2009). Sequence data for the ITS 5.8S region were excluded from the analysis because they were unavailable for several previously published taxa.

SeqMan (Burland 2000) was used to assemble DNA sequences and obtain consensus sequences. DNA sequences were aligned with ClustalX ver. 2.1 (Larkin et al. 2007) and then adjusted manually using MEGA7 (Kumar et al. 2016). Phylogenetic analyses of data were conducted by employing Maximum Likelihood (ML) and Bayesian Inference (BI) methods. Maximum Likelihood phylogenetic reconstruction was performed using RAxML-HPC ver. 8.2.10 under the GTR+G nucleotide substitution model and 1,000 rapid bootstraps. The BI analysis was performed in MrBayes version 3.2 (Ronquist et al. 2012). MrModeltest version 2.2 (Nylander 2004) was used for BI analysis to determine a best-fit model of nucleotide substitution. From a random starting tree, the BI analysis was run for 10 million generations and the trees were saved to a file every 1,000 generations. Posterior probabilities were approximated by sampling trees using a variant of the Markov Chain Monte Carlo (MCMC) method. The first 1,000 trees were discarded as "burn-in" and a majority-rule consensus tree was calculated based upon the remaining 9,000 trees resulting from Tracer 1.4 analysis (Drummond and Rambaut 2007).

## Results

### Plant morphology

The plant morphological characteristics of *Sinocarum* species are shown in Table 3 and Fig. 1, and we can know that *Sinocarum coloratum* typically possesses purplish stems, oblong-ovate sheaths, lanceolate ultimate segments of blades and white petals (Fig. 1A). *S. cruciatum* generally has torulose roots, subequal rays, triangular in outline and ternate-1–2-pinnate basal leaves, and reduced upward to 1-pinnate or 3-lobed cauline leaves (Fig. 1B). *S. filicinum* develops broadly ovate sheaths, linear-lanceolate bracts and bracteoles, triangular in outline and 2-pinnate blades, oblong-ovate blade ultimate segments with serrated margins, and sparsely pubescent petioles, rachides and the abaxial surface of segments (Fig. 1C). *S. schizopetalum* typically has conic taproot, broad-lanceolate sheaths, triangular in outline and ternate-1–2-pinnate blades with oblong-lanceolate ultimate segments, and white or violet petals (Fig. 1D). *S. vaginatum* generally possess ovate sheaths, unequal rays, entire petals with acute apexes, triangular and ternate-2–3-pinnate blades with elongate-linear ultimate segments, and reduced upwards to 1–2-pinnate cauline leaves (Fig. 1E).

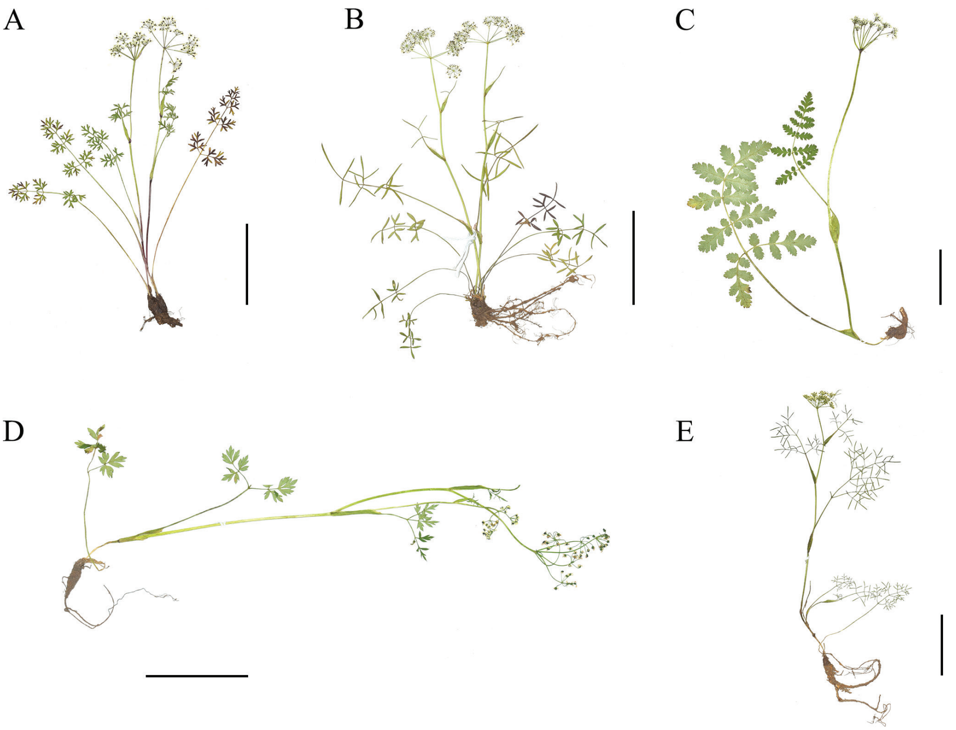

**Figure 1.** Specimens of *Sinocarum* **A** *S. coloratum* **B** *S. cruciatum* **C** *S. filicinum* **D** *S. schizopetalum* **E** *S. vaginatum*. Scale bars: 5 cm.

**Table 3.** Comparison of plant morphological characteristics of *Sinocarum*.

| Characteristics | *S. coloratum* | *S. cruciatum* | *S. filicinum* | *S. schizopetalum* | *S. vaginatum* |
|---|---|---|---|---|---|
| Root | taproot elongate, thickened at apex, branched | rootstock short, thick; roots torulose | taproot elongate, stout, often branched | taproot conic | taproot elongate, thick, often branched |
| Stems | characteristically purplish | green | green | green | green |
| Basal petioles | 2–10 cm | 5–7 cm | 8–15 cm, sparsely pubescent | 5–8 cm | 5–18 cm |
| Sheath | oblong-ovate | oblong-ovate | broadly ovate | broadly lanceolate | ovate |
| Basal leaves | blade ovate-lanceolate in outline, 1–2-pinnate; pinnae 4–5 pairs | triangular in outline, ternate-1–2-pinnate; pinnae 3–5 pairs | triangular in outline, 2-pinnate; pinnae 3–7 pairs | triangular in outline, ternate to 1- or 2-pinnate | triangular in outline, ternate-2–3-pinnate; pinnae 4–6 pairs |
| Ultimate segments of blade | linear-lanceolate | linear-lanceolate | oblong-ovate, margins serrate, abaxially sparsely pubescent along veins | oblong-lanceolate | elongate-linear |
| Cauline leaves | similar to the basal leaves | elongate-linear, reduced upwards becoming 1-pinnate or 3-lobed | similar to the basal leaves; upper leaves 1-pinnate | similar to the basal leaves | elongate-linear, 1–2-pinnate, reduced upwards |
| Bracts | absent or occasionally 1–2, linear | absent, occasionally 1 | 1–4, linear-lanceolate | absent, occasionally 1, linear-lanceolate | absent or occasionally 1 |
| Bracteoles | absent, rarely 1, linear | absent, occasionally 1 | 5–8, linear-lanceolate | 3–5, linear-lanceolate | absent |
| Rays | 5–8(–12), unequal | 4–7(–10), subequal | 2–8, 1–3 cm, unequal | (3–)5–6(–8), unequal | 10–12, unequal |
| Petals | ovate or broadly obovate, apex usually entire, occasionally 2–3-lobed, white | oblong-ovate or broadly obovate, apex obtuse to subacute, greenish-white | ovate or broadly obovate, apex subacute, white | apex palmately 3–4-lobed, lobes lanceolate or oblanceolate, white or violet | oblong-ovate or broadly obovate, apex subacute, white |

**Table 4.** Mature fruit morphological and anatomical characteristics of *Sinocarum* species and *Meeboldia yunnanensis*.

| Species | Fruit shape | | Transection | Endosperm concrescence | Development degree of ribs | Vittae number | |
|---|---|---|---|---|---|---|---|
| | L × W (mm) | Shape | | | | Furrow | Commissure |
| *Meeboldia yunnanensis* | 4.08 × 2.35 | ovoid | semicircle | cordate concave | unobvious | 2–3 | 4 |
| *S. coloratum* | 2.09 × 1.16 | ovoid | sub-pentagon | flat | obvious | 1–3 | 2–4 |
| *S. cruciatum* | 2.41 × 0.96 | oblong-ovoid | sub-pentagon | flat | obvious | 1–3 | 2–4 |
| *S. filicinum* | 2.81 × 1.97 | ovoid | semicircle | sub-cordate concave | unobvious | 2–3 | 4 |
| *S. schizopetalum* | 1.43 × 1.36 | broad-ovoid | semicircle | slightly concave | unobvious | 1–3 | 2 |
| *S. vaginatum* | 2.28 × 0.90 | oblong-ovoid | sub-pentagon | flat | obvious | 1–3 | 2–4 |

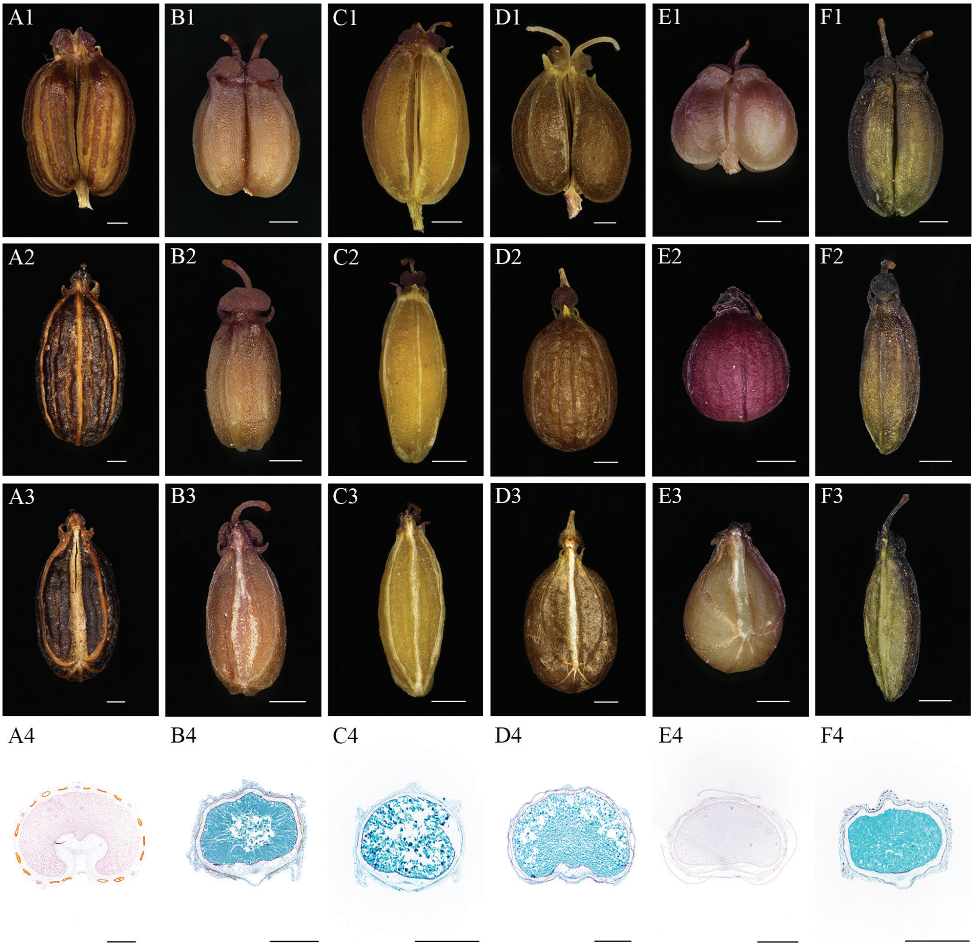

**Figure 2.** Morphological and anatomical characteristics of mature fruit **A** *Meeboldia yunnanensis* **B** *Sinocarum coloratum* **C** *S. cruciatum* **D** *S. filicinum* **E** *S. schizopetalum* **F** *S. vaginatum*. Scale bars: 0.5 mm.

## Fruit morphology and anatomy

The fruit morphological and anatomical characteristics of *Sinocarum* species and *Meeboldia yunnanensis* were studied. The results are shown in Table 4 and Fig. 2. We found that the mature fruits of *Meeboldia yunnanensis* are ovoid with 5 filiform inconspicuous ribs, 2–3 vittae in each furrow and 4 on commissure, semicircle transection of mericarp and cordate concave endosperm concrescence (Fig. 2A1–A4). The ovoid mature fruits of *S. coloratum* have 1–3 vittae in each furrow and 2–4 on commissure, sub-pentagon transection and flat endosperm concrescence (Fig. 2B1–B4). *S. cruciatum* typically has oblong-ovoid fruits with 5 filiform ribs, 1–3 vittae in each furrow and 2–4 on commissure, and sub-pentagon transection and flat endosperm concrescence (Fig. 2C1–C4). The mature fruits of *S. filicinum* are generally ovoid with slightly constricted apex, obscure ribs, 2–3 vittae in each furrow and 4 on commissure, semicircle transection and sub-cordate concave endosperm concrescence (Fig. 2D1–D4). The mature fruits of *S. schizopetalum* are broad-ovoid with obscure ribs, 1–3 vittae in each furrow and 2 on commissure, semicircular transection of mericarp, and slightly concave endosperm concrescence (Fig. 2E1–E4). The mature fruits of *S. vaginatum* are typically oblong-ovoid with 1–3 vittae in each furrow and 2–4 on commissure, sub-pentagon transection and flat endosperm concrescence (Fig. 2F1–F4).

## Palynology

The pollen morphology of the five *Sinocarum* species was studied by SEM, as shown in Table 5 and Fig. 3. The average ratio of the polar axis to the equatorial axis (P/E) of the pollen grains of *S. coloratum*, *S. cruciatum* and *S. vaginatum* is greater than 2, and the average size index was greater than 19. The pollen grains of these three species are super-rectangular in equatorial view, trilobate circular in polar view (Fig. 3A1–A3, B1–B3, E1–E3). The exine ornamentation of the polar area is cerebroid with a few perforations, and the equatorial area is cerebro reticulate (Fig. 3A3–A4, B3–B4, E3–E4). The pollen grains of *S. filicinum* are super-rectangular in equatorial view, trilobate circular in polar view (Fig. 3C1–C3). The exine ornamentation of the polar area is striate reticulate with a few perforations, and the equatorial area is cerebro reticulate (Fig. 3C3–C4). Compared with other *Sinocarum* species, the pollen size of *S. schizopetalum* is smaller, and its size index is 15.11(14.15~16.62). And its pollen grains are sub-rhomboidal in equatorial view, obtuse triangled in polar view (Fig. 3D1–D3). The exine ornamentation of the polar area is cerebroid, and the equatorial area is pitted reticulate (Fig. 3D3–D4).

**Table 5.** Pollen morphology of *Sinocarum* species in scanning electron microscope (SEM).

| Species | Type | Shape | | Size(μm) | Polar axis/ Equatorial axis(P/E) | Size index ($\sqrt{P \times E}$) | Exine ornamentation | |
|---|---|---|---|---|---|---|---|---|
| | | Equatorial view | Polar view | | | | Equatorial area | Polar area |
| *S. coloratum* | super-rectangular | super-rectangular | trilobate circular | (27.20~32.90)30.36×<br>(11.72~13.95)12.78 | 2.38<br>(2.16~2.59) | 19.69<br>(18.51~20.00) | cerebro reticulate | cerebroid with a few perforations |
| *S. cruciatum* | super-rectangular | super-rectangular | trilobate circular | (26.07~32.15)28.84×<br>(11.42~15.33)13.24 | 2.19<br>(1.80~2.56) | 19.52<br>(17.25~20.92) | cerebro reticulate | cerebroid with a few perforations |
| *S. filicinum* | super-rectangular | super-rectangular | trilobate circular | (22.46~27.08)24.95×<br>(11.12~13.57)12.56 | 1.99<br>(1.66~2.28) | 17.69<br>(15.80~18.68) | cerebro reticulate | striate reticulate with a few perforations |
| *S. schizopetalum* | sub-rhomboidal | sub-rhomboidal | obtuse triangle | (18.27~20.81)19.61×<br>(10.40~13.31)11.66 | 1.69<br>(1.56~1.92) | 15.11<br>(14.15~16.62) | pitted reticulate | cerebroid |
| *S. vaginatum* | super-rectangular | super-rectangular | trilobate circular | (25.83~29.30)27.44×<br>(10.92~14.62)13.58 | 2.03<br>(1.82~2.37) | 19.29<br>(16.79~20.20) | cerebro reticulate | cerebroid with a few perforations |

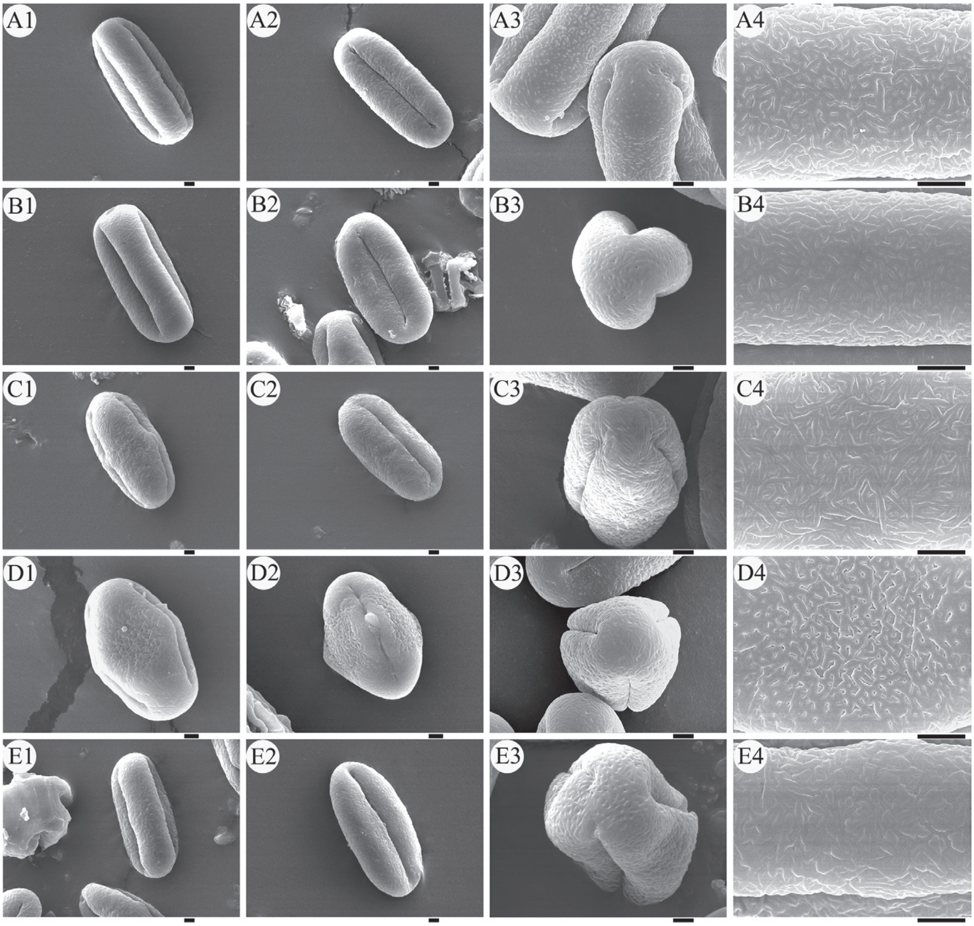

**Figure 3.** Pollen morphology in scanning electron microscope (SEM) **A** *Sinocarum coloratum* **B** *S. cruciatum* **C** *S. filicinum* **D** *S. schizopetalum* **E** *S. vaginatum*. Scale bars: 2 μm.

**Table 6.** Statistical summary for ITS and the cpDNA regions used to infer phylogenetic relationships of *Sinocarum*.

| | | **Apioideae** | ***Acronema* clade** | | **East Asia clade** | |
|---|---|---|---|---|---|---|
| | | **ITS** | **ITS** | **cpDNA(*rpl16+rps16*)** | **ITS** | **cpDNA(*rpl16+rps16*)** |
| No. of accessions | | 59 | 44 | 45 | 34 | 52 |
| Aligned length | | 472 | 476 | 1897 | 444 | 2128 |
| No. variable characters | | 349 (73.94%) | 288 (60.50%) | 318 (16.76%) | 252 (56.76%) | 278 (13.06%) |
| No. parsimony informative characters | | 276 (58.47%) | 205 (43.07%) | 143 (7.54%) | 184 (41.44%) | 163 (7.66%) |
| Model | ML | GTR+G | GTR+G | GTR+G | GTR+G | GTR+G |
| | BI | – | GTR+G | GTR+I+G | GTR+G | GTR+G |

ITS, internal transcribed spacer; ML, maximum likelihood; BI, Bayesian Inference.

## Sequence characteristics

The characteristics of the three DNA regions are summarized in Table 6. These results indicated that the aligned length of the background tree using 59 ITS sequences from

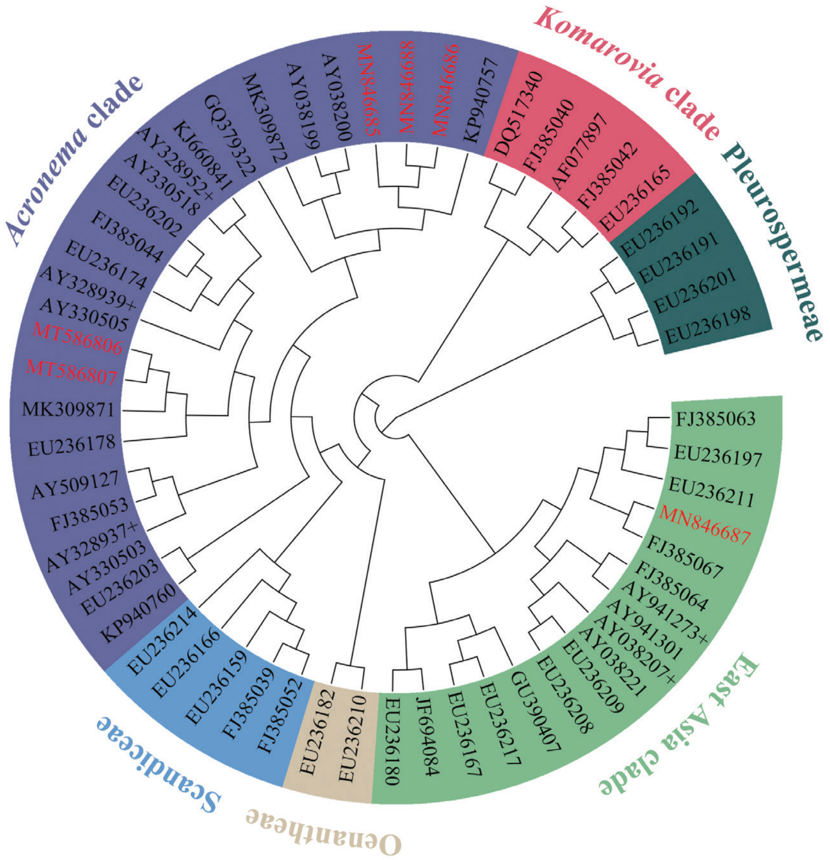

**Figure 4.** Phylogenetic relationships inferred from maximum likelihood (ML) analysis of 59 nrDNA ITS sequences from Apiaceae subfamily Apioideae. The tree is rooted with Pleurospermeae. The names of the clades are identified by Zhou et al. (2008, 2009) and Downie et al. (2010).

Apiaceae was 472, containing 73.94% average sequence divergence and 276 parsimony informative characters. In addition, average sequence divergence of ITS was more variable (60.50%; 56.76%) than cpDNA (16.76%; 13.06%) across the *Acronema* clade, East Asia clade and their outgroups. Overall, ITS was more variable than cpDNA, with greater average sequence divergence and more parsimony informative characters.

## Phylogenetic analyses

### ITS trees (Figs 4, 5A, 6A)

After phylogenetic analyses with comprehensive sequence data, we confirmed that the collected *Sinocarum* materials were from a polyphyletic group and fell into two different clades (i.e. *Acronema* clade, East Asia clade) according to the ITS tree inferred by ML approach (Fig. 4). The ITS trees of *Acronema* clade and its outgroups (Fig. 5A) inferred by ML and BI approaches were inconsistent for subclade topology. The ITS tree dem-

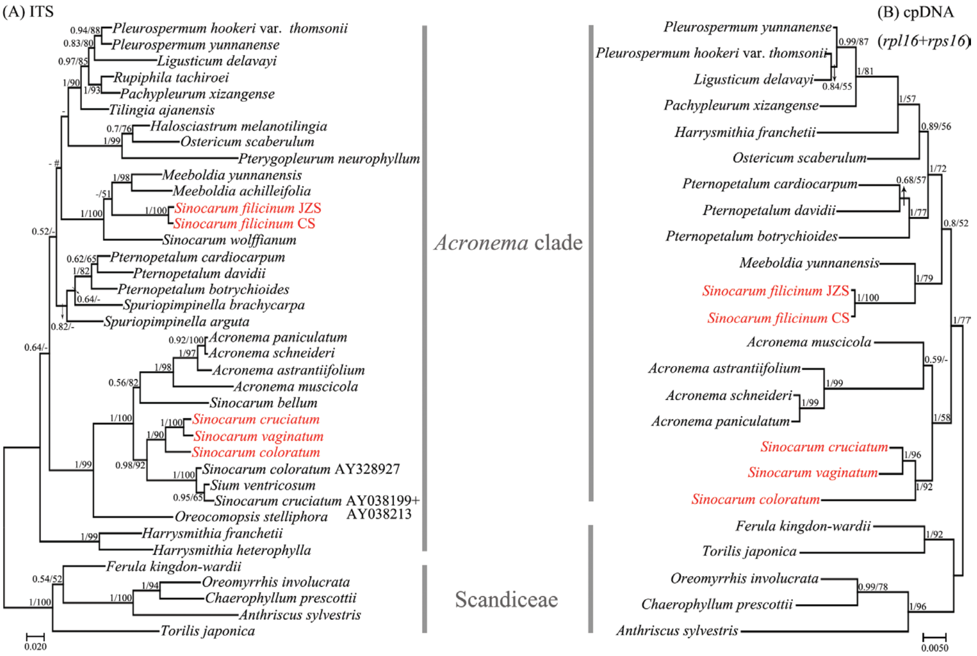

**Figure 5.** Bayesian 50% strict consensus trees of 44 nrDNA ITS sequences (**A**) and 45 combined cpDNA *rpl16* and *rps16* intron sequences (**B**) from *Acronema* clade and outgroups. Values on the branches indicate its support (Bayesian posterior probability/ bootstrap value). Those nodes not occurring in the ML strict consensus tree are indicated by pound symbols (#). Short line denotes values < 50%. The tree is rooted with Scandiceae. The names of the clades are identified by Zhou et al. (2008, 2009) and Downie et al. (2010).

onstrated again that *Sinocarum* was a polyphyletic group. Additionally, it indicated that the collected *Sinocarum* materials (*S. coloratum*, *S. cruciatum* and *S. vaginatum*), together with two previously sequenced *Sinocarum* species (*S. coloratum* AY328927; *S. cruciatum* AY038199; AY038213) and *Sium ventricosum* (H. Boissieu) Li S. Wang & M. F. Watson (2016: 266) (AY038200; AY038214) constituted a strongly supported group (Bayesian Inference (BI)–posterior probability (PP)=0.98/Maximum Likelihood (ML)–bootstrap value (BS)=92%), as sister group to four species of *Acronema* and *Sinocarum bellum* (C. B. Clarke) Pimenov & Kljuykov (2006: 122) (PP/BS = 1/100%). A newly recognized but weakly supported subclade in the ITS trees encompassed four species of *Acronema* and *Sinocarum bellum* (PP/BS = 0.56/82%). Of three *Sinocarum* species we collected, *S. cruciatum* was allied closer with *S. vaginatum* than *S. coloratum* (PP/BS = 1/100%). Two populations of *S. filicinum*, two species of *Meeboldia* H. Wolff (1924: 313) and *S. wolffianum* (Fedde ex H. Wolff) P. K. Mukh. & Constance (1991: 42) formed a strongly supported subclade (PP/BS = 1/100%), and *S. filicinum* had a closer relationship with *Meeboldia*. The ITS trees of the East Asia clade and its outgroups (Fig. 6A) inferred by ML and BI approaches were inconsistent for *Pimpinella* L. (1753: 263) subclade topology. In addition, the trees indicated that *S. schizopetalum* was allied distantly with other collected *Sinocarum* species, and closely related to *Trachydium* Lindley (1835: 232) subclade (PP/BS = 0.83/69%) in the strongly supported East Asia clade (PP/BS = 1/96%).

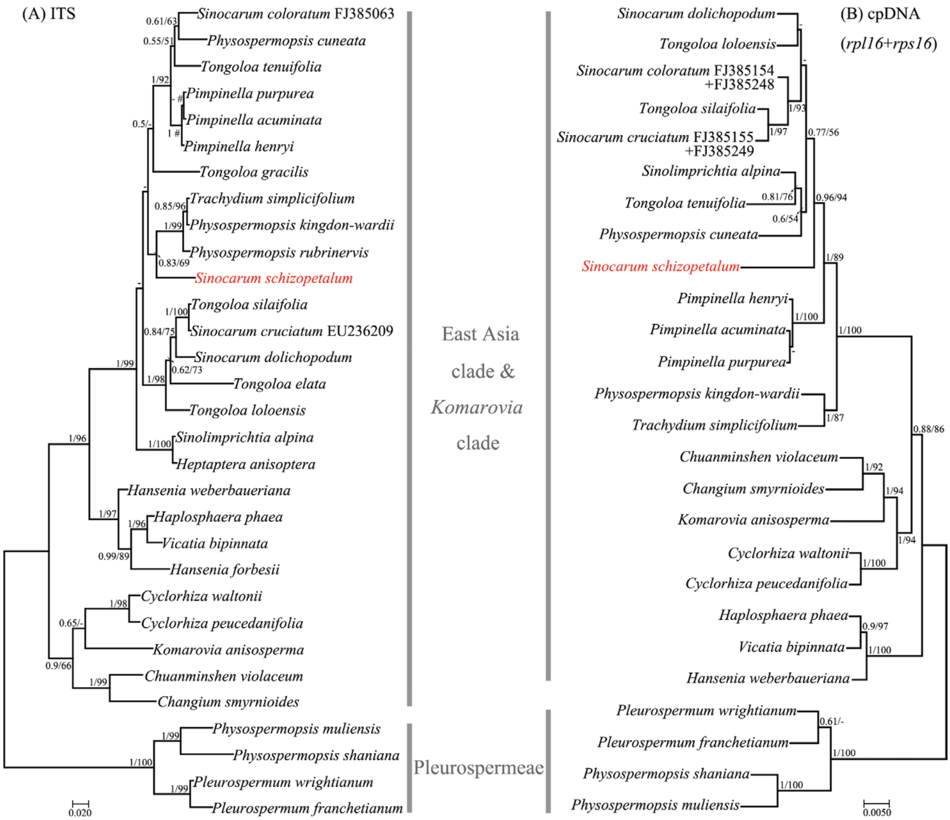

**Figure 6.** Bayesian 50% strict consensus tree of 34 nrDNA ITS sequences (**A**) and 52 combined cpDNA *rpl16* and *rps16* intron sequences (**B**) from East Asia clade & *Komarovia* clade and outgroups. Values on the branches indicate its support (Bayesian posterior probability/ bootstrap value). Those nodes not occurring in the ML strict consensus tree are indicated by pound symbols (#). Short line denotes values < 50%. The tree is rooted with Pleurospermeae. The names of the clades are identified by Zhou et al. (2008, 2009) and Downie et al. (2010).

## cpDNA trees (Figs 5B, 6B)

The cpDNA trees of *Acronema* clade and its outgroups (Fig. 5B) inferred by ML and BI approaches had consistent topologies. The cpDNA trees indicated that the generic type of *Sinocarum*, *S. coloratum*, together with *S. cruciatum* and *S. vaginatum* constituted a supported monophyletic group (PP/BS = 1/92%) as sister group to *Acronema* (PP/BS = 1/58%). Two populations of *S. filicinum* allied powerfully with the genus *Meeboldia* (PP/BS = 1/79%). The cpDNA trees of the East Asia clade and its outgroups (Fig. 6B) inferred by ML and BI approaches had consistent topologies and indicated that the position of *S. schizopetalum* differed from the ITS tree and was located in the East Asia clade.

# Discussion

## Morphology

We have studied the plant morphology, fruit morphological and anatomical characteristics, and palynology of five species of *Sinocarum*, and perfected the mature fruit characteristics of these species. Through the analysis of comprehensive morphological data, the five *Sinocarum* species can be divided into three groups. Group 1 includes *S. coloratum*, *S. cruciatum* and *S. vaginatum.* They are characterized by slender and glabrous plants, usually ovate or oblong-ovate sheath, mostly absent bracts and bracteoles, typically entire petals, ovoid or oblong-ovoid mature fruit with 1–3 vittae in each furrow and 2–4 on commissure, sub-pentagon transection and flat endosperm concrescence. And the pollen grains of these three species are super-rectangular in equatorial view, trilobate circular in polar view. Group 2 includes *S. filicinum*, whose morphological characteristics were significantly different from those of other *Sinocarum* species we collected, and the key identification features were the linear-lanceolate bracts and bracteoles, oblong-ovate blade ultimate segments with serration on the margins, sparsely pubescent petioles, rachides and the abaxial surface of segments. Group 3 includes *S. schizopetalum*, whose most prominent features are apex palmately 3–4-lobed and white or violet petals, broad-ovoid mature fruits and sub-rhomboidal pollen. Among them, petal characteristics are very special in the whole genus *Sinocarum*. It is concluded that plant morphology, fruit morphological and anatomical characteristics, and palynology have important taxonomic significance.

## Phylogenetic placement of *Sinocarum*

Previous studies have shown that *Sinocarum* is not a monophyletic group and the phylogenetic placement remains unclear (Valiejo-Roman et al. 2002; Zhou et al. 2008; Zhou et al. 2009; Downie et al. 2010). *S. coloratum* is the generic type of *Sinocarum*, its phylogenetic placement represents the phylogenetic placement of *Sinocarum.* In this study, we confirmed that *Sinocarum* is not a monophyletic group and used three sequences of *S. coloratum* (MN846685, AY328927, FJ385063), our sequenced specimen and two downloaded sequences, to determine the phylogenetic placement of *Sinocarum*. We found that one of the downloaded (FJ385063) was located in the East Asia clade, while our accession and the other downloaded accession were located in the *Acronema* clade but these two were not clustered together. The results of our field investigation, morphological study and specimen verification of *S. coloratum* obtained from the type locality (Mt. Yulong) showed that our collected material is highly consistent with the type specimen and the original literature description of *S. coloratum*. In conclusion, the true phylogenetic placement of *Sinocarum* is within the *Acronema* clade and the genus has a close affinity with *Acronema*.

## The relationship between *Sinocarum* and *Acronema*

This study's phylogeny results indicated that there was a close and complex relationship between *Sinocarum* and *Acronema*. Fusiform or elongate roots and apex slightly obtuse or rarely lobed petals are easily recognizable characteristics of *Sinocarum*, and an apex long-linear or long-aristate petal is the most prominent feature of *Acronema*. In fact, within each genus there are species that deviate in one or more morphological characteristics from the typical and the generic boundaries are blurred with a few species being easily confused as belonging to the other genus (Watson 1996; Watson et al. 2004; Pu et al. 2005). For example, *S. cruciatum* has torulose roots and several species of the genus *Acronema*, *A. chienii* R. H. Shan & S. L. Liou (1980: 197), *A. chinense* H. Wolff (1926: 309) have apex acute or obtuse-acute petals, characteristics typically observed in the other genus. Through a literature review, field investigation, morphological study and specimen examination, we found that the plants of *Sinocarum* and *Acronema* are all slender. In addition, *Sinocarum* and *Acronema* are both distributed in the high-elevation Sino-Himalayan region from Nepal to SW China. The habitat of the two genera is extremely similar as they are distributed in the humid environment of rock crevices, alpine meadows or shady forests. These conditions provide further evidence for the close and complex affinity between the two genera. This study and others have provided cumulative evidence to reduce phylogenetic uncertainty. Despite recent collections, the range of materials for the two genera is still limited and more field specimens will be required to provide a comprehensive revision of the phylogeny of *Sinocarum* and *Acronema* across their geographic range.

## Infrageneric relationships

Our ITS and cpDNA trees showed that *S. coloratum* (generic type), *S. cruciatum* and *S. vaginatum* clustered together, but *S. cruciatum* had a closer relationship with *S. vaginatum*, which was consistent with the results of morphological study. The closer relationship between *S. cruciatum* and *S. vaginatum* is supported by the ultimate segments of their blades being more slender than other *Sinocarum* species and forming a group of narrow-leaved taxa. However, *S. vaginatum* develops elongate-linear ultimate segments of basal leaves and cauline leaves, and more rays, about 10–12. Whereas *S. cruciatum* has subequal rays and torulose roots. These two species are recognizable by these major features. In addition, *S. cruciatum* and *S. vaginatum* were both collected from the Dali range, Dali, Yunnan, overlapping in their ranges. Consequently, the morphological evidence and geographical distribution are consistent with the phylogenetic analysis results.

*Sinocarum filicinum* H. Wolff (1929: 182) was originally described by Wolff (1929) based on G. Forrest n. 6863, 11691, 7230, and obtained from the eastern flank of the Dali Range in Yunnan. Since the description of *S. schizopetalum*, its phylogenetic placement has been controversial. Franchet (1894) originally described it as a new species as *Carum schizopetalum* Franch., and Wolff (1927) transferred it to *Sinocarum*, later Wu Zhengyi (1984) transferred this species to *Dactylaea* H. Wolff (1930: 304) as *Dactylaea schizopetala* (Franch.) Wu Zhengyi (1984: 910). Pu et al. (2005) accepted

Wolff's view in the description of *Sinocarum* in *Flora of China*. Pimenov (2017) also accepted Wolff's view that this species belongs to *Sinocarum* after studying the specimens of Apiaceae, especially the type specimens. In addition, no molecular phylogenetic studies have been carried out on *S. filicinum* and *S. schizopetalum.*

The results showed that the two populations of *S. filicinum* were not related to *S. coloratum* (*Sinocaurm* generic type) and allied most closely with the genus *Meeboldia*, according to the ITS and cpDNA trees. Our morphological results indicated that the morphological characteristics of *S. filicinum* are distinct from the three other *Sinocarum* species in the *Acronema* clade that we collected and are very consistent with the characteristics of *Meeboldia*. Among them, fruit characteristics play a key role of subfamily Apioideae classification (Kljuykov et al. 2004; Lyskov et al. 2017; Guo et al. 2018; Jia et al. 2019). And the fruit characteristics of *S. filicinum* are similar to those of *Meeboldia yunnanensis*, they are all ovoid, with 5 filiform inconspicuous ribs, 2–3 vittae in each furrow and 4 on commissure, semicircle transection of mericarp and cordate concave or sub-cordate endosperm concrescence (Fig. 2A, D). The molecular data and morphological evidence indicated that *S. filicinum* is closely related to *Meeboldia* and should be isolated from *Sinocarum*, but due to the lack of comprehensive samples, the phylogenetic placement will not be revised at present.

Our phylogenetic results showed that *S. schizopetalum* was distantly related to the other *Sinocarum* species we collected (*S. coloratum*, *S. cruciatum*, *S. filicinum* and *S. vaginatum*). We found that the exact phylogenetic placement of *S. schizopetalum* was inconsistent between the ITS tree and the cpDNA tree, but was nevertheless located in the East Asia clade. Morphologically, *S. schizopetalum* has apex palmately 3–4-lobed petals, broad-ovoid mature fruits and sub-rhomboidal pollen, and these features are clearly distinct from other species of *Sinocarum*. Unlike the other studied *Sinocarum* species, the plant morphology, fruit and pollen morphology of *S. schizopetalum* are more similar to species of the East Asia clade. According to the results of phylogeny and morphology studies, it is suggested *S. schizopetalum* should be isolated from *Sinocarum*. However, due to the complex taxonomic problems among genera in the East Asia clade, the phylogeny of *S. schizopetalum* cannot be resolved. Thus, *S. schizopetalum* needs revision pending expanded sampling and phylogenetic analyses to include more East Asia clade species from the Sino-Himalayan region.

## Acknowledgements

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## Reference

Altınordu F, Peruzzi L, Yu Y, He X (2016) A tool for the analysis of chromosomes: Karyo Type. Taxon 65(3): 586–592. https://doi.org/10.12705/653.9

Burland TG (2000) DNASTAR's Lasergene sequence analysis software. Methods in Molecular Biology (Clifton, N.J.) 132: 71–91. https://doi.org/10.1385/1-59259-192-2:71

Downie SR, Katz-Downie DS (1999) Phylogenetic analysis of chloroplast *rps16* intron sequences reveals relationships within the woody southern African Apiaceae subfamily Apioideae. Canadian Journal of Botany 77(8): 1120–1135. https://doi.org/10.1139/b99-086

Downie SR, Katz-Downie DS, Spalik K (2000a) A phylogeny of Apiaceae tribe Scandiceae: Evidence from nuclear ribosomal DNA internal transcribed spacer sequences. American Journal of Botany 87(1): 76–95. https://doi.org/10.2307/2656687

Downie SR, Katz-Downie DS, Watson MF (2000b) A phylogeny of the flowering plant family Apiaceae based on chloroplast DNA *rpl16* and *rpoC1* intron sequences: Towards a suprageneric classification of subfamily Apioideae. American Journal of Botany 87(2): 273–292. https://doi.org/10.2307/2656915

Downie SR, Spalik K, Katz-Downie DS, Reduron JP (2010) Major clades within Apiaceae subfamily Apioideae as inferred by phylogenetic analysis of nrDNA ITS sequences. Plant Diversity and Evolution 128(1–2): 111–136. https://doi.org/10.1127/1869-6155/2010/0128-0005

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analyses by sampling trees. BMC Evolutionary Biology 7(1): e214. https://doi.org/10.1186/1471-2148-7-214

Edgeworth MP (1846) III. Descriptions of some unpublished Species of Plants from North-Western India. Transactions of the Linnean Society of London 20(1): 23–91. https://doi.org/10.1111/j.1096-3642.1846.tb00410.x

Franchet A (1894) Notes sur quelques Ombellifères du Yunnan. Bulletin de la Société Philomathique de Paris 8: 106–146.

Guo XL, Wang CB, Wen J, Zhou SD, He XJ (2018) Phylogeny of Chinese *Chamaesium* (Apiaceae: Apioideae) inferred from ITS, cpDNA and morphological characters. Phytotaxa 376: 001–016. https://doi.org/10.11646/phytotaxa.376.1.1

Jia SB, Guo XL, Zhou SD, He XJ (2019) *Hansenia pinnatiinvolucellata* is conspecific with *H. weberbaueriana* (Apiaceae) based on morphology and molecular data. Phytotaxa 418: 203–210. https://doi.org/10.11646/phytotaxa.418.2.5

Jordan WC, Courtney MW, Neigel JE (1996) Low levels of intraspecific genetic variation at a rapidly evolving chloroplast DNA locus in North American Duckweeds (Lemnaceae). American Journal of Botany 83(4): 430–439. https://doi.org/10.1002/j.1537-2197.1996.tb12724.x

Kelchner SA, Clark LG (1997) Molecular evolution and phylogenetic utility of the chloroplast *rpl16* intron in Chusquea and the Bambusoideae (Poaceae). Molecular Phylogenetics and Evolution 8(3): 385–397. https://doi.org/10.1006/mpev.1997.0432

Kljuykov EV, Liu M, Ostroumova TA, Pimenov MG, Tilney PM, Wyk BEV (2004) Towards a standardised terminology for taxonomically important morphological characters in the Umbelliferae. South African Journal of Botany 70(3): 488–496. https://doi.org/10.1016/S0254-6299(15)30233-7

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Larkin MA, Blackshields G, Brown NP, Chenna R, Mcgettigan PA, Mcwilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics (Oxford, England) 23(21): 2947–2948. https://doi.org/10.1093/bioinformatics/btm404

Lindley J (1835) Illustrations of the botany and other branches of the natural history of the Himalayan Mountains. Royle, John Forbes, London, 232 pp.

Linnaeus C (1753) Species Plantarum (ed. 2, Vol. 1). Laurentii Salvii, Holmiae, 263 pp.

Liu SL, Shan RH (1980) A preliminary study on the Chinese *Acronema*. Acta Phytotaxonomica Sinica 18: 194–204.

Lyskov DF, Degtjareva GV, Samigullin TH, Pimenov MG (2017) The revision of *Prangos*, subsections *Koelzella* and *Fedtschenkoana*, (Apiaceae) with some notes to phylogeny and biogeography of the genus: Molecular and morphological evidences. Plant Systematics and Evolution 313(7): 815–826. https://doi.org/10.1007/s00606-017-1412-0

Mukherjee PK, Constance L (1991) New taxa and transfers in Indian Umbelliferae. Edinburgh Journal of Botany 48(1): 41–44. https://doi.org/10.1017/S0960428600003589

Nylander JAA (2004) MrModeltest, a program to evaluate the fit of several models of evolution to a given data and unrooted tree (version 2.2). Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Pimenov MG (2017) Updated checklist of Chinese Umbelliferea: Nomenclature, synonymy, typification, distribution. Turczaninowia 20(2): 106–239. https://doi.org/10.14258/turczaninowia.20.2.9

Pimenov MG, Kljuykov EV (2006) Two new species, two new nomenclatural combinations and a new name in Himalayan *Sinocarum* (Umbelliferae). Komarovia 4: 116–123.

Pu FT (1998) New names in Chinese Umbelliferae. Novon 8(1): 70–71. https://doi.org/10.2307/3391897

Pu FT, Watson MF, Holmes-Smith I (2005) *Sinocarum* H. Wolff ex R. H. Shan & F. T. Pu, Apiaceae. In: Wu ZY, Raven PH (Eds) Flora of China (Vol 14). Science Press, Beijing and Missouri Botanical garden Press, Saint Louis, 82–85.

Ronquist F, Teslenko M, Mark PVD, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Shan RH, Pu FT (1985) *Sinocarum* Wolff ex Shan & Pu. Flora Reipublicae Popularis Sinicae 55: 30–38.

Shan RH, She ML, Yuan CC, Wang TS, Pu FT, Chang HT (1980) New taxa of the Umbelliferae from Xizang (Tibet). Acta Phytotaxonomica Sinica 18: 374–379.

Shu P, She ML (2001) Pollen Photographs and Flora of Umbelliferae in China. Shanghai Science and Technology Press, Shanghai, 26 pp.

Valiejo-Roman CM, Terentieva EI, Samigullin TH, Pimenov MG (2002) nrDNA ITS sequences and affinities (Umbelliferae) of Sino-Himalayan Apioideae. Taxon 51: 685–701. https://doi.org/10.2307/3647333

Wang PL (1998) Practical Pollen in China. Sichuan Science and Technology Press, Chengdu, 110–111.

Wang LS, Zhang HR, Watson MF, Knapp S (2016) On the identity of *Sium frigidum* (Apiaceae). Phytotaxa 288(3): 265–272. https://doi.org/10.11646/phytotaxa.288.3.7

Watson MF (1996) Notes relating to the flora of Bhutan: XXXIII. Umbelliferae, I. Edinburgh Journal of Botany 53(1): 127–144. https://doi.org/10.1017/S0960428600002766

Watson MF, She ML, Pu FT, Pan ZH (2004) Nomenclatural novelties in the Apiaceae (Umbelliferae) for the Flora of China. Acta Phytotaxonomica Sinica 42: 561–565. https://doi.org/10.1088/1009-0630/6/5/011

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds) PCR Protocols: A Guide to Methods and Applications. Academic Press Inc, San Diego, California, 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Wolff H (1924) *Meeboldia*, genus novum Umbelliferarum Himalayicum. Repertorium novarum specierum regni vegetabilis 19(16–21): 313–313. https://doi.org/10.1002/fedr.19240191615

Wolff H (1926) Plantae sinenses a Dre. H. Smith annis 1921–22 lectae. XVI. Umbelliferae. Acta Horti Gothoburgensis 2: 288–328.

Wolff H (1927) Umbelliferae – Apioideae – Ammineae – Carinae, Ammineae-novemjugatae et genuinae. In: Engler A (Ed.) Das Pflanzenreich. Wilhelm Engelmann, Berlin, 90: 1–398.

Wolff H (1929) Umbelliferae Asiatiae novae relictae. II. Repert. Spec. Nov. Regni Veget 27: 179–192. https://doi.org/10.1002/fedr.4870270909

Wolff H (1930) Umbelliferae asiaticae novae relictae. III. Repertorium novarum specierum regni vegetabilis 27(16–25): 301–335. https://doi.org/10.1002/fedr.4870271612

Wu ZY [Ed.] (1984) The catalogue of seed plants in Yunnan Province. Yunnan People's Publishing House, Kunming, 910 pp.

Zhou J, Peng H, Downie SR, Liu ZW, Gong X (2008) A molecular phylogeny of Chinese Apiaceae subfamily Apioideae inferred from nuclear ribosomal DNA internal transcribed spacer sequences. Taxon 57: 402–416. https://doi.org/10.2307/25066012

Zhou J, Gong X, Downie SR, Peng H (2009) Towards a more robust molecular phylogeny of Chinese Apiaceae subfamily Apioideae: Additional evidence from nrdna its and cpDNA intron (rpl16 and rps16) sequences. Molecular Phylogenetics and Evolution 53(1): 56–68. https://doi.org/10.1016/j.ympev.2009.05.029

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# *Loncomelos koprulense* (Asparagaceae), a new species from southern Turkey

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## Abstract

A new species, *Loncomelos koprulense* (Asparagaceae), is described and illustrated from southern Turkey. It is a very rare endemic species growing on small semi-rocky escarpments within the Köprülü Kanyon in the province of Antalya. Morphologically for its hairy leaves, *L. koprulense* shows some relationships with *L. malatyanum* and *L. tardum*, species localized in Anatolia too. The chromosome number of the new species is $2n = 2x = 22$. Geographical distribution map for *L. koprulense*, *L. malatyanum* and *L. tardum* is provided.

## Keywords

Distribution, karyology, Mediterranean, *Ornithogalum s.l.*, Ornithogaleae, taxonomy

## Introduction

The genus *Ornithogalum* L., on account of its remarkable morphological and karyological variability, has been the object of various taxonomical treatments, which led to the recognition of several subgenera, sections and series or its splitting into different genera (Rafinesque 1840; Salisbury 1866; Speta 1998a, b, 2001; Pfosser and Speta 1999; Manning et al. 2009; Martínez-Azorín et al. 2009). Recently, phylogenetic investigations based on morphological and molecular approaches carried out by Martínez-Azorín et al. (2011)

emphasized that the hierarchical arrangement partly delineated by Speta (1998a) must be pursued, recognizing 19 monophyletic genera within the subfamily Ornithogaloideae Speta, all of which are morphologically well characterized. This approach was followed by Bogdanović et al. (2020) who widely analyzed the taxonomic aspect regarding these groups of Ornithogaleae J.C.Manning and Goldblatt. One of the accepted genera of this tribe, quite widespread in the Mediterranean territories, is *Loncomelos* Raf. showing in particular close relationships with *Ornithogalum* L. s.str. Morphologically, *Loncomelos* is mainly characterized by having inflorescence arranged in an elongated raceme, with pedicels more or less equal at maturity, capsule ovate-lanceolate, trigonous or trilobate with blunt or slightly retuse edges in cross section, seeds polygonal or irregularly compressed, with tuberculate, papillate or rugose testa, while *Ornithogalum* is differentiated by inflorescence corymbose or racemose-corymbose, capsule obovate or oblong, deeply trilobate with six evident ribs in cross section, seeds globose, with sinuous and prominent reticulate testa (Speta 1998a; Martínez-Azorín et al. 2011).

Currently, *Loncomelos* is represented by ca. 32 taxa, formerly mostly attributed to *Ornithogalum*, which are characterized by a very variable chromosome complement differing among the species, from diploid to polyploid and even aneuploid assets with 2n = 14, 16, 18, 20, 22, 24, 26, 28, 32, 36, 42, 44, 46, 52, 54, 88 (Cullen and Ratter 1967; Wittmann 1985; Speta 1998a, 2006, 2010, 2011; Mutlu and Karakuş 2012; Kypriotakis et al. 2018; Bogdanović et al. 2020). In the frame of taxonomic investigation on the genus *Loncomelos*, it is herein examined a very peculiar population collected in the southern Turkey, between Antalya and Manavgat. Based on careful morphological, anatomical and karyological observations, it was concluded that this new geophyte is taxonomically quite isolated, only showing some similarities in its hairy leaves with *L. tardum* Speta and *Ornithogalum malatyanum* Mutlu (here considered as a member of *Loncomelos*), both occurring in Turkey (Speta 2006; Mutlu and Karakuş 2012). Therefore, it is described herein as a new species and named *L. koprulense*.

## Materials and methods

The morphological study on the new species was carried out on living material collected from the locus classicus and cultivated in the Botanical Garden of Catania (Italy). Voucher specimens are deposited in the herbarium CAT (abbreviation follows Thiers 2020). Qualitative and quantitative morphological features were measured and scored on ten fresh plants, using a Zeiss Stemi SV11 Apo stereomicroscope at 6–66× magnification. Morphological comparison with the most related species was carried out using literature data (Speta 2006; Mutlu and Karakuş 2012). The diagnostic traits of the new species and its two allied ones are shown in Table 1.

Leaf anatomy was studied on cross-sections from cultivated plants, using fresh blades of minimum sized and maximum sized leaves in their optimal vegetative phase.

Karyological analyses were performed on root tip cells of cultivated bulbs, pre-treated with a 0.3% (w/v) colchicine water solution for 3 h at room temperature, fixed in Farmer's fixative (3:1 v/v, absolute ethanol: glacial acetic acid) for 12 h and stored in 70% ethanol

**Table 1.** Main morphological differences among *Loncomelos koprulense*, *L. tardum* and *L. malatyanum*.

| Characters | *L. koprulense* | *L. tardum* | *L. malatyanum* |
|---|---|---|---|
| Plant tall (cm) | up to 95 | up to 80 | up to 73 |
| Bulb shape | subglobose | ovoid | ovoid-globose |
| Bulb size (cm) | 2.5–3 × 3–3.6 | 2.5–4 × 2.5 | 2.5–2.7 × 1.5–2.5 |
| Bulb tunic colour | whitish | grey-brown | whitish |
| Scape height (cm) | 55–60 | 40–65 | 34–53 |
| Leaf number | 4 | 3–4 | 5–7 |
| Leaf length (cm) | 18–30 | up to 35 | (25) 28–40 |
| Leaf width (mm) | 3.5–8 | 4–5 | 3–10 (11) |
| Inflorescence length (cm) | 32–40 | 23–28 | 12–20 |
| Number of flowers | 50–55 | 40 | 18–30 (55) |
| Flower pedicel length (mm) | 12–25 | 14–25 | 5–15 |
| Bract shape | ovate-lanceolate | subulate | subulate |
| Bract length (mm) | 6–13(19) | 10–16 | 7–18 |
| Bract margin | smooth | smooth | 0–1 (2) toothed |
| Bract / pedicel ratio | shorter to subequal | about a half | equal or longer |
| Tepal size (mm) | 10–11 × 2.4–2.6 | 9.5–11.3 × 1.7–2.6 | 9–12 × 2.2–4 |
| Tepal shape | linear-oblong | linear | lanceolate to elliptical |
| Tepal colour | green, with white margin | greenish, with white margin | whitish, green in the centre |
| Tepal margin | undulate | slightly rolled, flat | flat |
| Staminal filament shape | oblong, narrowed and apiculate at the apex | oblong, apiculate at the apex | lanceolate, acuminate at the apex |
| Staminal filament size (mm) | 5–5.5 × 1.6–2 | 6 × 1.7–1.9 | 5.5–6 × 1.6 |
| Anther length (mm) | 2.5–2.7 | 2.8 | 2.2–3.2 |
| Anther colour | pale–green | greenish | yellowish-light green |
| Ovary shape | ovoid | ovoid | cylindrical |
| Ovary size (mm) | 3 × 2.3 | 3–3.5 × 2.2–2.5 | 2–3.3 × 2.2–2.8 |
| Style length (mm) | 2.2–2.3 | 3–3.8 | 4–5.3 |
| Capsule shape | ovoid | ellipsoid | ovoid to globose |
| Capsule size (mm) | 6.5–7 × 5 | 8–9 × 6 | (5) 7–11 × (4) 5–7 |
| Chromosome number (2n) | 22 | 20 | 24 |

water solution. Then, root tips were hydrolysed in 1 N HCl for 7 min at 60 °C and stained according to the Feulgen technique. Microphotographs of at least 10 good metaphase plates from different individuals were taken using a Zeiss PrimoStar microscope equipped with a Canon PowerShot G9 digital camera. Metaphase chromosomes were measured by the Zeiss Axiovision 4.8 image analysis software, while karyotyping was performed by CROMOLAB 1.1 software (Brullo 2002). The chromosome types were named according to the centromere position based on Levan et al. (1964) and Tzanoudakis (1983). All measured karyomorphometric parameters are provided in Table 2.

## Taxonomy

### *Loncomelos koprulense* Bogdanović, Brullo & Salmeri, sp. nov.

urn:lsid:ipni.org:names:77215897-1
Figs 1, 2, 5

**Type.** Turkey. Antalya: District of Manavgat, Köprülü Kanyon National Park, Bozyaka road, cultivated specimen, 15 June 2010, *Brullo s.n.* (Holotype: CAT!).

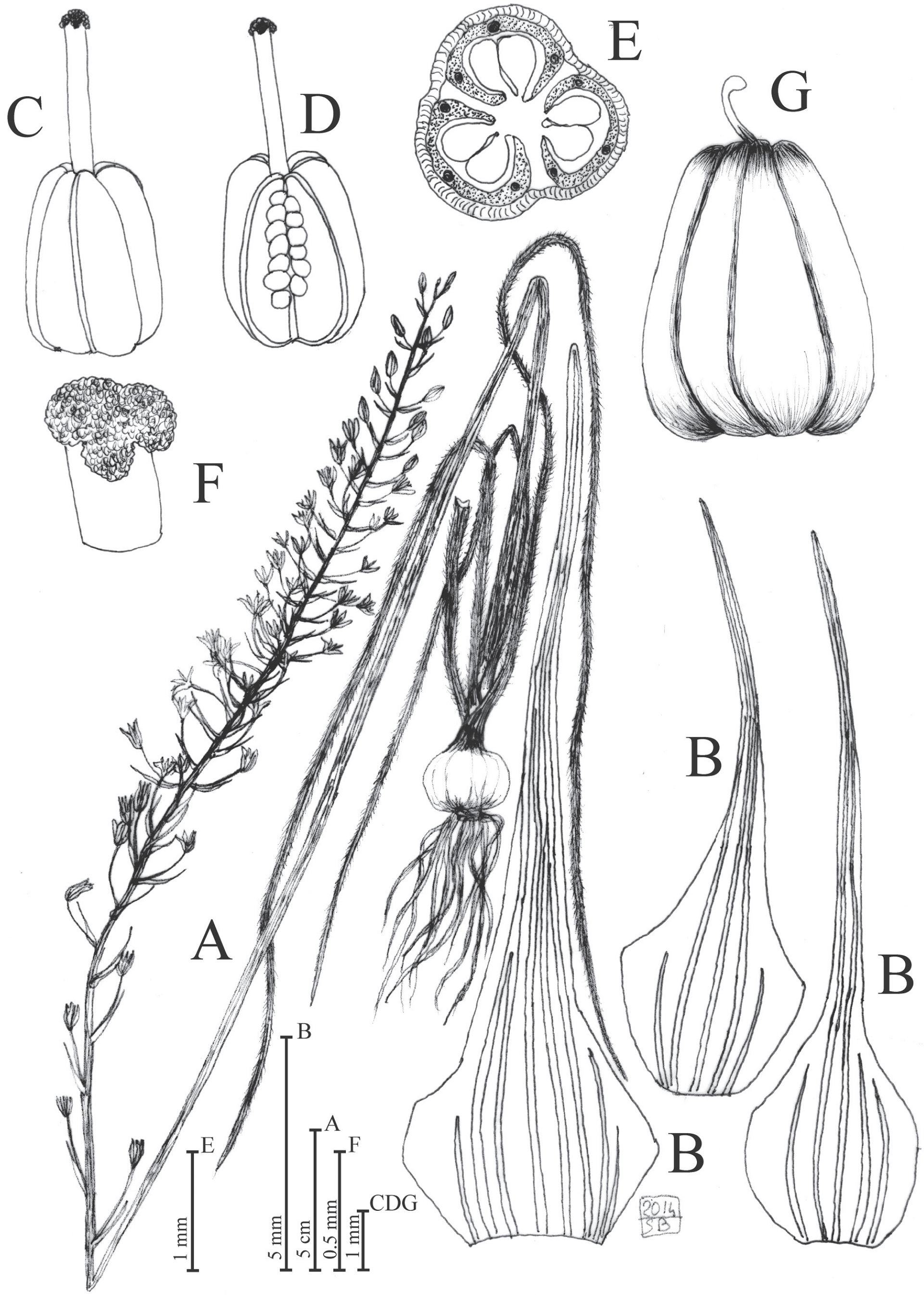

**Figure 1.** *Loncomelos koprulense* Bogdanović, Brullo & Salmeri, sp. nov. **A** habit **B** bracts **C** pistil **D** open pistil **E** ovary cross section **F** stigma **G** capsule. Drawing by S. Brullo from cultivated material coming from the type locality.

**Diagnosis.** Loncomelo malatyano affine, sed distinctum statura majore, bulbo subgloboso, latiore, 4–foliato, inflorescentia longiore, 50–55 floribus, bracteis ovato-lanceolatis, non dentatis, tepalis lineari-oblongis, viridibus, albis et undulatis margine, filamentis staminorum oblongis, latioribus, ovario ovoideo, stylo breviore, capsula minore.

**Description.** Plant up to 95 cm tall. Bulbs subglobose, 2.5–3 × 3–3.6 cm, outer tunics whitish, papery, without bulblets. Scape 55–60 cm long, glabrous, green-glaucous. Leaves 4, often withered at the anthesis, shorter than scape, linear, canaliculate, 18–30 × 0.35–0.8 cm, without white median line, abaxial face densely hairy, margins hairy, hairs 0.5–1.2 mm long, adaxial one glabrous. Raceme cylindrical, 32–40 cm long, 50–55 flowered. Pedicels 12–25 mm long, curved-divaricated, glabrous. Bracts membranous, ovate-lanceolate, 6–13(19) mm long, broadened at the base, long acuminate toward the apex, 5–8 nerved, shorter than pedicel or subequal, smooth at the margin, glabrous. Perigon stellate, 20 mm in diameter, tepals linear-oblong, 10–11 × 2.4–2.6 mm, glabrous, papillate-glandulose at the apex, markedly undulate at the margin, green with white margin. Staminal filaments white, oblong, abruptly narrowed and apiculate at the apex, 5–5.5 × 1.6–2 mm, thickened at the margin with central greenish midrib, anthers pale-green, 2.5–2.7 mm, dorsifixed. Ovary ovoid, green, 3 × 2.3 mm, with blunt lobes; each lobe with a distinct central nerve and two smaller, lateral ones. Style 2.2–2.3 mm long, stigma papillose. Capsule ovoid, 6.5–7 × 5 mm, erect. Seeds not seen. Chromosome number $2n = 2x = 22$.

**Phenology.** Flowering in June and fruiting in June-July.

**Etymology.** The species epithet is derived from the name of the Köprülü Kanyon, locality where this geophyte was collected.

**Karyology.** All investigated samples of *Loncomelos koprulense* from the type locality revealed a somatic chromosome complement with $2n = 22$ (Fig. 3A). The karyotype is rather asymmetrical, comprising 11 chromosome pairs (Fig. 3B), arranged in two size groups where the submedian type prevails, as highlighted by the values of different symmetric indices (Table 2). In particular, there are 3 metacentric pairs, 3 meta-submetacentric pairs (showing arm ratio exceeding 1.30), and 5 submetacentric pairs (3 big-sized and 2 small-sized). Thus, the chromosome formula can be expressed as $2n = 2x = 22 = 6\ m + 6\ msm + 10\ sm$. No evident satellites were detected. Absolute chromosome length varied from 11.1 ± 1.3 μm of the longest chromosome and 4.26 ± 0.3 μm of the shortest one, with a mean chromosome length of 6.99 ± 2.2 μm. Relative chromosome length varied from 7.24% ± 0.8 to 2.78% ± 0.2. Arm index varied on average from 1.03 to 2.76, while the centromeric index ranged from 49.3 to 26.6. All karyomorphometric parameters are given in Table 2.

**Leaf anatomy.** The known *Loncomelos* species are usually differentiated by their canaliculate leaves, uniformly colored with dorsi-ventral arrangement, presenting differences in size in the same individual. In particular, the leaf outline is smooth in adaxial faces and more or less ribbed in the abaxial one, with epidermal cells covered by a thickened cuticle; the pallisade tissues is one-layered and distributed

**Table 2.** Karyomorphometric parameters and karyotype symmetry indices of *Loncomelos koprulense*. Values come from 10 good metaphase plates from individuals of the type localities.

| **Chromosome** | **TAL (μm)** | | | **TRL%** | | | **AR** | **CI** | **Type** |
|---|---|---|---|---|---|---|---|---|---|
| **pairs** | **Mean ± SD** | **Max** | **Min** | **Mean ± SD** | **Max** | **Min** | | | |
| I | 10.8 ± 1.3 | 12.3 | 9.2 | 7.0 ± 0.7 | 7.9 | 6.1 | 1.06 | 48.6 | m |
| II | 10.0 ± 1.2 | 11.8 | 8.9 | 6.5 ± 0.3 | 6.6 | 6.4 | 1.13 | 46.9 | m |
| III | 8.8 ± 1.0 | 10.1 | 7.4 | 5.7 ± 0.2 | 6.8 | 6.0 | 1.22 | 45.1 | m |
| IV | 8.8 ± 1.1 | 10.0 | 7.4 | 5.7 ± 0.4 | 6.4 | 5.2 | 1.49 | 40.2 | msm |
| V | 6.9 ± 1.3 | 9.0 | 5.1 | 4.5 ± 0.4 | 5.1 | 3.8 | 2.49 | 28.6 | sm |
| VI | 6.5 ± 1.3 | 7.8 | 4.9 | 4.2 ± 0.6 | 5.1 | 3.6 | 1.39 | 41.8 | msm |
| VII | 5.9 ± 0.6 | 6.7 | 5.3 | 3.9 ± 0.3 | 4.4 | 3.5 | 2.69 | 27.1 | sm |
| VIII | 5.4 ± 0.8 | 6.7 | 4.5 | 3.5 ± 0.1 | 3.8 | 3.3 | 2.17 | 31.5 | sm |
| IX | 4.9 ± 0.6 | 5.7 | 4.2 | 3.2 ± 0.1 | 3.4 | 3.1 | 2.38 | 29.6 | sm |
| X | 4.5 ± 0.3 | 4.9 | 4.0 | 2.9 ± 0.2 | 3.3 | 2.6 | 1.80 | 35.7 | sm |
| XI | 4.5 ± 0.9 | 5.7 | 3.4 | 2.9 ± 0.5 | 3.4 | 2.2 | 1.38 | 42.0 | msm |

**TCL**: 153.9 ± 17.1 μm; **MCL:** 7.0 ± 2.2 μm; **d-value:** 32.5; **DRL%:** 4.5; **S%**: 38.3; **MAR**: 1.54; **MCI**: 38.7; **$Cv_{CL}$**: 32.2; **$Cv_{CI}$**: 21.2; **$M_{CA}$**: 24.2; **Stebbins' category**: 2B

**Abbreviations**: TAL = total absolute length; TRL = total relative length; AR = arm ratio index; CI = centromeric index; Type = chromosome nomenclature; TCL = total chromosome length; MCL = mean chromosome length; d-value = difference between long arms and short arms; DRL% = difference of relative length; S% = relative length of shortest chromosome; MAR = mean arm ratio index; MCI = mean centromeric index; $Cv_{CL}$ = coefficient of variation of chromosome length; $Cv_{CI}$ = coefficient of variation of centromeric index; MCA = mean centromeric asymmetry.

along the whole perimeter, while the inner part is occupied by the spongy tissue (Wittmann 1985; Tornadore 1985, 1986; Tornadore and Orza 1987; Lynch et al. 2006; Peruzzi et al. 2007; Öztürk et al. 2014; Bogdanović et al. 2020). The vascular bundles are arranged in two rows all along the mesophyll; large vascular bundles occur in the central part, which are alternated with other smaller one towards the abaxial side. The large bundles are interspersed with mucilage cells that are replaced by rhexigenetic lacunae in the mature leaves. Most species have completely glabrous leaves, except for *L. tardum*, *L. malatyanum* (see below for nomenclatural validation) and *L. koprulense*, showing a dense hairiness on the abaxial face. As a whole, the leaves of *L. koprulense* maintain the main features of the genus, revealing a marked variability in size; the largest leaves are characterized by 17–18 large vascular bundles, interposed among lacunae; these bundles decrease in number in the progressively narrower leaves up to a minimum of ca. 9, while the number of small vascular bundles coincides with that of the mesophyll lacunae (Fig. 2F). As far as hairs are concerned, they are irregularly distributed along the margin and on the abaxial face.

**Ecology and distribution.** *Loncomelos koprulense* seems to be a very rare species currently know only for a single locality of southern Turkey. One small and well circumscribed population was surveyed along the Bozyaka road within the Köprülü Kanyon at about 150 m of elevation (Fig. 4), where it grows on small semi-rocky escarpments covered by a scarce herbaceous vegetation. The woody vegetation near this habitat is represented by a thermophilous maquis characterized by *Quercus calliprinos* Webb, *Olea europaea* L. subsp. *sylvestris* (Mill.) Rouy ex Hegi, *Pistacia terebinthus* L., *Juniperus oxycedrus* L., *Myrtus communis* L., *Arbutus andrachne* L., *Cupressus sempervirens* L. etc.

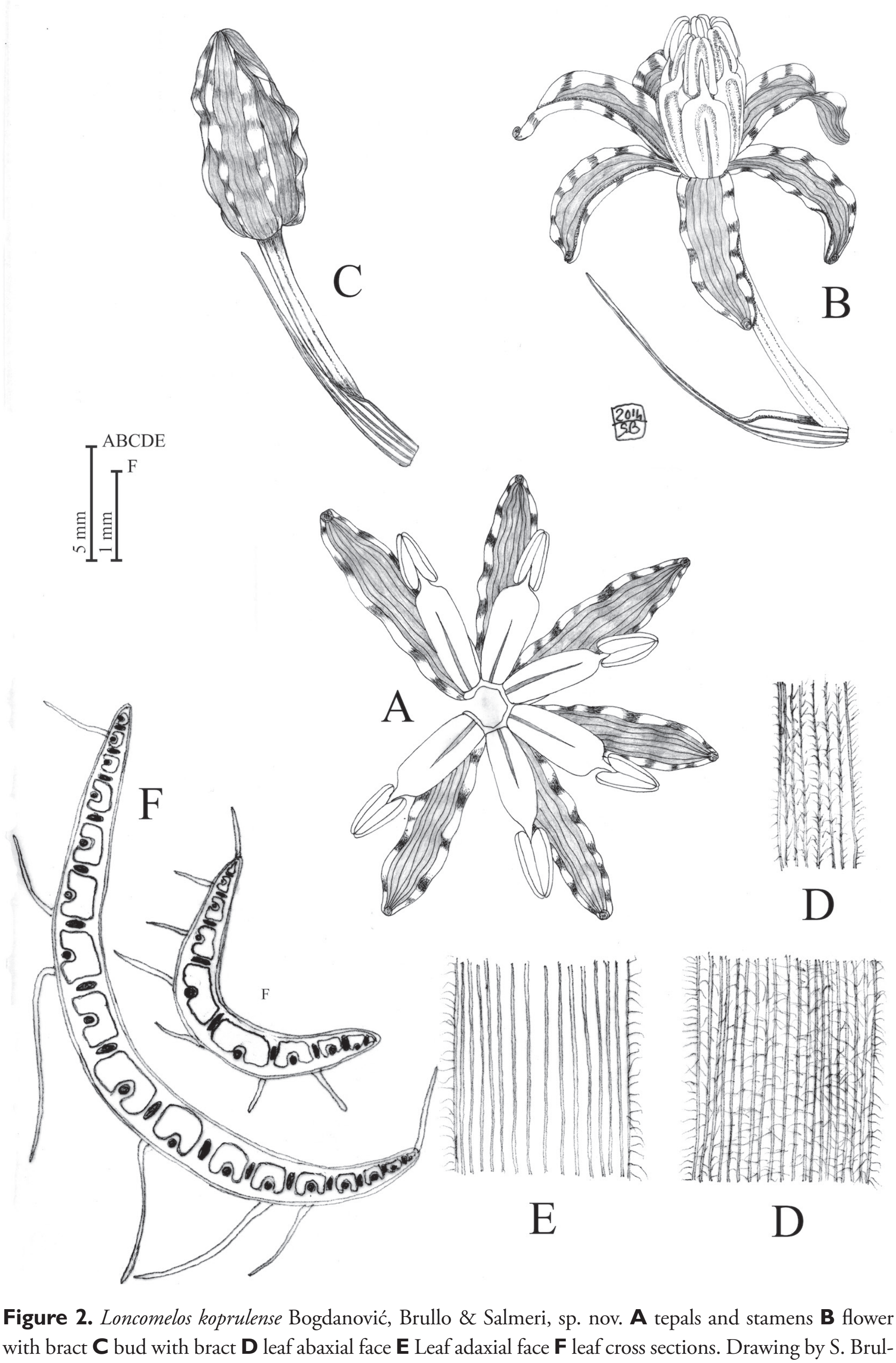

**Figure 2.** *Loncomelos koprulense* Bogdanović, Brullo & Salmeri, sp. nov. **A** tepals and stamens **B** flower with bract **C** bud with bract **D** leaf abaxial face **E** Leaf adaxial face **F** leaf cross sections. Drawing by S. Brullo from cultivated material coming from the type locality. Darker strips in tepal edges mark the undulations.

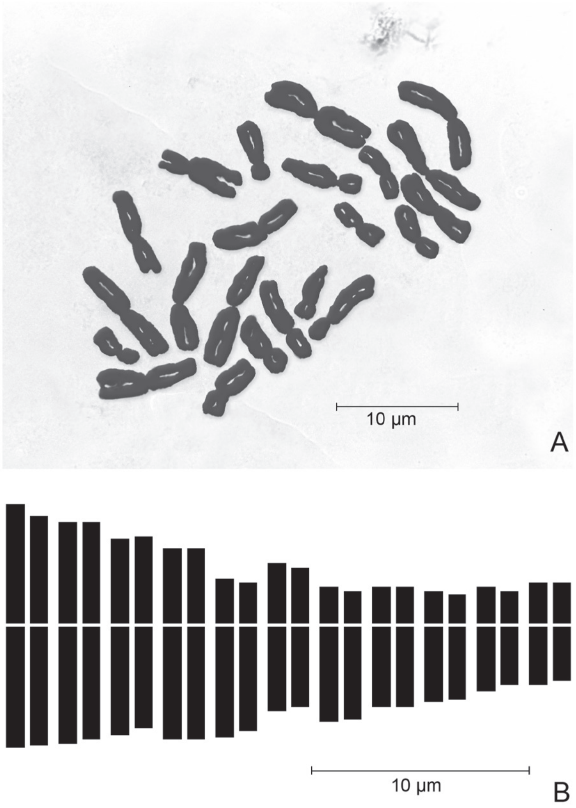

**Figure 3.** Chromosome complement ($2n = 2x = 22$) of *Loncomelos koprulense* **A** mitotic metaphase plate from the type locality **B** idiogram.

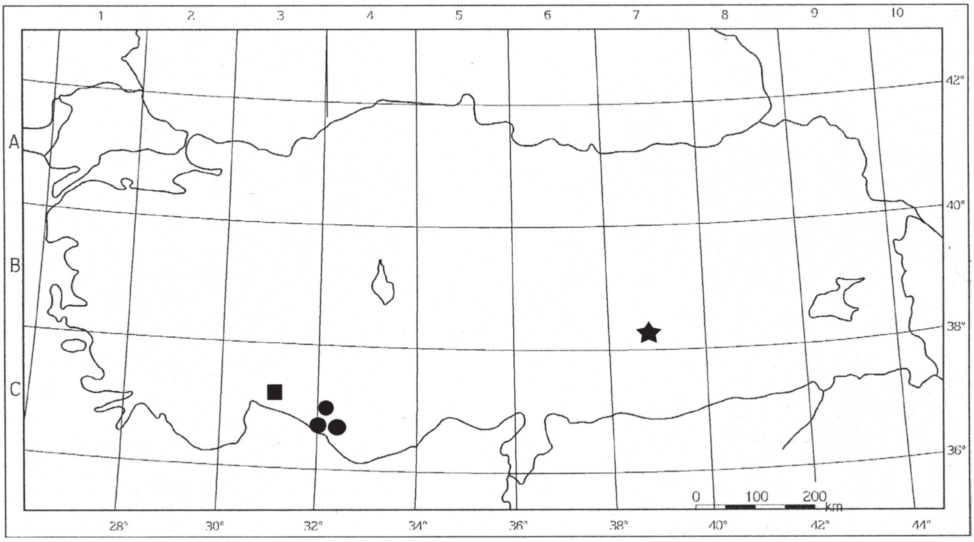

**Figure 4.** Distribution map of *Loncomelos koprulense* (square), *L. tardum* (circle) and *L. malatyanum* (star).

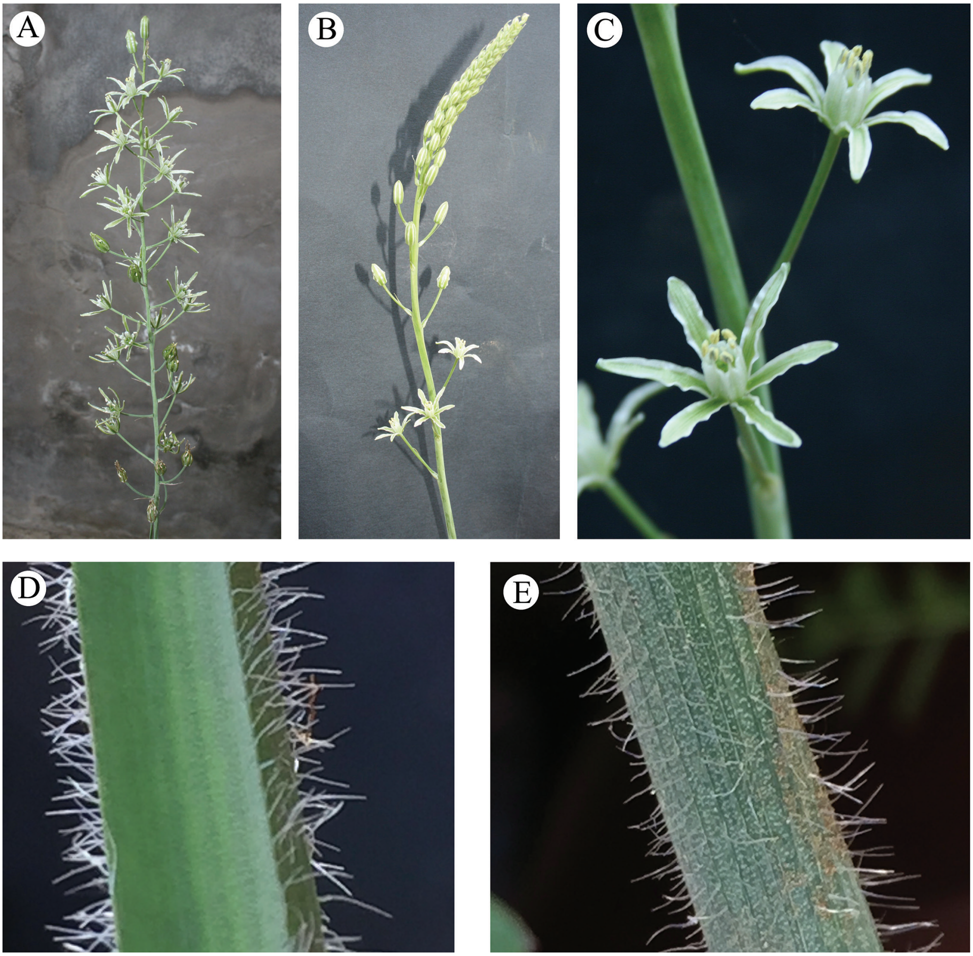

**Figure 5.** *Loncomelos koprulense* **A, B** inflorescence **C** flower **D** leaf abaxial face **E** leaf adaxial face from the type locality.

(Tavşanoğlu and Coşkun 2009). This area, falling within an important National Park and known as Köprülü Kanyon Milli Parkı between Antalya and Manavgat, is floristically highly rich in endemic species as emphasized by Özçelik (2018).

**Additional examined material.** Turkey. Antalya: District of Manavgat, Köprülü Kanyon National Park, Bozyaka road, cultivated specimen, 24 June 2013, *Brullo s.n.* (paratype: CAT!).

**Discussion.** From the literature data (Zahariadi 1977, 1980; Wittmann 1985; Martínez-Azorín 2008; Martínez-Azorín et al. 2009), the circumscription of the genus *Ornithogalum* within the tribe Ornithogaleae has always been problematic, emphasizing that the traditional morphological approach is not sufficient to discriminate the taxa at generic level. Recent phylogenetic studies carried out by Pfosser and Speta (1999) and Martínez-Azorín et al. (2011), based on cpDNA and nrDNA gene sequences, have provided a relevant support for a taxonomic arrangement of this tribe, validating the treatment previously proposed by Speta (1998a, b). As concern the genus *Loncomelos*, it is morphologically well differentiated from *Ornithogalum* s.str. by numerous and significant characters regarding the inflorescence, pedicel, capsule and seed. From the phytogeographical point of view, this genus is mainly distributed in the Mediterranean area with the higher concentration of species in the Balkan Peninsula and Anatolia. The last territory currently hosts 14 species (included the new one), that therefore can be considered the main centre of differentiation of the genus.

A very peculiar and significant morphological character occurring in *Loncomelos koprulense* is the densely hairy leaves (Fig. 5D–E). In fact, most species of the genus *Loncomelos* are characterized by glabrous leaves, while only *L. tardum* and *L. malatyanum* have hairs on the leaves. According to Speta (2006) and Mutlu and Karakuş (2012), both species occur in Anatolia too, where they are very rare and quite localized (Fig. 4). They differ from *L. koprulense* in some relevant morphological features (Table 1), such as the shape and size of the bulbs, number of leaves, inflorescence size, number of raceme flowers, bracts, tepal shape and colour, tepal margin, stamen filament, size and shape of ovary and capsule. Differences were also observed in karyological aspect, since *L. tardum* is characterized by a chromosome complement of $2n = 2x = 20$, reported by Speta (2006), while according to Mutlu and Karakuş (2012) the chromosome number of *L. malatyanum* is $2n = 2x = 24$. We found out that *L. koprulense* is also a diploid, but its chromosome number is $2n = 2x = 22$, which is quite rare in the genus *Loncomelos*, so far only previously counted in *L. fischerianum* (Krasch.) Speta by Agapova (1977).

**Nomenclatural note.** *Loncomelos malatyanum* (Mutlu) Bogdanović, Brullo & Salmeri comb. nov. ≡ *Ornithogalum malatyanum* Mutlu in Mutlu and Karakuş Turkish Journal of Botany 36: 126 (2012), basionym.

## References

Agapova ND (1977) Cytosystematic investigation of the European representatives of the genus *Ornithogalum* (Fam. Liliaceae) of the U.S.S.R. flora I. Bot. Zurn. 62: 970–983.

Bogdanović S, Brullo S, Ljubičić I, Rat M, Salmeri C (2020) Cytotaxonomical remarks on *Loncomelos visianicum* (Hyacinthaceae), a poorly known species endemic to Croatia. Phytotaxa 430: 095–108. https://doi.org/10.11646/phytotaxa.430.2.2

Brullo F (2002) Cromolab. Dipartimento di Botanica Università degli Studi di Catania.

Cullen I, Ratter JA (1967) Taxonomic and cytological notes on Turkish *Ornithogalum*. Notes from the Royal Botanic Garden Edinburgh 27(3): 293–339.

Kypriotakis Z, Antaloudaki E, Tzanoudakis D (2018) *Ornithogalum insulare* (Hyacinthaceae): A new species from the Cretan area (S. Aegean, Greece). Botanica Serbica 42: 117–122.

Levan A, Freda K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52(2): 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Lynch A, Rudall PJ, Cutler DF (2006) Leaf anatomy and systematics of Hyacinthaceae. Kew Bulletin 61: 145–159.

Manning JC, Forest F, Devey DS, Fay MF, Goldblatt P (2009) A molecular phylogeny and a revised classification of Ornithogaloideae (Hyacinthaceae) based on an analysis of four plastid DNA regions. Taxon 58(1): 77–107. https://doi.org/10.1002/tax.581011

Martínez-Azorín M (2008) Sistemática del género *Ornithogalum* L. (Hyacinthaceae) en el Mediterráneo occidental: implicaciones taxonómicas, filogenéticas y biogeográficas. Doctoral Thesis, Universidad de Alicante, Spain.

Martínez-Azorín M, Crespo MB, Juan A (2009) Taxonomic revision of *Ornithogalum* subg. *Beryllis* (Hyacinthaceae) in the Iberian peninsula and the Balearic islands. Belgian Journal of Botany 142: 139–161.

Martínez-Azorín M, Crespo MB, Juan A, Fay MF (2011) Molecular phylogenetics of subfamily Ornithogaloideae (Hyacinthaceae) based on nuclear and plastid DNA regions, including a new taxonomic arrangement. Annali di Botanica 107(1): 1–37. https://doi.org/10.1093/aob/mcq207

Mutlu B, Karakuş Ş (2012) A new species of *Ornithogalum* (Hyacinthaceae) from East Anatolia, Turkey. Turkish Journal of Botany 36: 125–133.

Özçelik H (2018) Flora inventory of Köprülü Kanyon National Park (Antalya-Isparta). Turk J Forst. 19: 40–50. https://doi.org/10.18182/tjf.338944

Öztürk D, Koyuncu O, Koray Yaylacı Ö, Özgişi K, Sezer O, Tokur S (2014) Comparative anatomical studies on twelve *Ornithogalum* (Asparagaceae) species (eleven nonendemic, one endemic) belonging to subgen. *Ornithogalum* and subgen. *Beryllis*, growing naturally in Eskişehir (Central Anatolia-Turkey). International Journal of Scientific Research and Reviews 3: 40–49.

Peruzzi L, Caparelli KF, Cesca G (2007) Contribution to the systematic knowledge of the genus *Ornithogalum* L. (Hyacinthaceae): Morpho-anatomical variability of the leaves among different taxa. Bocconea 21: 257–265.

Pfosser M, Speta F (1999) Phylogenetics of Hyacinthaceae Based on Plastid DNA Sequences. Ann Miss Bot Gard. 86(4): 852–875. https://doi.org/10.2307/2666172

Rafinesque CS (1840) Autikon Botanikon, Centuria V. Philadelphia, 55–71.

Salisbury RA (1866) The genera of plants: a fragment containing part of Liriogamae. John van Voorst, London, 143 pp.

Speta F (1998a) Hyacinthaceae. In: Kubitzki K (Ed.) The families and genera of vascular plants 3. Springer, Berlin, 261–285. https://doi.org/10.1007/978-3-662-03533-7_35

Speta F (1998b) Systematische analyse der Gattung *Scilla* L. s.l. (Hyacinthaceae). Phyton 38: 1–141.

Speta F (2001) Die Echte und die Falsche Meerzwiebel: *Charybdis* Speta und *Stellarioides* Medicus (Hyacinthaceae), mit Neubeschreibungen und Neukombinationen im Anhang. Stapfia 75: 139–176.

Speta F (2006) Die Gattung *Loncomelos* Raf. (Hyacinthaceae-Ornithogaloideae), vorgestellt anhand dreier neuer Arten. Phyton 46: 1–25.

Speta F (2010) Beitrag zur Kenntnis der *Loncomelos narbonensis*-Verwandtschaft (Hyacinthaceae – Ornithogaloideae). Verh Zool-Bot Ges Österreich 147: 125–157.

Speta F (2011) A remarkable new *Loncomelos* species from NE-Turkey: *L. erichpaschei* Speta spec. nova (Hyacinthaceae-Ornithogaloideae). Phyton 51: 153–160.

Tavşanoğlu Ç, Coşkun U (2009) Effect of goat browsing on growth form of maquis species in Köprülü Kanyon National Park (Antalya, Turkey). Ekoloji 18: 74–80.

Thiers B (2020) Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. http://sweet gum.nybg.org/ih/ [accessed 10 February 2020]

Tornadore N (1985) Il gen. *Ornithogalum* L. (Liliaceae). V. Osservazioni sulla citosistematica di *O. pyrenaicum* L. Atti della Società Toscana di Scienze Naturali – Memorie Serie B 92: 247–257.

Tornadore N (1986) Il genere *Ornithogalum* L. (Liliaceae). VI. *O. narbonense* L. Atti della Società Toscana di Scienze Naturali – Memorie Serie B 93: 111–120.

Tornadore N, Orza P (1987) Il gen. *Ornithogalum* L. (Liliaceae) in Italia. VIII. Il subgen. *Beryllis* (Salisb.) Baker con particolare riguardo ad *O. brevistylum* Wolfner. Atti della Società Toscana di Scienze Naturali – Memorie Serie B 94: 341–356.

Tzanoudakis D (1983) Karyotypes of ten taxa of *Allium* section *Scorodon* from Greece. Caryologia 36(3): 259–284. https://doi.org/10.1080/00087114.1983.10797667

Wittmann H (1985) Beitrag zur Systematik der *Ornithogalum* – Arten mit verlängert-traubiger Infloreszenz. Stapfia 13: 1–117.

Zahariadi C (1977) Notes on the intrageneric classification of the genus *Ornithogalum* L. (Liliaceae). Botanicheskii Zhurnal 62: 1624–1639. [in Russian]

Zahariadi C (1980) *Ornithogalum* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM (Eds) Flora Europaea 5. Cambridge University Press, Cambridge, 35–40.

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# *Heteropterys rosmarinifolia*, a new species of Malpighiaceae with verticillate leaves from savannas grasslands of central Brazil

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## Abstract

A new species of *Heteropterys* belonging to the Parabanisteria informal group is described for the savannas grasslands of the Serra do Tombador Natural Reserve, municipality of Cavalcante, State of Goiás, Brazil. *Heteropterys rosmarinifolia* is easily distinguished from the remaining Brazilian species of the Parabanisteria informal group due to its stems unbranched, leaves associated with the inflorescence verticillate, blades linear to very narrowly oblong, strongly conduplicate, ascending to upright, and sparsely sericeous to glabrous at age. We present a complete morphological description for the new species, photographic plates, comments on its distribution, ecology, and taxonomy, besides an identification key to the species of *Heteropterys* from the Parabanisteria group from the State of Goiás, Brazil.

## Keywords

Cerrado, Malpighiales, Neotropical flora, Tetrapteroid clade, xeric plants

## Introduction

*Heteropterys* Kunth is the largest genus of Neotropical Malpighiaceae, with ca. 160 species occurring in various open to closed habitats from northern Mexico to northern Argentina (Anderson et al. 2006; POWO 2021). A single species, *H. leona* (Cav.) L., is also widely and naturally distributed in western Africa (Anderson 2001). The genus includes a great deal of morphological variation in life forms (i.e., subshrubs, shrubs, lianas to small trees), gland position on the leaves (i.e., glands inserted at the base, middle or apex of petioles and/or along the leaf blade and/or on margins), inflorescence architecture (i.e., 1-flowered cincinni arranged in thyrsi, corymbs, or umbels), petal color (i.e., white, yellow, orange, pink, red to purple), petal shape when in bud (i.e., smooth or dorsally keeled), and shape of the style apex (i.e., rounded, truncate, uncinate, or hammer-shaped) (Amorim 2003; Pessoa et al. 2014; Almeida et al. 2021). The only morphological character shared by all species in the genus is the schizocarpic fruit with winged mericarps, in which the dorsal wing is more developed than the lateral ones, thickened on the inferior side, and curved towards the floral center (Almeida et al. 2021). This peculiar character is unique in Malpighiaceae and currently the sole synapomorphy of the monophyletic *Heteropterys* (Almeida & van den Berg, 2021).

The immense morphological variation in *Heteropterys* makes it one of the most taxonomically complex genera in the family, with no modern revision (Almeida pers. observ.). Nonetheless, seven informal groups, derived from different taxonomic ranks proposed in the past, are currently recognized in the literature: Apytchia, Madarophyllis, Metallophyllis, Parabanisteria, Rhodopetalis, Stenophyllarion, and Xanthopetalis (Amorim 2003). Out of those, the Parabanisteria group is the most diversified, with over 50 species confined to Neotropical savannas from the Amazon domain (i.e., campinaranas), Central Brazil and Bolivia, and the llanos from Colombia and Venezuela (POWO 2021; Almeida pers. observ.). Species of Parabanisteria are easily recognized by their large flowers arranged in thyrsi and covered in brown hairs, the apex of sepals revolute at anthesis, petals bright yellow and usually widely elliptic, and lateral wings of mericarps usually absent (Almeida et al. 2021).

During the preparation of the *Heteropterys* monograph for the Flora do Brasil 2020 project (Almeida et al. 2021), we came across two recently collected specimens of *Heteropterys* belonging to the Parabanisteria group with unusual verticillate leaves from Serra do Tombador, municipality of Cavalcante, State of Goiás. We describe these specimens as a new species, presenting detailed morphological descriptions, photo plates, and comments on its distribution, ecology, and taxonomy.

## Methods

Morphological data were based on herbaria samples (HUEFS, RB, and UFRN; acronyms according to Thiers, continuously updated). The indumentum terminology follows Anderson (1981), structure shapes follow Radford et al. (1974), inflorescence terminology and morphology follow Weberling (1965, 1989), and fruit terminology follows Spjut (1994) and Anderson (1981). The conservation status was proposed following the recommendations of IUCN Red List Categories and Criteria, Version 3.1 (IUCN 2012). GeoCAT (Bachman et al. 2011) was used for calculating the Extent of Occurrence (EOO) and the Area of Occurrence (AOO).

## Results

### *Heteropterys rosmarinifolia* R.F.Almeida & M.Pell., sp. nov.

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Figs 1–3

**Diagnosis.** *Heteropterys rosmarinifolia* differs from the remaining Brazilian species of the Parabanisteria group due to its stems unbranched (vs. branched), leaves associated with the inflorescence verticillate (vs. opposite to subopposite), blades linear to very narrowly oblong (vs. several shapes, but never linear or narrowly oblong), strongly conduplicate (vs. plane), ascending to upright (vs. patent).

**Type. Brazil. Goiás:** Cavalcante, Reserva Natural Serra do Tombador, road GO-241, estrada de terra para o Engenho II, a direita da estrada, 13°42'00"S, 47°48'00"W, fl., 25 Jul 2014, R. Sartin et al. 576 (holotype: UFRN barcode UFRN00024927!; isotype: RB barcode RB01408371!).

**Description.** *Subshrubs* 25–55 cm tall, unbranched. *Xylopodium* not seen. *Branches* densely rusty-sericeous, hairs adpressed; internodes 0.9–3.6 cm long. *Stipules* 15–20 mm long, interpetiolar, fused, both sides densely rusty-sericeous. *Leaves* subopposite at base in vegetative branches, becoming opposite at mid-length, and verticillate towards the apex of the vegetative branches and in flowering branches; petioles 0.2–0.3 cm long, canaliculate, densely rusty-sericeous, eglandular; blades 1.3–8.3 × 0.1–0.3 cm, linear to very narrowly oblong, strongly conduplicate, ascending to upright, coriaceous, eglandular, base obtuse, margins entire, plane to slightly involute, apex obtuse to rounded, sparsely rusty-sericeous to glabrous, abaxially rusty-sericeous, becoming only sparsely rusty-sericeous along the midvein with age. *Synflorescence* consisting of a solitary main florescence, leaves associated with the inflorescence much reduced, sessile, 3.1–14 × 0.8–1.0 mm. *Thyrsi* 4–16-flowered, terminal, pedunculate, many-branched; main axis 1.0–2.8 cm long, smooth, densely rusty-sericeous; bracts 2.0–4.3 × 0.9–1.3 mm, elliptic, concave, patent, eglandular, adaxially glabrous, abaxially rusty-sericeous; cincinni verticillate, 3–4 per node, 1-flowered; peduncle 3.0–5.1 mm long, rusty-sericeous; bracteoles opposite, inserted at the apex of the peduncle, 2.0–2.5 × 0.5–0.9 mm, elliptic to broadly elliptic, concave, patent, adaxially glabrous, abaxially rusty-sericeous. *Flowers* 1.0–1.2 cm diam. at anthesis; floral buds 3.9–5.0 × 2.8–4.0 mm, transversally widely oblongoid to widely depressed ovoid, apex obtuse to truncate; pedicel 5.0–7.0 mm long, rusty-sericeous. *Sepals* 3.0–4.8 × 1.0–1.9 mm, straight, smooth, apex rounded, strongly revolute at anthesis, adaxially glabrous, abaxially densely rusty-sericeous; the anterior eglandular, the latero-anterior 1-glandular, the posterior 2-glandular, glands 1.0–1.2 × 0.5–0.6 mm, green, elliptic. *Petals* bright yellow, dorsally smooth in bud, persistent at anthesis; lateral petals orbicular, plane, limb 3.4–4.2 × 2.7–3.5 mm, margin denticulate, eglandular, claws 1.4–2.2 × 0.4–0.6 mm, both sides glabrous; posterior petal orbicular, erect, limb 3.1–3.5 × 3.0–3.4 mm, base truncate, margin denticulate, eglandular, claws 1.0–2.1

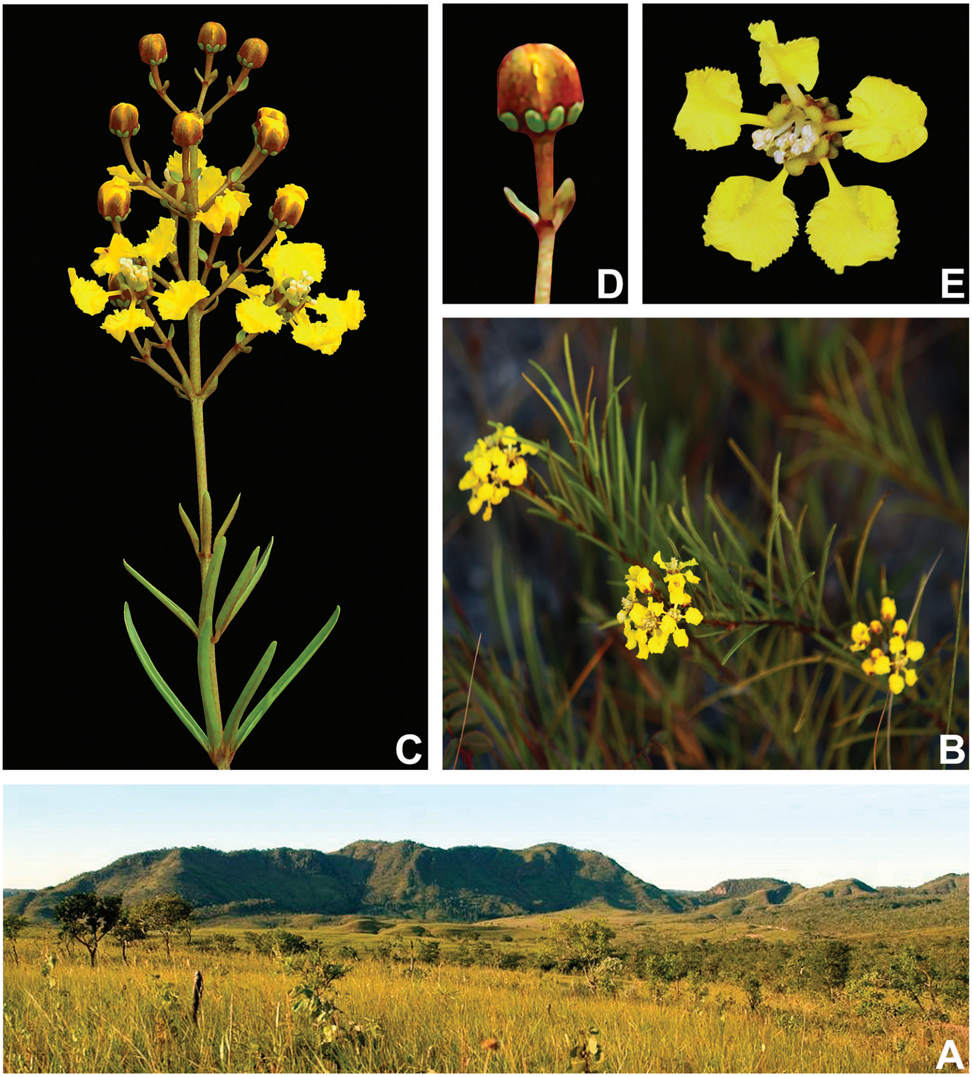

**Figure 1.** *Heteropterys rosmarinifolia* R.F.Almeida & M.Pell. **A** savannah grasslands from Reserva Natural Serra do Tombador **B** habit **C** detail of the apex of the branch, showing the verticillate leaves and solitary inflorescence **D** cincinnus, showing the concave bracteoles and the floral bud **E** front view of the flower. All photos by R. Sartin, except **A** by H. Palo-Jr.

× 0.6–0.8 mm, both sides glabrous. *Stamens* 10, all fertile, filaments 2.2–3.0 × 0.2–0.8 mm, basally connate for 0.3–1.2 mm, cylindrical, thicker at base, tapering towards the apex; connective glandular, white, glabrous; thecae 0.8–1.0 × 0.35–0.40 mm, white, glabrous. *Ovary* 1.4–1.8 × 1.1–1.5 mm, ovoid, densely sericeous; styles 3, erect, 2.4–2.9 × 0.5–0.7 mm, cylindrical, divergent at apex, glabrous, apex rounded, anterior style slightly shorter than posterior ones; stigmas lateral, pointing towards the center of the flower. *Fruits* not seen. *Seeds* not seen.

**Figure 2.** Holotype of *Heteropterys rosmarinifolia* R.F.Almeida & M.Pell. (R. Sartin et al. 576, UFRN barcode 00024927).

**Paratype. Brazil. Goiás:** Cavalcante, Reserva Natural Serra do Tombador, caminho para a cachoeira da Ave Maria, ponto onde se vê a cachoeira, 13°44'26"S, 46°52'46"W, 22 Sep 2015, fl., L. Rocha et al. 668 (HUEFS barcode HUEFS000273192!).

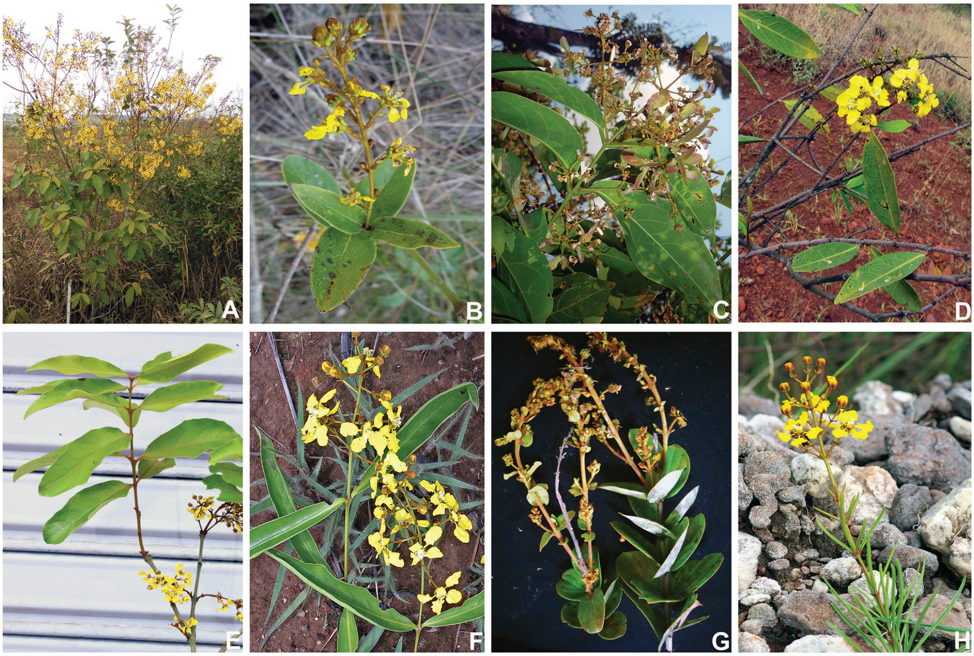

**Figure 3.** *Heteropterys* from the Parabanisteria group from the State of Goiás, Brazil **A** *H. byrsonimifolia* **B** *H. coriacea* **C** *H. eglandulosa* **D** *H. escalloniifolia* **E** *H. nervosa* **F** *H. pannosa* **G** *H. procoriacea* **H** *H. rosmarinifolia*. **A** by L.C. Marinho **B, G** by I. Morais **C, H** by R. Sartin **D, E** by R.F. Almeida **F** by R. Ripley.

**Distribution, habitat, and phenology.** *Heteropterys rosmarinifolia* is known only from savannas grasslands within the Serra do Tombador Natural Reserve in the State of Goiás, Brazil (Fig. 1). It blooms from July to September, but fruits are unknown.

**Conservation status.** *Heteropterys rosmarinifolia* is known only from two collections, probably from the same population within the limits of the Serra do Tombador Natural Reserve in the State of Goiás, Brazil. Until additional fieldwork can be done in the savannas of northern Goiás, this species is best categorized as data deficient (DD).

**Etymology.** The epithet makes reference to the leaves of the new species that resemble those of the widely cultivated aromatic herb, rosemary (*Salvia rosmarinus* Spenn., Lamiaceae).

**Notes.** *Heteropterys rosmarinifolia* is easily distinguished from the remaining species of the Parabanisteria group by the vegetative characters presented in the abovementioned diagnosis. It is most similar to *H. pannosa* Griseb. due to its small stature, delicate branches, and narrow leaf-blades. However, it can be differentiated by its stems distally unbranched (vs. branched in the upper half in *H. pannosa*), leaf-blades linear to very narrowly oblong, strongly conduplicate, ascending to upright, and abaxially rusty-sericeous (vs. narrowly elliptic, conduplicate, patent, and abaxially greyish-lanate-tomentose), leaves associated with the inflorescence verticillate (vs. opposite to ternate), cincinni verticillate (vs. subopposite to opposite), and anthers glabrous with connectives white (vs. pubescent and brown).

Despite fruits still being unknown for *H. rosmarinifolia*, they most likely are similar to the ones of *H. pannosa*, with the dorsal wing reduced to a crest, and nuts lacking lateral winglets (i.e., wingless nut). Alternatively, most species of the Parabanisteria group present a developed dorsal wing (Almeida et al. 2021). Floral and fruit characters are highly conserved in the Parabanisteria group and have been traditionally considered of little taxonomic relevance (Niedenzu 1928). Consequently, this group's taxonomy currently heavily relies on vegetative characters related to life form, branch, and leaf morphology, rendering it the most taxonomic convoluted in *Heteropterys* (Niedenzu 1928; Almeida pers. observ.). Further studies are still necessary to properly explore the relevance of floral and fruit characters in this group and review species boundaries. For the time being, we provide below an identification key for the species of *Heteropterys* from the Parabanisteria group from the State of Goiás, Brazil.

## Key to the species of the Parabanisteria group from Goiás

Modified from Pessoa et al. 2014.

1 Leaf-blades narrowly elliptic or linear to very narrowly oblong; thyrsi solitary ........ **2**
– Leaf-blades elliptic, ovate-lanceolate, ovate or oblanceolate; thyrsi arranged in thyrsi ........ **3**
2 Leaf-blades narrowly elliptic, conduplicate, patent, abaxially greyish-lanate-tomentose, the ones associated with the inflorescence ternate; cincinni subopposite to opposite; anthers pubescent, connectives brown ........ ***H. pannosa* (Fig. 3F)**
– Leaf-blades linear to very narrowly oblong, strongly conduplicate, ascending to upright, abaxially rusty-sericeous, the ones associated with the inflorescence verticillate; cincinni verticillate; anthers glabrous, connectives white ........ ***H. rosmarinifolia* (Fig. 3H)**
3 Lenticels lighter than the branch; leaf-blades abaxially sericeous to glabrescent ........ **4**
– Lenticels darker than the branch; leaf-blades abaxially glabrous ........ **5**
4 Treelets; leaf-blades ovate, base obtuse, rounded or subcordate, margins plane; anterior style the same length as the posterior ones, anterior stigma pointing towards the center of the flower, the posterior ones pointing towards the latero-posterior petals ........ ***H. byrsonimifolia* (Fig. 3A)**
– Lianas; leaf-blades elliptic to oblanceolate, base cuneate, rarely obtuse, margins revolute; anterior style longer than the posterior ones, all stigmas pointing towards the posterior petal ........ ***H. escalloniifolia* (Fig. 3D)**
5 Petioles sparsely tomentose to tomentose, sometimes glabrescent at age; anthers pubescent ........ ***H. coriacea* (Fig. 3B)**
– Petioles sparsely sericeous (glabrescent at age) or sericeous; anthers glabrous6

6 Leaf-blades lanceolate to ovate-lanceolate; inflorescences axillary; styles with truncate apex ........................................................ ***H. nervosa* (Fig. 3E)**

– Leaf-blades elliptic or ovate; inflorescences terminal; styles with uncinate apex ........................................................ **7**

7 Lianas; leaf-blades elliptic, base cuneate; sepals eglandular, styles with apex curved-uncinate ........................................................ ***H. eglandulosa* (Fig. 3C)**

– Subshrubs; leaf-blades ovate, base subcordate; sepals glandular, styles with apex straight-uncinate ........................................................ ***H. procoriacea* (Fig. 3G)**

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We would like to thank H. Palo-Jr., I. Morais, L.C. Marinho, R. Ripley, and R. Sartin for allowing us to use their images in this study; and James Lucas Costa Lima and an anonymous reviewer for their comments during the reviewing process of this manuscript.

## References

Almeida RF, van den Berg C (2021) Molecular phylogeny and character mapping support generic adjustments in the Tetrapteroid clade (Malpighiaceae). Nordic Journal of Botany 39(1): 1–27. https://doi.org/10.1111/njb.02876

Almeida RF, Francener A, Pessoa CS, Sebastiani R, Amorim AMA, Mamede MCH (2021) Malpighiaceae monograph in Flora do Brasil 2020. http://floradobrasil.jbrj.gov.br/ [Accessed 7 Jan 2021]

Amorim AMA (2003) Estudos taxonômicos em *Heteropterys* (Malpighiaceae). Doctoral thesis, Universidade de São Paulo, São Paulo, 280 pp.

Anderson WR (1981) Malpighiaceae. The botany of the Guayana Highland – Part XI. Memoirs of the New York Botanical Garden 32: 21–305.

Anderson CE (2001) The identity of two water-dispersed species of *Heteropterys* (Malpighiaceae): *H. leona* and *H. platyptera.* Contributions from the University of Michigan Herbarium 23: 35–47.

Anderson WR, Anderson CE, Davis CC (2006) Malpighiaceae website. https://webapps.lsa.umich.edu/herb/malpigh/ [Accessed: 7 Jan 2021]

Bachman S, Moat J, Hill AW, Torre J, Scott B (2011) Supporting Red List threat assessments with GeoCAT: Geospatial conservation assessment tool. e-Infrastructures for data publishing in biodiversity science. ZooKeys 150: 117–126. https://doi.org/10.3897/zookeys.150.2109

IUCN (2012) IUCN Red List Categories and Criteria: Version 3.1. Second edition. Gland, Switzerland and Cambridge. www.iucnredlist.org/technical-documents/categories-and-criteria. [Accessed: 7 Jan 2021]

Niedenzu F (1928) *Heteropterys*. In: Engler A (Ed.) Das Pflanzenreich, IV, 141, Part 2 (Heft 93). W. Engelmann, Leipzig, 290–385.

Pessoa CS, Costa JAS, Amorim AMA (2014) Flora da Bahia: Malpighiaceae 2 – *Heteropterys.* Sitientibus. Série Ciências Biológicas 14: 1–41. https://doi.org/10.13102/scb476

POWO – Plants of the World Online (2021) Malpighiaceae. http://www.plantsoftheworldonline.org/ [Accessed: 7 Jan 2021]

Radford AE, Dickison WC, Massey JR, Bell CR (1974) Vascular Plant Systematics. Harper and Row, New York, 891 pp.

Spjut RW (1994) A systematic Treatment of Fruit Types. The New York Botanical Garden, New York, 181 pp.

Thiers B (continuously updated) Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. http://sweetgum.nybg.org/ih/ [Accessed: 7 Jan 2021]

Weberling F (1965) Typology of inflorescences. Botanical Journal of the Linnean Society 59: 15–221. https://doi.org/10.1111/j.1095-8339.1965.tb00058.x

Weberling F (1989) Morphology of Flowers and Inflorescences. Cambridge University, Cambridge.

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# *Dolichos kongkandae* sp. nov. and lectotypification of *D. fragrans* (Leguminosae, Papilionoideae) from Asia

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**Abstract**

*Dolichos kongkandae* is described as a new species from Asia and includes a line drawing, photographs and information on its distribution and ecology. The morphological differences between *D. kongkandae* and the morphologically similar *D. tenuicaulis* are highlighted and clarified. Additionally, a lectotype for *D. fragrans* is designated.

**Keywords**

Fabaceae, lectotype, new species discovery, Phaseoleae, Phaseolinae, taxonomy

## Introduction

*Dolichos* L. is a large genus in subtribe Phaseolinae, tribe Phaseoleae, subfamily Papilionoideae in Leguminosae (Lackey 1981; Schrire 2005). Its approximately 55 species mostly occur in Africa and only 5–6 species are found in Asia, including the Indian subcontinent, South China, Indo-China and Malesia (Schrire 2005).

There are several important diagnostic features of *Dolichos*. These include basifixed stipules, trifoliolate pinnately compound leaves, purple, purplish-pink or white flowers, a standard petal with short cone-shaped appendages and inflexed auricles, incurved, but not twisted keels, a terete and incurved upward-bending style with a short pubescent or bearded inner side of the apex, a terminal and capitate stigma, compressed fruits and reticulate pollen (Verdcourt 1970, 1978, 1979; Lackey 1981; Moteetee and Van Wyk 2012).

Four species of *Dolichos* were reported for Thailand, including *D. lablab* L., *D. schomburgkii* Gagnep., *D. subcarnosus* Wight & Arn. and *D. tenuicaulis* (Baker) Craib (Craib 1928). Of these, two species have been transferred to other genera: *D. lablab* is now a synonym of *Lablab purpureus* (L.) Sweet and *D. schomburgkii* is now a synonym of *Dysolobium dolichoides* (Roxb.) Prain.

During fieldwork at Doi Chiang Dao Wildlife Sanctuary in 2013 and 2017, a population of *Dolichos* that presented distinct dark purple flowers was discovered along the Khun Huai Mae Kok Ranger Station (Den Ya Khat) nature trail. We studied and compared this plant with available herbarium specimens of *Dolichos* and found that it was similar to specimens identified as *D. tenuicaulis*. After thorough examination of the type specimens and first publication of *D. tenuicaulis*, we found that our newly-collected plant and several of the herbarium specimens identified as *D. tenuicaulis* were not similar to the type specimens of *D. tenuicaulis* and that they differed from all the known species of *Dolichos*.

Here, we describe these collections as a new species, *Dolichos kongkandae* R. Meeboonya, Ngerns. & Balslev, with descriptions of its morphological features and we provide a line drawing and fields photographs. As part of our investigation, we found that *D. fragrans* Kerr, an endemic species from the Doi Chiang Dao Wildlife Sanctuary of Thailand, needed a lectotypification, which is consequently provided here.

## Materials and methods

The morphological observations and description of this new species is based on field collections and herbarium specimens deposited at AAU, BK, BM, BKF, CMUB, E, K, L and P which were used to compare the new species to already-known species of *Dolichos*, especially the morphologically similar species and we provide the species' geographical information. Herbarium acronyms follow Index Herbariorum (Thiers 2019). Regarding the lectotypification of *Dolichos fragrans*, we designated a lectotype from five syntypes found at BM, E, K, L and P.

# Taxonomic treatment

## New species

***Dolichos kongkandae*** **Meeboonya, Ngerns. & Balslev, sp. nov.**
urn:lsid:ipni.org:names:77215900-1
Figs 1, 2

**Diagnosis.** *Dolichos kongkandae* is most similar to *D. tenuicaulis*, but differs in having a densely-pubescent stem (versus slightly pubescent), ovate or broadly elliptic stipules (versus lanceolate, elliptic or subtriangular), a longer axis of inflorescence, 1–3 cm long

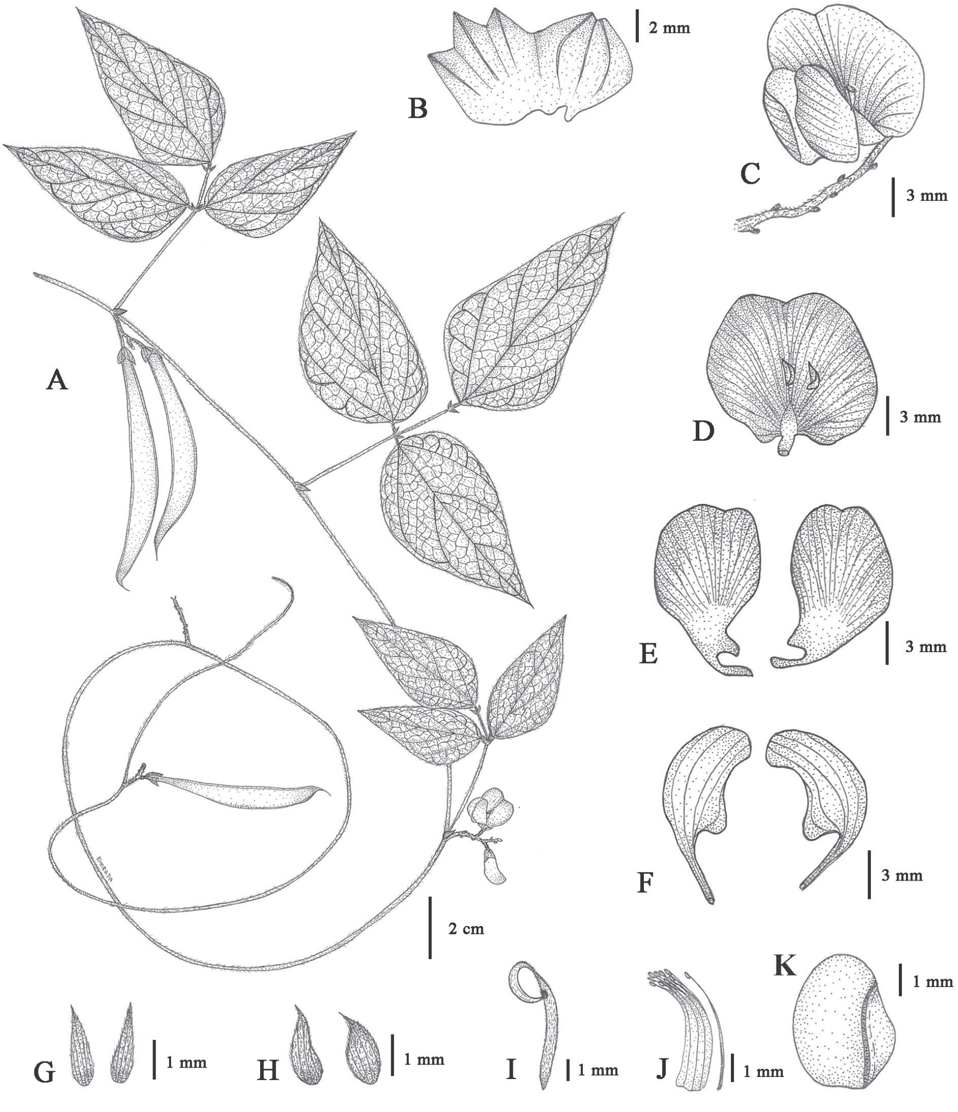

**Figure 1.** *Dolichos kongkandae* R. Meeboonya, Ngerns. & Balslev **A** stem with leaves, inflorescence and infructescences **B** calyx **C** inflorescence with a flower **D** standard petal **E** wing petals **F** keel petals **G** bracts of fascicle **H** bracteoles **I** pistil **J** stamens **K** seed. Drawn from *R. Meeboonya & P. Yodboplub 406* (BKF) by Rumrada Meeboonya.

(versus 0.3–0.5 cm long), the corolla dark purple turning blackish-purple when dried (versus purplish-pink or pale pink turning pale yellow when dried), a larger standard, ca. 12 × ca. 14 mm (versus 8–9 × 8.5–9 mm), wing petals, ca. 16 × ca. 8 mm (versus 10–11 × 3–4 mm), keel petals 11–12 × 3–4 mm (versus 9–10 × 2–2.5 mm) and a hirsute fruit stalk (versus slightly puberulous).

**Type.** Thailand. Chiang Mai, Chiang Dao, Doi Chiang Dao Wildlife Sanctuary, near the beginning of Khun Huai Mae Kok Ranger Station (Den Ya Khat) nature trail, 24 Nov 2017, *R. Meeboonya & P. Yodboplub 406* (holotype BKF!; isotypes AAU!, BK!).

**Description.** Perennial herb, stem slender, twining, densely pubescent. ***Stipules*** persistent, ovate or broadly elliptic, 3–5 × 1.5–3 mm, with striate veins, apex acute, base subcordate or truncate, abaxial surface pubescent, adaxial surface glabrous. *Leaves* trifoliolate pinnately compound, alternate, petiole 2.5–7 cm long, sparsely pubescent; rachis 0.8–1.8 cm long, sparsely pubescent. ***Leaflets*** densely pubescent on both surfaces, with 4–6 lateral veins on each side; terminal leaflet ovate, lanceolate or broadly ovate, 3.5–8 × 1.5–4.5 cm, apex acute and apiculate, base rounded, obtuse or subcordate, margin entire; lateral leaflets slightly obliquely ovate or obliquely lanceolate, 3.5–7 × 1.2–3.5 cm, apex acute and apiculate, base rounded, obtuse or subcordate, margin entire; stipels persistent, attached to the base of petiolules of leaflets, elliptic, lanceolate or obovate, 2–2.8 × 1–1.2 mm, with striate veins, apex acute, base truncate, abaxial surface hirsute, adaxial surface glabrous; petiolule 1.5–3 mm long, densely pubescent. ***Inflorescences*** axillary, with 2–10 fascicles arranged in nodose-pseudoracemes or nodose-pseudopanicles; peduncle 2–5 mm long, sparsely hirsute; axis 1–3 cm long, hirsute; bracts of fascicle 2, persistent, lanceolate, 2–2.5 × 0.5–1 mm, with striate veins, apex acute, base obtuse, margin ciliate, abaxial surface pubescent, adaxial surface glabrous. ***Flowers*** 1 in each fascicle; pedicel 2–3 mm long, densely hirsute; bracteoles 2, attached near the apex of pedicel, ovate, 1.8–2 × 0.8–1 mm, apex acute or acuminate, base obtuse, margin ciliate, abaxial surface pubescent, adaxial surface glabrous. ***Calyx*** greenish-purple, campanulate, 5-lobed, calyx tube ca. 4.5 × ca. 5.5 mm, 2 upper lobes connate, apex slightly divided to shallow lobes, the lowest of 3 lower lobes deltoid, ca. 2 × ca. 2.5 mm, apex acute, 2 lateral lobes deltoid, ca. 1 × ca. 1.5 mm, apex acute, abaxial surface sparsely pubescent, adaxial surface glabrous. ***Corolla*** pentamerous, dark purple, turning blackish-purple when dried, with clawed petals; standard petal, suborbicular, ca. 12 × ca. 14 mm, apex emarginate, near the centre with 2 appendages on adaxial surface, 1.5–2 mm long, base with 2 small auricles, ca. 1 mm long, the claw white, 1–2 mm long; wing petals, obovate, ca. 16 × ca. 8 mm, apex emarginate, base with 1 appendage, 1.5–2 mm long, the claw white, ca. 4 mm long; keel petals oblong, 11–12 × 3–4 mm, pubescent along the inner margins, apex truncate, the claw white, ca. 4 mm long. ***Stamens*** 10, diadelphous, 9 filaments connate and a vexillary filament free, filaments white, ca. 10 mm long; anthers uniform and dorsifixed, yellow, oblong, ca. 0.5 mm long. ***Pistil*** simple, ovary superior, ca. 9 mm long; ovary light green, linear, pubescent, base shortly stipitate; style flattened; stigma capitate with long hairs. ***Fruits*** oblong, slightly falcate, 5–7.5 cm × 6–8 mm, glabrous or slightly pubescent along both margins, apical beak 3–5 mm long, base stipitate, 2–3 mm long; fruit stalk 3–5 mm long, hirsute. ***Seeds*** 5–8, young seeds light green, dry seeds brown, elliptic or oblong, compressed, ca. 4 × 2.5–3 mm.

**Phenology.** Flowering and fruiting from August to December.

**Distribution.** Bhutan, India, Myanmar, China, Laos, Thailand.

**Ecology.** Open areas in montane rain forests, mixed deciduous forests, limestone ridges, 550–2150 m alt.

**Figure 2.** *Dolichos kongkandae* R. Meeboonya, Ngerns. & Balslev **A** habit **B** leaf **C** inflorescence with flower buds **D** inflorescence with young and mature flowers **E** inflorescence with a mature flower and a young fruit **F** infructescence with a mature fruit. Photographs from *R. Meeboonya & P. Yodboplub 406* (BKF).

**Vernacular name.** Thua doi dok muang kongkanda (ถั่วดอยดอกม่วงก่องกานดา), the name is here given by the authors. This vernacular name references legumes (*thua*), the hills or mountain regions of its origin (*doi*), purple corolla (*dok muang*), and our mentor (Dr. Kongkanda Chayamarit).

**Conservation status.** *Dolichos kongkandae* is widely distributed in its habitats. However, these areas are disturbed by the human activities. It is therefore considered as Near Threatened (NT), following the IUCN Red List Criteria and Categories version 14 (IUCN 2019).

**Etymology.** The specific epithet is named in honour of Dr. Kongkanda Chayamarit, the expert botanist of the Forest Herbarium and the Flora of Thailand Project. She was the former supervisor of Associate Professor Dr. Chatchai Ngernsaengsaruay in his master's and doctoral degrees and the thesis co-advisor of Dr. Rumrada Meeboonya in her master's and doctoral degrees. She has always encouraged and supported us.

**Additional specimens examined. Bhutan.** Kauchaw, Punakha, 21 Aug 1914, *R.E. Cooper & A.K. Bulley 3279* (BM!, E!); 26 Aug 1915, *R.E. Cooper & A.K. Bulley 4627* (BM!, E!). **India.** Assam, Karong, Manipur, 26 Sept 1950, *W.N. Koelz 26277* (L!); 9 Oct 1869, *C.B. Clarke s.n.* (K!); 3 Oct 1875, *C.B. Clarke 24925* (BM!, K!). **Myanmar.** Mandalay, Maymyo plateau, 3 Oct 1908, *J.H. Lace 4270* (E!, K!). **China.** Yunnan, Aug 1912, *G. Forrest 8821* (E!); ibid., Aug 1913, *G. Forrest 11966* (E!). **Thailand.** Chiang Mai: Doi Chiang Dao, 2 Nov 1922, *A.F.G. Kerr 6514* (BK!, BM!, E, K); ibid., 14 Oct 1926, *Put 322* (AAU!, BK!, BM!, E!, K!); ibid., 9 Nov 1962, *T. Smitinand, M.E.D. Poore & R.G. Robbins 7742* (BKF!); ibid., 25 Sept 1971, *G. Murata, K. Iwatsuki & C. Phengklai T-14920* (BKF!); ibid., 25 Sept 1971, *G. Murata, K. Iwatsuki & C. Phengklai T-14930* (AAU!, BKF!, L!); ibid., 25 Sept 1971, *J.E. Vidal 5161* (AAU!, P!); ibid., 27 Sept 1971, *J.E. Vidal 5233* (AAU!, P!); ibid, 27 Oct 1979, *T. Shimizu, H. Toyokuni, H. Koyama, T. Yahara, T. Santisuk & C. Niyomdham T-21180* (BKF!); ibid., 4 Nov 1995, *J.F. Maxwell 95-1051* (BKF!, CMUB!, L!); ibid., Dec 2000, *T. Rotjanadirok 81* (CMUB!); ibid., 12 Nov 2011, *R.P. Clark, P. Wilkin, P. Suksathan, A. Trias-Blasi & Phitak 211* (K!); ibid., 12 Nov 2011, *R.P. Clark, P. Wilkin, P. Suksathan, A. Trias-Blasi & Phitak 230* (K!); ibid., 11 Nov 2012, *V. Chamchumroon, M. Callmander, S. Christoph, C. Davidson, J. Regalado, S. Sirimongkol, N. Ritphet & S. Lai-lung 5564* (BKF!); ibid., 7 Nov 2013, *A.N. Egan, R.P. Clark, S. Sirimongkol, V. Chamchumroon & R. Meeboonya 13-0806* (K!); Mae Taeng, 30 Oct 1922, *A.F.G. Kerr 6491* (BK!, BM!, C!, K!).

**Notes.** *Dolichos kongkandae* has been usually confused with *D. tenuicaulis* (Fig. 3) because of some morphological similarities. This is especially true of dried herbarium specimens.

The type specimens of *Phaseolus tenuicaulis* Baker (the basionym of *D. tenuicaulis*), *Wallich Cat. no. 5598 D* (holotype K001121419!), has a slightly pubescent stem, lanceolate, elliptic or subtriangular stipules, shorter axis of the inflorescence and the corolla turning pale yellow when dried. It also has a smaller standard, wing and keel petals and a slightly puberulous fruit stalk all of which distinguish it from *D. kongkandae* (Table 1).

Craib (1912) published *D. tenuicaulis*, based on the specimens of *Lace 4270* and *Robertson 14*. Moreover, he cited *D. falcatus*, based on the specimens of *Housseus 45* and *Kerr 834*. We studied these specimens and found that *Housseus 45*, *Kerr 834* and *Robertson 14* are similar to *D. tenuicaulis*. However, the specimens of *Lace 4270* have a densely-pubescent stem and leaves, ovate or broadly-elliptic stipules, longer axis of the inflorescence, densely-hirsute pedicel and the corolla turns to blackish-purple when dried; it has a larger standard, wing and keel petals and a hirsute fruit stalk which is not similar to *D. tenuicaulis* and these specimens are similar to *D. kongkandae*.

Craib (1928) reported *D. tenuicaulis* as occurring in Thailand, based on the specimens *Hosseus 45*, *Kerr 834*, *Kerr 4569*, *Kerr 6491*, *Kerr 6514*, *Put 322* and *Winit 1542*.

**Figure 3.** *Dolichos tenuicaulis* (Baker) Craib **A** leaf **B, C** inflorescence with a mature flower. Photographs from *R. Meeboonya*, *K. Kommongkol*, *T. Napiroon*, *P. Wessapak & P. Yodboplub 372* (BKF).

**Table 1.** Morphological differences between *Dolichos kongkandae* and *D. tenuicaulis*.

| Characters | *D. kongkandae* | *D. tenuicaulis* |
|---|---|---|
| Stem indumentum | densely pubescent | slightly pubescent |
| Stipules shape and width | ovate or broadly elliptic, 1.5–3 mm wide | lanceolate, elliptic or subtriangular, 1–1.5 mm wide |
| Leaflets indumentum | densely pubescent | slightly pubescent |
| Stipels shape and width | elliptic, lanceolate or obovate, 1–1.2 mm wide | elliptic, up to 0.5 mm wide |
| Inflorescence axis length and indumentum | 1–3 cm long, hirsute | 0.3–0.5 cm long, slightly puberulous |
| Bracts of fascicle shape and size | lanceolate, 2–2.5 × 0.5–1 mm | lanceolate or ovate-lanceolate, 1.5–1.8 × ca. 0.5 mm |
| Corolla colour | dark purple, turn to blackish-purple when dried | purplish-pink or pale pink, turning to yellow when dried |
| Pedicel indumentum | densely hirsute | puberulous |
| Calyx colour, shape, size and apex of the lowest of 3 lower lobes | greenish-purple, deltoid, ca. 2 × ca. 2.5 mm, apex acute | green, deltoid, ca. 1 × ca. 1.5 mm, apex obtuse and apiculate |
| Standard petal size | ca. 12 × ca. 14 mm | 8–9 × 8.5–9 mm |
| Wing petals shape and size and appendage length | obovate, ca. 16 × ca. 8 mm, appendage at base 1.5–2 mm long | narrowly obovate, 10–11 × 3–4 mm, appendage at base, ca. 0.8 mm long |
| Keel petals size | 11–12 × 3–4 mm | 9–10 × 2–2.5 mm |
| Fruit stalk length and indumentum | 3–5 mm long, hirsute | 2–3 mm long, slightly puberulous |

We studied these specimens and found they can be separated into two groups. The specimens *Kerr 834* from Doi Suthep, Chiang Mai Province, *Hosseus 45* and *Kerr 4569* from Nakhon Sawan Province and *Winit 1542* from Lamphun Province have slightly pubescent stem and leaves, lanceolate, elliptic or subtriangular stipules, shorter axis of inflorescence, puberulous pedicel, the corolla turning to yellow when dried, has smaller standard, wing and keel petals and slightly puberulous fruit stalk, similar to *D. tenuicaulis*, whereas *Kerr 6491*, *Kerr 6514* and *Put 322* from Chiang Mai Province are *D. kongkandae*.

We thoroughly examined the specimens of *Dolichos* from Thai and foreign herbaria. We consistently found that the herbarium specimens of *D. kongkandae* were

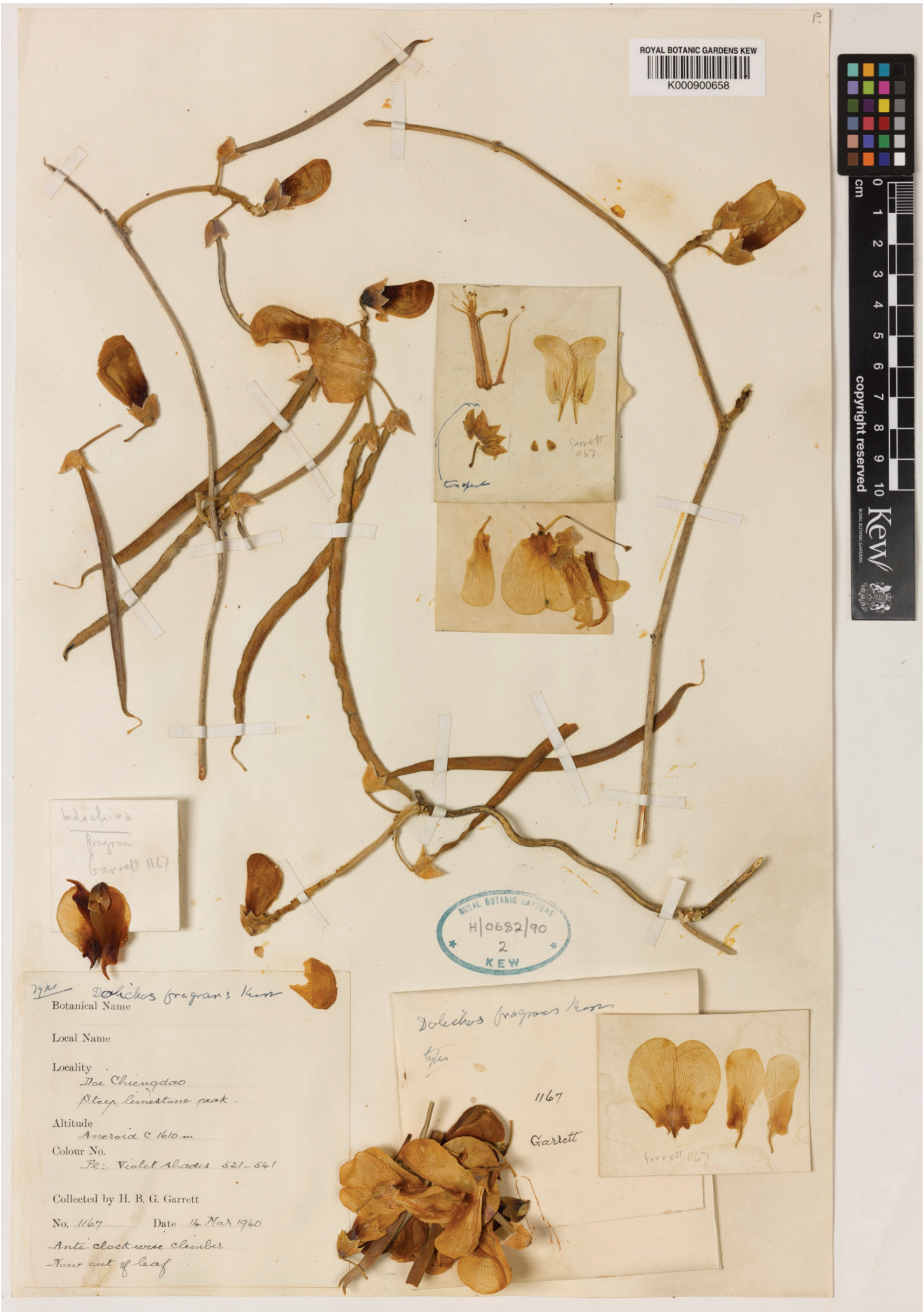

**Figure 4.** Lectotype of *Dolichos fragrans* Kerr, H.B.G. Garrett 1167 (K); see The Royal Botanic Gardens, Kew, Herbarium Catalogue (2020). http://specimens.kew.org/herbarium/ K000900658.

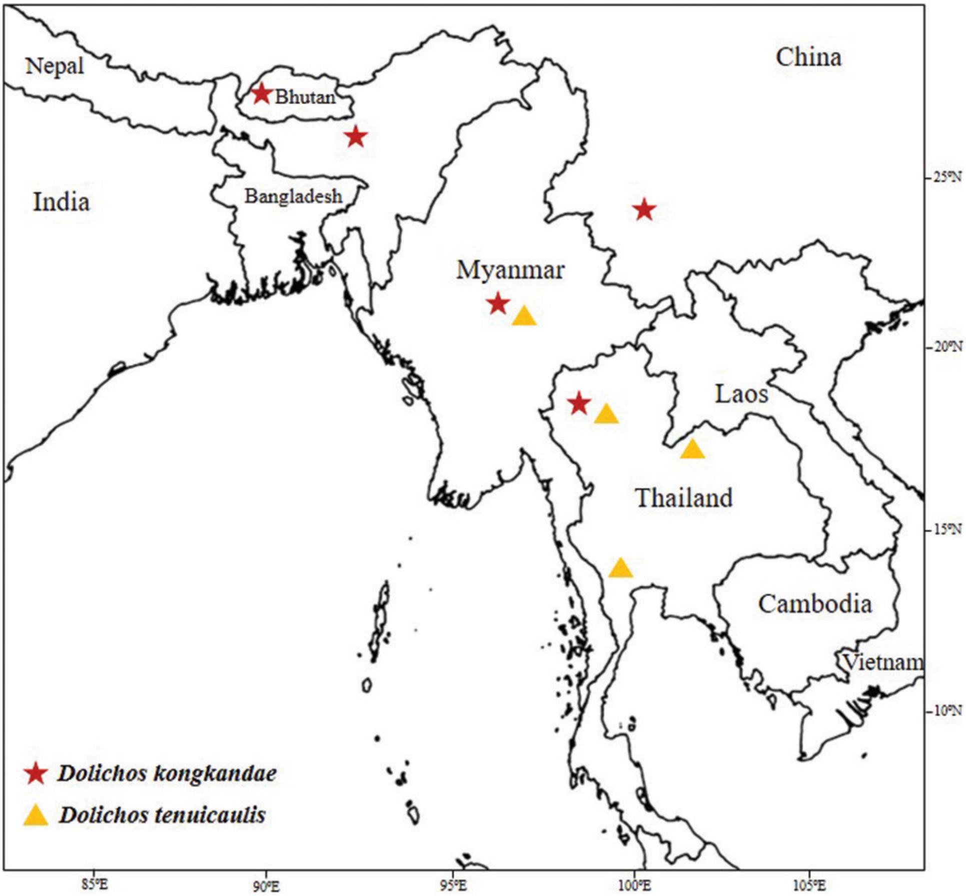

**Figure 5.** The geographical distribution of *Dolichos kongkandae* R. Meeboonya, Ngerns. & Balslev, sp. nov. and *D. tenuicaulis* (Baker) Craib.

misidentified as *D. tenuicaulis*. *Dolichos kongkandae* is distributed in Bhutan, India, Myanmar, Laos and Thailand. In Thailand, it is only found in Doi Chiang Dao and Mae Taeng, Chiang Mai Province. *Dolichos tenuicaulis* is distributed only in Myanmar and the northern, north-eastern and south-western regions of Thailand (Fig. 5).

## Lectotypification

***Dolichos fragrans* Kerr, Bull. Misc. Inform. Kew 1941(1): 9. 1941.**

**Type material.** **Thailand:** Doi Chiang Dao, Steep limestone peak, 14 Mar 1940, *H.B.G. Garrett 1167* (lectotype, designated here: K! [K000900658]; isolectotypes BM! [BM000839653], E! [E00275944], L! [L1952810], P! [P02775596]).

**Notes.** According to the protologue (Kerr 1941), the nomenclatural type of *D. fragrans* is *H.B.G. Garrett 1167*. Five syntypes of this gathering were deposited in BM, E, K, L and P and none of them has leaves (the leaves are often caducous when flowering). We designated the sheet K000900658 as the lectotype (Fig. 4), because it has many flowers and fruits, both of young and mature and also dissected flowers. The sheets, designated isolectotypes (BM000839653, E00275944, L1952810, P02775596), have fewer flowers and fruits than the sheet deposited at K and they have only flowers and young fruits, but no mature fruits.

The protologue also cites the specimens of *Kerr 2854*, *Put 4480* and *de Schauensee 719708*. These specimens were also collected from the limestone mountain of Doi Chiang Dao Wildlife Sanctuary and they must be regarded as paratypes, according to Article 9.7 (Turland et al. 2018).

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## References

Craib WG (1912) Contributions to the Flora of Siam (Dicotyledones). Aberdeen, UK. https://doi.org/10.5962/bhl.title.21865

Craib WG (1928) Florae Siamensis Enumeratio. V. 1 part 3. Siam Society, Bangkok.

Kerr AFG (1941) Contributions to the Flora of Thailand. Additamentum LIV. Bulletin of Miscellaneous Information Kew 1941(1): 9–10. https://doi.org/10.2307/4102538

Lackey JA (1981) Tribe Phaseoleae. In: Polhill RM, Raven PH (Eds) Advances in Legumes Systematics Part 1. Royal Botanic Gardens Kew, UK, 301–308.

Moteetee AN, Van Wyk BE (2012) A revision of the genus *Dolichos* (Fabaceae, Papilionoideae, Phaseoleae), including Lesotho and Swaziland. South African Journal of Botany 78: 178–194. https://doi.org/10.1016/j.sajb.2011.06.012

Schrire BD (2005) Tribe Phaseoleae. In: Lewis G, Schrire B, Mackinder B, Lock M (Eds) Legumes of the World. Royal Botanic Gardens Kew, UK.

IUCN (2019) Guidelines for Using the IUCN Red List Categories and Criteria. Version 14. Prepared by the Standards and Petitions Committee. http://www.iucnredlist.org/documents/RedListGuidelines.pdf

The Royal Botanic Gardens (2020) Herbarium Catalogue. http://specimens.kew.org/herbarium/K000900658 [accessed 09.03.2020]

Thiers B (2019) Index Herbariorum: a global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. The New York Botanical Garden. http://sweetgum.nybg.org/science/ih/

Turland NJ, Wiersema JH, Barrie FR, Greuter W, Hawksworth DL, Herendeen PS, Knapp S, Kusber W-H, Li D-Z, Marhold K, May TW, McNeill J, Monro AM, Prado J, Price MJ, Smith GF [Eds] (2018) International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code) adopted by the Nineteenth International Botanical Congress Shenzhen, China, July 2017. Regnum Vegetabile 159. Koeltz Botanical Books, Glashütten.

Verdcourt B (1970) Studies in the Leguminosae-Papilionoïdeae for the 'Flora of Tropical East Africa: III. Kew Bulletin 24(3): 379–447. https://doi.org/10.2307/4102845

Verdcourt B (1978) The classifications of *Dolichos* L. emend. Verdc., *Lablab* Adans., *Phaseolus* L., *Vigna* Savi and their Allies. In: Summerfield RJ, Bunting AH (Eds) Advances in Legume Science, Royal Botanic Gardens, Kew, UK, 45–48.

Verdcourt B (1979) A manual of New-Guinea legumes. Botany Bulletin, Department of Forests, Papua New Guinea 11: 488–490.

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# New insights into the phylogeny and taxonomy of Chinese *Physospermopsis* (Apiaceae)

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## Abstract

*Physospermopsis* (Apiaceae) comprises about 10 species, but its taxonomy and phylogeny are disputed. The genus is mostly distributed in the Himalayas and Hengduan Mountains at high elevation. Earlier molecular studies involving six species of *Physospermopsis* indicated that this genus is not monophyletic and is nested in the East Asia Clade. Therefore, the aims of this study were to re-assess the phylogenetic position of, and interspecific relationships within, *Physospermopsis* based on two chloroplast loci (*rpl16*, *rps16*) and one nuclear region, the internal transcribed spacers of ribosomal DNA (ITS). Eight species involving 13 populations of *Physospermopsis* were collected. These were sequenced and analyzed with the sequences of 31 other Apiaceae species obtained from the NCBI to determine phylogenetic relationships using Bayesian inference (BI) and Maximum likelihood (ML). Our study found that *Physospermopsis* is monophyletic, nested in Pleurospermeae of Apiaceae, sister to *Pleurospermum*. And we propose that the *Physospermopsis* clade should be replaced by the East Asia Clade. However, the interspecific relationships within *Physospermopsis* were not well resolved and the positioning of species was unclear. Diagnostic characteristics to distinguish *Physospermopsis* species in the field and laboratory are provided for future *Physospermopsis* phylogenetic studies.

## Keywords

Apiaceae, morphology, phylogeny, *Physospermopsis*, taxonomy

## Introduction

*Physospermopsis* H. Wolff (1925: 276) has been reported to contain about 10 species, with eight species distributed in China (Pan and Watson 2005). However, 11 species were reported by Pimenov and Kljuykov (Pimenov and Kljuykov 2000a, b, c; Pimenov 2017). There has been difficulty in interpretation of diverse morphology to diagnose species and even limits of the genus. Most species of this genus occur in the Himalayas and Hengduan Mountains, and of these, four are endemic to the Hengduan Mountains (Wang and Pu 1992). In China, most species of *Physospermopsis* grow in open forests, scrubs, grasslands and alpine meadows at elevations of 2250–4800 m (Wolff 1929; Mukherjee 1982; Farille and Malla 1985; Pan and Watson 2005). *Physospermopsis* is characterized by having a long, conic taproot, ribbed stem, pinnate, rarely entire leaf blade, prominent, leaf-like bracts, variable bracteoles, minute calyx teeth, emerald young fruits, ovoid to broadly ovoid mature fruit with slightly cordate base (Wolff 1925; Pan and Watson 2005). Based on an analysis of previous research (Wang and Pu 1992; Pu and Liu 2005, 2006), *Physospermopsis* species usually possess a pericarp with wavy stria or reticulate ornamentation, prominent or inconspicuous fruit ribs, diverse carpoderms and endosperms, and pollen morphology showing a trend from rhomboidal type to rectangular type.

Previous studies on *Physospermopsis* have been extensive, including on micromorphology, anatomy and pollen morphology (Wang and Pu 1992; Pu and Liu 2005, 2006). However, previous molecular phylogenetic analyses of *Physospermopsis* have only involved a small number of taxa mostly using internal transcribed spacer (ITS) sequences (Downie et al. 2000; Calviño et al. 2006; Zhou et al. 2008, 2009; Downie et al. 2010; Valiejo-Roman et al. 2012). Phylogeny of *Physospermopsis* has been disputed with Downie et al. (2000) placing *P. kingdon-wardii* (H.Wolff) C.Norman (1938: 231) and *P. rubrinervis* (Franchet) C.Norman (1938: 231) in the *Komarovia* clade based on the materials collected from Yunnan, China. Then, Calviño et al. (2006) provisionally positioned *Physospermopsis* in the *Physospermopsis* clade based on more comprehensive maximum parsimony (MP) analyses of ITS sequences, which arose as a weakly supported sister group to the *Komarovia* clade. Later, Zhou et al. (2008) studied five species of *Physospermopsis* and concluded that *Physospermopsis* was not a monophyletic group. Zhou et al. (2008) placed *P. kingdon-wardii* and *P. rubrinervis* in the East Asia clade and referred them to *Trachydium* J.Lindley (1835: 232). Zhou et al. (2008) also concluded that *P. cuneata* H.Wolff (1929: 126) was nested in Pimpinelleae and should be close to *Pimpinella* C.Linnaeus (1753: 263), while *P. muliensis* R.H.Shan & S.L.Liou (1979: 105) and *P. shaniana* C.Y.Wu & F.T.Pu (1993: 1285) were allied within Pleurospermeae. Additionally, the East Asia clade was proposed as the synonym of *Physospermopsis* clade due to almost all of its species being primarily distributed in East Asia (Zhou et al. 2008). The following year, Zhou et al. (2009) added *P. delavayi* (the nomenclatural type of *Physospermopsis*) to their previous analyses and placed it in Pleurospermeae. Downie et al. (2010) decided that *Physospermopsis* should be placed in the *Physospermopsis* clade (East Asia clade) and was not monophyletic, but did not include the type species in their analyses potentially influencing their conclusions. Valiejo-Roman et al. (2012) conducted a molecular phylogenetic analysis of the genus *Pleurospermum* G.F.Hoffmann (Hoffmann 1814) and its related genera, including three *Physospermopsis* species.

*Physospermopsis* is a taxonomically complex genus whose generic limits with *Pleurospermum*, *Tongoloa* H.Wolff (1925: 279), and *Trachydium* are problematic (Pan and Watson 2005). Therefore, misidentification was common due to the absence of convincing morphological evidence, limitations of collected materials and examinations of type specimens. Additionally, until now there has been no comprehensive analysis using molecular phylogenetics and morphology within the one study. Therefore, we aimed to determine an accurate phylogeny of *Physospermopsis* and infrageneric relationships within *Physospermopsis* based on molecular, morphology data and combined analysis linking phylogeny and morphology. We acquired accurate data by collecting field specimens of eight *Physospermopsis* species involving 13 populations from their type localities and adjacent areas. Species were identified by field observations, validation with herbarium specimens and primary literature.

## Material and methods

### Specimen examinations, field investigations and morphology observations

The taxonomic identification of *Physospermopsis* species was by field investigations and specimen examinations from herbaria BM, BNU, CDBI, CVH, E, HITBC, ILL, K, KUN, LBG, LE, MW, NAS, NHW, P, PE, PEY, SABG, SM, SZ, UC, WU, WUK.

In the field investigations, we sampled three populations of *P. delavayi*, two populations of *P. rubrinervis*, two populations of *P. shaniana*, one population of *P. obtusiuscula* (1938: 231) and one population of *P. nana* (2000: 538) in Yunnan Province. We sampled one population of *P. kingdon-wardii* and one population of *P. obtusiuscula* in Tibet. One population of *P. alepidioides* (H.Wolff & Hand.-Mazz.) R.H.Shan (1941: 187) and one population of *P. muliensis* were sampled in Sichuan Province. All populations were collected from the type locality and adjacent regions, and the features of every species were closely matched with the types and original descriptions (de Candolle 1830; Franchet 1894; Diels 1912; Wolff 1929; Wolff et Handel-Mazzetti 1933; Shan et Liou 1979). The specific collection information are listed in Appendix 1.

Fruits, leaf segments and specimens from these eight species of *Physospermopsis* were collected in the field for morphological study. Morphological analyses of leaves and fruits based on herbarium specimens or formaldehyde-acetic acid-alcohol (FAA) preserved material were photographed by a stereomicroscope Nikon SMZ25 (Japan). The morphological data were measured using KaryoType (Altnordu et al. 2016).

## Taxon sampling

We sampled 13 populations, representing eight species of *Physospermopsis* in our phylogenetic analysis, and obtained 31 sequences of other Apiaceae species from the NCBI (Appendix 1). Based on previous research (Zhou et al. 2009), *Bupleurum krylovianum* B.K.Schischkin (1935: 2010) and *Bupleurum rockii* H. Wolff (1929: 187) were selected as the outgroup for studying the phylogenetic position of *Physospermopsis*. We chose *Pl. franchetianum* W.B.Hemsley (1892: 307) and *Pl. wrightianum* H.Boissieu (1903: 847) as the outgroup for studying interspecific relationships within *Physospermopsis*. The DNA sequences of two chloroplast loci (*rpl16*, *rps16*) and one nuclear region, the internal transcribed spacers of ribosomal DNA (ITS), were used for phylogenetic analyses. According to the research to date (Zhou et al. 2008, 2009; Downie et al. 2010; Guo et al. 2018; Panahi et al. 2018), these three markers should be sufficient to obtain the general information about relationships within the genus and its phylogenetic position within the family Apiaceae.

## DNA extraction and sequencing

The fresh leaves were collected from field specimens in Yunnan, Sichuan and Tibet, China. Voucher specimens were deposited in the Herbarium of Sichuan University (SZ). Total genomic DNA was extracted from silica-dried leaves with plant genomic DNA kit (Cwbio Biosciences, Beijing, China). The universal primers ITS4 (5'-TCC TCCGCT TAT TGA TAT GC-3') and ITS5(5'-GGA AGT AAA AGT CGT AAC AAG G-3'; White *et al.* 1990) were used to amplify the entire internal transcribed sequences. The *rpl16* intron region was amplified using primers F71(5'-GCT ATG CTT AGT GTG TGA CTC GTT G-3') and R1516 (5'-CCC TTC ATT CTT CTA TGT TG-3') (Jordan et al. 1996; Kelchner and Clark 1997). The *rps16* sequences were amplified with primers *rps16* 3'exon (5'-CCT GTA GGY TGN GCN CCY TT-3') and *rps16* 5'exon (5'-AAA CGA TGT GGN AGN AAR CA-3')(Downie and Katz-Downie 1999). PCR amplification was implemented in a 30 μL volume reaction, including 3 μL total DNA, 1.5 μL forward primer, 1.5 μL reverse primer, 15 μL 2×Taq MasterMix (Cwbio, Beijing, China), and 9 μL ddH2O. The amplification of the ITS region was obtained by initial denaturation for 3 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 70 s at 54 °C, and 90 s at 72 °C, then final extension of 10 min at 72 °C. Amplification of cpDNA intron regions was obtained by initial denaturation for 3 min at 94 °C, followed by 36 cycles of 45 s at 94 °C,70 s at 58.5 °C, and 90 s at 72 °C, then final extension of 10 min at 72 °C. All PCR products were separated using a 1.5% (w/v) agarose TAE gel and sent to Sangon (Shanghai, China) for sequencing. New sequences obtained for this study have been deposited in GenBank. GenBank accession numbers are provided in the Appendix 1.

## Data analysis

We used SegMan7 (Burland 2000) to assemble ITS and cpDNA sequences. ClustalX (Jeanmougin et al. 1998) was used to align DNA sequences with manual adjustment. We then used MEGA7 (Kumar et al. 2016) to manually adjust and obtain ITS and cpDNA datasets. Gaps were positioned to minimize nucleotide mismatches. Bayesian inference (BI) and Maximum likelihood (ML) methods were used for phylogenetic analyses, using MrBayes v3.2 (Ronquist et al. 2012) and RAxML v8.2.4 (Stamatakis 2014), respectively. Before undertaking BI analyses, MrModeltest version 2.2 (Nylander 2004) was used to determine the best model of nucleotide substitution and the GTR+G model under the Akaike Information Criterion (Akaike 1974) was selected. Bayesian analyses were performed over 20 million generations with a variant of Markov Chain Monte Carlo (MCMC) method and the trees were saved to a file every 1,000 generations. The first 20% trees were discarded as "burn-in" and the remaining 80% trees were used to build a majority-rule consensus tree based on analysis of the program Tracer v1.4 (Drummond and Rambaut 2007). ML analyses were performed using RAxML v8.2.4 with the GTR+G model and 1,000 bootstrap replicates. We constructed the BI tree with ITS data from all 44 taxa to test the systematic position of *Physospermopsis*. And we mapped some valuable morphological characteristics of Physospermopsis on phylogenetic tree, including leaves, bracts and bracteoles, ribs of fruits. The BI and ML trees were constructed for analysis of interspecific relationships within *Physospermopsis* using ITS and plastid datasets from the 13 *Physospermopsis* populations we sampled, one *Physospermopsis* species and the two *Pleurospermum* species downloaded from NCBI. Detailed information on the investigated taxa can be found in the Appendix 1.

## Results

### Morphological characteristics of Physospermopsis

Through observations in the field, the most important characteristic to identify *Physospermopsis* species was prominent bracts and bracteoles. *Physospermopsis shaniana*, *P. nana*, *P. muliensis*, *P. rubrinervis*, *P. obtusiuscula* and *P. kingdon-wardii* usually have leaf-like bracts and bracteoles (Fig. 1A3–F3). While *P. alepidioides* and *P. delavayi* possess lanceolate or oblong bracts and bracteoles with a 2–3-lobed apex and dark purple margin (Fig. 1G3, H3). Furthermore, leaf shape varies with species and can be obovate-lanceolate (e.g. *P. alepidioides*), triangular (e.g. *P. rubrinervis*), obovate-orbicular (e.g. *P. delavayi*) or linear-lanceolate (e.g. *P. nana*) segments (Fig. 1B2, D2, G2, H2). Besides, the leaves of *P. kingdon-wardii* and *P. obtusiuscula* are 2-pinnate and ovate-oblong, and have 2–6 pairs of ovate pinnae with pinnatisect margin. *Physospermopsis muliensis* and *P. shaniana* possess 3–5 pairs pinnae with pinnatifid margin, narrowly winged petioles and narrow and purple-red sheaths. Fruit morphology was recorded prior to alcohol preservation because the alcohol altered the color slightly (as is seen in photographs). The fruits of *Physospermopsis* were emerald green or chartreuse, ovoid to broadly ovoid, and typically had a slightly cordate base, a gradually narrowed and laterally flattened apex, with filiform or prominent ribs. Fruit shape and size of all *Physospermopsis* species were similar except that *P. kingdon-wardii* had fruit half the size of other species and very prominent and sinuate ribs. *Physospermopsis nana* and

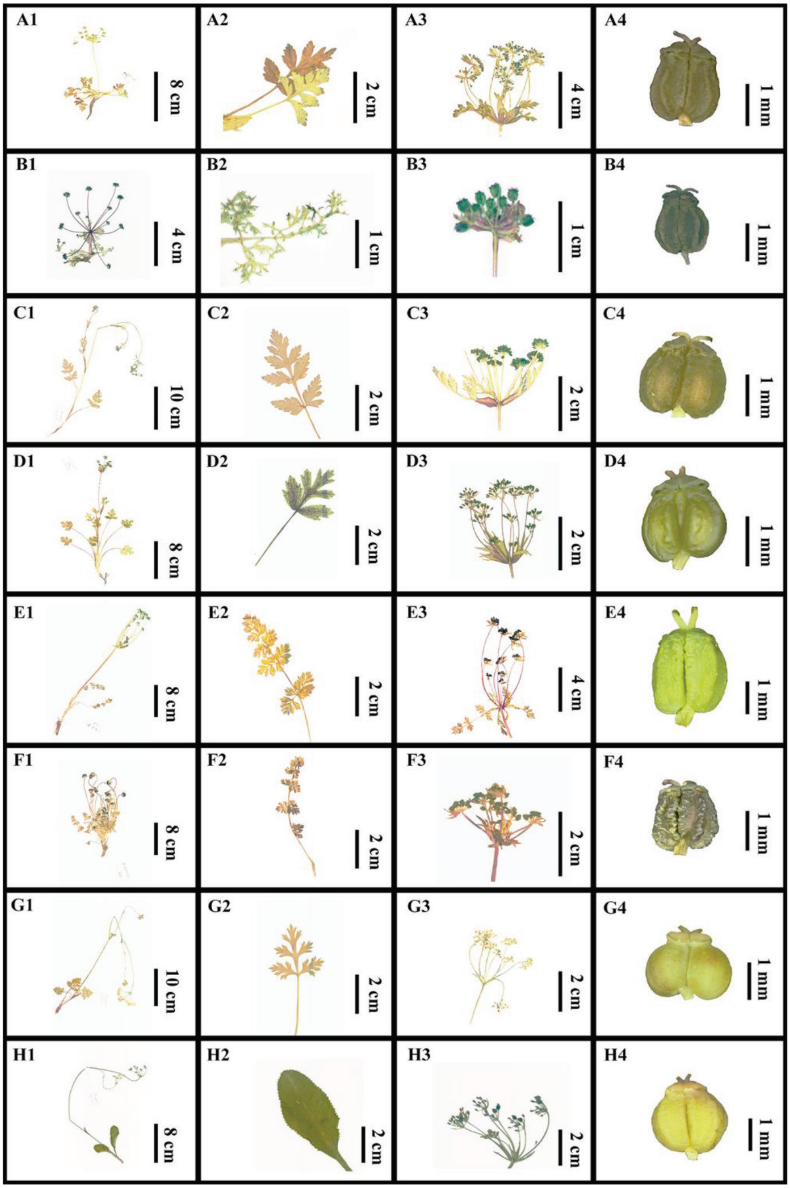

**Figure 1.** Morphological characters of *Physospermopsis* **A1–H1** habit **A2–H2** basal leaf **A3–H3** umbel **A4–H4** mericarps **A1** habit of *P. shaniana* **B1** habit of *P. nana* **C1** habit of *P. muliensis* **D1** habit of *P. rubrinervis* **E1** habit of *P. obtusiuscula* **F1** habit of *P. kingdon-wardii* **G1** habit of *P. delavayi* **H1** habit of *P. alepidioides* **A2** basal leaf of *P. shaniana* **B2** basal leaf of *P. nana* **C2** basal leaf of *P. muliensis* **D2** basal leaf of *P. rubrinervis* **E2** basal leaf of *P. obtusiuscula* **F2** basal leaf of *P. kingdon-wardii* **G2** basal leaf of *P. delavayi* **H2** basal leaf of *P. alepidioides* **A3** umbel of *P. shaniana* **B3** umbel of *P. nana* **C3** umbel of *P. muliensis* **D3** umbel of *P. rubrinervis* **E3** umbel of *P. obtusiuscula* **F3** umbel of *P. kingdon-wardii* **G3** umbel of *P. delavayi* **H3** umbel of *P. alepidioides* **A4** mericarps of *P. shaniana* **B4** mericarps of *P. nana* **C4** mericarps of *P. muliensis* **D4** mericarps of *P. rubrinervis* **E4** mericarps of *P. obtusiuscula* **F4** mericarps of *P. kingdon-wardii* **G4** mericarps of *P. delavayi* **H4** mericarps of *P. alepidioides*.

**Table 1.** The morphological characteristics of eight *Physospermopsis* species.

| Taxa | Bracts | Bracteoles | Fruits | Leaf shape | Ribs | Stems | Umbels |
|---|---|---|---|---|---|---|---|
| Physospermopsis delavayi | lanceolate or oblong | lanceolate | broadly ovoid, with obvious cordate base | winged, obovate-orbicular | filiform | branched above | 7–13 |
| *P. alepidioides* | lanceolate or oblong | ovate-lanceolate,entire | ovoid, with obscure cordate base | entire, obovate-lanceolate | prominent | branched above | 5–14 |
| *P. muliensis* | leaf-like | lanceolate, entire | broadly ovoid or ovoid | narrowly winged, pinnatifid | relatively prominent, with scattered warts | branched above, slender | 7–18 |
| *P. rubrinervis* | leaf-like | leaf-like, with purplish margin | ovoid, with slightly cordate base | triangular, with purple-red nerves | prominent | branched above, dark purple | 6–17 |
| *P. shaniana* | leaf-like | leaf-like, with purplish margin | ovoid, with slightly cordate base | narrowly winged, pinnatifid | prominent,with small warts | branched at the base, reduced | 6–15 |
| *P. obtusiuscula* | leaf-like | ovate-oblong | ovoid to broadly ovoid | ovate-oblong, pinnatisect | with narrowly winged, sinuolate | branched at the base, dark purple-green | 5–20 |
| *P. kingdon-wardii* | leaf-like | leaf-like, with purplish margin | broadly ovoid | ovate-oblong, pinnatisect | prominent, sinuate, with sparse minute warts | reduced, often acaulescent | 5–11 |
| *P. nana* | leaf-like | leaf-like, with membranous margin | broadly ovoid | linear-lanceolate | prominent, narrowly sinuolate-winged | reduced, slender | 4–13 |

*P. muliensis* fruits had relatively prominent and filiform ribs, but *P. muliensis* fruits had scattered warts especially on the ribs and *P. nana* had smaller fruit. *Physospermopsis obtusiuscula* fruits were ovoid with narrowly winged and sinuolate ribs. The fruit of *P. delavayi* had an obvious cordate base, and filiform and less prominent ribs. *Physospermopsis alepidioides*, *P. rubrinervis* and *P. shaniana* had ovoid, verucose fruits with prominent ribs, but *P. alepidioides* did not have a cordate base, while the other two species had a slightly cordate base. The fruit of *P. shaniana* had many small warts distinguishing it from *P. rubrinervis*. For easy reading and comparison, the main morphological characteristics were listed in Table 1.

## Phylogenetic analyses

Through comprehensive sampling, the ITS analyses indicated that the 13 populations of *Physospermopsis* we sampled and *P. muktinathensis* M.A.Farille & S.B.Malla (1985: 512) formed an individual clade. *Physospermopsis* was confirmed to be a monophyletic group and nested in Pleurospermeae. *Trachydium roylei* Lindl. (1835: 232) and *Pl. wilsonii* H.Boissieu (1906: 433) were the closest relatives of *Physospermopsis* (Fig. 2).

The ITS dataset tree topologies generated from BI and ML analyses were consistent. Therefore, only the BI tree with posterior probabilities (PP, 0–1) and bootstrap support values (BS, 0–100%) is illustrated in Fig. 3A. The first to differentiate from *Physospermopsis* was *P. muktinathensis*, which is distributed in Nepal. Three populations of *P. delavayi* and one of *P. alepidioides* united as a strongly supported (BI–PP = 1; ML–BS = 100%) group. *Physospermopsis obtusiuscula* was supported as a sister group to *P. kingdon-wardii* (BI–PP = 1; ML–BS = 100%). *Physospermopsis rubrinervis*, *P. muliensis*, *P. nana* and *P. shaniana* were allied in all trees (BI–PP = 1; ML–BS = 100%). However, clear interspecific relationships between *P. rubrinervis*, *P. muliensis*, *P. nana* and *P. shaniana* were not strongly supported by ML or BI analyses.

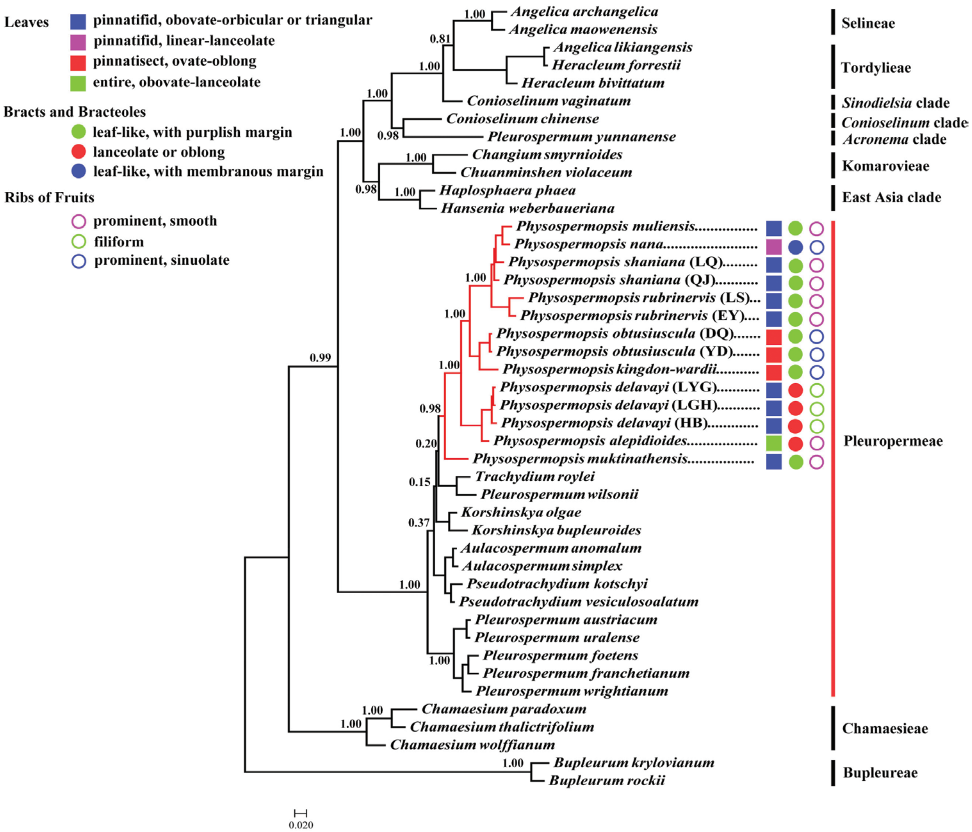

**Figure 2.** Bayesian tree inferred from the analysis of the 44 samples of ITS data. Branch lengths are proportional to the amount of character changes, scale = 0.02 substitutions per character. The tree is rooted with *Bupleurum*. The names of the clades identified are those of Zhou et al. (2008, 2009).

The cpDNA dataset tree topologies inferred by BI and ML analyses were consistent (Fig. 3B). However, results of a partition homogeneity test for the ITS and cpDNA datasets indicated that these genomes provide significantly different phylogenetic estimates. The taxa involved in this conflict are highlighted in Fig. 3. There was no chloroplast data for *P. muktinathensis*. The first to differentiate were *P. obtusiuscula* and *P. kingdon-wardii* (BI–PP = 1; ML–BS = 99%). The relationships of the three *P. delavayi* populations differed from the ITS dataset tree topology, although this cpDNA dataset relationship was not strongly supported. The cpDNA dataset tree topologies indicated that LYG population was closer to the HB population (BI–PP = 0.33; ML–BS = 47%), while LYG was closer to LGH in the ITS dataset tree topologies (BI–PP = 0.34; ML–BS = 70%). Additionally, the relationships between *P. rubrinervis*, *P. muliensis*, *P. nana* were not consistent with the ITS tree, where *P. nana* allied with *P. rubrinervis* in the cpDNA tree (BI–PP = 1; ML–BS = 72%), whereas *P. nana* allied with *P. muliensis* in the ITS tree (BI–PP = 1; ML–BS = 94%).

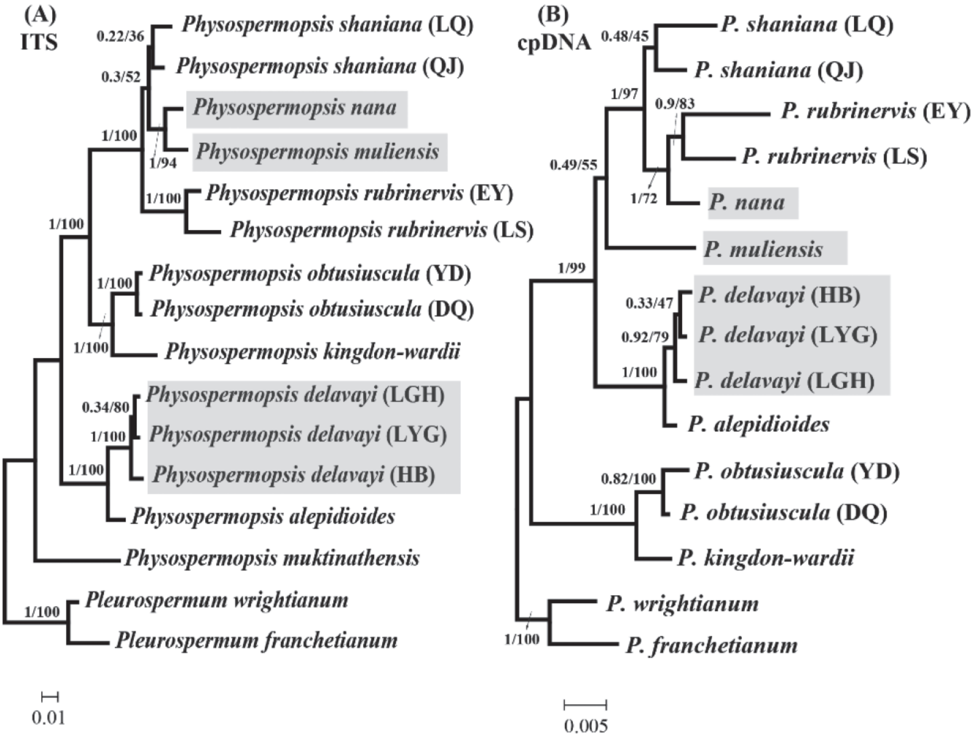

**Figure 3.** Bayesian trees of *Physospermopsis* and its related genus inferred from ITS (**A**) and plastid *rpl16+rps16* (**B**). Values on the branches indicate their support (Bayesian posterior probability/ Maximum-likelihood bootstrap). Branch lengths are proportional to the amount of character changes, scale = 0.01 (**A**), 0.005 (**B**) substitutions per character.

# Discussion

## The Phylogenetic position of *Physospermopsis* and relationship between *Physospermopsis* and *Pleurospermum*

*Physospermopsis* is monophyletic. The reasons for previous designations as a polyphyletic genus were likely attributable to the misidentification of several species (e.g. *P. rubrinervis*, *P. kingdon-wardii*, *P. cuneata*). Besides, *P. cuneata* is a poorly known species and unusual within the genus for its lack of conspicuous bracts and bracteoles, and therefore the phylogenetic placement of it is highly controversial. However, the most recent consensus is that *P. cuneata* should not be placed in *Physospermopsis* (Zhou et al. 2008; Zhou et al. 2009; Pimenov 2017). So previous molecular studies only involved five *physospermopsis* species which were widely accepted; we added another three *physospermopsis* species in this study, including *P. alepidioides*, *P. obtusiuscula*, and *P. nana*. Evidence obtained through more precise checking of generic type, infrageneric types and extensive herbarium specimens, literature and field investigations, analyzing morphological characters, and ITS and cpDNA evidence. This comprehensiveness allows us to be confident that *Physospermopsis* is monophyletic and nested in Pleurospermeae. In addition, we propose that the *Physospermopsis* clade should be replaced by the East Asia Clade.

The molecular results indicated that *Physospermopsis* is closest to *Pleurospermum*. Morphologically, *Pleurospermum* usually possess numerous bracts and bracteoles with white scarious margins, conspicuous or obsolete calyx teeth, white or purple-red petals with clawed base and narrow apex, prominent, acute ribs (Pan and Watson 2005). However, we found that *Physospermopsis* differed from *Pleurospermum* by less prominent and even inconspicuous fruit ribs, and the bracts and bracteoles did not have white scarious margins, resulting in an obvious, diagnostic boundary between *Pleurospermum* and *Physospermopsis*. The closeness of the two genera is also evidenced in pollen morphology. Wang and Pu (1992) found *P. alepidioides* and *P. muliensis* pollen to be rhomboidal and similar to several *Pleurospermum* species whereas other *Physospermopsis* species (*P. rubrinervis* and *P. delavayi*) have more advanced rectangular types. In addition, Pan and Watson (2005) identified several *Physospermopsis* species (e.g. *P. obtusiuscula*) with morphological similarities to *Pleurospermum* species, including having long fruit ribs and bulgy fruit walls, while other species had flattening of fruit and reduced wall thickness. Consequently, *Physospermopsis* is sister to *Pleurospermum*.

## Interspecific relationships within *Physospermopsis*

The morphological characteristics mapped on the phylogenetic tree indicated that most closely related species have similar morphological characteristics. For example, *P. rubrinervis*, *P. muliensis* and *P. shaniana* are highly consistent on leaves, bracts and bracteoles, ribs on fruits (Fig. 2). Similarly, these species are the geographically sympatric species (Fig. 4). Resolution of the relationships between these species will only be achieved through continued studies, which may be difficult due to their geographic and morphological similarities. However, we can learn that *P. nana*, *P. rubrinervis*, *P. muliensis* and *P. shaniana* are the more advanced species in *Physospermopsis*. The morphological characters of *P. nana* are the most particular; these might be caused by hybridization with *Pleurospermum* species.

The interspecific relationships between certain species within *Physospermopsis* are evident based on the consistencies between ITS and cpDNA trees. For instance, *P. alepidioides* showed a close affinity to *P. delavayi* in phylogenetic tree, and they have similar bracts and bracteoles (entire or 2–3-lobed at apex, with dark purple margin) (Figs 1, 2). However, differing leaf shapes can be used to easily distinguish these two species because *P. alepidioides* has an undivided leaf with sparsely serrated margin (Fig. 1H2) and *P. delavayi* has a pinnate leaf (Fig. 1G2). *Physospermopsis kingdon-wardii* is sister to *P. obtusiuscula*, which is consistent with their geographic closeness. *Physospermopsis kingdon-wardii* appears more morphologically similar to *P. obtusiuscula* (including the leaves, bracts and bracteoles), but differs in its reduced stem, small stature and small fruits with prominent and sinuate ribs (Fig. 1).

The topologies of the ITS and cpDNA trees differed in the positioning of *P. delavayi*, *P. muliensis* and *P. nana* (Fig. 3). This inconsistency between nrDNA ITS and cpDNA data has been reported in some studies of Apiaceae (Lee and Downie 2006; Zhou et al.

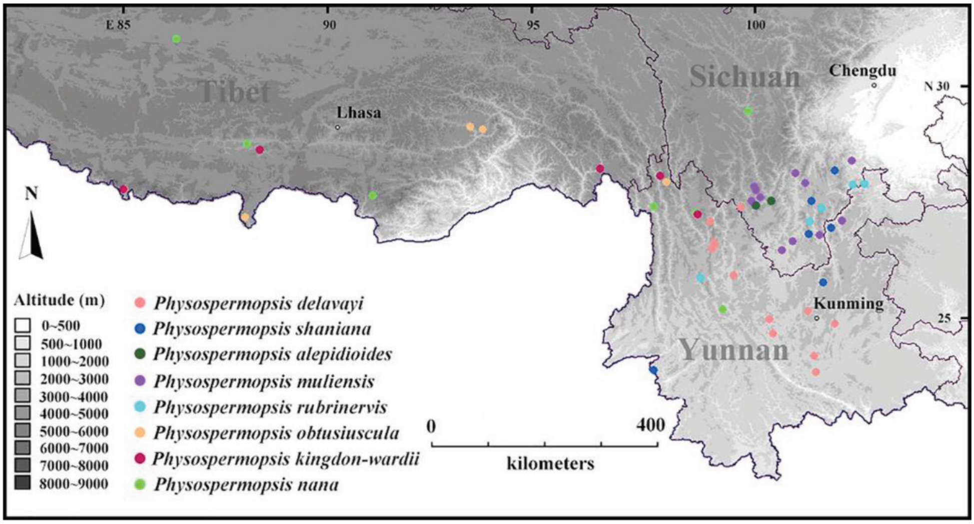

**Figure 4.** Geographic distribution of the eight Chinese *Physospermopsis* species in China. The altitude, scale, name of provinces and provincial capitals are also showed on the map.

2008, 2009; Spalik et al. 2009; Bone et al. 2011; Yi et al. 2015; Panahi et al. 2018). This difference generally has been caused by incomplete lineage sorting, hybridization, homoplastic substitutions and introgression. Since we did not sample by lineage and execute gene flow analysis, what caused the inconsistency cannot be determined. Previous studies have indicated that Pleurospermeae occupies a relative position in the base of the Apioideae (Zhou et al. 2008, 2009; Downie et al. 2010), the differentiation time should be earlier. Thus, for *Physospermopsis*, we infer the more effective reason for the inconsistency between nrDNA ITS and cpDNA data is hybridization. A further study based on widely sampling and deeper analysisis needed. However, several diagnostic characteristics can be utilized in the field and laboratory to separate them. *P. rubrinervis* can easily be recognized by purple-red nerves on the leaves, bracts and bracteoles with purple-red margin (Fig. 1). *Physospermopsis muliensis* possesses a slender, branched stem and narrowly winged basal petioles with narrow sheaths (Fig. 1). *Physospermopsis nana* has bracts and bracteoles with white scarious margins and linear-lanceolate segments with membranous-margined sheaths (Fig. 1). The stem of *P. shaniana* was reduced and branched at the base, and had prominent bracts 1–2-pinnate with developed, broad sheaths (Fig. 1).

## Taxonomy

### Chinese *Physospermopsis* species

#### ***Physospermopsis* H.Wolff (1925: 276)**

**Type.** *Physospermopsis delavayi* H.Wolff (1925: 278)

## Key to the Chinese *Physospermopsis* species

| | | |
|---|---|---|
| 1 | Leaves entire, margin sparsely serrate | ***P. alepidioides*** |
| – | Leaves pinnate or pinnatifid | 2 |
| 2 | Stems reduced, sometimes acaulescent | 3 |
| – | Stems developed | 5 |
| 3 | Bracteoles margin membranous | ***P. nana*** |
| – | Bracteoles margin purplish | 4 |
| 4 | Fruits ovoid, with slightly cordate base; ribs prominent, with small warts | ***P. shaniana*** |
| – | Fruits broadly ovoid; ribs prominent and sinuate, with sparse minute warts | ***P. kingdon-wardii*** |
| 5 | Nerves of leaves purple-red | ***P. rubrinervis*** |
| – | Nerves of leaves green | 6 |
| 6 | Bracts lanceolate or oblong; ribs filiform | ***P. delavayi*** |
| – | Bracts leaf-like; ribs prominent | 7 |
| 7 | Bracteoles ovate-lanceolate,entire; ribs with scattered warts | ***P. muliensis*** |
| – | Bracteoles ovate-oblong; ribs with narrowly winged, sinuolate | ***P. obtusiuscula*** |

### 1. *Physospermopsis alepidioides* (H.Wolff et Hand.-Mazz.) R.H.Shan, 1941: 187

≡ *Haploseseli alepidioides* H.Wolff et Hand.-Mazz., 1933: 722

**Type.** China. Sichuan: Yanyuan County, 2700–2800 m, 7 Oct 1914, *Handel-Mazzetti 5562* (holotype: WU [WU0060774]).

**Diagnostic characters.** *Physospermopsis alepidioides* usually possesses an entire leaf blade with a sparsely serrated margin. The shape of the entire leaf segment is an obvious diagnostic characteristic to distinguish it from other *Physospermopsis* species. The stem of it is velutinous.

**Distribution.** Endemic to China, Sichuan (Fig. 4).

**Habitat.** *Physospermopsis alepidioides* usually occurs in open forests and grasslands.

**Additional specimens examined. China. Sichuan Province:** Muli County, Hetaowan, 2300 m, 8 Aug 2019, *X.R.Xu XXR2019080801* (SZ); Muli County, Hetaowan, 2250 m, 23 Dec 1982, *Y.B.Yang & Y.L.Cao 400* (CDBI); Muli County, Liziping, 21 Sep 2011, *X.G.Ma m11092101* (SZ); unknown locality, 2650 m, 19 Jul 1983, *Anonymous 22* (HITBC); Yanyuan County, Mt. Huolu, 3950 m, 22 Jul 1983, *Anonymous 25* (HITBC).

### 2. *Physospermopsis rubrinervis* (A.R.Franchet) C.Norman, 1938: 231

≡ *Trachydium rubrinerve* A.R.Franchet, 1894: 112
≡ *Pleurospermum rubrinerve* (A.R.Franchet) M.Hiroe, 1979: 747

**Type.** China. Yunnan: Eryuan County, Mt. Luoping, 3200 m, 31 Aug 1888, *Delavay 3235* (holotype: P [P00245453]; lectotype, designated by Pimenov 2017, pg. 188: P [P00245453] ; isolectotypes: K [K001235378], P [P00245454, P00834665]).

**Diagnostic characters.** *Physospermopsis rubrinervis* usually possesses dark purple, sparsely branching stems. The basal blade is ovate to broadly ovate in outline, having almost purple-red nerves.

**Distribution in China.** Sichuan, Yunnan (Fig. 4).

**Distribution outside China.** India, Nepal.

**Habitat.** This species grows in the forest edge or rhododendron shrubs at an elevation of 2800–4800 m.

**Additional specimens examined. China. Sichuan Province:** Yanbian County, Yankou xiang, 3150 m, 20 Sep 2002, *X.F.Gao*, *Y.L.Peng & G.Sun 3753* (PE); Meigu County, Ligou xiang, 3600 m, 5 Aug 1959, *1591* (SM); Butuo County, Wuke pasture, 3500 m, 1 Jul 1976, *Vegetation expedition 13827* (CDBI); Dukou County, Mt. Dahei, 1400 m, 18 Jun 1983, *Qinghai-Tibet Expedition 11231* (KUN); Puge County, 9 Aug 1960, *Anonymous 25099* (SM); **Yunnan Province:** Eryuan County, Mt. Luoping, 3200 m, 17 Aug 2019, *X.R.Xu XXR2019081701* (SZ); Lushui County, 3000 m, 18 Oct 2019, *X.L.Guo G19101802* (SZ); unknown locality, 21 Sep 1959, *S.G.Wu 2715* (KUN).

### 3. *Physospermopsis kingdon-wardii* (H.Wolff) C.Norman, 1938: 231

≡ *Trachydium kingdon-wardii* H.Wolff, 1929: 124
≡ *Pleurospermum kingdon-wardii* (H.Wolff) M.Hiroe,1979: 747

**Type.** China. Yunnan: A-tun-tsi, screes, turf, 14000 ft. (ca. 4267 m), 7 Aug 1913, *Kingdon-Ward 992* (lectotype: E [E00000221]).

**Diagnostic characters.** *P. kingdon-wardii* is similar to *P. obtusiuscula* in shape of basal leaves, but the stem of *P. kingdon-wardii* is reduced. The fruits are smaller than other species, and the immature fruits sometimes have sparse minute warts. Additionally, the ribs are prominent, often sinuate.

**Distribution in China.** Tibet, Yunnan (Fig. 4).

**Distribution outside China.** Bhutan, Nepal, Sikkim.

**Habitat.** *Physospermopsis kingdon-wardii* usually grows in alpine meadows or scrubs at about 3900 m elevation.

**Additional specimens examined. China. Tibet Province:** Nyalam County, 4000 m, 24 Aug 2019, *X.L.Guo G19082407* (SZ); Bainang County, 4580 m, 24 Aug 1988, *Anonymous 8* (CDBI); Zayü County, 4180 m, 27 Sep 1982, *Qinghai-Tibet Expedition 10772* (PE); Zayü County, 4370 m, 31 Aug 2003, *X.F.Gao*, *W.G.Tu*, *H.He & Y.K.Qiao 6745* (CDBI); **Yunnan Province:** Dêqên County, 4300 m, 28 Sep 1981, *L.R.Xu 129* (WUK); Dêqên County, 3900 m, 18 Aug 1940, *K.M.Feng 6746* (PE); Dêqên County, 4500 m, 23 Sep 1986, *H.Sun & Z.G.Qian 751* (KUN); Zhongdian County, 4300 m, 2 Oct 1986, *H.Sun & Z.G.Qian 0980* (KUN).

### 4. ***Physospermopsis obtusiuscula*** **(DC.) C.Norman, 1938: 231**

≡ *Hymenolaena obtusiuscula* DC., 1830: 246
≡ *Trachydium obtusiusculum* (DC.) C.B.Clarke, 1879: 673
≡ *Pleurospermum obtusiusculum* (DC.) M.Hiroe, 1979: 741
≡ *Aulacospermum obtusiusculum* (DC.) A.R.Naqshi, U.Dhar et P.N.Kachroo, 1995: 107

**Type.** Nepal. "Ad Gossain-Than Nepalensium, Wallich [543]" (lectotype: G-DC; isolectotypes: BM [BM000622303, BM000944782], K [K000697363], K-WALLICH, LE).

**Diagnostic characters.** *Physospermopsis obtusiuscula* sometimes is flushed. The stems are dark purple-green, simple, and occasionally branched at the base. The fruit ribs are narrowly winged and sinuolate, which is a unique character in *Physospermopsis*.

**Distribution in China.** Sichuan, Tibet, Yunnan (Fig. 4).

**Distribution outside China.** Bhutan, India, Nepal, Sikkim.

**Habitat.** *Physospermopsis obtusiuscula* grows in shrubs or grassland at an elevation of ca. 4000 m.

**Additional specimens examined. China. Sichuan Province:** Xiangcheng County, 3900 m, 9 Aug 1981, *Qinghai-Tibet Expedition 3986* (PE); Xiangcheng County, 9 Aug 1981, *Qinghai-Tibet Expedition 3942* (PE); **Tibet Province:** Yadong County, 3500 m, 20 Aug 2019, *X.L.Guo G19082009* (SZ); Nyingchi County, 3400 m, 8 Aug 1983, *B.S.Li & S.Z.Cheng 6199* (PE); Nyingchi County, Mt. Shergyla, 3346 m, 13 Oct 2009, *J.Luo*, *S.L.Wang & G.Y.Wang LiuJQ-09XZ-388* (KUN); Nyingchi County, Mt. Shergyla, 3346 m, 13 Oct 2009, *J.Luo*, *S.L.Wang & G.Y.Wang LuoJian-ZX-0938* (PE); Yadong County, 3980 m, 14 Sep 1974, *Qinghai-Tibet Expedition 74-2505* (PE); Yadong County, 4000 m, 12 Sep 1974, *Qinghai-Tibet Expedition 2416* (PE); Nyalam County, 3800 m, 2 Sep 1972, *1736* (PE); Zayü County, 4300 m, 26 Sep 1982, *Qinghai-Tibet Expedition 10635* (PE); **Yunnan Province:** Dêqên County, Baimaxueshan, 4100 m, 15 Aug 2019, *X.R.Xu XXR2019081502* (SZ); Gongshan County, Dulongjiang, 2900 m, 15 Sep 1938, *T.T.Yü 20274* (PE); Gongshan County, Dulongjiang, 3800 m, 9 Aug 1938, *T.T.Yü 19829* (PE).

### 5. ***Physospermopsis muliensis*** **R.H.Shan et S.L.Liou, 1979: 105**

**Type.** China. Sichuan: Muli County, 4000 m, 20 Oct 1937, *T.T.Yu 14579* (holotype: PE [P01432306]).

**Diagnostic characters.** *Physospermopsis muliensis* usually possesses branching stems, ovate-oblong leaf blades, narrow sheaths, leaf-like bracts, lanceolate bracteoles, and ovoid fruits with filiform ribs with sparse scattered warts. Basal and lower petioles are narrowly winged.

**Distribution.** Endemic to China, Sichuan (Fig. 4).

**Habitat.** *Physospermopsis muliensis* usually grows in wet grasslands at 2500–4100 m elevation.

**Additional specimens examined.** **China. Sichuan Province:** Muli County, Kangwuliangzi, 3800 m, 9 Aug 2019, *X.R.Xu XXR2019080903* (SZ); Muli County, 3900 m, 20 Oct 1981, *L.R.Xu 0187* (WUK); unknown locality, 3600 m, 29 November 2005, *Anonymous 416* (PE); unknown locality, 2580 m, 28 November 2005, *Anonymous 418* (PE); unknown locality, 3650 m, 29 November 2005, *Anonymous 419* (PE); Muli County, 3800 m, 21 Sep 1955, *Anonymous 267* (PE); Muli County, 3620 m, 15 Oct 1982, *Y.B.Yang & G.Yao 174* (CDBI); Muli County, 3200 m, 3 Sep 1978, *Q.S.Zhao*, *K.H.Mou & Y.B.Yang 8395* (CDBI); Muli County, 3650 m, 25 Oct 1986, *Y.J.Li et al. 987* (CDBI); Muli County, 3150 m, 25 Oct 1982, *Vegetation expedition 29926* (CDBI); Muli County, 3900 m, 22 Aug 1983, *39* (HITBC); Dukou County, 21 Sep 1978, *412* (SM); Jinyang County, Mt. Shizi, 4000 m, 18 Aug 1978, *683* (SM); Mianning County, Juexingou, 3000 m, 11 Oct 1978, *Mianning expedition 667* (SM); Ningnan County, 3700 m, 24 Aug 1978, *Ningnan expedition 547* (SM); Dukou County, Yanbian, 28 Sep 1978, *Anonymous 435* (SM); Meigu County, 3700 m, 18 Aug 1979, *Anonymous 594* (SM); Yanbian County, 28 Sep 1978, *Anonymous 475* (SM); Zhaojue County, 4040 m, 13 Jul 1979, *Plants census expedition 179* (SM); Xide County, 29 Jun 1979, *Anonymous 495* (SM); Mabian County, 3400 m, 1 Jul 1978, *Mabian expedition 798* (SM); Muli County, 3900 m, 2 Aug 1978, *Muli expedition 577* (SM); Muli County, Changhaizi, 3669 m, 7 Oct 2009, *Z.L.Nie*, *Y.Meng & T.Deng SunH-07ZX-2330* (KUN).

### 6. *Physospermopsis shaniana* Z.Y.Wu et F.D.Pu, 1993: 1285

≡ *Trachydium forrestii* Diels, 1912: 291
≡ *Physospermopsis forrestii* (Diels) C.Norman, 1938: 231
≡ *Pleurospermum forrestii* (Diels) M.Hiroe, 1958: 123

**Type.** China. Yunnan: Lijiang range, shady, grassy openings in pine forests on the eastern flank, Aug 1906, *Forrest 2855* (lectotype, designated by Pimenov 2017, pg. 188: E [E00000219]; isolectotype: P [P00245432]).

**Diagnostic characters.** *Physospermopsis shaniana* usually possesses 2-pinnate/pinnatifid, ovate-oblong leaf blades, broad sheaths, broadly ovoid fruits, white petals, and leaf-like, 2-pinnate bracts. The pinnae are subsessile with pinnatifid margin. The stems of *P. shaniana* are reduced and branched at the base. The branches are longer than the main stem.

**Distribution in China.** Sichuan, Tibet, Yunnan (Fig. 4).

**Distribution outside China.** Myanmar.

**Habitat.** *Physospermopsis shaniana* usually grows in pasture and grassy slopes.

**Additional specimens examined.** **China. Sichuan Province:** Meigu County, Kongmingzhai, 3600 m, 5 Aug 1959, *Anonymous 1591* (KUN); Zhaojue County, Jiefanggou, 3200 m, 9 Jul 1976, *Vegetation expedition 12928* (PE); **Yunnan Province:** Qiaojia County, Yaoshan, 3200 m, 14 Jul 2019, *X.R.Xu XXR2019071404* (SZ); Luquan County, Jiaozixueshan, 3700 m, 17 Jul 2019, *X.R.Xu XXR2019071701* (SZ); Dongchuan County, Huizedahai, 3400 m, 30 Jul 1964, *Northeast Yunnan Expedition 434* (KUN); Qiaojia

County, *G.M.Yang SCSB-W-1237* (KUN); Zhenkang County, 8 Jul 1938, *T.T.Yu 17120* (PE); Zhenkang County, snow range, 3450 m, 4 Aug 1938, *T.T.Yu 17174* (PE); Dongchuan County, Mubanghai, 3360 m, 16 Aug 1964, *Northeast Yunnan Expedition 822* (LBG,KUN); Zhaotong County, 24Gang, 2300 m, 10 Aug 1974, *Anonymous 234* (KUN); Qiaojia County, Yaoshan, 3100 m, 16 Jul 1973, *B.X.Sun 1017* (KUN).

### 7. *Physospermopsis delavayi* (A.R.Franchet) H.Wolff, 1925: 278

≡ *Arracacia delavayi* A.R.Franchet, 1894: 115
≡ *Pleurospermum delavayi* (A.R.Franchet) M.Hiroe, 1958: 120

**Type.** China. Yunnan: Mosuoying, Yangyushan, 15 Sep 1885, *Delavay 2017* (lectotype, designated by Pimenov and Kljuykov 2000c, pg. 537: P [P00245424]).

**Diagnostic characters.** *P. delavayi* usually possesses a conspicuously winged rachis and yellow-green, round fruits. The bracts are usually smaller than other species of *Physospermopsis*. The basal leaves are obovate to obovate-orbicular with incised-serrate or lobed margin or cuneate with partite margin.

**Distribution.** Endemic to China, Hunan, Sichuan, Yunnan. (Fig. 4).

**Habitat.** *Physospermopsis delavayi* prefers to grow in the pine forest or open grasslands.

**Additional specimens examined. China. Sichuan Province:** Yanyuan County, Lugu Lake, 3200 m, 11 Aug 2019, *X.R.Xu XXR2019081102* (SZ); **Yunnan Province:** Lijiang County, Mt. Yulong, 3000 m, 13 Aug 2019, *X.R.Xu XXR2019081305* (SZ); Shangri-La, 3200 m, 18 Jul 2018, *X.L.Guo G18071802* (SZ); Lijiang County, Ganhaizi, 3100 m, 14 Feb 1968, *Anonymous s.n.* (HITBC); Lijiang County, Baishuihe, 2980 m, 4 Aug 1962, *Anonymous s.n.* (HITBC); Shuangbai County, Tuodian, 6 Oct 1958, *S.Q.Huang 0217* (LBG); Chuxiong County, Baomanjie, 19 Sep 1958, *S.Q.Huang 035* (LBG); Jianshui County, Yangjieba, 17 Mar 1941, *S.E.Liu 018312* (PE); Eryuan County, Chaijiaying, Mt. Longtou, 21 Jul 1929, *R.C.Qin 23338* (PE); unknown locality, *Anonymous 3365* (PE); unknown locality, *M.Chen 2460* (KUN); *W.R.He 2381* (KUN); Fumin County, Mt. Laoqing, 2300 m, 19 Oct1964, *B.Y.Qiu 596086* (KUN); Yiliang County, Qixingcun, 27 Aug 1975, *B.Y.Qiu 60727* (KUN); Xicheng County, Qingshuihe, 1900 m, 12 Oct1982, *B.T.Yue 2088* (KUN); Shangri-La County, Habacun, 2800 m, 3 Aug 1962, *A.L.Zhang 100750* (KUN); Lijiang County, Mt. Yulong, Baishuihe, 3000 m, 11 Jul 1962, *A.L.Zhang*, *S.W.Yu 100919* (KUN); Lijiang County, Yuhucun, 29 Aug 2010, *Z.X.Wang WZX2010082911* (SZ); Lijiang County, Yuhucun, 31 Aug 2010, *Z.X.Zhang WZX2010083102* (SZ); unknown locality, 29 Aug 2010, *P.Gao*, *S.Liu 10829-6* (SZ); unknown locality, 17 Sep 1938, *12366* (PEY); 22 Sep 1919, *K.K.TSOONG 387* (PEY).

### 8. *Physospermopsis nana* (A.R.Franchet) M.G.Pimenov et E.V.Kljuykov, 2000c: 538

= *Pleurospermum nanum* A.R.Franchet, 1894: 140. Type: CHINA. Yunnan: Dali County, Mt. Cang, 25 Sep 1884, *Delavay 197* (syntypes: P [P00834544, P00834545]).

**Type.** China. Yunnan: Mt. Cang, 4000 m, 30 Aug 1889, *Delavay 4066* (lectotype, designated by Pimenov and Kljuykov 2000c, pg. 538: P [P00834546]; isolectotype, designated by Pimenov and Kljuykov 2000c, pg. 538: P [P00834547]).

**Diagnostic characters.** *Physospermopsis nana* usually possesses reduced stem, membranous-margined sheaths, and leaf-like bracts. The bracteoles are pale green with whitish margin in lower half. The ultimate segments are linear-lanceolate. The characters mentioned above are sufficient to distinguish it from other *Physospermopsis* species.

**Distribution.** Endemic to China, Sichuan, Tibet, Yunnan. (Fig. 4).

**Habitat.** *Physospermopsis nana* usually grows on marshy meadows, under shrubs.

**Additional specimens examined. China. Sichuan Province:** Yajiang County, 3800 m, 9 Aug 1979, *Yajiangdui 293* (SM); Muli County, Sanqu, 3600 m, 13 Sep 1983, *Qinghai-Tibet Expedition 14043* (KUN); **Tibet Province:** Cona County, 4561 m, 10 Aug 2015, *L.Wei & J.C.Hao 15544* (BNU); Lahsa County, 5200 m, 3 Sep 1965, *G.C.Xia & T.K.Mi 2610* (KUN); Gê'gyai County, 5200 m, 21 Aug 1976, *Qinghai-Tibet Expedition 8710* (KUN); Lahsa County, 6000 m, 1 Sep 1965, *Y.T.Zhang & K.Y.Lang 2412* (KUN); Gê'gyai County, 5185 m, 9 Sep 2017, *Y.He BNU2017XZ325* (BNU); Shigatse, 4645 m, 23 Aug 2017, *Y.He & D.H.Liu BNU2017XZ064* (BNU); Gar County, 4360 m, 31 Aug 2008, *J.H.Chen et al YangYP-Q-0122* (KUN). **Yunnan Province:** Lanping County, 4200 m, 18 Oct 2019, *X.L.Guo G19101802* (SZ); Lijiang County, Mt. Yulong, 4400 m, 18 Aug 1976, *Y.Q.He 049* (WUK); Dêqên County, Baima snow range, 4400 m, 14 Jul 1981, *Qinghai-Tibet Expedition 2774* (HITBC); Gongshan County, Mt. Nanwan, 3400 m, 22 Sep 1997, *9604* (KUN); Zhongdian County, 4190 m, 2 Oct 2005, *Z.D.Fang et al PL-130* (SABG); Lijiang County, Yuhu, 4000 m, 8 Sep 1955, *G.M.Feng 21475* (KUN); Lijiang County, 4100 m, 31 Aug 1963, *C.Z.Bao 20229* (KUN); Zhongdian County, 4200 m, 2 Aug 1986, *H.Sun & Z.G.Qian 991* (KUN); Lijiang County, snow range, 15 Sep 1940, *R.C.Qin 31043* (KUN); Zhongdian County, Mt. Haba, 4000 m, 31 Aug 1962, *Zhongdian Expedition 1687* (KUN); Zhongdian County, Haba snow range, 26 Aug 1939, *G.M.Feng 2215* (KUN); Lijiang County, Yulong, 4000 m, 31 Aug 1963, *C.Z.Bao 20226* (KUN); Lijiang County, 2600 m, 27 Jul 1937, *D.J.Yu 15374* (KUN); Lijiang County, Mt. Yulong, 3200 m, 26 Aug 1961, *R.L.Xiong & Y.F.Qi 612715* (KUN); Dali County, 20 Aug 1945, *H.C.Wang 4507* (KUN); unknown locality, 1963, *J.S.Yang 2374* (KUN); Zhongdian County, 3400 m, 28 Jun 2009, *Z.D.Fang G-297* (SABG); Dali County, Mt. Cang, 3460 m, 15 Oct 1990, *CLD-90* (PE).

## Conclusion

*Physospermopsis* is monophyletic and nested in Pleurospermeae, sister to *Pleurospermum*. Although the interspecific relationships within *Physospermopsis* were not well resolved and the positioning of species was unclear, the relationships of *P. alepidioides* and *P. delavayi*, *P. kingdon-wardii* and *P. obtusiuscula* are close. Diagnostic characteristics for distinguishing the species in the field and laboratory are provided for necessary morphological and molecular research in future *Physospermopsis* phylogenetic studies.

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## References

Akaike H (1974) A new look at the statistical model identification. IEEE Transactions on Automatic Control 19(6): 716–723. https://doi.org/10.1109/TAC.1974.1100705

Altnordu F, Peruzzi L, Yu Y, He XJ (2016) A tool for the analysis of chromosomes: KaryoType. Taxon 65(3): 586–592. https://doi.org/10.12705/653.9

Boissieu H (1903) *Pleurospermum* Hoffm. Bulletin de l'Herbier Boissier, sér 2 3(10): 847–848.

Boissieu H (1906) *Pleurospermum* Hoffm. Bulletin de la Société Botanique de France 53: e433. https://doi.org/10.1080/00378941.1906.10831901

Bone TS, Downie SR, Affolter JM, Spalik K (2011) A phylogenetic and biogeographic study of the genus *Lilaeopsis* (Apiaceae tribe Oenantheae). Systematic Botany 36(3): 789–805. https://doi.org/10.1600/036364411X583745

Burland TG (2000) DNASTAR's Lasergene sequence analysis software. Methods in Molecular Biology (Clifton, N.J.) 132: 71–91. https://doi.org/10.1385/1-59259-192-2:71

Calviño CI, Tilney PM, Wyk BEV, Downie SR (2006) A molecular phylogenetic study of southern African Apiaceae. American Journal of Botany 93(12): 1828–1847. https://doi.org/10.3732/ajb.93.12.1828

Clarke CB (1879) Umbelliferae. In: Hooker JD (Ed.) The Flora of British India. L Reeve & Co, London, 673 pp.

de Candolle AP (1830) Cxlix. Hymenolaena. Prodromus Systematis Naturalis Regni Vegetabilis 4: e246.

Diels FLE (1912) Umbelliferae. Notes from the Royal Botanic Garden Edinburgh 5(25): e291.

Downie SR, Katz-Downie DS (1999) Phylogenetic analysis of chloroplast rps16 intron sequences reveals relationships within the woody southern African Apiaceae subfamily Apioideae. Canadian Journal of Botany 77(8): 1120–1135. https://doi.org/10.1139/b99-086

Downie SR, Watson MF, Spalik K, Katz-Downie DS (2000) Molecular systematics of Old World Apioideae (Apiaceae): Relationships among some members of tribe Peucedaneae sensu lato, the placement of several island-endemic species, and resolution within the apioid superclade. Canadian Journal of Botany 78(4): 506–528. https://doi.org/10.1139/b00-029

Downie SR, Spalik K, Katz-Downie DS, Reduron JP (2010) Major clades within Apiaceae subfamily Apioideae as inferred by phylogenetic analysis of nrDNA ITS sequences.

Plant Diversity and Evolution 128(1–2): 111–136. https://doi.org/10.1127/1869-6155/2010/0128-0005

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analyses by sampling trees. BMC Evolutionary Biology 7(1): e214. https://doi.org/10.1186/1471-2148-7-214

Farille MA, Malla SB (1985) Apiaceae Himalayenses, III. Candollea 40: 512–520.

Franchet AR (1894) Notes sur quelques Ombellifères du Yunnan. Bulletin de la Société Philomathique de Paris 8(6): 106–146.

Guo XL, Wang CB, Wen J, Zhou SD, He XJ (2018) Phylogeny of Chinese *Chamaesium* (Apiaceae: Apioideae) inferred from ITS, cpDNA and morphological characters. Phytotaxa 376(1): 1–16. https://doi.org/10.11646/phytotaxa.376.1.1

Hemsley WB (1892) Descriptions of new species. Journal of the Linnean Society. Botany 29(202): 307–308. https://doi.org/10.1111/j.1095-8339.1892.tb02268.x

Hiroe M (1958) Umbelliferae of Asia (excluding Japan). Eikodo (Akira Imagawa), Kyoto, 123–219.

Hiroe M (1979) Umbelliferae of World. Ariake Book Company, Tokyo, 741–747.

Hoffmann GF (1814) Genera plantarum Umbelliferarum eorumque characters naturales secundum numerum, figuram, situm et proportionem fructificationis et fructus partium. Mosquae.

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trends in Biochemical Sciences 23(10): 403–405. https://doi.org/10.1016/S0968-0004(98)01285-7

Jordan WC, Courtney MW, Neigel JE (1996) Low levels of intraspecific genetic variation at a rapidly evolving chloroplast DNA locus in North American Duckweeds (Lemnaceae). American Journal of Botany 83(4): 430–439. https://doi.org/10.1002/j.1537-2197.1996.tb12724.x

Kelchner SA, Clark LG (1997) Molecular evolution and phylogenetic utility of the chloroplast rpl16 intron in Chusquea and the Bambusoideae (Poaceae). Molecular Phylogenetics and Evolution 8(3): 385–397. https://doi.org/10.1006/mpev.1997.0432

Kumar S, Stecher G, Tamura K (2016) Mega7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Lee C-S, Downie SR (2006) Phylogenetic relationships within *Cicuta* (Apiaceae tribe Oenantheae) inferred from nuclear rDNA ITS and cpDNA sequence data. Canadian Journal of Botany 84(3): 453–468. https://doi.org/10.1139/b06-016

Lindley J (1835) Illustrations of the Botany and Other Branches of the Natural History of the Himalayan Mountains. Royle, John Forbes, London, 232 pp.

Linnaeus C (1753) Species Plantarum (Vol. 1). Laurentii Salvii, Holmiae, 263 pp.

Mukherjee PK (1982) Resume of Indian umbellifers. Monographs in Systematic Botany from the Missouri Botanical Garden 6: e59.

Naqshi AR, Dhar U, Kachroo PN (1995) Central Asia and Kashmir Himalaya-Archaeobotany and Floristics. Scientific Publishers, India, 107 pp.

Norman C (1938) *Physospermopsis* H. Wolff. Journal of Botany. British and Foreign 76: e231.

Nylander JAA (2004) MrModelTest 2.2. Program Distributed by the Author. Evolutionary Biology Centre, Uppsala University.

Pan ZH, Watson MF (2005) *Physospermopsis* H. Wolff. In: Wu ZY, Raven RH (Eds) Flora of China (Vol. 14). Science Press, Beijing and Missouri Botanical garden Press, Saint Louis, 31–33.

Panahi M, Banasiak Ł, Piwczyński M, Puchałka R, Kanani MR, Oskolski AA, Modnicki D, Miłobędzka A, Spalik K (2018) Taxonomy of the traditional medicinal plant genus *Ferula* (Apiaceae) is confounded by incongruence between nuclear rDNA and plastid DNA. Botanical Journal of the Linnean Society 188(2): 173–189. https://doi.org/10.1093/botlinnean/boy055

Pimenov MG (2017) Updated checklist of Chinese Umbelliferea: Nomenclature, synonymy, typification, distribution. Turczaninowia 20(2): 106–239. https://doi.org/10.14258/turczaninowia.20.2.9

Pimenov MG, Kljuykov EV (2000a) Taxonomic revision of *Pleurospermum* HOFFM. and related genera of Umbelliferae I. General part. Feddes Repertorium 111(7–8): 499–515. https://doi.org/10.1002/fedr.20001110717

Pimenov MG, Kljuykov EV (2000b) Taxonomic revision of *Pleurospermum* HOFFM. and related genera of Umbelliferae II. The genera *Pleurospermum*, *Pterocyclus*, *Trachydium*, *Keraymonia*, *Pseudotrachydium*, *Aulacospermum*, and *Hymenolaena.* Feddes Repertorium 111(7–8): 517–534. https://doi.org/10.1002/fedr.20001110718

Pimenov MG, Kljuykov EV (2000c) Taxonomic revision of *Pleurospermum* HOFFM. and related genera of Umbelliferae III. The genera *Physospermopsis* and *Hymenidium.* Feddes Repertorium 111(7–8): 535–552. https://doi.org/10.1002/fedr.20001110719

Pu GZ, Liu QX (2005) Comparative anatomical study on the genus *Physospermopsis* fruit from China and its systematic significance. Journal of Plant Resources and Environment 14: 1–6. https://doi.org/10.1111/j.1744-7909.2005.00141.x

Pu GZ, Liu QX (2006) The micromorphological features of pericarp surface of *Physospermopsis* and *Trachydium* (Apiaceae) in China and its taxonomic significance. Journal of Plant Resources and Environment 15: 1–6. https://doi.org/10.3969/j.issn.1674-7895.2006.03.001

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Systematic Biology 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Schischkin BK (1935) Flora Zapadnoi Sibiri. Tomsk State University, Tomsk, 2010 pp.

Shan RH (1941) On reduction of the genus *Haplesoseseli* WOLFF et HANDEL MAZZETTI. Sinensia 12(1–6): 185–187.

Shan RH, Liou SL (1979) *Physospermopsis* Wolff Apiaceae. In: Shan RH, She ML (Eds) Flora Reipublicae Popularis Sinicae. Science Press, Beijing, 105 pp.

Spalik K, Downie SR, Watson MF (2009) Generic delimitations within the *Sium* alliance (Apiaceae tribe Oenantheae) inferred from cpDNA *rps16*-5` *trnK*$^{(UUU)}$ and nrDNA ITS sequences. Taxon 58(3): 735–748. https://doi.org/10.1002/tax.583004

Stamatakis A (2014) RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics (Oxford, England) 30(9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Valiejo-Roman CM, Terentiev EI, Pimenov MG, Kljuykov EV, Samigullin TH, Tilney PM (2012) Broad Polyphyly in *Pleurospermum* s. l. (Umbelliferae-Apioideae) as Inferred from nrDNA ITS and Chloroplast Sequences. Systematic Botany 37(2): 573–581. https://doi.org/10.1600/036364412X635593

Wang PL, Pu FD (1992) Pollen morphology diversity and evolution trend of the genus *Physospermopsis* in the Hengduan Mountains of China. Yunnan Zhi Wu Yan Jiu 4: 413–417.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds) PCR Protocols: A Guide to Methods and Applications. Academic Press Inc, San Diego, California, 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Wolff H (1925) Neue umbelliferen-gattungen aus ostasien. Notizblatt Des Königl Botanischen Gartens Und Museums Zu Berlin-Dahlem 9(84): 275–280. https://doi.org/10.2307/3994550

Wolff H (1929) VII. Umbelliferae Asiaticae novae relictae. Repertorium Specierum Novarum Regni Vegetabilis 27: 121–192. https://doi.org/10.1002/fedr.4870270109

Wolff H, Handel-Mazzetti H (1933) Umbelliferae. Symbolae Sinicae 7(3): e722.

Wu CY, Pu FT (1993) Vascular Plants of the Hengduan Mountains. Science Press, Beijing, 1285 pp.

Yi T-S, Jin G-H, Wen J (2015) Chloroplast capture and intra- and inter- continental biogeographic diversification in the Asian-New World disjunct plant genus Osmorhiza (Apiaceae). Molecular Phylogenetics and Evolution 85: 10–21. https://doi.org/10.1016/j.ympev.2014.09.028

Zhou J, Peng H, Downie SR, Liu ZW, Gong X (2008) A molecular phylogeny of Chinese Apiaceae subfamily Apioideae inferred from nuclear ribosomal DNA internal transcribed spacer sequences. Taxon 57: 402–416. https://doi.org/10.2307/25066012

Zhou J, Gong X, Downie SR, Peng H (2009) Towards a more robust molecular phylogeny of Chinese Apiaceae subfamily Apioideae: Additional evidence from nrdna its and cpDNA intron (*rpl16* and *rps16*) sequences. Molecular Phylogenetics and Evolution 53(1): 56–68. https://doi.org/10.1016/j.ympev.2009.05.029

# Appendix 1

Voucher details and GenBank accession numbers of taxa used in this study. A n-dash (–) indicates unavailable information; new sequences are in bold.

| Taxa | Voucher | Locality | Genbank accession numbers | | |
|---|---|---|---|---|---|
| | | | ITS | *rpl16* | *rps16* |
| *Angelica archangelica* | Downie 79 (ILL) | cult. University of Joensuu Botanical Garden, Finland | AH003539 | AF094362 | AF110536 |
| *Angelica likiangensis* | 200421 (NHW ) | Lijiang, Yunnan, China | DQ263587 | FJ385074 | FJ385172 |
| *Angelica maowenensis* | ZJ0582 (KUN) | Mt. Gongga, Sichuan, China | EU236157 | FJ385075 | FJ385173 |
| *Aulacospermum anomalum* | 19932275 (E) | cult. Royal Botanic Garden, Edinburgh, United Kingdom | AF008641,<br>AF009120 | AF094440 | AF110558 |
| *Aulacospermum simplex* | Dingelstedt & Sovetkina 367 23-VII-1927 (LE) | Kazakhstan | GQ379339 | – | AF110557 |
| *Bupleurum krylovianum* | ZJ0726 (KUN) | KaNaSi Lake, Xinjiang, China | FJ385035 | FJ385082 | FJ385180 |
| *Bupleurum rockii* | J059 (KUN) | Ninglang, Yunnan, China | FJ385036 | FJ385083 | FJ385181 |
| *Chamaesium paradoxum* | ZJ0560 (KUN) | Daocheng-Litang, Sichuan, China | EU236161 | FJ385085 | FJ385184 |

| **Taxa** | **Voucher** | **Locality** | **Genbank accession numbers** | | |
|---|---|---|---|---|---|
| | | | **ITS** | ***rpl16*** | ***rps16*** |
| *Chamaesium thalictrifolium* | ZJ0607 (KUN) | Zhangla-Caowan, Sichuan, China | EU236162 | FJ385086 | FJ385185 |
| *Chamaesium wolffianum* | ZJ0525 (KUN) | Shudu Lake, Yunnan, China | EU236163 | FJ385087 | FJ385186 |
| *Changium smyrnioides* | J101 (KUN) | Jiangsu Institute of Botany, China | DQ517340 | FJ385088 | FJ385187 |
| *Chuanminshen violaceum* | J105 (KUN) | Cangxi, Sichuan, China | FJ385040 | FJ385089 | FJ385188 |
| *Conioselinum chinense* | Raiche 30046 (UC) | California, America | U78374 | AF094421 | GU395135 |
| *Conioselinum vaginatum* | ZJ0731 (KUN) | KaNaSi Lake, Xinjiang, China | FJ385041 | FJ385091 | FJ385190 |
| *Haplosphaera phaea* | ZJ0521 (KUN) | Shudu Lake, Yunnan, China | EU236167 | FJ385096 | FJ385194 |
| *Hansenia weberbaueriana* | ZJ0697 (KUN) | KIB nursery, Yunnan, China | EU236180 | FJ385115 | FJ385212 |
| *Heracleum bivittatum* | ZJ0611 (KUN) | MaoCountyg, Sichuan, China | EU236168 | FJ385098 | FJ385196 |
| *Heracleum forrestii* | ZJ091032 (KUN) | Shangri-La, Yunnan, China | EU236169 | FJ385099 | FJ385197 |
| *Korshinskya bupleuroides* | Pimenov et al. 106 (MW) | – | FJ489360,<br>FJ489391 | – | – |
| *Korshinskya olgae* | Pimenov et al. 228 (MW) | – | FJ489359,<br>FJ489390 | – | – |
| *Physospermopsis alepidioides* | XXR2019080801 (SZ) | Muli, Sichuan, China | **MT533355** | **MT542144** | **MT561011** |
| *Physospermopsis delavayi* (HB) | G18071802 (SZ) | Shangri-La, Yunnan, China | **MN658653** | **MN786490** | **MN786487** |
| *Physospermopsis delavayi* (LGH) | XXR2019081102 (SZ) | Lugu Lake, Sichuan, China | **MN658656** | **MN786488** | **MN786486** |
| *Physospermopsis delavayi* (LYG) | XXR2019081305 (SZ) | Lijiang, Yunnan, China | **MN658657** | **MN786489** | **MN786485** |
| *Physospermopsis kingdon-wardii* | G19082407 (SZ) | Nyalam, Tibet, China | **MN659655** | **MN786491** | **MN786484** |
| *Physospermopsis muktinathensis* | Pimenov & Kljuykov 22 (MW) | Annapurna, Nepal | FJ469961,<br>FJ483500 | – | – |
| *Physospermopsis muliensis* | XXR2019080903 (SZ) | Muli, Sichuan, China | **MT533356** | **MT542145** | **MT561012** |
| *Physospermopsis nana* | G19101802 (SZ) | Lanping, Yunnan, China | **MT542694** | **MT561018** | **MT561017** |
| *Physospermopsis obtusiuscula* (DQ) | XXR2019081502 (SZ) | Dêqên, Yunnan, China | **MT533361** | **MT542149** | **MT561016** |
| *Physospermopsis obtusiuscula* (YD) | G19082009 (SZ) | Yadong, Tibet, China | **MT533360** | **MT542148** | **MT561015** |
| *Physospermopsis rubrinervis* (EY) | XXR2019081701 (SZ) | Eryuan,Yunnan, China | **MN658654** | **MN786492** | **MN786483** |
| *Physospermopsis rubrinervis* (LS) | G19101802 (SZ) | Lushui, Yunnan, China | **MT533359** | **MT542143** | **MT561010** |
| *Physospermopsis shaniana* (LQ) | XXR2019071701 (SZ) | Luquan, Yunnan, China | **MT533357** | **MT542146** | **MT561014** |
| *Physospermopsis shaniana* (QJ) | XXR2019071404 (SZ) | Qiaojia, Yunnan, China | **MT533358** | **MT542147** | **MT561013** |
| *Pleurospermum austriacum* | Ghisa and Topa 2959 (MW) | – | FJ469962,<br>FJ483502 | – | – |
| *Pleurospermum foetens* | Chungtien 1181 (E) | Yunnan, China | FJ483482,<br>FJ469943 | AF094438 | AF110559 |
| *Pleurospermum franchetianum* | ZJ0573 (KUN) | Kangding, Sichuan, China | EU236198 | FJ385137 | FJ385232 |
| *Pleurospermum uralense* | LQX031 (NAS) | Liaoning, China | JF977839 | AF094439 | AF110560 |
| *Pleurospermum wilsonii* | ZJ0624 (KUN) | Hongyuan, Sichuan, China | EU236200 | FJ385139 | FJ385234 |

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# Plastome analysis unveils Inverted Repeat (IR) expansion and positive selection in Sea Lavenders (*Limonium*, Plumbaginaceae, Limonioideae, Limonieae)

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## Abstract

The genus *Limonium*, commonly known as Sea Lavenders, is one of the most species-rich genera of the family Plumbaginaceae. In this study, two new plastomes for the genus *Limonium*, viz. *L. tetragonum* and *L. bicolor*, were sequenced and compared to available *Limonium* plastomes, viz. *L. aureum* and *L. tenellum*, to understand the gene content and structural variations within the family. The loss of the *rpl16* intron and pseudogenisation of *rpl23* was observed. This study reports, for the first time, expansion of the IRs to include the *ycf1* gene in *Limonium* plastomes, incongruent with previous studies. Two positively selected genes, viz. *ndhF* and *ycf2*, were identified. Furthermore, putative barcodes are proposed for the genus, based on the nucleotide diversity of four *Limonium* plastomes.

## Keywords

Intron loss, IR expansion, positive selection, pseudogenisation, *ycf1*

## Introduction

The family Plumbaginaceae of the order Caryophyllales is highly diverse, rich in species and displays a cosmopolitan distribution with its maximum diversity in the temperate areas of the northern hemisphere (Kubitzki 1993). It is sister to Polygonaceae (Lledó et al. 1998; Chase et al. 2016) and further classified into two subfamilies: Limonioideae (formerly Staticoideae) and Plumbaginoideae (Lledó et al. 1998, 2001; Hernández-Ledesma et al. 2015). Limonioideae is further divided into two tribes, Limonieae (consisting of 24 genera) and the monotypic Aegialitideae, whereas Plumbaginoideae consists of four genera. Limonioideae is a sub-cosmopolitan group distributed mostly in the Mediterranean and Indo-Turanian regions, but a few genera have also diversified in the Southern Hemisphere. *Limonium* Mill., *Acantholimon* Boiss. and *Armeria* (DC.) Willd., all belonging to Limonioideae, are the most species-rich genera, comprising 80–90% of the species in Plumbaginaceae (Koutroumpa et al. 2018).

The genus *Limonium* Mill., popularly known as sea lavenders, belongs to the subfamily Limonioideae and tribe Limonieae (Kubitzki 1993; Lledó et al. 2005; Malekmohammadi et al. 2017). The genus is represented by ca. 600 species and is the sole genus of Plumbaginaceae exhibiting a sub-cosmopolitan distribution (Koutroumpa et al. 2018). The genus comprises several ornamental and medicinally-important species (Malekmohammadi et al. 2017). *Limonium* are herbs or shrubs growing in saline or metal-rich soils, mostly in coastal areas (Kubitzki 1993). Variation of reproductive systems (sexual and apomixis), as well as events like hybridisation and polyploidy, complicate the delimitation of most of the species of *Limonium* (Kubitzki 1993; Cowan et al. 1998; Palacios et al. 2000; Akhani et al. 2013; Róis et al. 2016).

Certain phylogenetic studies tried to resolve the relationships within *Limonium* at a global scale (Lledó et al. 2005); however, many others were confined to either a specific geographic area or some specific sections of *Limonium* (Palacios et al. 2000; Lledó et al. 2005; Lledó et al. 2011; Akhani et al. 2013; Róis et al. 2016). More recently, Malekmohammadi et al. (2017) tried to resolve the phylogenetic relationships within *Limonium* including 76 species of the Mediterranean region. They found two well-supported clades for subgenera *Limonium* and *Pteroclados* (Boiss.) Pignatti, which confirmed the earlier findings by Lledó et al. (2005, 2011). Their study was based on one nuclear and several plastid loci, but lacked comprehensive sampling. Koutroumpa et al. (2018) carried out a phylogenetic study of Plumbaginaceae which included 23 genera of the family with an emphasis on the genus *Limonium* (201 spp.), based on three chloroplast and one nuclear marker. The study again confirmed most of the previous molecular phylogenies and led to the proposal of new sections and altering some of the existing sections. However, taxonomic difficulties, diversity in reproductive modes and sub-cosmopolitan distribution of *Limonium* necessitate further studies on the genus.

With the advent of sequencing technologies, the availability of large genome-scale data has made it easier to understand phylogeny and detect polyploidy events (Gompert and Mock 2017; Thomas et al. 2017; McKain et al. 2018). Five plastomes have been sequenced for Plumbaginaceae thus far, viz. *Limonium sinense* (Girard) Kuntze (Li et al. 2020), *L. aureum* (L.) Chaz. (Zhang et al. 2020), *L. tenellum* (Turcz.) Kuntze (Yao et al. 2019) and one each of *Ceratostigma* Bunge and *Plumbago* L. The recent phylogenomic study to understand the evolution of Caryophyllales incorporated plastome sequences of *L. tenellum*, *Plumbago auriculata* Lam. and *Ceratostigma willmottianum* Stapf (Yao et al. 2019). The study reported that all Plumbaginaceae members, except *Limonium*, exhibit expanded IR to accommodate *ycf1*. However, no studies have been carried out to understand and compare the structure, composition and evolution of the plastome within Plumbaginaceae and *Limonium* in particular.

The present study reports the plastome sequences of two Asian *Limonium* species, viz. *L. tetragonum* (Thunb.) Bullock and *L. bicolor* (Bunge) Kuntze and compares the structure, composition and diversity within the genus by combining them with other available plastomes. *L. tetragonum* is a biennial species characterised by a spicate inflorescence, yellow corolla, acute calyx with pink at the base, white in upper parts and distributed in Japan, Korea, New Caledonia and Primorye (Ohwi 1965; POWO 2021). *Limonium bicolor* is a perennial species, characterised by a paniculate inflorescence, yellow corolla, somewhat rounded calyx, pink to purplish at base, white in upper parts and distributed in China and Mongolia (Wu and Raven 1996; POWO 2021). The former is known for its anti-cancerous properties (Kong et al. 2008; Bae et al. 2016), while the latter is most studied for its salt glands (Li et al. 2019; Lu et al. 2020). An attempt has also been made to unravel the structural variations within Plumbaginaceae plastomes and propose molecular markers for easier discrimination of *Limonium* species.

## Material and methods

### Sampling and sequencing

Leaf samples of *Limonium bicolor* and *L. tetragonum* were collected from Meneng steppe of Dornod Province of Mongolia (Voucher No. KRIB 0070251) in June 2015 and from the coastal area of Ulsan City of the Republic of Korea (Voucher No. KRIB 0086343) in April 2018, respectively. The samples were deposited at the Herbarium of Korea Research Institute of Bioscience and Biotechnology (KRIB). DNA extraction was carried out from dried leaves using the DNeasy Plant Mini Kit (QIAGEN, Cat. No. 69104) according to the manufacturer's protocol. For both the plastomes, a 550 bp DNA TruSeq Illumina (Illumina, San Diego, CA, USA) sequencing library was constructed. After the library preparation, the DNA samples were run in a single lane of an Illumina HiSeq 10X with a read length of 151 bp.

### Assembly and annotation

The raw reads obtained after Illumina sequencing were analysed using FastQC V0.11.7 (Andrews 2010) software to ensure the quality of the reads and Phred score. The assembly was carried out using NOVOPlasty V4.2 (Dierckxsens et al. 2017). Forward and reverse reads with a read length of 150 bp and insert size of 300 bp, as well as seed sequence (*rbcL* of *L. tetragonum*), were used as input. *De novo*, as well as reference-based assembly, were carried out. The plastome sequence of *L. aureum* (MN623109) was used as a reference for the assembly of both the plastomes. The assembly and orientation of the Inverted Repeats (IRs), Large Single Copy (LSC) and Small Single Copy (SSC) regions were confirmed by NCBI blast and graphic view using Geneious prime 2020.2.2 (https://www.geneious.com). The assembled plastomes were annotated using Geneious prime 2020.2.2 (https://www.geneious.com) and Geseq – Annotation of Organellar Genomes (Tillich et al. 2017). The transfer RNAs (tRNAs) were identified with tRNA-scan-SE (Lowe and Chan 2016). Graphical maps of both plastomes were visualised using OGDRAW (Lohse et al. 2013).

## Comparative plastome analysis

All Plumbaginaceae plastomes available so far were included for comparison. Four plastomes of *Limonium*, viz. *L. aureum* (MN623109), *L. tenellum* (MK397871), *L. tetragonum*, *L. bicolor* and *Ceratostigma willmottianum* (MK397862), as well as *Plumbago auriculata* (MH286308), were included in the analysis. The plastome of *L. sinense* was not included in any of the analyses due to ambiguities observed in the assembly. The selected six plastomes were aligned using a Geneious prime 2020.2.2 plugin MAFFT v.7.450 (Katoh and Standley 2013). The contraction and expansion of IRs at the junction sites were examined and plotted using IRscope (Amiryousefi et al. 2018). Percentage sequence identity for all six plastomes was plotted using MultiPIPMaker (http://pipmaker.bx.psu.edu/pipmaker/) considering *L. tetragonum* as a reference (Schwartz et al. 2003).

## Identification of Simple Sequence Repeats (SSRs) and repeats

Simple Sequence Repeats across the four *Limonium* plastomes were detected using MISA online server (Beier et al. 2017). The minimum repeat units for mono-, di-, tri-, tetra-, penta- and hexanucleotide repeats were set as 10, 5, 4, 3, 3 and 3, respectively, whereas forward, reverse, palindromic and complementary repeats were identified using the programme REPuter (Kurtz et al. 2001). The threshold for repeat length was set to 30 with more than 90% similarity and the hamming distance was set to 3.

## Nucleotide diversity

The four *Limonium* plastomes were aligned using Geneious prime 2020.2.2 plugin MAFFT v.7.450 (Katoh and Standley 2013). The aligned sequences were curated manually and, due to the presence of ambiguous *ycf1* region in *L. tenellum* at IRb/SSC junction, the sequences were trimmed for all four taxa before the analysis. Nucleotide diversity and sliding window analysis were performed using the software DnaSP v.6.12.01 (Rozas et al. 2017).

### Codon usage

The percentage codon usage of protein-coding regions of all four *Limonium* plastomes was calculated using Geneious prime 2020.2.2. To examine the frequency and uniformity of Synonymous codon and codon biases, the Relative Synonymous Codon Usage (RSCU) was also determined in DnaSP v.6.12.01 software (Rozas et al. 2017).

### Positive selection analysis

In order to detect protein-coding genes under selection in the genus *Limonium*, sequences of each gene were aligned using the MAFFT v.7.450 plugin of Geneious prime 2020.2.2. The aligned sequences were again manually checked for an end-to-end alignment. The phylogenetic tree for each protein-coding gene was constructed using the FastTree plugin (Price et al. 2010) of Geneious prime 2020.2.2. The site-specific model was performed using CODEML algorithms (Yang 1998) implemented in EasyCodeML software (Gao et al. 2019). Seven codon substitution models (M0, M1a, M2a, M3, M7, M8 and M8a) were investigated and compared to identify positively selected sites, based on likelihood ratio tests.

### Phylogenomic analysis

A total of 39 plastome sequences, including six from Plumbaginaceae were considered as ingroups for phylogenomic analysis. The outgroup was composed of members of Amaranthaceae. All the plastome sequences were aligned using the MAFFT v.7.450 plugin of Geneious prime 2020.2.2. Maximum Likelihood analysis, based on the best fit model GTR+F+R5, was performed using IQtree 1.6.12-MacOSX (Nguyen et al. 2015). The best likelihood score for the consensus tree was -979751.011.

## Results

### General features of the plastomes

The average organelle coverage for the plastomes of *L. tetragonum* and *L. bicolor* was 1014X and 1009X, respectively. Plastomes of *L. tetragonum* and *L. bicolor* exhibited a typical quadripartite structure (Fig. 1). Total plastome lengths of *L. tetragonum* and *L. bicolor* were 154,691 bp and 154,617 bp with LSC (84,568 bp and 84,541 bp), SSC (12,997 bp and 12,964 bp) and a pair of IRs (28,563 bp and 28,556 bp), respectively (Table 1). The GC content of both plastomes was 37%. Both the plastomes exhibited 83 protein coding genes, 37 tRNA genes and four rRNA genes (duplicated in IR region) (Tables 1, 2). The assembled and annotated plastomes of *L. tetragonum* and *L. bicolor* were deposited to the NCBI database with accession numbers MW085088 and MW085089, respectively.

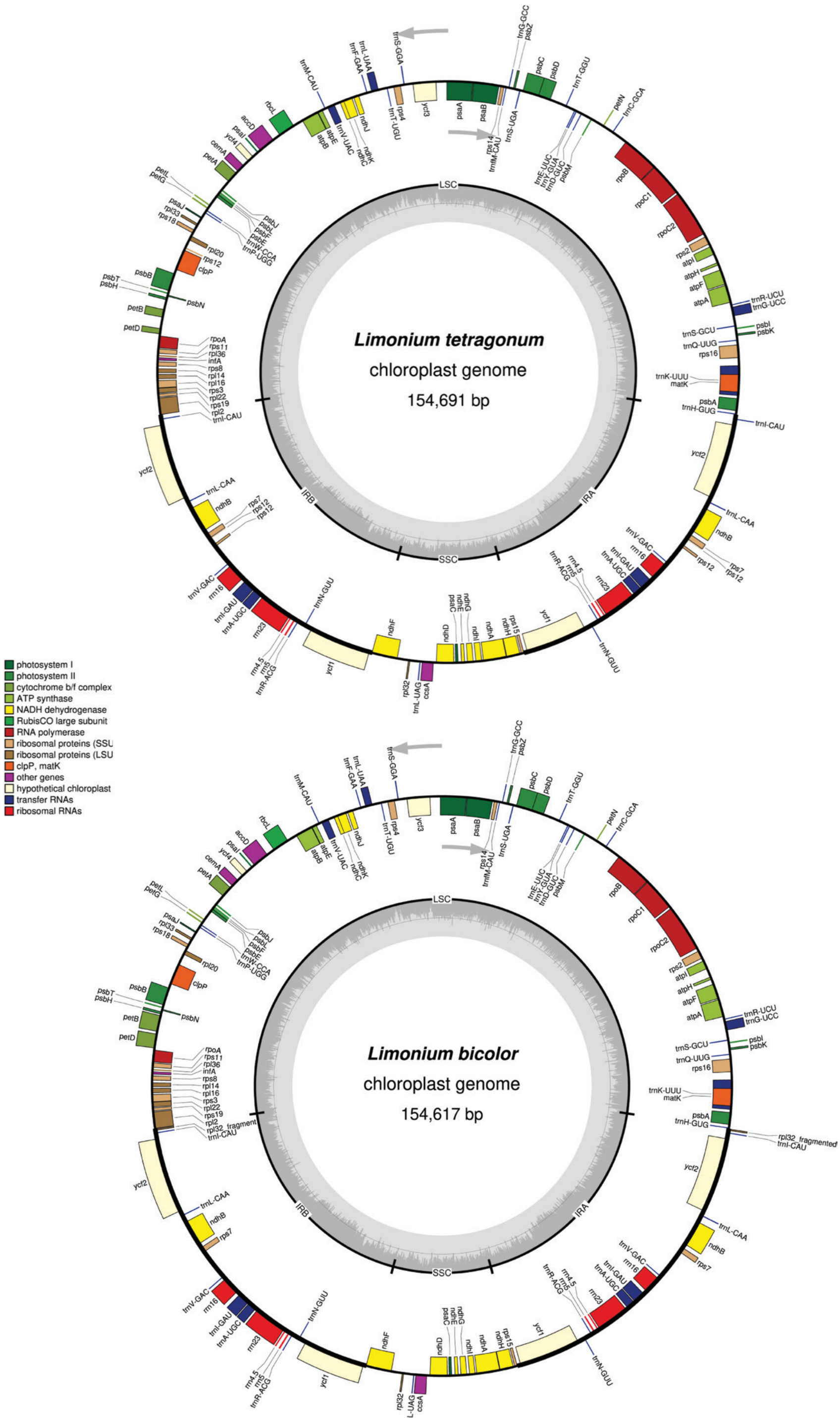

**Figure 1.** Circular gene map of plastomes of *L. tetragonum* and *L. bicolor*. Genes drawn inside the circle are transcribed clockwise and those outside are counter-clockwise. Genes belonging to different functional groups are shown in different colours. The innermost circle denotes GC content across the plastome.

**Table 1.** Comparison of plastome features of Plumbaginaceae members.

| Species | _Limonium tetragonum_ | _Limonium bicolor_ | _Limonium aureum_ | _Limonium tenellum_ | _Ceratostigma willmottianum_ | _Plumbago auriculata_ |
|---|---|---|---|---|---|---|
| Accession No. | MW085088 | MW085089 | MN623109 | MK397871 | NC041261 | NC041245 |
| Genome size (bp) | 154691 | 154617 | 154661 | 150515 | 164999 | 168765 |
| LSC length (bp) | 84568 | 84541 | 84546 | 84634 | 89454 | 91912 |
| SSC length (bp) | 12997 | 12964 | 12980 | 23755 | 13491 | 13331 |
| IR length (bp) | 28563 | 28556 | 28568 | 21063 | 31027 | 31761 |
| No. of genes duplicated in IR | 15 | 15 | 16 | 10 | 15 | 19 |
| No. of genes | 128 | 128 | 130 | 124 | 127 | 132 |
| No. of protein coding genes | 83 | 83 | 83 | 82 | 82 | 84 |
| No. of tRNA genes | 37 | 37 | 37 | 36 | 37 | 37 |
| No. of rRNA genes | 8 | 8 | 8 | 6* | 8 | 8 |
| Total GC content (%) | 37 | 37 | 37.1 | 37.1 | 37.5 | 37.2 |

*indicates annotations available as per NCBI database.

**Table 2.** List of genes in the newly-sequenced plastomes of _L. tetragonum_ and _L. bicolor_.

| Category | Group | Name |
|---|---|---|
| Photosynthesis-related genes | Rubisco | _rbcL_ |
| | Photosystem 1 | _psaA, psaB, psaC, psaI, psaJ_ |
| | Photosystem 2 | _psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, psbZ_ |
| | APT synthase | _atpA, atpB, atpE, atpF†, atpH, atpI_ |
| | Cytochrome b/f complex | _petA, petB, petD, petG, petL, petN_ |
| | NADPH Dehydrogenase | _ndhA†, ndhB†*, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK_ |
| Transcription and translation-related genes | Transcription | _rpoA, rpoB, rpoC1†, rpoC2_ |
| | Ribosomal proteins | _rps2, prs3, rps4, rps7*, rps8, rps11, rps12†*, rps14, rps15, rps16†, rps18, rps19_ |
| | | _rpl2†, rpl14, rpl16, rpl20, rpl22, rpl23#*, rpl33, rpl36_ |
| | Translation initiation factor | _infA_ |
| RNA genes | Ribosomal RNA | _rrn5*, rrn4.5*, rrn16*, rrn23*_ |
| | Transfer RNA | _trnA-UGC†*, trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnfM-CAU, trnG-UCC, trnH-GUG, trnI-GAU†*, trnK-UUU†, trnL-CAA*, trnL-UAA, trnL-UAG†, trnM-CAU, trnN-GUU*, trnP-UGG, trnQ-UUG, trnR-ACG*, trnR-UCU, trnS-GCU, trnS-GGA, trnS-UGA, trnT-GGU, trnT-UGU, trnV-GAC*, trnW-CCA, trnY-GUA_ |
| Other genes | RNA processing | _matK_ |
| | Carbon metabolism | _cemA_ |
| | Fatty acid synthesis | _accD_ |
| | Proteolysis | _clpP†_ |
| Genes of unknown function | Conserved reading frame | _ycf1†*, ycf2*, ycf3†, ycf4_ |

*duplicated genes, †genes with introns, #pseudogenised genes.

## IR expansion and contraction

The IR regions of plastomes are divided by four junctions viz., IRb/LSC, IRb/SSC, IRa/SSC and IRa/LSC. All six plastomes of Plumbaginaceae (including four _Limonium_) were compared for their IR boundaries. The annotations available on NCBI database were used for _Limonium aureum_, _L. tenellum_, _Ceratostigma willmottianum_ and _Plumbago auriculata_.

The IRb/LSC junction of three _Limonium_ species viz. _L. tetragonum_, _L. bicolor_ and _L. aureum_ was characterised by the presence of the _rpl2_ gene (Fig. 2). The gene also extends in IRb with 11 bp, 10 bp and 11 bp, respectively. However, in _L. tenellum_ and

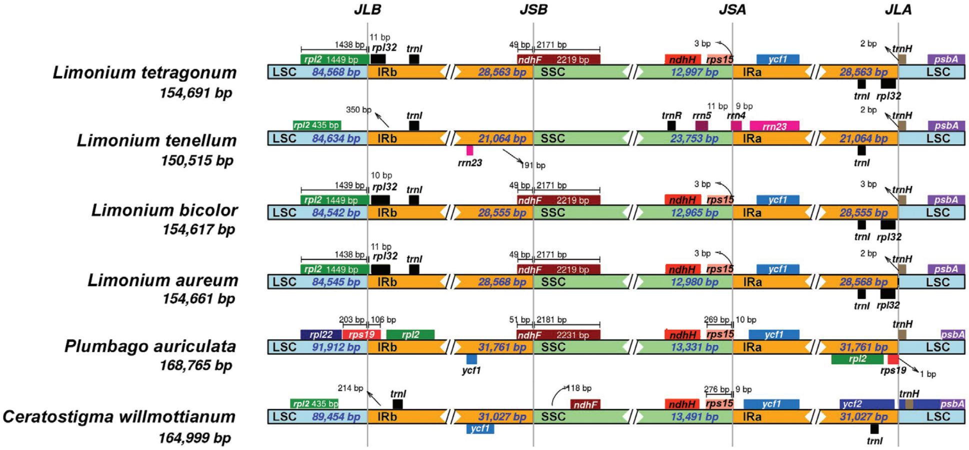

**Figure 2.** Comparison of IRs of Plumbaginaceae plastomes.

*C. willmottianum*, it was exclusively present in the LSC region, 937 bp and 1,041 bp away from the junction, respectively. In *Plumbago*, IR was expanded to include the *rps19* gene at the junction, while *rpl2* was duplicated in the IR region.

The next junction, i.e. IRb/SSC, was characteried by the presence of the gene in *L. tetragonum*, *L. bicolor*, *L. aureum* and *P. auriculata* (49 bp of all *Limonium* species and 51 bp of *Plumbago* in IR region). In *L. tenellum*, the junction exhibited *rrn23* (IR) and *trnR-ACG* (SSC), which could probably be due to wrong assembly or annotation. In *Ceratostigma*, *ndhF* appeared to be shifted to SSC, 118 bp away from IRb border.

IRa/SSC junction of all compared species was characterised by the presence of *rps15* and *ycf1* genes, except in *L. tenellum*. The IRa/LSC junction was characterised by *rpl32* and *trnH* in *L. tetragonum*, *L. bicolor* and *L. aureum*, while in *L. tenellum* and *C. willmottianum*, it was characterised by *ycf2* and *trnH*. In *P. auriculata*, the junction was bordered by *rps19* and *trnH* (Fig. 2).

Plastomes of *L. aureum*, *L. bicolor* and *L. tetragonum* exhibited two copies of *ycf1*, except for *L. tenellum* which exhibited a single copy. All three plastomes are characterised by the *ycf1* gene having a length of 5,298 bp and IR has been expanded to accommodate the *ycf1* gene. *Plumbago* and *Ceratostigma* also exhibited the *ycf1* gene duplicated in IRs. However, the annotation provided for *L. tenellum* (MK397871) exhibits a single copy of *ycf1*.

## Structural comparison

Plastomes of *Limonium tetragonum*, *L. tenellum*, *L. bicolor* and *L. aureum* were compared with two Plumbaginaceae plastomes, keeping *L. tetragonum* as a reference. Sequence divergence amongst the four compared *Limonium* plastomes was similar as compared to *Plumbago* and *Ceratostigma*. *Limonium tenellum* exhibited partial deletion at the IRa/LSC junction in the *ycf1* gene (Fig. 3). Our results suggest that the gene *rpl23* has been pseudogenised in all the studied plastomes of the three genera of Plumbaginaceae. The length of *rpl23* in all four *Limonium* plastomes was observed to be 171 bp, 270 bp in *Plumbago* and 50 bp in *Ceratostigma*. A similar loss of intron was observed in *L. aureum*,

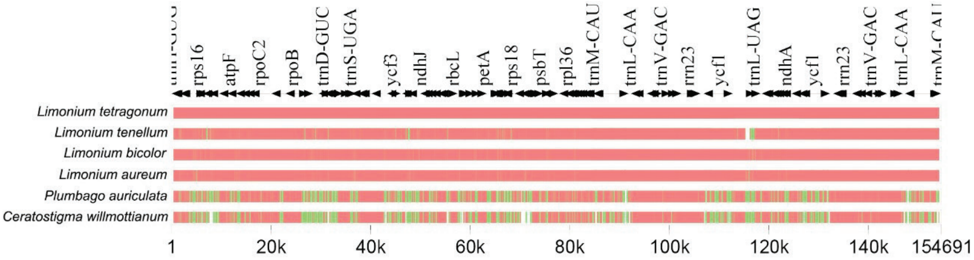

**Figure 3.** Plastome alignment of Plumbaginaceae members.

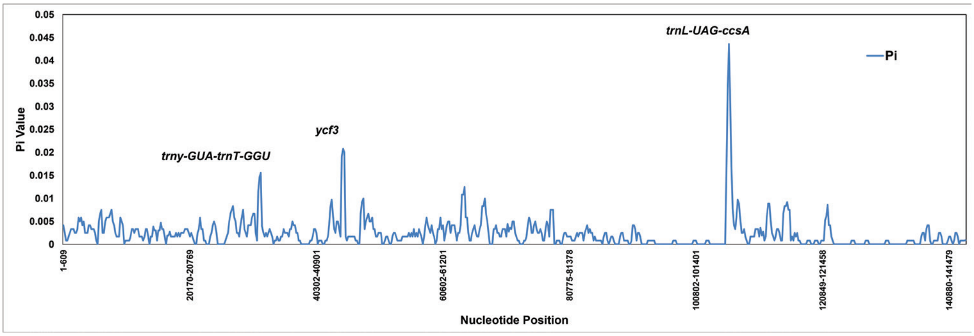

**Figure 4.** Nucleotide diversity and hotspot regions between *Limonium* plastomes. The X-axis represents the nucleotide position and Y-axis represents nucleotide diversity (Pi).

*L. bicolor* and *L. tetragonum* in our study. In all four compared species of *Limonium*, the length of *rpl16* was 408 bp without any intron. However, the two other genera of Plumbaginaceae, i.e. *Plumbago* and *Ceratostigma*, exhibited the presence of intron. The length of the gene was 1,449 bp in the former, while in the latter, it was 1,469 bp.

## Nucleotide diversity

The nucleotide diversity (Pi) of four *Limonium* plastomes was analysed, except for the *ycf1* region, which was removed due to ambiguous alignment. Sliding window analysis yielded some regions with higher Pi values. High nucleotide diversity was found in two spacer regions viz. *trnY-GUA-trnT-GGU*, *trnL-UAG-ccsA* and one gene *ycf3* with Pi values 0.015, 0.043 and 0.02, respectively (Fig. 4).

## SSR and repeat analyses

The plastomes of *L. bicolor* exhibited 6 compound, 26 mono-, 9 di-, 5 tri- and 5 tetranucleotide repeats, while *L. tetragonum* exhibited 4 compound, 31 mono-, 9 di-, 4 tri-, 6 tetra- and 1 hexanucleotide repeats (Fig. 5a). The size of repeats ranged from 10 to 168 in *L. bicolor* and 10 to 109 in *L. tetragonum* (Fig. 5b). Repeats in both the plastomes were found to be AT-rich. The highest number of SSRs was found in the LSC region, followed by IR and SSC regions in both the plastomes (Fig. 5c).

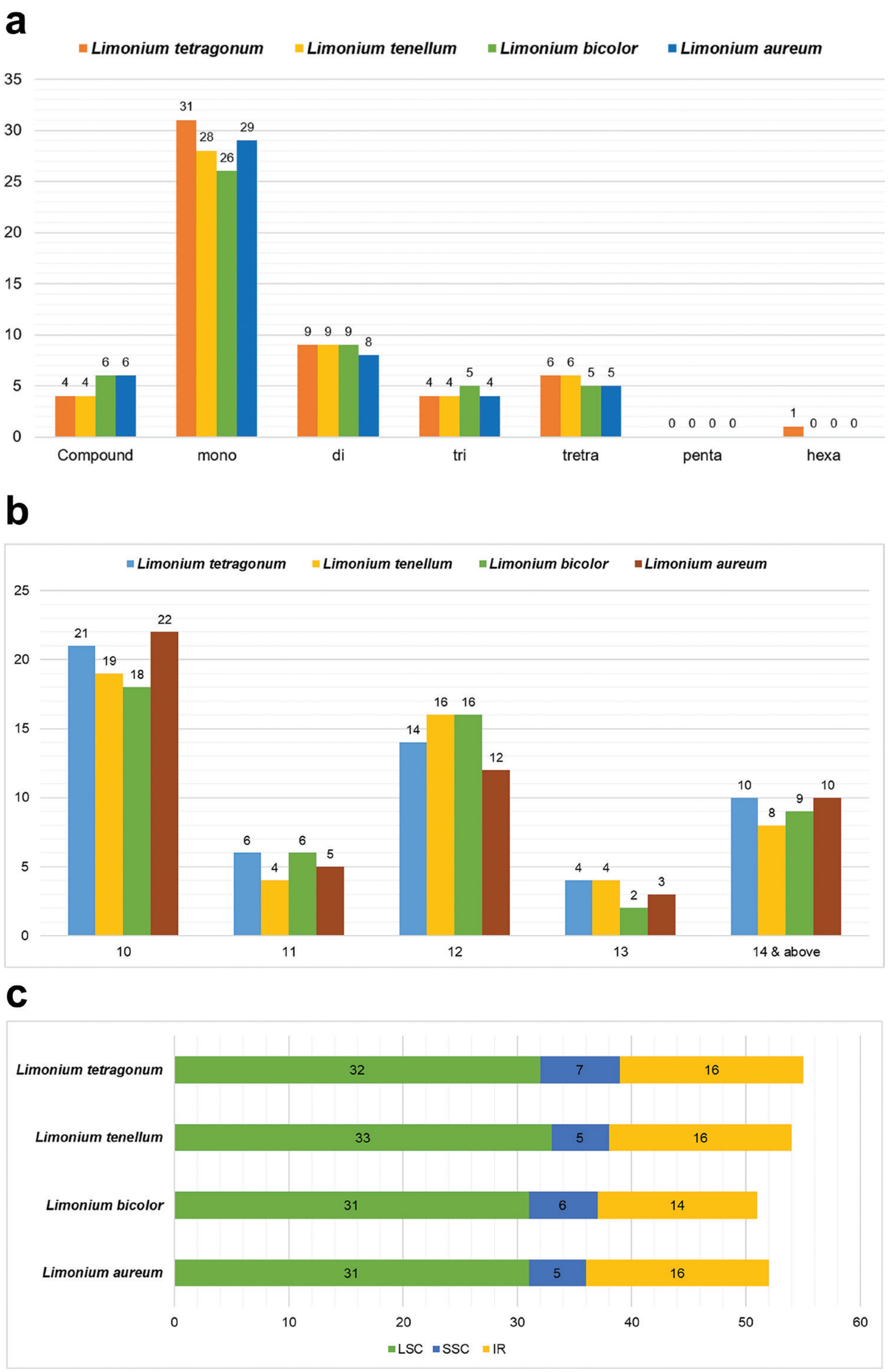

**Figure 5.** Repeat analysis of four *Limonium* plastomes. a. Types of repeats, b. Size of repeats, c. Position of repeats.

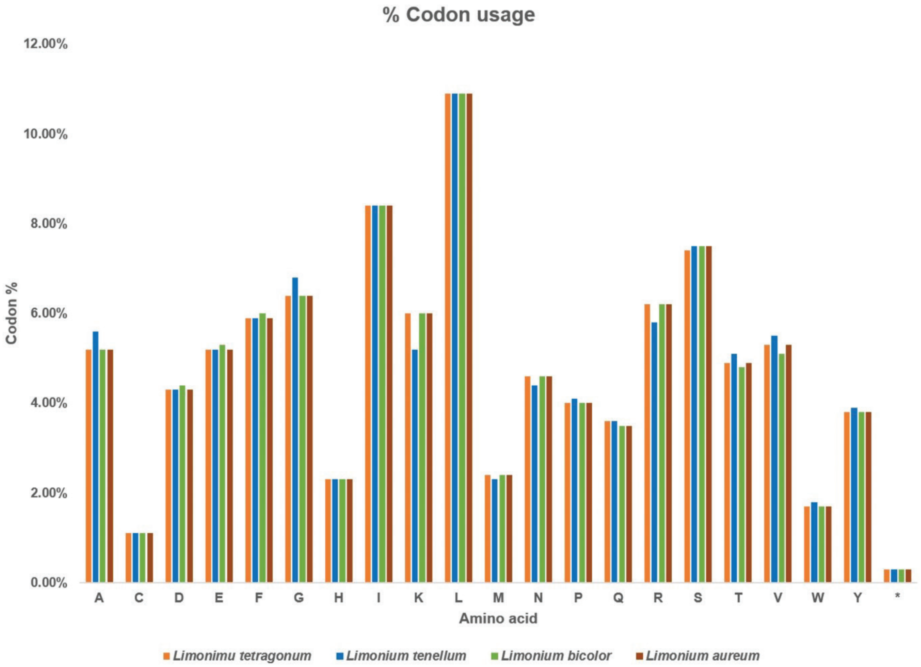

**Figure 6.** Codon usage of four *Limonium* species. X-axis: Amino acid, Y-axis: codon usage in percentage. * indicates the stop codon.

All four species of *Limonium* exhibited only forward and palindrome type in REPuter analysis (Suppl. material 1).

## Codon usage

The four *Limonium* plastomes were compared for their codon usage. The plastomes of *L. tetragonum*, *L. tenellum*, *L. bicolor* and *L. aureum* exhibited 27,290, 24,093, 26,682 and 27,308 codons, respectively. Leucine was the most abundant while Cysteine was the least abundant amino acid in all the compared plastomes (Fig. 6). Codon usage was biased towards A and T in all the compared plastomes. The highest codon preference was 1.88, 1.89, 1.82 and 1.91 while the lowest was 0.33, 0.36, 0.37 and 0.36, respectively, in *L. tetragonum*, *L. tenellum*, *L. bicolor* and *L. aureum* (Suppl. material 2). Codon usage was biased towards A and T in all the compared plastomes.

## Positive selection analysis

A total of 79 consensus protein-coding genes of four *Limonium* species were evaluated with respect to selective pressure. Two genes were found to have undergone positive selection viz. *ndhF* and *ycf2* with ω values 2.2278 and 19.657, respectively (Suppl. material 3).

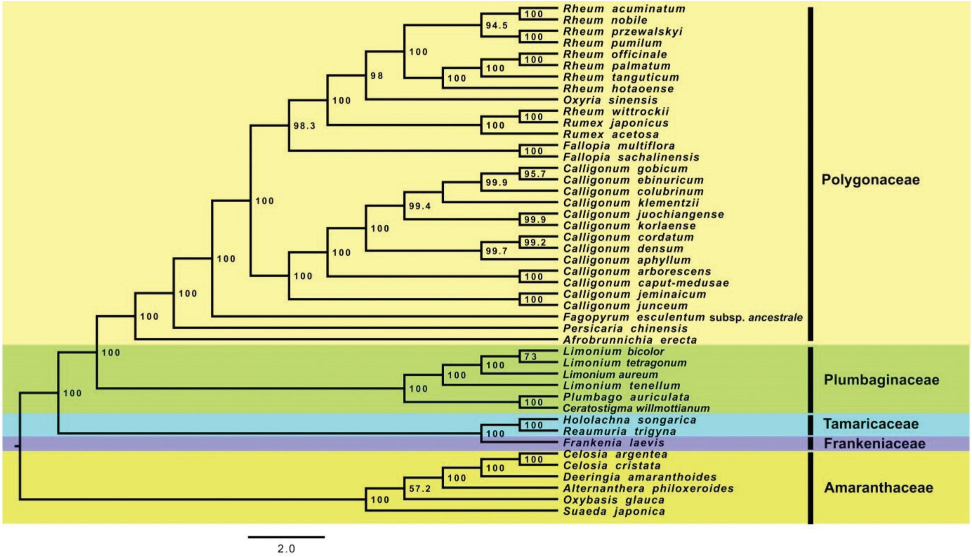

**Figure 7.** Phylogenomic tree from the Maximum Likelihood analysis showing the placement of the studied four *Limonium* species. Nodal support is represented by bootstrap percentages.

## Phylogenomic analysis

In the phylogenomic analysis, the representatives of *Limonium* formed a strongly-supported monophyletic group (BS = 100), in which *L. bicolor* was recovered as sister to *L. tetragonum* (BS = 73), with *L. aureum* and *L. tenellum* being successive sisters (BS = 100) to the clade of *L. bicolor* and *L. tetragonum*. *Plumbago auriculata* and *Ceratostigma willmottianum* also formed a monophyletic group (BS = 100), sister to the *Limonium* clade (BS = 100). All these made Plumbaginaceae a strongly-supported (BS = 100) monophyletic group (Fig. 7).

# Discussion

In this study, two *Limonium* plastomes were assembled and the structure and composition of four *Limonium* plastomes were compared. The plastomes were conserved in terms of size and structure ranging from 154,617 to 154,691 bp, except for *L. tenellum* with 150,515 bp. Expansion and contraction of IRs/SSC account for huge variation, evolutionary events and also affect the plastome sizes (Zhu et al. 2016; Darshetkar et al. 2019; Maurya et al. 2020). Yao et al. (2019) reported the expansion of IRs in Polygonaceae and Plumbaginaceae members to accommodate *ycf1*, except for the genus *Limonium*. However, the study included a single plastome sequence of *Limonium tenellum*. In all the *Limonium* plastomes, except *L. tenellum*, IR has been expanded to accommodate *ycf1*, unlike that reported by Yao et al. (2019). We assume the single copy of *ycf1* in *L. tenellum* could be a result of erroneous assembly and annotation. Sequencing more plastomes for the genus will help to better understand the arrangement and position of *ycf1* in *Limonium*.

Ribosomal Protein L23 is a protein component of the 60s large ribosomal subunit. The comprehensive study of plastomes of Caryophyllales (Yao et al. 2019) reported that the gene has undergone pseudogenisation at least 11 times in the order. Pseudogenisation has also been reported in the family Polygonaceae, a sister family of Plumbaginaceae (Logacheva et al. 2008). The results of our study corroborate these earlier studies. Transfer of the functional copy of the gene to the nucleus has been reported for many plastid genes in angiosperms (Millen et al. 2001; Jansen et al. 2011; Daniell et al. 2016). The functional copy of *rpl23* might have been transferred to the nucleus, even in the members of Plumbaginaceae as predicted by Yao et al. (2019). Ribosomal Protein L16 codes for a protein component of the 50s ribosomal subunit. The loss of *rpl16* intron was reported in *Limonium gmelinii* Kuntze and *Limonium latifolium* Kuntze (Campagna and Downie 1998). Recently, Yao et al. (2019) reported the loss of *rpl16* intron in *L. tenellum* plastome. Hence, our study confirms the loss of *rpl16* intron on the branch leading to *Limonium* as reported by Yao et al. (2019).

The value of the ratio of synonymous and nonsynonymous substitutions (Ka/Ks or ω) above 1 indicates that the corresponding genes experience positive selection, however, ω values ranging from 0.5 to 1 indicate relaxed selection (Tomoko 1995). The *ndhF* gene was found to be positively selected in the genus *Rheum* L. of Polygonaceae. The study reported that the higher expression levels of *ndhF* were observed in *Rheum* under environmental stress conditions (Li et al. 2016). Most of the *Limonium* species also grow in saline soils, which could be the reason behind the adaptive evolution of the gene. Positive selection of the *ycf2* gene has already been reported in several angiosperms (Jiang et al. 2018; Zhong et al. 2019).

The sampling for the phylogenomic analysis followed the studies of Walker et al. (2018) and Yao et al. (2019). The ingroup consisted of plastomes belonging to the FTPP clade, i.e. Frankeniaceae, Tamaricaceae, Plumbaginaceae and Polygonaceae (they were all recovered as strongly-supported monophyletic groups), while the outgroup consisted of species belonging to Amaranthaceae. The position of the four *Limonium* species studied fits with the earlier studies (Yao et al. 2019). In the present study, phylogenetic position of four *Limonium* species belonging to subgenus *Limonium* (Koutroumpa et al. 2018) was studied. A study based on *rbcL*, *trnL* intron and *trnL-F* intergenic spacer included two species viz. *L. tenellum* and *L. tetragonum*, which were resolved in the same clade, but were placed into two different subsections, according to Boissier (1848), *Rhodanthae* and *Chrysanthae*, respectively. However, the study treated them under subgenus *Limonium* (Lledo et al. 2005). Later, Malekmohammadi et al. (2017) studied the phylogeny for the genus, based on several plastid and single nuclear (ITS) markers. The study included *L. tetragonum* and *L. aureum* and both the species were placed in the "*L. aureum* clade". Recently, Koutroumpa et al. (2018) carried out phylogenetic studies of the genus, based on three chloroplast (*trnL-F*, *matK* and *rbcL*) and one nuclear marker (ITS). Three species, *L. tetragonum*, *L. aureum* and *L. tenellum* were included in the study. These three species were all resolved in the same clade. These species were earlier placed under sect. *Plathymenium*, *L. tetragonum* and *L. aureum* belonging to subsect. *Chrysanthae* and *L. tenellum* under subsect. *Rhodanthae* (Boissier 1848). Earlier studies reported plastome data dealing with single *Limonium* species and placed them as sister to *Plumbago* species (Yao et al. 2019; Li et al. 2020; Zhang et al. 2020). The present phylogenetic study, for the first time, included *L. bicolor* and analysed four *Limonium* plastomes together. However, inclusion of more *Limonium* plastomes would help in understanding the intrageneric relationships.

## Conclusions

The present study has made an effort to understand the structural changes in the plastomes of Plumbaginaceae by including two newly-generated *Limonium* plastome sequences. The study also confirms the loss of *rpl16* intron in the genus *Limonium* and pseudogenisation of the *rpl23* gene in Plumbaginaceae. Our results also revealed, for the first time, the expansion of the IRs to accommodate the *ycf1* gene in *Limonium* as in other Plumbaginaceae members. The annotation available for *L. tenellum* exhibits *ycf1* in the SSC region. Hence, the sequencing of more plastomes would aid in identifying the exact position of *ycf1*. Two positively-selected genes were identified, viz. *ndhF* and *ycf2*. The positive selection of these genes could be linked to the evolution of *ndhF* to adapt to extreme environmental conditions, such as salt stress. It would be interesting to identify the adaptive sites in the *ndhF* amino acid by adding more *ndhF* sequences of *Limonium* species, while the expansion of IRs and accommodation of *ycf2* genes could be related to the re-arrangement of the plastome. The function of *ycf2* is still not clear, but it would be interesting to study the *ycf2* evolution and re-arrangement in the whole order. High nucleotide diversity was observed in two spacer regions *trnY-GUA--trnT-GGU*, *trnL-UAG--ccsA* and one gene *ycf3*, which could be used as potential DNA barcodes for the genus. Future studies will focus on identifying adaptive codon sites in positively-selected genes and correlating those with the habitats and environmental conditions and validation of the proposed barcodes by including more *Limonium* species.

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## References

Akhani H, Malekmohammadi M, Mahdavi P, Gharibiyan A, Chase MW (2013) Phylogenetics of the Irano-Turanian taxa of *Limonium* (Plumbaginaceae) based on ITS nrDNA sequences and leaf anatomy provides evidence for species delimitation and relationships of lineages. Botanical Journal of the Linnean Society 171(3): 519–550. https://doi.org/10.1111/boj.12015

Amiryousefi A, Hyvönen J, Poczai P (2018) IRscope: An online program to visualize the junction sites of chloroplast genomes. Bioinformatics 34(17): 3030–3031. https://doi.org/10.1093/bioinformatics/bty220

Andrews S (2010) Babraham bioinformatics-FastQC a quality control tool for high throughput sequence data. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Bae M-J, Karadeniz F, Lee S-G, Seo Y, Kong C-S (2016) Inhibition of MMP-2 and MMP-9 Activities by *Limonium tetragonum* Extract. Preventive Nutrition and Food Science 21(1): 38–43. https://doi.org/10.3746/pnf.2016.21.1.38

Beier S, Thiel T, Münch T, Scholz U, Mascher M (2017) MISA-web: A web server for microsatellite prediction. Bioinformatics 33(16): 2583–2585. https://doi.org/10.1093/bioinformatics/btx198

Boissier E (1848) Plumbaginales. In: de Candolle AP (Ed.) Prodromussystematis naturalis regni vegetabilis, Treuttel et Wurz, Paris, France 12: 617–696.

Campagna ML, Downie SR (1998) The Intron in Chloroplast Gene rpl16 is missing from the flowering plant families Geraniaceae, Goodeniaceae, and Plumbaginaceae. Transactions of the Illinois State Academy of Science. Illinois State Academy of Science 91: 1–11.

Chase MW, Christenhusz MJM, Fay MF, Byng JW, Judd WS, Soltis DE, Mabberley DJ, Sennikov AN, Soltis PS, Stevens PF (2016) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Botanical Journal of the Linnean Society 181(1): 1–20. https://doi.org/10.1111/boj.12385

Cowan RMJ, Ingrouille M, Lledó MD (1998) The taxonomic treatment of agamosperms in the genus *Limonium* Mill. (Plumbaginaceae). Folia Geobotanica 33(3): 353–366. https://doi.org/10.1007/BF03216212

Daniell H, Lin C-S, Yu M, Chang W-J (2016) Chloroplast genomes: Diversity, evolution, and applications in genetic engineering. Genome Biology 17(1): e134. https://doi.org/10.1186/s13059-016-1004-2

Darshetkar AM, Datar MN, Tamhankar S, Li P, Choudhary RK (2019) Understanding evolution in Poales: Insights from Eriocaulaceae plastome. PLoS ONE 14(8): e0221423. https://doi.org/10.1371/journal.pone.0221423

Dierckxsens N, Mardulyn P, Smits G (2017) NOVOPlasty: De novo assembly of organelle genomes from whole genome data. Nucleic Acids Research 45: e18–e18. https://doi.org/10.1093/nar/gkw955

Gao F, Chen C, Arab DA, Du Z, He Y, Ho SYW (2019) EasyCodeML: A visual tool for analysis of selection using CodeML. Ecology and Evolution 9(7): 3891–3898. https://doi.org/10.1002/ece3.5015

Gompert Z, Mock KE (2017) Detection of individual ploidy levels with genotyping-by-sequencing (GBS) analysis. Molecular Ecology Resources 17(6): 1156–1167. https://doi.org/10.1111/1755-0998.12657

Hernández-Ledesma P, Berendsohn WG, Borsch T, Von Mering S, Akhani H, Arias S, Castañeda-Noa I, Eggli U, Eriksson R, Flores-Olvera H (2015) A taxonomic backbone for the global synthesis of species diversity in the angiosperm order Caryophyllales. Willdenowia 45(3): 281–383. https://doi.org/10.3372/wi.45.45301

Jansen RK, Saski C, Lee S-B, Hansen AK, Daniell H (2011) Complete Plastid Genome Sequences of Three Rosids (*Castanea*, *Prunus*, *Theobroma*): Evidence for at least two independent transfers of rpl22 to the nucleus. Molecular Biology and Evolution 28(1): 835–847. https://doi.org/10.1093/molbev/msq261

Jiang P, Shi F-X, Li M-R, Liu B, Wen J, Xiao H-X, Li L-F (2018) Positive selection driving cytoplasmic genome evolution of the medicinally important Ginseng plant genus *Panax.* Frontiers of Plant Science 9: e359. https://doi.org/10.3389/fpls.2018.00359

Katoh K, Standley DM (2013) MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Molecular Biology and Evolution 30(4): 772–780. https://doi.org/10.1093/molbev/mst010

Kong C-S, Um Y-R, Lee J-I, Kim Y-A, Lee J-S, Seo Y-W (2008) Inhibition effects of extracts and its solvent fractions isolated from *Limonium tetragonum* on growth of human cancer cells. KSBB Journal 23: 177–182.

Koutroumpa K, Theodoridis S, Warren BH, Jiménez A, Celep F, Doğan M, Romeiras MM, Santos-Guerra A, Fernández-Palacios JM, Caujapé-Castells J, Moura M, de Sequeira MM, Conti E (2018) An expanded molecular phylogeny of Plumbaginaceae, with emphasis on *Limonium* (sea lavenders): Taxonomic implications and biogeographic considerations. Ecology and Evolution 8(24): 12397–12424. https://doi.org/10.1002/ece3.4553

Kubitzki K (1993) The Families and Genera of Vascular Plants: Flowering Plants: Dicotyledons, Magnoliid, Hamamelid and Caryophyllid Families. Springer, Berlin, Heidelberg & New York, 653 pp.

Kurtz S, Choudhuri JV, Ohlebusch E, Schleiermacher C, Stoye J, Giegerich R (2001) REPuter: The manifold applications of repeat analysis on a genomic scale. Nucleic Acids Research 29(22): 4633–4642. https://doi.org/10.1093/nar/29.22.4633

Li J, Liu H, Mao S (2016) Adaptive evolution of ndhF gene in the genus *Rheum* (Polygonaceae). Guihaia 36: 101–106.

Li J, Zhao C, Zhang M, Yuan F, Chen M (2019) Exogenous melatonin improves seed germination in *Limonium bicolor* under salt stress. Plant Signaling & Behavior 14(11): 1659705. https://doi.org/10.1080/15592324.2019.1659705

Li J, Xu B, Yang Q, Wang T, Zhu Q, Lin Y, Liu Z-L (2020) The complete chloroplast genome sequence of *Limonium sinense* (Plumbaginaceae). Mitochondrial DNA. Part B, Resources 5(1): 556–557. https://doi.org/10.1080/23802359.2019.1710286

Lledó MD, Crespo MB, Cameron KM, Fay MF, Chase MW (1998) Systematics of Plumbaginaceae based upon cladistic analysis of *rbcL* sequence data. Systematic Botany 23(1): 21–29. https://doi.org/10.2307/2419571

Lledó MD, Karis PO, Crespo MB, Cameron KM, Fay MF, Chase MW (2001) Phylogenetic position and taxonomic status of the genus *Aegialitis* and subfamilies Staticoideae and Plumbaginoideae (Plumbaginaceae): Evidence from plastid DNA sequences and morphology. Plant Systematics and Evolution 229(1–2): 107–124. https://doi.org/10.1007/s006060170021

Lledó MD, Crespo MB, Fay MF, Chase MW (2005) Molecular phylogenetics of *Limonium* and related genera (Plumbaginaceae): Biogeographical and systematic implications. American Journal of Botany 92(7): 1189–1198. https://doi.org/10.3732/ajb.92.7.1189

Lledó MD, Karis PO, Crespo MB, Fay MF, Chase MW (2011) Endemism and evolution in Macaronesian and Mediterranean *Limonium*. In: Bramwell D, Caujapé-Castells J (Eds) The Biology of Island Floras. Cambridge University Press, Cambridge, 325–337. https://doi.org/10.1017/CBO9780511844270.014

Logacheva MD, Samigullin TH, Dhingra A, Penin AA (2008) Comparative chloroplast genomics and phylogenetics of *Fagopyrum esculentum* ssp. *ancestrale* – A wild ancestor of cultivated buckwheat. BMC Plant Biology 8(1): e59. https://doi.org/10.1186/1471-2229-8-59

Lohse M, Drechsel O, Kahlau S, Bock R (2013) OrganellarGenomeDRAW – A suite of tools for generating physical maps of plastid and mitochondrial genomes and visualizing expression data sets. Nucleic Acids Research 41(W1): W575–W581. https://doi.org/10.1093/nar/gkt289

Lowe TM, Chan PP (2016) tRNAscan-SE On-line: Integrating search and context for analysis of transfer RNA genes. Nucleic Acids Research 44(W1): W54–W57. https://doi.org/10.1093/nar/gkw413

Lu C, Feng Z, Yuan F, Han G, Guo J, Chen M, Wang B (2020) The SNARE protein LbSYP61 participates in salt secretion in *Limonium bicolor*. Environmental and Experimental Botany 176: e104076. https://doi.org/10.1016/j.envexpbot.2020.104076

Malekmohammadi M, Akhani H, Borsch T (2017) Phylogenetic relationships of *Limonium* (Plumbaginaceae) inferred from multiple chloroplast and nuclear loci. Taxon 66(5): 1128–1146. https://doi.org/10.12705/665.8

Maurya S, Darshetkar AM, Datar MN, Tamhankar S, Li P, Choudhary RK (2020) Plastome data provide insights into intra and interspecific diversity and ndh gene loss in *Capparis* (Capparaceae). Phytotaxa 432(2): 206–220. https://doi.org/10.11646/phytotaxa.432.2.10

McKain MR, Johnson MG, Uribe-Convers S, Eaton D, Yang Y (2018) Practical considerations for plant phylogenomics. Applications in Plant Sciences 6(3): e1038. https://doi.org/10.1002/aps3.1038

Millen RS, Olmstead RG, Adams KL, Palmer JD, Lao NT, Heggie L, Kavanagh TA, Hibberd JM, Gray JC, Morden CW, Calie PJ, Jermiin LS, Wolfe KH (2001) Many parallel losses of infA from chloroplast DNA during angiosperm evolution with multiple independent transfers to the nucleus. The PLANT Cell 13(3): 645–658. https://doi.org/10.1105/tpc.13.3.645

Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ (2015) IQ-TREE: A fast and effective stochastic algorithm for estimating Maximum-Likelihood phylogenies. Molecular Biology and Evolution 32(1): 268–274. https://doi.org/10.1093/molbev/msu300

Ohwi J (1965) Flora of Japan (rev. ed.). Shibundo Co. Ltd., Tokyo, 344 pp.

Palacios C, Rosselló JA, González-Candelas F (2000) Study of the evolutionary relationships among *Limonium* Species (Plumbaginaceae) using nuclear and cytoplasmic molecular markers. Molecular Phylogenetics and Evolution 14(2): 232–249. https://doi.org/10.1006/mpev.1999.0690

POWO (2021) Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew. http://www.plantsoftheworldonline.org/ [accessed 15 February 2021]

Price MN, Dehal PS, Arkin AP (2010) FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. PLoS ONE 5(3): e9490. https://doi.org/10.1371/journal.pone.0009490

Róis AS, Sádio F, Paulo OS, Teixeira G, Paes AP, Espírito-Santo D, Sharbel TF, Caperta AD (2016) Phylogeography and modes of reproduction in diploid and tetraploid halophytes of *Limonium* species (Plumbaginaceae): Evidence for a pattern of geographical parthenogenesis. Annals of Botany 117(1): 37–50. https://doi.org/10.1093/aob/mcv138

Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A (2017) DnaSP 6: DNA sequence polymorphism analysis of large data sets. Molecular Biology and Evolution 34(12): 3299–3302. https://doi.org/10.1093/molbev/msx248

Schwartz S, Elnitski L, Li M, Weirauch M, Riemer C, Smit A, Program NCS, Green ED, Hardison RC, Miller W (2003) MultiPipMaker and supporting tools: Alignments and analysis of multiple genomic DNA sequences. Nucleic Acids Research 31(13): 3518–3524. https://doi.org/10.1093/nar/gkg579

Thomas GWC, Ather SH, Hahn MW (2017) Gene-Tree Reconciliation with MUL-Trees to resolve polyploidy events. Systematic Biology 66(6): 1007–1018. https://doi.org/10.1093/sysbio/syx044

Tillich M, Lehwark P, Pellizzer T, Ulbricht-Jones ES, Fischer A, Bock R, Greiner S (2017) GeSeq – versatile and accurate annotation of organelle genomes. Nucleic Acids Research 45(W1): W6–W11. https://doi.org/10.1093/nar/gkx391

Tomoko O (1995) Synonymous and nonsynonymous substitutions in mammalian genes and the nearly neutral theory. Journal of Molecular Evolution 40(1): 56–63. https://doi.org/10.1007/BF00166595

Walker JF, Yang Y, Feng T, Timoneda A, Mikenas J, Hutchison V, Edwards C, Wang N, Ahluwalia S, Olivieri J, Walker-Hale N, Majure LC, Puente R, Kadereit G, Lauterbach M, Eggli U, Flores-Olvera H, Ochoterena H, Brockington SF, Moore MJ, Smith SA (2018) From cacti to carnivores: Improved phylotranscriptomic sampling and hierarchical homology inference provide further insight into the evolution of Caryophyllales. American Journal of Botany 105(3): 446–462. https://doi.org/10.1002/ajb2.1069

Wu Z, Raven PH [Eds] (1996) Flora of China. Science Press (Beijing) & Missouri Botanical Garden Press (St. Louis), 15: 1–387.

Yang Z (1998) Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. Molecular Biology and Evolution 15(5): 568–573. https://doi.org/10.1093/oxfordjournals.molbev.a025957

Yao G, Jin J-J, Li H-T, Yang J-B, Mandala VS, Croley M, Mostow R, Douglas NA, Chase MW, Christenhusz MJM, Soltis DE, Soltis PS, Smith SA, Brockington SF, Moore MJ, Yi T-S, Li D-Z (2019) Plastid phylogenomic insights into the evolution of Caryophyllales. Molecular Phylogenetics and Evolution 134: 74–86. https://doi.org/10.1016/j.ympev.2018.12.023

Zhang X, Xu Y, Liu X (2020) Complete plastome sequence of *Limonium aureum*, a medicinal and ornamental species in China. Mitochondrial DNA. Part B, Resources 5(1): 333–334. https://doi.org/10.1080/23802359.2019.1703608

Zhong Q, Yang S, Sun X, Wang L, Li Y (2019) The complete chloroplast genome of the Jerusalem artichoke (*Helianthus tuberosus* L.) and an adaptive evolutionary analysis of the ycf2 gene. PeerJ 7: e7596. https://doi.org/10.7717/peerj.7596

Zhu A, Guo W, Gupta S, Fan W, Mower JP (2016) Evolutionary dynamics of the plastid inverted repeat: The effects of expansion, contraction, and loss on substitution rates. The New Phytologist 209(4): 1747–1756. https://doi.org/10.1111/nph.13743

## Supplementary material 1

**The output of the repeat analysis of four *Limonium* plastomes**

Authors: Ashwini M. Darshetkar, Satish Maurya, Changyoung Lee, Badamtsetseg Bazarragchaa, Gantuya Batdelger, Agiimaa Janchiv, Eun Ju Jeong, Sangho Choi, Ritesh Kumar Choudhary, Soo-Yong Kim

Data type: molecular analysis

Link: https://doi.org/10.3897/phytokeys.175.61054.suppl1

## Supplementary material 2

**Relative Synonymous Codon Usage**

Authors: Ashwini M. Darshetkar, Satish Maurya, Changyoung Lee, Badamtsetseg Bazarragchaa, Gantuya Batdelger, Agiimaa Janchiv, Eun Ju Jeong, Sangho Choi, Ritesh Kumar Choudhary, Soo-Yong Kim

Data type: molecular data

Link: https://doi.org/10.3897/phytokeys.175.61054.suppl2

## Supplementary material 3

**List of Positively selected genes**

Authors: Ashwini M. Darshetkar, Satish Maurya, Changyoung Lee, Badamtsetseg Bazarragchaa, Gantuya Batdelger, Agiimaa Janchiv, Eun Ju Jeong, Sangho Choi, Ritesh Kumar Choudhary, Soo-Yong Kim

Data type: molecular data

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# Taxonomic revision of *Habenaria josephi* group (sect. *Diphyllae* s.l.) in the Pan-Himalaya

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## Abstract

Species of the *Habenaria josephi* group in the Pan-Himalaya region are revised, based on their morphological characters and results of previous molecular phylogenetics. Eight distinctive species are recognised; key to the species, taxonomic descriptions, illustrations and distribution maps are provided. *Habenaria josephi* is re-instated, based on morphological and molecular evidence; *H. wolongensis* is synonymised with *H. aitchisonii*, a neotype for *H. tibetica* and the lectotypes for *H. balfouriana*, *H. fargesii*, *H. glaucifolia* and *H. clarkei* are designated.

## Keywords

Distribution, lectotypes, morphological characters, neotype, taxonomy

## Introduction

*Habenaria* Willd. is a large genus in the Orchidaceae (Orchidoideae, Orchideae, Orchidinae), with about 891 species (Govaerts et al. 2020), most of which are terrestrial plants. The genus is distributed along the tropical, subtropical and temperate zones of the Old and New Worlds (Pridgeon et al. 2001; Batista et al. 2011) and has three main centres of diversity, i.e. eastern Asia, central and southern Africa and Brazil (Kurzweil and Weber 1992). The plants are characterised by frequently having simple or bifid petals, a tripartite lip, long rostellar arms, stalked stigmas and a well-developed nectariferous spur (Dressler 1993; Pridgeon et al. 2001).

Of about 208 species of *Habenaria* occurring in the south to east Asian biodiversity hotspot, roughly one fifth are represented in the Pan-Himalaya (Pearce and Cribb 2002; Chen and Cribb 2009; Rajbhandari and Rai 2017; Govaerts et al. 2020). Amongst the Asian clades of Old World *Habenaria*, one group with two basal leaves and with a temperate or alpine distribution is of particular taxonomic interest: the *Habenaria josephi* Rchb.f. complex, as the species belonging to this group formed a close alliance (clade XXIV) in a recent molecular study (Jin et al. 2017). Additionally, they share many morphological traits (e.g. pubescent scape and floral parts, mostly 2-lobed petals) and similar habitats, which often renders the species delimitation difficult; owing to this, some species were either misidentified or assigned different ranks in the past (Lang 1984; Pearce and Cribb 2002; Lucksom 2007; Chen and Cribb 2009; Choudhury et al. 2011; Maity et al. 2019). To address these taxonomic inconsistencies, here we attempt to revise the group in light of the recent molecular works (Jin et al. 2017; Raskoti and Ale 2019) and a broader examination of the herbarium specimens and literature. Our study shows eight species from the group occurring in the region, for which general morphological features, taxonomic description and illustrations are provided. Furthermore, a brief history of *Habenaria* section *Diphyllae* Kränzl. and an artificial key to the species are also given.

## Materials and methods

### Study area

The study area Pan-Himalaya (also referred to as the PH hereafter) ranges from parts of Afghanistan in the west to the Yunnan Province of China in the east, forming a natural phytogeographic unit; it is further divided into 17 subregions (Hong 2015, Fig. 1).

This work is based on the review of relevant literature and examination of herbarium specimens, supplemented with observations made on living plants in natural habitats. The specimens of *Habenaria* collected from the PH (Fig. 1), preserved at the herbaria AMES, B, CAL, E, K, KATH, KUN, LD, LE, P, PE, S, TI, TUCH, UPS, W and WU (herbarium acronyms according to Thiers 2020) were thoroughly examined; CAL, KATH, PE and TUCH were personally visited and for others, online catalogues were utilised (e.g. 'www.cvh.ac.cn' for Chinese herbaria) and high-resolution images of putative type materials were requested.

More than 500 specimens were sorted and about 200 of them, occurring within the PH, were considered for taxonomic characterisation. Species descriptions are based on vegetative and reproductive features as observed directly and/or through stereomicroscopes (Nikon SMZ1000 and Leica S8 APO) for details. Floral parts were rehydrated in boiling water before their observation and measurements were made under the microscopes. Lip and spur morphology was particularly regarded as taxonomically-informative characters. For species delimitation, morphological species concept

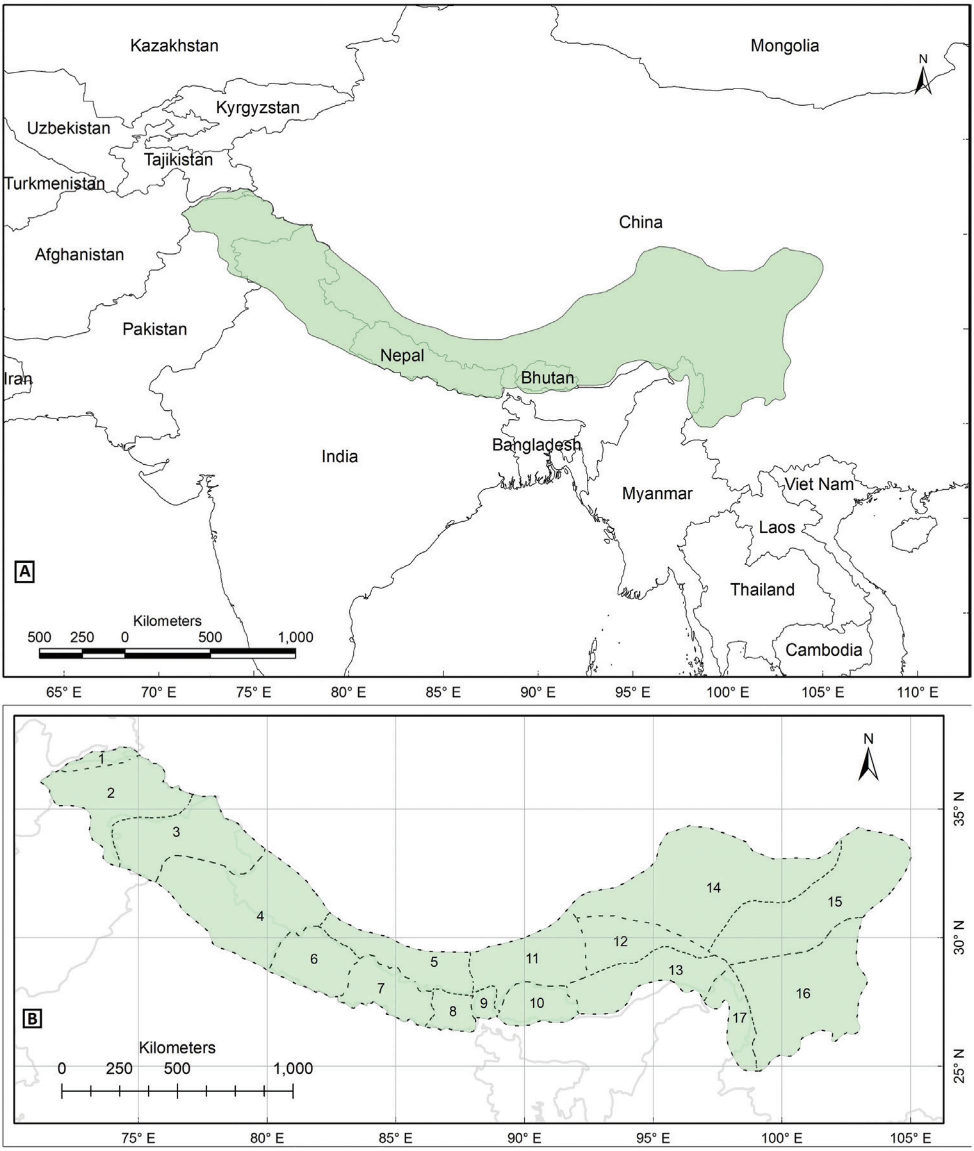

**Figure 1.** Biogeographical context of the study, the Pan-Himalaya **A** geographic location of the Pan-Himalaya (shaded with green colour) **B** subregions of the Pan-Himalaya (Hong 2015) **1** Vakhan **2** N Pakistan **3** Jammu and Kashmir **4** U Ganga and Indus **5** U Yarlung Zangbo **6** W Nepal **7** C Nepal **8** E Nepal **9** Sikkim and Darjiling **10** Bhutan **11** M Yarlung Zangbo **12** L Yarlung Zangbo **13** Yarlung Zangbo-Brahmaputra **14** Tangut **15** N Hengduan **16** S Hengduan **17** U Irrawaddy.

(Cronquist 1978; Stuessy 2009), along with recent molecular phylogeny (Jin et al. 2017), was taken into consideration.

Data on phenology, habitat and distribution were derived from specimen labels and the distribution maps were prepared from the occurrence locations approximated to the corresponding PH county or district; the list of examined specimens is arranged in the geographical order of the PH (Hong 2015). Information on distribution outside the PH and on illustrations available from literature are also provided.

## Results

### The taxonomic history of *Habenaria* sect. *Diphyllae*

*Habenaria* sect. *Diphyllae* is one of the 32 sections established by Kränzlin (1892) in his first worldwide revision of *Habenaria*, including 17 species from Africa and Asia. The sections he assigned were based on the degree of dissection of the petals and labellum and on the structure of the gynostemium. The presence of 3-lobed labellum, simple or bilobed broad petals and thick fleshy stigmatic processes were taken as the diagnostic characters for the section *Diphyllae* (Kränzlin 1892; Kränzlin 1901). Later, Summerhayes (1968) designated *H. diphylla* as the type for the section and ascribed 24 species from East Africa to it. Several sectional treatments of *Habenaria* are available for Neotropics and Africa (Batista et al. 2013; Summerhayes 1968), but comprehensive accounts of Asian species are lacking, though country-level treatments were attempted previously (e.g. Pearce and Cribb 2002). The present study has uncovered the occurrence of 11 species of *H.* sect. *Diphyllae* in the PH. Eight of these species are found in high mountain habitats (i.e. collected at an altitude above 1500 m) and represent a monophyletic clade, which is here designated as the *H. josephi* group. The three remaining species (*H. reniformis*, *H. diphylla* and *H. acianthoides*), in contrast, inhabit the tropical climate and are morphologically distinct (e.g. glabrous scape and floral parts, petals always simple) and will not be considered here.

### General morphology

**Tuber:** One or two, globose, oval or elliptic, underground, with few roots dispersed around their junction to the stem. **Stem:** Erect, terete and slender (sometimes robust in *Habenaria aitchisonii*), pubescent, often papillate in *H. diplonema*, *H. aitchisonii*, *H. szechuanica* and *H. tibetica*. **Leaves:** Typically two, opposite to sub-opposite, appressed to the ground, glabrous (densely papillate hairy in *H. diplonema*). Leaf blades orbicular or ovate-orbicular, with amplexicaul base and acute or mucronate apex (acuminate in *H. diplonema*). **Inflorescence:** Racemose, with few (two to six in *H. josephi*; up to eight in *H. glaucifolia*, *H. fargesii* and *H. szechuanica*; up to 14 in *H. diplonema*, *H. balfouriana* and *H. tibetica*) to many-flowered peduncle (sometimes reaching up to 40 flowers in *H. aitchisonii*). Floral bracts are mostly lanceolate with acuminate apex and densely pubescent. **Ovary and pedicel:** Pubescent, sometimes papillate (e.g. *H. aitchisonii*, *H. balfouriana*, *H. szechuanica* and *H. tibetica*); curved and twisted. **Flower:** Generally small-sized (smallest in *H. diplonema*, larger in *H. glaucifolia*, *H. szechuanica* and *H. tibetica*), greenish to greenish-yellow (Fig. 2), sometimes fragrant (e.g. *H. diplonema* and *H. josephi*). Dorsal sepal forms the hood

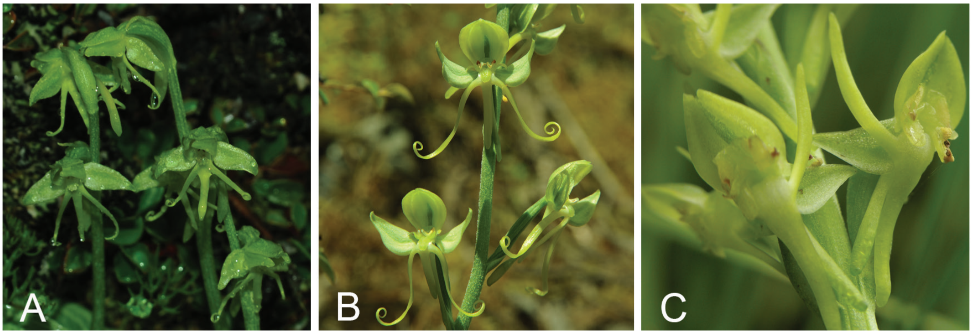

**Figure 2.** Floral structure in *Habenaria josephi* group **A** *H. josephi* **B** *H. glaucifolia* **C** *H. aitchisonii* (Photographs by X.H. Jin).

together with the petals, while lateral sepals are deflexed. Six of the species are with distinct 2-lobed petal; *H. fargesii* has filiform, long anterior lobe; in *H. glaucifolia*, anterior lobe is lanceolate and smaller than posterior lobe and in *H. aitchisonii*, *H. balfouriana*, *H. szechuanica* and *H. tibetica*, it is represented by a small tooth at the base of the petals. *H. diplonema* and *H. josephi* are with entire petals, albeit the basal part is broadened. Lip shape is a taxonomically-informative feature in this group; though all the species possess 3-lobed lips, the relative size and orientation of especially the lateral lobe is quite variable; *H. glaucifolia*, *H. szechuanica* and *H. tibetica* have much longer lateral lobes which ultimately are coiled at the tip; lateral lobes of *H. aitchisonii* and *H. balfouriana* are reflexed up, while those in *H. josephi*, *H. diplonema* and *H. fargesii* are deflexed. A prominent needle-like appendage present at the base of the lip near the opening of the spur is characteristic of *H. szechuanica*. Spur, too, exhibits remarkable variation; *H. diplonema* bears a very short spur, in *H. aitchisonii*, spur is shorter than ovary, while in the rest of the species, the spur is robustly longer than the ovary. The column is well demarcated in all of the species with parallel anthers and stout caudicles; *H. josephi* and *H. glaucifolia* have parallel, closely lingulate stigmatic process; the stigma of *H. aitchisonii* are concave surrounding the opening of the spur.

**Ecology:** All species in the *Habenaria josephi* group are terrestrial herbs growing on moist grasslands, rocky surfaces and alpine gullies. Generally, they occur in the elevation range of 2000–5000 m (Fig. 3).

## Taxonomic synopsis

### *Habenaria* sect. *Diphyllae* Kränzl., Bot. Jahrb. Syst. 16(2): 147. 1892.

### Key to the species of *Habenaria*. sect. *Diphyllae* from the Pan-Himalaya

1a Plants 4–20 cm tall; petals entire .......... **2a**
2a Rachis and ovary pubescent; sterile bracts absent .......... **3a**
3a Leaves glabrous; floral bracts ca. 10 mm long; ovary and pedicel 8–14 mm long; lateral lobes of lip linear with coiled tips, slightly longer than median lobe; spur 8–15 mm long.......... **1. *H. josephi***

3b Leaves papillate; floral bracts ca. 3 mm long; ovary and pedicel ca. 7 mm long; lateral lobes of lip filiform with straight tips, much longer than median lobe; spur ca. 4 mm long .......... **2. *H. diplonema***

2b Rachis and ovary glabrous; sterile bracts present .......... **4a**

4a Lip 3-lobed; spur distinct and always present .......... **5a**

5a Leaves usually 2; ovary ca. 10 mm long; petals falcate linear-lanceolate .......... **9. *H. diphylla***

5b Leaf usually one; ovary 4–5 mm long; petals obliquely ovate .......... **10. *H. aciantholdes***

4b Lip simple, linear; spur indistinct or absent .......... **11. *H. reniformis***

1b Plants 10–50 cm tall; petals distinctly 2-lobed .......... **6a**

6a Petals deeply 2-lobed, lower lobe linear to linear lanceolate, more than 4 mm long; lateral lobe of lip linear, with circinate tip .......... **7a**

7a Petal upper lobe falcate-oblong, ca. 4 mm long; lower lobe ca. 10 mm long; floral bracts ca. 7 mm long; ovary cylindric, 12–20 mm long... **3. *H. fargesii***

7b Petal upper lobe spatulate-oblong, 12–15 mm long, lower lobe ca. 4 mm long; floral bracts ca. 15 mm long; ovary terete, 22–35 mm long.... **4. *H. glaucifolia***

6b Petals shallowly 2-lobed, lower lobe like a tooth, less than 2 mm long; lateral lobe of lip linear, retrorse, with bent, but not circinate tip .......... **8a**

8a Sepals 3–7 mm long, 2.5–4 mm broad; petals glabrous; spur reflexed, pendulous .......... **9a**

9a Inflorescence laxly to densely many flowered; ovary with pedicel 7–12 mm long; spur 7–8 mm long .......... **5. *H. aitchisonii***

9b Inflorescence subdensely 3–12 flowered; ovary with pedicel 8–10 mm long; spur 12–20 mm long .......... **6. *H. balfouriana***

8b Sepals 7–11 mm long, 3–5 mm broad; petals ciliate; spur spreading horizontally .......... **10a**

10a Leaves with green veins adaxially; lip base with a needle-like appendage near entrance of spur .......... **7. *H. szechuanica***

10b Leaves with white veins adaxially; lip base lacking appendage near entrance of spur .......... **8. *H. tibetica***

**Note.** Of the 11 species of sect. *Diphyllae* from the Pan-Himalaya, only the eight species that belong to the high mountain clade (Jin et al. 2017, Fig. 3) are presented here.

## 1. *Habenaria josephi* Rchb. f., Trans. Linn. Soc. London, Bot., ser. 2, 3: 114 (1888).

≡ *Habenaria aitchisonii* var. *josephi* (Rchb.f.) Hook.f., Fl. Brit. India 6: 152 (1890).

≡ *Habenaria diphylla* var. *josephi* (Rchb.f.) N. Pearce & P.J. Cribb, Edinburgh J. Bot. 58: 114. 2001. Type. INDIA, Sikkim, 1849, *J.D. Hooker 42* [holotype: K (K000247480 image!); isotypes: K, AMES (00256484 image!), P (P00370608 image!), LE n.v.].

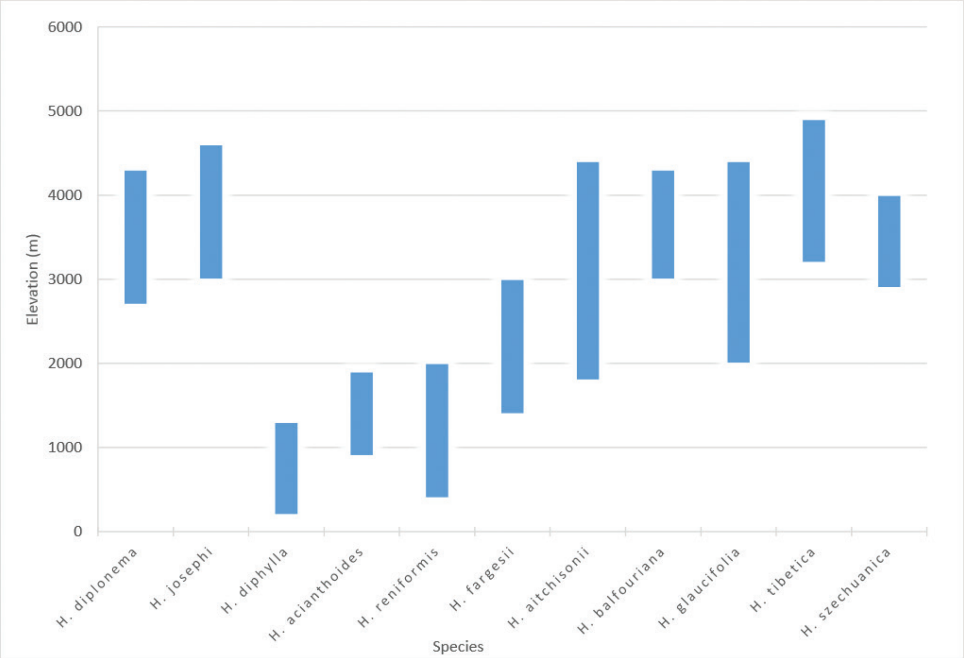

**Figure 3.** Altitudinal range of *Habenaria* sect. *Diphyllae* in the Pan-Himalaya.

*Habenaria clarkei* Kränzl., Bot. Jahrb. Syst. 16: 148 (1892). Type. INDIA, Sikkim, *J. D. Hooker 42* [lectotype designated here: K (K000247480 image!); isolectotypes: K, AMES (00256484 image!), P, LE n.v.].

**Description.** Terrestrial herbs, 5–20 cm tall. Tubers ovoid-fusiform. Stems pubescent. Leaves 2, opposite, basal; sheathing at base; leaf blade broadly ovate-orbicular to weakly cordate, 1.5–3.1 cm long, 1–2.5 cm broad, apex apiculate. Inflorescences 4–15 cm long, laxly to subdensely 2- to 6-flowered; rachis minutely glandular, pubescent, 1.5–3.8 cm long; floral bracts narrowly lanceolate, ca. 1 cm long, pubescent, apex acuminate. Flowers green, fragrant; ovary and pedicel curved, 7–13 mm long, pubescent. Dorsal sepal ovate, 5.5–6 mm long, 2.5–3 mm broad, apex acute, forming hood with petals; lateral sepals ovate, reflexed to spreading, 5–7 mm long, ca. 2.6 mm broad, apex acute. Petals obliquely ovate-triangular, base broad, 5–6 mm long, 2–2.5 mm wide, apex acute; lip 3-lobed, clawed, spurred; lateral lobes linear, apex recurved-coiled, 6–9 mm long, ca. 0.6 mm wide; mid-lobe linear, ca. 5 mm long, ca. 0.5 mm broad; spur curved, clavate, 8–15 mm long. Column stout; anther locules parallel; pollinia globose-ovoid; caudicle stout; stigma processes closely parallel, united above mouth of spur, lingulate. (Fig. 4).

**Phenology.** Flowering from July to September.

**Habitat.** Moist grassy hillsides, stream banks, in *Betula* forest; 3000–4600 m elev.

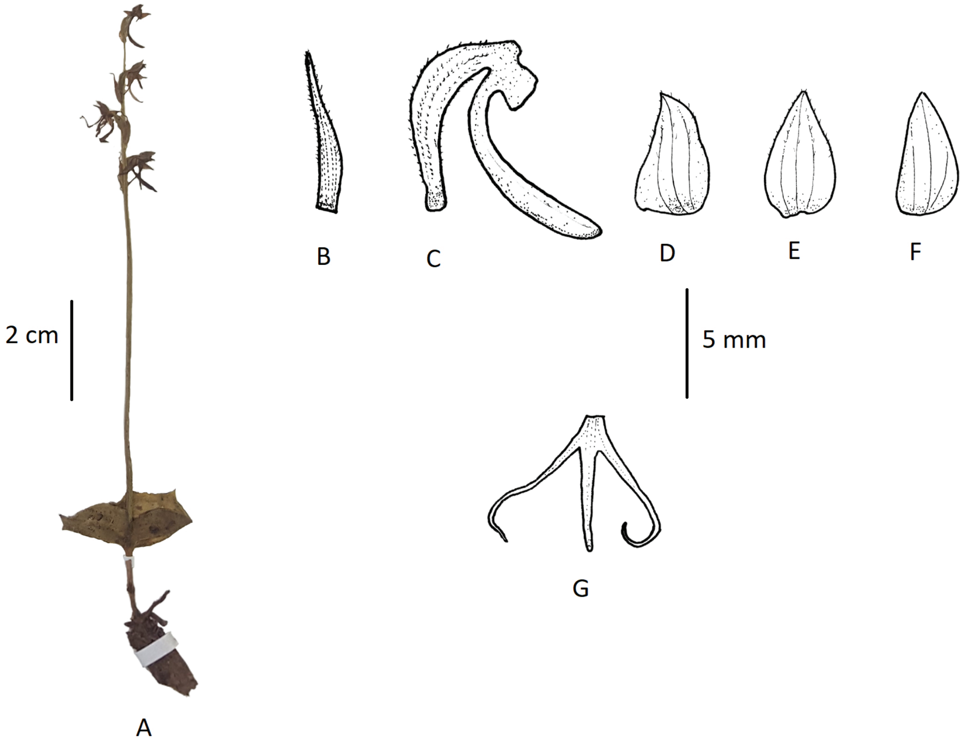

**Figure 4.** *Habenaria josephi* **A** habit **B** floral bract **C** pedicellate ovary with spur **D** petal **E** dorsal sepal **F** lateral sepal **G** lip (**A** photographed from *FLPH Expedition 13-0845*, PE **B–G** drawn from the same specimen by T.R. Pandey).

**Distribution.** Endemic to the Pan-Himalaya; Sikkim and Darjeeling, Bhutan, M Yarlung Zangbo, Yarlung Zangbo-Brahmaputra and S Hengduan. (Fig. 5).

**More illustrations.** Pearce and Cribb (2002, fig. 41, a–m; as *H. diphylla* var. *josephi*).

**Additional specimens examined. SIKKIM and DARJEELING: Sikkim,** Tungu, 3648–3952 m elev., 1849, *J.D. Hooker 42* (K). **BHUTAN: Bumthang**, Marlungm Tsampa, 4400 m elev., 1949, *F. Ludlow*, *G. Sherriff & J.H. Hicks 19397* (AMES 01946674); **Gasa**, Upper Mo Chu, 4120 m elev., 1984, *I.W.J. Sinclair & D.G. Long 5289* (RENZ); **Gasa**, Gafoo-la, Upper Pho Chu, 4000 m elev., 1949, *F. Ludlow*, *G. Sherriff & J.H. Hicks 16725* [CAL (CAL0000056823), AMES (01946675)]; **Jomolhari** (GLORIA site), 4514 m elev., 2010, *S. Den et al.* 193 (National Biodiversity Centre, Bhutan). **M YARLUNG ZANGBO: Yadong,** Phari, 1879, *Dungboo s. n.* [K (K000247481), CAL (CAL0000092705)]; **Yadong**, North of Phari, 1882, *Dr. King's collectors s. n.* (CAL0000092702); **Yadong**, Tem-la, North of Phari, 1882, *G. King's collectors s. n.* (CAL0000092703). **YARLUNG ZANGBO-BRAHMAPUTRA: Cona**, 3641 m elev., 2013, *FLPH Tibet Expedition 13-0957* (PE); **Zayü**, 4100 m elev., 2013, *FLPH Tibet Expedition 13-0845* (PE). **S HENGDUAN: Yulong**, eastern flank of Lichiang range, 3500 m elev., 1906, *G. Forrest 2739* (CAL0000055843).

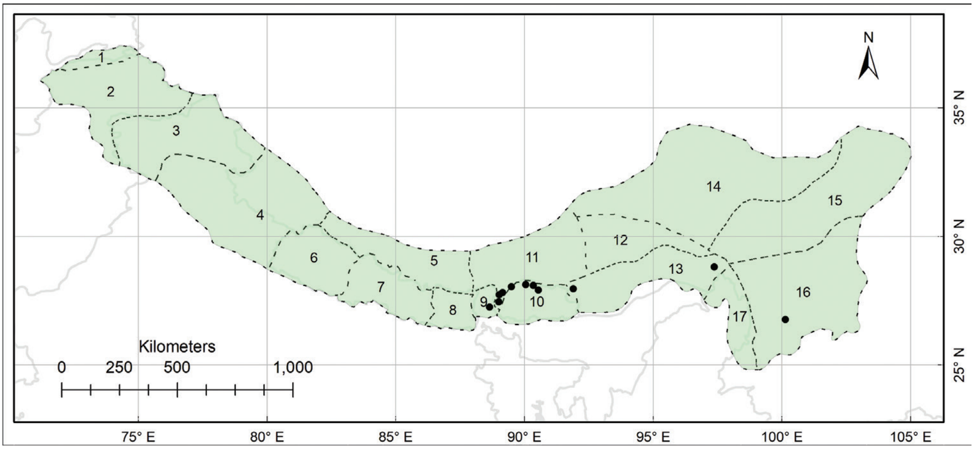

**Figure 5.** Distribution of *Habenaria josephi* (black circles) in the Pan-Himalaya.

**Note.** Hooker (1890) tentatively placed some of the *Habenaria* species, such as *H. reniformis* (D. Don) Hook.f., *H. diphylla* Dalz. and *H. aitchisonii* Rchb.f., into the section *Trimeroglossa*. However, *H. josephi* was assigned a varietal rank under *H. aitchisonii*. Later, Kränzlin (1892, 1901) proposed the section *Diphyllae* to accommodate the species with two basal leaves and included *H. clarkei*, *H. glaucifolia*, *H. diphylla*, *H. reniformis* and *H. aitchisonii* in the section along with a few other *Habenaria* species. He followed Hooker's view regarding the position of *H. josephi* as a variety of *H. aitchisonii*, albeit with a note "*Die var. Josephi Hook. f. ist nur eine Form, aus den höchsten der oben angegebenen Standorte stammend* (the variety *josephi* is only a form occurring in the highest of the above-mentioned locations)" suggesting that the plant is merely a higher elevation variety of *H. aitchisonii*. Paradoxically, Kränzlin described *H. clarkei* in the same publication, based on a duplicate of *Hooker 42* (the type specimen of *H. josephi*) from Sikkim, which was kept at B (distributed from Hooker's Herbarium at K). After an extensive search, it now appears that *Hooker 42* had at least six duplicates: two at K and one each at P, AMES, B and LE, of which the specimen at B was lost during the Second World War, while at LE, the specimen could not be found during a recent search (fide Petr Efimov). The remaining four duplicates are still extant.

In the past, the taxonomic identity of *Habenaria josephi* became doubtful, often shifting from one name to another, sometimes as *Habenaria aitchisoni* var. *josephi* (Rchb.f.) Hook.f. or as *H. diphylla* var. *josephi* (Rchb.f.) Pearce & Cribb. Even in recent literature on the orchid species of Sikkim, its type locality is not uniform in this regard. Some botanists treat it as a variety of *H. diphylla* (Pearce et al. 2001; Pearce and Cribb 2002; Lucksom 2007; Choudhury et al. 2011), while Maity et al. (2019) regard it merely as another synonym of *H. diphylla*. A closer look at this species reveals it to be not only distinct morphologically, but also well characterised in terms of habitat and distribution. Whereas *H. diphylla* is predominantly a tropical species of moderate size (10–40 cm tall), broadly distributed from peninsular India to the Philippines, *H. josephi* is a small-sized (5–20 cm tall) high-elevation temperate to alpine species occurring from Sikkim eastwards to the Hengduan Mountains, i.e. it is endemic to the Pan-Himalaya. Pearce et al. (2001) provided an elaborate discussion on the phenetic variations that delineate this taxon from the other species, yet they assigned it to a varietal rank under *H. diphylla*. Examination of the type and other dried specimens, as well as living individuals, clearly shows that it is distinct. Short stature, scape without sterile bracts, 2–6-flowered rachis, curved, pubescent ovary, deflexed lateral sepals and lip with stooping (reflexed) lateral lobes that ultimately coil around terminally are amongst the unique diagnostic morphological features. Furthermore, recent molecular studies (Jin et al. 2017; Raskoti and Ale 2019) have consolidated its distinction from similar-looking species. Therefore, a specific rank seems fully justified here.

### 2. *Habenaria diplonema* Schltr., Notes Roy. Bot. Gard. Edinburgh 5: 100. 1912.

**Type.** China, Yunnan, 3300–3600 m elev., 1906. *G. Forrest 2812* [holotype: E (E00381985 image!); isotypes: IBSC (0635875!), CAL (CAL0000000748!), P (P00426408 image!)].

**Description.** Terrestrial herbs, 4–15 cm tall. Tubers globose-oblong. Stems densely papillate-pubescent. Leaves 2, opposite, basal; sheathing at base; leaf blade ovate to orbicular, 1–2.4 cm long, 1–2.2 cm broad, adaxially with yellowish-white venation, densely papillate, apex acute to acuminate. Inflorescence 3–12 cm long, sparsely 2–14-flowered; rachis 2–5.5 cm long, pubescent; floral bracts lanceolate, 3–6 mm long, apex acuminate. Flowers green, faintly fragrant; ovary and pedicel curved, 6–8 mm long, pubescent. Dorsal sepal broadly ovate, ca. 3.5 mm long, ca. 3 mm broad, glabrous, apex obtuse; lateral sepals oblique, ovate-elliptic, deflexed, ca. 4 mm long, ca. 2.5 mm broad, glabrous, apex obtuse. Petals obliquely falcate-ovate, ca. 3.5 mm long, 2–2.5 mm broad, glabrous, entire; lip 3-lobed, spurred; lateral lobes filiform, ca. 10 mm long; mid-lobe linear-lingulate, ca. 3 mm long; spur pendulous, clavate, 1–4 mm long. Column short; anther apex retuse; caudicles short; stigma processes clavate. (Fig. 6).

**Phenology.** Flowering from July to September.

**Habitat.** Shady cliffs and rocks; 2700–4300 m elev.

**Distribution.** S Hengduan; also in N Fujian of China. (Fig. 7).

**More illustrations.** Wu et al. (2010, fig. 192, 7–9).

**Additional specimens examined. S. HENGDUAN: Muli**, Rangetzantze, 3500 m elev., 1937, *T.T. Yü 14014* (KUN, PE); **Yulong (Lijiang)**, 2800 m elev., 1935, *C.W. Wang 70748* (PE, KUN); **Yulong (Lijiang)**, 4200 m elev., 1914, *C. Schneider 2459* (K).

**Note.** The photograph of the type specimen housed at the herbarium of Royal Botanic Garden Edinburgh (*G. Forrest 2812*, E00381985) was published along with the protologue, therefore this specimen is the holotype (Art. 9.1, Note 1 (b), Turland et al. 2018).

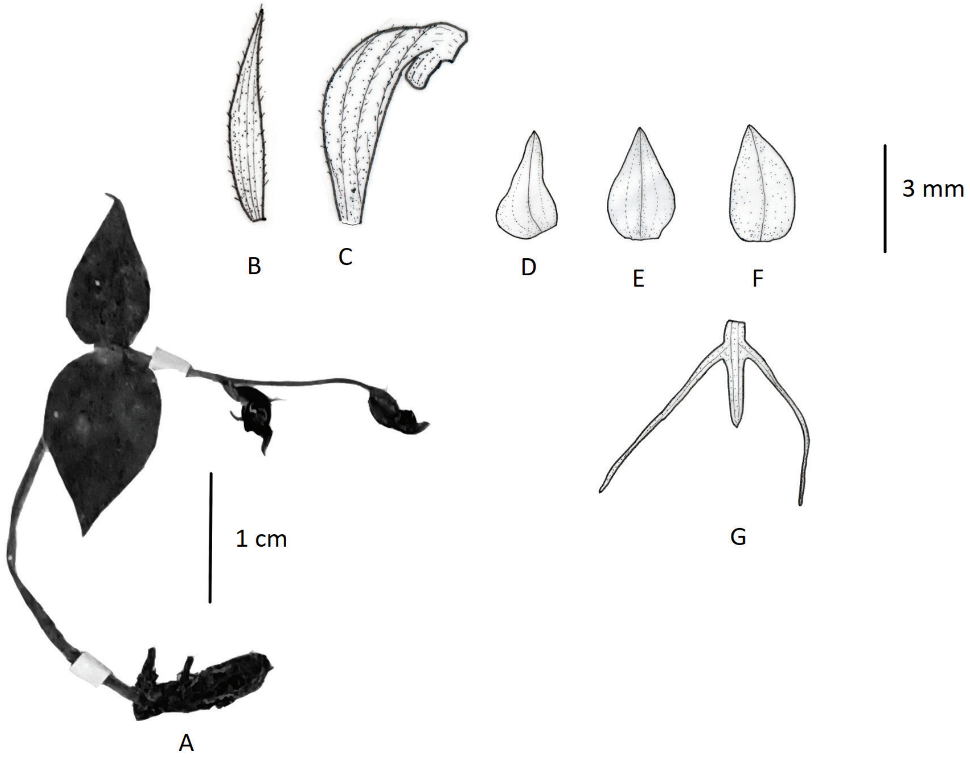

**Figure 6.** *Habenaria diplonema* **A** habit **B** floral bract **C** pedicellate ovary with spur **D** petal **E** dorsal sepal **F** lateral sepal **G** lip (**A** photographed from *T.T. Yu 14014*, PE **B–G** drawn from the same specimen by T.R. Pandey).

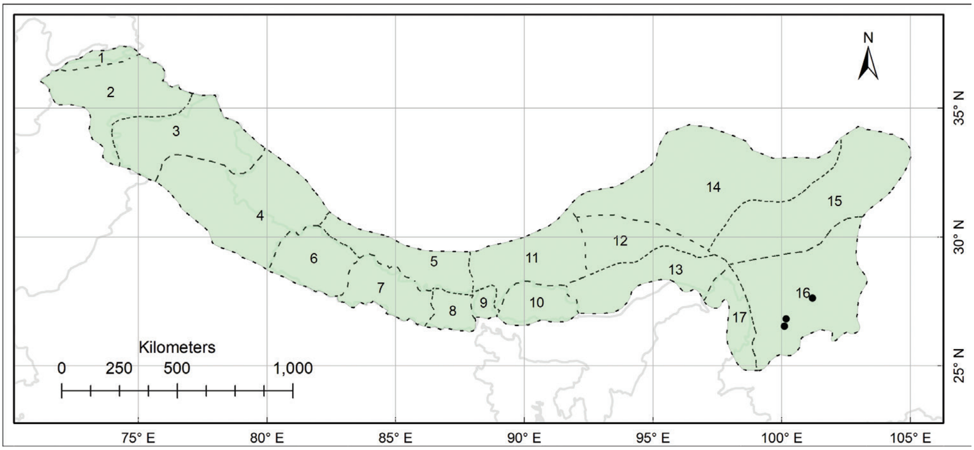

**Figure 7.** Distribution of *Habenaria diplonema* (black circles) in the Pan-Himalaya.

### 3. *Habenaria fargesii* Finet, Rev. Gen. Bot. 13: 528, t. 18A. 1–8. 1901.

**Type.** China, Sichuan, 1400 m elev., 1893, *Farges 1279* [lectotype designated here: P (P00426411 image!)].

**Description.** Terrestrial herbs, 13–37 cm tall. Tubers ovoid or oblong. Stems erect or ascending, finely papillate-pubescent. Leaves 2, opposite, basal; base narrowed and amplexicaul; leaf blade spreading horizontally, adaxially yellowish-white marked, ovate or orbicular, 4–6 cm long, 3.5–6 cm broad, apex acute. Inflorescence 10–30 cm long, 4–9-flowered; rachis 5–11 cm long, finely papillate-pubescent; floral bracts lanceolate, ca. 7 mm long, apex acuminate. Flowers yellowish-green; ovary and pedicel twisted, 1.2–2 cm long, pubescent. Dorsal sepal ovate, 3–3.5 mm long, ca. 2.5 mm broad, margins ciliate, apex acute; lateral sepals strongly reflexed, obliquely ovate, 5–7 mm long, ca. 4 mm broad, margins ciliate, apex acute. Petals connivent with dorsal sepal, deeply 2-lobed; upper lobe falcate-oblong, ca. 4 mm long; lower lobe linear, ca. 10 mm long; lip deeply 3-lobed above the base, spurred; lateral lobes divaricate, filiform, ca. 1.5 cm long, apex curled; mid-lobe linear, ca. 1 cm long; spur pendulous, 2–2.5 cm long. Column short and broad, anther connective wide; pollinia granular; caudicles linear; stigmatic processes elongated. (Fig. 8).

**Phenology.** Flowering in July to September.

**Habitat.** Montane forests, grassy valleys; 1400–3000 m elev.

**Distribution.** N and S Hengduan; also in Chongqing and Gansu of China (Fig. 9).

**More illustrations.** Finet (1901, fig. 18A, 1–8).

**Additional specimens examined. N HENGDUAN:** Sichuan, *T.T. Li 644* (PE). **S HENGDUAN: Yanyuan**, 3000 m elev., 2015, *FLPH Sichuan Expedition 152179* (PE).

### 4. *Habenaria glaucifolia* Bureau & Franch., J. Bot. (Morot) 5: 152. 1891.

≡ *Senghasiella glaucifolia* (Bureau & Franch.) Szlach. J. Orchideenfreund 8: 365. 2001.

**Type.** China, Sichuan, *Prince Henry D'Orleans s. n.* [lectotype designated here: P (P00426784 image!)].

**Description.** Terrestrial herbs, 12–50 cm tall. Tubers oblong or ovoid. Stems erect, pubescent. Leaves 2, opposite, basal; base obtuse-rounded and amplexicaul; leaf blade spreading horizontally, abaxially tinged with greyish-white, adaxially purplish-green, ovate-orbicular, 2–5 cm long, 1–4.7 cm broad, apex acute-acuminate. Inflorescence 8–45 cm long, 2–8-flowered; rachis 4–18 cm long, pubescent; floral bracts lanceolate or ovate, apex acuminate. Flowers yellowish to yellowish-white; ovary and pedicel twisted, 2.2–3.5 cm long, pubescent. Dorsal sepal forming a hood with petals, erect, ovate or oblong, concave, 10–13 mm long, 6–7 mm broad, apex obtuse; lateral sepals reflexed, obliquely ovate or oblong, 11–14 mm long, 7–7.5 mm broad, apex acute. Petals deeply 2-lobed; upper lobe spatulate-oblong, 12–15 mm long, ca. 6 mm broad, margin ciliate, apex obtuse; lower lobe linear-lanceolate, ca. 4 mm long, ca. 1 mm broad, apex acute; lip reflexed, base with a short claw, deeply 3-lobed, spurred; lateral

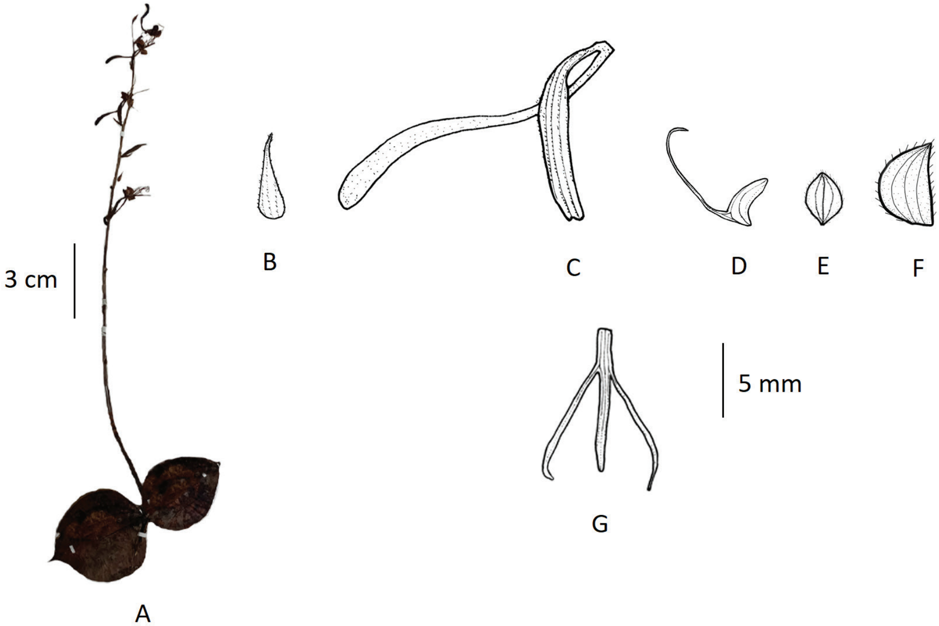

**Figure 8.** *Habenaria fargesii* **A** habit **B** floral bract **C** pedicellate ovary with spur **D** petal **E** dorsal sepal **F** lateral sepal **G** lip (**A** photographed from *FLPH Sichuan Expedition 152179*, PE **B–G** drawn from the same specimen by T.R. Pandey).

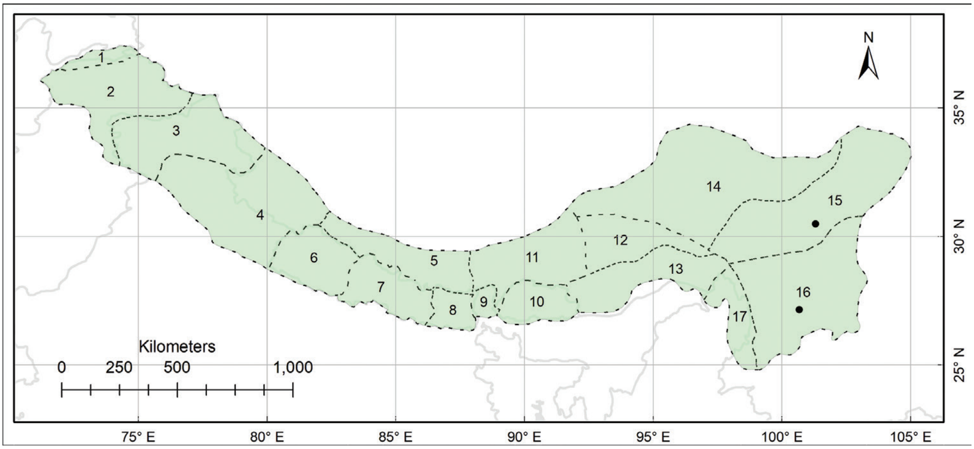

**Figure 9.** Distribution of *Habenaria fargesii* (black circles) in the Pan-Himalaya.

lobes divaricate, linear, 2.5–4 cm long, circinate toward apex; mid-lobe straight, linear, 1.1–1.4 cm long; spur pendulous, cylindrical-subclavate, 2–3 cm long. Column short; anther parallel, connective wide; pollinia granular; caudicles slender, elongated; stigmatic processes closely parallel, lingulate. (Fig. 10).

**Phenology.** Flowering from July to September.

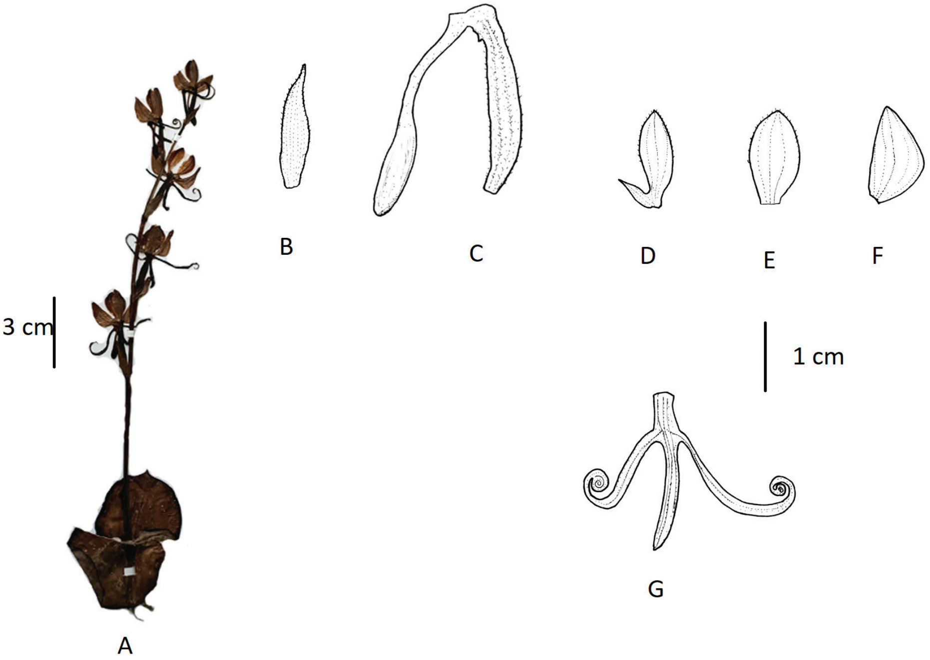

**Figure 10.** *Habenaria glaucifolia* **A** habit **B** floral bract **C** pedicellate ovary with spur **D** petal **E** dorsal sepal **F** lateral sepal **G** lip (**A** photographed from *K.Y. Lang et al. 945*, PE **B–G** drawn from the same by T.R. Pandey).

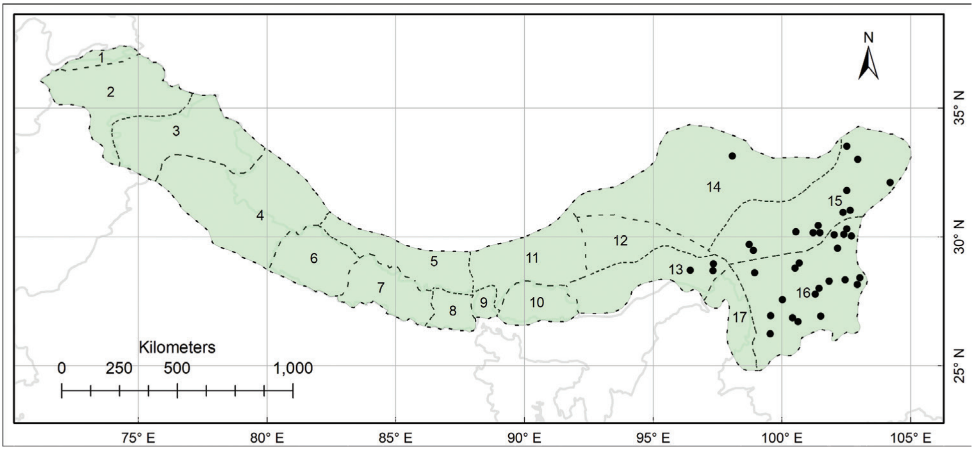

**Figure 11.** Distribution of *Habenaria glaucifolia* (black circles) in the Pan-Himalaya.

**Habitat.** Montane forests, grasslands; 2000–4400 m elev.

**Distribution.** Yarlung Zangbo-Brahmaputra, Tangut, N and S Hengduan; widely spread in eastern part of Pan-Himalayan Region. Also found in Gansu, Guizhou and Shaanxi of China. (Fig. 11).

**More illustrations.** Wu et al. (2010, fig. 194, 1–2).

**Additional specimens examined. YARLUNG ZANGBO-BRAHMAPUTRA: Mishmi Hills**, 1911, *Bailey s. n.* (E); **Zayü**, Guyu, Luoma, 2996 m elev., 2009, *X.H. Jin et al. SET-ET344* (PE); **Zayü**, Shang, 3400 m elev., 2013, *Jin et al. ST-2554* (PE). **TANGUT: Sêrxü**, 3900 m elev., 1974, *Vegetation Team of Sichuan 5771* (PE). **N HENGDUAN: Danba**, 3000 m elev., 1940, *Qu 7523* (PE); **Heishui**, 3200 m elev., 1957, *Li 73181* (PE); **Heishui**, 1959, *Chuan 1432* (PE); **Hongyuan**, 2900 m elev., 1957, *Zhang & Zhou 22665* (PE); **Jinchuan**, 2450 m elev., 1957, *Li 75398* (PE); **Litang-Yalong**, 1921, *F. Kingdon-Ward 4466* (E); **Maoxian**, 1952, *He & Zhou 13230* (PE); **Markam**, 2700 m elev., 1957, *s. coll. 22969* (PE); **Markam**, 2900 m elev., *W.L. Chen 766* (PE); **Xiaojin**, Gasiling, 3100 m elev., 1957, *J. Zhou 204* (IBSC); **Xiaojin**, 2450 m elev., 1958, *Zhang & Wang 5907* (PE); **Yajiang**, 3700 m, elev.1961, *Jiang 9863* (PE); **Yajiang**, 2875 m elev., 2006, *Boufford et al.* 35947 (PE, KUN). **S HENGDUAN: Daocheng**, 3100 m elev., 1973, *Sichuan Vegetation Survey Team 2391* (PE): **Daocheng**, 4300 m elev., 1973, *Sichuan Vegetation Survey Team 3645* (PE): **Dêqên**, 3100 m elev., 1937, *T.T. Yü 9867* (PE); **Eryuan**, 2900 m elev., 1963, *NW Yunnan Team 6339* (PE); **Ganluo**, 2000 m elev., 1959, *Chuan 4191* (PE); **Kangding**, 1951, *Hu & He 10401* (PE); **Kangding**, 3500 m elev., 1963, *Guan & Wang 808* (PE); **Kangding**, 3650 m elev., 1982, *K.Y. Lang et al. 945* (PE); **Luding**, 2420 m elev., 1959, *Jiang & Jin 1949* (PE); **Meigu**, 2100 m elev., 1959, *Chuan 1113* (PE); **Meigu**, 2300 m elev., 1976, *s. coll. 13083* (PE); **Mianning**, 3300 m elev., 1940, *Qu 7353* (PE); **Muli**, 1921, *F. Kingdon-Ward 4571* (E); **Muli**, Ye-tze, 3100 m elev., 1937, *T.T. Yü 7022* (PE); **Shangri-la**, 3200 m elev., 2010, *Kham Expedition 10-3079* (PE); **Tianquan**, 1959, *Chuan 853* (PE); **Weixi**, 3200 m elev., 1935, *C.W. Wang 68011* (PE); **Yanyuan**, 2500–2600 m elev., 1960, *Jiang 5991* (PE); **Yulong (Lijiang)**, 2900 m elev., 1981, *Hengduanshan Team of Beijing Institute 02666* (PE); **Yulong (Lijiang)**, 1910, *G. Forrest 6050* (PE).

## 5. *Habenaria aitchisonii* Rchb.f., Trans. Linn. Soc. London, Bot., ser. 2, 3: 113. 1888.

*Habenaria wolongensis* K.Y. Lang, Acta Phytotax. Sin. 22(4): 314, 1984, syn. nov. Type. CHINA, Sichuan, Wolong, 2200 m elev., 1982, *K. Y. Lang, L. Q. Li & Y. Fei 1528* [lectotype designated by Pandey and Jin (in press): PE (01147127!), isolectotype: PE (01147128!)].

*Habenaria diceras* Schltr., Notes Roy. Bot. Gard. Edinburgh 5: 101, t. 78. 1912. Type. CHINA, Yunnan, Lijiang Range eastern flank, 2700–3000 m elev., 1906, *G. Forrest 3074* [holotype: E (E00381986 image!), isotype: P (P00426380 image!].

*Habenaria bihamata* Kränzl., Repert. Spec. Nov. Regni Veg. 17: 106. 1921. Type. CHINA, Yunnan, Pe yen tsin, *S. Ten s. n.* (type: B n.v., probably lost).

*Habenaria pubicaulis* Schltr., Acta Horti Gothob. 1: 139. 1924. Type. CHINA, Sichuan, ca. 3900 m elev., 1922, *Harry Smith 3858* [holotype: UPS (V-091292 image!); isotypes: PE (01516965!), E (E00381983 image!), LD (1073030 image!), S (S07-285 image!)].

**Type.** Afghanistan (now Pakistan), Darban Valley, Kuram District, 2280 m elev., 1880, *Aitchison 413* [holotype: K (K000247484 image!), isotype: AMES (00256482 image!)].

**Description.** Terrestrial herbs, 10–50 cm tall. Tubers oblong or ellipsoid. Stems erect, papillate-pubescent. Leaves 2, opposite, basal; base narrowed and amplexicaul; blade ovate or ovate-orbicular, 2–7 cm long, 1.5–6 cm broad, apex acute. Inflorescence 8–45 cm long, laxly to densely many (up to 40) flowered; rachis 1.5–8 cm long, papillate-pubescent; sterile bracts ovate to lanceolate, 0.7–1.5 cm long, acuminate; floral bracts ovate-lanceolate, margins ciliate, ca. 0.7 cm long, apex acuminate. Flowers bright green, with often faintly yellowish lip; ovary and pedicel arcuate, 0.7–1.2 cm long, papillate-hairy. Dorsal sepal forming a hood with petals, erect, ovate, concave, 3–5 mm long, 2.5–3.5 mm broad, apex obtuse or acute; lateral sepals reflexed, obliquely ovate-oblong, 3.5–5.5 mm long, 2.5–3 mm broad, apex obtuse or acute. Petals 2-lobed, glabrous; upper lobe obliquely falcate-lanceolate, 3–5 mm long, 1.5–2 mm broad; lower lobe a tooth at the base of upper lobe, ca. 0.5 mm long; lip deeply 3-lobed near the base, spurred; lateral lobes linear, retrorse, embracing erect sepals, 6–12 mm long, apex slightly bent; mid-lobe reflexed, linear, 5–9 mm long, ca. 1 mm broad; spur pendulous, cylindrical-clavate, 7–8(-10) mm long. Column stout, anthers diverging, connective wide; pollinium granular; caudicles short, stout; stigmatic processes converging, subclavate. (Fig. 12).

**Phenology.** Flowering from July to September.

**Habitat.** Open *Juniperus*/*Larix* montane forests, thickets, grasslands; 1800–4400 m elev.

**Distribution.** N Pakistan, U Ganga and Indus, U Yarlung Zangbo, W, C and E Nepal, Sikkim and Darjeeling, Bhutan, M Yarlung Zangbo, L Yarlung Zangbo, Yarlung Zangbo-Brahmaputra, N and S Hengduan. (Fig. 13).

**More illustrations.** Wu et al. (2010, fig. 192, 4–6).

**Additional specimens examined. N PAKISTAN: Hazara**, Rawalpindi district, 2500 m elev., 1975, *J. Renz 10800* (RENZ). **U GANGA & INDUS**: **Mussoorie**, 2128 m elev., 1898, *P.W. Mackinnon s. n.* (CAL0000092710); **Mussoorie**, 1824 m elev., 1899, *P.W. Mackinnon 22991* (CAL0000092691); **Mussoorie**, 2300 m elev., 1983, *J. Renz & Y.P.S. Pangtey 13641* (RENZ). **U YARLUNG ZANGBO**: **Dinggyê**, 4151 m elev., 2013, *PE-Tibet team 3421* (PE); **Gyirong (Jilong)**, 3700 m elev., 2013, *PE-Tibet team 3825* (PE); **Gyirong (Jilong)**, 2950 m elev., 1975, *Qinghai-Tibet Team 7034* (PE). **W NEPAL**: **Karnali**, Mugu, Pina-Ghurchi, 2800 m elev., 1985, *P.R. Shakya*, *M.N. Subedi*, *R.K. Uprety 8783* (KATH); Jumla to Dori Lekh, 3200 m elev., 1979, *K.R. Rajbhandari & B.Roy 3378* (KATH). **C NEPAL**: **Dhawalagiri,** Mustang, Muktinath-Thorungse, 3900 m elev., 1983, *K.R. Rajbhandari 8173* (KATH); Mustang, Dhampus, 2450 m elev., 1988, *M. Suzuki et al. 8881592* (KATH); Mustang, Larjung, 2550 m elev., 1996, *T. Hoshino et al. 9668062* (KATH); Mustang, 1999, *S. Ishizawa et al. 990912020* (TI). **E NEPAL**: **Sagarmatha**, Solukhumbu, 3570 m elev., 2005, *Watson et al. DNEP3 BX107* (KATH); Solukhumbu, Thame, 2800 m elev., 2013, *B.B. Raskoti 00987* (KATH). **SIKKIM and DARJEELING**: **Sikkim**, Lachen Valley, 3648 m elev., 1895, *R. Pantling 398* (CAL); **Lachen**, 2584 m elev., 1909, *Smith & Cave 2669* (CAL0000092699). **BHU-**

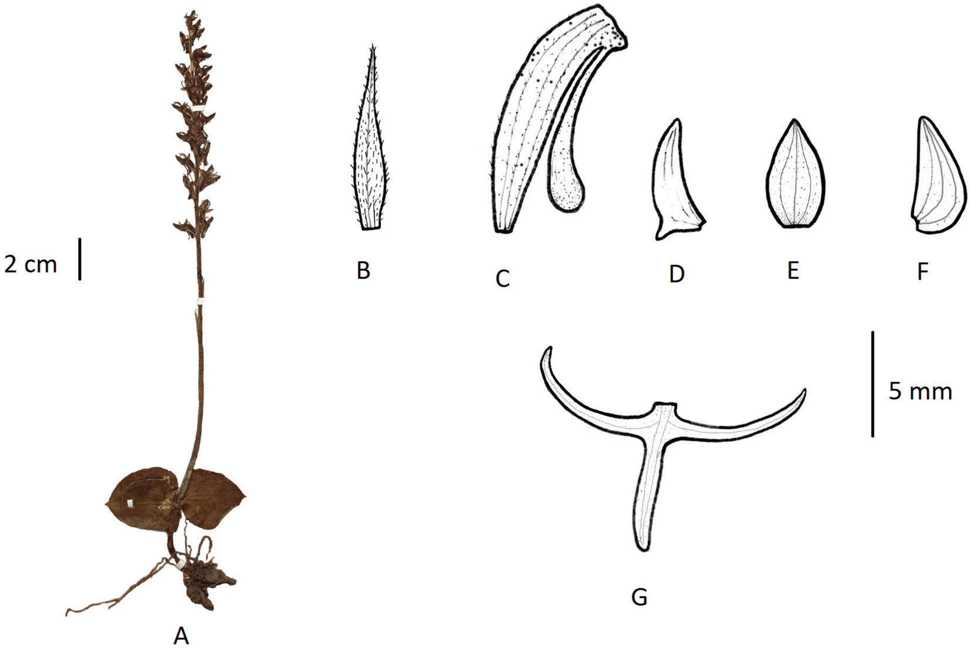

**Figure 12.** *Habenaria aitchisonii* **A** habit **B** floral bract **C** pedicellate ovary with spur **D** petal **E** dorsal sepal **F** lateral sepal **G** lip (**A** photographed from *K.Y. Lang et al. 944*, PE **B–G** drawn from the same specimen by T.R. Pandey).

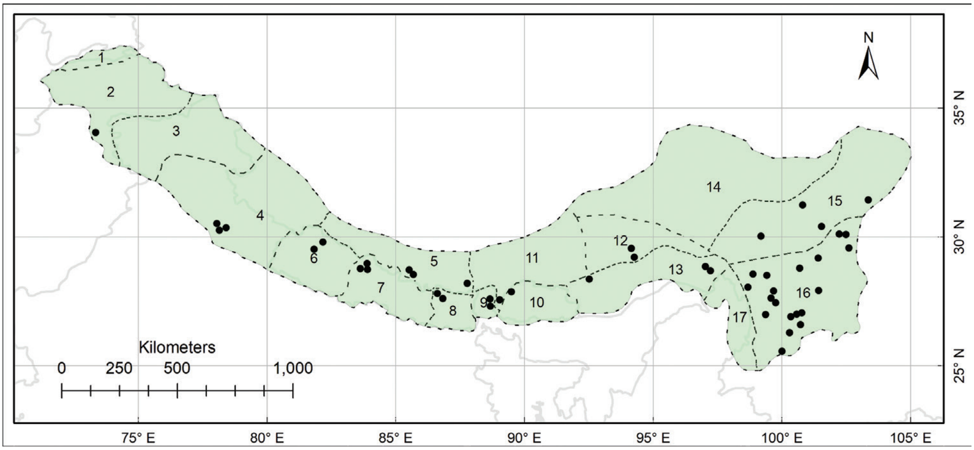

**Figure 13.** Distribution of *Habenaria aitchisonii* (black circles) in the Pan-Himalaya.

**TAN**: **Gasa**, Upper Mo Chu, Laya, 3950 m elev., 1983, *C. Sargent 114* (RENZ 16003). **M YARLUNG ZANGBO**: **Yadong**, Phari, Kang me, 1882, *Dr. King's Collector s. n.* (CAL0000092702). **L YARLUNG ZANGBO**: **Mainling**, 3100 m elev., 1972, *Tibet Chinese Medicinal Plants Survey team 3858* (PE); **Nyingchi**, near township, 3200 m elev., 1964, *Anonymous 793* (PE). **YARLUNG ZANGBO-BRAHMAPUTRA**: **Lhünzê**, 3889 m elev., 2013, *FLPH Tibet Expedition 13-0890* (PE); **Zayü**, 4100 m elev., 1973, *Qinghai-Tibet Team 1218* (PE); **Zayü**, 3200 m elev., 1935, *C.W. Wang 65772* (PE). **N HENGDUAN**: **Batang,** 3520 m elev., 1983, *K.Y. Lang et al. 2410* (PE); **Danba**, 3000 m elev., *G.L. Qu 7522* (PE); **Luhuo Xian,** NW of Luhuo along highway 317, 3385 m elev., *D.E. Bufford et al. 33485* (K); **Wenchuan**, Wolong Nature Reserve, 2220 m elev., 1982, *Lang et al. 1528* (type of *H. wolongensis*, PE). **S HENGDUAN**: **Cawarong**, 3450 m elev., 1982, *Qinghai-Tibet Team 10547* (PE); **Dali**, 3000 m elev., 2001, *H.K. Kadoorie Team s. n.* (PE); **Daocheng,** 3100–3200 m elev., 1981, *Qinghai-Tibetan Team 4235* (PE); **Dêqên**, 2200 m elev., 1935, *C.W. Wang 69920* (PE); **Heishui**, 3200 m elev., *Q. Li 73191* (PE); **Heqing**, 2730 m elev., 1996, *Y.B. Luo 40* (PE); **Jinyang**, 3500 m elev., *K.Y. Lang 14900* (PE); **Jiulong**, Tanggu Xiang, 3700 m elev., 2005, *D.E. Bufford 33420* (K); **Kangding**, 3470 m elev., 1982, *Lang et al. 1534* (PE); **Kangding**, 3650 m elev., 1982, *K.Y. Lang et al. 944* (PE); **Luding**, 2500 m elev., 1938, *Z.B. Wang 9772* (PE); **Muli**, 3200–3400 m elev., 1983, *Qinghai-Tibet Team 14063* (PE); **Shangri-la**, 4200–4300 m elev., 1981, *Hengduan Team 3322*, *2832* (PE); **Shangri-la**, Napa Hai, amongst shrubs, 3350 m elev., 2002, *H. Sun 08* (K); **Shangri-la** (Zhongdian), 3000 m elev., 1937, *T.T. Yu 12539* (PE); **Weixi**, 3600 m elev., 1935, *C.W. Wang 68356* (PE); **Xiangcheng**, 3500 m, elev. *Qinghai-Tibetan Team 004810* (PE); **Yulong (Lijiang)**, 3040 m elev., 1922, *G. Forrest 22208* (K); **Yulong (Lijiang)**, 2700–3000 m elev., 1913, *G. Forrest 10985* (PE); **Yulong (Lijiang)**, 2750 m elev., 1981, *Qinghai-Tibet Team 213* (PE).

**Note.** This species is distributed along the whole range of the Himalaya up to the Hengduan Mountains at elevations between 2000 and 4500 m (temperate to alpine) and thus is the most widespread *Habenaria* species in the Pan-Himalaya. The type material was collected from the Darban Valley along the Pakistan-Afghanistan border in the western end of the distribution (Aitchison 1888). Similar plants from the Hengduan Region were described as new species by Schlechter (1912, 1924) and Kränzlin (1921), respectively, but were later reduced to synonyms (Chen and Cribb 2009). There is considerable variation in the size of the plant, in the colouring of the leaves, with yellowish-white markings that occasionally give a false impression of this being a new species; the peduncle is also quite variable in length as is the density of the inflorescence, from subdensely few-flowered to densely many-flowered. *Habenaria wolongensis* from Sichuan also falls within the range of variation of *H. aitchisonii* and is here reduced to synonymy.

### 6. *Habenaria balfouriana* Schltr., Repert. Spec. Nov. Regni Veg. 20: 381 (1924).

**Type.** China, Yunnan, 1910, *G. Forrest 6149* [lectotype designated here: E (E00381989 image!); isolectotypes: PE (00340644!), IBSC (0636129!), K (K000796932 image!), P (P00370551 image!)].

**Description.** Terrestrial herbs, 10–24 cm tall. Tubers oblong. Stems densely pubescent. Leaves 2, opposite, basal; leaf blade ovate or ovate-orbicular, 2–4.5 cm long,

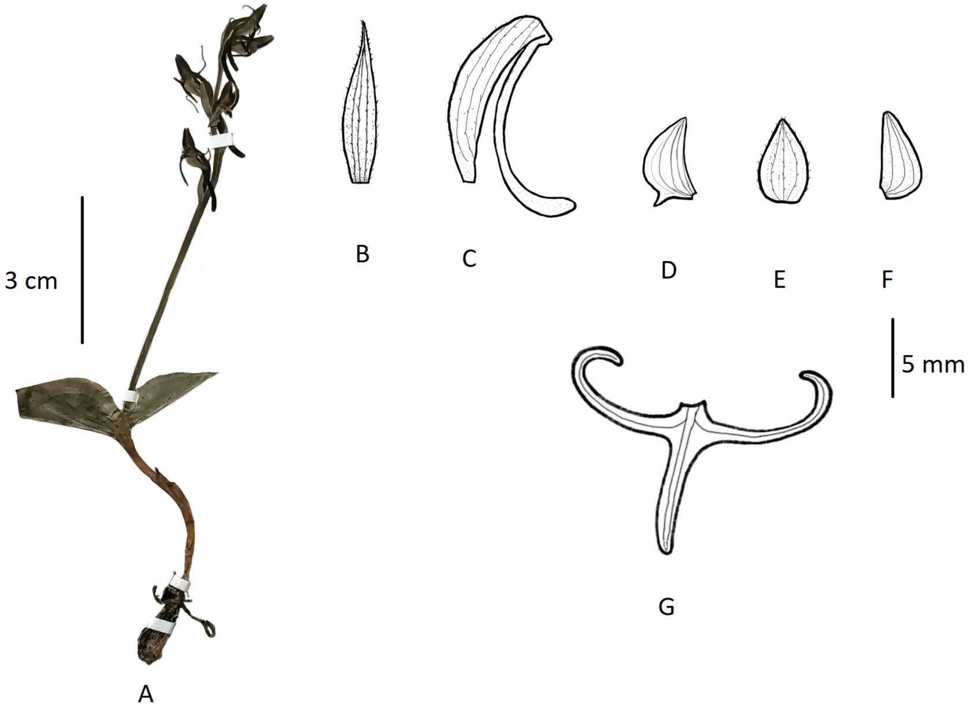

**Figure 14.** *Habenaria balfouriana* **A** habit **B** floral bract **C** pedicellate ovary with spur **D** petal **E** dorsal sepal **F** lateral sepal **G** lip (**A** photographed from the isolectotype specimen *G. Forrest 6149*, PE **B–G** drawn from the same specimen by T.R. Pandey).

2–4 cm broad, fleshy, apex acuminate or acute. Inflorescence 8–20 cm long, subdensely 3–12-flowered; rachis 5–10 cm long; floral bracts lanceolate, apex acuminate. Flowers yellowish-green; ovary and pedicel arcuate, fusiform, 0.8–1 cm long, finely papillate-hairy. Dorsal sepal forming a hood with petals, erect, ovate, concave, 5–6 mm long, 3.5–4 mm broad, margin ciliate-denticulate, apex obtuse; lateral sepals oblique, ovate-oblong, reflexed, 6–7 mm long, 3.5–4 mm broad, apex subacute. Petals 2-lobed, glabrous; upper lobe obliquely ovate-lanceolate, 5–6 mm long, 2–2.2 mm broad; lower lobe a tooth at base of upper lobe, ca. 0.5 mm long; lip deeply 3-lobed above base, spurred; lateral lobes linear, retrorse, almost embracing ovary, linear, 1–1.2 cm long, apex bent; mid-lobe linear, reflexed, ca. 1 cm long; spur pendulous, slightly curved, clavate, 1.2–2 cm long. Column stout, anthers parallel, connective wide; stigmatic processes sub-oblong. (Fig. 14).

**Phenology.** Flowering in July and August.

**Habitat.** Montane forests, shrubby grasslands, alpine meadows; 3000–4300 m elev.

**Distribution.** Endemic to the Pan-Himalaya, found only in Hengduan Mountains; S Hengduan. (Fig. 15).

**More illustrations.** Wu et al. (2010, fig. 194, 3–4).

**Additional specimens examined. S HENGDUAN**: **Daocheng**, 4236 m elev., 2007, *X.H. Jin 9194* (PE); **Yanyuan**, 3600 m elev., 1983, *Qinghai-Tibet Team 12529*

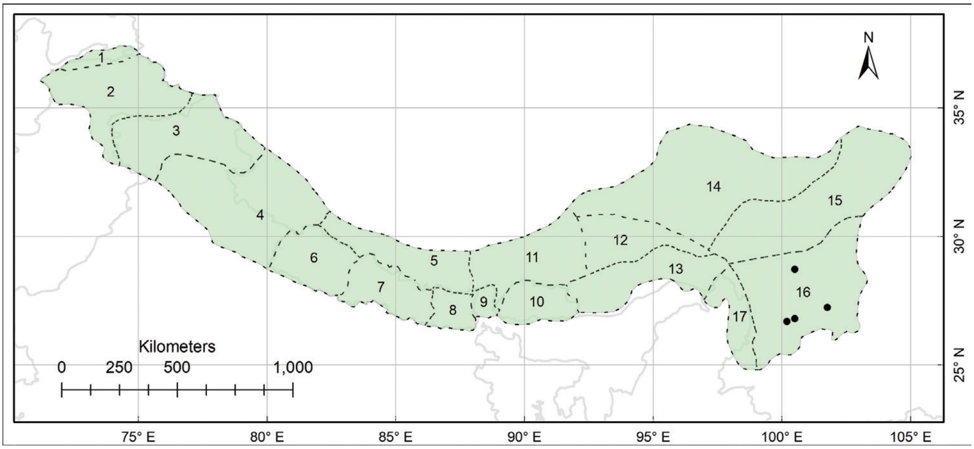

**Figure 15.** Distribution of *Habenaria balfouriana* (black circles) in the Pan-Himalaya.

(PE); **Yulong (Lijiang)**, 3000 m elev., 1937, *T.T. Yu 15316* (PE); **Yulong (Lijiang)**, mountain meadows, 3344 m elev., 1906, *G. Forrest 2739* (K).

**Note.** This species grows above 3000 to 4300 m in grassy alpine meadows. *Habenaria balfouriana* has a restricted distribution in the southern Hengduan Mountains, sharing the habitat with the similar-looking *H. aitchisonii*. Though *H. aitchisonii* and *H. balfouriana* were found closely allied in a recent molecular study (Jin et al. 2017), they are distinct morphologically; both have yellowish-green flowers with bilobed petals, but the former has an elongated spur that exceeds the length of the ovary and pedicel (versus spur shorter than ovary in *H. aitchisonii*).

## 7. *Habenaria szechuanica* Schltr., Acta Horti Gothob. 1: 140. 1924.

**Type.** China, Sichuan, 3200 m elev., 1922, *Harry Smith 2916* [holotype: UPS (UPS-V-109140 image!); isotypes: E (E00381982 image!), LD (1072390 image!), PE (01516964!), S (S-G-7344 image!)].

**Description.** Terrestrial herbs, 18–30 cm tall. Tubers subglobose or ellipsoid. Stems erect, finely papillate-hairy. Leaves 2, opposite, basal; base obtuse-rounded, abruptly narrowed and amplexicaul; leaf blade broadly ovate or orbicular, 3–5 cm long, 3–5 cm broad, apex shortly acuminate or acute. Inflorescence 15–26 cm long, 3–8-flowered; rachis 5–12 cm long, papillate-hairy; floral bracts linear or lanceolate, apex acuminate. Flowers yellowish-green; ovary and pedicel twisted, fusiform, 1.2–1.5 cm long, papillate-hairy. Dorsal sepal forming a hood with petals, erect, ovate, concave, 7–8 mm long, 3–4 mm broad, apex obtuse; lateral sepals reflexed, obliquely ovate, 7–9 mm long, 3.5–4 mm broad, apex subobtuse. Petals shallowly 2-lobed; upper lobe obliquely oblong-lanceolate, 7–9 mm long, 2–2.5 mm broad, margin papillate-ciliate, apex obtuse; lower lobe a tooth at the base of upper lobe, ca. 1.5 mm long; lip reflexed, spurred,

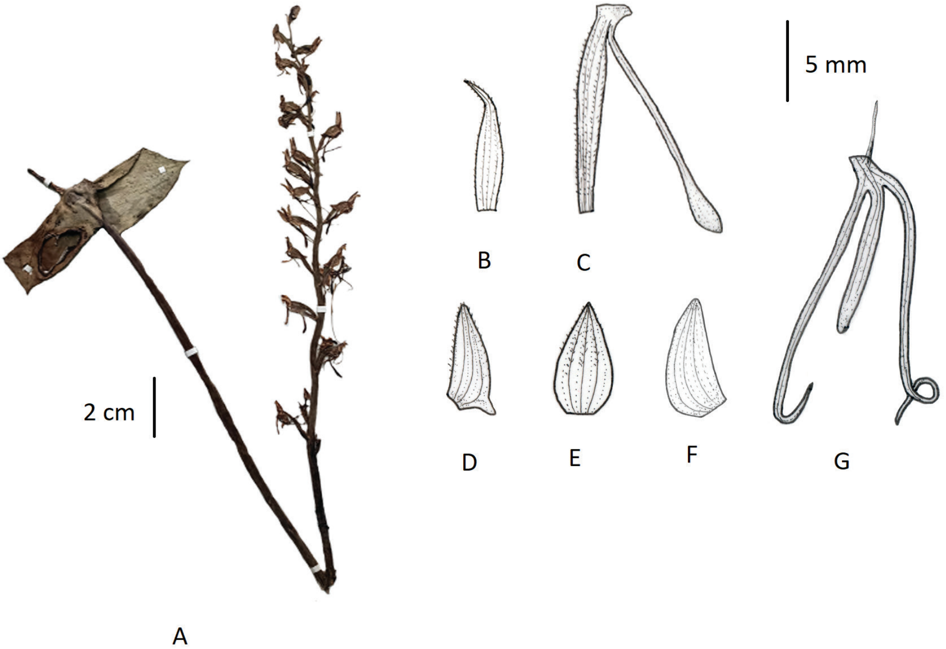

**Figure 16.** *Habenaria szechuanica* **A** habit **B** floral bract **C** pedicellate ovary with spur **D** petal **E** dorsal sepal **F** lateral sepal **G** lip (**A** photographed from *Hengduan Mountain Team 02687*, PE **B–G** drawn from the same specimen by T.R. Pandey).

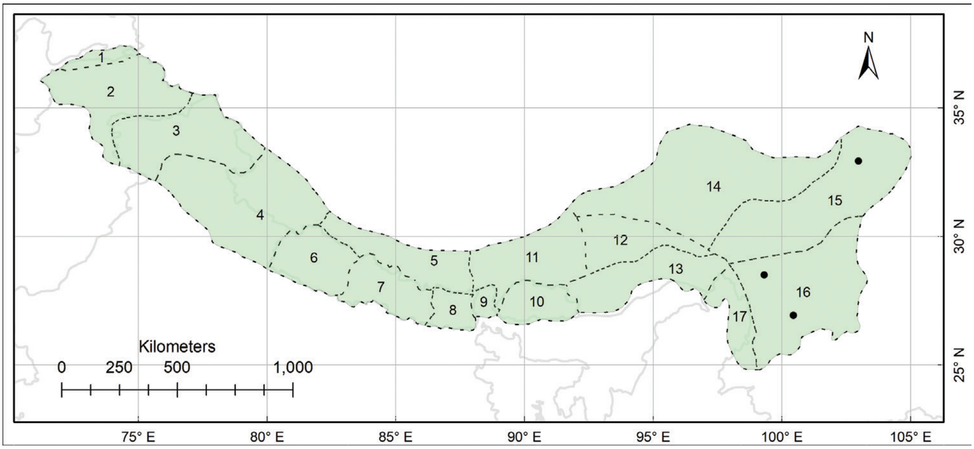

**Figure 17.** Distribution of *Habenaria szechuanica* (black circles) in the Pan-Himalaya.

adaxially with a needle-like 5–7 mm long appendage near the entrance of spur, deeply 3-lobed; lateral lobes linear-filiform, 2.4–2.8(–4) cm long, apex often curled; mid-lobe linear, 1.2–2 cm long; spur cylindrical-clavate, 1.6–2.4 cm long, horizontally curved. Column stout, anther resupinate; stigma processes narrow, lingulate. (Fig. 16).

**Phenology.** Flowering in July and August.

**Habitat.** Montane forests with *Pinus yunnanensis* Franch. and *Picea* sp.; 2900–4000 m elev.

**Distribution.** N and S Hengduan; also in Shaanxi of China. (Fig. 17).

**More illustrations.** Wu et al. (2010, fig. 195, 1–4).

**Additional specimens examined. N HENGDUAN**: **Songpan**, 3450 m elev., 2002, *Y.B. Luo 850* (PE). **S HENGDUAN**: **Xiangcheng**, 3900 m elev., 1981, *Team of Qinghai-Tibetan Plateau 4782c* (PE). **Yulong (Lijiang)**, 2900 m elev., 1981, *Hengduan Mountain Team 02687* (PE, 4 duplicates).

## 8. *Habenaria tibetica* Schltr. ex Limpricht, Repert. Spec. Nov. Regni Veg. Beih. 12: 338. 1922.

**Type.** China, Kangding, 3600 m elev., 13 July 1953, *X.L. Jiang 36260* (neotype designated here: PE 00340847!) (Fig. 18).

**Description.** Terrestrial herbs, 12–40 cm tall. Tubers narrowly oblong to ellipsoid. Stems mostly underground, papillate-hairy. Leaves 2, nearly opposite, basal; prominent white veins on adaxial surface, base abruptly narrowed and amplexicaul; leaf blade wide ovate or orbicular, 2.5–6.5 cm long, 2.5–7 cm wide, apex obtuse or acute. Inflorescence 10–35 cm long, laxly 2–10-flowered; rachis 2–15 cm long, papillate-hairy; floral bracts lanceolate or linear-lanceolate, apex acuminate. Flowers yellowish-green; ovary and pedicel strongly arcuate, 1.5–2 cm long, pubescent. Dorsal sepal forming hood with petals, ovate, concave, 7–9 mm long, 4–5 mm wide, apex subobtuse; lateral sepals reflexed, obliquely ovate, 8–11 mm long, 4–5 mm wide, apex acuminate. Petals shallowly 2-lobed; upper lobe oblique, oblong-lanceolate to ovate-lanceolate, 8–10 mm long, 3–4 mm wide, margin papillate-ciliate, apex subacute; lower lobe ca. 1.5 mm long; lip deeply 3-lobed, spurred, lobes reflexed over base; lateral lobes linear-filiform, 2–4 cm long, curled at apex; mid-lobe linear, 1–2 cm long; spur cylindrical-clavate, 1.5–2.5 cm long, often horizontal and curved upwards. Column stout, anthers parallel; pollinia granular; caudicles stout, elongated; stigma processes lingulate. (Fig. 19).

**Phenology.** Flowering from June to August.

**Habitat.** Thickets, alpine grasslands; 3200–4900 m elev.

**Distribution.** N and S Hengduan, also in NE Qinghai of China. (Fig. 20).

**More illustrations.** Wu et al. (2010, fig. 195, 5–7).

**Additional specimens examined. N HENGDUAN**: **Gonjo**, 3200 m elev., *Vegetation Team of Qinghai-Tibet Plateau 9671* (PE); **Gonjo**, 3800 m elev., 2010, *Kham Expedition 10-1872* (PE); **Songpan**, 3835 m elev., 2002, *Y.B. Luo 827* (PE); **Xiaojin**, Hanniu Region, 3400 m elev., 1959, *Z.G. Liu 0359* (PE). **S HENGDUAN**: **Kangding,** Muya Region, A-Tai Xiang, 3560 m elev., 1982, *Lang et al. 981* (PE, KUN).

**Note.** According to the protologue, *Habenaria tibetica* was described by Schlechter, based on two specimens from China: East Tibet, Ta tsien lu, 3600 m elev., *Limpricht 2303*

中国科学院植物研究所
植物标本馆（PE）
条码号：00340847

科　名：Orchidaceae
学　名：Habenaria tibetica Schltr. ex Limpricht
鉴定人：郎楷永　鉴定日期：1982. 02. 11
产　地：四川省 康定县
木雅区阿太乡毛家沟路边
生　境：
海　拔：3600 m　经纬度：
采集人：蒋兴麟
采集号：36260　采集日期：1953. 07. 13

中国科学院植物研究所
植物标本室
采集者 蒋兴麐
号数 36260
学名 Habenaria tibetica Schltr ex Limpricht
附记

川康植——
川康植物採集記錄 12
地點及產地 康定木雅区阿太鄉毛家沟路边
海拔高度 3,600 公尺
喬木，灌木，草本或藤本 高15—30公分
莖綠色被短柔毛
葉 叶互生于茎基部几乎对生心臟形肉质全缘 叶面淺白花绿色 叶背淺绿脉平行 基部鞘状将茎部包围
花 花序總狀花序每花具一披針形苞片花被六瓣外輪萼片状绿色具紋 内輪花瓣状绿黄色 中間裂片三细長線形深裂 基部成细長矩 小蕊两条花丝极细 药黄色
果 子房下位三室被毛细披
特別記錄 習見
主持人：方文培：採集人：蔣興麐
號數 36260　53年 7月 13日
俗名　科名
學名

第17卷
中国植物志

269590

中國科學院
植物研究所
標本室

CHINESE NATIONAL HERBARIUM (PE)
00340847

中国植物志依据标本
Habenaria tibetica Schltr. ex Limp.
定名人 郎楷永 (Lang Kai-yong)　1982 年 2 月 11 日

**Figure 18.** Neotype of *Habenaria tibetica* Schltr. ex Limpricht *X.L. Jiang 36260* (PE 00340847) (Photographed by T.R. Pandey).

and Batang-Litang, 4800–4900 m elev., *Limpricht 2277* (Limpricht 1922); consequently, both of these are syntypes as per Art. 9.6 of the ICN (Turland et al. 2018). Many of the type specimens, described by Schlechter together with *H. tibetica* (e.g. *Platanthera minax*

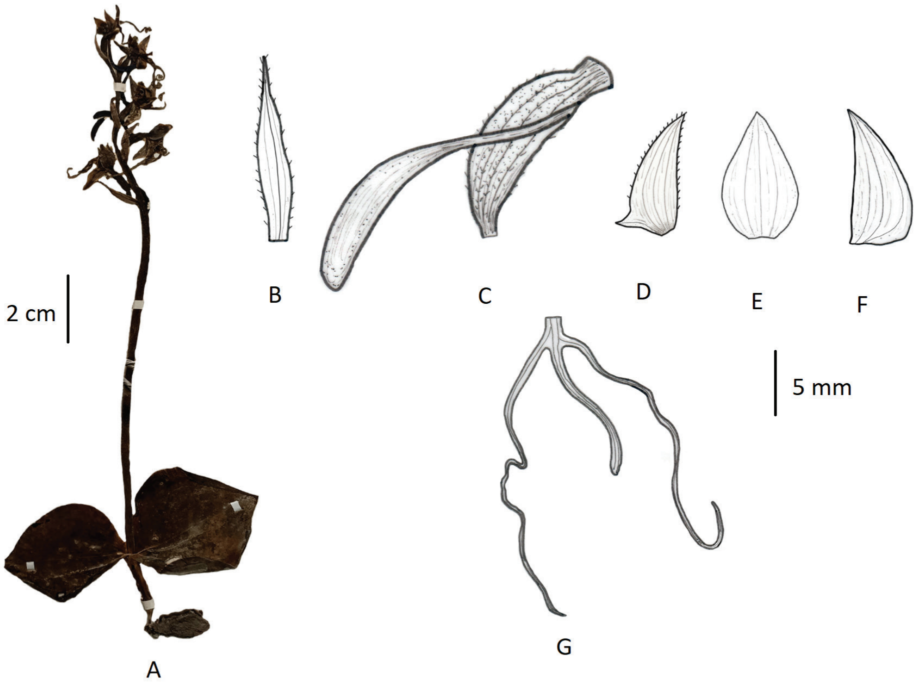

**Figure 19.** *Habenaria tibetica* **A** habit **B** floral bract **C** pedicellate ovary with spur **D** petal **E** dorsal sepal **F** lateral sepal **G** lip (**A** photographed from the neotype *X.L. Jiang 36260*, PE **B–G** drawn from the same specimen by T.R. Pandey).

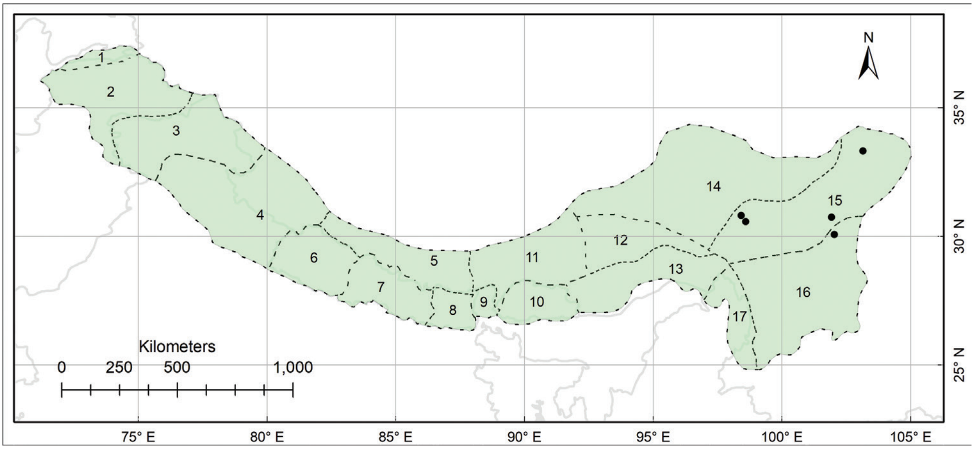

**Figure 20.** Distribution of *Habenaria tibetica* (black circles) in the Pan-Himalaya.

Schltr. & *P. winkleriana* Schltr.), were believed to be kept at B and later, their duplicate specimens were recovered at other European herbaria WU and WRSL; however, despite an extensive search, none of the type materials of *H. tibetica* could be located in any of the world's major herbaria and could have been destroyed during the Second World War at B. Furthermore, we were unable to find any other original material related to the species. Thus, assuming that all the original material of *H. tibetica* is lost, it warrants designating a neotype, which is here accomplished. For that purpose, *X.L. Jiang 36260* (PE) is designated the neotype according to Art. 9.8 of the ICN (Turland et al. 2018); this specimen was also collected from the original type locality (Kangding, Sichuan). The designated neotype specimen conforms to the description in the protologue and is consistent with the current application of the taxon name (e.g. Chen and Cribb 2009).

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## References

Aitchison JET (1888) The Botany of the Afghan Delimitation Commission. In: The Transactions of the Linnean Society of London, Series 2. Botany 3: 1–139. https://doi.org/10.1111/j.1095-8339.1888.tb00623.x

Batista JAN, Bianchetti LB, González-Tamayo R, Figueroa XMC, Cribb PJ (2011) A synopsis of new world *Habenaria* (Orchidaceae) I. Harvard Papers in Botany 16(1): 1–47. https://doi.org/10.3100/025.016.0101

Batista JAN, Borges KS, Faria MW, Proite K, Ramalho AJ, Salazar GA, van den Berg C (2013) Molecular phylogenetics of the species-rich genus *Habenaria* (Orchidaceae) in the New World based on nuclear and plastid DNA sequences. Molecular Phylogenetics and Evolution 67(1): 95–109. https://doi.org/10.1016/j.ympev.2013.01.008

Chen SC, Cribb PJ (2009) *Habenaria*. In: Wu ZY, Raven PH, Hong DY (Eds) Flora of China (Vol. 25). Science Press, Beijing and Missouri Botanical Garden Press, St. Louis, 144–160.

Choudhury S, Mukherjee SK, Chowdhery HJ (2011) Distribution and diversity of the genus *Habenaria* Willdenow (Orchidaceae) in India (conference paper). In: Ghosh C, Das AP (Eds) Recent Studies in Biodiversity and Traditional Knowledge in India. Gour Mahavidyalaya, Malda, 81–90.

Cronquist A (1978) Once again, what is a species? In: Romburger JA (Ed.) Beltsville Symposia in Agricultural Research. [2] Biosystematics in Agriculture. Invited papers presented at a symposium held May 8–11, 1977, at the Beltsville Agricultural Research Center (BARC), Beltsville, Maryland 20705. Allenheld, Osmun & Co., Montclair, 3–20.

Dressler RL (1993) Phylogeny and classification of the orchid family. Dioscorides Press, Portland, Oregon.

Finet AE (1901) Les Orchidées de L'Asie Orientale. Revue Générale de Botanique 13: 497–534. [t. 18A. 1–8.]

Govaerts R, Bernet P, Kratochvil K, Gerlach G, Carr G, Alrich P, Pridgeon AM, Pfahl J, Campacci MA, Holland Baptista D, Tigges H, Shaw J, Cribb P, George A, Kreuz K, Wood J (2020) World checklist of Orchidaceae. Facilitated by the Royal Botanic Gardens, Kew. http://wcvp.science.kew.org/

Hong D-Y (2015) Flora of Pan-Himalaya (Vol. 47). Science Press, Beijing.

Hooker JD (1890) The Flora of British India, Orchideae to Cyperaceae (Vol. 6), Part 17. L. Reeve & Co., London, 224 pp. https://doi.org/10.5962/bhl.title.678

Jin, WT, Schuiteman A, Chase MW, Li JW, Chung SW, Hsu TC, Jin XH (2017) Phylogenetics of subtribe Orchidinae s.l. (Orchidaceae; Orchidoideae) based on seven markers (plastid matK, psaB, rbcL, trnL-F, trnH-psba, and nuclear nrITS, Xdh): implications for generic delimitation. BMC Plant Biology 17(1–14): e222. http://dx.doi.org/10.1186/s12870-017-1160-x

Kränzlin F (1892) Beiträge zu einer Monographie der Gattung *Habenaria* Willd. Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie 16: 52–223.

Kränzlin F (1901) Orchidacearum genera et species (Vol. 1). Mayer & Müller, Berlin.

Kränzlin F (1921) Orchidaceae Ténianae Yunnanense. Repertorium Specierum Novarum Regni Vegetabilis 17(4–7): 99–112. https://doi.org/10.1002/fedr.19210170411

Kurzweil H, Weber A (1992) Floral morphology of southern African Orchideae II. Habenariinae. Nordic Journal of Botany 12(1): 39–61. https://doi.org/10.1111/j.1756-1051.1992.tb00200.x

Lang KY (1984) New species of Orchidaceae from western Sichuan, China. Zhiwu Fenlei Xuebao 22(4): 312–315.

Limpricht W (1922) Botanische Reisen in den Hochgebirgen Chinas und Ost-Tibets. Repertorium Specierum Novarum Regni Vegetabilis. Beihefte 12: 1–515.

Lucksom SZ (2007) The Orchids of Sikkim and North East Himalaya. S.Z. Lucksom, Siliguri.

Maity D, Ghosh J, Pradhan N, Mukherjee SK, Maiti GG (2019) Enumeration of Orchids of Sikkim. Pleione 13(2): 355–384. https://doi.org/10.26679/Pleione.13.2.2019.355-384

Pandey TR, Jin XH (in press) Taxonomic notes and typification of some *Habenaria* species in the Pan-Himalaya. Phytotaxa.

Pearce N, Cribb PJ (2002) The Orchids of Bhutan. Flora of Bhutan (Vol. 3(3)). Royal Botanic Garden, Edinburgh and Royal Government of Bhutan.

Pearce N, Cribb PJ, Renz J (2001) Notes relating to the Flora of Bhutan: XLIV. Taxonomic notes, new taxa and additions to the Orchidaceae of Bhutan and Sikkim (India). Edinburgh Journal of Botany 58(1): 99–122. https://doi.org/10.1017/S0960428601000488

Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN (2001) Genera Orchidacearum (Vol. 2). Orchidoideae, part 1. Oxford University Press, New York.

Rajbhandari KR, Rai SK (2017) A Handbook of the Flowering Plants of Nepal (Vol. 1). Department of Plant Resources, Kathmandu, Nepal.

Raskoti BB, Ale R (2019) Molecular phylogeny and morphology reveal a new epiphytic species of *Habenaria* (Orchidaceae; Orchideae; Orchidinae) from Nepal. PLoS ONE 14(10): e0223355. https://doi.org/10.1371/journal.pone.0223355

Schlechter R (1912) Enumeration and Description of Species of Orchidaceae. Notes from the Royal Botanic Garden Edinburgh 5: 99–113.

Schlechter R (1924) Acta Horti Gothoburgensis 1(3): 139–140. https://doi.org/10.1111/j.1651-2227.1924.tb14521.x

Stuessy TF (2009) Plant taxonomy: The Systematic Evaluation of Comparative Data. Second Edition. Columbia University Press, New York.

Summerhayes VS (1968) Orchidaceae (Part 1). In: Milne-Redhead E, Polhill RM (Eds) Flora of Tropical East Africa. Crown Agents for Overseas Governments and Administrations, London, 236 pp.

Thiers B (2020) Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. http://sweetgum.nybg.org/science/ih/ [accessed: 10 September 2020]

Turland NJ, Wiersema JH, Barrie FR, Greuter W, Hawksworth DL, Herendeen PS, Knapp S, Kusber WH, Li DZ, Marhold K, May TW, McNeill J, Monro AM, Prado J, Price MJ, Smith GF [Eds] (2018) International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code) adopted by the Nineteenth International Botanical Congress Shenzhen, China, July 2017. Regnum Vegetabile 159. Glashütten, Koeltz Botanical Books. https://doi.org/10.12705/Code.2018

Wu ZY, Raven PH, Hong DY [Eds] (2010) Flora of China Illustrations (Vol. 25). Science Press, Beijing and Missouri Botanical Garden Press, St. Louis.

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RESEARCH ARTICLE

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# A new synonym of *Polygonatum* in China, based on morphological and molecular evidence

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## Abstract

*Polygonatum kingianum* Collett et Hemsl. var. *grandifolium* D.M. Liu & W.Z. Zeng (1981), which sprouts twice a year, once in spring and once in autumn, differs from *Polygonatum kingianum* in leaves, bracts, perianth and filaments. Morphological comparison and molecular phylogeny indicate that it is identical to the newly-published *Polygonatum hunanense* H.H. Liu & B.Z. Wang (2021). Hence, we propose that *P. kingianum* var. *grandifolium* should be recognised as a new synonym of *P. hunanense*. In addition, phylogenetic analyses confirmed that *P. hunanense* is sister to *Polygonatum* sect. *Polygonatum*, rather than *P. kingianum* of *Polygonatum* sect. *Verticillata*.

## Keywords

*Polygonatum hunanense*, *Polygonatum kingianum* var. *grandifolium*, phylogeny, plastome

## Introduction

The genus *Polygonatum* Mill. (Asparagaceae, tribe Polygonateae), commonly known as 'Solomon's Seal', contains more than 60 species widespread in the Northern Hemisphere, with Himalayas to southwest China and north-eastern Asia as diversification centres (Tamura et al. 1997a; Jeffrey 1980, 1982; Wang et al. 2016). Species in *Polygonatum* are perennial herbs with rhizome, stems erect, arching or sometimes scandent, leaves alternate, opposite or whorled, inflorescences an umbel, corymb or raceme (Chen and Tamura 2000). Rhizomes of some species, like *Polygonatum sibiricum* Redouté, *Polygonatum cyrtonema* Hua and *Polygonatum kingianum* Collett et Hemsl., are widely used in traditional Chinese medicine. Tamura et al. separated *Heteropolygonatum* M.N. Tamura et Ogisu from *Polygonatum* (Tamura et al. 1997a) and found the topological difference of *Heteropolygonatum* and its relative taxa: (1) (*Heteropolygonatum* + *Disporopsis*) + *Polygonatum* and (2) (*Heteropolygonatum* + *Polygonatum*) + *Disporopsis* (Tamura et al. 1997b). Later, the former case was supported by Meng et al. (2014) and Wang et al. (2016), whereas the latter one was supported by Floden and Schilling (2018) and Zhao et al. (2019). The genus *Polygonatum* was divided into three sections, based on four chloroplast molecular markers, leaf arrangement and basic chromosome number: (1) *Polygonatum* sect. *Polygonatum*, which is characterised by alternate leaves and basic chromosome number x = 9–11; (2) *Polygonatum* sect. *Sibirica* only includes *Polygonatum sibiricum* with whorled leaves and x = 12; and (3) *Polygonatum* sect. *Verticillata* shows variable phyllotaxy and x = 13–15 (Meng et al. 2014). This is widely accepted and confirmed by multiple molecular phylogenetic studies (Wang et al. 2016; Floden and Schilling 2018; Zhao et al. 2019). However, due to hybridisation and polyploidisation in this genus, classification of some species with large morphological variations and wide distribution range remains controversial (Tamura 1990; Tamura 2008; Floden 2015; Floden and Schilling 2018; Zhao et al. 2019).

During fieldwork in the last few years, we found several populations of a unique *Polygonatum* species in Sichuan Province, Chongqing Municipality and Hubei Province of China (Figure 1). The plants are 1–3 m high with 3–5 whorled leaves per round, yellowish-white or greenish-white flowers and yellow or orange berries (Figure 2). It sprouts twice a year, once in spring (March to April) and once in autumn (September), whereas other species sprout only once in spring. It is likely belonging to the section *Verticillata*, according to the phenotype. After carefully checking the protologue and type specimens (Figure 3), we found that our collections matched the description of *Polygonatum kingianum* Collett et Hemsl. var. *grandifolium* D.M. Liu et W.Z. Zeng, which was published in Flora Sichuanica and is differing from other *P. kingianum* varieties by having broader leaves ((1.5–) 2.4–5 cm wide) with green leaf base (vs. 0.2–1.0 (–1.5) cm wide, leaf base red), 2-5 mm long bracts at base of pedicel (vs. 1-2 mm long, on pedicel), yellowish- or greenish-white perianth (vs. pink, red or white) (Figures 2, 4; Xu 1981). In addition, it is also similar to the newly-published *Polygonatum hunanense* H.H. Liu & B.Z. Wang from Hunan, China (Liu et al. 2021). In this study, molecular phylogenetic analyses were performed to reveal the phylogenetic relationships amongst *P. kingianum* var. *grandifolium*, *P. hunanense* and *P. kingianum*.

## Materials and methods

### Morphologic observation

Morphological characters of the living individuals from Mt. Emei, Sichuan Province, China were observed. In addition, 16 herbarium specimens of *Polygonatum kingianum* var. *grandifolium* in IMC, CDCM and CDBI were examined. Subsequently, mor-

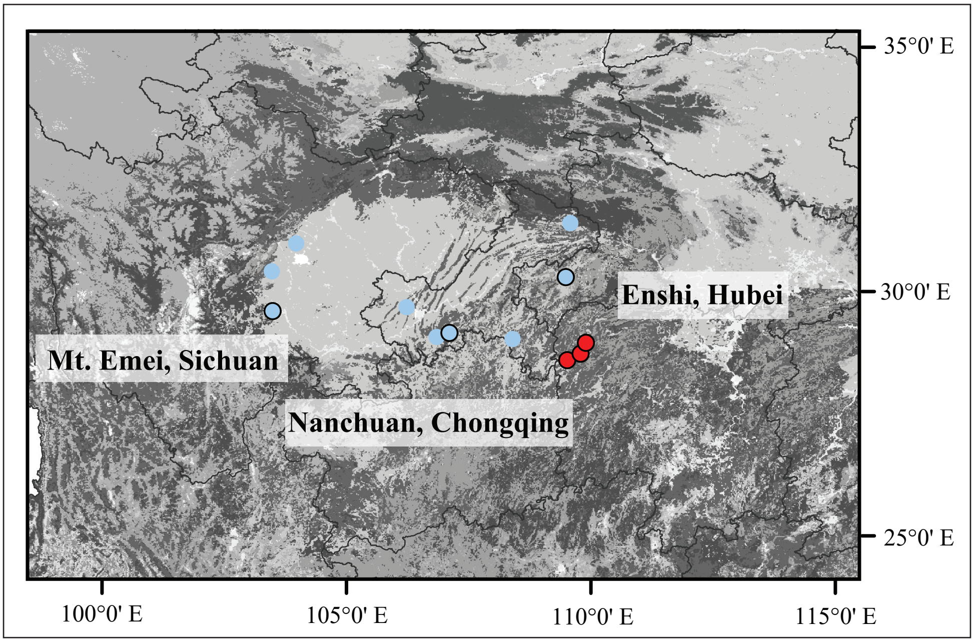

**Figure 1.** Distribution map of *Polygonatum hunanense* (red dots with black circle, based on Liu et al. 2021) and *Polygonatum kingianum* var. *grandifolium* (blue dots with black circle: three populations investigated in this study; blue dots: previous specimen records).

phological comparisons were conducted with the living individuals, specimens and descriptions of *Polygonatum hunanense* and *Polygonatum kingianum* from flora and previous research (Jeffrey 1980; Xu 1981; Chen and Tamura 2000; Liu et al. 2021).

## Sequencing, plastome assembly and annotation

In order to determine the phylogenetic status of the taxon, we sequenced three samples from Nanchuan, Chongqing Municipality, Enshi, Hubei Province and Mt. Emei, Sichuan Province, respectively (Figure 1), as well as two *Polygonatum kingianum*, one *Polygonatum sibiricum* and one *Polygonatum zanlanscianense* (Table 1). Representative voucher specimens are currently deposited at the Herbarium of Zhejiang University (HZU). Genomic DNAs were extracted from silica-gel dried leaves using DNA Plantzol Reagent (Invitrogen), following the manufacturer's instructions. The libraries were prepared and sequenced using paired-end 150 bp at Beijing Genomics Institute (BGI, Shenzhen, China) on a BGISEQ-500 sequencing platform. Approximately 3G raw data were generated for each sample. Raw data were trimmed by removing adapters and low-quality reads and then a de novo approach was applied to assemble plastomes using the NOVOPlasty v.3.8.3 (Dierckxsens et al. 2017) with K-mer = 39. The plastome and *rbc*L gene sequences of *Polygonatum stenophyllum* Maxim. (KX822773) were adopted as reference and seed sequence, respectively. DOGMA (Wyman et al. 2004) was used for plastome annotation with manually checking the start/stop codons in Geneious 10.2.3

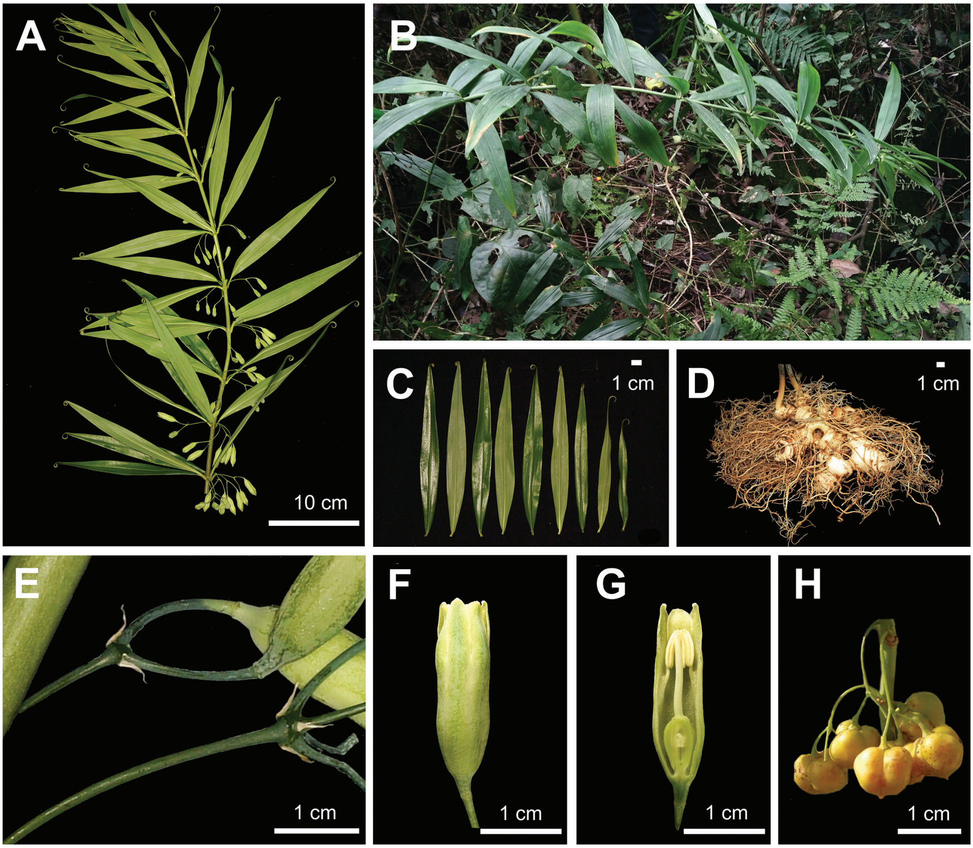

**Figure 2.** *Polygonatum kingianum* var. *grandifolium* **A** stem **B** plant habit **C** leaves **D** rhizome **E** bracts **F** flower **G** longitudinal section of flower **H** fruit.

(http://www.geneious.com). In addition, plastome data of *Polygonatum* and outgroups (*Heteropolygonatum* and *Disporopsis* Hance) from Floden & Schilling (2018) were used for phylogenetic analyses (Table 1). To study the phylogenetic relationship between *P. kingianum* var. *grandifolium* and *P. hunanense*, *rbc*L, *trn*K, *psb*A-*trn*H and *trn*C-*pet*N, sequences from Liu et al. (2021) were downloaded for further phylogenetic analyses.

## Phylogeny of *Polygonatum*

The sequence of 78 protein coding genes (CDS) shared by all plastomes were aligned using MAFFT v.7 (Katoh and Standley 2013) in Geneious 10.2.3. The *rbc*L, *trn*K, *psb*A-*trn*H and *trn*C-*pet*N sequences from Liu et al. (2021) and those from the seven newly-reported plastomes were aligned using MUSCLE in Geneious 10.2.3. DNASP6 was used to do statistics of site information (Rozas et al. 2017). The phylogenetic trees were constructed using both Maximum Likelihood (ML) and Bayesian Inference (BI) methods, implemented on CIPRES Science Gateway website (https://www.phylo.org, Miller et

**Table 1.** GenBank accessions of plastomes involved in this study. Samples in bold were newly sequenced in this study.

| Species name | GenBank number | Length |
|---|---|---|
| *Disporopsis jinfushanensis* Z.Y. Liu | MH891733 | 155,188 |
| *Heteropolygonatum altelobatum* (Hayata) Y.H. Tseng, H.Y. Tzeng et C.T. Chao | MH891734 | 155,534 |
| *Heteropolygonatum alternicirrhosum* (Hand.-Mazz.) Floden | MH891737 | 155,510 |
| *Heteropolygonatum marmoratum* (H. Lév.) Floden | MH891735 | 155,447 |
| *Heteropolygonatum pendulum* (Z.G. Liu et X.H. Hu) M.N. Tamura et Ogisu | MH891736 | 155,436 |
| *Polygonatum acuminatifolium* 2 Komarov | MH891751 | 155,304 |
| *Polygonatum annamense* Floden | MH891738 | 155,277 |
| *Polygonatum arisanense* Hayata | MH891752 | 155,340 |
| *Polygonatum biflorum* (Walter) Elliott | MH891756 | 155,470 |
| *Polygonatum cathcartii* Baker | MH891745 | 155,970 |
| *Polygonatum govanianum* Royle | MH891755 | 155,089 |
| **_Polygonatum kingianum_ var. _grandifolium_ 1** | **MW373518** | **155,609** |
| **_Polygonatum kingianum_ var. _grandifolium_ 2** | **MW373529** | **155,632** |
| **_Polygonatum kingianum_ var. _grandifolium_ 3** | **MW373520** | **155,609** |
| *Polygonatum huanum* H. Lév. | MH891743 | 155,545 |
| **_Polygonatum kingianum_ 1 Collett et Hemsley** | **MW373516** | **155,810** |
| **_Polygonatum kingianum_ 2** | **MW373517** | **155,824** |
| *Polygonatum mengtzense* 1 F.T. Wang et Tang | MH891740 | 155,498 |
| *Polygonatum mengtzense* 2 | MH891741 | 155,492 |
| *Polygonatum oppositifolium* (Wall.) Royle | MH891746 | 155,760 |
| *Polygonatum orientale* Desf. | MH891753 | 155,386 |
| *Polygonatum punctatum* Royle ex Kunth | MH891739 | 155,333 |
| **_Polygonatum sibiricum_ 1 Redouté** | **MW373521** | **155,549** |
| *Polygonatum stenophyllum* Maxim. | KX822773 | 156,028 |
| *Polygonatum stewartianum* Diels | MH891749 | 155,867 |
| *Polygonatum tessellatum* F.T. Wang et Tang | MH891747 | 155,724 |
| *Polygonatum uncinatum* Diels | MH891744 | 155,694 |
| *Polygonatum urceolatum* (J.M.H. Shaw) Floden | MH891742 | 155,504 |
| *Polygonatum verticillatum* 1 (L.) Allioni | MH891748 | 155,878 |
| *Polygonatum verticillatum* 2 | MH891750 | 155,502 |
| *Polygonatum yunnanense* H. Lév. | MH891754 | 155,363 |
| **_Polygonatum zanlanscianense_ 1 Pampanini** | **MW373522** | **155,911** |

al. 2010) with the best-fit model of DNA substitution estimated by jModelTest v.2.1.4 (Darriba et al. 2012). ML analysis was conducted using RAxML-HPC BlackBox 8.2.12 with 1000 bootstrap replicates (Stamatakis 2014). Bayesian analysis was constructed using MrBayes XSEDE 3.2.7 with two independent Markov Chain Monte Carlo chains for 10,000,000 generations and sampling every 1000 generations (Ronquist and Huelsenbeck 2003). The first 25% of calculated trees were discarded as burn-in and the remaining trees were used to construct a consensus tree to estimate the posterior probability (PP).

## Results and discussion

Morphological comparisons showed that *P. kingianum* var. *grandifolium* is almost the same as *P. hunanense*, except that the latter has narrower leaves (Table 2). However, they both differ from other *P. kingianum* varieties in leaves, bracts, perianth and fila-

**Table 2.** Comparison of morphological characters amongst *P. hunanenses*, *P. kingianum* var. *grandifolium* and *P. kingianum* varieties. The dashed line indicates the characters are the same as the original variety.

| Characters | *P. hunanense* | *P. kingianum* var. *grandifolium* | *P. kingianum* | | | |
|---|---|---|---|---|---|---|
| | | | var. *kingianum* | var. *cavaleriei* | var. *ericoideum* | var. *uncinatum* |
| Rhizomes | moniliform or ginger-like, 1–4 cm thick | moniliform or ginger-like, 1–3.5 cm thick | subterete or submoniliform, 1–3 cm thick | – | – | – |
| Stem | 1–3.5 m, apex subscandent | 1–3 m, apex subscandent | 1–3 m, apex subscandent | – | – | ca. 60 cm |
| Phyllotaxy | whorled, 3–6 (–10) per round | whorled, 3–5 per round | whorled, 3–10 per round | – | – | whorled, 4–5 per round |
| Leaf | 5–20 (–27) cm × 0.5–2.5 (–3.2) cm, linear to lanceolate, apex strongly cirrose or curved | 13–27 cm × (1.5–) 2.4–5 cm, linear to lanceolate, apex strongly cirrose or curved | 6–16 (–20) cm × 0.2–1.0 (–1.5) cm, linear to lanceolate, membranous, apex cirrose | linear to lanceolate, somewhat coriaceous | narrow linear | short lanceolate, somewhat coriaceous, 5–6 cm × 1–1.4 cm |
| Inflorescence | (1–) 2–5 | (1–) 2–5 | (1–) 2–4 (–6) | 1–2 | 2–4 | 1–2 |
| Peduncle | 1.7–3.5 cm | 1–4 cm | 1–2 cm | stout, strongly deflexed | 2–3 cm | decurved |
| Pedicel | 0.7–1.8 cm | 0.4–1.7 cm | 0.5–1.5 cm | | | – |
| Bract | subulate to lanceolate, 3–4 mm, at base of pedicel | linear, 2–5 mm, at base of pedicel | linear, 1–2 mm, on proximal part of pedicel | | | |
| | white or pale yellowish-green | yellow or greenish-white | pink, red | white, tinged purple | white | white |
| Perianth | cylindrical campanulate | cylindrical | cylindrical–campanulate | – | – | – |
| | 17–22 mm | 15–27 mm | 18–25 mm | – | – | 10–13 mm |
| Lobes | 5–6.5 mm | 4–6.5 mm | 3–5 mm | – | – | – |
| Filament | 2–3 mm, flat | 2.5–3.5 mm, stout and no thickening | 1.7–5 mm, slender | – | – | – |
| Anthers | ca. 5 mm | 2.5–5.5 mm | 4–6 mm | – | – | – |
| Ovary | 5–7 mm | ca. 4 mm | 4–6 mm | – | – | – |
| Style | ca. 9–12 mm | ca. 8–14 mm | (8–) 10–14 mm | – | – | – |
| Fruits | berries pale yellowish-green or orange, 1–1.8 cm | berries yellow or orange with black spots, 1.4–1.8 cm | berries yellow, orange or red, 1–1.5 cm | – | – | – |
| Seeds | 3–12 | 5–15 | 7–12 | – | – | – |
| Distribution | Hunan, China | Southwest China | Southwest China, Myanmar, Thailand, Vietnam | China: Sichuan, Yunnan | China: Yunnan | China: Yunnan |
| Altitude | 200–700 m | 600–1200 m | 700–3600 m | – | – | – |

**Figure 3.** Lectotype of *Polygonatum kingianum* var. *grandifolium*, *Hao Zhang 1231* (CDCM 00044013).

ments (Table 2). In addition, we have observed stout and no thickening filaments in *P. kingianum* var. *grandifolium* (Figure 2G and Suppl. material 1: Figure S1) and slender filaments in *P. kingianum* (Figure 4G and Suppl. material 1: Figure S1). Tamura has reported that species of sect. *Verticillata* has slender filaments, whereas sect. *Polygonatum* has stout filaments and filaments of the ser. *Bracteata*, ser. *Polygonatum*

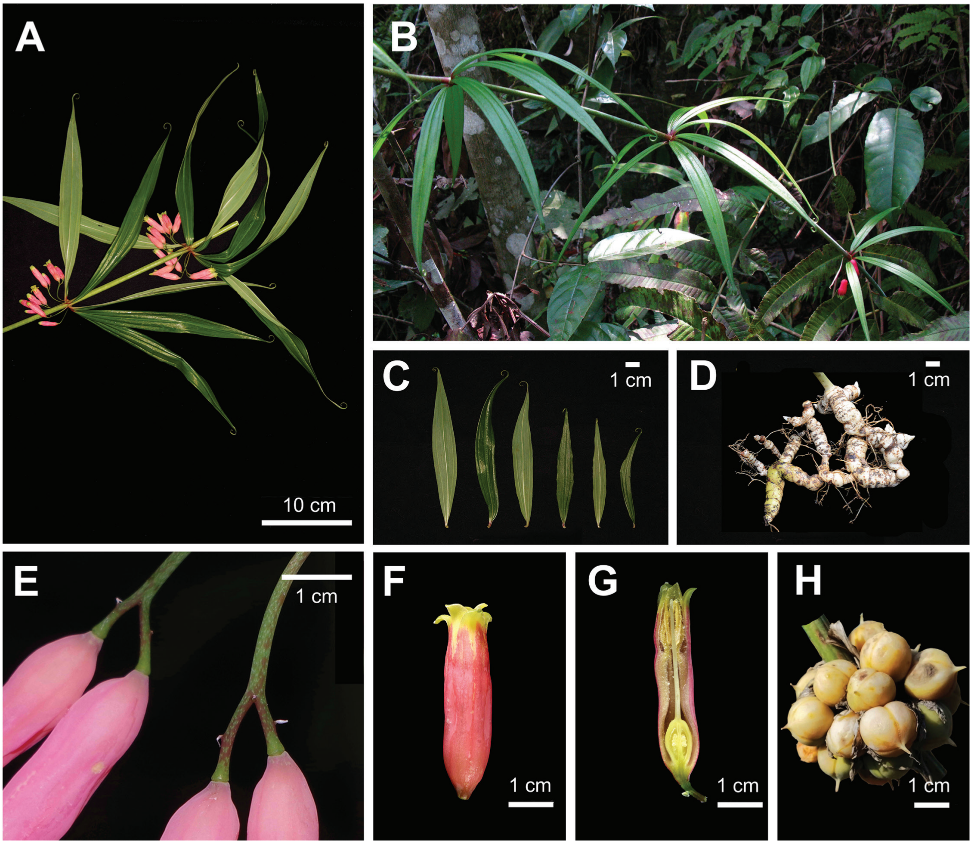

**Figure 4.** *Polygonatum kingianum* **A** stem **B** plant habit **C** leaves **D** rhizome **E** bracts **F** flower **G** longitudinal section of flower **H** fruit.

and ser. *Inflata* are thickening in the upper part, thickening in the middle or without thickening and thickening in the lower part, respectively (Tamura 1991, 1993; Tamura et al. 1997b).

The length of seven plastomes ranged from 155,549 bp to 155,911 bp, the accession numbers being MW373516–MW373522 (Table 1). They displayed the typical quadripartite structure with 132 genes in the same order, of which 112 were unique genes including 78 protein-coding genes, 30 tRNA genes and 4 rRNA genes. The alignment CDS matrix has 66,589 characters in length, in which 1827 are variable (polymorphic) sites and 512 are parsimony-informative sites. In addition, the alignment matrix of four plastid fragments has 4,652 characters in length, of which 85 are variable (polymorphic) sites and 58 are parsimony-informative sites. The phylogenetic tree of 78 CDS supports a robust monophyletic clade of three samples of *P. kingianum* var. *grandifolium* (BS = 100, PP = 1; Figure 5). However, it was not closely related to *P. kingianum* of section *Verticillata*, but was sister to the section *Polygonatum* (BS = 100, PP = 1; Figure 5). Additionally, the phylogeny, based on four plastid fragments, supported the monophyly of *P. hunanense* and

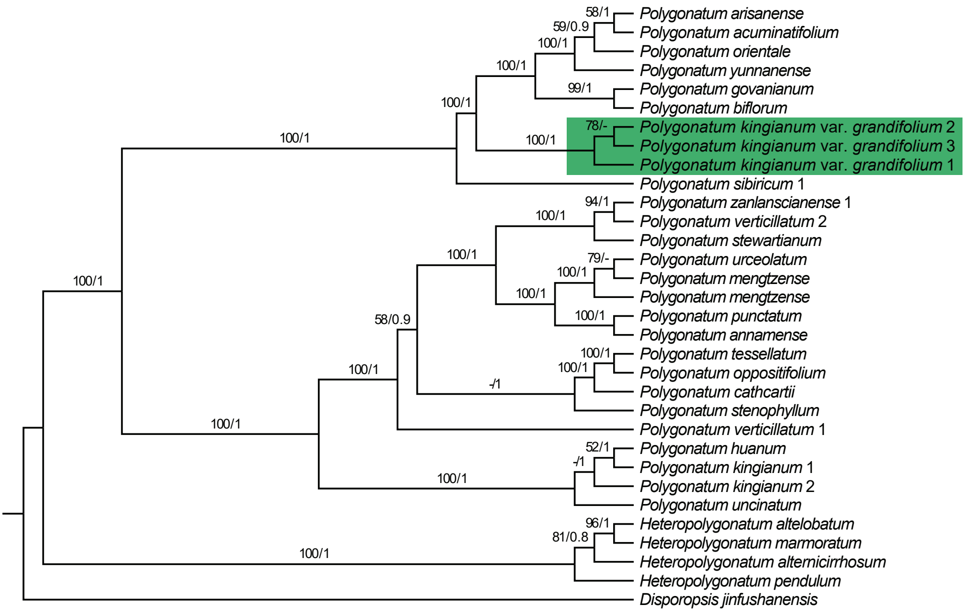

**Figure 5.** Phylogeny of *Polygonatum*, based on 78 protein coding genes (CDS) of plastome. Numbers above branches are Maximum Likelihood bootstrap values (BS)/Bayesian posterior probability (PP). The dash indicates support values of less than 50%. The phylogenetic position of *Polygonatum kingianum* var. *grandifolium* is highlighted in green.

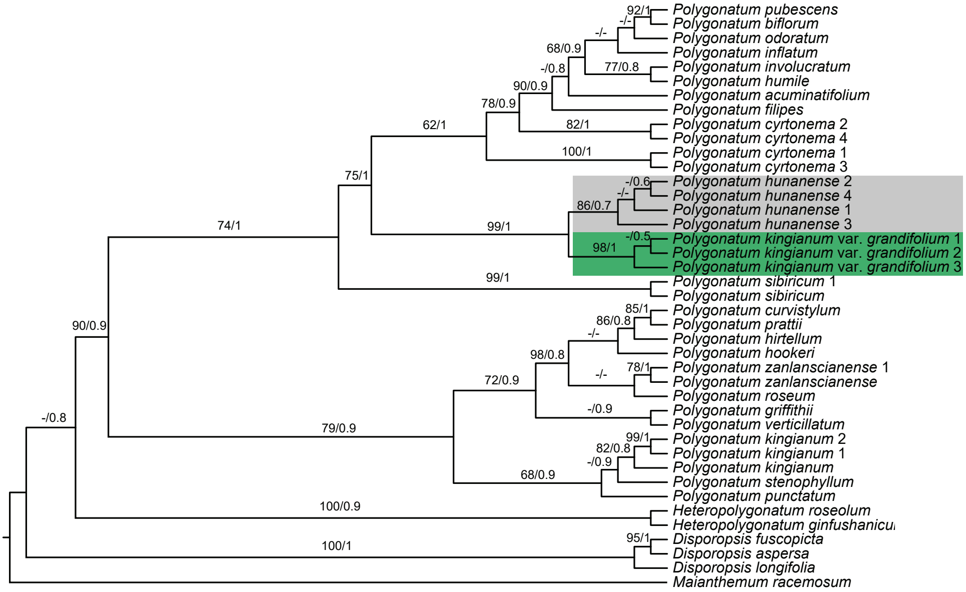

**Figure 6.** Phylogeny of *Polygonatum*, based on *rbc*L, *trn*K, *psb*A-*trn*H and *trn*C-*pet*N sequences. Numbers above branches are Maximum Likelihood bootstrap values (BS)/Bayesian posterior probability (PP). The dash indicates support values of less than 50%. The phylogenetic position of *Polygonatum kingianum* var. *grandifolium* and *P. hunanense* are highlighted in green and grey, respectively.

*P. kingianum* var. *grandifolium* (BS = 99, PP = 1; Figure 6), which suggested they should be conspecific.

Therefore, we propose that *P. kingianum* var. *grandifolium* should be recognised as a new synonym of *P. hunanense*. In addition, both morphology and phylogeny showed that *P. hunanense* is different from *P. kingianum* and has a close relationship with sect. *Polygonatum*.

## Taxonomic treatments

### ***Polygonatum hunanense*** **H.H. Liu & B.Z. Wang**

=*Polygonatum kingianum* Collett & Hemsl. var. *grandifolium* D.M. Liu et W.Z. Zeng, Flora Sichuanica. 7: 230–231. 1981. Type: CHINA. Sichuan: Guan County, 900 m alt., 8 June 1978, *Hao Zhang 1231* (lectotype, designated here: CDCM [barcode 00044013]!, Figure 3; isolectotype: CDCM [barcode 00044022]!).

**Modified description of *P. hunanense*.** Perennials, rhizome moniliform or ginger-like, 1–4 cm in diam. Stem straight or apex subscandent, 1–3.5 m. Leaves in whorls of 3–6 (–10), sometimes alternate or opposite near base and/or apex of stem, sessile, elliptic to oblong-lanceolate, 5–20 (–27) cm long, 0.5–5 cm wide, apex strongly cirrose or curved. Inflorescences (1–) 2–5 flowered; peduncle 1–4 cm long, pendulous; bracts at base of pedicel, subulate to lanceolate, 2–5 mm. Pedicel 0.4–1.8 cm. Perianth yellowish- or greenish-white, cylindrical campanulate, slightly constricted in the middle, 1.5–2.7 cm long, perianth segments 6, arranged into 2 whorls, each 3 lobes 4–6.5 mm. Stamens 6, filaments 2–3.5 mm long, stout and no thickening, anthers 2.5–5.5 mm long. Ovary superior, globose 4–7 mm in diameter. Style 8–14 mm long. Berries pale yellowish-green or orange, 1–1.8 cm in diam., 3–15 seeds.

**Phenology.** It sprouts twice a year, in spring (March to April) and autumn (September). The spring-sprouting individual flowers from April to May and fruits from December to next February. The autumn-sprouting individual flowers from November to December.

**Distribution and habitat.** *Polygonatum hunanense* is relatively common in Chongqing Municipality, Sichuan, Hubei and Hunan Provinces, China (Figure 1). It grows in evergreen broad-leaved forests, thickets or on moist grassy slopes, at an elevation of 200 m to 1200 m. In addition, it is also widely cultivated in those areas for harvesting the rhizomes.

**Conservation status.** To our knowledge, this species is widely distributed in low elevations of southwest China. Therefore, we propose to treat it as Least Concern (LC) according to the IUCN Red List Categories and Criteria version 14 (August 2019). However, due to the medicinal value of the genus, many of its populations are destroyed by unmanaged exploitation.

**Other specimens examined.** China. **Chongqing Municipality**: Nanchuan District, Sanquan, 24 January 1984, *Zhengyu Liu 4958* (fl., IMC!); *ibidem*, 10 July 1991, *Zhengyu Liu 917801* (fr. IMC!); Nanchuan District, Jinfo Mountain, 13 September 1985, *Zhengyu Liu 851732* (fr. IMC!); *ibidem*, 28 June 1999, *Zhengyu Liu 974488* (fl., IMC!); *ibidem*, 28 June 1999, *Zhengyu Liu 974498* (fl., IMC!); *ibidem*, 28 June 1999, *Zhengyu Liu 990498* (fl., IMC!); *ibidem*, 28 August 1999, *Zhengyu Liu 975059* (fr. IMC!); Jiangjin District, Simian Mountain, 16 July 2000, *Zhengyu Liu 2004036* (fl., IMC!); Pengshui County, Longmenxia, 24 June 1988, *Fading Fu & Yaling Cao 0264* (CDBI!); Qijiang District, Zhongfeng Town, Lianghekou, 22 October 2012, *The Qijiang team 13-500222-LY-411-01* (IMC!); *ibidem*, 22 October 2012, *The Qijiang team 13-500222-LY-411-02* (fr., IMC!); Qijiang District, Shihao & Wanlong, 12 October 2014, *The Qijiang team Qianjiang-0310* (IMC!); Wuxi County, Shuangyang, 16 July 1996, *Zhengyu Liu 760044* (fl., IMC!). **Hubei Province**: Enshi City, Hegongwei Village, 06 November 2016, *Jinping Si & Jingjing Liu 33-4* (HZU!). **Sichuan Province**: Pengzhou City, 22 June 1978, *Tianfu Yang & Yunjin Chen 1231* (CDCM!); Qionglai City, 11 June 1979, *Chengdu University of TCM 0668* (fl., CDCM!).

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## References

Chen XQ, Tamura MN (2000) *Polygonatum* Mill. In: Wu ZY, Raven PH (Eds) Flora of China (Vol. 24). Science Press, Beijing & Missouri Botanical Garden Press, Beijing/St. Louis, 225–235.

Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: More models, new heuristics and parallel computing. Nature Methods 9(8): 772–772. https://doi.org/10.1038/nmeth.2109

Dierckxsens N, Mardulyn P, Smits G (2017) NOVOPlasty: De novo assembly of organelle genomes from whole genome data. Nucleic Acids Research 45. https://doi.org/10.1093/nar/gkw955

Floden A (2015) Three new Solomon's Seals (*Polygonatum*: Asparagaceae) from the Eastern Himalaya. Phytotaxa 236(3): 273–278. [2): 125–131.] https://doi.org/10.11646/phytotaxa.236.3.8

Floden A, Schilling EE (2018) Using phylogenomics to reconstruct phylogenetic relationships within tribe Polygonateae (Asparagaceae), with a special focus on *Polygonatum*. Molecular Phylogenetics and Evolution 129: 202–213. https://doi.org/10.1016/j.ympev.2018.08.017

Jeffrey C (1980) The genus *Polygonatum* (Liliaceae) in eastern Asia. Kew Bulletin 34(3): 435–471. https://doi.org/10.2307/4109822

Jeffrey C (1982) Further note on eastern Asian *Polygonatum* (Liliaceae). Kew Bulletin 37(2): 335–339. https://doi.org/10.2307/4109978

Katoh K, Standley DM (2013) MAFFT Multiple sequence alignment software version 7: Improvements in performance and usability. Molecular Biology and Evolution 30(4): 772–780. https://doi.org/10.1093/molbev/mst010

Liu HH, Ma YS, Wang BZ, Jie HD, Xiang SJ, Yang JN, Jie YC (2021) Morphological and molecular characters on a new species of *Polygonatum hunanense* from Hunan, China. Crop Research 35(1): 88–94.

Meng Y, Nie ZL, Deng T, Wen J, Yang YP (2014) Phylogenetics and evolution of phyllotaxy in the Solomon's seal genus *Polygonatum* (Asparagaceae: Polygonateae). Botanical Journal of the Linnean Society 176(4): 435–451. https://doi.org/10.1111/boj.12218

Miller AM, Pfeiffer P, Schwartz T (2010) Creating the CIPRES science gateway for inference of large phylogenetic trees. Gateway Computing Environments Workshop 14: 1–8. https://doi.org/10.1109/GCE.2010.5676129

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics (Oxford, England) 19(12): 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A (2017) DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. Molecular Biology and Evolution 34(12): 3299–3302. https://doi.org/10.1093/molbev/msx248

Stamatakis A (2014) RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics (Oxford, England) 30(9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Tamura MN (1990) Biosystematic studies on the genus *Polygonatum* (Liliaceae) I. Karyotype analysis of species indigenous to Japan and its adjacent regions. Cytologia 55(3): 443–466. https://doi.org/10.1508/cytologia.55.443

Tamura MN (1991) Biosystematic studies on the genus Polygonatum (Liliaceae) II. Morphology of staminal filaments of species indigenous to Japan and its adjacent regions. Acta Phytotaxonomica et Geobotanica 42: 1–18.

Tamura MN (1993) Biosystematic studies on the genus Polygonatum (Liliaceae) III. Morphology of staminal filaments and karyology of eleven Eurasian species. Bot. Jahrb. Syst. 115: 1–26.

Tamura MN (2008) Biosystematic studies on the genus *Polygonatum* (Asparagaceae) V. Taxonomic revision of species in Japan. Acta Phytotaxonomica et Geobotanica 59: 15–29.

Tamura MN, Ogisu M, Xu JM (1997a) *Heteropolygonatum*, a new genus of the tribe Polygonateae (Convallariaceae) from West China. Kew Bulletin 52(4): 949–956. https://doi.org/10.2307/4117821

Tamura MN, Schwarzbach AE, Kruse S, Reski R (1997b) Biosystematic studies on the genus *Polygonatum* (Convallariaceae) IV. Molecular phylogenetic analysis based on restriction site mapping of the chloroplast gene *trn*K. Feddes Repertorium 108(3–4): 159–168. https://doi.org/10.1002/fedr.4921080306

Wang JJ, Yang YP, Sun H, Wen J, Deng T, Nie ZL, Meng Y (2016) The biogeographic South-North divide of *Polygonatum* (Asparagaceae tribe Polygonateae) within Eastern Asia and its recent dispersals in the Northern Hemisphere. PLoS ONE 11: e0166134. https://doi.org/10.1371/journal.pone.0166134

Wyman SK, Jansen RK, Boore JL (2004) Automatic annotation of organellar genomes with DOGMA. Bioinformatics (Oxford, England) 20(17): 3252–3255. https://doi.org/10.1093/bioinformatics/bth352

Xu JM (1981) *Polygonatum* Mill. In: Sichuan Flora Editorial Board (Eds) Flora Sichuanica. Sichuan People's Publishing House, Chengdu, 7, 230–232.

Zhao LH, Zhou SD, He XJ (2019) A phylogenetic study of Chinese *Polygonatum* (Polygonateae, Asparagaceae). Nordic Journal of Botany 37(2): njb.02019. https://doi.org/10.1111/njb.02019

## Supplementary material 1

**Figure S1. Longitudinal section of flower of *P. hunanense* and *P. kingianum***

Authors: Maoqin Xia, Ying Liu, Jingjing Liu, Donghong Chen, Yan Shi, Zhicong Bai, Yu Xiao, Chen Peng, Jinping Si, Pan Li, Yingxiong Qiu

Data type: species data

Link: https://doi.org/10.3897/phytokeys.175.63383.suppl1

PhytoKeys 175: 151–174 (2021)
doi: 10.3897/phytokeys.175.61467
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CHECKLIST

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# A checklist of vascular plants of Ewe-Adakplame Relic Forest in Benin, West Africa

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## Abstract

Covering 560.14 hectares in the south-east of Benin, the Ewe-Adakplame Relic Forest (EARF) is a microrefugium that shows insular characteristics within the Dahomey Gap. It is probably one of the last remnants of tropical rain forest that would have survived the late Holocene dry period. Based on intensive field investigations through 25 plots (10 × 50 m size) and matching of herbarium specimens, a checklist of 185 species of vascular plant belonging to 54 families and 142 genera is presented for this forest. In addition to the name for each taxon, we described the life form following Raunkiaer's definitions, chorology as well as threats to habitat. The Rubiaceae family was the richest (20 species) followed by the Fabaceae (15 species). Life forms showed the preponderance of phanerophytes (88%). The Chorological spectrum was dominated by Guineo-Congolean species (66%). Species richness estimated were 200.52 ± 9.2808 for *Bootstrap*; 217.62 ± 14.5972; 224.16 ± 15.3725 and 242.67 respectively for *Chao*, *Jacknife1* and *Jacknife2*. *Bootstrap* appears to be the estimation closer to the field records. In Benin, EARF is home for *Rinorea* species described as West African forest bio-indicators and single location for *Nesogordonia papaverifera*, *Mansonia altissima*, *Englerophytum oblanceolatum*, *Octolobus spectabilis*, *Vitex micrantha* and most of *Drypeteae* tribe species (*Drypetes aframensis*, *Drypetes afzelii*, *Drypetes gilgiana* and *Drypetes leonensis*) recorded in Benin. Our results provides baseline information for further in-depth analysis of vegetation history in Benin by raising the question on the past floristic connection of the Dahomey gap and community engagement in conservation.

**Keywords**
Dahomey Gap, Flora, Kétou, Range-restricted species, Refugial

## Introduction

At the continental level, African rain forests, primarily those of the Guineo-Congolean block, are the main centres of species diversity (White 1983; Lebrun 2001; Sosef et al. 2017; Droissart et al. 2018; Couvreur et al. 2021). However, in some regions of this block, landscape changes have been so severe that particular areas would have functioned as refugia, while diversity in surrounding areas would have experienced losses. This is probably the case with forest islands within the Dahomey Gap which is the dry corridor separating the West African rain forest into the Upper Guinean and Lower Guinean blocks (White 1983; Jenik 1994; Poorter et al. 2004). During the late Holocene dry period (3000–2500 yrs BP), the once continuous rain forest belt became fragmented and was reduced to isolated patches that would have persisted and survived as "small isolated humid pockets" (Dupont and Weinelt 1996; Salzmann and Hoelzmann 2005). In addition to these historical climatic oscillations, archaeologists and ecologists highlighted several proofs of human footprint such as metallurgy (cast iron), agriculture, pottery that would have gradually caused deforestation and may explain the phytogeographic status of the Dahomey gap in West Africa (Richards 1973; Paradis 1977; Wantchecon 1983; Brand 2001; Garnier et al. 2018). Indeed, this savanna intermingled with small forest patches within the Dahomey gap, is also seen as a cultural landscape, produced by humans for subsistence, security and/or worship uses of religious traditions (Juhé-Beaulaton 2010; Cousin 2018). Consequently, the vegetation of Benin which is supposed to be luxuriant is today largely dominated by farms, fallows and grasslands (Adjanohoun 1966; Jenik 1994; Sosef et al. 2017). One fifth of the original forests remain, fragmented into isolated patches (Poorter et al. 2004). Today, Benin is home to 2807 plant species in terms of floristic composition (Akoègninou et al. 2006). It is among the best explored botany countries of sampling completeness with 2460 species theoretically estimated between 2864 and 2889 species (Sosef et al. 2017). Despite this sampling effort, some species that have not yet been collected or reported may not be listed and therefore omitted. Either because they may have disappeared, following their habitat degradation, as was the case of *Chrysobalanus icaco* L. (Syn. *C. atacoriensis* A. Chev.), which would have disappeared following earthworks (Adjanohoun et al. 1989).

However in Benin, most of these remaining forest patches, although playing the role of a high conservation priority area for heritage plants, are still experiencing severe threats due to the lack of adequate conservation strategies (Oloukoï et al. 2007; Adomou et al. 2010). This is probably the case with Ewe and Adakplame Relic Forest (EARF) in the south east of Benin. Up to now, this relic still persists on a community land while showing insular characteristics with some rare and poorly known plant species. Earlier botanists who worked on this refugium include Chevalier (1910), Aubréville (1937) and Adjanohoun (1966). They noted the typical feature of the Ewe-Adakplame Relict Forest (EARF) described as a Timber-refuge of the time of tribal wars, as one having a considerable number of African rain forest species of Guineo-Congolean region comparable to those of Côte d'Ivoire (Adjanohoun et al. 1989, P. 20).

In this context, floristic details on EARF may be very useful for conservation purposes through restoration and rehabilitation of degraded land with native trees. Such information is necessary for further studies in biogeography and phylogeny on the one hand, to address the main speciation models and mechanisms that may apply across tropical Africa (Demenou et al. 2016, 2018; Couvreur et al. 2021), and on the other hand to reconstruct the history of Tropical Africa vegetation.

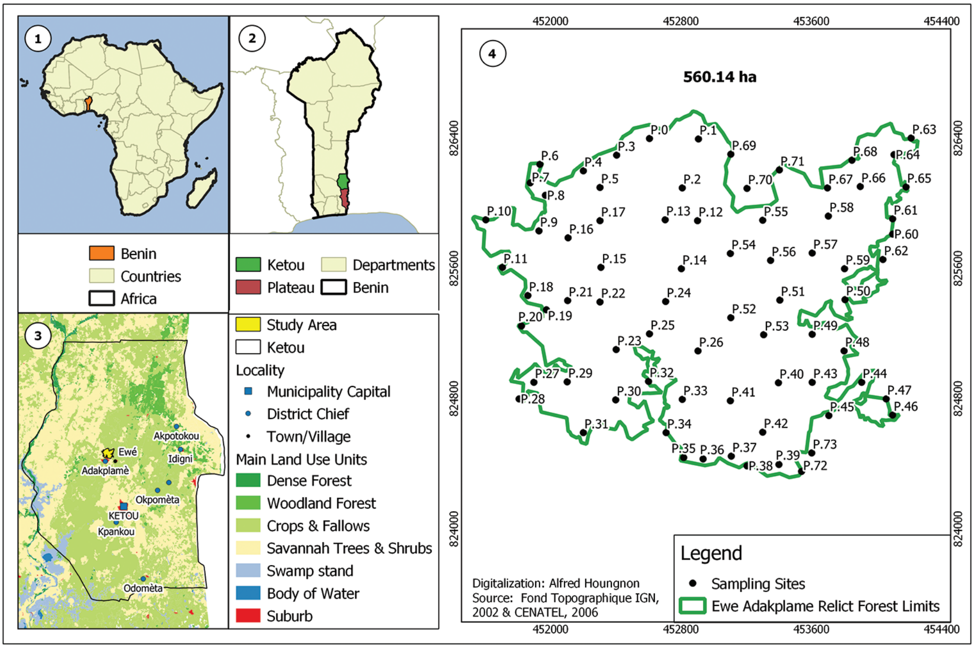
This paper aims to provide a comprehensive checklist of vascular plants occurring in EARF that will serve as baseline for understanding the history of this vegetation over millennia. By exploring the floristic composition of EARF, we can better appreciate the biogeographic status of some species previously reported by Achoundong (1996; 2000) as bio indicators in other West African forests located on either side of the Dahomey gap. The results could help (1) to better understand how the Dahomey Gap has affected the vegetation of this area and (2) to catalyze long-lasting conservation actions toward EARF.

## Methods area

### Study site

The EARF covers 560.14 hectares in the Kétou District in the south-east of Benin Republic at 07°27'59.195"N, 002°34'29.395"E (Fig. 1). This part of the country belongs to the Guineo-Congolean Region (White 1983; Adomou et al. 2006). The forest relic is located at the north-east of the depression of "Co" or "Lama" on the plateaus of low altitude that evolved on the pre-Cambrian base rocks (Adjanohoun et al. 1989). In Benin, the most important national protected areas are in the north. There are several other forests (albeit small) in the southern part of the country which are within the national protected areas network (e.g. gazetted forests of Dogo-Ketou, Pobe, Lama, Pahou) which are well-managed (Adomou et al. 2006). There is also the recent Transboundary biosphere reserve of Mono which is now part of the national protected areas network. However, EARF has not yet been included in this national protected areas network.

The mean annual rainfall in the EARF is between 900–1300 mm (Adjanohoun et al. 1989; CARDER 2002; Adomou et al. 2006) which contrasts to other similar African dense semi-deciduous forests. The rainfall recorded in Upper Guinea is between 1750–1900 mm (Martin 2008) in Côte d'Ivoire (West Africa) and annual rainfall measured around the Kakamega rain forest in East Africa was approximately 2215 mm (Cords 1987) and 1956 mm (Greiner 1991). Table 1 provides parameters such as temperature, relative humidity, vegetation and soil types of the study site. The



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Source: Fond Topographique IGN, 2002 & CENATEL, 2006

**Figure 1.** Location of the Ewe-Adakplame Relict Forest in Benin and positions of the sampling stands.

**Table 1.** Ecological characteristics of the study region.

| | | |
|---|---|---|
| Location | 6°25–7°30N | Adjanohoun et al. (1989)<br>Adomou et al. (2006) |
| Annual rainfall | 900–1100 mm | CARDER 2002, Adomou et al.(2006) |
| Rainfall trend | Bimodal | Adjanohoun et al. (1989) |
| Rainy season(s) | March–July & September–October | Adjanohoun et al. (1989)<br>Adomou et al. (2006) |
| Dry season | August and November–February | Bani (2006) |
| Temperature | 24–37 °C | CARDER (2002), Bani (2006) |
| Insolation | 2135 h | CARDER (2002), Bani 2006 |
| Relative humidity | 78–95% | CARDER (2002), Bani (2006) |
| Climate type | Sub-equatorial | Adomou et al. (2006) |
| Length of plant growing season | 240 days | CARDER (2002), Bani (2006) |
| Vegetation | Mosaic of Savanna | Adjanohoun et al. (1989)<br>Adomou et al. (2006) |
| Soil types | Ferralitic soils without concretion | Adjanohoun et al. (1989), Bani (2006) |
| Altitude | 200–286 m above sea level | Bani (2006) |

landscape surrounding EARF is dominated by fallows, cultivation areas and housing. The vegetation is a mosaic of savanna with species of the Sudanian transition zone such as *Adansonia digitata* L., *Stereospermum kuntianum* Cham., *Trichilia emetic* Vahl, *Annona senegalensis* Pers., *Vitex doniana* Sweet, *Parkia biglobosa* (Jacq.) R.Br. ex G. Don, *Dichrostachys cinerea* (L.) Wight & Arn., *Pterocarpus erinaceus* Poir., *Pericopsis laxiflora* (Benth.) Meeuwen, *Daniellia oliveri* (Rolfe) Hutch. & Dalziel, *Vitellaria paradoxa* C.F. Gaertn., *Sarcocephalus latifolius* (Sm.) E.A. Bruce, *Uvaria chamae* P. Beauv., *Vitex grandifolia* Gürke and *Andropogon gayanus* Kunth (Biaou 2009; Armani et al. 2018).

The total population of the villages of Ewe and Adakplame is 13,623 individuals with 2,078 households (INSAE 2016). The main activity is agriculture, followed by hunting, livestock breeding and local commerce.

## Sampling and data collection

The inventory of EARF plant species was conducted from February 2014 to December 2017. The forest investigation was based on a vegetation map divided into 250,000 m$^2$ (500 × 500 m) grids following 6 transects, each of 500 m width and 3000 m length. Transects were oriented south-north. The floristic sampling covered different components of the EARF (Fig. 1). At each stand, a topometer (Chaining Buddy, Fremaco Devices, Canada) with disposable filament was used to delimit quadrats of 10 × 50 m. The observation stands were set out at intervals of 100 m along each transect line and there was one quadrat per plot of 250,000 m$^2$. In total, 25 forest quadrats of 500 m$^2$ were floristically surveyed. To set a preliminary list of EARF flora, species identification was first based on our self-background during the sampling field work with photo captures (Olympus Digital Camera SP-620 UZ Silver and Samsung Galaxy S7 Android 6.0.1). This approach was combined with description session (on field and at the National Herbarium). Voucher specimens were systematically collected for specimens whose determination is confused. They were compared with voucher specimens of the national Herbarium. To access the systematic information notes, the botanical nomenclature followed the Analytical Flora of Benin (Akoègninou et al. 2006). The list of plant species recorded was compared to online resources such as the "Catalog of life" (Hassler 2020) and the Benin National Red List (Neuenschwander et al. 2011) in order to access botanical information notes and the conservation status of species.

## Data analysis

The Angiosperm Phylogeny Group (APG IV 2009) and the legume subfamilies currently accepted by the legume phylogeny working group (LPWG 2017) were used to update the list of the vascular plants recorded in EARF. The taxonomic plant diversity was assessed in terms of species, genus, and family richness. The species richness (S) corresponds to the number of species recorded from sampling plots ($n$ = 25). We used the functions "specpool" and "estimateR" in R software (R-Core-Team 2016) for the main reason that S is sensitive to sample size and this may introduce bias in our estimations based on the field record. To circumvent this, we use species accumulation curve and different estimation methods in order to appreciate in the case of our field study, the best estimator which is closest to our field record (Palmer 1990; Colwell and Coddington 1994; Chiu et al. 2014). This approach also helps to assess the completeness of our sampling effort. Chao, first order jackknife, second order jackknife and bootstrap were then used to estimate the total number of species surveyed and to draw species accumulation curves (R-Core-Team 2016; Oksanen et al. 2017).

Life forms assessment followed Raunkiaer (1934); Hutchinson and Dalziel (1954–1972): Ph: phanerophytes subdivided into meg: megaphanerophyte (> 30 m tall), mes: mesophanerophyte (8–30 m), mph: microphanerophyte (2–8 m), nph: nanophanerophyte (0.5–2 m); Ch: chamaephyte, Hc: hemicryptophyte; Th: therophyte; G: geophyte (Gb: with bulb, Gr: with rhizome and Gt: with tuber); Ep: epiphyte and their climbing forms L: liana (Lmph, Lnph and Lmes, LGr, LHc).

The Chorology types were established after Hutchinson and Dalziel (1954–1972) and White (1983), as follows: GC: Guineo-Congolean, SG: Sudano/Guinean transition, GE: Lower Guinean, GO: Upper Guinean, TA: Tropical Africa, AM: Afro-Malagasy, Pan: Pantropical.

## Data resources

The data underpinning the analysis reported in this paper are deposited in the Dryad Data Repository at https://doi.org/10.5061/dryad.z8w9ghxbg (Houngnon and Adomou 2021).

# Results

## Floristics

Fig. 2A, B shows a panoramic view around and inside of EARF. Table 2 gives an overview of the vascular plant species recorded in the EARF. A total of 185 plant species distributed over 143 genera and 54 families was recorded (Table 2). With this figure, EARF conserves 6.59% of the national flora over 560.14 hectares of a community land. Of these, Rubiaceae was the most speciose family (20 species), followed by Fabaceae (15), Malvaceae (13), Apocynaceae (12), Sapindaceae (8) and Annonaceae (7). Capparaceae, Celastraceae, Dioscoreaceae, Putranjivaceae, Violaceae

**Figure 2.** Panoramic view of Ewe-Adakplame Relict Forest **A** forest ecosystem in contact with Ewe village's (Olympus photo A. Houngnon 2014) **B** forest gap with *Momordica charantia* carpet (Samung photo A. Houngnon 2016).

**Table 2.** Vascular plants of Ewe-Adapklame relict forest in Benin with their binomial, family life-forms and Chorotypes [Life-forms are meg: megaphanerophyte (> 30 m tall), mes: mesophanerophyte (8–30 m), mph: microphanerophyte (2–8 m), nph: nanophanerophyte (0.5–2 m); Ch: chamaephyte, Hc: hemicryptophyte; Th: therophyte; G: geophyte (Gb: with bulb, Gr: with rhizome and Gt: with tuber); Ep: epiphyte and their climbing forms L: liana (Lmph, Lnph and Lmes, LGr, LHc) and chorotypes are GC: Guineo-Congolean, SG: Sudano/Guinean transition, GE: Lower Guinean, GO: Upper Guinean, TA: Tropical Africa, AM: Afro-Malagasy and Pan: Pantropical].

| Scientific name | Life forms | Chorology types | Voucher specimens |
|---|---|---|---|
| **Acanthaceae** | | | |
| *Rhinacanthus virens* (Nees) Milne. Readh. var. *virens* | Ch | GC | Houngnon 3860 |
| **Amaranthaceae** | | | |
| *Cyathula prostrata* (L.) Blume | Th | Pan | Houngnon 3383 |
| **Amaryllidaceae** | | | |
| *Scadoxus multiflorus* (Martyn) Raf. subsp *multiflorus* | Gb | TA | Houngnon 6724 |
| **Anacardiaceae** | | | |
| *Lannea nigritana* (Sc. Elliot) Keay var. *nigritana* | mes | GO | De Souza 1971 a |
| *Spondias mombin* L. | mes | Pan | Maesen 7705 |
| **Annonaceae** | | | |
| *Artabotrys dahomensis* Engl. & Diels. | Lnph | GE | Houngnon 97e |
| *Artabotrys velutinus* Sc. Elliot | Lnph | GC | Maesen 6612 |
| *Monanthotaxis parvifolia* (Oliv.) Verdc. | Lnph | GE | Houngnon s.n. |
| *Monodora tenuifolia* Benth. | mph | GC | Éq. Bot. 105d |
| *Uvariodendron angustifolium* (Engl. & Diels) R.E.Fr | mph | GC | Houngnon 5571 |
| *Uvariopsis tripetala* (Baker f.) G.E.Schatz Syn. *Dennettia tripetala* Baker f. | mph | GE | Akoègninou 2201 |
| *Xylopia longipetala* De Wild. & T. Durand | mph | GC | Houngnon 4524 |
| **Apocynaceae** | | | |
| *Alafia barteri* Oliv. | Lmph | GC | Chevalier 22841 |
| *Ancylobotrys scandens* (Schumach. & Thonn.) Pifchon | Lmph | GC | Chevalier 23456 |
| *Baissea zygodioides* (K. Schum.) Stapf | Lmph | GC | Houngnon 118c |
| *Cryptolepis nigrescens* (Wennberg) L. Joubert & Bruyns Syn. *Parquetina nigrescens* (Afzel.) Bullock | Lmph | GC | Le Testu 297 |
| *Holarrhena floribunda* (G. Don) Dur. & Schinz | mph | TA | Houngnon 6574 |
| *Hunteria umbellata* (K. Schum.) Hall. f. Syn. *H. eburnea* Pichon | mph | GC | Aké Assi 20284 |
| *Landolphia hirsuta* (Hua) Pichon | Lmes | GC | Chevalier 23922 |
| *Marsdenia latifolia* (Benth.) K. Schum., | Lmph | TA | Akoègninou 5438 |
| *Mondia whitei* (Hook. f.) Skeels | Lmph | TA | Adjakidjè 3007 |
| *Motandra guineensis* (Thonn.) A. DC. | Lmph | TA | Adjanohoun 102 |
| *Saba thompsonii* (A. Chev.) Pichon | Lmes | GC | Chevalier 22967 |
| *Secamone afzelii* (Schultes) K. Schum. | Lmph | GC | Essou 3208 |
| **Araceae** | | | |
| *Anchomanes difformis* (Blume) Engl. (Syn. *A. welwitschii* Rendle) | Gt | GC | Essou 1554 |
| *Cercestis mirabilis* (N. E. Br.) Bogner Syn. *Rhektophyllum mirabile* N.E.Br. | Ep | GE | Akoègninou 3299. |
| **Aristolochiaceae** | | | |
| *Pararistolochia goldieana* (Hook. f.) Hutch. & Dalz. | LGr | GC | Houngnon 4605 |
| **Asparagaceae** | | | |
| *Dracaena arborea* Bak | mph | GC | Maesen 6340 |
| **Asteraceae** | | | |
| *Chromolaena odorata* (L.) R. King & H. Robinson | nph | AM | Sokpon B14 |
| *Gymnanthemum coloratum* (Willd.) H. Rob. & B.Kahn | mph | SZ | Ayichédéhou 395 |
| *Laggera crispata* (Vahl) Hepper & J. R. I. Wood. | Th | TA | Maesen 6746 |
| **Bignoniaceae** | | | |
| *Newbouldia laevis* (P. Beauv.) Seem. ex Bureau | mph | GC | Houngnon 3087 |
| **Boraginaceae** | | | |
| *Ehretia cymosa* Thonn. | mph | GC | Houngnon 5081 |
| **Cannabaceae** | | | |
| *Celtis mildbraedii* Engl. | mes | GC | Essou 1648 |
| *Celtis philippensis* Blanco Syn. *C. brownii* Rendle | mph | GC | Houngnon 2783 |

| **Scientific name** | **Life forms** | **Chorology types** | **Voucher specimens** |
|---|---|---|---|
| *Celtis zenkeri* Engl. | meg | GC | Sokpon 852 |
| *Trema orientalis* Syn. *T. guineensis* | mph | GC | Houngnon 1714d |
| **Capparaceae** | | | |
| *Capparis brassii* DC. Syn. *C. thonningii* Schum. | Lnph | GC | Maesen 6701 |
| *Capparis erythrocarpos* Isert var. *erythrocarpos* | nph | GC | Esson 1087 |
| *Maerua duchesnei* (De Wild.) F. White Syn: *Ritcheia duchesnei* (De Wild.) Keay | mph | GC | Houngnon 229a |
| *Ritchiea capparioides* (Andr.) Britten var. *capparoides* | Lnph | GC | Houngnon 4200 |
| *Ritchiea erecta* Hook. f. Syn. *R. pentaphylla* Gilg & Bened. | nph | GE | Aké Assi 20288 |
| **Celastraceae** | | | |
| *Loeseneriella africana* (Willd.) N.Hallé var. *africana* Syn. *Hippocratea Africana* (Willd.) Loes. | Lmph | Pan | Houngnon 6573 |
| *Reissantia indica* (Willd.) N. Hallé | Lnph | Pan | Akoègninou 4026 |
| *Salacia longipes* (Oliv.) N. Hallé | nph | TA | Akoègninou 3291 |
| *Salacia pallescens* Oliv. | nph | GC | Sokpon 2221 |
| *Simicratea welwitschii* (Oliv.) Syn. *S. welwitschii* (Oliv.) N. Hallé | Lmph | GC | Essou 1467 |
| **Combretaceae** | | | |
| *Combretum racemosum* P. Beauv. | Lmph | GC | Le Testu 191 |
| **Commelinaceae** | | | |
| *Cyanotis lanata* Benth. | Ch | SG | Morton A4570 |
| **Connaraceae** | | | |
| *Cnestis ferruginea* Vahl ex DC. | nph | GC | Houngnon 3051 |
| *Cnestis corniculata* Lam. Syn. *Cnestis longiflora* Schellenb. | Lmph | GO | Chevalier 22828 |
| *Rourea coccinea* (Bak.) Jongkind syn. *Byrsocarpus coccineus* Thonn. & Schumach. | nph | TA | Chevalier 22798b |
| **Convolvulaceae** | | | |
| *Calycobolus africanus* (G. Don) heine | Lnph | GC | Adjakidjè 4111 |
| *Ipomoea mauritiana* Hall. f. | Lmph | Pan | Oumorou 740 |
| **Cucurbitaceae** | | | |
| *Coccinia grandis* (L.) Voigt | Lnph | GC | De Souza & Paradis 444a |
| *Lagenaria breviflora* (Benth.) Roberty Syn. *Adenopus breviflorus* Benth. | Lmes | TA | Houngnon 443a, 1518a |
| *Luffa cylindrica* (L.) M. J. Roem syn. *Luffa aegyptiaca* Mill | Lnph | Pan | Houngnon 453a |
| *Momordica charantia* L. | Lnph | GC | Houngnon 1676 |
| **Dichapetalaceae** | | | |
| *Dichapetalum madagascariense* Poir. Syn. *D guineense* (DC.) Keay | Lmph | GC | Adomou 95 |
| *Tapura fischeri* Engl. | mph | GC | Houngnon 1878a |
| **Dioscoreaceae** | | | |
| *Dioscorea bulbifera* L. var. bulbifera | Gt | Pan | Essoun 3316 |
| *Dioscorea lecardii* De Wild. | Gt | SZ | Pauwels 8139 |
| *Dioscorea odoratissima* Pax Syn. *D. praehensilis sensu* F.T.A, F.W.T.A | Gt | SG | Chevalier 24154 |
| *Dioscorea quartiniana* A. Rich. | Gt | SZ | Sokpon 2329 |
| *Dioscorea sagittifolia* Pax syn. *D. abyssinica* Hochst. ex Kunth | Gt | SZ | Paradis et Houngnon: 619d |
| **Ebenaceae** | | | |
| *Diospyros abyssinica* (Hiern) White | mes | GC | Houngnon 627b |
| *Diospyros monbuttensis* Gürke | mph | GC | Houngnon 629c |
| *Diospyros soubreana* F. White | nph | GC | Houngnon 2824 |
| **Euphorbiaceae** | | | |
| *Erythrococca anomala* (Juss. ex Poir.) Prain | nph | GC | Houngnon 3345 |
| *Mallotus oppositifolius* (Geisel.) Müell. Arg. var. *oppositifolius* | nph | AM | Adjakidjè & Akoègninou 590c |
| *Tragia senegalensis* Müll. Arg. | Lnph | SG | Adjakidjè 2803 |
| **Fabaceae** | | | |
| **Caesalpinoideae (Mimosoid clade)** | | | |
| *Acacia pennata* (L.) Willd. | Lmph | TA | Essou 1672 |
| *Acacia polyacantha* Willd. subsp. *Campylacantha* (Hochst. ex A. Rich.) Brenan | mes | SZ | Maesen 6703 |
| *Albizia adianthifolia* (Schum.) W. Wight var. *adianthifolia* | mes | GC | Adjakidjè 4163 |
| *Albizia glaberrima* (Schum. & Thonn.) Benth. | mph | GC | Houngnon 6532 |
| *Albizia ferruginea* (Guill. & Perr.) Benth. | mes | GC | Paradis & Houngnon 933a |

| Scientific name | Life forms | Chorology types | Voucher specimens |
|---|---|---|---|
| *Albizia zygia* (DC.) J. F. Macbr. | mes | GC | Houngnon 936d |
| *Mezoneuron benthamianum* (Baill.) Herend. & Zarucchi | Lmph | GC | Paradis & Houngnon 277c |
| **Detarioideae** | | | |
| *Detarium senegalense* J.F. Gmel. | mes | GC | Houngnon 268[e] |
| **Dialioideae** | | | |
| *Dialium guineense* Willd. | mes | GC | Spire 118 |
| **Faboideae/ Papilionoideae** | | | |
| *Abrus precatorius* L. | Lnph | Pan | Houngnon 1423g |
| *Dalbergia lactea* Vatke | Lmph | GE | De Souza & Paradis 1239e |
| *Dalbergia melanoxylon* Guill. Perr. | mph | SG | Adomou 167 |
| *Desmodium salicifolium* (Poir.) DC. var. *salicifolium* | nph | GC | Frahm-Leliveld 57139 |
| *Dolichos trilobus* | Lnph | SZ | Adomou 80 |
| *Millettia thonningii* (Schum. & Thonn.) Bak. | mph | GC | Essou 1164 |
| **Icacinaceae** | | | |
| *Stachyanthus occidentalis* (Keay & Miège) Boutique syn. *Neostachyanthus occidentalis* Keay & Miège | Lnph | GO | Essou 1102 |
| **Lamiaceae** | | | |
| *Clerodendrum capitatum* (Willd.) Schum. & Thonn. | Lmph | GC | Lisowski 0-929 |
| *Hoslundia opposita* Vahl | nph | AM | Pauwels 8286 |
| *Premna quadrifolia* Schum. & Thonn. | nph | GO | Sokpon 1068 |
| *Vitex micrantha* Gürke* | mes | GC | Adomou s.n. |
| **Linaceae** | | | |
| *Hugonia platysepala* Welw. ex Oliv. | Lmph | GC | Paradis & Houngnon 831a |
| **Loganiaceae** | | | |
| *Strychnos barteri* Soler. | Lmes | GC | Paradis & Houngnon 838a |
| *Strychnos floribunda* Gilg | Lmes | GC | Maesen 6821 |
| *Strychnos nigritana* Bak. | Lmes | GC | Akoègninou 3289 |
| *Strychnos splendens* Gilg | Lmes | GC | Houngnon 835b |
| **Malvaceae** | | | |
| *Abutilon mauritianum* (Jacq.) Medic. | Ch | TA | De Souza & Paradis 851a |
| *Ceiba pentandra* (L.) Gaertn. | meg | Pan | Houngnon 188a |
| *Hibiscus lunariifolius* Willd. | Lmph | Pan | Adomou s.n. |
| *Hibiscus owariensis* P. Beauv. | nph | GC | Paradis et Houngnon 856a |
| *Cola milfenii* K. Schum. | mph | GC | Houngnon 4399 |
| *Glyphaea brevis* (Spreng.) Monachino | mph | GC | Houngnon 2036[e] |
| *Grewia carpinifolia* Juss. | mph | GC | Houngnon 1446f |
| *Mansonia altissima* (A. Chev.) A. Chev. var. *altissima** | mes | GC | Houngnon 1309a ; 4322 |
| *Nesogordonia papaverifera* (A. Chev.) syn *N. kabengaensis* (K.Schum.)* | mph | GC | Houngnon 13l0a |
| *Octolobus spectabilis* Welw. Syn. *O. angustatus* Hutch.* | nph | GC | Adomou s.n. |
| *Pterygota macrocarpa* K. Schum *. | mph | GC | Houngnon 4321 |
| *Sterculia tragacantha* Lindl. | mes | GC | De Souza & Houngnon 188d |
| *Triplochiton scleroxylon* K. Schum. | meg | GC | Chevalier 22819 |
| **Melastomataceae** | | | |
| *Memecylon afzelii* G. Don var. *afzelii* | Lnph | GC | Houngnon 897c |
| *Warneckea memecyloides* (Benth.) Jac. Fél Syn. *Memecylon memecyloides* (Benth) | Lmph | GC | De Souza & Paradis 900a |
| **Meliaceae** | | | |
| *Trichilia prieureana* A. Juss. subsp. *prieureana* | mph | GC | Adomou 90 |
| **Menispermaceae** | | | |
| *Dioscoreophyllum cumminsii* (Stapf) Diels | Lnph | GC | Houngnon 919a |
| *Rhigiocarya racemifera* Miers | Lnph | GC | Maesen 6820 |
| *Tiliacora funifera* (Miers) Oliv. | Lmph | GC | De Souza 92li |
| *Triclisia subcordata* Oliv. | Lnph | GC | Sokpon 31 |

| Scientific name | Life forms | Chorology types | Voucher specimens |
|---|---|---|---|
| **Moraceae** | | | |
| *Antiaris toxicaria* Lesch. | meg | GC | Essou 1547 |
| *Ficus recurvata* De Wild. Syn. *Ficus goliath* A. Chev. | mes | GC | Adomou s.n. |
| *Ficus ovata* Vahl, | Ep | GC | Adomou s.n. |
| *Milicia exelsa* (Welw.) Berg Syn. *Chlorophora excelsa* (Welw.) benth. | meg | GC | Chevalier 23169 |
| **Olacaceae** | | | |
| *Olax subscorpioidea* Oliv. var. *subscorpioidea* | mph | GC | Houngnon 7652 |
| **Oleaceae** | | | |
| *Schrebera arborea* A. Chev. | mes | GC | Akoègninou 2187 |
| **Opiliaceae** | | | |
| *Opilia amentacea* Roxb. Syn. *O. celtidifolia* (Guill. & Perr) Endl., | Lmph | SZ | Adjakidjè 1477 |
| **Pandaceae** | | | |
| *Microdesmis keayana* J. Léonard, syn. *M. puberula* Hook. f. | mph | GC | Pauwels 8298 |
| **Passifloraceae** | | | |
| *Adenia cynanchifolia* (Benth.) Harms | Lmph | GE | Adomou s.n. |
| *Adenia lobata* (Jacq.) Engl. | Lmph | GC | Essou 1637 |
| **Phytolaccaceae** | | | |
| *Hilleria latifolia* (Lam.) H. Walt. | Ch | AM | Adomou 129 |
| **Poaceae** | | | |
| *Acroceras gabunense* (Hack.) Clayton* | Th | GC | Mission *ACCT/Bénin 2165* |
| *Olyra latifolia* L. | nph | GC | Houngnon 720c |
| *Oplismenus hirtellus* (L.) P. Beauv. subsp. *Hirtellus* | Ch | SG | De Souza & Paradis 722a |
| *Streptogyna crinita* P. Beauv. | Gr | GC | Houngnon 765b |
| **Polygalaceae** | | | |
| *Carpolobia lutea* G. Don | mph | GC | Maesen 6617 |
| **Putranjivaceae** | | | |
| *Drypetes aframensis* Hutch.* | mph | GO | Adomou s.n. |
| *Drypetes afzelii* (Pax) Hutch.,* | mes | GO | Houngnon 177 la |
| *Drypetes floribunda* (Müll. Arg.) Hutch. | mph | GC | Houngnon 4266 |
| *Drypetes gilgiana* (Pax) Pax & Hoffm.* | nph | GC | Akoègninou 2196 |
| *Drypetes leonensis* Pax,* | mes | GC | Houngnon 1771b |
| **Rhamnaceae** | | | |
| *Lasiodiscus mannii* Hook. f. | mph | GC | Houngnon 1329b |
| **Rubiaceae** | | | |
| *Aidia genipiflora* (DC.) Dandy | mph | GC | Maesen 6611 |
| *Chassalia kolly* (Schumach.) Hepper | nph | GC | Maesen 6358 |
| *Coffea ebracteolata* (Hiern) Brenan | Lnph | GC | Lejoly&Ganglo 2 |
| *Cremaspora triflora* (Thonn.) K. Schum. | Lmph | GC | Maesen 6284 |
| *Leptactina involucrata* Hook. f. | Lnph | GC | Adomou s.n. |
| *Gardenia nitida* Hook. | mph | GC | Adomou 73 |
| *Hymenodictyon floribundum* (Steud. & Hochst.) B.L.Rob. | mes | GC | Sinsin 2863 |
| *Keetia hispida* (Benth.) Bridson | Lmph | GC | Adomou s.n. |
| *Morinda lucida* Benth. | mph | Pan | Maesen 6651 |
| *Oxyanthus pallidus* Hiern | nph | GC | Adomou s.n. |
| *Oxyanthus speciosus* DC. subsp. *speciosus* | nph | GC | Essou 2496 |
| *Pavetta corymbosa* (DC.) F. N. Williams | mph | SG | Sokpon 1884 |
| *Pouchetia africana* DC. | nph | GC | Houngnon 6659 |
| *Psydrax horizontalis* (K. Schum. & Thonn.) Bridson | Lmph | SG | Maesen 6710 |
| *Psydrax parviflora* (Afzel.) Bridson | nph | GO | Maesen 6287 |
| *Rothmannia longijlora* Salisb | mph | GC | Le Teslu 101 |
| *Rothmannia urcelliformis* (Hiern) Bullock ex Robyns | mph | GC | Dansi TW 50799 |
| *Rytigynia canthioides* (Benth.) Robyns | mph | GC | Adomou s.n. |
| *Vangueriella nigerica* (Robyns) Verdc. Syn. *Vangueriopsis nigerica* Robyns | mph | SZ | Maesen 6315 |
| *Vangueriella spinosa* (Schumach.&Thonn.)Verdc. Syn. *Vangueriopsis spinosa* Hepper | mph | SZ | Adomou 32 |
| **Rutaceae** | | | |
| *Zanthoxylum leprieurii* Guill. & Perr. Syn. *Fagara angolensis* Engl. | mph | GC | Houngnon 535a |
| *Zanthoxylum zanthoxyloides* (Lam.) Zepernick & Timber | mph | SG | Essou 2396 |

| Scientific name | Life forms | Chorology types | Voucher specimens |
|---|---|---|---|
| **Salicaceae** | | | |
| *Dovyalis zenkeri* Gilg (+) Syn. *D. afzelii* Gilg. (+) | nph | GO | Houngnon 1364a |
| *Flacourtia indica* (Burm. f.) Merr. Syn. *Flacourtia flavescens* Willd. | mph | GC | Houngnon 6606 |
| **Sapindaceae** | | | |
| *Allophylus africanus* P. Beauv. | mph | GC | Houngnon 4037 |
| *Allophylus spicatus* (Poir.) Radlk. | mph | GC | Houngnon 4037 |
| *Blighia sapida* Koenig | mPh | Pan | Houngnon 5472 |
| *Blighia unijugata* Bak. | mph | GC | Paradis & Houngnon 1693d |
| *Deinbollia pinnata* (Poir.) Schumach. & Thonn. | nph | GC | Maesen 6397 |
| *Lecaniodiscus cupanioides* Planch. | mph | GC | Maesen 6310 |
| *Majidea forsteri* (Sprague) Radlk. | meg | GC | Houngnon 1254a |
| *Pancovia bijuga* Willd. | mph | GC | Houngnon 4978 |
| **Sapotaceae** | | | |
| *Chrysophyllum welwitschii* Engl.* (+) | Lnph | GC | Adomou s.n. |
| *Englerophytum oblanceolatum* (S.Moore) T.D.Penn. syn. *Bequaertiodendron oblanceolatum** (S.Moore) Heine & J. H. Hemsl. | nph | TA | Maesen 6154 |
| *Pouteria alnifolia* (Baker) Roberty Syn. *Malacantha alnifolia* (Baker) | mph | GC | Sokpon 1915 |
| **Smilacaceae** | | | |
| *Smilax anceps* Willd.Syn. *S. kraussiana* Meissner | LGr | TA | Chevalier 24225 |
| **Solanaceae** | | | |
| *Solanum terminale* Forssk. Subsp inconstans (C.H. Wright) Heine | Lmph | GC | Yédomonhan 173 |
| **Ulmaceae** | | | |
| *Chaetachme aristata* Planch. | mph | GC | Houngnon 1784c |
| **Violaceae** | | | |
| *Rinorea batesii* Chipp, Kew Bull. 293 (1923). | nph | GC | Adomou 83 |
| *Rinorea brachypetala* (Turcz.) Kuntze | nph | GC | Adomou s.n. |
| *Rinorea dentata* (P.Beauv.) Kuntze | mph | GC | Houngnon |
| *Rinorea ilicifolia* (Welw. ex Oliv.) Kuntze * | nph | GC | Adomou 109 |
| *Rinorea kibbiensis* Chipp. | nph | GC | Paradis & Houngnon 1347a |
| **Vitaceae** | | | |
| *Cissus glaucophylla* Hook. f. | Lnph | GC | Adomou s.n. |
| *Cissus petiolata* Hook. f. | Lnph | GC | Adjakidjè 2976 |
| *Cissus populnea* Guill. & Perr. var. *populnea* | LHc | SZ | Houngnon 68d |
| *Cissus quadrangularis* L. | Lmph | SZ | Houngnon 5105 |

*: Plant species restricted to Ewe Adakplame Relict Forest (+): Non recorded

were each represented by five species. Among the 185 vascular plants of EARF 12% of the families (22) were represented by one species each. The genera *Dioscorea*, *Drypetes and Rinorea*, are represented by five species followed by genera *Albizia*, *Cissus*, *Strychnos* (4 species each) and *Celtis* and *Diospyros* (3 species each). EARF also provides habitat for *Chrysophyllum welwitschii*, (Not mentioned in the Flora), *Cissus glaucophylla* (Not mentioned in the Flora), *Drypetes aframensis*, (Not mentioned in the Flora), *Drypetes afzelii*, *Drypetes gilgiana*, *Drypetes leonensis*, *Englerophytum oblanceolatum*, *Mansonia altissima*, *Nesogordonia papaverifera*, *Octolobus spectabilis*, (Not mentioned in the Flora), *Rinorea batesii*, *Rinorea brachypetala* (Not mentioned in the Flora), *Rinorea dentata*, *Rinorea ilicifolia*, *Rinorea kibbiensis*, *Vitex micrantha*), (Not mentioned in the Flora) which appear to be confined to EARF that can be seen as the single location of their occurrence in Benin. Table 2 also describes the community data set using family, binomial, life-forms and Chorotypes. Some of these plant species are featured in Fig. 3A–K.

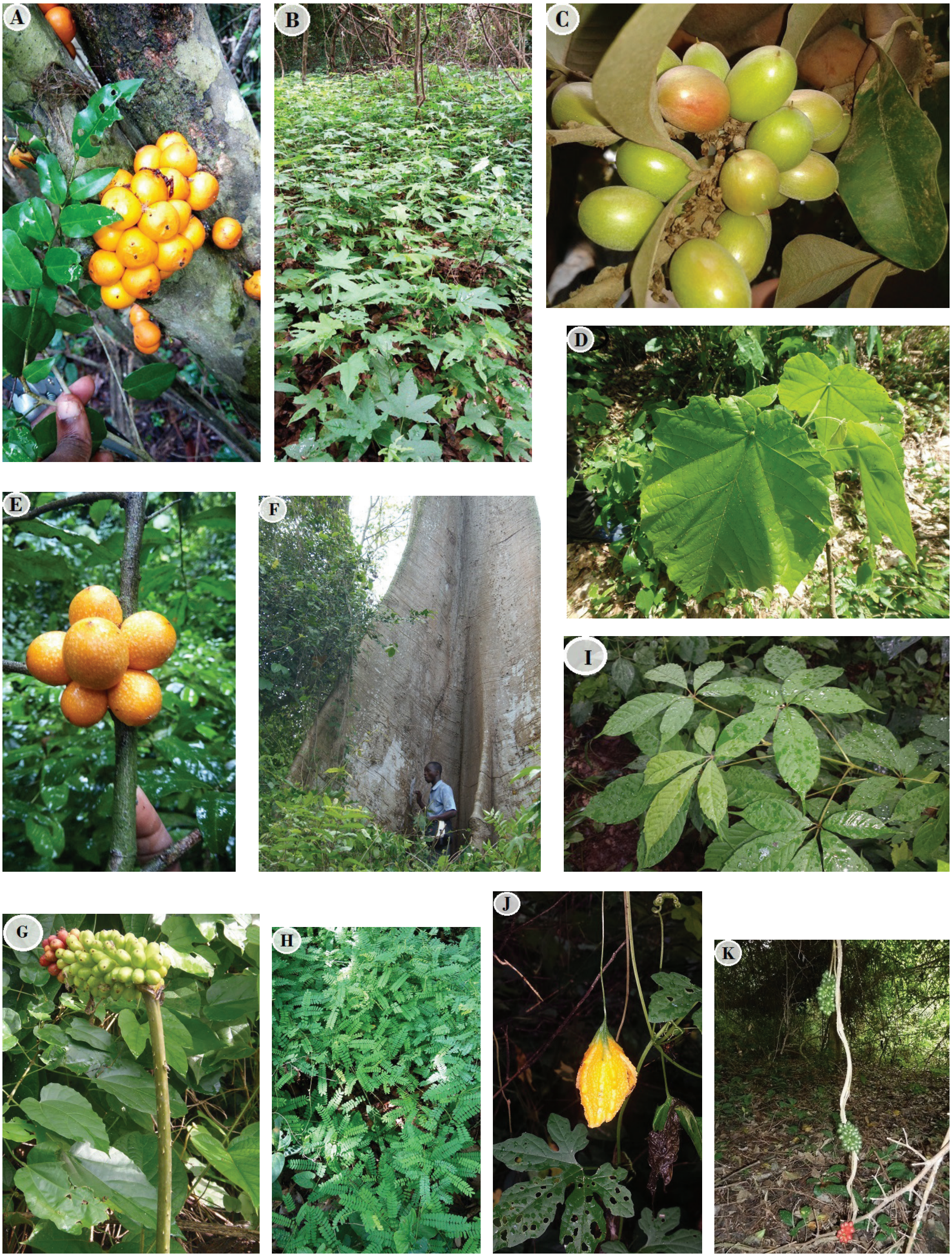


**Figure 3.** Common species of Ewe-Adakplame Relict Forest **A** *Drypetes gilgiana* (Photo of Alfred Houngnon 2017) **B** *Triplochiton scleroxylon* (Samsung photo A. Houngnon 2017) **C** *Englerophytum oblanceolatum* (Olympus photo A. Houngnon 2014) **D** *Mansonia altissima* (Olympus photo A. Houngnon 2014) **E** *Uvariopsis tripetala* **F** *Ceiba pentandra* (Olympus photo A. Houngnon 2014) **G** *Anchomanes welwitschii* (Samsung photo A. Houngnon 2015) **H** *Abrus precatorius* (Samsung photo A. Houngnon 2016) **I** *Vitex micrantha* (Samsung photo A. Houngnon 2016) **J** *Momordica charantia* (Samsung photo A. Houngnon 2016) **K** *Dioscoreophyllum cumminsii* (photo A. Adomou 2011).

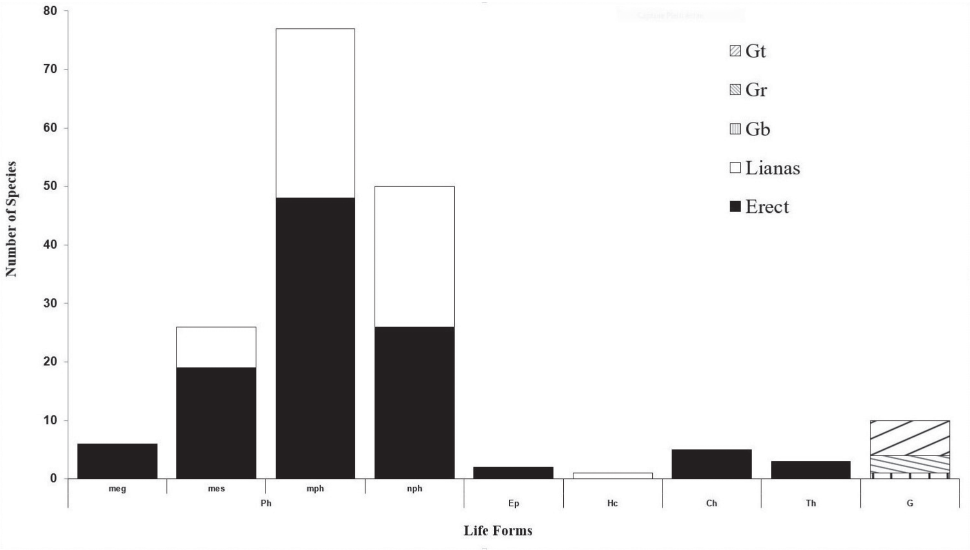


**Figure 4.** Life form spectrum of the Ewe-Adakplame relict forest. Erects are represented by Ph: Phanerophytes including megaphanerophytes (meg), mesophanerophytes (mes), microphanerophytes (mph), nanophanerophytes (nph), G: Geophytes are: Gb: with bulb, Gr: with rhizome and Gt: with tuber Ch: Chamaephytes, Th: Therophytes, Ep: Epiphytes, Hc: Hemicryptophytes. Climbing forms are L: Lianas (mph, nph and mes, Gr, Hc).

## Life form spectrum

The most common life forms were phanerophytes (88%), containing, 3% of mega phanerophytes (meg) which are very large forest trees, 14% of mesophanerophytes (mes) or medium-sized forest trees, 43% of microphanerophytes (mph) or small forest trees and 28% of shrubs gathered into nanophanerophytes (nph). We recorded 33% of lianas, 6% of geophytes and 6% for chamaephyte, therophyte, epiphyte, hemicryptophyte (Fig. 4). The microphanerophytes were most representative among phanerophytes. The tree layer was discontinuous and composed of *Celtis mildbraedii* (Cannabaceae), *Triplochiton scleroxylon* (Malvaceae), *Antiaris toxicaria* (Moraceae), *Celtis zenkeri* (Cannabaceae), *Dialium guineense* (Fabaceae), *Ceiba pentandra* (Malvaceae), *Mansonia altissima* (Malvaceae), *Milicia exelsa* (Moraceae), and *Nesogordonia papaverifera* (Malvaceae).

## Chorology of Ewe-Adakplame relict forest

The most representative chorotypes (Fig. 5) included Guineo-Congolean species (66%), followed by 14% of wide distribution species including Tropical Africa (TA) and Pantropical (Pan) plant species. Upper Guinea species included plants such as *Uvariopsis tripetala* Syn. *Dennettia tripetala* (Annonaceae), *Drypetes aframensis* (Putranjivaceae tribe Drypeteae), *Stachyanthus occidentalis* Syn.

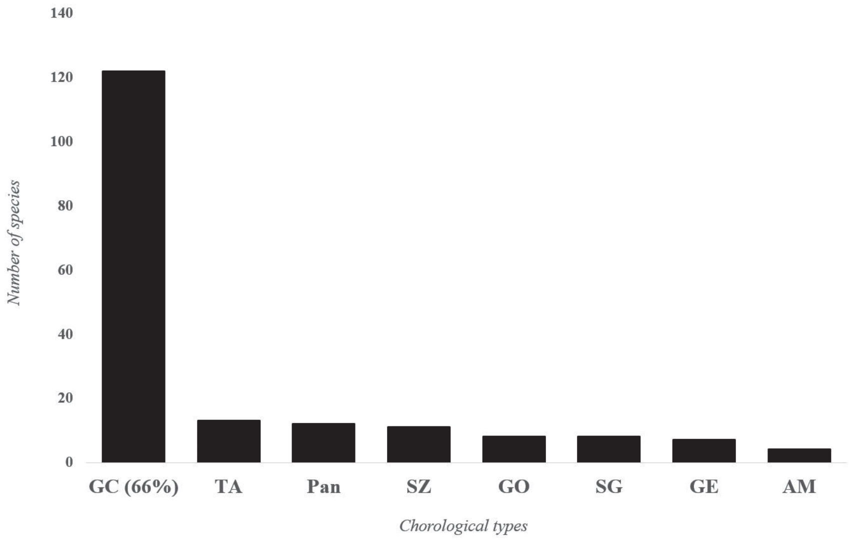


**Figure 5.** Chorological spectrum of the Ewe-Adakplame Relict Forest. GC: Guineo-Congolean, SG: Sudano/Guinean transition, GE: Lower Guinean, GO: Upper Guinean, TA: Tropical Africa, AM: Afro-Malagasy and Pan: Pantropical.

*Neostachyanthus occidentalis* (Icacinaceae), *Lannea nigritana* var. *nigritana* (Anacardiaceae), *Psydrax parviflora* (Rubiaceae), *Premna quadrifolia* (Lamiaceae), *Cnestis corniculata* (Connaraceae). *Monanthotaxis parvifolia* (Annonaceae), *Artabotrys dahomensis* (Annonaceae), *Dalbergia lactea* (Fabaceae – Faboideae), *Ritchiea erecta* Syn. *R. pentaphylla* (Capparaceae) and *Cercestis mirabilis* Syn. *Rhektophyllum mirabile* (Araceae) are among Lower Guinea species.

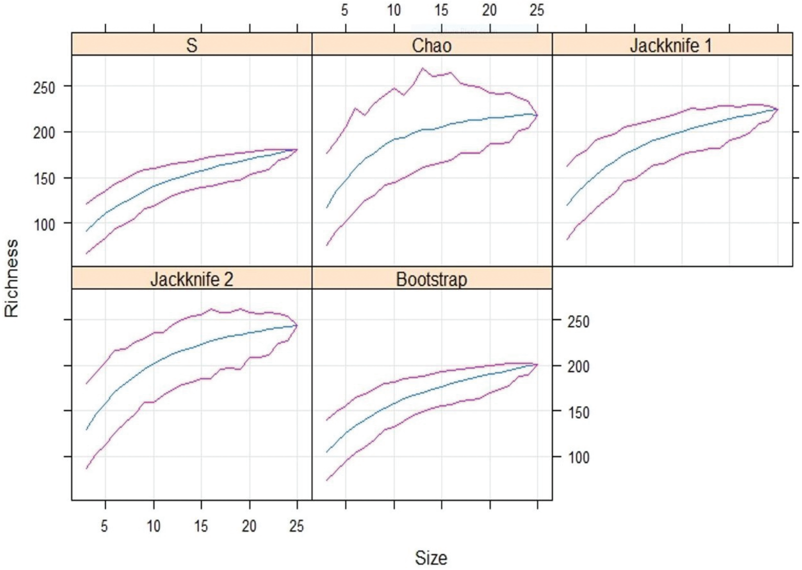
## Species richness estimations

The counted number of plant species for the EARF was 185. This corresponds to the species richness (S) or the number of species that has been recorded from plot sampling and listed in Table 2. The species richness estimations as per *Bootstrap*, *Chao*, *Jacknife1* and *Jacknife2* were respectively 200.52 ± 9.2808; 217.62 ± 14.5972, 224.16 ± 15.3725 and 242.67. We can see that the species richness estimates differ strongly giving a range of 200.52–242.67 species. The species accumulation curves in Fig. 6 show that they were hardly tending towards the asymptote and are still climbing at the right-hand end signifying that the sampling effort was insufficient. This suggests that the sampling has not captured nearly all the species in EARF and that many species were missed (on average approximately 16 species (± 9.2808) for *Bootstrap*, 33 species (± 14.5972) for *Chao*, 39 species (± 15.3725) for *Jacknife1* and 58 species for *Jacknife2*.

## Discussion

The Ewe-Adakplame Relict Forest corresponds to the semi-deciduous forest type, which was described in Benin as the only *Drypetes aframensis-Nesogordonia papaverifera* community (Adomou et al. 2009). Guineo-Congolean species are abundant (66%) although the EARF is located in a matrix of savanna-dominated vegetation. Guineo-Congolean species accounted for 33% in riparian forests of Benin (Natta 2003), 61.7% in gallery forests of the Hippopotamus Pond Biosphere Reserve at Burkina Faso (Bélem and Guinko 1998), and 70 to 75% in gallery forests at Lamto (southern Côte d'Ivoire) (Devineau 1975). The overall plant species composition makes EARF floristically comparable to the moist semi-deciduous forest of Nigeria (Lower Guinea) (Richards 1939) and the *Celtis* spp.-*Mansonia altissima* community of Côte d'Ivoire (Upper Guinea) (Guillaumet and Adjanohoun 1971). Guillaumet and Adjanohoun (1971) also pointed out that the dominance of Cannabaceae (previously Ulmaceae) and Malvaceae (previously Sterculiaceae) in the Upper and Lower Guinea forests offers evidence that West African semi-deciduous forests are at climatic climax. These indicator families were also reported as characteristics of the semi-deciduous forests in Ghana (Vooren and Sayer 1992) and Côte d'Ivoire (Swaine 1996) within the Upper Guinea zone. This high proportion of Guineo-Congolean species shows the floristic uniqueness of EARF and highlights its great importance in Benin which landscape is savanna dominated.

Unlike Palmer (1990) who showed that *Jack 1* is the most precise and least biased, it is rather the *Bootstrap* estimator (200.52 ± 9.2808) that seems to be closer to our field results (S = 185 species). The *Bootstrap* value is also close to estimates of Adomou et al. (2010) who assessed the specific richness of EARF around 200 species. This is what justifies the calculation of the four estimation methods (*Chao*, *first order jackknife*, *second order jackknife and bootstrap*) and not only one as Palmer (1990) would have demonstrated. In our study, the species accumulation curves were calculated with estimators (*Chao*, *Jack 1*, *Jack 2* and *Bootstrap*) and showed a change in species richness without flatten off at the right hand. The *Bootstrap* appears to be the best estimator which is closest to EARF plant richness estimated by Adomou et al. (2010). The Fig. 6 show that species accumulation curves were hardly tending towards the asymptote and are still climbing at the right hand end signifying that the sampling effort was insufficient. This suggests that the sampling did not capture all the species in EARF. The shape of the species accumulation curves should plateau for large numbers of sites sampled. However, the number of observed species will typically be smaller than the true number of species. Since species richness depends on sample size, we can expect that we will not have recorded all the species that occur in the survey area. So, the Species accumulation curves were used to ascertain range in values obtained with the different methods and we can expect that the correct total richness lies somewhere within that range. Many species will always remain unseen or undetected in a



| Number of observation (N) | Richness observed (S) | Chao | Chao.se | Jack1 | Jack1.se | Jack2 | Boot | Boot.se |
|---|---|---|---|---|---|---|---|---|
| 25 | 185 | 217.62 | 14.5972 | 224.16 | 15.3725 | 242.67 | 200.52 | 9.2808 |

**Figure 6.** Species richness and richness estimations (Chao, first order jackknife, second order jackknife and bootstrap) (y-axis) in relation to sample size (x-axis) at the Ewe-Adakplame relict forest.

collection of sample plots. It is like the case of *Chrysophyllum welwitschii* (Sapotaceae) and *Drypetes aframensis* which, although reported in EARF (Adomou 2005), has not been recorded since 2014. *Drypetes aframensis* (Salicaceae) is also not mentioned in the Benin Flora. From a physiognomic point of view, it is important to underline that it is sometimes extremely difficult to survey some areas because of the thickness of the vegetation which can be very inaccessible. Other places are severely affected by human presence leading to very sparse vegetation or gap areas in the heart of the forest with completely isolated forest tree species. This is also the case of the periphery cleared by human boundaries encroachment and so many activities often carried out illegally. These constraints have often influenced the layout and the number of sampled sites. This also proves that despite the botanical sampling effort of Benin (Sosef et al. 2017), some priority species for conservation have either not yet been collected or reported and therefore could be omitted.

The connection of EARF with the West African forests blocks located on both side of the Dahomey Gap is emphasized here by the high proportion of Guineo-Congolean species recorded (66%). The high rate of phanerophytes and their phytochories testifies to the floristic originality of EARF in a crop and savanna dominated landscape. This justifies the physiognomic and floristic links of EARF with the two Guinean and Congolia forest blocks and substantiates the hypothesis that EARF is a remnant of the dense forests which were once a continuous block from west to central Africa as demonstrated by numerous palynological studies (Tossou 2002; Salzmann and Hoelzmann 2005) and also phylogeography (Demenou et al. 2016). The floristic link of the EARF with the West African semi-deciduous forest is also best highlighted by the high representation of many Upper Guinean endemic species belonging to the families of Cannabaceae (*Celtis mildbraedii*, *C. zenkeri* and *C. prantlii*), Malvaceae (*Triplochiton scleroxylon*, *Nesogordonia papaverifera*, *Mansonia altissima*, *Pterygota macrocarpa*, *Octobolus spectabilis* and *Sterculia tragacantha*), and Putranjivaceae (*Drypetes floribunda*, *D. gilgiana* and *D. aframensis*). This record provides strong evidence for past floristic connections with the West African rain forest zone which is consistent with paleovegetation reconstructions indicating that the tropical African rain forest formed a single block during the Holocene Humid Period (c. 9000–4500 yr BP) (Tossou 2002; Salzmann and Hoelzmann 2005; Demenou et al. 2018). Furthermore, the richness of EARF in *Rinorea* species (*R. batesii*, *R. brachypetala*, *R. dentata*, *R. kibbiensis* and *R. ilicifolia*) and their abundance are unique in the country. According to Akoègninou et al. (2006), *Rinorea batesii* Chipp, *Rinorea ilicifolia* (Welw. Ex OIiv.) Kuntze, *Rinorea dentata* are common in West, Central and East Africa and some of them in Madagascar. However, in Benin most of them are rare and are only found in EARF. *Rinorea brachypetala*, although it has been reported by Adomou (2005), was not mentioned in the National Flora (Akoègninou et al. 2006). *Rinorea* species are described as good indicators for West African semi-deciduous forests in a climax state (Achoundong 2000). The strong representation of *Rinorea* spp., the Cannabaceae (previously Ulmaceae) and the Malvaceae (previously Sterculiaceae) substantiate the view of Guillaumet and Adjanohoun (1971), who considered this forest type as the climatic climax or primeval type of semi-deciduous forest in West Africa. With this floristic composition, EARF can be seen as a unique West African rain forest refuge in a matrix of savanna-dominated vegetation in Benin.

In contrast, the surrounding vegetation at the immediate edge of EARF is composed of savanna species (Fig. 7A–J) from the Guineo-Sudanian transition zone such as: *Adansonia digitata* L. (Malvaceae), *Stereospermum kunthianum* Cham. (Bignoniaceae), *Trichilia emetic* Vahl (Meliaceae), *Annona senegalensis* Pers. (Annonaceae), *Vitex doniana* Sweet (Lamiaceae), *Parkia biglobosa* (Jacq.) R.Br. ex G. Don (Fabaceae-Caesalpinioideae (mimosoid clade)), *Dichrostachys cinerea* (L.) Wight & Arn. (Fabaceae- Caesalpinioideae (mimosoid clade)), *Pterocarpus erinaceus* Poir. (Fabaceae-Faboideae), *Pericopsis laxiflora* (Benth.) Meeuwen (Fabaceae-Faboideae), *Daniellia oliveri* (Rolfe) Hutch. & Dalziel (Fabaceae-Detarioideae), *Sarcocephalus latifolius* (Sm.) E.A. Bruce (Rubiaceae) and *Andropogon gayanus* Kunth (Poaceae). The landscape is characterized by two basin ecosystems with clear dissimilarity combining forest/savanna and crop areas in stable equilibrium (Figs 8A, B). This makes EARF a special site of rich biodiversity and emphasizes the vital role the forest plays as a corridor of transition conducive to resilience and the flow of genes for ecosystem equilibrium.

**Figure 7.** Species collected in the surrounding zone of Ewe-Adakplame Relict Forest **A** *Adansonia digitata* (Samsung photo A. Houngnon 2015) **B** *Pericopsis laxiflora* (Samsung photo A. Houngnon 2016) **C** *Trichilia emetic* (Samsung photo A. Houngnon 2016) **D** *Annona senegalensis* (Olympus photo A. Houngnon 2014) **E** *Vitex doniana* (Samsung photo A. Houngnon 2016) **F** *Parkia biglobosa* (Samsung photo A. Houngnon 2016) **G** *Dichrostachys cinerea* (Samsung photo A. Houngnon 2016) **H** *Pterocarpus erinaceus* (Samsung photo A. Houngnon 2016) **I** *Daniellia oliveri* (Samsung photo A. Houngnon 2016) and **J** *Andropogon gayanus* (Samsung photo A. Houngnon 2017).

In total, we counted thirteen species restricted to one site in EARF. This is higher than the nine species previously reported by Adomou et al. (2010) in the EARF. Our record represents 15.4% of species with high conservation priority, thus describing

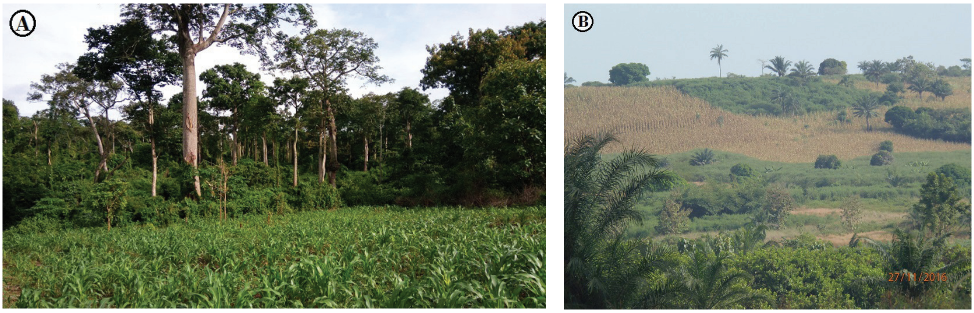


**Figure 8.** Ewe Adakplame Relict Forest in a matrix of savanna and agriculture landscape **A** forest edge affected by anthropogenic activities (Samsung photo A. Houngnon 2017) **B** Landscape of fallow and field around the edge of the forest ecosystem (Olympus photo A. Houngnon 2016).

EARF as being one of the richest sites in range-restricted plant species of Benin. This increase in range-restricted species can be explained by the fact that species with a high scarcity index are vulnerable and could disappear if biodiversity sanctuaries that protect them disappear (Juhé-Beaulaton 2010). Indeed, during the last three decades in Benin, some forests areas have been cleared with an annual degradation rate of 70,000 hectares per year (PNF Bénin 2004). This habitat loss has considerably narrowed the survival places where rare species were previously recorded.

Some of these species found in the single location of EARF within Benin (e.g., *Acroceras gabunense*, *Chrysophyllum welwitschii*, *Dovyalis afzelii*, *Drypetes aframensis*, *Drypetes gilgiana*, *Englerophytum oblanceolatum*, *Mansonia altissima*, *Nesogordonia papaverifera*, *Octolobus spectabilis*, *Pterygota macrocarpa*, *Rinorea ilicifolia*, *Rinorea kibbiensis* and *Vitex micrantha*) may gain more attention in the National Red List (Neuenschwander et al. 2011). Among them, there are many globally threatened species as the case of *Nesogordonia papaverifera* and *Mansonia altissima*, respectively reported as vulnerable (VU) and endangered (EN) by IUCN (2002) and later, were both assessed in Benin as critically endangered (CR) by Adomou et al. (2010) who considered EARF as sites with high concentration of threatened plant species in Benin. Moreover, the impact of harvesting on the survival of the most endangered species has been long ignored while many of them are not domesticated and many species uncharacterized. The case of *Mansonia altissima* is of urgent concern because its population is almost completely depleted, since it is locally used for roofing poles. *Englerophytum oblanceolatum* (Sapotaceae), which is not listed on the National Red List of Benin also tends to be concentrated in EARF (Houngnon 2014). Unfortunately, most of Benin Forest is still under severe threat due to expansion of towns, agricultural and fallow (Oloukoï et al. 2007) that are narrowing the natural habitat and leading to a massive loss of many of the local biodiversity taxa. To this end, since 2014, we have been trying to raise awareness among local communities from the villages of Ewe and Adakplame through participative action toward nursery establishment (Houngnon 2014) and vegetative propagation of native tree (Houngnon 2015) in order to rehabilitate the degraded lands and areas that could potentially be sensitive for the EARF durability.

## Conclusion

The importance of the flora of EARF testifies to its role in conserving forest biodiversity in the Dahomey gap corridor. This justifies its peculiarity and the relevance of this baseline vegetation information that could be used as complete range taxa that may allow us to test the forest refuge hypothesis against alternative speciation models across ecological gradients. As it happens, the management of the forest of this type, also raises the question of deepening interactions linking human environment in order to better understand the actual role that humans would have played in shaping ecosystems in the Dahomey gap since millennia. So, it would be interesting to understand the interplay between locals and EARF in order to explain its persistence in this savanna dominated landscape. Therefore, the actions to be considered following this checklist of EARF must take into account the community's engagement in rehabilitating the degraded lands inside and around EARF.

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## References

Achoundong G (1996) Les *Rinorea* comme indicateurs des grands types forestiers du Cameroun. In: van der Maesen LJG, van der Burgt XM, van Medenbach de Rooy JM (Eds) The Biodiversity of African Plants. Kluwer Academy Publishing, The Netherlands, 536–544. https://doi.org/10.1007/978-94-009-0285-5_69

Achoundong G (2000) Les *Rinorea* et l'étude des refuges forestiers en Afrique. In: Servant M, Servant-Vildary S (Eds) Dynamique à long terme des écosystèmes forestiers intertropicaux. Mémoire UNESCO, Paris, 19–29.

Adjanohoun E (1966) Existence au Dahomey de savanes côtières analogues à celles de la Côte d'Ivoire. Etudes dahoméennes 6–7: 48–71.

Adjanohoun E, Adjakidjè V, Ahyi MRA, Aké Assi L, Akoègninou A, Dalmeda J, Akpovo F, Boukef K, Chadaré F, Cusset G, Dramane K, Eyme J, Gassita J-N, Gbaguidi N, Goudoté E, Guinko S, Houngnon P, Issa L, Keita A, Kiniffo HV, Koné Bamba D, Musampa Nseyya A, Saadou N, Sodogandji TH, De Souza S, Tchabi A, Zinsou Dossa C, Zohoun Th (1989) Contribution aux études ethnobotaniques et floristiques en République Populaire du Bénin. ACCT, Paris, 895 pp.

Adomou A (2005) Vegetation patterns and environmental gradients in Benin. PhD thesis. Wageningen University, Wageningen, 133 pp.

Adomou AC, Sinsin B, van der Maesen JLG (2006) Phytosociological and chorological approaches to phytogeography: A meso-scale study in Benin. Systematics and Geography of Plants 76: 155–178.

Adomou AC, Akoègninou A, Sinsin B, de Foucault B, van der Maesen JLG (2009) Semi-deciduous forest remnants in Benin: Patterns and floristic characterization. Acta Botanica Gallica 156(2): 159–171. https://doi.org/10.1080/12538078.2009.10516148

Adomou AC, Sinsin B, Akoégninou A, van der Maesen JLG (2010) Plant species and ecosystems with high conservation priority in Benin. In: van der Burgt X, van der Maesen J, Onana J-M (Eds) Systematics and Conservation of African Plants. Royal Botanic Gardens, Kew, 429–444.

Akoègninou A, van der Burg WJ, van der Maesen LJG (2006) Flore analytique du Bénin. Backhuys Publishers, Leiden, 1034 pp.

APG IV (2009) An Update of the Angiosperm Phylogeny Group Classification for the Orders and Families of Flowering Plants: APG III. Botanical Journal of the Linnean Society 161(2): 105–121. https://doi.org/10.1111/j.1095-8339.2009.00996.x

Armani M, Langevelde FV, Tomlinson KW, Adu-Bredu S, Djagbletey GD, Veenendaal EM (2018) Compositional patterns of overstorey and understorey woody communities in a forest–savanna boundary in Ghana. Plant Ecology & Diversity 11(4): 451–463. https://doi.org/10.1080/17550874.2018.1539133

Aubréville A (1937) Les forêts du Dahomey et du Togo. Bulletin de communication et Histoire des Sciences en Afrique Occidentale Française 20: 1–112.

Bani G (2006) Monographie de la Commune de Kétou. Afrique Conseil, Cotonou.

Bélem OM, Guinko S (1998) La biodiversité des galleries forestières du Burkina Faso: cas de la reserve de la biosphere de la mare aux hippopotames. In: Ba AT, Madsen JE, Sambou B (Eds) Atelier sur flore, vegetation et Biodiversité au Sahel. Aarhus University Press, Aarhus Denmark, AAU Reports 39: 75–86.

Bénin PNF (2004) Plan national forestier du Bénin, Cotonou, Bénin. http://www.un.org/esa/forests/pdf/national_reports/unff4/benin.pdf

Biaou SSH (2009) Tree recruitment in West African dry woodlands: The interactive effects of climate, soil, fire and grazing. PhD thesis. Wageningen University, Wageningen.

Brand R (2001) Diversité ethnique et appropriation des cultes Vodoun dans une population de réfugiés au Sud Bénin. Outre-mers économiques: de l'Histoire à l'actualité du XXIe siècle, 269–297. https://doi.org/10.3406/outre.2001.3852

CARDER (2002) Plan de campagne agricole 2001–2002. Ouémé – Plateau Secteur Kétou. Secteur agricole, Kétou Benin, 69 pp.

Chevalier A (1910) Le Pays des Hollis et les régions voisines. 1 fascicule, Paris.

Chiu CH, Wang YT, Walther BA, Chao A (2014) Improved nonparametric lower bound of species richness via a modified Good-Turing frequency formula. Biometrics 70(3): 671–682. https://doi.org/10.1111/biom.12200

Colwell RK, Coddington JA (1994) Estimating terrestrial biodiversity through extrapolation. Philosophical Transactions of the Royal Society of London – Series B, Biological Sciences 345(1311): 101–118. https://doi.org/10.1098/rstb.1994.0091

Cords MC (1987) Demography of Blue Monkeys in the Kakamega Forest, Kenya. International Journal of Primatology 8: 1–546.

Cousin S (2018) Etat de l'art de Gou. Pérégrinations d'un dieu du progrès, hier et aujourd'hui. In: Murphy M, Houénoudé D (Eds) Création contemporaine et patrimoine royal au Bénin: autour de la figure du Dieu Gou. HICSA et Presse de l'Université d'Abomey Calavi, 22–43.

Couvreur TLP, Dauby G, Blach-Overgaard A, Deblauwe V, Dessein S, Droissart V, Hardy OJ, Harris DJ, Janssens SB, Ley AC, Mackinder BA, Sonké B, Sosef MSM, Stévart T, Svenning J-C, Wieringa JJ, Faye A, Missoup AD, Tolley KA, Nicolas V, Ntie S, Fluteau F, Robin C, Guillocheau F, Barboni D, Sepulchre P (2021) Tectonics, Climate and the Diversification of the Tropical African Terrestrial Flora and Fauna. Biological Reviews of the Cambridge Philosophical Society 96(1): 16–51. https://doi.org/10.1111/brv.12644

Demenou BB, Piñeiro R, Hardy OJ (2016) Origin and History of the Dahomey Gap Separating West and Central African Rain Forests: Insights from the Phylogeography of the Legume Tree Distemonanthus Benthamianus. Journal of Biogeography 43(5): 1020–1031. https://doi.org/10.1111/jbi.12688

Demenou BB, Doucet J-L, Hardy OJ (2018) History of the Fragmentation of the African Rain Forest in the Dahomey Gap: Insight from the Demographic History of Terminalia Superba. Heredity 120(6): 547–561. https://doi.org/10.1038/s41437-017-0035-0

Devineau JL (1975) Etude quantitative des forêts galeries de Lamto (Moyenne Côte d'Ivoire). PhD Thesis, Université Pierre et Marie Curie, Paris VI.

Droissart V, Dauby G, Hardy OJ, Deblauwe V, Harris DJ, Janssens S, Mackinder BA, Blach-Overgaard A, Sonké B, Sosef MSM, Stévart T, Svenning J-C, Wieringa JJ, Couvreur TLP (2018) Beyond Trees: Biogeographical Regionalization of Tropical Africa. Journal of Biogeography 45(5): 1153–1167. https://doi.org/10.1111/jbi.13190

Dupont LM, Weinelt M (1996) Vegetation history of the savanna corridor between the Guinean and Congolean rain forest during the last 150 000 years. Vegetation History and Archaeobotany 5(4): 273–292. https://doi.org/10.1007/BF00195296

Garnier A, Eichhorn B, Robion-Brunner C (2018) Impact de l'activité métallurgique au cours du dernier millénaire sur un système fluvial soudano-guinéen. Étude multi-proxy des archives sédimentaires de la vallée du Tatré (pays bassar, Togo). Géomorphologie: relief, processus, environnement 24(3): 257–276. https://doi.org/10.4000/geomorphologie.12446

Greiner A (1991) Nature Conservation and local people at Kakamega Forest. ASA Project. Stuttgart.

Guillaumet JL, Adjanohoun E (1971) La végétation de la Côte d'Ivoire. In: Avenard JM, Eldin E, Girard G, Sircoulon J, Touchebeuf P, Guillaumet JL, Adjanohoun E, Perraud A (Eds) Le milieu naturel de la Côte d'Ivoire. Mémoire ORSTOM, Paris 50: 156–263.

Hassler M (2020) World Plants: Synonymic Checklists of the Vascular Plants of the World. In: Roskov Y et al. (Eds) Species 2000 & ITIS Catalogue of Life, 2020-09-01 Beta, Leiden. www.catalogueoflife.org/col

Houngnon A (2014) Community Based Actions to Benefit a Threatened Plant Species: Case Study of *Bequaertiodendron oblanceolatum* in Ewe-Adapklame Remnant Forests in Benin. http://www.rufford.org/projects/alfred_houngnon [accessed 15.09.2014]

Houngnon A (2015) Propagation of native trees by local communities to provide food for primates in the forests of Ketou: a new challenge for habitat restoration in Benin Republic. http://www.rufford.org/projects/alfred_houngnon_0 [accessed 12.12.2015]

Houngnon A, Adomou AC (2021) Vascular plants of Ewe-Adakplame Relic Forest at Kétou in Benin, West Africa, Dryad, Dataset. https://doi.org/10.5061/dryad.z8w9ghxbg

Hutchinson J, Dalziel JM (1954–1972) Flora of West Tropical Africa (2nd ed. Vol. I–III). Crown Agents for Oversea Governments and Administrations, London.

INSAE (2016) Effectif de la population des villages et quartiers de ville du Bénin. RGPH-4 de 2013, Cotonou Bénin, 20 pp.

IUCN (2002) Evaluation of Nominations of Natural and Mixed Properties to the World Heritage List, Gland.

Jenik J (1994) The Dahomey Gap: An important issue in African phytogeography. Mémoire Société Biogéographie 4(3): 125–133.

Juhé-Beaulaton D (2010) Forêt sacrées et sanctuaires boisés. Edition Karthala, Paris, 280 pp.

Lebrun J-P (2001) Introduction à la flore d'Afrique. Paris: Cirad, Ibis Press, 213 pp.

LPWG (2017) Phylogeny and classification of the Leguminosae (Legume Phylogeny Working Group): A new subfamily classification of the Leguminosae based on a taxonomically comprehensive phylogeny. Taxon 66(1): 47–77. https://doi.org/10.12705/661.3

Martin P (2008) Influence de la fragmentation forestière sur la régénération des espèces arborées dans le Sud-Ouest de la Côte d'Ivoire. University of Geneva, Geneva.

Natta AK (2003) Ecological assessment of riparian forests in Benin. Phytodiversity, phytosociology and spatial distribution of tree species. PhD Thesis, Wageningen University, The Netherlands.

Neuenschwander P, Sinsin B, Goergen G (2011) Protection de la Nature en Afrique de l'Ouest: Une Liste Rouge pour le Bénin. Nature Conservation in West Africa: red list for Benin. International Institute of Tropical Agriculture, Ibadan, 365 pp.

Oksanen J, Guillaume Blanchet F, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H (2017) Vegan: Community Ecology Package. Version 2.4-2. https://CRAN.R-project.org/package=vegan [accessed 30.06.2017]

Oloukoï J, Mama VJ, Agbo FB (2007) Modélisation de la dynamique de l'occupation des terres dans le département des collines au Bénin. Télédétection 6(4): 305–323.

Palmer MW (1990) The estimation of species richness by extrapolation. Ecology 71(3): 1195–1198. https://doi.org/10.2307/1937387

Paradis G (1977) Observation sur l'Holocène récent du Sud-Bénin (ex-Dahomey). Bulletin de liaison de l'Association Sénégalaise de l'Etude du Quaternaire 51: 49–73.

Poorter L, Bongers F, Kouamé FN, Hawthorne WD (2004) Biodiversity of West African Forests: An ecological atlas of woody plants species. CABI Publishing, Cambridge, 521 pp. https://doi.org/10.1079/9780851997346.0000

R-Core-Team (2016) R: a language and environment for statistical computing. R. Foundation for Statistical Computing, Vienna. version 3.3.2. http://www.R-project.org [accessed 31.10.2016]

Raunkiaer C (1934) The Life Forms of Plants and Statistical Plant Geography. Clarendon Press, London, 632 pp.

Richards PW (1939) Ecological studies on the rain forest of southern Nigeria. N°1 the structure and floristic composition of the primary forest. Journal of Ecology 27(1): 1–61. https://doi.org/10.2307/2256298

Richards PW (1973) Africa, the "Odd man out." In: Meggers BJ, Ayensu ES, Duckworth WD (Eds) Tropical Forest Ecosystems in Africa and South America: A Comparative Review. Washington: Smithsonian Institution Press, 212–239.

Salzmann U, Hoelzmann P (2005) The Dahomey Gap: An abrupt climatically induced rain forest fragmentation in West Africa during the late Holocene. The Holocene 15(2): 1–10. https://doi.org/10.1191/0959683605hl799rp

Sosef MSM, Dauby G, Blach-Overgaard A, van der Burgt X, Catarino L, Damen T, Deblauwe V, Dessein S, Dransfield J, Droissart V, Duarte MC, Engledow H, Fadeur G, Figueira R, Gereau RE, Hardy OJ, Harris DJ, de Heij J, Janssens S, Klomberg Y, Ley AC, Mackinder BA, Meerts P, van de Poel JL, Sonké B, Stévart T, Stoffelen P, Svenning J-C, Sepulchre P, Zaiss R, Wieringa JJ, Couvreur TLP (2017) Exploring the Floristic Diversity of Tropical Africa. BMC Biology 15(1): e15. https://doi.org/10.1186/s12915-017-0356-8

Swaine MD (1996) Rainfall and soil fertility as factors limiting forest species distribution in Ghana. Journal of Ecology 84(3): 419–428. https://doi.org/10.2307/2261203

Tossou MG (2002) Recherche palynologique sur la végétation Holocène du Sud-Bénin (Afrique de l'Ouest). PhD Thesis, Université de Lomé, 136 pp.

Vooren F, Sayer J (1992) Côte d'Ivoire. In: Sayer JA, Harcourt CS, Collins NM (Eds) The Conservation Atlas of Tropical Forests: Africa. IUCN and Macmillan Publishers, UK, 133–142. https://doi.org/10.1007/978-1-349-12961-4_16

Wantchecon AM (1983) Contribution à l'histoire des technologies anciennes du Bas-Bénin. L'exemple du, travail du fer à Allada des origines du royaume à la conquête aboméenne en J.E.R., Maîtrise en Histoire, U.N.B., E.N.S., 48 pp.

White F (1983) The vegetation of Africa. A descriptive memoir to accompany the Unesco/AETFA/UNSO. Natural Resources Research 20: 1–356.