RESEARCH ARTICLE



Typification of 14 names in the Dianthus virgineus group (Caryophyllaceae)

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Abstract

The nomenclature of 14 taxa from Central and Southern Europe within the *Dianthus virgineus* group is discussed. *Dianthus aggericola* Jord., *D. collivagus* Jord., *D. consimilis* Jord., *D. orophilus* Jord., *D. saxicola* Jord., *D. juratensis* Jord. are here lectotypified by specimens from the Jordan herbarium in LY, while *D. godronianus* Jord. by a specimen in P. *Dianthus subacaulis* Vill. is neotypified by a specimen collected on Mont Ventoux (S. France) and housed in MPU. For *D. sylvestris* Wulfen, a lectotype is here designated and its previous neotypification is discussed. *Dianthus caryophyllus* var. *tenuifolius* Moris, *D. caryophyllus* f. *minor* Moris and *D. sylvestris* var. *garganicus* Ten. are lectotypified by specimens housed in herbarium Moris (TO) and herbarium Tenore (K). *Dianthus virgineus* var. *tergestinus* Rchb. is lectotypified by a drawing from the *Icones florae Germanicae* & *Helveticae*, while *D. contractus* var. *evolutus* Lojac. is neotypified by a specimen in P. For each taxon the currently accepted name is provided including new synonymies. The type indication is followed by nomenclatural and taxonomic notes, in which the original material found is commented and the reasons for the identification of the types are discussed.

Keywords

Dianthus, France, Italy, Slovenia, nomenclature

Introduction

Dianthus L. (Caryophyllaceae) includes about 300 species from the temperate regions of the Old World, many of which are narrow endemics (Hardion et al. 2020). This genus still shows challenging systematics. A large part of recent taxonomic research, in fact, has been focused on the description of new taxa. Since 2000, 64 new species and subspecies have been described for the Euro-Mediterranean area, and a total of 98 new names have been published (IPNI 2021). Conversely, the taxonomic contributions on *Dianthus* that have taken into consideration groups of species with statistical analyses of morphological data or molecular investigations are very few (Domina et al. 2017; Hardion et al. 2020). Furthermore, the genus-level taxonomic treatments date back to more than 50 years ago (Williams 1890, 1893; Pax and Hoffmann 1934; Reeve 1967), and the recently published treatments of *Dianthus* in national flora (Bernal et al. 1990; Tison and de Foucault 2014; Vangjeli 2015; Brullo and Guarino 2017; Nikolić 2020) have not brought any significant change to the taxonomy of this genus. In several cases, the nomenclatural types for these taxa are not yet designated (Domina & al. 2021). This created a situation of taxonomic uncertainty. Dianthus is an interesting genus, both from a biological and economical point of view (Hardion et al. 2020). Hence, an integrated approach to the taxonomy of this genus is needed.

The *D. virgineus* L. group can be considered as one of the richest and most complex in the genus. Many taxa have been described from Central and Southern Europe, albeit their actual taxonomical value is often doubtful. The genus has undergone extensive taxonomic investigations since the 18th century (Smith 1794), but in many cases the original material used for the description of the taxa is not known and the nomenclatural types have not been designated yet. The lectotype of *Dianthus virgineus* L., the oldest available name that applies to wild plants in this group, has been designated only recently (Domina et al. 2021). The nomenclature and morphology of the large part of taxa described from Southern Italy, Sardinia, and Sicily have been investigated by Bacchetta et al. (2010). Other typifications were published by Camarda and Corrias (1987), Brullo et al. (2000), Arrigoni (2006), and Clementi et al. (2015). However, most of the taxa described in this group are still to be investigated.

In this study, the nomenclature of 14 taxa is discussed: *Dianthus aggericola* Jord., *D. collivagus* Jord., *D. consimilis* Jord, *D. godronianus* Jord., *D. orophilus* Jord., *D. saxicola* Jord., *D. juratensis* Jord., and *D. subacaulis* Vill. from S. France; *D. sylvestris* Wulfen from NE Italy/SW Slovenia; *D. virgineus* var. *tergestinus* Rchb. from NE Italy; *D. caryophyllus* var. *tenuifolius* Moris and *D. caryophyllus* f. *minor* Moris from Sardinia; *D. sylvestris* var. *garganicus* Ten. from S Italy; and *D. contractus* var. *evolutus* Lojac. from Sicily.

As part of an ongoing project aimed to push forward the taxonomic knowledge on selected genera of the Italian vascular flora, this study aims to lay the foundations for further taxonomic investigations by an integrated approach based on morphometric and molecular data (Domina et al. 2021; Giacò et al. 2021).

Material and methods

We examined the scientific literature for the effective place of publication of surveyed Dianthus names described from Central and South Europe. The bibliographic data was searched in the available digital sources and in the libraries of the European institutions, while the original material of the investigated species was searched in the main European herbaria: B, C, FI, G, K, MA, MPU, NAP, P, PAL, RAB, RO, TO, W, WU, and ZA; herbarium acronyms follow Thiers (2021). A start-up online screening was possible thanks to digital herbarium specimens' images provided by GBIF (https://www.gbif.org), Jstor (http://plants.jstor.org), and ReColNat (https://www.recolnat.org/fr/). More thorough investigations were conducted in the Jordan herbarium at LY. The articles of the International Code of Nomenclature for Algae, Fungi, and Plants (hereafter ICN) follow Turland et al. (2019). Until more in-depth, integrated morphometric, genetic, karyological, and ecological information comes to light, our judgments should be considered provisionally accepted, according to current knowledge. In this group, the characters that have been proven to best discriminate species (Bacchetta et al. 2010) are: leaf length and width, number of flowers per scape, shape and length of outer and inner bracts. These characters have been used to check the morphological features of the selected types. The anther and petal length have been proposed as additional discriminant characters, but these can be easily appreciated on fresh plants and not on herbarium samples.

Typification of the names

Dianthus aggericola Jord. in Billot, Annot.: 48. 1856. [December 1856]

= D. virgineus L., Sp. Pl. 1: 412. 1753. Ind. Loc.: "du Reculet (Ain)".

Type. (lectotype here designated): *Dianthus aggericola* Jord., du Reculet, 8 July 1854 [A. Jordan], LY0079734!

Note. No other original material was found in the surveyed herbaria. According to the label, this plant was originally collected in Reculet and then grown in Jordan's garden, where it was collected in July. As a matter of fact, besides his huge herbarium and library, Alexis Jordan owned a one-hectare experimental garden. We know that he used it to sow most species every year, while maintaining alive perennial ones, and that he regularly made herbarium sheets from these cultivated plants. In this context, the notion of original material requires particular attention (Thiébaut and Tison 2016).

The lectotype designated here matches the protologue and corresponds to the current application of the name, which is considered a heterotypic synonym of *D. godronianus* Jord. in Kerguélen (1993), in turn, currently considered a heterotypic synonym of *D. virgineus* (Domina et al. 2021). The lectotype of *D. aggericola* and that of *D. virgineus*, have the same leaf length and width, uniflowered scapes, the same length and shape of outer and inner bracts, the same calyx length and shape. We, therefore, confirm this synonymy.

Dianthus caryophyllus var. tenuifolius Moris, Fl. Sardoa 1: 231. 1837. [April 1837]

- \equiv *D. siculus* subsp. *tenuifolius* (Moris) Arrigoni, Parlatorea 7: 20. 2005.
- = D. genargenteus Bacch., Brullo, Casti & Giusso, Nordic J. Bot. 28(2): 145. 2010.
 - Ind. Loc.: "In sterilibus frequens [Sardinia]".

Type. (lectotype here designated): *Dianthus caryophyllus tenuifolia*, prope Belvì, July, inter rupe / Mus. Bot. Horti Taurinensis, Herb. Moris Barbey Cat Sard. N.156, TO!

Note. Three herbarium sheets are kept in TO, with several individuals each. All three specimens bear labels handwritten by Moris but lack the year of collection. Two of them come from generic localities ("in arenis maritimis" and "in collibus"), while one is from Belvì in the centre of Sardinia (Nuoro). All the specimens are complete and in good condition but refer to different collections: two specimens have been collected in inner localities, whereas another one comes from the coast. Moris reports that the scape bears a single flower and that another taxon (*D. caryophyllus* var. *tenuifolius* f. *minor*) grows in arenosis maritimis [sandy coast]. Thus, here we propose the specimen from Belvì as lectotype, despite not being dated, assuming that the herbarium in TO hosts the original material by Moris as already done by Arrigoni (1979), Rizzotto (1989), Escobar García et al. (2010) in other similar cases,

Based on the specimen in TO coming from the coast (referring actually to f. *minor*), Valsecchi (1985), and then Bacchetta et al. (2010), refer *D. caryophyllus* var. *tenuifolius* to *D. morisianus* Vals. Based on the diagnosis and the lectotype designated here, *D. caryophyllus* var. *tenuifolius* does not belong to *D. morisianus*. The former taxon shows short scapes bearing one or few flowers and epicalyx scales with mucro 0.5–1.5 mm long, while the latter shows longer multiflowered scapes with epicalyx scales with mucro 2.0–3.5 mm long. This interpretation agrees with Arrigoni (2010). According to the lectotype of *D. caryophyllus* var. *tenuifolius*, which shows woody stocks contracted with branches, epicalyx scales with an evident mucro, and small calyx, this taxon is a heterotypic synonym of *D. genargenteus* Bacch., Brullo, Casti & Giusso.

Dianthus caryophyllus f. minor Moris, Fl. Sardoa 1: 231. 1837. [April 1837]

= D. morisianus Vals., Boll. Soc. Sarda Sci. Nat. 24: 333. 1985. Ind. Loc.: "In arenosis maritimis [Sardinia]".

Type. (lectotype here designated): *Dianthus caryophyllus* var *tenuifolia*, in arenosis maritimis, S. Nicolai flumini major Majo junio / Mus. Bot. Horti Taurinensis, Herb. Moris Barbey Cat Sard. N.156, TO!

Note. A single sheet was found in TO. Albeit it may represent the holotype, it is cautiously designated here as a lectotype.

The selected specimen, uniflorous, has fixed seven portions of plants whose leaves and flower scapes are smaller than those of the typical form. All other characters of the flowers correctly match the protologue. This taxon is a heterotypic synonym of *D. morisianus*, a species described by Valsecchi (1985) for the same area and habitat (Peruzzi et al. 2015), that shows the same leaf length and width and, albeit with multiflowered scapes, the same length and shape of outer and inner bracts, and the same calyx length and shape.

Dianthus collivagus Jord. in Billot, Annot.: 46. 1856. [December 1856]

- ≡ D. caryophyllus var. collivagus (Jord.) Cariot & St-Lager Étude Fl., éd. 8, 2: 104. 1889.
- =? *D. sylvestris* Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: "abonde sur les côteaux du Rhône près de Lyon".

Type. (lectotype here designated): *Dianthus scheuchzeri* Rchb., *Dianthus sylvestris* auct. Gall. ex parte non Wulf, Lyon a Néron, Jordan, odor levis, folia ramis trigemina semper angustissima; *Dianthus scheuchzeri* Jord. non Rchb., *Dianthus collivagus* Jord., Lyon à Neron, ex herbis Jordan, July 1854, CLF056818!

Note. Other six specimens collected by Jordan are preserved at LY, but they are not original material, since they are lacking a date or reporting dates later than the protologue.

The lectotype designated here matches the protologue and corresponds to the current application of the name, which is considered as a heterotypic synonym of *D. sylvestris* subsp. *sylvestris* in Kerguélen (1993). The lectotype of *D. collivagus*, concerning the shape of calyx teeth, is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which in turn is currently included within the variability of *D. sylvestris* (Domina et al. 2021).

Dianthus consimilis Jord. in Billot, Annot. 47. 1856. [December 1856]

 \equiv D. caryophyllus var. consimilis (Jord.) Rouy & Foucaud in Rouy, Fl. Fr. 3: 195. 1896.

=? *D. sylvestris* Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: "Alpes de l'Oisans".

Type. (lectotype here designated): *Dianthus consimilis* Jord., June-July 1855, [A. Jordan] Roux, Herbier Jordan, LY0079676!

Note. At LY we found another specimen citing "Lautaret (H. Alpes, May, June-July 1855, ex Horto Alexis Jordan, LY0079674!" but lacking basal leaves.

The lectotype designated here matches the protologue and corresponds to the current application of the name, which is considered as a heterotypic synonym of *D. sylvestris* subsp. *sylvestris* in Kerguélen (1993). The lectotype of *D. consimilis*, concerning the shape of calyx teeth, is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which, in turn, is currently included within the variability of *D. sylvestris* (Domina et al. 2021).

Dianthus contractus var. evolutus Lojac., Fl. Sicul. 1(1): 165. 1888. [September 1888]

= D. arrostoi C.Presl, Delic. Prag. 60. 1822. Ind. Loc.: "Sulle più alte vette delle Nebrodi sui terreni ghiaiosi o sulle rupi calcaree di Serre di Quacedda. Juntera Minà Pal!".

Type. (neotype here designated): *Dianthus contractus* Jan., *Dianthus constrictus* Janka, In asperis calcareis elatioribus montis Nebrodes, Julio, M. Lojacono Pojero, P05052873 (photo!).

Note. Neither the original material nor traces of this taxon were found in the herbaria consulted and among the documents accompanying the centuries distributed by Lojacono (Aghababyan et al. 2012; Domina et al. 2014). We chose to designate as a neotype the single specimen found, which is at least collected by Lojacono.

The neotype designated here matches the protologue and allows to consider this name as an heterotypic synonym of *D. arrostoi* C.Presl. Compared to the lectotype of *D. contractus* designated by Bacchetta et al. (2010: 151: s.l., s.d., Jan, NAP-GUSS!), and to the lectotype of *D. arrostoi* designated by Camarda and Corrias (1987: 417), this variety differs only by the more elongated scapes.

Dianthus godronianus Jordan in Mém. Acad. Roy. Sci. Lyon, Sect. Sci., ser. 2, 1: 241. 1851. [January 19851]

- ≡ D. caryophyllus subsp. godronianus (Jord.) P.Martin, Soc. Ech. Pl. Vasc. Eur. Bassin Médit. 19: 93. 1984.
- ≡ *D. sylvestris* var. *godronianus* (Jord.) Kerguélen, Lejeunia, Nouv. Sér., 120: 81. 1987.
- = D. virgineus L., Sp. Pl. 1: 412. 1753. Ind. Loc.: Coteaux stériles de la région des oliviers. Provence, Hyères, Marseille, Toulon, Apt, mont Ventoux, Vaucluse, Villeneuve; Dauphinée, Rabou près de Gap, Valence, Avignon, Languedoc, Viviers, pont du Gard, Uzés, Montpellier, Mende, Perpignan Corse, Calvi, Bastia, Cervione, Evisa, Otta, Campitello.

Type. (lectotype here designated): Soleirol, Herb. Cors., 959 *Dianthus virgineus* L. (Gren. et Godr.), *Dianthus sylvestris* Duby, Bastia - mai 1823, P05000349 (photo!).

Note. – Jordan (1851, 1856) believed that the plants referred by Godron (1847, 1848) to *D. virgineus* L. actually represent a different species, which he renamed *D. godronianus*. According to Godron (1848), this species grows in the surroundings of Montpellier, South France, and Corsica. A duplicate of the collection no. 959 by Soleirol, explicitly cited as seen by Godron (1848), was chosen as lectotype.

This specimen corresponds with the protologue and with the current application of the name. In Kerguélen (1993), this taxon is considered accepted at varietal rank (*D. sylvestris* subsp. *longicaulis* var. *godronianus*). In Jauzein (2014), this taxon is instead included in *D. caryophyllus* subsp. *longicaulis* (Ten.) Arcang., but the author argues that it could constitute a distinct subspecies (*D. caryophyllus* subsp. *godronianus* (Jord.) P.Martin). *Dianthus godronianus* is instead considered a distinct species by Tison and

de Foucault (2014), although these authors note that some coastal populations in Provence differ for a few morphological features. According to the lectotype features and the recent lectotypification of the latter name (Domina et al. 2021), this species can be regarded as a heterotypic synonym of *D. virgineus*.

Dianthus orophilus Jord. in Billot, Annot.: 43. 1856 [December 1856]

- ≡ D. caryophyllus var. orophilus (Jord.) Rouy & Foucaud, Fl. Fr. 3: 195. 1896 [July-August 1896]
- =? *D. sylvestris* Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: "schistes au Lautaret et dans le province de Maurienne (Savoie)".

Type. (lectotype here designated): *Dianthus orophilus, Dianthus sylvestris* an var. *gracilior*, du Lautaret May [18]53-June [18]55 [...], LY0825955!

Note. Two syntypes from Col de Lautaret are housed at LY: LY0825955 and LY0087623, both in good condition. We have designated here the most complete one as lectotype. The selected type comes from Jordan's garden, where it was cultivated since its first collection in 1853.

This specimen conforms to the description of the protologue and corresponds to the current application of the name, which is considered as a heterotypic synonym of *D. sylvestris* subsp. *sylvestris* in Kerguélen (1993). The lectotype of *D. orophilus* concerning the shape of calyx teeth, is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which in turn is currently included within the variability of *D. sylvestris* (Domina et al. 2021).

Dianthus saxicola Jord., Pugill. Pl. Nov.: 29. 1852 [October 1852]

D. caryophyllus var. *saxicola* (Jord.) Cariot & St-Lager, Étude Fl., éd. 8, 2: 103. 1889.
P. sylvestris Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: "in lapidosis et rupestribus calcareis Beugesi et Delphinatus prope Lyon ubi eum legi".

Type. (lectotype here designated): *Dianthus saxicola* Jord., Serrières (Ain) près de Lyon, 7 June 1852, A. Jordan, LY0682162!

Note. Two specimens belonging to the original material are housed at LY: LY0682162 and LY0088790. Both are in good condition. We have designated here the most complete one as the lectotype.

This specimen conforms to the description of the protologue and corresponds to the current application of the name, which is considered a distinct species by Tison and de Foucault (2014). The lectotype of *D. saxicola* has 10–15 cm long basal leaves and multiflorous scapes; concerning the shape of calyx teeth, it is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which in turn is currently included within the variability of *D. sylvestris* (Domina et al. 2021). Further research is needed to clarify the relationships between these two taxa.

Dianthus juratensis Jord. in Billot, Annot.: 47. 1856. [December 1856]

≡ D. caryophyllus var. *juratensis* (Jord.) Gren., Fl. Chaîne Jurass.: 105. 1865.

=? D. sylvestris Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: "du Mont Reculet (Ain)".

Type. (lectotype here designated): *Dianthus juratensis* Jord., mont Reculet (Ain), 24 August 1854, [A. Jordan], LY0083755!

Note. Another herbarium sheet (LY08259243) is preserved at LY; it contains plants collected in 1855 in Villeurbanne, where they were cultivated after being originally collected in the wild at Reculet (Ain).

The lectotype designated here matches the protologue and corresponds to the current application of the name, which is considered as a heterotypic synonym of *D. sylvestris* subsp. *sylvestris* in Kerguélen (1993). The lectotype of *Dianthus juratensis*, concerning the shape of calyx teeth, is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which in turn is currently included within the variability of *D. sylvestris* (Domina et al. 2021).

Dianthus subacaulis Vill., Hist. Pl. Dauphiné 3(2): 597. 1789. [September-October 1789]

- ≡ D. sylvestris var. subacaulis (Vill.) W.D.J.Koch, Syn. Fl. Germ. Helv. 1: 97. 1835.
- ≡ D. virgineus var. subacaulis (Vill.) Ser., Prodr. [A. P. de Candolle] 1: 361. 1824.
- ≡ D. pungens subsp. subacaulis (Vill.) Bernal, Laínz, Muñoz Garmendia & Pedrol, Anales Jard. Bot. Madrid 44(2): 571. 1987.
- =? *D. sylvestris* Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]. Ind. Loc.: "aux environs du Buis, sur le Mont Ventoux".

Type. (neotype here designated): Herbier A. Dubuis, *Dianthus subacaulis* Vill. subsp. *subacaulis*, Pentes rocailleuses dénudées près du somment du Mont Ventoux (1912 m). (Vaucluse), 7 July 1955, MPU329773 (photo!).

Note. No original material was found in GRM and in the other surveyed herbaria. Also A. P. V. Mutel's Herbarium was checked because he used to include Villars specimens in his own herbarium (M. Lefebvre, pers. comm.).

The neotype designated here matches the protologue and corresponds to the current application of the name, which is accepted by both Kerguélen (1993) and Tison and de Foucault (2014). This species is characterized by having 1 cm long basal leaves, very short, 1–5 cm long single-flowered scapes and epicalyx scales lanceolate with a linear mucro. Concerning the shape of calyx teeth, it is very similar to the lectotype of *D. inodorus* (L.) Gaertn., which in turn is currently included within the variability of *D. sylvestris* (Domina et al. 2021). Further research is needed to clarify the relationships between these two taxa.

Dianthus sylvestris Wulfen in Jacq., Coll. 1: 237. 1786. [January-September 1786]

≡ D. caryophyllus subsp. sylvestris (Wulfen) Rouy & Foucaud, Fl. France 3: 193. 1896. Ind. Loc.: – "in montibus illis prope Ponewitsch Baronis Wolkensberg in Carniolia, tum in M. Utocsek prope Pillichgraz; in iis. Vallis Rablensis; denique & in iis Vallis Canalensis &c.".

Types. (lectotype here designated): The water-coloured iconography published by Jacquin (1781–1786, t. 82, the small individual on the right).

Note. The iconography designated by Bacchetta et al. (2010) as neotype is actually part of the original material as uncited illustration (Art. 9.12 of the ICN), since Jacquin's Icones and Collectanea work are interrelated. Therefore this neotypification must be corrected in lectotypification. This illustration depicts two individuals: one small with a 2 branched single-flowered stem and one large, unbranched but with multiflowered stems and basal leaves three times longer, exemplifying morphological variation in this species. In the protologue, it is clearly stated that the larger plant was seen only once in Monte Re, near Lake of Predil, NE Italy ("Uno duntaxat, quod miratus sum, loco Montis regii Rablensis, giganteum inveni, caulibus cubitalibus bi- & trifloris"), while smaller plants are common elsewhere in Carniola. Accordingly, we can conclude that the two drawings depict plants originating from two different areas, thus belonging to two different gatherings. Consequently, the type designated by Bacchetta et al. (2010: 143), neotype or lectotype, belongs to more than one gathering and cannot be accepted as a type (Art. 8.1, 8.2, 9.3 of the ICN). Thus, the name remains to be typified. No other original material for this name exists (de Langen et al. 1984), so that we select here as lectotype only the small specimen of the water-coloured iconography published by Jacquin at table 86 that better fits the description "folia ... pollicari aut circiter longitudine... Caulis subquinquepollicaris... Flos plerumque unicus [Leaves ... one inch or about one inch long, stem less than 5 inches ... flower generally single]".

The lectotype here selected agrees with the current application of the name by numerous authors, e.g., Kerguélen (1993), Bacchetta et al. (2010), Tison and de Foucault (2014), Brullo and Guarino (2017), who consider *D. sylvestris* as an accepted species. The overall size of the plant, and the length of the leaves are not stable characters for taxonomic discrimination. The shape and relative size of calyx and epicalyx scales are better discriminating taxonomic characters and are evident in the lectotype. These features allow to distinguish *D. sylvestris* subsp. *sylvestris* from *D. sylvestris* subsp. *tergestinus* (Bacchetta et al. 2010).

Dianthus sylvestris var. *garganicus* Ten., Fl. Napol. Syll.: 208. 1831. [July-August 1831]

≡ D. caryophyllus subsp. garganicus (Ten.) Grande, Boll. Soc. Bot. Ital. 1912: 178. 1912.

- \equiv *D. caryophyllus* var. *garganicus* (Ten.) Fiori, Nuova Fl. Italia 1: 512. 1924.
- ≡ D. sylvestris subsp. garganicus (Ten.) Pignatti, Giorn. Bot. Ital. 107: 211. 1973.
- \equiv *D. garganicus* (Ten.) Brullo, Braun-Blanquetia 2: 31. 1988.
- = D. tarentinus Lacaita, Nuovo Giorn. Bot. Ital. n.s., 18(4): 511. 1911. Ind. Loc.: "Gargano".

Type. (lectotype here designated): *Dianthus sylvaticus*, *D. sylvestris* Ten. Fl. Neap. Prodr. (1811) p. xxv. - Eiusd. Fl. Nap. I (1811–1815) p. 231, Gargano, Tenore misit Nov 1827 / Herb. J. Gay., Presented by Dr. Hooker, February 1868, K000725365 (photo!).

Note. In the same herbarium sheet three herbarium specimens, sent by Michele Tenore to Jaques Étienne Gay, are mounted. K000725363 was collected by Tenore from Calmaldoli (Campania, Italy) in November 1825; K000725364 by Nicolas Bové from La Calle (Algeria) in June 1839, and K000725365 by Tenore from Gargano (Apulia, Italy) in November 1827. In NAP there is a specimen from Gargano with the handwriting by Michele Tenore, lacking a date.

The lectotype designated here matches the protologue and corresponds to the current application of the name, which is considered as a heterotypic synonym of *D. tarentinus* Lacaita (Bacchetta et al. 2010; Brullo and Guarino 2017; Bartolucci et al. 2018). This synonymy is here confirmed based on the shape and size of the leaves, of the scales of the epicalyx and of the calyx which are observable on the types of the two taxa.

Dianthus virgineus var. *tergestinus* Rchb., Icon. Fl. Germ. Helv. 6: 47, pl. 266 fig. 5049β?. 1842–1844. [1844 publ. 1842–1844]

- \equiv D. tergestinus (Rchb.) A.Kern., Sched. Fl. Exs. Austro-Hung. 2: 71. 1883.
- \equiv *D. caryophyllus* var. *tergestinus* (Rchb.) Tanfani in Caruel, Fl. Ital. 9(2): 283. 1892.
- \equiv *D. sylvestris* subsp. *tergestinus* (Rchb.) Hayek, Repert. Spec. Nov. Regni Veg. Beih. 30(1, 2): 247. 1924. Ind. Loc.: none [but Trieste, Italy, can be easily inferred from the epithet "tergestinus" that means "from Trieste"].

Type. (lectotype here designated): Rchb., Icon. Fl. Germ. Helv. 6: pl. 266 fig. 5049β. 1842–1844.

Note. The main text (Icon. Fl. Germ. Helv. 6: 47. 1842–1844. [1844 publ. 1842–1844]) lacks a written diagnosis or description, and, in any case, it is not clear if the plate was published simultaneously with the main text. Stafleu and Cowan (1983) reports that the volume 6 was published between 1842 and 1844, even though the title page shows 1844. However, this name was validly published on plate CCLXVI (= 266) by an illustration with analysis (Arts. 38.7 and 38.8 of the ICN), which is obviously part of the original material.

This taxon is considered as a subspecies of *D. sylvestris* by Vangjeli (2015), Brullo and Guarino (2017), Bartolucci et al. (2018), Peruzzi et al. (2019), and Nikolić (2020). It differs from *D. sylvestris* subsp. *sylvestris* by having a poorly developed mucro of the epicalyx scales and entire petals. Its distribution (Trieste area and along the north-eastern Adriatic coast), separated from the main range of *D. sylvestris* subsp. *sylvestris*, is compatible with the rank of subspecies.

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RESEARCH ARTICLE



Primulina scutellifolia, a new species of Gesneriaceae from southern Vietnam

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Abstract

Primulina scutellifolia is described as a new species from Khanh Hoa Province, southern Central Vietnam. It is distinct in the genus in having scutellate leaves that make it a highly potential ornamental plant. The new species looks like *P. annamensis* in general shapes, sizes and colours of habit, inflorescence, flower, and leaf but is distinguishable by adaxially glabrous and abaxially strigose leaves with serrate margins, scutellate leaf blade and appressed downwards tomentose petiole, sparsely glandular hairs on apical 1/2 of the gynoecium and trapeziform one-lipped stigma with slightly emarginated apex.

Keywords

Gesneriaceae, new species, Primulina, scutellate leaves, Vietnam

Introduction

Primulina Hance (Gesneriaceae) is known to have more than 220 species, with the center of species diversity in south and southwest China and North Vietnam (Weber et al. 2011; Möller and Clark 2013; Möller et al. 2016; Xu et al. 2017; Li et al. 2019; Möller 2019; IPNI 2021). As more than 40 new species have been described over the past five years, we now expect that more species will be discovered for the genus if further explorations are employed (Möller 2019; Ge et al. 2020; Tong et al. 2020; Xin et al. 2021).

In Vietnam, *Primulina* was last revised to have 23 species (Vu 2017), but this work ignored *P. crassirhizoma* F.Wen, Bo Zhao & Xin Hong (Zhao et al. 2013). Based on molecular, morphological and cytological characters, Möller et al. (2016) moved *P. cycnostyla* (B.L.Burtt) Mich.Möller & A.Weber, *P. eberhardtii* (Pellegr.) Mich.Möller & A.Weber, *P. minutihamata* (D.Wood) Mich.Möller & A.Weber and *P. tamiana* (B.L.Burtt) Mich.Möller & A.Weber to *Deinostigma* W.T.Wang & Z.Y.Li. Since then, three other new species (*P. elegans* B. M. Wang, Y. H. Tong & N. H. Xia; *P. malipoensis* L.H. Yang & M. Kang; *P. xuansonensis* W.H.Chen & Y.M.Shui) have been described from northern Vietnam (Yang et al. 2018; Chen et al. 2020; Tong et al. 2020). Therefore, prior to this paper, the total number of *Primulina* species known in Vietnam is 23.

In 2013, the last author of this paper collected a *Primulina* species in an evergreen broadleaf forest in Khanh Hoa Province. It was misidentified as *P. annamensis* (Pellegr.) Mich.Möller & A.Weber (Weber et al. 2011) – a species that grows popularly in many forests in Khanh Hoa Province and the neighbour Bidoup – Nui Ba National Park in Lam Dong Province. During our ongoing study of Gesneriaceae in southern Vietnam, we have carefully examined the plant and found that its scutellate leaves are distinct in the genus, and therefore we describe it here as a new species. Measurements of morphological characters were based on living plants whose photographs were taken with Canon EOS 7D digital camera. Morphological comparison with the close species was based on in situ observation and consultation with published literature.

Taxonomy treatment

Primulina scutellifolia Luu, N.L.Vu & T.Q.T.Nguyen, sp. nov. urn:lsid:ipni.org:names:77234377-1 Figure 1

Type. VIETNAM. Khanh Hoa Province, Khanh Vinh District, Son Thai Commune, 12°11'39"N, 108°43'30"E, at ca. 1485 m elevation, 01 November 2013, *Luu Hong Truong* KH0945 (holotype SGN!; isotypes SGN!; PHH!; VNMN!).

Diagnosis. *Primulina scutellifolia* differs from other congeners in having scutellate leaves.

Description. Herb, perennial, rosulate, acaulescent. Rhizome terete, woody, to 9 cm long, 3 mm in diameter. Leave 3–13, all basal; petioles cylindrical, appressed downwards tomentose, 6–9 cm long, 0,3 cm in diameter; leaf bade scutellate, 3–5.5 cm long, 2–4 cm wide, adaxially glabrous, shining, plain dark green or dark green with yellowish-greenish spots, leathery, abaxially pale green, reticulate-foveate, sparsely strigose; margins serrate; apex obtuse; base slightly sinuate; venation sunken adaxially, prominent and strigose abaxially; lateral veins 4–6 paired. Inflorescences cymose, axillary, 1–3 flowered. Peduncle reddish brown or greenish, 10–12 cm long, 2–3 mm in diameter, sparsely hirsute. Bracts narrowly triangular, ca 2–3 mm long, ca. 0.5 mm wide at base, same color with peduncle. Calyx 5-lobed from base; lobes equal,



Figure 1. *Primulina scutellifolia* **A**, **B** habit **C** inflorescence **D** leaf blade **E** leaf blade, abaxial surface **F** petiole **G** flower, longitudinal dissection **H** corolla, abaxial lip **I** staminodes **J** anthers **K** opened anther **L** stigma **M** opened fruit **N** calyx lobes, abaxial surface **O** calyx lobes, adaxial surface **P** fruit, cross section **Q** seeds.

lanceolate-oblong 8-10 mm long, 1-2 mm wide, abaxially reddish brown to light green and sparsely glandular hairy, adaxially yellowish green, margin entire, apex acute. Corolla infundibuliform, 4-5 cm long, 1-1.2 cm in diameter at mouth, ca. 0.5 cm in diameter at base, white, violet or white at base and gradually turning to violet towards the apex, outside sparsely glandular hairy, inside smooth and with two yellow stripes on lower part of the corolla. Limb distinctly 2-lipped; upper lip 2-lobed, lobes broadly ovate, 3.5–4 mm long × 7–8 mm wide; lower lip 3-lobed, central lobe orbicular, 7–8 mm long \times 7–8 mm wide, lateral ones broadly ovate, 7–8 mm long \times 8–9 mm wide. Stamens 2; filaments 29-32 mm long, adnate to the corolla tube base for 16-18 mm, free part 13–14 mm long and slightly curved, apically sparsely glandular hairy; anthers fused by their entire adaxial surfaces, elliptic, ca. 3 mm long, yellowish. Staminodes 3, linear, translucent; apex capitate, yellowish, glabrous, lateral ones 19-21 mm long, adnate to the corolla for 15-16 mm, free part 4-5 mm long, middle one 16-17 mm long, ca. 1 mm long, adnate to the corolla tube base for 16 mm. Disc ca. 1 mm high, slightly 5-lobed. Ovary linear, 3-3.5 cm long, ca. 2 mm in diameter at base, glabrous on basal 1/2, glandular hairy on apical 1/2. Stigma of only lower lip developing, translucent white, trapeziform, finely hairy, with emarginated apex. Capsule linear, slightly falcate, oblique in relation to the pedicel, reddish brown or greenish and turning to light yellowish, sparsely hairy on the apical part, 65-70 mm long, 5-7 mm in diameter, opening along the dorsal side. Seeds long ellipsoid, translucent brownish.

Phenology. Flowering was found in August to November and fruiting in September to January.

Etymology. The specific epithet is derived from its special scutellate leaves.

Vietnamese names. Báo xuân đón lộc.

Distribution and habitat. *Primulina scutellifolia* is currently only known from the type location. It grows scattered on humid fertile soils in the evergreen broadleaf forest at elevations of 1,450 to 1,950 m. Our surveys throughout forests of Khanh Hoa and Lam Dong Provinces, which have now been ongoing for more than ten years, confirm its distribution is confined to the eastern slopes of the Hon Giao Range. This is a locally endemic plant.

Preliminary conservation status. The plant has been recorded in one population at the type location, with Extent of Occurrence <100 km² that is impacted by continued logging and not effectively protected. Therefore, we suggest the species to be categorized as Critically Endangered (A1a or B1a,b) (IUCN Standards and Petitions Subcommittee 2019).

Discussion

Primulina scutellifolia is unique in the genus by its scutellate leaves. It may be confused with *P. annamensis* (Figure 2) which has similar general shapes, sizes and colours of habit, inflorescence, flower, and leaf (Pellegrin 1930; Wood 1974; Pham-Hoang 1993;



Figure 2. *Primulina annamensis* A habit B stigma C leaf blade, adaxial surface D leaf blade, abaxial surface.

Characters	P. annamensis	P. scutellifolia
Petiole	densely pilose	appressed downwards tomentose
Lamina	flat, with obviously cordate base and entire or cre-	scutellate, with slightly sinuate base and serrate
	nate margins, abaxially and adaxially densely silky	margins, adaxially glabrous, abaxially sparsely
		strigose
Gynoecium	densely glandular hairy on apical >2/3	glandular hairy on apical 1/2
Stigma	bi-lipped; lips bifid with round lobes	upper lip absent; lower lip with slightly emarginated
		truncate apex

Table 1. Key morphological differences between Primulina annamensis and P. scutellifolia.

Pham-Hoang 2003; Weber et al. 2011; Vu 2017). However, the latter taxon can be distinguished from our species in having abaxially and adaxially denser silky leaves with obviously cordate base, flat blade and entire or crenate margins, denser pilose petioles, denser glandular hairs on more than apical 2/3 of the gynoecium, bi-lipped stigma and bifid stigma lips with round lobes (Table 1). Both species sometimes grow sympatrically but the latter is much more abundant. In dried specimens, the leaves of the new species look somehow subpeltate, which may be reminiscent of those in *Deinostigma tamiana* (B.L.Burtt) D.J.Middleton & H.J.Atkins from northern Vietnam (Burtt 1999; Möller et al. 2016), but the latter is distinguishable by its slightly peltate leaves with hairs on both surfaces, short petioles, hooked hairs on pedicel and 4–9-flowered inflorescences. The scutellate leaf blades, often with yellowish-greenish spots and beautiful flowers of the new taxon, render it an ornamental plant of great potential.

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New and poorly known "araphid" diatom species (Bacillariophyta) from regions near Lake Titicaca, South America and a discussion on the continued use of morphological characters in "araphid" diatom taxonomy

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Abstract

Based on two Andean Altiplano samples and on light and scanning electron microscopy analyses, we present six new species of "araphid" diatoms in the genus *Pseudostaurosira*, *P. aedes* **sp. nov.**, *P. frankenae* **sp. nov.**, *P. heteropolaris* **sp. nov.**, *P. oblonga* **sp. nov.**, *P. occulta* **sp. nov.**, and *P. pulchra* **sp. nov.** Additional data are provided for four other known taxa, *Nanofrustulum cataractarum*, *N. rarissimum*, *P. sajamaensis* and *P. vulpina*, the latter species corresponding to a stat. nov. based on a variety of *P. laucensis*. Each taxon is described morphologically and compared with closely related published taxa, using characters such as axial area, virgae, vimines, areolar shape, volae, internal striae depositions, spines, flaps and apical pore fields, which are not usually used for species distinction within the genus. It is our intention that the detailed morphological descriptions of each taxon and the elaborate comparative tables we provide serve as a basis for correction of neo and paleo-databases for the Altiplano to produce a better account of autecological data and ecological change in the region. Some arguments for our continued use of a morphologically based approach are given in the context of rapid environmental degradation in the Andes and the difficulties in applying molecular approaches in countries such as Bolivia.

Keywords

Andean mountains, fragilarioid diatoms, morphology, South America, traditional taxonomy

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Introduction

In the last two decades, many new "araphid" taxa have been described, clarifying the morphological concepts of existing genera or better delimiting the boundaries of widely reported species (e.g. Lange-Bertalot and Ulrich 2014; Wetzel and Ector 2015; Wengrat et al. 2016; Almeida et al. 2017; García et al. 2017; Van de Vijver et al. 2020a). The study of type material helped in the latter endeavor, which coupled with illustrated reports and newly found populations, gave a clearer view of diagnostic characters and added other features that had not been used before for recognition of purportedly well-known species (e.g. Edlund et al. 2006; Cejudo-Figueiras et al. 2011; Wetzel et al. 2013a, b; Talgatti et al. 2014; Delgado et al. 2015; Van de Vijver et al. 2020a, b). Such is the case, for example, with *Pseudostaurosira brevistriata* (Grunow) D.M. Williams & Round (Morales et al. 2013a, 2019b) and *Staurosirella pinnata* (Ehrenberg) D.M. Williams & Round (Morales et al. 2013a, 2019a). The literature for both is extensive, revealing a history of taxonomic drift, lumping and imprecise reports of their autecology (Morales et al. 2013a, 2014c, 2019b).

These morphological studies continue to be important in the resolution of taxonomic issues and taxa delimitation. New morphological descriptions and taxonomic revisionary work provide a series of testable hypotheses that constitute the grounds upon which further progress can be made in fields such as systematics, ecology, conservation, etc. (de Carvalho et al. 2007; Haszprunar 2011).

Though molecular studies are becoming increasingly important in the resolution of taxonomical issues at the species level, both sources of information, morphological and molecular, ought not to be divorced and are rather complementary since morphological studies generate hypotheses based on the phenotype, while molecular studies do it based on the genotype. One dataset can be used as a confirmation of the other. The concatenation of both sources of information could produce a stronger and bettersupported taxonomic system that can be translated, for example, into a practical tool to be used at the bench during routine identification analyses (Kahlert et al. 2019).

However, the colossal task that represents the production of fully operating barcode datasets (Zimmermann et al. 2014; Kelly et al. 2018) that are applicable to nature deems "traditional" morphological analyses a continued fast and viable way to produce data and hypotheses on taxa identities and distinctiveness. The same can be said for reliable phylogenies that aim to express natural classifications (see Li et al. 2018 and discussion in Morales et al. 2019b) but production of phylogenies is another matter, a different stage in the process of studying biodiversity that we are not concerned with in the present contribution. Here we deal only with a first stage of discovery, description of traits and a comparative analysis to justify the hypothetical placement of the treated taxa under given genera.

The study of "araphid" diatoms from high Andean ecosystems is important since they are frequent in current and paleoecological samples. Their abundance and distribution have been used to determine past climate, water level and precipitation changes, salinity and ionic composition, and temperature variations (e.g. Servant-Vildary 1978; Servant-Vildary and Roux 1990; Fritz et al. 2004; Tapia et al. 2006). But also from the taxonomic standpoint, it becomes relevant to describe species and produce inventories and autecological data for Andean "araphids" and other diatoms, especially because mountainous areas are affected more rapidly by climate change than any other land ecosystems (Marengo et al. 2011; Cuesta et al. 2012; Michelutti et al. 2015). Coupled with land use effects, threats to ecological stability with the anticipated negative effects on human and other populations are beginning to be observed in these areas (Suárez et al. 2011). A thorough knowledge of the taxonomy and autecology of diatoms could provide an aid in the conservation of Andean areas and their communities.

The examination of recent samples from Bolivia and Argentina has shown that the Andes contains hot spots for "araphid" species diversity (e.g. Morales et al. 2012b, 2019b; Grana et al. 2018; Seeligmann et al. 2018; Guerrero et al. 2019). Study of these sites could facilitate the taxonomic clarification of several known taxa, quickly produce new species and generate inventories that can then be applied to other Andean regions.

The present paper aims to continue the morphological description of diatom taxa present in the region contiguous to Lake Titicaca, concretely in the Desaguadero River and adjacent zones. This area is affected by natural soil erosion, typical of the Bolivian Altiplano, but also by land use and water use changes that have been affecting the area for several decades (UNEP-United Nations Environmental Program 1996). The recent international news about the drying of neighboring Lake Poopó, the site where a substantial amount of fauna and flora thrived and from where human groups, descendants of the millenary Urus tribes, have been displaced (Richard and Contreras 2015), furnished evidence of the urgency of basic studies such as the present one. The long-term goal is to improve the paleolimnological/paleoclimatic characterizations conducted in the area (e.g. Servant-Vildary 1978; Servant-Vildary and Blanco 1984; Tapia et al. 2003) and to provide baseline data for conservation and management practices in the region.

Concretely, we present six new species together with comparative analyses with published morphologically closely related taxa, and additional morphological information and comparative data for other four species described from the Andes and elsewhere. For all ten taxa, a pertinent discussion is presented to aid in their distinction and identification.

Methods

The study area, the southern region contiguous to Lake Titicaca, was already described in a geographical and ecological context by Morales et al. (2012b, p. 42–44) and Grana et al. (2015, table 1, fig. 4 and p. 815). Epipsammon material used in the present study is the same as that described in Grana et al. (2015), collected from rivers Desaguadero and Sajama with the aid of a turkey baster and fixed with 20 drops of 40% formaldehyde in the field.

For LM analysis, subsamples of 20–30 mL were mixed with a similar volume of 70% HCl. The mixture was boiled for 45 min and rinsed 8 times using distilled H_2O . Drops of cleaned slurry were dried on coverslips overnight at room temperature. Perma-

nent slides were mounted using the synthetic medium Naphrax. Slides were analyzed using a Zeiss Universal microscope equipped with differential interference contrast optics, a 1.25 optivar, and a Plan 100X, 1.25 NA, immersion objective. Images were taken using a Jenoptik CF color digital camera and ProGres CapturePro ver. 2.8 software.

For SEM analysis, about 10 to 20 mL aliquots of raw samples were digested with concentrated H_2O_2 and heated for 24 h using a sand bath. Then, samples were allowed to cool and settle (ca. 1 cm/h) and 80 to 90% of supernatant was eliminated by vacuum aspiration. A volume of 1 mL of HCl acid (37%) was added and the preparation was allowed to settle for 2 h. Subsequently, the sample was rinsed and decanted three times using deionized water. Approximately 100 mL aliquots of clean material were filtered and rinsed with deionized water through glass fiber filters with a 3 μ m pore diameter. Coating with platinum was accomplished using a BAL-TEC MED 020 Modular High Vacuum Coating System for 30 s at 100 mA. A Hitachi SU-70 electron microscope operated at 5 kV and 10 mm distance was used for SEM analysis. Micrographs were digitally manipulated and plates containing LM and SEM pictures were mounted using Photoshop CS3.

Identification of taxa was performed using literature published for South America (Metzeltin and Lange-Bertalot 1998, 2007; Rumrich et al. 2000; Metzeltin et al. 2005). Taxonomic articles by Frenguelli (1939), Patrick (1961), Manguin (1964), Hohn (1966), Servant-Vildary (1986), Morales and Vis (2007) and Morales et al. (2007) were also used. Additionally, non-South American references were used such as the general floras of Patrick and Reimer (1966, 1975), Krammer and Lange-Bertalot (1986, 1988, 1991a, b), Simonsen (1987), Lange-Bertalot (1993), Hofmann et al. (2011), and Lange-Bertalot et al. (2017), as well as references specialized in certain genera such as Navicula (Lange-Bertalot 2001), Pinnularia (Krammer 2000) and cymbelloids (Krammer 1997a, b). Descriptions of the new taxa are based on measurements of 30 valves and observation of more than 100 individuals of each taxon under both LM and SEM. Morphological terminology follows Anonymous (1975), Ross et al. (1979) [both references used for terminology applied to striae, areolae and spines], Barber and Haworth (1981) [for terminology related to valve shape and striae orientation], Williams and Round (1987) and Round et al. (1990) [both references used for terminology on areolar substructures, girdle band features and apical pore field characteristics].

Results

Nanofrustulum cataractarum (Hustedt) C.E. Wetzel, E. Morales & Ector in Morales et al. 2019b, Plant Ecology and Evolution 152, p. 275. Figs 1A–E (LM), 2A, B (SEM)

Basionym. *Melosira cataractarum* Hustedt 1938, Archiv für Hydrobiologie, Supplement 15, p. 142, pl. 9, figs 6–7.

Most current illustrations of type material: Wetzel et al. 2013a, figs 1A–AB, 2A–G; Beauger et al. 2019, figs 93, 94.



Figure I. A–M' LM images of little known and new "araphid" diatoms from the Bolivian Altiplano A–E Nanofrustulum cataractarum F–L N. rarissimum M–S Pseudostaurosira sajamaensis T–Z P. pulchra sp. nov. (Fig. 1U is the holotype) A'–G' P. aedes sp. nov. (Fig. 1A' is the holotype). H'–M' P. heteropolaris sp. nov. (Fig. 1I' is the holotype). Scale bar: 10 μm.



Figure 2. A–F SEM images of little known "araphid" diatoms from the Bolivian Altiplano **A**, **B** *Nanofrustulum cataractarum* **A** specimen from the Desaguadero River, showing quasifract girdle elements with prominent ligulae (white arrows) **B** specimen from the Sajama River showing common internal depression into which the areolae open (dotted arrows) and the blister-like depositions of silica at the abvalvar edge of the mantle **C**, **D** *N*. *rarissimum* from the Desaguadero River **C** small, spineless valve **D** internal view showing apical and foot pole pore fields (black arrows) and internal depressions containing all areolae within a stria (dotted arrow) **E**, **F** *Pseudostaurosira sajamaensis* from the Desaguadero River **E** top view showing gradual transition of valve face to mantle and the reduced apical pore fields (black arrow) **F** side view of two half cells still attached by heavily silicified spines. Notice open girdle elements (white arrows). Scale bars: 1 μm (**B**); 3 μm (**C**, **E**); 4 μm (**A**, **D**); 5 μm (**F**).

Synonym. *Pseudostaurosira cataractarum* (Hustedt) C.E. Wetzel, E. Morales & Ector in Wetzel et al. 2013a Acta Nova 6(1–2), p. 60.

Comment. The taxon was first described for insular Asia, specifically from Java, Indonesia, by Hustedt (1938). Type material was reanalyzed by Wetzel et al. (2013a)

and Beauger et al. (2019) and regional and worldwide distributions were presented in Wetzel et al. (2013a) and Grana et al. (2015).

As presented in Table 2 in Grana et al. (2015), N. cataractarum from Bolivia (Figs 1A-E, 2A, B) are smaller (length and width: 4.5-5 µm) than specimens in Asian type material (length 5.8-8.2, width 5.4-7.2), and the stria density of the Bolivian population is higher than that from Asia (18–20 and 15–28 in 10 μ m, respectively). Regarding the areola density there is a complete overlap between both populations (2.5–3.5 in Bolivian specimens and 1–4 per 1 μ m in Asian ones). Other features, such as the pattern of areolation in both valve face and mantle, the ample, round to oval axial area, the round to slightly elongated base and flattened body of the spines with small lateral projections, are similar in both populations. Also, the depression into which the areolae from valve face and mantle open internally is similar in Bolivian and Asian specimens (Fig. 2B). The features of the girdle elements with short but wide body and prominent ligula is also comparable in both populations. The Bolivian specimens tended to have more prominent blister depositions at the abvalvar edge of the mantle (Fig. 2B). All populations reported from around the world lack apical pore fields, and areolae flaps or spine stipules have not been reported either.

Taking into account all the above-mentioned reports, the dimensions for this taxon are length: 2.8–8.2 μ m; width: 2.7–7.2; stria density: 15–29 in 10 μ m; areola density: 1–4 in 10 μ m.

In Bolivia, the taxon has been found in the Desaguadero and Sajama rivers. Fig. 2A is the first illustration of the taxon for the Desaguadero River.

Nanofrustulum rarissimum E. Morales, Novais, C.E. Wetzel & Ector in Morales et al. 2019b, Plant Ecology and Evolution 152, p. 269, figs 1A–K, 2A–D. Figs 1F–L (LM); 2C, D (SEM)

Comment. This taxon was first described by Morales et al. (2019b) from the Desaguadero River. Here we present illustrations of specimens from the Sajama River for the first time (Fig. 1F–L). Thus far, this diatom has only been seen in samples from these two sites.

The specimens found in the Sajama sample fit the dimensions of the type population, except for the length, with Sajama River specimens being shorter (5.1–9.7 μ m). At the SEM levels, no differences were noted between specimens from both sites.

Our reanalysis of Desaguadero River material yielded small valves that are spineless (Fig. 2C), but that had all the other features similar to those of larger, spiny specimens. Also, we were able to capture the apical and foot pole pore fields from an internal view (Fig. 2D), confirming that both are developed, but that the one at the foot pole is larger. Additionally, we were able to confirm the raised nature of the axial area and virga in internal view (Fig. 2D), which leaves all the areolae within a stria open into a single internal depression.

The smaller specimens found in the Sajama River sample expand the length range of this taxon which now has the following diagnostic measurements length: 5.5-9.5; width 2.5-3.3; stria density 12-13 in $10 \mu m$.

Pseudostaurosira sajamaensis E. Morales & Ector in Morales et al. 2012b, Fottea 12, p. 45, figs 12–26, 45–56.

Figs 1M–S (LM); 2E, F (SEM)

Comment. This taxon was first described from the Desaguadero River; here we also report its finding in the Sajama River. The population found in the latter falls well within the features described by Morales et al. (2012b) based on the Desaguadero River sample.

At the LM level, the narrowly elliptical valves with pointy ends and coarser striation can be used to recognize the taxon in a first instance. At the SEM level, the transapically elongated and wide areolae (Fig. 2F) are present in the majority of specimens from both sites reported here and the valve face typically and gradually transitions into the mantle, making the striae on the mantle partially visible in top outer views (Fig. 2E, and also see LM images in Figs 1M–S). The areolae vary in shape from round to trapezoid on the valve face and there is usually one very large trapezoid areola on the mantle. The volae are conspicuous and form an entangled structure. The spines have a flattened body, but they look sagittate in lateral view due to the presence of well-developed stipules. These spines sometimes have a V-shaped cleft on its back, and the tips terminate in a single or two ends (diapason-shaped) that have serrate borders pointing downward. The stipules are well-developed giving the spines a profile resembling an arrow (sagittate).

As was the case with the Desaguadero population, the Sajama River specimens lack or have weakly developed apical pore fields. Regarding the girdle elements, the valvocopula is conspicuously wider than the rest of the elements and all are open (Fig. 2F).

No changes in valve diagnostic measurements were yielded by our observations of Sajama River material.

Pseudostaurosira pulchra E. Morales, C.E. Wetzel & Ector, sp. nov.

Figs 1T–Z (LM), 3A–F (SEM)

Holotype. Slide ANSP GC 26815, Fig. 1U. Diatom Herbarium, Academy of Natural Sciences, Philadelphia (ANSP). **Isotype.** Slide DBOL-0246a, Diatomotheca Boliviensis (before HCUCB), Cochabamba, Bolivia.

Type locality. Bolivia. Sajama Province, Department of Oruro, Desaguadero River, epipsammon, 17°23'51"S; 68°14'33"W, 3701 m elev., *leg.* G. Chávez, 05.07.2009.

Description. Frustules rectangular in girdle view (Fig. 3D), joined together by interlocking spines (Figs 3C, F). Valves narrowly lanceolate, isopolar, with abrupt transition from valve face to mantle. Rostrate valve ends in larger specimens, broad-



Figure 3. A–F SEM micrographs of *Pseudostaurosira pulchra* sp. nov. from the Bolivian Altiplano **A**, **B** outer views of valves showing axial area, striae and spines features **C** inner view of valve still attached to neighboring cell. Black arrow indicates absence of apical pore field. Dotted arrow points to depression into which the stria opens internally **D** tilted view of frustule. Notice larger valvocopula, lateral growth of spines and blister-like depositions on abvalvar edge of mantle **E** outer view of valve tip. Black arrow denotes absence of apical pore field. Notice round to elliptical spine base and larger areola on valve mantle. **F** frustule attached to neighboring valve by means of heavily silicified spines. Notice open girdle elements (white arrow) and depositions along abvalvar mantle edge. Scale bars: 2 μ m (**E**); 4 μ m (**C**, **F**); 5 μ m (**A**, **B**, **D**).

ly rounded in smaller ones (Figs 1T–Z). Axial area narrowly lanceolate (Figs 1T–Z, 3A, B) and externally and internally depressed with respect to virgae (Fig. 3A, B). Internally, striae open in small trapezoid, transapically elongated depressions (Fig. 3C). Vimines short and wide, restricted to the valve face/mantle junction;

additional ones rarely present on either valve face or valve mantle (Fig. 3C). Striae typically composed by two narrow, round to elliptic areolae, one on valve face and a larger one on the valve mantle (Figs 3E, F). Well-developed volae, arising from the areolar inner periphery and projecting inwards forming a loose mesh-like structure (Figs 3C, E). Flaps usually present in various stages of development, typically single and disk-like on valve face and two or more on mantle areolae (Fig. 3A). Spines originating from vimines at the valve face/mantle junction; solid, with round to elliptical base, wider that the vimines they sit on; flattened, with biconcave sides and spatulate body, truncated (cut) at the top or with a short bifurcation (Fig. 3A, B, D, E, F). Stipules absent. Apical pore fields absent (Fig. 3C, E). Well-developed blister-like depositions present on abvalvar edge of mantle also covering both apices (Fig. 3D–F). Girdle elements variable in number, open, lacking pores, ligulated, with larger valvocopula (Fig. 3D, F).

Dimensions (n > 50): Length 5–22 μ m; width 2.4–3.0 μ m; striae 13–16 in 10 μ m.

Etymology. The epithet makes reference to the neat and eye-catching morphology of the frustules.

Distribution. Found in the Desaguadero and Sajama rivers.

Pseudostaurosira aedes E. Morales, C.E. Wetzel & Ector, sp. nov.

Figs 1A'-G' (LM), 4A-F (SEM)

Holotype. Slide ANSP GC 26815, Fig. 1A', Diatom Herbarium, Academy of Natural Sciences, Philadelphia (ANSP). **Isotype.** Slide DBOL-0246a, Diatomotheca Boliviensis (before HCUCB), Cochabamba, Bolivia.

Type locality. Bolivia. Sajama Province, Department of Oruro, Desaguadero River, epipsammon, 17°23'51"S; 68°14'33"W, 3701 m elev., *leg.* G. Chávez, 05.07.2009.

Description. Frustules rectangular in girdle view (Fig. 4B, D-F), joined together by interlocking spines (Fig. 4F). Valves narrowly elliptic with rounded ends, isopolar, with abrupt transition from valve face to mantle (Fig. 1A'-G'). Axial area narrowly lanceolate (Figs 1A'-G', 4A, B, D), externally only slightly below the virga (Fig. 3A, D, E). Internally, axial area and virgae raised, leaving the striae in large elliptic or 8-shaped, transapically elongated depressions (Fig. 4C). Vimines shorter than virgae and wide, restricted to the valve face/mantle junction; additional ones rarely present on valve mantle (Fig. 4B). Striae typically composed by two narrow, elliptic to trapezoid areolae, one on valve face and a slightly larger one on the valve mantle (Fig. 4A, B, D, E). Volae arising from the areolar inner periphery and projecting inwards forming a tightly packed mesh-like structure (Fig. 4B, C). Flaps frequently present in various stages of development, typically one disk-like or bilobate on valve face and two or more of different shape on valve mantle areola (Fig. 4A, B, D-F). Spines originating from vimines at the valve face/mantle junction, solid, with elliptic to rectangular base, as wide as the vimines; conical body with a roughly triangular profile and serrate, pointy tips. Spines have a general arrowhead-like appear-



Figure 4. A–F SEM images of *Pseudostaurosira aedes* sp. nov. from the Bolivian Altiplano **A** top, tilted view of valve showing axial area, slightly raised virga, valve face areolae covered with bilobed or disk-like flaps, and slightly larger valve mantle areola covered with two or more flaps **B**, **D**, **E**, **F** girdle views showing features of the valve mantle, open girdle elements with larger valvocopula (white arrows in **D** and **E**). Notice serrate spines with well-developed stipules in **F** which have varying patterns **C** inner view showing reduced apical pore field (black arrow) and single depression into which the areolae open internally (dotted arrow). Scale bars: 4 μ m (**D**); 5 μ m (**A–C**, **E**, **F**).

ance when seen form their posterior ends. (Fig. 4A–F). Stipules well-developed giving spines a sagittate shape and having themselves varying shapes in girdle view (Fig. 4D–F). Apical pore fields very reduced with no more than 3 round poroids, usually externally obliterated by an apical blister (Fig. 4C, D, E). Well-developed blister-like depositions present on abvalvar edge of mantle (Fig. 4B–F). Girdle elements variable in number, open, lacking pores, ligulated, with larger valvocopula (Fig. 4B, D–F).



Figure 5. A–F SEM images of *Pseudostaurosira heteropolaris* sp. nov. from the Bolivian Altiplano **A**, **B** Valve views showing striation pattern and features of the axial area, virgae, vimines, and spine location **C** side view of complete frustule and neighboring cell showing girdle bands (white arrows denote open copulae). White arrows point to open girdle elements **D** close up of **C** showing details of spines with bifurcations with pinnatifid projections, characteristics of the striae on valve mantle and the features of the blisters **E** broken frustule with girdle bands. Pattern of volae within areolae is also shown **F** frustule in side, tilted view. Notice open copulae (white arrow) and reduced apical pore field with cavernous poroids (black arrow). Scale bars: 1 μ m (**D**); 3 μ m (**A–C, E**); 4 μ m (**F**).

Dimensions (n > 50): Length $2.9-12.3 \mu m$; width $2.1-2.3 \mu m$; striae 15 in 10 μm . **Etymology.** The species epithet makes reference to the difficulty in the LM distinction of this diatom from co-occurring species with similar outline.

Distribution. Found in the Desaguadero River.

Pseudostaurosira heteropolaris E. Morales, C.E. Wetzel & Ector, sp. nov. Figs 1H'-M' (LM), 5A-F (SEM)

Holotype. Slide ANSP GC 26815, Fig. 1I', Diatom Herbarium, Academy of Natural Sciences, Philadelphia (ANSP). **Isotype.** Slide DBOL-0246a, Diatomotheca Boliviensis (before HCUCB), Cochabamba, Bolivia.

Type locality. Bolivia. Sajama Province, Department of Oruro, Desaguadero River, epipsammon, 17°23'51"S; 68°14'33"W, 3701 m elev., *leg.* G. Chávez, 05.07.2009.

Description. Frustules rectangular in girdle view (Fig. 5C, D), joined together by interlocking spines (Fig. 5C). Valves ovoid to elliptic, heteropolar, with gradual transition from valve face to mantle (Figs 1H'-M', 5A-F). Axial area elliptic (Figs 1H'-M', 5A, B, F), externally slightly depressed with respect to virgae, internally at the same level as virgae (Fig. 5A, D, E). Virgae much wider than striae (Fig. 5A, D-F). Vimines shorter than virgae and wide (Fig. 5A, B, D-F). Striae composed of narrow, apically elongated, rectangular to semi-elliptic areolae (Fig. 5A-F). Areolae diminish in size from valve face/mantle junction towards striae extremes at about the same rate (Fig. 5F). Volae arising from up to two points (typically one) within the areolar inner periphery, projecting inwards (Fig. 5A, B, D-F). Base of volae thick and giving areolae a C-shape (Fig. 5A, B). Flaps absent. Spines originating from vimines at the valve face/mantle junction, solid, with elliptic to rectangular base, wider than the vimines they sit on; cylindrical body with biconcave sides, spatulate tips with pinnatifid (with deep lateral) bifurcations (Fig. 5C, D). Stipules absent (Fig. 5D). Apical pore fields very reduced with no more than 3 cavernous poroids in external view; not seen in internal view (Fig. 5F). Small blister-like depositions present on abvalvar edge of mantle, including at the valve apices (Fig. 5C-F). Girdle elements variable in number, open, lacking pores, ligulated, with larger valvocopula (Fig. 5C, E, F).

Dimensions (n > 50): Length 3.0–4.3 μ m; width 2.6–3.3 μ m; striae 13–16 in 10 μ m. **Etymology.** The epithet of this species refers to its typical heteropolar valve outline. **Distribution.** Found in the Desaguadero River.

Pseudostaurosira vulpina (Lange-Bertalot & U. Rumrich) E. Morales, stat. nov. Figs 6A–D (LM), 7A–F (SEM)

Basionym. *Staurosira laucensis* var. *vulpina* Lange-Bertalot & U. Rumrich *in* Rumrich et al. 2000, Diatoms of the Andes from Venezuela to Patagonia/Tierra Del Fuego, Iconographia Diatomologica 9, p. 223–224, Plate 10, Figs 1–11.

Comment. This taxon was first described from the Chilean Altiplano and was found mixed with the nominate variety *Pseudostaurosira laucensis* (Lange-Bertalot & Rumrich) E. Morales & Vis (in Rumrich et al. 2000, p. 222, figs 10–20, 22, 23; Morales and Vis 2007, p. 25). This was the probable reason why Lange-Bertalot and Rumrich (in Rumrich et al. 2000) decided to describe it as a variety. However, we found the



Figure 6. A–V LM images of little known and new "araphid" diatoms from the Bolivian Altiplano **A–D** *Pseudostaurosira vulpina* stat. nov. **E–I** *P. frankenae* sp. nov. (Fig. 6E is the holotype) **J–O** *P. occulta* sp. nov. (Fig. 6K corresponds to the holotype) **P–V** *P. oblonga* sp. nov. (Fig. 6R corresponds to the holotype). Scale bar: 10 µm.


Figure 7. A–**F** SEM images of *Pseudostaurosira vulpina* sp. nov. **A**, **B** valve views showing striation pattern, features of the axial area, spine position and presence of depressed apical pore fields (black arrows) **C**, **D** close-ups with details of apical pore fields areolae and spines **E** tilted view of a frustule showing open girdle bands with overlapping extremes (white arrows) **F** internal view showing apical pore fields and the internal disk-like areolar depositions. Scale bars: 2 μ m (**C**, **D**); 5 μ m (**A**, **B**, **E**, **F**).

var. *vulpina* isolated from the nominate variety in the Desaguadero River sample. This population, like the one reported from Chile, exhibits a range of sizes which is probably showing that it is undergoing asexual reproduction and its size is most probably being re-established through sexual reproduction.

At the LM level, this taxon is distinguished by its typical triradiate shape (Fig. 6A–D). Between each of the arms there is also a central inflation that becomes more pronounced as the valve decreases in size (Fig. 6C, D). At the SEM level, the axial area is depressed in external view with respect to the virgae, while internally it

is at the same level as the latter. Each of the arms has an apical pore field that lies within a shallow, irregular depression (Fig. 7A–C) and opens to the valve interior as a plain plate of pores (Fig. 7F). The transapically elongate areolae bear well-developed volae (Fig. 7A–E), which allow inorganic deposition of an inverted cone-like structure internally covering the areolae, sometimes filled with extra depositions in their hollow interior (Fig. 7F). The spines are conical, but also it is common to find them as incipient, shapeless spines that are generated from the virgae and the vimines (Fig. 7E). The yalvocopula is wider. At the open side, each element has its terminations super-imposing each other (Fig. 7E).

Dimensions (n > 10): Length (from the extreme of one arm to the other) 4.8–13.0 μ m; width (from one swollen central area to its opposite side) 4.1–5.6 μ m; stria density (measured from arm to arm) 14–16 in 10 μ m. The dimensions are given here for the first time since the original description in Rumrich et al. (2000) did not include them. Table 3 contains additional characteristics that are used below for comparative purposes in Discussion.

Pseudostaurosira frankenae E. Morales, C.E. Wetzel & Ector, sp. nov.

Figs 6E–I (LM), 8A–F (SEM)

Holotype. Slide ANSP GC 26815, Fig. 6E, Diatom Herbarium, Academy of Natural Sciences, Philadelphia (ANSP). **Isotype.** Slide DBOL-0246a, Diatomotheca Boliviensis (before HCUCB), Cochabamba, Bolivia.

Type locality. Bolivia. Sajama Province, Department of Oruro, Desaguadero River, epipsammon, 17°23'51"S; 68°14'33"W, 3701 m elev., *leg.* G. Chávez, 05.07.2009.

Description. Frustules rectangular with a curved middle portion in girdle view, joined together by interlocking spines. Valves cruciform, isopolar, with abrupt transition from valve face to mantle. Broadly rounded valve ends (Fig. 6E–I). Axial area lanceolate with a broad central inflation (Fig. 6E-I), externally and internally depressed with respect to virgae (Fig. 8A-C). Vimines short and wide (Fig. 8A, B, E, F). Striae typically composed round to elliptic areolae, decreasing in size towards the axial area (Fig. 8A, B); a single elliptical areola present on valve mantle (Fig. 8A–C, E, F). Welldeveloped volae, arising from the areolar inner periphery and projecting inwards (not shown here). Internally, depositions on volae forming round to elliptic structures, sealing areolae (Fig. 8C, D). Flaps persistent, a single disk-like one covering each areola in external view (Fig. 8A, B, E, F), 1-3 in enlarged mantle areolae (Fig. 8C). Spines originating from vimines at the valve face/mantle junction; solid, with round to elliptical base (Fig. 8E), wider that the vimines they sit on (Fig. 8F); flattened, with shallow biconcave sides, triangular in side view (Fig. 8A, B, E, F), and with spatulate body, bifurcate at the top (Fig 8C). Stipules absent. Apical pore fields of cavernous appearance in external view, occluded by heavy silica deposition to the point only one row of



Figure 8. A–**F** SEM images of *Pseudostaurosira frankenae* sp. nov. **A**, **B** titled frustules showing external features; notice open girdle elements in B (white arrows) **C**, **D** internal view of valves showing internal elliptic depositions on striae (dashed arrows) and depression of the apical pore field (black arrows) **E** close-up of frustule apex (**B**) showing the externally occluded apical pore field, showing only a single row of poroids (black arrow) **F** close-up of frustule tip (**A**) showing open girdle bands (white arrow). Scale bars: 2 μ m (**E**, **F**); 3 μ m (**C**); 5 μ m (**A**, **B**, **D**).

pores can be seen (Fig. 8A, B, E, F). Internally, apical pore field opening into roundish depression, revealing several rows of round poroids (Fig. 8C, D). Well-developed blister-like depositions present on abvalvar edge of mantle also covering both apices (Fig. 8A–C, D–F). Girdle elements variable in number, open, lacking pores, ligulated, with larger valvocopula (Fig. 8A, B, F).

Dimensions (n > 30): Length 8.7–12.0 μ m; width 6.7–7.7 μ m; striae 14 in 10 μ m.

Etymology. The species is dedicated to the late Dr. Margot Franken, Professor and Researcher from the Ecology Institute, University Mayor de San Andrés, La Paz, Bolivia. Dr. Franken, originally from Germany, worked in Bolivia from 1985 to 2021, focusing on bioindication, urban ecology, water management and ecological architecture.

Distribution. Found only in the Desaguadero River.

Pseudostaurosira occulta E. Morales, C.E. Wetzel & Ector, sp. nov. Figs 6J–O (LM), 9A–F (SEM)

Holotype. Slide BR-4679, Fig. 6K, Meise Botanic Garden, Belgium. Isotype. Slide DBOL-0249a, Diatomotheca Boliviensis (before HCUCB), Cochabamba, Bolivia.

Type locality. Bolivia. Sajama Province, Department of Oruro, Sajama River, epipsammon, 17°30'33"S; 68°20'35"W, 4000 m elev., *leg.* G. Chávez, 05.07.2009.

Description. Frustules rectangular in girdle view, joined together by interlocking spines. Valves lanceolate, isopolar with semi-gradual transition from valve face to mantle. Valve apices subrostrate with broadly rounded, somewhat squarish ends (Figs 6J-O, 9A-D). Axial area lanceolate (Fig. 6J-O), externally and internally faintly depressed with respect to virgae (Fig. 9A-D). Vimines short and wide (Fig. 9A, B). Striae composed of round to elliptic areolae, decreasing in size towards the axial area (Fig. 9A, B); wide elliptical areolae present toward valve face/mantle transition before and after the spine, sometimes accompanied by a narrower additional areola on valve mantle (Fig. 9E, F). Striae contained in a single depression in internal view (Fig. 9C, D). Well-developed volae, arising from the areolar inner periphery and projecting inwards (Fig. 9B–D). Flaps small and present on some valve face areolae (Fig. 9A, B), more commonly on larger mantle areolae (Fig. 9D-F). Spines originating from vimines at the valve face/ mantle junction; solid, with round to elliptical base (Fig. 9A, B), as wide as the vimines they sit on (Fig. 9A, B, E); with cylindrical body and shallow concave sides (Fig. 9E, F), and spatulate tip with lateral projections and a serrate border pattern (Fig. 9D–F). Stipules incipient and subtending a small circular depression on the spine upper body (Fig. 9D, F). Apical pore fields covered by external flaps (Fig. 9A, E, F). Internally, apical pore field opening into roundish depression, revealing several rows of round poroids (Fig. 9D). Well-developed blister-like depositions present on abvalvar edge of mantle also covering both apices (Fig. 9D-F). Girdle elements variable in number, open, lacking pores, ligulated, with larger valvocopula (Fig. 9E, F).

Dimensions (n > 30): Length 6.7–35.6 μ m; width 3.3–3.8 μ m; striae 14–16 in 10 μ m.

Etymology. The species epithet alludes to the fact that this diatom has remained unidentified thus far and has been confused with morphologically similar taxa (see Discussion).

Distribution. Found in the Sajama River.



Figure 9. A–**F** SEM images of *Pseudostaurosira occulta* sp. nov. **A**, **B** external views of valves showing apical pore fields (black arrow in A) and other features **C** internal view of valve showing depressions containing the striae (dashed arrow) **D** internal close-up showing apical pore field depression (black arrow) and striae depression (dashed line) **E** close-up on valve apex showing flaps on apical pore field (black arrow) and open girdle element (white arrow) **F** close-up on cell-cell connection showing apical pore field covered with flaps (black arrow) and open girdle element (white arrow). Scale bars: 2 μ m (**D**, **E**); 3 μ m (**F**); 5 μ m (**A–C**).

Pseudostaurosira oblonga E. Morales, C.E. Wetzel & Ector, sp. nov. Figs 6P–V (LM), 10A–F (SEM)

Holotype. Slide BR-4680, Fig. 6R, Meise Botanic Garden, Belgium. Isotype. Slide DBOL-0249a, Diatomotheca Boliviensis (before HCUCB), Cochabamba, Bolivia.



Figure 10. A–F SEM images of *Pseudostaurosira oblonga* sp. nov. **A**, **B** external views showing apical pore fields covered with small flaps (black arrow in **A**) and other characteristics **C**, **E** internal valve features stressing on apical pore fields and striae in a depression (black arrows in **C** and dashed arrow in **C** and **E** respectively) **D**, **F** titled views of valves showing girdle bands (white arrow in **F**) and apical pore field (black arrow in **F**). Scale bars: $4 \mu m$ (**B**, **C**, **E**, **F**); $5 \mu m$ (**A**, **D**).

Type locality. Bolivia. Sajama Province, Department of Oruro, Sajama River, epipsammon, 17°30'33"S; 68°20'35"W, 4000 m elev., *leg.* G. Chávez, 05.07.2009.

Description. Frustules rectangular in girdle view, joined together by interlocking spines. Valves oblong, isopolar, with abrupt transition from valve face to mantle and broadly rounded apices (Figs 6P–V, 10A–F). Axial area lanceolate (Figs 6P–V), externally and internally depressed with respect to virgae (Figs 10A–F). Vimines short and wide (Fig. 10A–F). Striae composed of round to elliptic areolae, decreasing in size towards the axial area (Fig. 10A, B); wide trapezoid areolae present near the valve face/ mantle transition at the base of the spine, sometimes accompanied by an additional narrower, round areola on valve mantle (Fig. 10A–F). Striae contained in a single depression in internal view (Fig. 10C, E). Developed volae, arising from the areolar inner periphery and projecting inwards (Fig. 10A, C, E). Flaps little-developed on valve face, developed on valve mantle, more commonly on larger mantle areolae (Fig. 10A, F). Spines originating from vimines at the valve face/mantle junction; solid, with elliptic base (Fig. 10F), as wide as the vimines they sit on (Fig. 10A, B, F); with a somewhat cylindrical body, concave sides, in the shape of a trapezium in side view (Fig. 10D, F), and widely spatulate tip with wide lateral projections (Fig. 10F). Stipules incipient or absent (Fig. 10A, D, F). Apical pore fields reduced, covered by small external flaps (Fig. 10A, B, F). Internally, apical pore field opening by means of a few very narrow, round poroids (Figs 10C). Small blister-like depositions present on abvalvar edge of mantle, absent from apices (Fig. 10A, D, F). Girdle elements variable in number, open, lacking pores, ligulated, with larger valvocopula (Fig. 10D, F).

Dimensions (n > 30): Length 6.9–13.5 μ m; width 3.8–4.8 μ m; striae 13–14 in 10 μ m. **Etymology.** The species epithet refers to the widely ellipsoidal valve outline typical of this taxon.

Distribution. Found in the Sajama River.

Discussion

Nanofrustulum cataractarum, as seen in samples from the Desaguadero and Sajama rivers, is very similar to the type and other populations reported from around the world (Wetzel et al. 2013a; Grana et al. 2015; Beauger et al. 2019; Genkal 2021). The smaller dimensions of Bolivian specimens only expanded the initial measurements given by Hustedt (1938).

The more noticeable blisters on the mantle for Bolivian specimens could be due to the state of preservation of the material (more recent collection from Bolivia) and the possible higher availability of silica in the environment.

The lack of apical pore fields, stipules and flaps are typical in this taxon, but the most noticeable characteristic at the time of its identification under LM is the round shape of its valves and areolation pattern of the valve face and mantle, which resemble the smallest members of *Aulacoseira* Thwaites.

Nanofrustulum rarissimum, as discussed by Morales et al. (2019b), belongs in *Nanofrustulum* due to the quasifract nature of its girdle elements. The valvocopula in this case, however, is entire and ligulate. This difference with *N. cataractarum* that has all girdle elements quasifract has not been assessed in detail (Morales et al. 2019b), especially regarding the consequences for classification at the genus and species levels. It is known that other species can form morphologically different girdle elements (see the case of *Nitzschia transtagensis* E. Morales, Novais, C.E. Wetzel, Morais & Ector in Morales et al. [2020, p. 34, figs 2–26], and other examples therein). A more detailed

study of the variation of this character within the species currently assigned to *Nanof-rustulum* is required.

Pseudostaurosira sajamaensis has large areolae proportional to its size (Morales et al. 2012b), which together with the gradual valve face/mantle transition, the sagittateprofiled spines with single or diapason-shaped tips, bearing serrate borders pointing downward are the main features to look for at the SEM level (Morales et al. 2012b, Fig. 2E, F, Table 1). Also characteristic at the latter level is the infrequent presence of a Vshaped cleft in the posterior side of the spine body. In the context of the taxa contrasted in Table 1, these are the features that are unique to this taxon.

Pseudostaurosira sajamaensis is found in the same Desaguadero River sample together with *P. aedes* sp. nov. and *P. pulchra* sp. nov. However, at the LM level, the elliptic valves with pointy ends, the proportionately larger areolae, and the gradual transition between valve face and mantle in *P. sajamaensis* readily differentiate this taxon from the other two. The SEM features mentioned above, and which are defining for this taxon, can also be used to distinguish it from *P. altiplanensis* at this level (Lange-Bertalot & Rumrich) E. Morales (Rumrich et al. 2000, p. 220, pl. 14, figs 1–8; García et al. 2017, p. 112) (Table 1).

Pseudostaurosira linearis (Pantocsek) E. Morales, Buczkó & Ector (in Morales et al. 2019b, p. 276, figs 3, 4) is another taxon with a gradual valve face/mantle transition, but it has very different features to those taxa included in Table 1. This taxon is fossil, and tends to produce longer valves; the axial area is at the same level as virgae in external and internal views, has spines with T-shaped tips and well-developed M or V-shaped stipules, and the apical pore fields are more- or less-developed with cavernous appearance externally and opening internally into a non-depressed area.

Pseudostaurosira sajamaensis has been recorded at the LM level from the Tunari Cordillera, in the Department of Cochabamba (E. Morales pers. obs.) located more than 200 km to the east of the Sajama and Desaguadero rivers. This cordillera is part of a long range that branches off the main Andes mountains, penetrating Bolivian territory and receiving the name of Eastern Cordillera (see "Study area" description in Morales 2020). These records, however, need to be confirmed with SEM. If confirmed, the range of this diatom would be extended to the eastern limits of the Bolivian Altiplano and their confluence with the Bolivian Dry Valleys.

Pseudostaurosira pulchra sp. nov. has the main distinguishing feature of *Pseudostaurosira*, the short and wide vimines (Morales et al. 2019b). Because the striae are mostly composed of two areolae, the vimines are mostly restricted to the valve face/mantle junction. The characteristics of the spines interrupting the striae, the areolae and associated structures (volae and flaps), the blister-like depositions and girdle elements are all in accordance with species currently ascribed to this genus.

Table 1 shows that the diagnostic features of *P. pulchra* sp. nov. are the narrow lanceolate valves with rostrate apices in larger specimens, becoming broadly rounded in smaller ones. Also the virgae wider than the striae, that are raised with respect to the axial area in internal and external views are typical in this species. Finally, the spines having a base that is wider than the vimines they sit on, and a body that is flattened

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 Comparison 	Features in bo
Table I	outline.

Feature/species	Reudostaurosira aedes sp. nov.	<i>P. altiplanensis</i> (Lange-Bertalot & Rumrich) E. Morales	<i>R pulchra</i> sp. nov.	<i>P. sajamaensis</i> E. Morales & Ector	Pseudostaurosiropsis connecticutensis E. Morales	<i>R geocollegarum</i> (Witkowski) E. Morales
Valve dimensions (µm)	L: 2.9–12.3 W: 2.1–2.6	L: 4.5–8.0 W: 2.8–3.6	L: 5.0–22.0 W: 2.4–3.0	L: 2–18 W: 2–4	L: 1.9–13.5 W: 1.5–4.7	L: 5–16 W: 2–4
Stria density (in 10 µm)	15	14-15	13–16	10-14	17–20	12-16
Valve outline; axial area; virgae	Narrowly elliptic with rounded ends with abrupt transition of valve face to mande; narrowly lanceolare; wide and slightly raised with respect to axial area, both elevated in internal view	Broadly elliptic with abrupt transition of valve face to mantle: linear to narrowly lanceolate, <i>wide and slightly</i> ratised together with axial area in both external and internal views	Narroudy lanceolate with rostrate to broadly rounded ends with abrupt transition of variable face to mande, narowly lancoolate, wide and raised with respect to axial area in external and internal view	Narrowly elliptic in smaller specimens to elliptic with pointy ends in larger valves, <i>utib gradual transition from ualue face</i> <i>to mantle</i> , widely lanccolate; wide and raised with respect to virgae in external view, both elevated in internal view	Round to narrowly elliptic, broadly elliptic, wide, at the same level of axial area in external view, slightly raised in internal view	Lanceolate with subrostrate ends, widely alarcoolate, wide, raised with respect to axial area in external view, at the same level as axial area in internal view
Arcolae; volae; striae	Narrow, elliptic to trapezoid; well- developed forming a tight mesh-like structure visible externally and internally; with 2, rarely 3 arcolae, ustally larget on valve mande	Wide, often transaptically elongated; well-developed forming a loose mesh-like structure visible externally and internally; with 2 or more areale of de the same size change away from the value face/mantle junction	Narrow, round to elliptic; well-developed forming a loose mesh-like structure visible externally and internally; typically composed by 2 areolae, usually larger on valve mande	Very uide, round to trapezzoid on valve face, trapezzoid to elongate on mantle, well-developech, forming a tight mesh-like structure as even in internal view, usually composed of 2 arcolate mande, additional smaller ones more mande, additional smaller ones more	Narrow, round, of about the same size on valve face and mantle; absent, rotae present; typically composed of 2 arcolae of similar size, additional arcolae more frequent on mande	Narrow, round, of about the same size on valve face and mantle; absent, rotae present; typically composed of 2 areolae of similar size, additional areolae more frequent on mantle
Spines; stipules; flaps	Solid, with elliptic to rectangular base, as wide as basal vimen, conical body, with a somewhat triangular profile, with serate and pointy tips. well-developed, like posterior profile; well-developed, like posterior profile; well-developed, aisk-like or bibloate on valve face, smaller, usually more than 2 on mande	Solid, with elliptic base, narrower than basal vimen, cylindrical body with straight sides, spatulate tips with small and thin lateral projections, very little developed developed	Solid, round to elliptic base, <i>wider than basal vimen,</i> <i>flatteneid obdy with concave</i> <i>stales, straighty cut or slightly</i> <i>bifurcute apiese</i> ; absent, well- developed, circular on valve face, smaller, usually more than face, and the straight of the stra	Solid, heavily silicified with elliptic base as wide as basal vimen, flattened body, sometimes <i>unith a V-shaped eleft in its</i> <i>posterior side, unith diapason-shaped tips</i> with serrate borders and <i>pointy,</i> <i>downuard, lateral projections,</i> well- developed, giving the <i>gpine a agittate</i> <i>lateral profile</i> , well-developed, bilobate on valve face, less developed on mantle	Hollow, with elliptic base as wide as basal vimen, flattened, bicomcare - spatulate body with bifurcate ends , absent, absent	Hollow, round to elliptic base as wide as vimen, <i>pyramidal us the</i> <i>body starts, becoming</i> <i>cylindrical toward the top</i> , with bifurcate ends; absent absent
Apical pore fields; mantle abvalvar blisters	Present and very reduced, usually no more than 3 round poroids, <i>opening</i> <i>in a single linear depression in</i> <i>internal view</i> , well-developed, covering the apical pore fields	Present or absent, composed of up to 3 rows of round poroids opening into a single, roundish internal depression, small , covering the apical pore fields	Absent, well-developed, covering the apices	Absent or reduced, composed of a single row of round poroids opening in a single roundah depression in internal view; developed, present at apices but not covering the apical pore fields	Present or reduced, with small round poroids opening into a single roundish depression in internal view; small, present at apices but not covering apical pore fields	Composed of up to three caternous porvids, opening as large over at the value interior, small, present at apices but not covering apical pore fields
References	This study	Rumrich et al. (2000), Seeligmann et al. (2018)	This study	Morales et al. (2012b), This study	Morales (2001), Beauger et al. 2019)	Morales (2001, 2002), Beauger et al. (2019), Radhakrishnan et al. (2020)

New and poorly known araphid diatoms from Bolivia

with concave margins and has a flat top, or small bifurcate projections are also diagnostic in this taxon.

The taxon with the most similar morphology to *P. pulchra* sp. nov. is *Pseudos-taurosiropsis geocollegarum* (Witkowski & Lange-Bertalot) E. Morales (Witkowski et al. 1995, p. 734, figs 16–22; Morales 2002, p. 104, pl. 1, figs 1–9) (Table 1). At the LM level both have lanceolate valves, but in *P. pulchra* sp. nov. they are narrower (2.4–3.0 vs. 2–4 μ m in *P. geocollegarum*), and the ends vary from rostrate to broadly rounded (subrostrate in *P. geocollegarum*). At the SEM level the most conspicuous difference is that *P. geocollegarum* does not possess volae and has rotae, a structure completely lacking in all *Pseudostaurosira*. *Pseudostaurosiropsis geocollegarum* also has spines, the body of which starts with a pyramidal shape, becoming cylindrical toward the top, bearing bifurcate ends, a defining feature for the species (Table 1). Additionally, the apical pore fields in *P. geocollegarum* have a cavernous appearance with few pores that open as large isolated pores at the valve interior; this is also a defining feature for the species.

Pseudostaurosira pulchra sp. nov. has not been observed in other samples from the Altiplano and seems to be restricted to the Desaguadero and Sajama regions.

Pseudostaurosira aedes sp. nov. has short and wide vimines, which place it in *Pseudostaurosira*. The vimines are mostly restricted to the valve face/mantle junction since the striae are commonly composed of only two areolae, one on the valve face and the other, usually larger, on the valve mantle. The other features such as spines located along the striae, the areolae and subareolar structures (volae and flaps), the blisters and girdle elements, are all in accordance with species currently placed in *Pseudostaurosira*.

Despite the apparent difficulty in the distinction of this diatom from other similar taxa (Table 1), it has distinctive features that set it apart from them. The narrowly elliptic shape with rounded ends, the arrowhead-like spines (configuration given by the well-developed stipules) with a conical body and serrate tips, and the apical pore fields composed of only a few round poroids and opening internally into a single linear depression are all diagnostic features of this species.

At the LM level, the most similar taxon to *P. aedes* sp. nov. is *Pseudostaurosiropsis* connecticutensis E. Morales (2001, p. 117, figs 7a-l) (Table 1). Especially for smaller specimens, both present elliptic valves but the stria density is much higher in *P. connecticutensis* (17–20 vs. 15). Also, the spines in the latter are flattened with a biconcave-spatulate body and bifurcate ends. As a member of *Pseudostaurosiropsis*, *P. connecticutensis* has rotae and lacks volae. Additionally, this taxon has virgae that are at the same level of the axial area in internal view, while they are slightly raised in internal view. Spines with a flattened, spatulate body, concave on the sides, and with bifurcate ends. These characteristics of the virgae with respect to the axial area, and the spines are defining features for *P. connecticutensis*.

Smaller representatives of *P. aedes* sp. nov. can also resemble *Pseudostaurosira altiplanensis* (Lange-Bertalot & Rumrich) E. Morales. At the LM level, however, *P. altiplanensis* is much wider (2.8–3.6 vs. 2.1–2.6 in *P. aedes* sp. nov.), the valves are broadly elliptic instead of narrowly elliptic with rounded ends and the striae are long, composed of transapically very elongate areolae. At the SEM level, the virgae are wide and slightly raised together with axial area in both external and internal views; the spines are narrower than vimines, with a cylindrical body with straight sides, spatulate tips with small and thin lateral projections, bearing little-developed stipules. The blisters on the abvalvar side of the mantle are comparatively smaller. All these SEM features are defining characters for *P. altiplanensis* (Table 1).

Pseudostaurosira aedes sp. nov. was only found in the Desaguadero River in the present study, but it was illustrated before by Rumrich et al. (2000, pl. 13, fig. 26, only figure that is not numbered in the plate) and identified as "*Staurosira brevistriata* Grunow" (*Pseudostaurosira brevistriata* (Grunow) D.M. Williams & Round, 1987, p. 276; for figures of type material refer to Morales et al. 2015, figs 107–143 since figures in Williams & Round and incorrectly labeled). The valve shape, the features of the are-olae, spines and blisters are all confluent with what is described here as *P. aedes* sp. nov.

Pseudostaurosira ushkaniensis Kulikovskyi & Lange-Bertalot (in Kulikovskyi et al. 2015, p. 28, pl. 16, figs 1–12, pl. 17, figs 1–8) resembles both *P. pulchra* sp. nov. and *P. aedes* sp. nov. under LM. However, the mantle areolae in *P. ushkanenis*, being large and occupying almost the entire shallow, curved mantle in a pervalvar direction, are clearly visible showing two rows of these structures per stria in valve view. At the SEM level, the spines are located on virgae, have a cylindrical body and a spatulate tip. The apical pore fields are well-developed with neatly arranged rows of poroids in external view and a depressed elliptic plate with clearly round poroids in internal view. All of these features differ from the two Bolivian taxa (see them in Table 1).

Pseudostaurosira heteropolaris sp. nov. has wide and short vimines, a character that places it in *Pseudostaurosira*. This species is distinguished by its short, ovoid to elliptic, heteropolar valves, the wide base of the volae which give the areolae a C-shape appearance, the pinnatifid profuse bifurcations of the spine tips and the small blisters on the abvalvar edge of the mantle (Table 2).

The evident heteropolar configuration of *P. heteropolaris* sp. nov. is shared with *P. clavatum* E. Morales (2002, p. 107, pl. 1, figs. 22–34, pl. 4, figs 1–6), but this is a very different species with larger valves (8–20 μ m), coarser striae (11–12), wide, elliptic to trapezoid areolae on valve face and mantle (one on each), profusely bifurcate volae, flat, hollow spines with serrated borders and two ligulae projected laterally, and well-developed apical pore fields on both valve apices.

Another species with evident heteropolar shape is *P. conus* Kulikovskyi & Lange-Bertalot (in Kulikovskyi et al. 2015, p. 26, pl. 15, figs 15–18). At the LM level the valves are clavate with rostrate, sometimes subcapitate head pole and a very fine foot pole. External views of this taxon under SEM are unknown, but from the single figure presented by Kulikovskyi et al. (2015) it can be seen that the spines are small and conical and that the apical pore fields are well-developed and composed of several rows of poroids, features absent in *P. heteropolaris* sp. nov.

The same authors presented *P. gomphonematoidea* Kulikovskyi & Lange-Bertalot (in Kulikovskyi et al. 2015, p. 26, pl. 19, figs 1–3), another clavate taxon with a broadly rounded head pole and a thinner foot pole. The areolae are round to elliptical and the vol-

Feature/ species	<i>P. alvareziae</i> Cejudo- Figueiras, E. Morales	P. americana E. Morales	<i>P. bardii</i> Beauger, C.E. Wetzel & Ector	P. heteropolaris sp. nov.	P. oblonga sp. nov.
•	& Ector				
Valve	L: 10–18 W: 3.6, 5.0	L: 6.0-38.0	L: 4.0-6.5	L: 3.0–4.3 W: 2.6, 3.3	L: 6.9–13.5
(um)	W. 5.0 5.0	W. 1.9 9.0	W. 5.0 5.5	W. 2.0 5.5	W. 5.0 1.0
Stria density	13-15	16-18	12-16	13-16	13-14
(in 10 µm)	-5 -5				-5
Valve outline;	Elliptic with faintly	Lanceolate with cuneate	Elliptic to round with	Ovoid (sometimes	Oblong with widely
axial area;	subrostrate to broadly	apices, isopolar; linear, <i>at</i>	broadly rounded ends,	elliptic in small	rounded apices,
virgae	rounded apices,	same level as virgae in	isopolar; lanceolate to	specimens), heteropolar;	isopolar; lanceolate,
	isopolar; narrowly	external and internal	elliptic, depressed with	elliptic, externally slightly	depressed in external
	lanceolate, faintly	<i>view</i> ; wider than striae	respect to virgae in external	depressed with respect to	and internal view
	depressed in external		view, at the same level as	virgae, internally at the	with respect to virgae;
	view with respect to		wider than striae	same level as the latter;	wider than striae
	as virgae in internal		wider than striac	inden wider than striac	
	view; much wider than				
	striae				
Areolae; volae;	Wide, round to	Wide, round to elliptic;	Wide, round to elliptic;	Narrow, elliptic to round	Narrow, round to
striae	elliptic; well-developed	well-developed and	developed and directed	or hemispherical at the	elliptic; well-developed
	and forming a tight	forming a tight mesh-like	toward valve interior;	axial area; well-developed	forming a tight
	mesh-like structure	structure visible externally	composed of up to 5	and directed toward	mesh-like structure
	seen in external	and internally; composed	areolae decreasing in size	valve interior, <i>base</i>	visible externally and
	and internal view;	of up to / areolae with	away from valve face/	of volae wide giving	internally; composed
	areolae decreasing in	from value face/mantle	areola on mantle transcuot, first	composed by up to 7	decreasing in size away
	size away from valve	junction on valve face.	and as large as first areola	areolae decreasing in	from valve face/mantle
	face/mantle junction,	valve mantle with larger	on valve face	size away from valve	transition, first areola
	larger areolae	areolae varying from		face/mantle transition,	on mantle trapezoid
	contiguous to spines	elliptic to trapezoid		first areola on mantle as	and larger
	on valve face and			large as first areola on	
	mantle			valve face	
Spines;	Solid, elliptic base, as	Solid, elliptic base, as	Solid, elliptic base, wider	Solid, elliptic to	Solid, elliptic base, as
stipules; flaps	wide as basal vimen,	wide as basal vimen,	than basal vimen, flattened	rectangular base, wider	wide as basal vimen,
	widely spatulate tips	Vshapad middla opening	cida view spatulate tips	cylindrical body with	with concave sides
	with broad lateral	and openly concave	with servate horders	concave sides spatulate	trapezium-shaped in
	projections; small,	sides, widely spatulate	developed; developed but	tips with pinnatifid	<i>side view</i> , spatulate
	conical; incipient on	tips with serrate borders;	more frequent on mantle	<i>bifurcations</i> ; absent;	tips with wide lateral
	valve face, developed	well-developed, almost	areolae	absent	projections; <i>incipient</i>
	on valve mantle	covering entire first			or absent; little
		areola on mantle; well-			developed on valve
		developed, more common			face, larger on valve
A	Wall davalage d	Wall developed of	View and in a defined a	Vana and and and and aller	Dadward annual ha
fields: mantle	externally with wide	cavernous appearance	completely externally	up to 3 cavernous	small external flaps
abyalyar	round poroids and	externally. <i>poroids lie</i>	covered by flaps, only a	poroids: <i>small present</i>	internally opening
blisters	covered by contorted	at bottom of troughs,	pair of poroids can be seen,	at apices	through few, narrow,
	<i>flaps</i> ; small, absent	internally round	of cavernous appearance,	^	unsunk poroids; small
	from apices	poroids open into a	internally only 3 narrow,		absent from apices
		shallow depression;	round poroids can be seen,		
		well-developed, present	which open into a shallow		
		at apices	depression; developed and		
Defense	Cainda Ein staars 1	Chindle Einertennen 1	Present at apices	TL:1	This are 1
references	(2011)	(2011) Morales et al	Deauger et al. (2019)	Inis study	Inis study
	(2011)	(2013b)			
		(

Table 2. Comparison of *Pseudostaurosira heteropolaris* sp. nov and *P. oblonga* sp. nov. with morphologically similar, congeneric species. Features in bold italic font are defining for each taxon.

ae are not as developed as in *P. heteropolaris* sp. nov. The spines are completely flat and the apical pore fields are well-developed, features not present in the new taxon from Bolivia.

As evident in Table 2, *P. heteropolaris* sp. nov. is different from morphologically similar species in the genus, from which it can be distinguished in a first instance under LM by the features cited above, especially by the small size and heteropolar valves. The rest of the

diagnostic features need to be revealed by SEM. Among the latter, the features of the areolae and spines completely separate this taxon from the others in the present manuscript.

Pseudostaurosira vulpina stat. nov. has a triradiate form, the first key feature to its identification under LM. This combined with the swellings mid-way between arms most surely give a positive identification. Confirmation at the SEM level is given by the apical pore fields, somewhat depressed into the three valve apices and opening to the valve interior by a single non-depressed porous plate. In Table 3, we also annotate that the externally depressed axial area with respect to the virgae, and internally at the same level as the latter is an exclusive feature of *P. vulpina* among triradiate forms. Within the latter, *P. vulpina* is also the only one possessing small conical spines.

Depositions in the internal surface of the striae have also been reported in *Pseu-dostaurosira decipiens* E. Morales, G. Chávez & Ector (in Morales et al. 2012b, p. 44, figs 2–10, 39–44). However in *P. decipiens* there are two superimposed disks on each areolar opening, while in *P. vulpina* there is only one, inverted cone-like structure, hollow at the center or with extra siliceous deposition in its interior (Fig. 7F). These cones seem to appear as material accumulates over the copiously branched volae. Since the areolae are elongated in *P. vulpina*, the base of the cones is distorted, assuming a somewhat triangular or ovoid configuration (Fig. 7F).

P. vulpina as presented in Rumrich et al. (2000) lacks the internal cone-like depositions, but this seems to be due to erosion on the valves since the volae seem to have also been lost to a great extent from the areolae. These cone-like depositions are also found in the recently described fossil *Pseudostaurosira crateri* Marquardt & C.E. Wetzel (in Marquardt et al. 2021, p. 107, figs 1–57). However, this latter taxon is very different

E	D'	Dutition	D 1
reature/species	P. iztaccimuatiti V.H. Salinas & D. Mora	<i>P. vulpina</i> stat. nov.	Pseudostaurostropsis triradiatum (E.
			Morales) Kulikovskiy, Glushchenko &
			B. Karthick
Valve	L: 6.2–10.8	L: 4.8–13.0	L: 7–10
dimensions	W: ND	W: 4.1–5.6	W: ND
(µm)			
Stria density (in	12-18	14–16	14-16
10 μm)			
Valve outline;	Triradiate; irregularly triangular, externally	Triradiate; irregularly triangular and	Triradiate; <i>triangular, with concave</i>
axial area;	and internally depressed with respect to	externally depressed with respect to the	sides, depressed with respect to the virgae
virgae	the virgae; wide	virgae, internally at the same level as the	in external and internal view; <i>much</i>
-	_	<i>latter</i> ; wide	wider than striae
Areolae; volae;	Roundish to transapically elongate; well-	Roundish to transapically elongated;	Circular; absent, with rotae instead;
striae	developed and <i>with ring-like depositions</i>	well-developed and <i>with inverted cone-</i>	composed of up to 4 areolae 2 on valve
	distorted in different ways at the valve	like accumulations of different shape at	face and 2 on the mantle
	<i>interior</i> ; composed of several areolae, a	the valve interior; composed of several	
	single one on the valve mantle	areolae, a single one on the valve mantle	
Spines; stipules;	Large, conical base, spatulate tip, slightly	Small, conical, slender than basal vimen,	Small, conical, wider that basal vimen;
flaps	slender than basal vimen; absent; absent	sometimes shapeless and occurring on	absent; absent
		virgae and vimines; absent; absent	
Apical pore	Depressed, not known from valve interior	Depressed, opening into a non-depressed	Not depressed, opening internally into
fields; mantle	view; large and extending to the apical	porous plate at the valve interior; large and	a circular depressed zone; ND
abvalvar blisters	portions but below the apical pore field	extending to the apical portions but below	
		the apical pore field	
References	Salinas et al. (2020)	Rumrich et al. (2000), this study	Morales (2005)

Table 3. Comparison of <i>P. vulpina</i> stat. nov. with most similar triradiate species in <i>Pseudostaurosira</i> and
Pseudostaurosiropsis that have LM and SEM information available. ND=not determined. Features in bold
italic font are defining for each taxon.

from the triradiate *P. vulpina* in that it has a lanceolate shape, very narrow striae with more areolae on the valve mantle than on the valve face, which results in a wide axial area, depressed externally and internally, and apical pore fields in internal view reminiscent of *P. parasitica* (W. Smith) E. Morales (2003a, p. 287, figs 27–43, 54–54, 60, 64), i.e. an elevated plate that contains several round poroids. Externally, these apical pore fields resemble those in *P. vulpina*, although they are much larger proportionately to the valve size in *P. crateri*.

Pseudostaurosira iztaccihuatlii V.H. Salinas & D. Mora (in Salinas et al. 2020, p. 196, figs 18–35) is most probably conspecific with *P. vulpina*. The only two differences between the population from Mexico and the Andean population are the larger spines interrupting the striae of the former and the shape of the internal striae depositions (Table 3, features that have not been traditionally used for separation of species within the genus). The structure of the areolae and volae structure is similar in both taxa and there is no external or internal velum, as misinterpreted by Salinas et al. (2020); if a velum were present, *P. iztaccihuatlii* would have to be placed in a different genus. Both the structure of the areolae and volae are extensively used to separate *Pseudostaurosira* from other genera and to separate its species, as we have done herein. The internal striae depositions are ring-like in the latter and cone-like in *P. vulpina*.

Also from Table 3, it is clear that *Pseudostaurosiropsis triradiatum* (E. Morales) Kulikovskiy, Glushchenko & B. Karthick (Morales 2005, p. 129, Figs 74–79, 127–132; in Radhakrishnan et al. 2020, p. 173) is different from *P. vulpina* due to its axial area in the shape of a triangle with concave sides, the much wider virgae than striae, the circular areolae with rotae, small conical spines wider than vimines, and the apical pore fields that are not depressed exteriorly, but they do internally.

A third taxon that could be compared with P. vulpina is Staurosira mercedes Lange-Bertalot & Rumrich (in Rumrich et al. 2000, p. 224, pl. 10, figs 12-14), a taxon that had been introduced under the name Fragilaria brevistriata var. trigona Lange-Bertalot nom. inval. without a diagnosis in Krammer & Lange-Bertalot (1991a, pl. 117, fig. 7B). No SEM images of *S. mercedes* have been published, but at the LM level the valves are triangular with concave sides (boomerang-like) and the ends are cuneate rather than rostrate as they are in P. vulpina. Lange-Bertalot also introduced the name Staurosira pseudoconstruens var. trigona (in Rumrich et al. 2000, pl. 15, figs 1, 2), but this name was not accompanied by a diagnosis either. The LM figure the authors presented (fig. 2) seems to be a teratological form and has a similar general appearance in the lower side of the triradiate shape as a medium-sized valve of *P. vulpina*. The SEM image presented in Rumrich et al. (2000, pl. 15, fig. 1) confirms this. The shape of the areolae, the position of spines and the sunken apical pore fields in the close-up image are all similar to P. vulpina. We also note that Metzeltin & Lange-Bertalot (1998, pl. 2, fig. 5) presented a valve of *P. vulpina* (judging by valve shape and features of striae, areolae, spines and apical pore fields) that they identified as "Fragilaria brevistriata (Grunow s. lato) var. trigona Lange-Bertalot" a name that appears only in this text and without a formal description.

The change in status of *P. vulpina* is here justified by the finding of a population with mixed frustule sizes, a probable indication that the species is reproducing asexu-

ally and sexually, independently from the nominate variety, *P. laucensis*, and growing isolated from it in the Desaguadero River.

This taxon has been reported and illustrated from the Argentinian (Tchilinguirian et al. 2018), Bolivian (Morales et al. 2007) and Chilean Altiplano (Rumrich et al. 2000), with possible records from Europe still to be confirmed by SEM. It is possible that it was also identified with other names. For example, Servant-Vildary (1978, p. 3, pl. 2, fig. 13, see also LM image in Servant-Vildary 1986, pl. 2, fig. 27) presented a drawing that closely resembles our Figs 6C and D, with triangular shape and inflated sides and mammillate ends, which the author named "*Fragilaria construens* (Ehr) Grun var. *exigua* (W. Smith) Schuls[sic]" (*=Staurosira construens* var. *exigua* (Ehrenberg) H. Kobayasi (in Mayama et al. 2002, p. 90, for illustrations refer to Krammer and Lange-Bertalot 1991a, pl. 117, figs 4–7 (LM) and Potapova 2014, p. 80, fig. 98 (SEM, under "*Staurosira construens* f. *exigua*")). From the latter references the var. *exigua* is distinguished by subcapitate ends, incipient spines developing on virgae, and the unbroken striation pattern, since the striae are formed by small, apically elongate areolae. Additionally, the apical pore fields are not sunken and contain several rows of neatly arranged poroids.

Pseudostaurosira frankenae sp nov. is an additional cruciform species included in the genus (Table 4). It has the main feature of species currently assigned to it, namely the wide and short vimines, but it also shares with them the features of the areolae, spines and flaps, apical pore fields and girdle elements.

This new species has several distinguishing features that set it apart from other congeneric taxa with cruciform valve outline. The virgae are slender than striae, internally the striae bear a single elliptic, occluding disk (a character unique in the entire genus), Spines have a triangular basal configuration when viewed from the side; the areolae bear persistent flaps (Table 4). Additionally, the apical pore fields are covered by a siliceous deposition that only reveals a single row of poroids. We have not studied variation of this latter feature, it seems to be constant in the species but it requires confirmation.

Due to their cruciform shape, *P. australopatagonica* M.L. García, L.A. Villacís, Maidana & E. Morales (in García et al. 2021, p. 3, figs 2–35), *P. caballeroae* V.H. Salinas, D. Mora, R. Jahn & N. Abarca (2020, p. 199, figs 36–50) and *P. pseudoconstruens* (Marciniak) D.M. Williams & Round (1987, p. 277, figs 28–31 mistakenly under "*Pseudostaurosira brevistriata*") are the closest morphological relatives to *P. frankenae* sp. nov. However, from Table 4, it can be seen that *P australopatagonica* has the volae forming an internal dendritic pattern, which is a character unique for the species. Additionally, this latter species has incipient spines forming arched, convex structures on the vimines, interrupting the striae at the valve face/mantle junction. The species from Argentina lacks stipules as *P. frankenae* sp. nov. does, but the latter has flaps, which are absent in *P. australopatagonica*.

In the case of *P. caballeroae*, the spines are thin and flat, forming an undulate dentate pattern over vimines (where most of the spine base and lower body lie) and virgae. While this species and *P. frankenae* sp. nov. lack stipules, the latter has flaps, which are lacking in *P. caballeroae*. Table 4. Comparison of Pseudostaurosina frankenae sp. nov. with selected, similar, congeneric, and cruciform to broadly lanceolate taxa. Features in bold italic font are defining for each taxon. *Internal view of P. caballeroae is unknown.

Feature/ species	<i>P. australopatagonica</i> M.L. García, L.A. Villacís, Maidana & E. Morales	<i>P. caballeroae</i> V.H. Salinas, D. Mora, R. Jahn & N. Abarca*	<i>P. decipiens</i> E. Morales, G. Chávez & Ector	P.frankenae sp. nov.	<i>P. laucensis</i> Lange-Bertalot & Rumrich	<i>P. parasitica</i> (W. Smith) E. Morales	<i>P. pseudoconstruens</i> (Marciniak) D.M. Williams & Round
Valve dimensions	L: 20–25 W: 6.5–9.0	L: 13.8–17.7 W: 6.9–8.5	L: 4–29 W: 4–6	L: 8.7–12.0 W: 6.7–7.7	L: 5.5–20.0 W: 3.5–5.5	L: 9–18 W: 4.5–5.0	L: 4–22 W: 3–7
Stria density (in 10 µm)	10-12	13–14	13–15	14	14-15	19–21	15–18
Valve outline; axial area; virgae	Cruciform to rhomboid with subcapitate apices; lanceolate, wider at central area, dearly depressed with respect to virgae in outer view, slightly depressed in internal view; much wider than striae	Cruciform with narrowly rounded ends; lanceolate, wider at central area; dearly depressed with respect to striae in external view; wide	Lanceolate with rostrate ends; lanceolate wider at central area, externally slightly depressed with respect to virgae, internally at the same level as the latter; wide	Cruciform with broadly rounded ends: lanceolate, wider at central area; clearly depressed with respect to striae in external and internal view. <i>slender</i> <i>than striae</i>	Lanceolate to rhomboid with narrowly subrostrate to cuncate ends; lanceolate, faintly depressed with respect to virgae in outer view, flat in internal view; wide	Lanccolate with narrowly rounded to subcapitate ends; lanccolate, wider at central area, deatly depressed with respect depressed with respect to virgae in external and internal view, <i>as wide</i>	Cucciform with broadly rounded to subcaptitate ends; lanceolate wider at central area, slightly depresed with respect to virgae in external and internal view; much wider than striae
Areolae; volae; striae	Round at apices to elliptically elongate; large generally growing opposite from shorter axis of arcolae, <i>internally</i> <i>forming a dendritic pattern</i> : composed of 1–2 arcolae on valve face, 1 large, round to ovoid on valve mande	Round at apices to elliptically clongate, sometimes only 1 very long on valve face; smallet, phitrustate and growing from longer areolar axis; composed of 1–2 areolae on valve face, valve mande areola not clearly illustrated	Round to ovoid; diapason shaped and further bifurcate at the valve interior, allowing the internal deposition of two concentric disks of inorganic anterth, composed of 1–2 areolae on valve face and 1 large, trapezoid on mantle, sometimes an extra round one present on mantle	Round to elliptic; smaller, bifurcate, allowing the internal <i>deposition of an elliptic disk of</i> <i>inorganic material</i> , composed of 1–4 areolae on valve face and a single, large, trapezoid one on mande on mande	Round to elliptic; developed with ring-like depositions distorted in different unys at the value interior; composed of 1–2 arealae on valve face, decreasing in size toward axial area and a single, slightly larger, elliptic to trapzoid on the mande	Round at apices to elliptically elongates small, usually originating from smaller axis of valve; composed typically of 1, unusually 2, areolae on valve face, sppically one smaller areola on undue mantle	Round to elliptic; smaller, bifurcate, originating from inner arcolar perimeter; composed of $1-4$ arcolae on valve face, grame size as undue face,sme size as undue face
Spines; stipules; flaps	Incipient, forming a short arch-like structure extending from virgae to virgae; absent ; absent	Very thin, flattened, extending from virgae to virgae, forming an undulate to dentate pattern on value face/ manile transition; absent absent	Solid, elliptic base, as wide as basal vimen, flattened body with concave sides, sputtare or heart- shaped top; absent; absent, orly mineral depositions resembling floating disks on outer arrolar pening	Solid, round to elliptic base, wider than basal vimen, <i>triangular in side view</i> , flattened upper body with bifurcate tip; absen; <i>disk-like</i> , <i>1 persistent in oulde face</i> <i>areolae</i> ; <i>1–3 in mantle areolae</i>	Incipient and occurring as whitish depositions along vulve facemantle transition, absent, absent	Absent; absent; absent	Solid, with long elliptic base, shorter than basal virgue on which they grow, flattened body with concave sides, highly branched tips; absent; absent
Apical pore fields; mantle abvalvar blisters	Cavernous and large, almost covering entire valve apex with poroids at the base of troughs internally opening into a single elliptic depression; small, absent from the apices	Cavernous and large, almost covering entire valve apex with pomoids at the base of troughs, internaty unknown; excess derpositions impede visualization in original illustrations	Cavernous, from 1 to several rows of poroids can be seen externally, internally a roundish depression contains several rows or poroids; developed, present including at valve apices	Cavernous, only one transapical row of poroids can be seen externally, internally, around depression contains various rows of poroids; developed, present including at valve apices	Non-carnermous, sunken onto valve apex in external view, internal view unknown; small, not covering apices	Cavernous, visibly sunken onto valve apex and occupying almost its entirety, porce lie at bottom of troughs, <i>internally plaque of</i> <i>internally plaque of</i> <i>pores is raised</i> ; very small and absent from apices	<i>Very reduced externally</i> , internally opening into a small depressed circular area; small and present at apices
References	García et al. (2021)	Salinas et al. (2020)	Morales et al. (2012b)	This study	Servant-Vildary (1986), Rumrich et al. (2000)	Morales (2003a, 2010)	Williams and Round (1987)

Pseudostaurosira pseudoconstruens has the closest overall valve shape to *P. frankenae* sp. nov. However, *P. pseudoconstruens* has the spines on the virgae and not on vimines as the rest of the species discussed in the present work (Williams and Round 1987). The base of those spines is shorter and they possess highly branched tips. Both stipules and flaps are lacking in this species. Additionally, the apical pore fields are highly reduced and are not cavernous as in *P. frankenae* sp. nov.

Pseudostaurosira decipiens E. Morales, G. Chávez & Ector (in Morales et al. 2012b, p. 44, figs 2–11, 39–44), *P. laucensis* and *P. parasitica* (W. Smith) E. Morales (2003a, p. 287, figs 27–43, 54–58, 60, 64) have a lanceolate valve shape, this being the first distinguishing feature to separate them from *P. frankenae* sp. nov.

From Table 4, it is worth noticing that *P. laucensis*, a taxon that can be distinguished from morphological related species by its incipient spines occurring on virgae and vimines along the entire valve face/mantle transition, its internal ring-like depositions on the striae and its non-cavernous apical pore fields, had already been shown by Servant-Vildary (1986, pl. 3, figs 31b–36), identified as *"Fragilaria brevistriata* Grunow". Both references show populations with the same valve features, although the latter reference shows a valve interior with the typical ring-like depositions on the striae. These depositions are unique to *P. laucensis* among taxa with cruciate to lanceolate valves. The ring-like configuration, however, is shared with the triradiate *P. iztaccihuatlii* (see Table 3 and discussion above). These two taxa also share the apical pore field configuration and the difference in areolar width between valve face and valve mantle areolae.

Regarding the incipient spines in *P. laucensis*, these also occur in *P. vulpina* as shown in Fig. 7E, but also in Rumrich et al. (2000, pl. 10, figs 8, 10, 11). Both taxa also share the apical pore field configuration.

Pseudostaurosira occulta sp. nov. is distinguished from similar species under LM by its lanceolate shape with subrostrate, somewhat square and broadly rounded apices (Table 5). In this latter table it can be seen that there are several distinguishing features typical of this new species at the SEM level. From these, the circular depression on the spine body, subtended by an incipient stipule, stands out. Also, the flap coverings, twisted, almost externally completely obstructing the apical pore fields are characteristic in this taxon. The remaining species in Table 5 have their own features that separate them from their morphologically close relatives. Valve dimensions are not very useful to differentiate these species and, apart from valve shape, SEM features should be used to distinguish them.

Pseudostaurosira subsalina (Hustedt) E. Morales (in Cejudo-Figueiras et al. 2011, p. 69, figs 2–33, 94–99, 107, 109, 111) is perhaps the closest species to *P. occulta* sp. nov. at the morphological level (Table 5). However, the former taxon has valves with parallel sides, poorly-developed volae and a single valve mantle areola, larger than the rest of areolae along the same stria. In the case of spines, these have an overall flared shape, somewhat resembling an ice cream cone, spatulate body and tip, the latter bearing two small lateral projections. The apical pore fields in *P. subsalina* sit on a step-like apex.

Likewise, the remaining species in Table 5 can be readily separated from *P. occulta* sp. nov. For each taxon we highlight the salient distinguishing features. *Pseudostaurosira polonica* (Witak & Lange-Bertalot) E. Morales & M.B. Edlund (2003, p. 235, figs 25–32, 45–50) has broadly elliptic valves, sometimes clavate, the areolae are very wide, and the spines are hollow. *Pseudostaurosira oliveraiana* Grana, E. Morales, Maidana & Ector (in Grana et al. 2018, p. 63, figs 2–15, 16–24) has valves with subcapitate to cuneate ends, trumpet shaped spines, and externally sunken apical pore fields that open interiorly into an elliptical depression but with a raised central area. In turn, *P. zolitschkae* M.L. García, S. Bustos, Maidana & E. Morales (in García et al. 2021, p. 11, figs 81–91, 103–109) has the widest range of morphological variability of all taxa included in Table 5 producing smaller valves with clearly biconvex sides and pointy ends. The areolae acquire an 8-shape configuration due to the thick origin of the volae that arise from the longer (transapical) axes of the areolae. The spines in this taxon are T-shaped and the apical pore fields are cavernous and small.

Pseudostaurosira linearis has a similar overall shape to *P. occulta* sp. nov. However, as stated above when comparing it to *P. sajamaensis*, *P. linearis* is a fossil species, and it has much wider areolae, has more coarsely striated valves (12–14 striae in 10 m versus 14–16 in *P. occulta* sp. nov.) and has all the other features cited in the comparison to *P. sajamaensis* that *P. occulta* sp. nov. does not possess.

Pseudostaurosira occulta sp. nov. has been reported before under the name "*Fragillaria zeilleri* Heribaud[sic]" by Servant-Vildary and Roux (1990, p. 276, fig. 29). The shape of the valve imaged under SEM, the characteristics of the areolae and spines resemble what we are describing here as the new species *P. occulta*. Type material of *Fragilaria zeilleri*, now *Pseudostaurosira zeilleri* (Héribaud) D.M Williams & Round (1987, p. 276, no figures), was studied by Serieysol (1988), who showed a taxon with widely elliptic valves and cuneate ends, axial area varying from linear to elliptic, striae composed of narrower areolae, usually one larger after the spine, on the valve mantle. The spines in this taxon have an overall flat, trumpet-like shape without lateral projections or serrate borders. The apical pore fields in *P. zeilleri* are clearly visible, are somewhat cavernous but lack any external coverings.

Pseudostaurosira oblonga sp. nov. can be distinguished by clearly oblong valve shape, the externally and internally depressed axial area, the trapezium-shaped profile of the spines and the incipient stipules that appear to be facultative (Table 2). This species is clearly different from *P. heteropolaris* sp. nov. simply based on the small size of the latter and its heteropolar valve shape, although we present other distinguishing features in Table 2.

The remaining species differ from *P. oblonga* sp. nov in the following salient features, selected from Table 2. *Pseudostaurosira alvareziae* Cejudo-Figueiras, E. Morales & Ector (in Cejudo-Figueiras et al. (2011), p. 69, figs 34–73, 100–105, 106, 108, 110) characteristically has two larger areolae before and after the spines along the same stria, the stipules are small and conical and the apical pore fields are typically covered by twisted flaps. *Pseudostaurosira americana* E. Morales (in Cejudo-Figueiras et al. 2011, p. 70, figs 74–93, 112–115. See also Morales et al. 2013b) has a V-shaped middle opening in the spine body, large stipules covering the subtending areolae on

Feature/species	P. polonica (Witak	P. occulta sp. nov.	P. oliveraiana Grana,	P. subsalina (Hustedt)	P. zolitschkae M.L.
	& Lange-Bertalot)		E. Morales, Maidana	E. Morales	García, S. Bustos,
	E. Morales & M.D.		& Ector		Iviaidana & E. Morales
Value	Linund L. 8, 23	I.67 356	I · 19 0 39 5	L 10 36	1.85.280
dimensions	W· 3_4	W-33-38	W: 3 5-5 5	W: 4 0-5 3	W: 3 5-5 0
(μm)		11.515 516		110 915	
Stria density (in	13-15	14-16	11-17	13-14	11–14
10 µm)					
Valve outline;	Broadly elliptic,	Lanceolate with	Lanceolate with	Lanceolate with parallel	Lanceolate with
axial area; virgae	rarely clavate, broadly	subrostrate , <i>squarish</i> ,	subcapitate to	sides and subrostrate	subrostrate apices,
	rounded apices, larger	broadly rounded apices;	<i>cuneate ends</i> ; broadly	apices; linear to narrowly	smaller valves biconvex
	specimens slightly	lanceolate, faintly	lanceolate, at same	lanceolate, faintly	with cuneate, pointy
	constricted in the	depressed with respect	level as virgae in both	depressed with respect	ends; broadly lanceolate,
	middle; lanceolate, at	to virgae in external and	external and internal	to virgae in external and	at the same level as
	same level as virgae	internal view; wider than	views; wider than striae	internal view; wider	virgae in external view,
	in both external and	striae		than striae	slightly depressed in
	internal views; wider				internal view; much
4 1 1		NT 1 10 1	NT 11: -	NT 1	wider than striae
Areolae; volae;	Wide, elliptic; well-	Narrow, round to elliptic;	Narrow, elliptic to	Narrow, round	Narrow, elliptic to
striae	from longer areolar	from the inner areolar	originating from	developed originating	value mentles developed
	axis and forming a	periphery forming a tight	longer axis of areolae	from inner areolar	and forming a mesh-like
	tight mesh in internal	mesh-like structure visible	with up to 3 areolae	periphery and projecting	pattern in inner view:
	view: rarely formed by	internally; with up to 4	larger on valve face	inwards: with up to 5	with 2 areolae of about
	more than 3 areolae,	areolae on valve face and	8	areolae, decreasing in size	the same size on face
	larger on valve	up to 2 on valve mantle,		towards the axial area,	and mantle
		usually larger near valve		a single larger areolae	
		face/mantle transition		present on valve mantle	
Spines; stipules;	Hollow, with elliptic	Solid, with elliptic to	Solid, round to elliptic	Solid, round to elliptic	Hollow, with round to
flaps	base, narrower than	rectangular base, as wide	base, as wide as basal	base, as wide as basal	elliptic base, narrower
	basal vimen, flattened	as basal vimen, <i>cylindrical</i>	vimen, flattened	vimen, flattened body	<i>than basal vimen</i> , with
	body with very	body with open concave	body and <i>tips with</i>	with somewhat straight	flattened body and
	openly concave sides,	<i>sides</i> , with spatulate	an overall inverted	but flaring sides and	typically T-shaped tips;
	spatulate tips with	tips that bear two lateral	trumpet shape, tips	spatulate tip bearing	well-developed and
	somewhat straight	projections and <i>serrate</i>	bifurcate once or	two small lateral	lobed; developed on
	bifurcate projections;	borders; incipient,	<i>twice</i> ; absent; absent	projections with serrate	valve mantle, lobed
	davalanadi naarliy	depression on spine hoder		borders, with an	
	developed on valve	poorly developed on valve		shape: incipient: poorly	
	face and mantle	face, well-developed on		developed	
		valve mantle, spatulate			
Apical pore	Somewhat developed,	Developed, externally	Somewhat developed,	Somewhat developed,	Small, externally
fields; mantle	with a few, <i>large</i> ,	covered by contorted	externally sunken	sitting on a step-like	reduced to large
abvalvar blisters	round pores, sunken	flaps in external view,	into an elliptic	transition between	pores with cavernous
	into an irregular	internally opening	depression at the	valve face and mantle,	appearance, internally,
	depression at the	in a single ca. elliptic	<i>valve apex</i> , internally	externally large roundish	small round pores lie in
	<i>valve apex</i> , internally	depression, revealing	opening into a ca.	pores lie in an irregular	a roundish depression;
	opening into a small	several rows of round	elliptic depression	depression, internally,	developed, absent from
	roundish depression;	poroids; well-developed,	with an elevated	the narrow, round	apices
	well-developed,	also present at valve apices	<i>central area</i> , and	pores open into a single	
	apparently not present		showing several round	roundish depression;	
	at apices		poroids; developed but	developed but present	
D.f	Manalas &r Edla 1	Th:	Creans at al. (2019)	Cainda Eigeneigen 1	Carría et al. (2021)
References	(2003)	This study	Grana et al. (2018)	(2011)	Garcia et al. (2021)

Table 5. Comparison of *Pseudostaurosira occulta* sp. nov. with morphologically similar species within the genus. Features in bold italic font are defining for each taxon.

the valve mantle, and an apical pore field in which the external openings of the poroids lie in troughs. *Pseudostaurosira bardii* Beauger, C.E. Wetzel & Ector (in Beauger et al. 2019, p. 4, figs 2–56), on its part, has spines with a triangular profile, spines that have tips with serrate borders.

As it has been seen here, defining *Pseudostaurosira* as a genus distinguished by short (apical extension) and wide (transapical extension) vimines (relative to the size of the areolae) (Morales et al. 2019b), allows the analysis of the variability of other features for the discrimination of species. That is, there is a large chance that the features of the vimines result in an evolutionary character that defines and separates the genus from others; however, this possibility for vimines to be synapomorphic requires demonstration, and we will perform the necessary cladistic analyses once a fair amount of species have been described and the type material of remaining key species has been studied (e.g. *P. pseudoconstruens, P. microstriata* (Marciniack) Flower [2005, p. 65], etc.).

As expressed in the descriptions of the new species and the comparative tables presented herein, the salient features that can be used to distinguish species are the features of the axial area, virga and vimines, the areolae and subareolar features (volae, rotae, flaps and internal depositions), the spines (base, body and tips) and stipules, and apical pore fields. We have tried to find differences in other features such as valve shape, morphometric measurements, stria density, blisters and girdle bands, but we have been unsuccessful in finding sufficient variability across a large number of species. As more species are described and type material is re-analyzed, it is possible that the latter characters take more importance in defining species.

A note on our morphologically based approach to "araphid" diatom taxonomy

Biodiversity conservation is a crucial endeavor in the face of climate change, pollution and habitat loss (Prathapan and Rajan 2020). It is already recognized that to carry out this conservation process "a good and constantly updated taxonomical knowledge is fundamental" (Khuroo et al. 2007). The problem in South America, as in many parts of the world, is that the taxonomic impediment (Wilson 1985; Wheeler et al. 2004) is even more vexing since very few universities and research groups in the continent are actively trying to solve it (perhaps not true, at least in some countries, for insects, fish and higher plants, as argued by de Carvalho et al. 2014a). And this lack of attention currently happens in a region grouping several countries declared as biodiversity hotspots and high-biodiversity wilderness areas (IUCN - International Union for Conservation of Nature 2013), but also as the least caring about nature and the environment. For example, Brazil, Bolivia, Peru and Colombia (in that order) are among the top 10 countries with the highest loss of primary forest in the world (Weisse and Goldman 2021).

In Bolivia, habitat loss is a deeply preoccupying problem since there is a lack of strong environmental policies and even the Government itself constantly breaks the existing law in order to expand the agricultural frontier, exploit oil, minerals, timber, etc. (Castro et al. 2014). The situation of the aquatic systems in Bolivia, those associated with urban development or even those in protected areas that are now being damned, is also worrying (U.S. Army Corps of Engineers 2004). The recent loss of Lake Poopó to the mining industry and contamination is one of the largest recent environmental catastrophes in South America and an example of the degree of degradation of aquatic resources in the country (Richard and Contreras 2015). It is evident, therefore, that

there is a tremendous need for discovering, describing, identifying and cataloguing the diversity present in the affected areas in order to provide a historic record of what is (was) present in these sites for conservation and restoration purposes. In particular, the Sajama and Desaguadero regions are currently being affected by mining and urbanization of some of their areas, though the effects of both have not yet been officially reported.

Although there is a growing body of literature, the main diatom treatises for the region have been conducted by foreign authors (e.g. Frenguelli 1939; Servant-Vildary 1986; Rumrich et al. 2000) and not always reflecting topographic, bioclimatic and ecosystem variability, resulting in an incomplete account that often manifests in skewed conclusions regarding the richness and composition of diatom communities in high and lowlands (see discussions in Morales et al. 2012b, 2014c, 2020). For the existing literature, besides the shortcomings in sampling and geographic coverage, at least for small "araphids", there is a history of taxonomic drift, misidentification and a severe lack of pictorial support for floristic surveys in different regions of the continent (Morales et al. 2014c; García et al. 2021). Misidentification and poor illustration are problems that we have also shown here in the case of *P. vulpina* appearing in the literature under *Fragilaria construens* var. *exigua* (Servant-Vildary 1978, 1986). Other examples are those of *P. occulta* sp. nov. lumped under the name *P. zeilleri* (Servant-Vildary and Roux 1990), and *P. laucensis* being mistaken for *P. brevistriata* (Servant-Vildary 1986).

Whether this diatom biodiversity account should be done using molecular or morphological approaches is (for now and given the urgency to document as much of that diversity as possible in a short time) a matter of availability of funds and equipment, which are scant in the country. Currently, the cheapest route to diatom biodiversity reporting is to concatenate LM and SEM approaches via international collaboration.

But besides the reality of research conditions in the country, there is also the more general matter of whether morphological or molecular taxonomy should be used in the urgent endeavor to solve the biodiversity crisis (Wilson 1985). What we have done here is to produce hypotheses on distinctiveness based on morphological characters, by comparison among morphologically closely related species, breaking down features that are currently underexplored in "araphid" diatoms. This breakdown produces a substantial amount of information, as seen in Tables 1–5, that could later be used to support barcoding and/or DNA data, which in turn can be used to test the hypotheses we raised here.

The ongoing debate on whether molecular or morphological data should prevail over another has revealed important pros and cons of both approaches (Savolainen et al. 2005; Evans et al. 2007; Pires and Marinoni 2010; Zimmermann et al. 2011). But as implied by Lipscomb et al. (2003), Teletchea (2010), and Kahlert et al. (2019), it is much more productive to think of a fusion of both approaches than to think that either of them, in isolation, could produce a reliable identification system or even fairly approximate the actual number of species present in nature. For diatoms, a first attempt to concatenate morphological and molecular datasets has been tried already as in the case of the marine epizoic *Tursiocola* spp. (Frankovich et al. 2018), and in the case of freshwater "araphids" in the genus *Fragilaria* (Kahlert et al. 2019), although a

uniform protocol for the treatment of both datasets and consensus trees fusing molecular and morphological data have not yet been achieved. But the latter is not surprising since nowadays very little has been done in terms of translating molecular data into functional perspectives of the diatom phenotype (i.e. we know very little about what genes produce which characters, a process that could be beneficial for the establishment of homologous traits and recognition of diagnostic features (see e.g. Cox 2010)), although considerable progress, albeit still weak regarding the molecular connection, is expressed by Hale and Mitchell (2001) and Aitken et al. (2016). Even now, it is interesting to note that current molecular analyses, outstandingly exemplified by the construction of barcode databases, is undoubtedly a type of morphological analysis, i.e. the analysis of the morphology of the DNA molecule.

These and other shortcomings highlighted by Morales et al. (2019b) have determined that an integrative taxonomy (Pires and Marinoni 2010) in diatom research is still not in clear sight and that much more work is still required to produce reliable and practical accounts of the biodiversity of these organisms. Kahlert et al. (2019) also point out historic shortcomings of morphological data, referring to the lack of uniformity in morphological descriptions of taxa, the fact that old descriptions are based on LM, and that it is not always possible to observe all diagnostic features during routine analyses. We have been trying to solve these issues pointed by Kahlert et al. (2019) for the past decade-and-a half. Through the revision of type material, we have attempted the documentation of traditional and new diagnostic features, expanding original descriptions and confirming or re-ascribing taxa into newly erected genera (such as those in Williams and Round 1987). We have also discovered new taxa, described and documented them following the current standards (Morales 2001, 2002, 2003a, b, 2005, 2006; Morales and Edlund 2003; Edlund et al. 2006; Morales and Manoylov 2006a, b; Morales et al. 2005, 2009, 2010a, b, c, 2012a, b, 2013a, 2014a, b, 2015, 2019a, b, 2021; Siver et al. 2006; Cejudo-Figueiras et al. 2011; Wetzel et al. 2013a, b; Rioual et al. 2014; Talgatti et al. 2014; Van de Vijver et al. 2014, 2020a, b; Almeida et al. 2015, 2016, 2017; Grana et al. 2015, 2018; Wetzel and Ector 2015, 2021; Wengrat et al. 2016; García et al. 2017, 2021; Beauger et al. 2019; Seeligmann et al. 2018; Guerrero et al. 2019; Marquardt et al. 2021). The amalgamation of LM and SEM has been crucial in our work, even though type materials were not always in a good state of preservation.

The growing amount of morphological and re-analyzed historical data, and the relative easiness and low cost of the methods employed in their collection, continue to be a convenient way to contribute data for the study of biodiversity (e.g. elaboration of species inventories, numbers and distribution, morphological variation), ecology (e.g. autecology, assemblages and their relations to their environment, biogeography) and applied fields such as biostratigraphy, paleoecology, bioindication, bioprospection, climate change research and preservation/conservation/recuperation practices). Therefore, the resolution of historic taxonomic entanglements, description of new species and clarification of taxonomic boundaries based on morphological analyses continue to be valid and they are a very much needed practice.

Regarding the standardization of terminology and the format of the descriptions, we have been putting forward expanded diagnoses of taxa (e.g. descriptions provided herein) which, although they tend to be repetitive in the case of shared features among taxa, constitute a deep account of as many observable features under LM and SEM as it has been possible for us to collect. Also, regarding the provision of good comparative analyses, we have provided tables contrasting key diagnostic features, that we are herein expanding even further to include previously underexplored characters (Tables 1–5, see also Table 1 in Morales et al. 2019b for a comparison of small "araphids" at the genus level).

The revisionary work and study of type material we have been doing, which result in the morphological redefinition of taxa boundaries, is not only descriptive work (Haszprunar 2011). Descriptions, by means of accurate, standard terminology, and concatenation of LM and SEM are valuable records that constitute the taxonomic history of an entity. Thorough descriptions do not only reveal the original author's intentions and appreciation of the importance of certain characters, but they also offer guidance to subsequent interpretation in the context of what was known about morphology and key characters of taxa at the time of the first description of a taxon.

These revisionary activities and the results we have achieved over the years for the small "araphid" diatoms provide concrete evidence that much more work is still needed to describe in morphological terms the diversity of these diatoms and that this process is completely justified given the current needs and the state of the art in diatom diversity studies (Mann and Vanormelingen 2013). Meanwhile, molecular studies continue their parallel advancement, not without problems similar to those encountered by morphologists (Bailet et al. 2020), nevertheless augmenting the chance that in the near future we will be able to produce a more complete molecule-phenotype system that allows building a natural compendium of "araphid" diatom biodiversity, perhaps even meeting the goals of the Grand Linnean Enterprise (Wheeler et al. 2012; Prathapan and Rajan 2020). But, a compendium must also be translated into a classification system that reflects evolutionary history (de Carvalho et al. 2014b), a systematic approach that is of outmost importance in sustainable conservation practices for populations and communities are not static groupings, but rather they have evolutionary trajectories in the context of their environment, which are important to consider for their preservation, conservation and recuperation (Olivieri et al. 2015).

In the context of the paleolimnological research done in the Bolivian Altiplano on "araphid" diatoms, and in the face of the taxonomic inconsistencies encountered in some publications, paleolimnological data must be reviewed, but for this to take place there must be a fair knowledge of the current biodiversity of the group. These can be accomplished by wider surveys than the one we presented here, based only on two sites and referencing a few others. As discussed by Morales (2020) under-representativeness and under-sampling of a rather varied geographical landscape are serious flaws in the knowledge of Andean diatoms. Thus, much work needs to be devoted to better represent the composition of the diatom flora present in this region. Our effort expressed here and in Morales et al. (2012b) only represents the first steps to unravel the diatom community

developing at these localities. As explained by the authors, the sample from Desaguadero contains more than 200 species with restricted distribution and more than half are unknown taxa in multiple genera, not only "araphids" (see also discussion in Morales et al. 2014c). Thus, wider surveys may yield a high number of new taxa completely changing the current view of the Andean diatoms as being dominated by cosmopolitan taxa, but based on sampling of easily accessible, human-influenced areas (Rumrich et al. 2000).

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RESEARCH ARTICLE



Vicia mingyueshanensis (Fabeae, Papilionoideae, Fabaceae), a new species from western Jiangxi, China

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Abstract

Vicia mingyueshanensis, a new species from the Mingyue Mountain Region of western Jiangxi, China, is described and illustrated. It is a perennial climbing liana that always links to riparian woods. A morphological comparison indicated that the new species is closely similar to *Vicia taipaica* K. T. Fu and *Vicia dichroantha* Diels; however, it differs from the other two species by several salient characters, such as plant indumentum, stipule shape, corolla colour, bractlet shape and calyx shape. Photographs, a preliminary conservation assessment, table of morphological characters and distribution map comparing this new species to two morphologically-similar species are also provided.

Keywords

Flora of China, Leguminosae, new taxon, taxonomy, Vicia

Introduction

The genus *Vicia* Linn. (Fabeae, Papilionoideae, Fabaceae) comprises about 180–200 annual or perennial herbaceous species, which are mainly distributed throughout the temperate regions of Europe, Asia, Africa, North, and South America (Kupicha 1976; Gunn 1979; Hanelt and Mettin 1989). This genus is widely distributed throughout China. Till now, 40 species of *Vicia* have been reported in China (Xia 1996; Bao and

Turland 2010). Over many years, due to its high biological yield and high content of the crude protein within a short growth period, *Vicia* is considered with potential value as forage and it is extensively planted globally (Maršalkienė 2016).

During field surveys carried out in May 2019, a population of a perennial *Vicia* species was discovered in the Mingyue Mountain Region (Jiangxi Province, China). Detailed comparisons showed that the specimens and living plant materials were different from the type of specimens and protologues of some related known *Vicia* species. Moreover, the shapes of its leaf and rhizomes were most similar to those of *Vicia taipaica* K. T. Fu and *Vicia dichroantha* Diels. The three species are perennial herbs with branched stems that climb by means of tendrils on the ends of their paripinnate leaves. However, the new species can easily be distinguished from the latter two by several morphological characters (Table 1).

On the basis of careful investigations of herbarium specimens and living material and after the observation and cultivation in two years, the new species *Vicia mingyueshanensis* is described in this paper. The genus *Vicia* is divided into two large subgenera, subgen. *Cracca* and subgen. *Vicia*. Due to the perennial herbaceous and climbing habit of the new species, as well as the presence of tendrils, it belongs to subgen. *Cracca*.

Materials and methods

This study was mainly based on field surveys, the detailed examinations of herbarium specimens and literature. Herbarium specimens were examined in PE, KUN

Characters	Vicia mingyueshanensis	Vicia taipaica	Vicia dichroantha
Plant indumentum	totally glabrous	totally glabrous	densely hairy
Stem height (m) and	0.5–1.8, relatively thin	0.6–1.0, relatively thick	0.6–2.0, relatively thin
appearance	decumbent	decumbent	erect
Leaf length (cm), tendril	8-15	8-12	7–16
excluded			
Leaflet pairs per leaf	4–6	3–5	46
Leaflet shape	elliptic to ovate-oblong	elliptic to ovate-oblong	linear to linear-lanceolate
Leaflet size (cm)	$2.3 - 3.8 \times 0.7 - 1.5$	1.3–5.0 × 0.6–1.5	2.5-5.0 × 0.6-0.9
Stipule shape and size (cm)	hastate or lanceolate,	semi-ovate or lanceolate,	fan-shaped or lanceolate,
	opposite, unequal, margins	margin entire, 0.5–0.9 long	margin 2–3 toothed
	entire, 0.4-0.7 × 0.2-0.3		
Raceme (number of flowers)	10-20	5-15	20-25
Corolla colour	light yellow or dull orange	yellow or brown-yellow	yellow, dark yellow or dull
			orange, marked purple at
			the apex of standard
Bractlet shape	subulate	absent	absent
Calyx shape	5 lateral teeth acute, some	shortly and unequally	5 lateral teeth acute, hairy
	calyces are cleft	toothed	
Seed colour and size (cm)	brown-green	oblong	oblate-spheroid
	$0.3 - 0.4 \times 0.3$	$0.3 - 0.4 \times 0.2$	$0.3 - 0.4 \times 0.4$
Seed numbers	4–6	2–5	2–4

Table 1. Detailed comparison of Vicia mingyueshanensis and its two morphologically-similar species.
and **JJF** and from online specimen images from the International Plant Name Index (IPNI, https://www.ipni.org), Jiangxi Virtual Herbarium (JVH, http://site.nsii.org. cn/api/site.ashx?id=JXVH&a=app&app=VHForeword) and the Chinese Virtual Herbarium (CVH, https://www.cvh.ac.cn/index.php), National Specimen Information Infrastructure (NSII, http://www.nsii.org.cn/2017/home-en.php) and NYBG Steere Herbarium (http://sweetgum.nybg.org/science/vh/). Specimens collected from the field were deposited at the CSFI and NF. Detailed observations and measurements of the collected individuals were undertaken and micromorphological features were analysed using a Leica MZ16 stereomicroscope.

Taxonomic treatment

Vicia mingyueshanensis Z.Y.Xiao & X.C.Li, sp. nov. urn:lsid:ipni.org:names:77234378-1 Figures 1, 2, Table 1

Type. China. Jiangxi Province, Yichun County, Hongjiang Township, Dongnan Village, under bamboo forests, beside the river ditch, 328 m elevation, 8 May 2019, *Z.Y. Xiao & X.C.Li*, CSFI076074 (holotype: CSFI; isotypes: NF).

Diagnosis. Sepal lobes and bractlets, completely glabrous. Most similar to *Vicia taipaica*, but differs from it by its hastate or lanceolate stipules and subulate bractlets (stipules semi-ovate or lanceolate and bractlets absent in *Vicia taipaica*). Similar to *Vicia dichroantha* as well, but differs from it by the light yellow or dull orange colour of the corolla and subulate bractlet (yellow, dark yellow or dull orange corolla, marked purple at apex of standard and braclets, absent in *Vicia dichroantha*). The new species is restricted to western Jiangxi Province (Figs 1, 2, Table 1).

Description. Perennial herb, strongly climbing, 0.5-1.8 m tall, glabrous throughout. Root robust, woody, branched, well-developed in depth. Stems flexuous, subquadrangular, striate, branched. Leaves paripinnate, 8-15 cm (excluding the tendril), with 4-6 pairs alternate leaflets, provided with a terminal twining tendril, 2–3 branched; leaflets elliptic to ovate-oblong, margin entire, not toothed, papery, 2.3-3.8 cm long, 0.7-1.5 cm wide, broadly cuneate or suborbicular at the base, mucronulate at the apex, subsessile or shortly petiolulate (to ca. 1 mm long), lateral veins 7-12 paired. Stipules opposite, unequal, margin entire, hastate or lanceolate, 0.4-0.7 × 0.2-0.3 cm. Racemes 10-20 flowered, shorter or nearly as long as the subtending leaves, with peduncle up to 4-8 cm long. Flowers slightly pendent, 1.6-2.0 cm long, bractlet, subulate, 0.2-0.3 × 0.1 cm. Calyx membranaceous, obliquely campanulate, 0.4–0.5 cm long, tubular, gibbous at the base, zygomorphic, with 5 lateral teeth acute, some calyces are cleft. Corolla light yellow or dull orange, standard with $1.3-1.4 \times 0.4-0.5$ cm, subequalling to wings and keels, apex retuse. Staminal tube 1.2–1.4 cm long, vexillary staminal filament free, anther greenish-yellow. Ovary 0.5-0.6 cm long, with 4-6 ovules. Style geniculate



Figure 1. Distribution map of *Vicia mingyueshanensis* (blue dots) and its closest similar species Vicia taipaica (red dots) and Vicia dichroantha (yellow dots).

at the base, cylindrical, 0.3 cm long, evenly hairy under the stigma. Pod stipitate, falcate, often apiculate, smooth, $3.0-3.5 \times 0.3$ cm. Seeds 4–6, oblate-spheroid, brown-green, $0.3-0.4 \times 0.3$ cm, hilum circumlinear, up to the middle of the circumference long.

Phenology. Flowering time from May to early June; fruiting in July and defoliation from late July to early August.

Etymology. The species epithet is derived from the name of the mountain range (Mingyueshan) where the species had been discovered.

Vernacular name. The Chinese name '明月山野豌豆' (Ming Yue Shan Ye Wan Dou) Distribution and habitat. Vicia mingyueshanensis is only known in western Jiangxi Province, Yichun County, Hongjiang Town, Dongnan Village, Mingyue Mountain Region, located in an open area of Phyllostachys edulis J. Houzeau forests with Castanopsis tibetana Hance and Lithocarpus litseifolius (Hance) Chun as associated tree species. The observed population is very small, with fewer than 200 plants growing along roadsides and ditches, accompanied by Oreocnide frutescens (Thunb.) Miq. and Rubus tephrodes Hance. Elevation is 300–650 m above sea level.

Preliminary conservation assessment. *Vicia mingyueshanensis* is currently only known from a small population in a habitat that is subject to logging and disturbance, thus, it is very rare and distributed in a few patches. On the basis of our field observa-



Figure 2. *Vicia mingyueshanensis* **A**, **B** habitat **C** habit **D** the whole plant **E** pods **F** pods and seeds **G** root **H** inflorescences **I** leaflets and stipules **J** bractlet **K** calyx **L** tendrils.

tions, this species is represented by no more than 200 large and mature individuals, along a road where bamboo was being cut. Due to its rarity and a low number of individuals, *Vicia mingyueshanensis* is considered to be **Critically Endangered** (**CR**, **B1**), according to the IUCN (2019).

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RESEARCH ARTICLE



A new combination in *Pseudolappula* (Boraginaceae, Rochelieae) based on morphological, molecular and palynological evidence

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Abstract

Lappula sinaica was recently transferred to the monotypic genus Pseudolappula based on phylogenetic studies, while the related species, L. occultata, has remained in the genus Lappula. In this study, morphological, molecular, and palynological evidence supports that L. occultata should be transferred to the genus Pseudolappula. Both L. occultata and P. sinaica share a combination of nutlets features that distinguish them from Lappula: a longer adaxial keel and a linear attachment scar. Phylogenetic analysis based on ITS and trnL-F strongly supports L. occultata as the sister taxon of P. sinaica. In addition, pollen grains of these two species are 3-syncolporate with 3 alternating pseudocolpi, which is significantly different from the grains of Lappula taxa. Based on the above evidence, the new combination Pseudolappula occultata is proposed.

Keywords

Boraginaceae, Lappula occultata, new combination, Pseudolappula

Introduction

Recent phylogenetic studies on the Rochelieae (Boraginaceae) have greatly advanced our understanding of this plant group, and the circumscription of some genera has been changed (Huang et al. 2013; Saadati et al. 2017; Khoshsokhan-Mozaffar

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et al. 2018), the genus *Lappula* being one of these. Phylogenetic analyses indicate that *Lappula* is not monophyletic, with the species of this genus placed in three different lineages (Khoshsokhan-Mozaffar et al. 2018). The systematic position of *Lappula sinaica* (A.DC.) Asch. & Schweinf. was distinctive in occurring on a separate branch of the subtribe Eritrichiinae, while the other taxa of *Lappula* were clustered in different clades. After considering both molecular results and morphological comparisons, Khoshsokhan-Mozaffar et al. (2018) transferred the species *L. sinaica* to a new monotypic genus, *Pseudolappula*.

Echinospermum sinaicum A.DC. was described by Candolle (1846), based on two collections from the Sinai Peninsula, Egypt. This species was subsequently transferred to the genus *Lappula* by Ascherson and Schweinfurth (1887) and has since been regarded as a member of that genus (Candolle 1846; Gürke 1894; Brand 1931; Popov 1953; Riedl 1967; Wang 1989; Zhu et al. 1995; Ovczinnikova 2005a, 2009; Ovchinnikova et al. 2017).

The related species, *Lappula occultata* Popov (1951), was described based on specimens from Sary-tau mountains, Tajikistan. The type specimen of this species was designated by Ovchinnikova et al. (2020). In the protologue, the author stated that *L. occultata* differs from *L. sinaica* in its erect pedicel and long calyx. Both *L. sinaica* and *L. occultata* share morphological features of the nutlets and the two species have been viewed as sister taxa by most authors (Popov 1953; Riedl 1967; Wang 1989; Ovczinnikova 2005a, 2009). From 1953 to 2009, these two species were classified under the same section, subsection, and series. The systematic position of *L. sinaica*, *L. occultata*, and their congeneric relatives is presented in Table 1.

Although *L. sinaica* has now been formally placed in the new genus *Pseudolappula* by Khoshsokhan-Mozaffar et al. (2018), the related species, *L. occultata*, has remained in the genus *Lappula*. Current phylogenetic studies do not support the two taxa as allied species, as *L. sinaica* forms a distinct monospecific clade, while *L. occultata* is nested in the *Lappula* clade (Huang and Zhang 2012; Huang et al. 2013; Khoshsokhan-Mozaffar et al. 2018). Because of this obvious conflict between previous taxonomic treatments for *L. occultata* and the aforementioned molecular studies, further examination of these two species is needed, including past voucher specimens used.

The specimens used in the previous phylogenetic analyses (Huang et al. 2013; Khoshsokhan-Mozaffar et al. 2018) were examined. After comparing these with

	Popov (1953)	Riedl (1967)	Wang (1981)	Ovczinnikova	Khoshsokhan-
				(2005a)	Mozaffar et al.
					(2018)
Genus	Lappula	Lappula	Lappula	Lappula	Pseudolappula
Section	Eulappula	Lappula	Lappula	Sinaicae	-
Series/Subsection	Sinaicae	Sinaicae	Sinaicae	-	-
Species	L. sinaica	L. sinaica	L. sinaica	L. sinaica	P. sinaica
_	L. occultata	L. sessiliflora	L. occultata	L. occultata	_
-	L. lipschitzii	-	-	L. mogoltavica	-

Table 1. Historical classifications of L. sinaica with L. occultata and its congeneric relatives.

both the protologue and type specimens, we discovered that almost all the specimens of *L. occultata* preserved in Chinese herbaria were misidentified. In the protologue (Popov 1951), *L. occultata* is described has bearing small flowers, with the corolla limb 1–1.5 mm wide. However, in *Flora Reipublicae Popularis Sinicae* (Wang 1989) and *Flora of China* (Zhu et al. 1995), this species is described as having large flowers, with the corolla limb 5–6 mm wide, indicating a different, possibly incorrect, circumscription of *L. occultata* in Chinese Flora, and this may cause misunderstanding of *L. occultata*.

Therefore, it was necessary to revise the circumscription of *L. occultata* based on a more appropriate understanding and identification of that species. In this study, morphological, molecular, and palynological analyses are conducted to clarify the systematic position of *L. occultata*.

Materials and methods

Morphological observation

Specimens at PE, XJU, XJA, XJBI, MW, NSK, and TASH were examined critically, including type specimens. Field observations were carried out in Xinjiang province, China. Morphological studies were made of living plants in the field and of pressed specimens, with particular attention to the mature nutlets which were photographed by a ZEISS V8 stereoscopic microscope.

Molecular taxon sampling

In order to verify the evolutionary relationships between *Pseudolappula* and *L. occultata*, 46 taxa within the tribe Rochelieae were sampled, including 5 genera of subtribe Eritrichiinae (*Hackelia, Pseudolappula, Eritrichium, Rochelia,* and *Lappula*), which covered all main clades of this lineage. *Pseudoheterocaryum subsessile* (Vatke) Kaz.Osaloo & Saadati was selected as an outgroup according to the previous studies (Huang et al. 2013; Chacón et al. 2016; Khoshsokhan-Mozaffar et al. 2018). The DNA sequences obtained from this study were deposited in GenBank, with all accession numbers listed in Appendix 1.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from silica-gel dried leaves using the Plant Genomic DNA Kit (Tiangen, Beijing, China), following the manufacturer's instructions. The ITS (White et al. 1990; Sang et al. 1995) and trnL-F (Taberlet et al. 1991) regions were amplified using primer pairs of the cited authors. The amplification profile followed Huang et al. (2013). Products of amplification reactions were sequenced with an ABI3730XL automated DNA sequencer (BGI Tech. Solutions Beijing Liuhe Co., Limited, Beijing, China).

Sequence alignment Phylogenetic analysis

Sequences of ITS and *trnL-F* were aligned with MAFFT online version 7 and manually adjusted (Katoh et al. 2019). A combined matrix of ITS and *trnL-F* was generated by SequenceMatrix (Vaidya et al. 2011). Combinability of ITS and *trnL-F* were assessed using the incongruence length difference test (Farris et al. 1994), as implemented in PAUP*4.0 (Swofford 2002). According to jModeltest (Darriba et al. 2012), the best evolutionary model was TrN+I+G. Molecular phylogeny reconstruction was performed in the CIPRES Science Gateway (Miller et al. 2015) with Maximum Likelihood analysis employing RAxML-HPC2 on XSEDE (8.2.12) (Stamatakis 2014) and Bayesian inference using MrBayes on XSEDE (3.2.7a) (Ronquist et al. 2012). For MrBayes, four Markov chains were run for 50,000,000 generations. The trees were sampled every 1,000 generations, while the first 25% of trees were discarded as burn-in. The rest of the trees were used to generate a majority-rule consensus tree. Trees were visualized using Fig Tree v. 1.4.4.

Pollen sampling and scanning electron microscopy

Samples of taxa were obtained from field surveys during 2019–2021 and from voucher specimens were preserved in the BNU herbarium (Appendix 2). Pollen grains of these samples were mounted on metallic stubs with conductive adhesive tape, then coated with gold using an E-1045 ion sputter. The prepared samples were observed with a Hitachi S-4800 scanning electron microscope at 5 kV. Terminology follows Punt et al. (2007) and Ovczinnikova (2021).

Results

Morphological studies

The results of morphological comparisons indicated that *Pseudolappula sinaica* (A.DC.) Khoshsokhan, Sherafati & Kaz.Osaloo and *L. occultata* are quite similar and likely closely related. The two taxa exhibited a special combination of characters in both the nutlet attachment scar and adaxial keel (Fig. 1), and these characters are congruent with the description of section *Sinaicae* (Ovczinnikova 2005a). Both *P. sinaica* and *L. occultata* possessed no obvious attachment scar on the nutlets (Figs 1D, H, M, N), and the shape of cicatrix was linear (Fig. 1N). In addition, the length of adaxial keel was the same as the nutlet (Figs 1D, H). However, taxa in *Lappula* developed an ovoid, triangular-ovoid or narrow lanceolate attachment scar (Figs 1L, O, P), and the adaxial keel was shorter than the nutlet (Figs 1L, O, P). Detailed comparison of *Pseudolappula*, *L. occultata* and *Lappula* is provided in Table 2.



Figure 1. Nutlet morphology of *Pseudolappula* and *Lappula* **A–D** *Pseudolappula sinaica* **A** fruit lateral view **B** fruit polar view (growth direction of nutlets indicated by arrows) **C** nutlet abaxial view **D** nutlet adaxial view **E–H** *Lappula occultata* **E** fruit lateral view **F** fruit polar view (arrangement of nutlets indicated by cross lines) **G** nutlet abaxial view **H** nutlet adaxial view **I–L** *Lappula patula* **I** fruit lateral view **J** fruit polar view (arrangement of nutlets indicated by cross lines) **K** nutlet abaxial view **L** nutlet adaxial view **M** adaxial keel of *P. sinaica* (adaxial view) **N** adaxial keel of *L. occultata* (lateral view) **O** attachment scar of *Lappula balchaschensis* **P** attachment scar of *Lappula balchaschensis* **1** mm.

Phylogenetic analyses

The phylogeny was rooted with *Pseudoheterocaryum subsessile* from the tribe Rochelieae, and the tree showed subtribe Eritrichiinae as monophyletic. Eritrichiinae comprised 5 major clades (Fig. 2). *Pseudolappula* was the first diverging clade (pp=1, ML-BS=100), followed by *Hackelia* (pp=1, ML-BS=100). *Lappula* resolved as sister to the clade that includes *Rochelia* and *Eritrichium*. Notably, the new sample of *L. occultata* from the present study was clearly clustered into a monophyletic clade with *P. sinaica* (Fig. 2), which was consistent with our morphological analysis.

	Characters	Pseudolappula	L. occultata	Lappula
Life form	1	annual	annual	annual, biennial, perennial
Leaves	basal leaves	petiolate	petiolate	sessile
Bracts	lower shape	lanceolata	narrowly ovate	leaflike or linear
Calyx	shape	oblong-linear	linear	linear, oblong
Corolla	color	blue	blue	blue, white
	size (mm)	$2-3 \times 1-2$ (3)	$2-2.5 \times 1-1.5$	$2.5-4 \times 1.5-12$
	limb/tube (ratio)	ca. 1	ca. 1	ca. 1–3
Stamens	pollen aperture type	3-syncolporate 3 pseudocolpi	3-syncolporate 3 pseudocolpi	3-colporate 3 pseudocolpi
	polar axis	12.3–15 μm	13.6–15.5 μm	8.9–19.4 μm
	equatorial axis	4.3–5.6 μm	4.7–7.1 μm	2.2–9.1 μm
Style	-	exceeding the nutlets	exceeding the nutlets	exceeding or not the nutlets
Nutlets	disk shape	narrowly oblong	oblong	lanceolata to ovate
	attachment scar (cicatrix)	not obvious, linear	not obvious, linear	obvious, narrow lanceolata to ovate
	adaxial keel	as long as the nutlets	as long as the nutlets	shorter than the nutlets

Table 2. Comparisons of Pseudolappula, L. occultata and Lappula.

Palynological studies

The palynological data also supported that *P. sinaica* and *L. occultata* are closely related to each other. We examined the pollen morphology of *Pseudoheterocaryum*, *Pseudolappula*, *Lappula*, and *Rochelia* taxa. Pollen grains were isopolar, dumbbell-shaped or oblong in equatorial view and sub-circular in polar view (Fig. 3). Their sizes ranged from $8.9-19.4 \times 2.2-9.1 \mu m$.

The pollen apertures of the studied taxa were of three types: 3-colporate alternating with 3 pseudocolpi (Figs 3E, G, I, K, M), 3-syncolporate alternating with 3 pseudocolpi (Figs 3B, D) and 3-colporate types (Fig. 3O). Specifically, the true apertures of *P. sinaica* and *L. occultata* were 3-syncolporate, which is unique in the subtribe Eritrichiinae (Carr 1973; Díez and Valdés 1991; Mazari et al. 2018). The shapes of true apertures and pseudocolpi were narrowly linear. The length of pseudocolpi were nearly equal (Figs 3E, G, I, K) or shorter (Figs 3A, C, M) compared to colpi.

Discussion

Nutlets are always important for identification and classification of Boraginaceae, especially for *Lappula* (Popov 1953; Riedl 1967; Zhu et al. 1995; Ovczinnikova 2005a, 2005b, 2006, 2009; Ovchinnikova et al. 2017, 2020). Traditionally, the classification of *Lappula* is heavily based on the abaxial characters of nutlets (number of rows of glochids, the length of glochids, the shape of the eremocarp disk, and the confluent degree of glochids). However, information on nutlets beyond well studied abaxial characters deserves more attention. *Pseudolappula sinaica* is such a case, although Khoshsokhan-Mozaffar et al. (2018) provided the key to distinguish *Pseudolappula* and *Lappula*. After examining the types and specimens for *P. sinaica* and species of *Lappula*, we consider these characters are not effective for recognizing each of the two genera.



Figure 2. Maximum Likelihood tree of subtribe Eritrichiinae inferred from ITS + *trnL-F*. The tree topology was constructed using RAxML. Bootstrap values and Bayesian posterior probabilities are indicated above branches. Note that the *Lappula occultata* (Huang et al. 2013) is a misidentified voucher from previous studies (Huang et al. 2013).



Figure 3. Scanning electron micrographs. A, B Pseudolappula sinaica C, D Lappula occultata E, F L. shanhsiensis G, H L. redowskii I, J L. macrantha K, L L. tianschanica M, N Rochelia bunge O, P Pseudoheterocaryum rigidum. Scale bars: 5 µm.

First, the length of nutlets between the two genera is overlapping (2 mm vs 2–5 mm). Second, the nutlets of *P. sinaica* possess a distinct margin (Fig. 1C) different from the description by Khoshsokhan-Mozaffar et al. (2018), whose use of immature nutlets may have led to differences and inaccurate descriptions. In addition, the character of the fruit pedicel is not reliable to differentiate the two genera, because not all the sampled individuals of *P. sinaica* have recurved pedicels on the herbarium specimens. Seemingly, some *Lappula* species, such as *L. semiglabra*, also have recurved pedicels. Even with the combined nutlets and pedicel features, it is challenging to distinguish the two genera.

Lappula sinaica and L. occultata were placed in the section Eulappula, series Sinaicae by Popov (1953) based on the nutlets characters, notably the adaxial keel. Since then, the systematic position of L. sinaica and its congeneric relatives has been relatively independent (Riedl 1967; Wang 1989; Ovczinnikova 2005a), and our results basically agree with previous treatments. Based on critical morphological comparisons, we find that the attachment scar and adaxial keel of nutlets are useful to separate P. sinaica and L. occultata from species in Lappula. Our morphological results are consistent with the findings of Ovczinnikova (2005a), who described the new section Sinaicae according to the above-mentioned characters. Additionally, the arrangement of 4 nutlets is unusual in both *L. occultata* and *P. sinaica* (Figs 1B, F). This arrangement pattern is in accordance with the view of Hilger (2014) concerning *Lappula spinocarpos* (Forssk.) Asch. ex Kuntze, and some other researchers hold the view that *L. spinocarpos* should be separated from *Lappula* and raised to genus level (Brand 1931; Sadat 1989). Therefore, adaxial features of nutlets may be more critical than abaxial characters for the identification of *Pseudolappula*.

To better resolve relationships of *P. sinaica* with *L. occultata* and the *Lappula* species within subtribe Eritrichiinae, we newly sequenced the ITS and *trnL-F* regions from a 'real' specimen of L. occultata, which was determined to match the initial species description after careful specimen examination. As a result, the phylogenetic framework within tribe Rochelieae is highly congruent with previous work (Khoshsokhan-Mozaffar et al. 2018). However, our study cannot corroborate the recent authors' treatment of L. occultata (Huang et al. 2013; Khoshsokhan-Mozaffar et al. 2018). Our phylogenetic result shows that L. occultata is more closely related to P. sinaica than to any member of the Lappula. The incongruous systematic position of L. occultata (Fig. 2) is based on misidentification due to the incorrect description of L. occultata in Flora Reipublicae Popularis Sinicae and Flora of China. Consequently, specimens of Lappula brachycentra (Ledeb.) Gürke are incorrectly identified as L. occultata. Furthermore, we carefully examined Lappula specimens at the same location that Huang et al. (2013) sampled. These specimens possess very short marginal glochids and large flowers. In some individuals, there are no visible glochids, but only marginal ribs on the nutlets. These characters are more in line with the inaccurate descriptions of L. occultata in Chinese flora (Wang 1989; Zhu et al. 1995) and frequently cause misidentifications.

Boraginaceae are a palynologically heterogeneous family (Erdtman 1969; Nowicke and Miller 1990), and pollen grains also could be useful for lower taxonomic levels, such as genera (Bigazzi et al. 2006; Liu et al. 2010; Sutorý 2013; Noroozi et al. 2021). The subtribe Eritrichiinae is one of the major clades of tribe Rochelieae with 6 genera and over 200 species (Chacón et al. 2016), but palynological studies of this subtribe are insufficient. In the present research, pollen morphology of 8 taxa of *Pseudoheterocaryum*, *Pseudolappula*, *Lappula*, and *Rochelia* were studied. Their pollen grains are mostly heterocolpate, which is consistent with previous studies (Díez and Valdés 1991; Khatamsaz 2001). Specifically, the pollen apertures of *L. occultata* and *P. sinaica* are 3-syncolporate alternating with 3 pseudocolpi (Figs 3B, D), which is unique, and the characters of pollen apertures shared by these two species are not found in other members of subtribe Eritrichiinae (Carr 1973; Díez and Valdés 1991; Khatamsaz 2001; Mazari et al. 2018).

Although *Lappula* species have been studied in terms of nutlet morphology (Wu et al. 2014; Ovczinnikova 2006, 2021), palynology (Ahn and Lee 1986; Díez and Valdés 1991; Khatamsaz 2001), cytology (Löve 1975, 1983; Luque 1992; Kobrlová and Hroneš 2019), and phylogeny (Huang et al. 2013; Khoshsokhan-Mozaffar et al. 2013, 2018), there are still limits due to insufficient sampling. On the one hand, taxonomic and phylogenetic studies require very broad sampling. On the other hand, correct identification of species is fundamental for the various research. *Lappula* is a

taxonomically difficult genus, and nutlet characters are essential for the proper identification of species in this genus. However, we must be careful not to rely too much on one feature for taxonomic delimitation. More characters should be closely investigated and integrated into further work.

Taxonomic treatment

Pseudolappula occultata (Popov) Q.R.Liu & D.H.Liu, comb. nov. urn:lsid:ipni.org:names:77234443-1

Basionym. Lappula occultata Popov (1951: 331).

Type. Tajikistan: Sary-tau mountains. 25 June 1920, Popov 697 (TASH003719!). Description. Annual herbs. Stems erect, 15-40 cm tall, frequently branched from base or above middle, with appressed or semi-appressed white hairs. Basal leaves oblong with obvious petiole, 2-3 cm long, 5-8 mm wide; cauline leaves sessile, oblong to lanceolate, 2-4 cm long, 4-9 mm wide, with spreading hairs, hairs discoid at base. Inflorescences to 10-15 cm in fruit; bracts small, narrowly ovate to linear. Pedicels erect, the lower 5-6 mm long. Calyx lobes linear, erect, 2 mm long, to 4-5 mm in fruit, enclosing the nutlets. Corolla blue, 2–3 mm long, the tube shorter than calyx; throat appendages yellow, trapeziform, ca. 0.3 mm; limb 1-1.5 mm wide, lobes ovata-rounded. Stamen 5, filaments short, pollen grains isopolar, dumbbell-shaped in equatorial view and subcircular in polar view, 3-syncolporate apertures alternating with 3 pseudocolpi, with 6 orae. Coenobium 2-3 mm in diameter, homomorphic nutlets. Style surpassing the nutlets by ca. 0.5 mm. Nutlets ovoid, shiny, ca. 2 mm long, not easily separated from gynobase; disk ovate, weakly keeled, densely with rounded granulose, margin prominent and forming a narrow smooth rim. Cicatrix linear, not obvious, adaxial keel ca. 2 mm long.

Phenology. Flowering and fruiting from May to July.

Distribution and habitat. China, Kazakhstan, Tajikistan, Uzbekistan, Kyrgyzstan, Afghan, Mongolia (Vvedensky 1961; Sharashova 1962; Goloskokov 1964; Chukavina 1984; Ovczinnikova 2005a; Ovchinnikova et al. 2017). It grows on rocky slopes at elevations of 600–2400 m (Chukavina 1984).

Note. The section *Sinaicae* (Riedl) Ovczinnikova was proposed by Ovczinnikova (2005a). This small section is comprised of 3 species, *L. sinaica*, *L. occultata*, and *Lappula mogoltavica* Popov ex Czukav. *L. sinaica* has been transferred to the new monotypic genus, and our study supports that *L. occultata* should also be combined into the genus *Pseudolappula*. Then, the systematic position of *L. mogoltavica* needs to be settled. The species *L. mogoltavica* was published by Czukavina (1983). Ovchinnikova et al. (2017) conducted a detailed study on this species and its congeneric relatives, and morphological comparisons demonstrate that the length of the adaxial keel and the shape of cicatrix are uniform in section *Sinaicae*. These characters suggest that the three species have a close relationship.

Additional specimens examined. China. Xinjiang: Yining county, 29 April 1977, suzenan 613 (XJU!); Gongliu county to Tekes county, 9 August 1996, wangai 96-047 (XJA00057876!); Nilak county, 43°41'18.28"N, 82°20'4.70"E, alt. 1287 m, 17 May 2020, Dan-Hui Liu BNU2020XJ088(BNU!); Huocheng county, 44°18'35.61"N, 81°6'6.07"E, 21 May 2021, Dan-Hui Liu BNU2021XJ095 (BNU!). Kazakhstan. Alma-Ata region, Enbekshikazakh district, 43°39'N, 78°56'E, 17 May 2003, A.Yu. Korolyuk, I.A.Khrustaleva s.n. (NSK0005820!); East Kazakhstan region, Urdzhar district, alt. 660 m, 30 May 2003, A.Yu.Korolyuk 45 (NSK0005868!); Karaganda region, 47°42994'N, 74°81770'E, 13 May 2014, A.L.Ebel s.n. (NSK0008730!); Baidibek district, 25 April 2015, N.N.Lashchinsky s.n. (NSK0008234!). Uzbekistan. Viloyat Surxondaryo, 15 km W from city Boysun, 38°14'12"N, 67°1'54"E, alt. 1140 m, 7 May 2013, D.Lyskov s.n. (MW0895220!). Afghan. Kabul, 20 km SE, alt. 2000-2400 m, 21 May 1968, Freitag 2641 (KUFS!); Kapisa, Unteres Panjsher Tal gegenueber Korawa, alt. 1700 m, 24 May 1973, O. Anders 9764 (KUFS!); Takhar, Gebirgshange 12 km SE Eshkamesh, alt. 1300-2100 m, 24 May 1970, O. Anders 6751 (KUFS!); Upper Maidan-valley near Takona, alt. 2700 m, 11 June 1968, Freitag 2970 (KUFS!); Loghar, Dobandaytal, alt. 2800 m, 4 May 1970, O. Anders 3356 (KUFS!); Paktya, Saydkhelo Lgad, alt. 1620 m, 12 May 1972, O. Anders 8877 (KUFS!); Nangahar, N Haenge des Safeed Koh bei Baghdara, alt. 1200-1400 m, 23 March 1973, O. Anders 9511 (KUFS!).

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Appendix I

Taxa used for molecular analyses (Taxon, GenBank accession no. (ITS, *trnL-F*), source and collector/collection number).

OUTGROUP TAXA: Pseudoheterocaryum subsessile Vatke, AB758297, AB758326, Iran, Faghihna & Zangooei 28193 (TMUH). INGROUP TAXA: Eritrichium aretioides DC. KU927709, KC542591, U.S.A., Weigend 9126 (BSB); Eritrichium canum (Benth.) Kitam. AB758294, AB758323, Germany, cultivated in Munich Botanical Garden; Eritrichium nanum Schrad. Ex Gaudin, KU927711, KC542483, Switzerland, Zippel & al. s.n. (B); Eritrichium pamiricum B.Fedtsch. KU927712, KC542564, Afghanistan, Anders 8098 (M); Eritrichium sericeum DC. JQ388500, Russia, West Chukotka, Petrovsky & Plieva s.n. (O); Eritrichium splendens Kearney, JQ388501, Alaska, Noatak Quad, Solstad & Elven 03/1216 (O); Eritrichium thymifolium (DC.) Y.S.Lian & J.Q.Wang, JX976807, JX976913, China, Xinjiang, B. C. Han et J. F. Huang 201007004 (PE); Hackelia bella I.M.Johnst., KU927715, KC542497, U.S.A., Merello & al. 702 (MO); Hackelia floribunda I.M.Johnst., JQ513445, Reveal 2390 (SD 103849) KC542513, USA Miller et al. 6966 (MO) ; Hackelia micrantha (Eastw.) J.L.Gentry, JQ388504, JQ388584, U.S.A, Oregon, Grant Co., Hinchliff 869 (WS); Hackelia revoluta I.M.Johnst., KF849119, KF849224, Argentina, C. Aedo 15407 (MA); Hackelia sharsmithii I.M.Johnst., KU927717, KC542498, U.S.A., Hilger U.S.A. 94/18 (BSB); Hackelia velutina I.M.Johnst., KU927719, KC542530, U.S.A., Hilger 411/1997 (BSB); Hackelia deflexa (Wahlenb.) Opiz, JX976808, JX976914, China, Xinjiang, J. F. Huang 20090109 (XJBI); Lappula albiflora (Riedl) Khoshsokhan & Kaz.Osaloo, KF287982, KF288065, Rechinger 31424 (TARI); Lappula anocarpa Ching J.Wang, JQ388505, JQ388585, China, Xinjiang, Juan Qiu 08-0007 (XJA); Lappula balchaschensis M.Popov ex Golosk., JX976776, JX976890, China, Xinjiang, J. F Huang et B. C. Han 201008016 (XJBI); Lappula brachycentra (Ledeb.) Gürke, JX976777, JX976891, China, Xinjiang, J. F. Huang et B. C. Han 201008042 (XJBI); Lappula consanguinea (Fischer et C.A. Mey.) Gürke, JX976779, JX976893, China, Xinjiang, J. F. Huang 20090213 (XJBI); Lappula consanguinea var. cupuliformis Ching J.Wang, JX976780, JX976894, China, Xinjiang, J. F. Huang et B. C. Han 201009011-2 (XJBI); Lappula duplicicarpa

Pavlov, JX976781, JX976895, China, Xinjiang, J. F. Huang 20090181 (XJBI); Lappula lipschitzii Popov, JX976787, JX976899, China, Xinjiang, W. Zhai 087 (SHI); Lappula microcarpa (Ledeb.) Gürke, IX976788, IX976900, China, Xinjiang, J. F. Huang et B. C. Han 201009003 (XJBI); Lappula myosotis Moench, JX976789, JX976901, China, Shanxi, J. F. Huang 2010050 (XJBI); Lappula occultata Popov, JX976791, JX976902, China, Xinjiang, J. F. Huang et B. C. Han 201008043 (XJBI); OK135686, OL364176, China, Xinjiang, Dan-Hui Liu BNU2020XJ088 (BNU); Lappula patula (Lehm.) Menuharth, JX976792, JX976903, China, Xinjiang, J. F. Huang 20090056-A (XJBI); Lappula persica (Boiss.) Khoshsokhan & Kaz. Osaloo, AB758312, AB758339, Iran, Assadi and Maassumi 51278 (TARI); Lappula redowskii (Hornem.) Greene, KP027121; KF288063, U.S.A., Cohen 161; Lappula semiglabra (Ledeb.) Gürke, JX976793, JX976904, China, Gansu, J. F. Huang et B. C. Han 2010055 (XJBI); Lappula semiglabra var. heterocaryoides Popov ex Ching J.Wang, JX976784, JX976896, China, Xinjiang, J. F. Huang et B. C. Han 201008054 (XJBI); Lappula shanhsiensis Kitag., KU927725, KU927725, China, Kürschner & al. 634 (BSB); Lappula spinocarpos (Forssk.) Asch. ex Kuntze, JX976795, JX976906, China, Xinjiang, W. Zhai 120 (SHI); Lappula stricta (Ledeb.) Gürke, JX976798, JX976907, China, Xinjiang, J. F. Huang et B. C. Han 201008052 (XJBI); Lappula tadshikorum Popov, JX976799, JX976908, China, Xinjiang, W. Zhai 12 (SHI); Lappula tenuis (Ledeb.) Gürke, JX976800, JX976909, China, Xinjiang, J. F. Huang et B. C. Han 201008049 (XJBI); Lappula wendelboi (Riedl) Khoshsokhan & Kaz.Osaloo, AB758314, AB758340, Iran, Kazempour Osaloo 2008-7 (TMUH); Rochelia bungei Trautv., AB564695, AB564705, Iran, Assadi & Massoumi 55785 (TARI); Rochelia cancellata Boiss. & Balansa, AB564702, AB564712, Turkey, Bani 4971 (TMUH); Rochelia cardiosepala Bunge, AB564701, AB564711, Iran, Kazempour Osaloo 2006-1 (TMUH); Rochelia disperma (L.) Wettst., AB564698, AB564708, Iran, Kazempour Osaloo 2007-2 (TMUH); Rochelia mirheydari Riedl & Esfand., AB564696, AB564706, Iran, Faghihnia & Zangooei 23477 (TMUH); Rochelia peduncularis Boiss., AB564699, AB564709, Iran, Abdolzadeh 20447 (FUMH); Rochelia persica Bunge ex Boiss., AB564697, AB564707, Iran, Kazempour Osaloo 2007-1 (TMUH); Pseudolappula sinaica (A.DC.) Khoshsokhan, Sherafati & Kaz.Osaloo, AB758308, AB758336, Iran, Kazempour Osaloo 2007-7 (TMUH);

Appendix 2

Sources of the pollen materials.

Species	Voucher
Pseudoheterocaryum rigidum (A.DC.) Kaz.Osaloo & Saadati	China. Xnjiang, Dan-Hui Liu BNU2020XJ079 (BNU)
Lappula macrantha (Ledeb.) Gürke	China. Xnjiang, Dan-Hui Liu BNU2020XJ147 (BNU)
<i>L. occultata</i> Popov	China. Xnjiang, Dan-Hui Liu BNU2020XJ088 (BNU)
L. redowskii (Hornem.) Greene	China. Gansu, Dan-Hui Liu BNU2020GS038 (BNU)
L. shanhsiensis Kitag.	China. Shanxi, Dan-Hui Liu BNU2020GS117 (BNU)
L. tianschanica Popov & Zakirov	China. Xinjiang, Dan-Hui Liu BNU2019XJ346 (BNU)
Rochelia bungei Trautv.	China. Xinjiang, Dan-Hui Liu BNU2021XJ076 (BNU)
Pseudolappula sinaica (A.DC.) Khoshsokhan, Sherafati & Kaz.Osaloo	China. Xinjiang, Dan-Hui Liu BNU2021XJ095 (BNU)

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RESEARCH ARTICLE



An image dataset of cleared, x-rayed, and fossil leaves vetted to plant family for human and machine learning

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Abstract

Leaves are the most abundant and visible plant organ, both in the modern world and the fossil record. Identifying foliage to the correct plant family based on leaf architecture is a fundamental botanical skill that is also critical for isolated fossil leaves, which often, especially in the Cenozoic, represent extinct genera and species from extant families. Resources focused on leaf identification are remarkably scarce;

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however, the situation has improved due to the recent proliferation of digitized herbarium material, liveplant identification applications, and online collections of cleared and fossil leaf images. Nevertheless, the need remains for a specialized image dataset for comparative leaf architecture. We address this gap by assembling an open-access database of 30,252 images of vouchered leaf specimens vetted to family level, primarily of angiosperms, including 26,176 images of cleared and x-rayed leaves representing 354 families and 4,076 of fossil leaves from 48 families. The images maintain original resolution, have user-friendly filenames, and are vetted using APG and modern paleobotanical standards. The cleared and x-rayed leaves include the Jack A. Wolfe and Leo J. Hickey contributions to the National Cleared Leaf Collection and a collection of high-resolution scanned x-ray negatives, housed in the Division of Paleobotany, Department of Paleobiology, Smithsonian National Museum of Natural History, Washington D.C.; and the Daniel I. Axelrod Cleared Leaf Collection, housed at the University of California Museum of Paleontology, Berkeley. The fossil images include a sampling of Late Cretaceous to Eocene paleobotanical sites from the Western Hemisphere held at numerous institutions, especially from Florissant Fossil Beds National Monument (late Eocene, Colorado), as well as several other localities from the Late Cretaceous to Eocene of the Western USA and the early Paleogene of Colombia and southern Argentina. The dataset facilitates new research and education opportunities in paleobotany, comparative leaf architecture, systematics, and machine learning.

Keywords

Angiosperms, cleared leaves, data science, fossil leaves, leaf architecture, paleobotany

Introduction

General patterns of angiosperm leaf architecture, the shape and venation characters of leaves, are well known for very few of the more than 400 angiosperm families. The development of a standard descriptive terminology (von Ettingshausen 1861; Hickey 1973, 1979; Ellis et al. 2009) has catalyzed increased detail and reproducibility in species descriptions of both living and fossil leaves. However, despite the use of numerous visual examples (e.g., Ellis et al. 2009), publications to date do not inform the reader how to accomplish the fundamental task of identifying leaves that, as for the great majority of leaf fossils, are isolated from the rest of the plant and missing diagnostic information from stipules, leaf organization, and reproductive and other organs.

To build their knowledge of leaf architecture, researchers still rely primarily on "oral tradition" from a dwindling number of knowledgeable colleagues and a handful of survey papers and field guides that emphasize purportedly diagnostic leaf features (Hickey and Wolfe 1975; Gentry 1993; da Ribeiro et al. 1999; Keller 2004). There is significant literature on the leaf architecture and leaf-fossil records of various taxa (among many others, von Ettingshausen 1858; Hill 1982; Jones 1986; Manchester 1987; Todzia and Keating 1991; Gandolfo and Romero 1992; Premoli 1996; Fuller and Hickey 2005; Martínez-Millán and Cevallos-Ferriz 2005; Doyle 2007; Kellner et al. 2012). However, many of the most diverse and ecologically significant groups of angiosperms have virtually no documentation of diagnostic leaf-blade features (e.g., Asteraceae, Rubiaceae), and thus their leaf fossils remain largely unrecognized, though probably hidden in plain sight in museum collections (see Wilf 2008; Wilf et al. 2016). More than half of fossil-leaf species in many older monographs are thought to have been misclassified (see Dilcher 1971), and most of the millions of leaf fossils in the general stratigraphic collections of the world's museums are not yet identified.

Machine-vision algorithms, as seen in popular applications such as LeafSnap (Kumar et al. 2012), Pl@ntNet (Bonnet et al. 2018), and iNaturalist (Van Horn et al. 2018), are making spectacular breakthroughs in automated species identification of live plants; however, they provide little, if any, feedback about the diagnostic features they detect. Few algorithms have attempted to generalize above the species level (Wilf et al. 2016; Carranza-Rojas et al. 2018), and so far the methods do not work on leaf fossils, which mostly represent extinct species and often extinct genera.

Increasing general knowledge of leaf architecture for both human and machine learners depends on the development of customized, accessible, vetted visual libraries that allow rapid morphological comparisons of a high phylogenetic diversity of extant and fossil leaves. The recent proliferation of digitized plant-image resources comprises an invaluable reference for plant morphology, already including tens of millions of digitized herbarium sheets on portals and aggregator sites such as IStor Global Plants (https://plants.jstor.org), iDigBio (https://www.idigbio.org), RecolNat (https://www. recolnat.org), and many others, as well as servers located at numerous individual herbaria worldwide (e.g., Bakker et al. 2020). However, studying leaf comparative morphology is not simple because leaves only represent part of the visual field of a herbarium sheet and appear, with overlaps, at many different angles and sizes. Computer-vision algorithms that blur text or segment leaves from background or from other plant material are likely to help solve this issue (Hussein et al. 2020, 2021; Weaver et al. 2020; de Lutio et al. 2021). However, many visual distractors remain, and critical details of higherorder venation are often not visible in digitized herbarium sheets. Assessing leaf architecture at family level from digital herbaria also requires examination of extremely large numbers of specimens for all but the most species-poor families. In this regard, JStor Global Plants stands out for prioritizing type specimens collated digitally from across the world's herbaria, thus allowing rapid surveys of the taxa in a family based on protologue voucher material. Finally, digitization efforts are far more advanced in resource-rich countries, whereas many significant collections are located in developing nations where herbarium digitization is occurring at a slower pace.

Cleared or x-rayed leaves from phylogenetically diverse taxa, selectively sampled from vouchered herbarium sheets, remain the most valuable visual reference for comparative study of leaf architecture because they have a similar visual presentation, with high capture of venation detail and comparatively few distractors. Existing collections of this type are fragile, mostly made decades ago as references for fossil leaf identification by selecting leaves from herbarium sheets, then either chemically clearing the specimens of most tissues other than veins and mounting them on glass slides or x-ray imaging them, in either case with extreme care and effort. Most cleared-leaf collections suffer from deterioration of the mounting media, which obscures large areas of the leaves; thus, photographic archiving offers a form of visual preservation before further degradation occurs. The largest and best-known cleared-leaf collections are those of the late Drs. Jack A. Wolfe and Leo J. Hickey, together now forming the National Cleared Leaf Collection (NCLC; NCLC-W and NCLC-H, respectively), housed in the Division of Paleobotany of the Smithsonian Institution National Museum of Natural History (NMNH, repository acronym USNM, Washington, D.C.).

For the many users who may find it challenging to visit these collections in person for suitable lengths of time, many cleared and x-rayed leaf collections are already accessible from various websites or in print. These valuable resources include the NCLC-W and other collections in the Cleared Leaf Image Database (http://clearedleavesdb. org; Das et al. 2014); the NCLC-H served from the Yale Peabody Museum (https:// collections.peabody.yale.edu/pb/nclc); the Daniel I. Axelrod cleared-leaf collections of the University of California Museum of Paleontology (UCMP; https://ucmp.berkeley. edu/collections/paleobotany-collection/ucmp-cleared-leaf-collection); the National Museum of Nature and Science (NMNS, Ibaraki, Japan) Cleared Leaf Database by Drs. Toshimasa Tanai and Kazuhiko Uemura (https://www.kahaku.go.jp/research/db/ geology-paleontology/cleared_leaf/database/?lg=en); leaf x-ray images of Australian rainforest plants by the late Dr. David C. Christophel and colleagues (Christophel and Hyland 1993; Christophel and Rowett 1996), some of which are maintained in the online Australian Tropical Rainforest Plants identification system (https://apps.lucidcentral.org/rainforest/text/intro/index.html); and the late Dr. Edward P. Klucking's book series illustrating cleared leaves from selected families (Klucking 1986-2003). We also note an open-access image dataset of cleared leaves from Borneo, consisting of small (1 cm²) lamina samples (Blonder et al. 2019; Xu et al. 2021). In most of the online image sets, bulk downloads are not easily done, images are downsampled to low resolution, and the filenames are not standardized, requiring significant manual effort to re-organize and collate them for a particular project. Adding further complications to data modularity, taxonomic data have often become partially obsolete.

Isolated fossil leaves present an additional set of challenging problems (e.g., Wilf 2008), including incomplete preservation, morphological convergence, and the wellknown legacy of innumerable taxonomic misidentifications in older publications (see Dilcher 1971, 1974; Hill 1982). Numerous high-quality systematic treatments have become available for many leaf-fossil taxa, especially over the last few decades, but the images are dispersed across publications and are usually of low resolution. An increasing number of images of vouchered fossil-leaf collections is available online from natural history museums. Examples include aggregator sites such as GBIF (gbif.org) and individual institutions such as the Yale Peabody Museum, (https://peabody.yale.edu/ collections/paleobotany), the Burke Museum (www.burkemuseum.org/collectionsand-research/geology-and-paleontology/collections-database/images.php), the University of Colorado Boulder Museum of Natural History (https://www.colorado.edu/ cumuseum/research-collections/paleontology/invertebrates-plants), and the UCMP (https://ucmpdb.berkeley.edu). Nevertheless, museum servers and project sites (e.g., Traiser et al. 2018) usually retain the taxonomy as published, which is vital for the nomenclatural stability of type specimens but well known to be problematic, especially

for the many older collections that have not been revised under modern standards. All these issues make it very difficult for researchers, students, and non-specialists to form a reliable base of knowledge about fossil-leaf identification and have perhaps engendered an overreliance on methods that do not require taxonomy at all, such as leaf morphotyping (see Wilf 2008).

Here, we meet the community need for a specialized dataset of leaf images by consolidating a set of original-resolution photographs of vouchered extant and fossil specimens (Fig. 1, Table 1), primarily of angiosperms, vetted to family level and relabeled to user-friendly filenames, into an open-access archive in a single, standard file format (jpeg, at minimum possible compression). A principal goal, based on many years of practical experience using leaf-image datasets in our research, is maximum and sustained ease of use with rapid access to the entire library. Thus, instead of creating an interactive database that may become obsolete and limit resolution or user flexibility, we simply provide the image files in labeled folders that can easily be downloaded, then viewed and searched using any visual browser (e.g., Adobe Bridge, Adobe Lightroom, Windows Explorer) on any suitable device, such as a personal computer.

The full image dataset and supporting data files are available open-access for download in a single Figshare Plus data collection at https://doi.org/10.25452/figshare. plus.14980698 (hereafter, "the Figshare archive"). The components described below

Collection	Collection	#Images	#Families	#Genera,	#Species,	Repository	Collection	Other data and images‡
	type			approx.	approx.		numbers†	
NCLC-Wolfe	cleared	16,249	267	3,893	12,439	USNM	secondary	http://clearedleavesdb.org
	leaves							
NCLC-	cleared	6,861	313	1,678	5,723	USNM	secondary	https://collections.peabody.yale.
Hickey	leaves							edu/pb/nclc
Axelrod	cleared	832	89	270	641	UCMP	primary	https://ucmpdb.berkeley.edu/
Cleared	leaves						· ·	photos/cleared_leaf.html
Leaves								-
Wing X-Rays	x-ray	2,234	26	416	890	USNM	secondary	n/a
	negatives							
Total extant		26,176	354	4,573	17,385			
Florissant,	fossil leaves	666	23	47	73	several	secondary	https://flfo-search.colorado.edu
Meyer et al.								-
(2008) project								
Florissant,	fossil leaves	2,654	21	40	70	FLFO	primary	https://www.flickr.com/
FLFO							· ·	photos/155340198@N06
General fossil	fossil leaves	756	39	93	135	several	primary	n/a
collection								
Total fossil		4,076	48	129	222			

 Table 1. Summary of component datasets.

Abbreviations: FLFO, Florissant Fossil Beds National Monument. NCLC, National Cleared Leaf Collection. UCMP, University of California Museum of Paleontology. USNM, National Museum of Natural History, Smithsonian Institution.

†As used in records specific to these collections and our image filenames. Secondary inventory numbers are those assigned by the creators of collections that were assembled from several primary collections. Examples are the cleared and x-rayed leaf samples physically gathered from primary herbarium sheets and the Meyer et al. (2008) Florissant photographic collection of specimens housed at numerous primary repositories. The Meyer et al. secondary (photograph) numbers have an informal "CU" prefix added here to the filenames, merely to distinguish them easily from the FLFO set in searches and not to indicate primary primary repository. See text for more details.

‡Images, specimen inventories, and other supporting data are available in the Figshare item accompanying this article: https://doi. org/10.25452/figshare.plus.14980698. Fossil taxa and references are listed in Appendix 1.



Figure 1. Selected image pairs of confamilial extant and fossil (see Appendix 1) leaves from the dataset A Batesia floribunda Spruce ex. Benth. (Fabaceae), NCLC-W 6417, showing typical layout of a cleared-leaf slide with original annotations (other examples are cropped in this figure); source voucher Froes 12074, DS 291771 (at CAS), Amazonas, Brazil B Fabaceae sp. CJ1, SGC-ICP-10173; Cerrejón mine, middle-late Paleocene of Guajira Peninsula, Colombia C Crataegus viridis L. (Rosaceae), NCLC-W 11951b; H. Meyer s/n (collected 1974, no other voucher), cultivated, California, USA D Crataegus copeana (Rosaceae), UCMP 3610; Florissant, late Eocene of Colorado, USA; H. Meyer photograph number 0420 E Tetracentron sinense Oliv. (Trochodendraceae), S. Wing negative 71-002; E.H. Wilson 659, US 599036, Szechuan, China F Ziziphoides flabellum (Trochodendraceae), USNM 560134; Mexican Hat, early Paleocene of Montana, USA G Quercus prinus L. (Fagaceae), NCLC-W 6137; H. Foster 8223, US 1730249, Florida, USA H Fagopsis longifolia (Fagaceae), FLFO 003432A; Florissant, late Eocene of Colorado, USA | Eucalyptus astringens (Maiden) Maiden (Myrtaceae), NCLC-W 10489; J.H. Maiden (9 November 1909), Western Australia, UC 437518 J Eucalyptus frenguelliana (Myrtaceae), MPEF-Pb 2344; Laguna del Hunco, early Eocene of Chubut, Argentina K Cercidiphyllum obtritum (Cercidiphyllaceae), DMNH 25061; Republic, early Eocene of Washington, USA L Cercidiphyllum japonicum Siebold & Zucc. ex J.J.Hoffm. & J.H.Schult.bis (Cercidiphyllaceae), Axelrod cleared leaf 166; UCMP (no other voucher) M Platanus racemosa Nutt. (Platanaceae), NCLC-H 6631; Handel s/n (collected 1985, no other voucher), California, USA N Erlingdorfia montana (compound-leaved Platanaceae), DMNH 7642; Hell Creek Formation, Late Cretaceous of North Dakota, USA. Scale bars: centimeters as labeled (A, B, L, M); 1 cm when not labeled (C-K, N).

are summarized in Table 1, along with relevant online resources where many of the specimens can already be searched, usually at lower resolution. Although individual linkage of each specimen with online resources would be desirable, it is highly impractical at present because the necessary tags and lookup tables have never been compiled and vetted for most of the collections used here. For readability, we use "leaves" to refer to all specimens discussed here, whether they are leaves, leaflets, or other plant organs that are included in small numbers.

Cleared and x-rayed leaves

The cleared and x-rayed leaf-image collections included here were chosen for availability of a large number of botanically diverse, high-quality images, accessible voucher data, and open-access re-use permissions. The collections primarily represent non-monocot ("dicot") angiosperm leaves, with minor representation of monocots, other vascular plant groups, and non-foliar plant organs. Several other large cleared and x-rayed leaf collections exist (see Introduction) but were not used in the dataset presented here for various reasons. For example, the significant cleared-leaf atlas series by Klucking (1986–2003) was manually scanned, cropped, and made into a dataset as part of a machine-learning study (Wilf et al. 2016); however, that dataset is not retained here due to comparatively low resolution, moiré patterns, and other artifacts of printing (Wilf et al. 2016). In addition, the University of California Berkeley collection of over 800 vouchered cleared leaves (distinct from the Axelrod collection) has not been included here because it has not yet been digitized.

A master inventory of the 26,176 images of cleared and x-rayed specimens from >4,500 genera and >17,300 extant species in 354 plant families (Table 1) is provided in the accompanying Figshare archive. A small number of specimens are represented by multiple images, such as close-ups or lighting variants. Taxonomic fields include the family, genus, and species as provided in the respective collection catalog, with additional fields for updated Angiosperm Phylogeny Group (APG) family and order (APG IV 2016). Taxonomic and geographic coverage are uneven, constrained by the general availability of herbarium materials to the creators of the collections (similar issues occur even in recent, large herbarium-image datasets; e.g., de Lutio et al. 2021). Eight families are represented by more than 1,000 images each (Fabaceae, Sapindaceae, Rosaceae, Fagaceae, Annonaceae, Rubiaceae, Ulmaceae, and Malvaceae), whereas 173 families have fewer than ten images apiece. Photographs were taken by many people (see Acknowledgments) over an extended period of time and at different institutions, with a wide variety of cameras and methods that we do not attempt to detail; however, the original camera or scanner EXIF (Exchangeable Image File Format) metadata remain embedded in most of the images and are viewable in standard file browsers. We have also maintained the original pixel resolution and image dimensions in all photographs.

Catalog numbers of cleared or x-rayed leaves in the master inventory (available in the accompanying Figshare archive) refer to a unique glass slide (for the cleared leaves) or a film-negative number (for the x-rays) used to organize the respective collection, as designated by the creator of the collection. The catalog numbers of the cleared and x-rayed leaf collections are usually secondary, i.e., specific to the collection but linked in museum records (as legacy data and thus without hyperlinks) to a primary source voucher at a herbarium (Table 1). Thus, the collection-specific secondary numbers are usually the information needed to search the specimens online (using resources listed in Table 1 and further described below) or in paper catalogs to locate the primary source-voucher data. In some cases there is no herbarium or other voucher besides the mount-ed slide, and then the curated cleared-leaf specimen, usually specially collected for the purpose, is a primary collection. We also provide in the accompanying Figshare archive a catalog file containing the voucher data for the Wing X-Ray Collection, for which data are not otherwise available online. In publications, specimens should be formally cited by primary voucher as well as secondary catalog number if possible (see Fig. 1).

Family and order updates were done iteratively by first doing automatic lookups to family of the catalog genera and species, using the tables provided in The Plant List (www.theplantlist.org) and its successor, World Flora Online (WFO; www. worldfloraonline.org; Borsch et al. 2020). These resources include standardized lookups to family and order for most generic and species names, modified slightly to include a few taxa not listed in WFO. Failed lookups were flagged and corrected manually. Most lookup failures resulted from typographical errors of generic and species names in the catalog data, and these were manually corrected. Others resulted from genera not being listed or having ambiguous or unverified family status in WFO; these taxa were then manually vetted using other standard resources such as Tropicos (www.tropicos.org), the International Plant Names Index (www.ipni.org), and the Angiosperm Phylogeny Website (www.mobot.org/MOBOT/research/APweb). For consistency, the WFO was designated as the priority lookup for conflicting results among taxonomic databases.

For reference, we note other online resources for batch-vetting plant names that we did not use, including Taxonomic Names Resolution Service (tnrs.iplantcollaborative. org), taxize (github.com/ropensci/taxize), and the Kew Vascular Plant Families and Genera database (data.kew.org/vpfg1992/vascplnt.html). In addition, an automated tool, the WORLDFLORA R package, is now available for batch lookups from the WFO taxonomic backbone file (Kindt 2020), although this would not have resolved the large number of taxa with uncertain status in WFO that required manual vetting.

Due to the intensive labor that would be required to update the large number of names below family level, even with the aid of batch services, and the emphasis here on family-level vetting, generic and species names were for the most part not updated except to correct misspellings that would hinder future lookups. A full vetting below family level would also require manually consulting and hyperlinking all the primary herbarium records to check for new determinations, a process of several years. However, any user can easily find taxa of interest using the specimen list provided (accompanying Figshare archive) and access updated nomenclature and voucher data using the resources listed.

The resulting master inventory of cleared and x-rayed leaves was manually inspected repeatedly to eliminate variant spellings and other inconsistencies, until no more were found. Even after this stage, many issues remained from duplicate and corrupt files, invalid paths, labeling errors, ghost folders of problem images, and other common legacy database errors. Automated and reproducible data analysis and cleaning was done (by J. Rose and R. Saha) largely in Jupyter Notebooks and scripted in Python. In an iterative process, we used the Pandas library to load, sort, and filter the dataset in the form of a table, mapping metadata values in each column to unique specimens in each row. From there, we verified each file path's full compliance with a pair of requirements, namely that it be both (a) a unique absolute path, and (b) a valid path specifying an existing, uncorrupted image file that can be successfully opened and closed. Rows that failed this test were flagged and taken out for manual review.

Further file path cleaning included the use of a fuzzy matching algorithm, through which all possible matches between a flagged query file path q and a possible near-duplicate reference path f_R were compared by calculating the Levenshtein Distance (e.g., https://xlinux.nist.gov/dads/HTML/Levenshtein.html). This distance serves as a measure of the character-level similarity between two strings, from which all pairs are sorted in order of decreasing similarity to the flagged file q. Several duplicated source files that had evaded detection in previous stages were identified in this way, by manually scanning the top few most similar matches and searching for signs of typos. This procedure for automating the identification of the tens of thousands of species in thousands of genera, hundreds of families, and dozens of orders included any artificial categories created by a misspelling. An example could be two samples from the same family, where one's family was spelled "Fabaceae" (correct), whereas the other was accidentally entered as "Fabeceae." This is an easy typo to miss, but it can skew downstream analyses.

Once all taxonomic and archival fields were validated, we assigned each sample a new filename that accomplishes both (a) directly encoding multiple levels of metadata into human-readable format within the filename, and (b) allowing easy sorting and searching of files on disk, without any additional alterations or struggling with a full relational database. The new filename format is constructed in the form: "Family_Genus_species_Collection_Catalog number". This user-friendly format facilitates, for the first time, rapid alphabetic sorting, visual inspection, and searching of all the merged images from multiple sources in standard personal-computer windows and visual browsers. In the filenames, as just described, the family is updated to APG standard according to World Flora Online and other resources, whereas the genus and species fields are usually not updated except to correct spelling errors, especially those that could cause lookup failures.

National cleared leaf collection – NCLC-W and NCLC-H

The National Cleared Leaf Collection is derived from parallel, broadly collaborative efforts supervised by the late Drs. Jack A. Wolfe (NCLC-W) and Leo J. Hickey (NCLC-H), beginning in the late 1960s. The NCLC is the world's largest and most phylogenetically comprehensive assembly of cleared, stained, and mounted leaves sampled primarily from vouchered herbarium sheets. The collections underpinned the scientists' research on fossil leaves and leaf architecture, including their landmark evolutionary survey (Hickey and Wolfe 1975). Hickey and Wolfe (1975) reported that the clearing techniques they used were those of Foster (1952), as adapted by Hickey (1973); Dilcher (1974) further described the techniques of Hickey and Wolfe. More recent work has improved the methods for clearing and mounting leaves without deterioration and provided historical methods reviews (Vasco et al. 2014; García-Gutiérrez et al. 2020). The Wolfe and Hickey cleared-leaf collections, kept separately during the scientists' lifetimes and without any intention to merge them to our knowledge, are now curated together in the Division of Paleobotany, Department of Paleobiology, NMNH as the National Cleared Leaf Collection, constituting a monumental resource for leaf architecture that is combined here digitally for the first time. Physically, the two sub-collections are adjacent but not merged because Wolfe and Hickey used somewhat different family delimitations as they assembled their collections, and these are retained in the organization of the slides at NMNH (their systems were standardized and merged digitally for this contribution, as described earlier). The slides are organized alphabetically by family within each sub-collection.

The Wolfe contribution (NCLC-W) is the larger of the two parts, comprising over 18,000 specimens, from which 16,249 images are available here (Table 1). As described at the Cleared Leaf Image Database website (http://clearedleavesdb.org), the largest contributing source for NCLC-W was the University of California Herbarium (UC), Berkeley. Other significant sources were the California Academy of Sciences (CAS; including the Dudley Herbarium, DS, formerly of Stanford University), the Herbarium of the Arnold Arboretum (A) of the Harvard University Herbaria, the Missouri Botanical Garden (MO), the New York Botanical Garden (NY), the Field Museum of Natural History (F), and the National Herbarium of the Smithsonian Institution (US). Wolfe kept his collection for many years as a core reference for his voluminous body of work on fossil angiosperm leaves (see Upchurch et al. 2007), first at the United States Geological Survey (USGS) in Menlo Park, then at USGS Denver. Various photographic projects to document the collection advanced during the 1980s and 1990s, though none of these was published.

Following Dr. Wolfe's retirement in 1992, S. Wing supervised the moving and curation of the cleared-leaf collection from Denver to NMNH, as well as, after Dr. Wolfe's passing in 2005, a small portion of the collection that Wolfe had kept in his emeritus position at the University of Arizona. The collection was re-assembled, loaded into NMNH cabinetry, partially repaired, photographed, and placed under curation in the Division of Paleobotany, Department of Paleobiology, NMNH, officially as NCLC-W. A registry kept on paper by Dr. Wolfe and his team, containing the herbarium voucher data for all slides, is also kept with the collection; the registry was professionally transcribed into a digital format, then updated and corrected by E. González-Akre and several other Smithsonian staff members and volunteers. The photographs used in this contribution were made by another large group of Smithsonian staff and

volunteers (see Acknowledgments). Most slides have approximately the same physical dimensions, although some are oversize to accommodate large leaves; scale bars are included on most photographs (e.g., Fig. 1A). The photographs and collections data for NCLC-W were separately archived several years ago in the Cleared Leaves Image Database (http://clearedleavesdb.org; Das et al. 2014), also under open-access but at lower resolution than we provide here. We refer the reader to that useful platform to look up primary specimen metadata online using Wolfe's (secondary; Table 1) catalog numbers, including the herbarium-voucher data. Exact nomenclature may vary from what is presented here, following our separate vetting process.

The NCLC-W has been used extensively as a reference library, especially by paleobotanists; one notable example is its service as a principal reference for identifying leaf fossils from the oldest Neotropical paleorainforests, the Paleocene Cerrejón and Bogotá formation floras of Colombia (Herrera et al. 2008; Wing et al. 2009; Carvalho et al. 2021a, 2021b). Many images from NCLC-W (and NCLC-H) were used to illustrate leaf characters in the Manual of Leaf Architecture (Ellis et al. 2009), and the collection was used in a study of leaf rank and areole size (Green et al. 2014). A selection of more than 5,000 NCLC-W images was used for training and testing for family recognition as part of a machine-learning study that also included computer-marked heat maps, showing diagnostic regions for machine identification (Wilf et al. 2016).

Professor Leo J. Hickey supervised the assembly of a parallel cleared-leaf collection to Wolfe's during his time as curator of paleobotany at NMNH (Wing et al. 2014), comprising more than 7,000 slides, from which 6,861 images are included here (Table 1). Dr. Hickey made a successful effort to sample complementary taxa to Dr. Wolfe, thus increasing the combined diversity of their collections considerably (Table 1). Hickey targeted a larger number of herbaceous taxa, partly reflecting his interest in herbaceous early angiosperms (e.g., Taylor and Hickey 1992). Nearly all specimens were sampled at US, with minor contributions from MO, NY, and several other herbaria, along with a small amount of freshly sampled or fluid-preserved material. Dr. Hickey borrowed the collection that he made when he relocated to the Yale Peabody Museum of Natural History (YPM) in 1982. Web access to images of NCLC-H and additional information about the collection are still provided by the Yale Peabody Museum (https://collections.peabody.yale.edu/pb/nclc/), where slides were imaged and inventoried by a large team (see Acknowledgments) under the direction of Drs. Hickey and S. Hu. The same photographs are aggregated here as summarized in the master inventory (available in the accompanying Figshare archive), and full metadata and source voucher information for each slide are available at https://collections.peabody. yale.edu/pb/nclc and from YPM staff. In NCLC-H, primary herbarium-voucher data are usually visible in the photographs on labels that were mounted with the leaves. The physical size of the slides varies, and scale bars are included on most photographs. Following Dr. Hickey's passing in 2013, NCLC-H was returned to NMNH, where it is now curated in the Division of Paleobotany, Department of Paleobiology, adjacent to NCLC-W as just mentioned.

Axelrod cleared leaf collection

The Daniel I. Axelrod Cleared Leaf Collection at UCMP includes about 1,300 specimens that are in exceptionally good condition, compared with the NCLC, because the late Dr. Axelrod (Barbour et al. 1998) mounted them in plexiglass with a medium, possibly clear epoxy, that has remained clear for over 50 years. The slides mostly represent the California flora. They are a self-standing primary collection not linked to herbarium vouchers, and only general locality data are given on the slide labels, but nevertheless the material comprises a well-curated museum collection with good preservation and high image quality in the photographs. The UCMP has provided the Axelrod images online for many years through several portals linked from the UCMP Cleared Leaf Collection web page (https://ucmp.berkeley.edu/collections/paleobotany-collection/ucmp-clearedleaf-collection). Scale bars are included in all photographs, of which 832 are used here (Table 1). A selection of images from the Axelrod collection was used for training and testing of automatic leaf recognition in the Wilf et al. (2016) machine-learning study.

Wing X-ray collection

In the early 1990s, S. Wing developed an x-ray scanning technique (Wing 1992) and used it to capture leaf and other organ images of selected families on large-format (8 by 10 inches, or 20.3 by 25.4 cm) x-ray negatives. The specimens are mostly from US, along with a variety of other herbaria and living collections; the negatives are now archived in the Division of Paleobotany, Department of Paleobiology, NMNH, and a separate data item is made available in the accompanying Figshare archive that matches the negative numbers (preserved in the current filenames) with their vouchers. The 1200-dpi cropped scans of the negatives by S. Wing are made available here digitally for the first time. Although the images lack embedded scales, the direct contact method of imaging with x-rays means that the images on physical negatives are the same size as the original specimens, and the negatives were scanned 1:1 as well. Thus, measurements can be made directly from the images or calibrated, if needed, using the post-crop image dimensions in the image metadata. Grayscale values of the scanned negatives were batch-inverted to positive here (easily reversed with a second inversion), to provide light backgrounds and improve comparability with the other image sets. The reverse grayscale tends to accentuate the visual impact of large differences in exposure caused by variation in leaf density; however, we found that standard image level and contrast adjustments are sufficient to make fine details more visible when needed. The collection includes a sizable number of x-rays of reproductive organs, especially Sapindaceae fruits, which are retained here for their general interest.

Fossil leaves

We provide 4,076 vouchered leaf-fossil images of specimens that are assigned to family level, in total covering 44 angiosperm and four non-angiosperm families from a variety of sites in the Americas that are well known to the authors (Table 1; Appendix 1).

Although far from comprehensive, this image set nevertheless covers at least a majority of angiosperm families that are reliably known in the fossil record from nearly-complete leaf remains; it provides a starter set both for comparative learning in angiosperm paleobotany and training machine-learning algorithms.

Unlike the images from the cleared and x-rayed collections, which were not adjusted except for cropping of the x-rays, the fossil-leaf images were all manually and reversibly rotated, close-cropped, and contrast- and temperature-adjusted (all whole-image adjustments, other than cropping) in Adobe Camera Raw so that they are approximately similar in relative frame alignment and overall contrast, with emphasis on making vein features visible (for some photographs taken on early-model digital cameras with barrel distortion in macro mode, the lens distortion was corrected manually using Adobe Camera Raw). This procedure minimizes strong distractors such as rock matrix for machine learning of fossil leaves, an interest of several of the authors (Wilf et al. 2016), and we found that it also enhances human learning for the same reason, by increasing visual comparability of the leaf features and eliminating distractors and variable orientation. In all cases, we have maintained the full pixel resolution and (post-crop) dimensions of the original image and resaved processed images from Camera Raw to jpeg format only once (usually with tiff format as a lossless intermediate step), using the minimum compression ratio to maintain image quality. A cost of this approach was removal of most of the scale bars. However, nearly all scaling information can be accessed if needed from online (usually much lower resolution but suitable for scaling) versions of the images or sets of uncropped originals that we have included where necessary (see General Collection, below). In addition, all physical voucher specimens can be accessed at their respective repositories. As for the cleared and x-rayed leaves, original camera or scanner EXIF data remain embedded in the image metadata.

The fossil set of 4,076 images is comprised of two parts (Table 1, Appendix 1): first, a concentrated collection of 3,320 images from a single prolific site, the late Eocene Florissant fossil beds of Colorado; and second, a smaller general collection of 756 images from a variety of latest Cretaceous and Paleogene sites in North and South America (Appendix 1; accompanying Figshare archive). Appendix 1 annotates and lists authorities and taxonomic references for the ca. 222 species used in the fossil dataset, and individual catalog numbers are embedded in the filenames of the images. Appendix 1 also lists references for site-specific collections that pertain directly to the specimens used here, if the latter are different from the taxonomic references. Some specimens are represented by multiple images, such as close-ups or lighting variations (but not by duplicate images), and many images of counterparts are included. Although the major target for the collection was "dicot" leaves, images of a few species of monocots, ferns, and conifers that were readily available were included to help seed future expansions. Several generic names that may be botanically doubtful are left in as-published or ascataloged form (Appendix 1), but all included material is considered reliably placed at family level. Informal morphotypes are included if they have reliable features at family level. Filenames, as for the cleared and x-rayed leaves, embed taxonomy to enable rapid auto-sorting and searching in standard PC windows, followed by collection data.

Florissant collection

The late Eocene Florissant Fossil Beds Lagerstätte of Colorado is known worldwide for its long history of collection and investigation, its outstanding diversity of plant and animal fossils, and its seminal role in the conservation movement (MacGinitie 1953; Evanoff et al. 2001; Meyer 2003; Leopold et al. 2008; Veatch and Meyer 2008; Leopold and Meyer 2012). Florissant's diverse fossil flora has a long history of study, resulting in an exceptional level of taxonomic understanding (e.g., Lesquereux 1873, 1883; MacGinitie 1953; Manchester and Crane 1983; 1987; Manchester 1989a, 2001a; Jia and Manchester 2014; Herendeen and Herrera 2019). The late Harry D. MacGinitie's (1953) landmark monograph of the Florissant flora was outstanding among comparable works of the time for the high quality and botanical accuracy of his descriptions and identifications (Manchester 2001a).

Among its many distinctions, the Florissant biota was one of the first large fossil assemblages of any kind to be photographed, cataloged comprehensively, and made openly available in an internet database (Meyer et al. 2008). This massive effort by H. Meyer and associates, beginning in the 1990s, has two components as described below. The Florissant images were manually filtered and prepared by X. Zou from an initial set of 13,691 images of plant, animal, and geological specimens, of which 7,798 are of plants and 6,122 are of leaves, from which we further filtered and prepared the 3,320 images used here of leaf specimens that can be confidently placed in a plant family (Appendix 1). Vetting to plant family followed Manchester (2001a) and other publications as listed in Appendix 1.

The first of two components of the Florissant image set (Table 1) comes from the Meyer et al. (2008) project to capture high-resolution images of all type, published, and related Florissant collections, representing 5,663 specimens of ca. 1800 fossil plant and animal species as described in >300 scientific papers from a total Florissant collection of ca. 50,000 specimens. Much of this material had never been illustrated or was illustrated poorly by modern standards. The fossils are held in about 15 museums around the world as listed by Meyer et al. (2008); the largest three Florissant type and published collections are at the Smithsonian National Museum of Natural History, the Harvard University Museum of Comparative Zoology (almost entirely insects and spiders), and the University of Colorado Museum of Natural History. The original photographs on Kodachrome slides are archived at Florissant Fossil Beds National Monument (FLFO), and they were scanned twice about 12-15 years apart to take advantage of improving technology, the second time at high resolution. The resulting image database of the more recent scans (Meyer et al. 2008) was hosted on a National Park Service server initially and then moved several years ago to the University of Colorado Museum of Natural History, where it can be searched online using the Florissant Fossil Beds Collection Search at https://flfo-search.colorado.edu. That website provides full specimen metadata and reduced-resolution image files (with scale bars), which can be searched using the secondary inventory (photograph) numbers from the Meyer et al. (2008) project that are here embedded in the filenames (Table 1). To help distinguish images in this collection from the others rapidly in searches, we have also

attached an informal "CU" prefix (for the University of Colorado) to the secondary catalog numbers in the filenames.

The second component of the Florissant image collection provided here (Table 1) is a selection of fully digital images from the collections at Florissant Fossil Beds National Monument (primary acronym FLFO, specimen number embedded in the filename), assembled by H. Meyer and numerous interns and assistants. The images can also be searched and viewed (with scale bars but at lower resolution) by FLFO number on the park's flickr page, located at https://www.flickr.com/photos/155340198@N06. The corresponding, full-resolution, uncropped images that were processed here from both Florissant image sets are archived at the University of Colorado Museum of Natural History and FLFO, respectively, and available on request to collections management.

General collection

The general collection of 756 fossil leaf images provided here (Appendix 1; specimen data in the accompanying Figshare archive) draws from a set of Late Cretaceous to Eocene fossil floras from the Americas. The general collection diversifies the phylogenetic, preservational, temporal, and geographic coverage of the overall fossilimage dataset and forms a base to encourage other teams to make similar efforts. Repositories of the material are indicated in the filenames and supplemental data files in the accompanying Figshare archive; they include the Denver Museum of Nature & Science (repository acronym DMNH); Museo Paleontológico Egidio Feruglio (MPEF-Pb, Trelew, Argentina); National Museum of Natural History, Smithsonian Institution (USNM-PAL, abbreviated here as USNM, Washington, D.C.); Colombian Geological Survey and Colombian Petroleum Institute (combined as SGC-ICP, Bogotá, Colombia); Florida Museum of Natural History (UF, Gainesville); and University of California Museum of Paleontology (UCMP, Berkeley). Filenames embed the taxonomy as well as primary repository numbers or unique field numbers, if a formal repository number is not assigned (some MPEF, USNM specimens). In a few cases, where more than one image of the same fossil is included (i.e., close-ups or unlabeled parts and counterparts), an informal tag is included in the filename in brackets to ensure uniqueness of filenames. Some material lacks formal taxonomy but is considered reliably identified at family level; there are also a few cases of historic generic identifications considered incorrect and indicated by quotations (e.g., "Acer," "Ficus") but still assignable to a (often different) family (Appendix 1). Because very few fossils from the general collection are otherwise viewable online to check scale bars, we provide a parallel folder of the corresponding uncropped images in the accompanying Figshare archive.

Major contributions to the general collection are briefly listed here for paleobotanists, with additional taxonomic and occurrence references listed in Appendix 1. The fossils come from (1) a suite of latest Cretaceous (late Maastrichtian, Hell Creek Formation) and early Paleocene (early Danian, Fort Union Formation) sites from western North Dakota and South Dakota USA that have been used extensively for studies of the end-Cretaceous extinction (e.g., Johnson et al. 1989; Johnson 2002; Wilf and Johnson 2004); (2) the early Paleocene Salamanca Formation (early Danian) and Las Flores (late Danian) floras of Chubut, Argentina, known for diverse and well-preserved fossil plants and insect-feeding damage following the end-Cretaceous extinction (e.g., Iglesias et al. 2007, 2021; Clyde et al. 2014; Donovan et al. 2017; Stiles et al. 2020); (3) the early Paleocene (Danian, Fort Union Formation) Mexican Hat site in southeastern Montana, USA, known for diverse insect herbivory traces preserved in its fossil leaves (Wilf et al. 2006; Winkler et al. 2010; Donovan et al. 2014); (4) the middle-late Paleocene (Selandian-Thanetian, Cerrejón Formation) Cerrejón flora from the Guajira Peninsula, Colombia and Bogotá Formation flora of Sabana de Bogotá, central Colombia, together preserving the remains of the oldest known Neotropical rainforests (e.g., Doria et al. 2008; Herrera et al. 2008, 2019; Gómez-Navarro et al. 2009; Wing et al. 2009; Carvalho et al. 2011, 2021a, 2021b); (5) a suite of sites spanning the late Paleocene (Fort Union Formation) through early Eocene (Wasatch Formation and Little Mountain locality of the Green River Formation) of southwestern and northwestern Wyoming that have been used in many studies of floristic and plant-insect associational responses to climate change (e.g., Gemmill and Johnson 1997; Wilf et al. 1998, 2006; Wilf and Labandeira 1999; Wilf 2000; Donovan et al. 2014); (6) the early Eocene Laguna del Hunco Lagerstätte in Chubut, Argentina (Huitrera Formation), known for its outstanding diversity of fossil plants and animals, varied biogeographic connections, and large number of unique taxon occurrences for South America (e.g., Wilf et al. 2003, 2013, 2019; Gandolfo et al. 2011); (7) the late early Eocene flora of Republic, Washington (Wolfe and Wehr 1987; DeVore et al. 2005; Greenwood et al. 2016; Klondike Mountain Formation) and the middle Eocene Green River Formation flora (MacGinitie 1969; Smith et al. 2008) of Bonanza, Utah, specifically using images of field-censused collections at DMNH from both sites led by K. Johnson that were used previously for analyses of insect herbivory, fossil-leaf economics, and digital leaf physiognomy (Wilf et al. 2001, 2005b; Cariglino 2007; Royer et al. 2007; Peppe et al. 2011).

Concluding remarks

The dataset presented here consolidates thousands of hours of labor by many people (see Acknowledgments) into a single accessible platform. Due to the extraordinary effort involved, it is unlikely that many new, large-scale cleared and x-rayed leaf collections will ever be assembled and digitally processed. Thus, the future prospects for significantly increasing the overall sample size and improving the coverage of taxonomy and geography in digital leaf-reference collections most likely lie elsewhere. The greatest potential appears to come from the advancing techniques for segmenting and enhancing leaf images from the enormous, widely available resource of digitized herbarium sheets (Hussein et al. 2020; Weaver et al. 2020), which have the significant additional advantage of direct linkage to the global data infrastructure for biodiversity (e.g., Bakker et al. 2020). To reach comparability with cleared leaves, segmented leaf
images will require high pixel resolution, optimized contrast for the capture of venation details, and the careful retention of significant edge features such as the leaf margin. For leaf fossils, increasing the sample size of well-identified specimens is straightforward in principle but will require efforts far beyond the resources of a single collaboration. Thus, we plan a community initiative for this purpose.

We look forward to seeing the assembled image dataset catalyze advances in research, education, and outreach. The images and supporting data are available openaccess on Figshare Plus at https://doi.org/10.25452/figshare.plus.14980698. Some mistakes are inevitable in a first-version database of this nature; please report any errors observed to the corresponding author. Corrections and updates may be applied to the Figshare archive under new version numbers; the version precisely corresponding to this article will remain preserved as version 1.0.

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Appendix I

Species list for fossil-leaf images.

Species	#Images	References				
Drvopteridaceae	88					
Dryopteris guyottii (Lesquereux) MacGinitie	64	Florissant, late Eocene, Colorado, USA	MacGinitie 1953			
Cupressaceae						
Chamaecyparis linguaefolia (Lesquereux)	100	Florissant, late Eocene, Colorado,	MacGinitie 1953; Manchester 2001a			
MacGinitie, Chamaecyparis sp.		USA				
Sequoia affinis Lesquereux, Sequoia sp.	153	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a			
Pinaceae						
Pinus florissantii Lesquereux	9	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a			
Pinus macginitieii Axelrod	1	Florissant, late Eocene, Colorado, USA	Axelrod 1986; Manchester 2001a			
Pinus wheeleri Cockerell, Pinus sp.	55	Florissant, late Eocene, Colorado, USA	Cockerell 1908; MacGinitie 1953; Manchester 2001a			
Taxaceae						
<i>Torreya geometrorum</i> (Cockerell) MacGinitie, <i>Torreya</i> sp.	9	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a			
Araceae						
Araceae sp. CJ80	2	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Wing et al. 2009			
<i>Limnobiophyllum scutatum</i> (Dawson) Krassilov	2	Florissant, late Eocene, Colorado, USA	see Manchester 2001a			
Montrichardia aquatica Herrera et al.	4	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Herrera et al. 2008			
Petrocardium cerrejonense Herrera et al.	1	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Herrera et al. 2008			
Petrocardium wayuuorum Herrera et al.	1	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Herrera et al. 2008			
Arecaceae						
Arecaceae spp. CJ67, CJ68	4	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Gómez-Navarro et al. 2009; Wing et al. 2009			
Rhipogonaceae						
<i>Ripogonum americanum</i> R.J. Carpenter et al.	2	Laguna del Hunco, early Eocene, Chubut, Argentina	Carpenter et al. 2014			
Zingiberaceae						
Zingiberaceae spp. CJ49, CJ65	3	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Wing et al. 2009			
Adoxaceae		·				
Sambucus newtoni Cockerell	33	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a			

Species	#Images	es Source, age, region† References			
Akaniaceae	0				
Akania patagonica Gandolfo et al.	9	Laguna del Hunco, early Eocene, Chubut, Argentina	Gandolfo et al. 1988; Wilf et al. 2005a‡		
Altingiaceae					
<i>"Acer" lesquereuxi</i> Knowlton	2	Little Mountain & Bonanza, early (LM) and middle (B) Eocene, Wyoming (LM) & Utah (B), USA	MacGinitie 1969; Johnson and Plumb 1995; Wilf et al. 2005b‡		
Anacardiaceae					
Anacardiaceae sp. CJ34	4	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Wing et al. 2009		
Anacardiaceae sp. TY203	1	Laguna del Hunco, early Eocene, Chubut, Argentina	P. Wilf, unpubl. obs.		
Rhus lesquereuxi Knowlton & Cockerell	21	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Rhus malloryi Wolfe & Wehr	5	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡; Flynn et al. 2019		
Rhus nigricans (Lesquereux) Knowlton	27	Little Mountain & Bonanza, early and middle Eocene, Wyoming & Utah, USA	MacGinitie 1969; Wilf 2000‡; Wilf et al. 2001‡		
Rhus obscura (Lesquereux) MacGinitie	22	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
<i>Rhus stellariaefolia</i> (Lesquereux) MacGinitie, <i>Rhus</i> sp.	175	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Schmalzia (Rhus) vexans (Lesquereux) Cockerell	5	Florissant, late Eocene, Colorado, USA	MacGinitie 1953		
Apocynaceae					
Apocynaceae sp. RR17	1	Wasatch Fm., early Eocene, Wyoming, USA	Wing 1998; Wilf 2000‡		
Atherospermataceae					
Atherospermophyllum guinazui (E.W. Berry) C.L. Knight	16	Laguna del Hunco, early Eocene, Chubut, Argentina	Knight and Wilf 2013		
Berberidaceae					
Mahonia marginata (Lesquereux) Arnold	15	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Mahonia subdenticulata (Lesquereux) MacGinitie, Mahonia sp.	11	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Betulaceae					
Alnus parvifolia (E.W. Berry) Wolfe and Wehr	33	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡		
Alnus sp. RR14	1	Wasatch Fm., early Eocene, Wyoming, USA	Wilf 2000		
Betula leopoldae Wolfe and Wehr	4	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡		
<i>Betula</i> sp. RP21	1	Republic, early Eocene, Washington, USA	Wilf et al. 2005b		
Corylites spp. BB05, FW01	3	Fort Union Fm., several sites, late Paleocene, Wyoming, USA	Manchester and Chen 1996; Wilf 2000‡		
Paracarpinus fraterna (Lesquereux) Manchester & Crane Paracarpinus sp	58	Florissant, late Eocene, Colorado, USA	Manchester and Crane 1987; Manchester 2001a		
Cannabaceae					
Celtis aspera (Newberry) Manchester	3	Fort Union Fm., several sites, late	Wilf 2000‡: Manchester et al. 2002		
et al.§	5	Paleocene, Wyoming, USA	2018; Wilf et al. 2006‡		
Cercidiphyllaceae		, , , , , , , , , , , , , , , , , , , ,			
Archeampelos lobatocrenata (Lesquereux) Doweld	2	Fort Union Fm., several sites, late Paleocene, Wyoming, USA	McIver and Basinger 1993; Wilf 2000‡; Wilf et al. 2006‡; Manchester 2014; Doweld 2016		

Species	#Images	Source, age, region†	References		
Cercidiphyllum obtritum (Dawson) Wolfe and	12	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡		
Wehr					
<i>Trochodendroides genetrix</i> (Newberry) Manchester	13	Fort Union Fm., several sites, early & late Paleocene, Montana, North Dakota, & Wyoming, USA	Wilf 2000‡; Johnson 2002‡; Wilf et al. 2006‡; Manchester 2014		
Cornaceae					
<i>Beringiaphyllum cupanioides</i> (Newberry) Manchester et al.	3	SW Wyoming, several sites, late Paleocene, Wyoming, USA	Manchester et al. 1999; Wilf 2000‡; Wilf et al. 2006‡		
<i>Browniea serrata</i> (Newberry) Manchester & Hickey	6	Fort Union Fm., several sites, late Paleocene, Wyoming, USA	Wilf 2000‡; Wilf et al. 2006‡; Manchester and Hickey 2007		
Cornus sp. RP62	3	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡		
Cornus swingii Manchester et al.	2	Fort Union Fm., several sites, late Paleocene, Wyoming, USA	Wilf 2000‡; Wilf et al. 2006‡; Manchester et al. 2009		
<i>Davidia antiqua</i> (Newberry) Manchester	4	Fort Union Fm., several sites, late Paleocene, Wyoming, USA	Wilf 2000‡; Manchester 2002; Wilf et al. 2006‡		
Cunoniaceae			· · · · · · · · · · · · · · · · · · ·		
Cunoniaceae sp. SA020	13	Salamanca Fm., early Paleocene, Chubut, Argentina	Iglesias et al. 2007, 2021		
Cunoniaceae sp. TY116	9	Laguna del Hunco, early Eocene, Chubut, Argentina	Wilf et al. 2005a; Merkhofer et al. 2015		
Ericaceae					
Ericaceae sp. RP41	1	Republic, early Eocene, Washington, USA	Wilf et al. 2005b‡		
Rhododendron sp. RP53	1	Republic, early Eocene, Washington, USA	Wilf et al. 2005b‡		
Euphorbiaceae					
Euphorbiaceae sp. CJ24	4	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Wing et al. 2009		
Stillingia casca Hickey	1	Wasatch Fm., early Eocene, Wyoming, USA	Hickey 1977; Wilf 2000‡		
Fabaceae		, ,			
Caesalpinia pecorae Brown	9	Little Mountain & Bonanza, early and middle Eocene, Wyoming & Utah, USA	MacGinitie 1969; Wilf 2000‡; Wilf et al. 2001‡		
<i>Caesalpinites acuminatus</i> (Lesquereux) MacGinitie	13	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Caesalpinites coloradicus MacGinitie	21	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Cercis parvifolia Lesquereux	23	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Jia and Manchester 2014		
<i>Conzattia coriacea</i> MacGinitie, Fabaceae sp.	21	Florissant, late Eocene, Colorado, USA	MacGinitie 1953		
Fabaceae sp. SA045	1	Salamanca Fm., early Paleocene, Chubut, Argentina	Iglesias et al. 2007, 2021; Brea et al. 2008		
Fabaceae sp. TY117	14	Laguna del Hunco, early Eocene, Chubut, Argentina	Wilf et al. 2005a		
Fabaceae spp. CJ1, CJ19, CJ55	15	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Wing et al. 2009; Herrera et al. 2019		
Fabaceae spp. GR554, GR567	3	Little Mountain, early Eocene, Wyoming, USA	Wilf 2000		
Fabaceae spp. morphotypes 2, 3	3	Cogua & Nemocón mines, late Paleocene, Cogua, Cundinamarca, Colombia	Herrera et al. 2019		
<i>Gymnocladus hesperia</i> (Brown) MacGinitie	1	Little Mountain, early Eocene, Wyoming, USA	MacGinitie 1969; Wilf 2000‡		
Leguminosites lespedezoides MacGinitie	1	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		

Species	#Images	Source, age, region†	References
Leguminosites lesquereuxiana	6	Bonanza, middle Eocene, Utah, USA MacGinitie 1969; Wilf et al.	
(Knowlton) Brown			
Parvileguminophyllum coloradensis	30	Little Mountain & Bonanza, early	Call and Dilcher 1994; Wilf 2000‡; Wilf
(Knowlton) Call & Dilcher		and middle Eocene, Wyoming & Utah, USA	et al. 2001‡
<i>"Prosopis" linearifolia</i> (Lesquereux) MacGinitie	23	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Robinia lesquereuxi (Ettingshausen)	39	Florissant, late Eocene, Colorado,	MacGinitie 1953; Manchester 2001a
MacGinitie		USA	
Fagaceae			
Castanea dolichophylla Cockerell	15	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
<i>Castaneophyllum patagonicum</i> Wilf et al.	17	Laguna del Hunco, early Eocene, Chubut, Argentina	Wilf et al. 2019
cf. Quercus sp. GR522	2	Little Mountain, early Eocene, Wyoming, USA	Wilf 2000
Fagaceae spp. RP060, RP154	2	Republic, early Eocene, Washington, USA	Manchester and Dillhoff 2004; Wilf et al. 2005b
Fagopsis longifolia (Lesquereux) Hollick, Fagopsis sp., cf. Fagopsis sp.	521	Florissant, late Eocene, Colorado, USA	Manchester and Crane 1983; Manchester 2001a
Quercus dumosoides MacGinitie	9	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Quercus lyratiformis Cockerell	1	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Quercus mohavensis Axelrod	9	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Quercus orbata MacGinitie	11	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Quercus peritula Cockerell	12	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Quercus predayana MacGinitie	8	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Quercus scottii (Lesquereux) MacGinitie	37	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Quercus scudderi Knowlton, Quercus sp.	46	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Grossulariaceae			
Ribes errans MacGinitie	3	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Hamamelidaceae			
Langeria magnifica Wolfe and Wehr	2	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡
Hydrangeaceae			
Philadelphus minutus MacGinitie	1	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Iteaceae			
Itea sp. RP19	3	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡; Hermsen 2013
Juglandaceae			
<i>Carya libbeyi</i> (Lesquereux) MacGinitie, <i>Carya</i> sp.	53	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 1987; 2001a
Juglandaceae sp. BB09	1	Bison Basin, late Paleocene, Wyoming, USA	Gemmill and Johnson 1997; Wilf 2000
Juglandaceae sp. GR519	1	Little Mountain, early Eocene, Wyoming, USA	Wilf 2000
<i>Juglandiphyllites glabra</i> (R. Brown) Manchester and Dilcher	7	Fort Union Fm., several sites, early & late Paleocene, Montana & Wyoming, USA	Manchester and Dilcher 1997; Wilf 2000‡; Wilf et al. 2006‡

Species	#Images	Source, age, region†	References
Platycarya americana Hickey	1	Wasatch Fm., early Eocene, Wyoming, USA	Wing and Hickey 1984; Manchester 1987; Wilf 2000‡
Platycarya castaneopsis (Lesquereux) Wing & Hickey	1	Wasatch Fm., early Eocene, Wyoming, USA	Wing and Hickey 1984; Manchester 1987; Wilf 2000‡
Lauraceae			
"Ficus" planicostata Lesquereux	1	Battleship, Maastrichtian, North Dakota, USA	Johnson 2002‡
Lauraceae sp. RR19	1	Wasatch Fm. several sites, early Eocene, Wyoming, USA	Wilf 2000
Lauraceae sp. SA010	17	Salamanca Fm., early Paleocene, Chubut, Argentina	Iglesias et al. 2007, 2021
Lauraceae sp. TY084	21	Laguna del Hunco, early Eocene, Chubut, Argentina	Wilf et al. 2005a
Lauraceae spp. CJ5. CJ22	8	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Wing et al. 2009
Lauraceae spp. FW02, FW03, FW28	6	Fort Union Fm., several sites, late Paleocene, Wyoming, USA	Wilf 2000
Laurophyllum piatnitzkyi E.W. Berry	12	Salamanca & Peñas Coloradas fms., early Paleocene, Chubut, Argentina	Iglesias et al. 2007, 2021‡
Lindera coloradica MacGinitie	4	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Lindera varifolia MacGinitie	18	Little Mountain, early Eocene, Wyoming, USA	MacGinitie 1969; Wilf et al. 2001‡
Marmarthia pearsoni K. Johnson	5	Hell Creek Fm., several sites, Maastrichtian, North Dakota, USA	Johnson 1996, 2002
Marmarthia trivialis K. Johnson	2	Madeline's Bank, Hell Creek Fm., Maastrichtian, North Dakota, USA	Johnson 1996, 2002
Persites argutus Hickey	11	Fort Union Fm., several sites, late Paleocene, Wyoming, USA	Hickey 1977; Wilf 2000‡; Wilf et al. 2006‡
"Sassafras" hesperia E.W. Berry	2	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
"Sassafras" hesperia E.W. Berry	6	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡
Magnoliaceae			
Liriodendrites bradacii K. Johnson	1	Madeline's Bank, Hell Creek Fm., Maastrichtian, North Dakota, USA	Johnson 1996, 2002
Malvaceae			
"Dombeya" novi-mundi Hickey	1	Wasatch Fm., several sites, early Eocene, Wyoming, USA	Hickey 1977; Wilf 2000‡; Carvalho et al. 2011
Malvaceae sp. TY023	16	Laguna del Hunco, early Eocene, Chubut, Argentina	Wilf et al. 2005a; Wilf 2008
Malvaceae spp. CJ25, CJ36	3	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Wing et al. 2009; Carvalho et al. 2011
<i>Malvaciphyllum macondicus</i> M. Carvalho	6	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Wing et al. 2009; Carvalho et al. 2011
<i>Tilia johnsoni</i> Wolfe & Wehr	1	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡
<i>Triumfetta ovata</i> MacGinitie	4	Little Mountain, early Eocene, Wyoming, USA	MacGinitie 1969; Wilf 2000‡
Meliaceae			
Cedrela lancifolia (Lesquereux) Brown	35	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a
Meliaceae sp. CJ2	3	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Wing et al. 2009
Menispermaceae			
Menispermaceae sp. GR507	1	Little Mountain, early Eocene, Wyoming, USA	Wilf 2000
Menispermites cerrejonensis Doria et al.	6	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Doria et al. 2008

Species	#Images	Source, age, region†	References		
Menispermites cordatus Doria et al.	1	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Doria et al. 2008		
Menispermites horizontalis Doria et al.	1	Cerrejón mine, middle-late Paleocene, Guajira Peninsula, Colombia	Doria et al. 2008		
Wilkinsoniphyllum menispermoides Jud et al.	1	Salamanca Fm., early Paleocene, Chubut, Argentina	Iglesias et al. 2007, 2021; Jud et al. 2018		
Monimiaceae					
<i>Monimiophyllum callidentatum</i> C.L. Knight	1	Laguna del Hunco, early Eocene, Chubut, Argentina	Knight and Wilf 2013		
Myricaceae					
Comptonia columbiana Dawson	1	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡		
Myrtaceae					
<i>Eucalyptus frenguelliana</i> Gandolfo & Zamaloa	13	Laguna del Hunco, early Eocene, Chubut, Argentina	Gandolfo et al. 2011; Hermsen et al. 2012		
<i>Eugenia arenaceaeformis</i> (Cockerell) MacGinitie	16	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Myrtaceae sp. TY41	1	Laguna del Hunco, early Eocene, Chubut, Argentina	Wilf et al. 2005a		
<i>Syzygioides americana</i> (Lesquereux) Manchester et al.	5	Little Mountain, Bonanza, & Wasatch Fm., early and middle Eocene, Wyoming & Utah, USA	Manchester et al. 1998; Wilf 2000‡; Wilf et al. 2005b‡		
Platanaceae					
<i>Erlingdorfia montana</i> (Brown) K. Johnson	15	Hell Creek Fm., several sites, Maastrichtian, North Dakota, USA	Johnson 1996, 2002		
Grewiopsis saportana Lesquereux	8	Hell Creek Fm., several sites, Maastrichtian, North Dakota, USA	Johnson 2002‡		
Leepierciea preartocarpoides K. Johnson	5	Hell Creek Fm., several sites, Maastrichtian, North Dakota, USA	Johnson 1996, 2002		
Macginitiea gracilis (Lesquereux) Wolfe and Wehr	4	Fort Union Fm., several sites & Republic, late Paleocene, early Eocene, Washington & Wyoming, USA	Wolfe and Wehr 1987; Wilf 2000‡		
Macginitiea wyomingensis (Knowlton & Cockerell) Manchester	9	Little Mountain & Bonanza, early and middle Eocene, Wyoming & Utah, USA	Manchester 1986; Wilf 2000‡; Wilf et al. 2005b‡		
<i>Platanites marginata</i> (Lesquereux) K. Johnson	7	Hell Creek Fm., several sites, Maastrichtian, North Dakota, USA	Johnson 1996, 2002		
<i>Platanites raynoldsii</i> (Newberry) Manchester	21	Fort Union Fm., several sites, early & late Paleocene, Montana, North Dakota, & Wyoming, USA	Wilf 2000‡; Johnson 2002‡; Wilf et al. 2006‡; Manchester 2014		
Platanus sp. GR506	1	Little Mountain, early Eocene, Wyoming, USA	Wilf 2000		
Proteaceae					
Lomatia occidentalis (E.W. Berry) Frenguelli	32	Laguna del Hunco, early Eocene, Chubut, Argentina	González et al. 2007		
Lomatia preferruginea E.W. Berry	9	Laguna del Hunco, early Eocene, Chubut, Argentina	González et al. 2007		
Proteaceae gen. et sp. indet. (sp. TY208)	1	Laguna del Hunco, early Eocene, Chubut, Argentina	González et al. 2007		
Rhamnaceae					
Hovenia cf. H. oregonensis Meyer & Manchester	1	Wasatch Fm., several sites, early Eccene, Wyoming, USA	Meyer and Manchester 1997; Wilf 2000‡		
Rhamnaceae sp. TY025	15	Laguna del Hunco, early Eocene, Chubut, Argentina	Wilf et al. 2005a		
<i>Rhamnica cleburnii</i> (Lesquereux) Doweld	1	Battleship, Maastrichtian, North Dakota, USA	McIver and Basinger 1993; Johnson 2002‡; Doweld 2016		
Suessenia grandensis Jud et al.	4	Rancho Grande, early Paleocene, Chubut, Argentina	Jud et al. 2017 (source of images used)		

Species	#Images	Source, age, region†	References		
<i>Zizyphus florissantii</i> (Lesquereux) MacGinitie.	11	Florissant, late Eocene, Colorado, MacGinitie 1953; Mancheste USA			
Rosaceae					
Amelanchier scudderi Cockerell, Amelanchier sp.	10	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Cercocarpus myricaefolius (Lesquereux) MacGinitie, Cercocarpus sp.	146	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Wolfe and Schorn 1990; Manchester 2001a		
Crataegus copeana (Lesquereux) MacGinitie	33	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
<i>Crataegus hendersoni</i> (Cockerell) MacGinitie	4	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
<i>Crataegus nupta</i> (Cockerell) MacGinitie, <i>Crataegus</i> sp.	10	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Crataegus sp., aff. Crataegus spp. RP32, RP42	6	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡		
Holodiscus lisii Schorn	2	Florissant, late Eocene, Colorado, USA	Schorn 1998; Manchester 2001a		
<i>Malus florissantensis</i> (Cockerell) MacGinitie	2	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
<i>Malus pseudocredneria</i> (Cockerell) MacGinitie	1	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Photinia pageae Wolfe & Wehr	3	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡		
Prunus gracilis (Lesquereux) MacGinitie	4	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Prunus sp. RP54	2	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡ DeVore and Pigg 2007		
Rosa hilliae Lesquereux, Rosa sp.	23	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Rosaceae sp. RP61	2	Republic, early Eocene, Washington, USA	Wilf et al. 2005b		
<i>Rubus coloradensis</i> (MacGinitie) Wolfe & Tanai	2	Florissant, late Eocene, Colorado, USA	Wolfe and Tanai 1987		
<i>Spiraea</i> sp. RP29	8	Republic, early Eocene, Washington, USA	Wolfe and Wehr 1987; Wilf et al. 2005b‡; DeVore and Pigg 2007		
<i>Vauquelinia coloradensis</i> (Knowlton) MacGinitie	52	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
<i>Vauquelinia lineara</i> MacGinitie, <i>Vauquelinia</i> sp.	7	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Salicaceae					
Populus cinnamomoides (Lesquereux) MacGinitie	2	Little Mountain, early Eocene, Wyoming, USA	MacGinitie 1969; Wilf 2000‡; Manchester et al. 2006		
Populus crassa (Lesquereux) Cockerell, Populus sp.	93	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Manchester 2001a		
Populus tidwellii Manchester et al.	3	Bonanza, middle Eocene, Utah, USA	MacGinitie 1969; Wilf et al. 2001‡; Manchester et al. 2006		
Populus wilmattae Cockerell	4	Bonanza, middle Eocene, Utah, USA	MacGinitie 1969; Wilf et al. 2001‡		
Populus wyomingiana (E.W. Berry) MacGinitie	1	Wasatch Fm., several sites, early Eocene, Wyoming, USA	MacGinitie 1974; Wilf 2000‡		
Salix cockerelli Brown	8	Bonanza, middle Eocene, Utah, USA	MacGinitie 1969; Wilf et al. 2001‡		
<i>Salix ramaleyi</i> Cockerell, <i>Salix</i> sp.	15	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; J.A. Wolfe pers. comm. in Manchester 2001a		
Salix taxifoliodes MacGinitie	2	Florissant, late Eocene, Colorado, USA	MacGinitie 1953		
Sapindaceae					
Acer florissantii Kirchner, Acer sp.	36	Florissant, late Eocene, Colorado, USA	MacGinitie 1953; Wolfe and Tanai 1987		
Aesculus hickeyi Manchester	3	Fort Union Fm., several sites, late Paleocene, Wyoming, USA	Wilf 2000‡; Manchester 2001b; Wilf et al. 2006‡		

Species	#Images	Source, age, region†	References
Allophylus flexifolia (Lesquereux)	10	Little Mountain & Bonanza, early	MacGinitie 1969; Wilf 2000‡; Wilf et
MacGinitie		and middle Eocene, Wyoming &	al. 2001‡
		Utah, USA	
Athyana haydenii (Lesquereux0	106	Florissant, late Eocene, Colorado,	MacGinitie 1953
MacGinitie		USA	
Bohlenia insignis (Lesquereux) Wolfe	16	Florissant, late Eocene, Colorado,	Wolfe and Wehr 1987; Manchester
& Wehr		USA	2001a; McClain and Manchester 2001
"Cardiospermum" coloradensis	5	Little Mountain & Bonanza, early	MacGinitie 1969; Wilf 2000‡; Wilf et al.
(Knowlton) MacGinitie		and middle Eocene, Wyoming &	2001‡; Jud et al. 2021
		Utah, USA	
"Cardiospermum" terminalis	41	Florissant, late Eocene, Colorado,	MacGinitie 1953; Manchester 2001a;
(Lesquereux) MacGinitie,		USA	Jud et al. 2021
"Cardiospermum" sp.			
Koelreuteria allenii (Lesquereux)	28	Florissant, late Eocene, Colorado,	MacGinitie 1953; Manchester 2001a;
Edwards		USA	Wang et al. 2013
Sapindaceae sp. TY018	18	Laguna del Hunco, early Eocene,	Wilf et al. 2005a
		Chubut, Argentina	
Schoepfiaceae			
Schoepfia republicensis (La Motte) Wolfe	2	Republic, Wasatch Fm., early Eocene,	Wolfe and Wehr 1987; Wilf 2000‡; Wilf
& Wehr		Washington & Wyoming, USA	et al. 2005b‡
Theaceae			
Theaceae sp. RP49	2	Republic, early Eocene, Washington,	Wolfe and Wehr 1987; Wilf et al.
		USA	2005b‡
Trochodendraceae			
Trochodendron nastae Pigg et al.	1	Republic, early Eocene, Washington,	Pigg et al. 2001; Wilf et al. 2005b‡
		USA	Manchester et al. 2018
Ziziphoides flabellum (Newberry) Crane	9	Fort Union Fm., several sites, early	Crane et al. 1991; Johnson 2002‡; Wilf
et al.		Paleocene, Montana, North Dakota,	et al. 2006‡
		& Wyoming, USA	
Ziziphoides sp. RP37	1	Republic, early Eocene, Washington,	Pigg et al. 2001; Wilf et al. 2005b‡
		USA	
Ulmaceae			
Cedrelospermum lineatum (Lesquereux)	978	Florissant, late Eocene, Colorado,	Manchester 1989a, 2001a
Manchester, Cedrelospermum sp.		USA	
Cedrelospermum nervosum (Newberry)	30	Little Mountain & Bonanza, early	Manchester 1989a; Wilf 2000‡; Wilf et
Manchester		and middle Eocene, Wyoming &	al. 2001‡
		Utah, USA	
Ulmites microphylla (Newberry)	1	Wasatch Fm. several sites, early	Wilf 2000‡; Manchester 2014
Manchester		Eocene, Wyoming, USA	
Ulmus sp. RP17, Zelkova sp. RP50	8	Republic, early Eocene, Washington,	Wolfe and Wehr 1987; Wilf et al.
		USA	2005b‡ Denk and Dillhoff 2005
Ulmus tenuinervis Lesquereux,	30	Florissant, late Eocene, Colorado,	Manchester 1989b, 2001a
Ulmaceae sp., Ulmus sp.		USA	
Vitaceae			
"Vitis florissantella"	11	Florissant, late Eocene, Colorado,	MacGinitie 1953, 1969; Manchester
		USA	2001a

† Fossil sites listed are only those that sourced the fossils in this dataset and may not include type localities and other occurrences of the species.

‡ Occurrence reference for the dataset in this paper, when different from the taxonomic reference.

§ Doweld (2016) proposed revision of *Celtis aspera* to *Rhamnites asperus* (Newberry) Doweld, which we acknowledge but do not adopt here.

RESEARCH ARTICLE



Achnanthidium gladius sp. nov. (Bacillariophyceae) – a new monoraphid diatom species from Indonesia

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Abstract

A new monoraphid diatom species *Achnanthidium gladius* **sp. nov.** is described from Indonesia. The description is based on molecular data (18SV4), morphological analysis and comparison with similar species. According to molecular data, *Achnanthidium gladius* sp. nov. is closely related to *Achnanthidium minutissimum*. Morphologically, the new species differs from similar species by the absence of a fascia on raphe valve, cell size, and striae density and pattern. The new species is only known from the type locality in Indonesia. Comparison with close related species is given.

Keywords

Achnanthidium, diatoms, Indonesia, new species, molecular investigation

Introduction

The genus *Achnanthidium* Kützing is one of the largest genera of monoraphid diatoms. It contains more than two hundred species which are widely distributed worldwide in various types of water bodies. *Achnanthidium* was first suggested by F. Kützing (1844), who included in it all monoraphid species that do not form colonies. Later, P.T. Cleve (1895) changed the status of this taxon and made it a subgenus of *Achnanthes* s.l. Most of monoraphid diatoms were included in *Achnanthes* s.l., and this system was used by many authors for a long time.

Round et al. (1990) reinstated *Achnanthidium* as a separate genus, with the explanation that *Achnanthes* and *Achnanthidium* differ significantly in the structure of areolae, girdle, raphe, and plastids. Round and Bukhtiyarova (1996) suggested an improved diagnosis for the genus which included the following features: radiate uniseriate striae, small linear-lanceolate to elliptic-lanceolate valves, external distal raphe ends straight or curved to one side, and sternum widened at the centre. The species that belonged to *Achnanthidium* but did not correspond with this diagnosis were moved to other genera.

Since the beginning of the 21st century many descriptions of new *Achnanthidium* species have been published (Monnier et al. 2004; Cantonati and Lange-Bertalot 2006; Ivanov and Ector 2006; Potapova 2006; Wojtal et al. 2010; Liu et al. 2021). Furthermore, the type materials of earlier described species are studied using light microscopy (LM) and scanning electron microscopy (SEM) (Hlúbiková et al. 2011; Van de Vijver et al. 2011). This data is used for clearer definition of species boundaries and separation of species complexes. Also, there have been attempts to separate species complexes using morphometric methods (Potapova and Hamilton 2007) and a complex of morphological, ecological and geographic studies (Jüttner et al. 2011).

Many authors note that the taxonomy of *Achnanthidium* is quite complicated for a number of reasons. First of all, most species of this genus are quite small, which makes light microscopy studies more difficult. Most of the features used for species identification are ultrastructural, so that SEM is required for precise identification. Also, the species boundaries may be unclear, since there are no criteria for species separation and values of quantitative features often overlap in different species, which complicates identification even further. Moreover, many species are quite similar in terms of morphology and require the usage of molecular methods. There are few molecular studies of *Achnanthidium* species. In a recent article (Pinseel et al. 2017) 12 different lines have been identified in the *Achnanthidium minutissimum* (Kützing) Czarnecki species complex on the basis of molecular studies. The representatives of one of these lines have been described as a separate species *Achnanthidium digitatum* Pinseel, Vanormelingen, Hamilton & Van de Vijver (Pinseel et al. 2017).

In Indonesia, *Achnanthidium* has not been studied extensively. Most existing works concern the ecology and general biodiversity of diatoms in different water bodies (Hustedt 1937; Bramburger et al. 2004; Sabo et al. 2008; Soeprobowati et al. 2016) but not the taxonomy of the genus. Indonesia is a less studied region with a high level

of endemicity (Bramburger et al. 2004; Kapustin et al. 2017, 2019, 2020; Kapustin and Kulikovskiy 2018; Kociolek et al. 2018; Kulikovskiy et al. 2020), which indicates a high probability of finding new diatom species there.

Materials and methods

Sample collection

The sample used in this study was collected from Indonesia by Ivanov I.I. on 23.09.2011. It was designated as no. I241 and was collected from the Lake Matano (02°31.985'S, 121°26.279'E), epipsammon, pH 8.36, conductivity 187 μ S cm⁻¹.

Culturing

Monoclonal strains were established by micropipetting single cells under an inverted microscope Zeiss Axio Vert. A1. The culture was maintained in the liquid medium WC (Guillard and Lorenzen 1972) in a lightbox with the photoperiod day:night 12:12 hours and temperature = 22–25 °C. The culture was grown for a month. The strain was designated Ind391.

Preparation of slides and microscopic observation

The monoclonal culture was boiled in 30% hydrogen peroxide at the temperature 150–160 °C for 8 hours to dissolve organic matter. After decanting and refilling up to 100 ml with deionized water, the suspension was spread onto coverslips and left to dry at room temperature. Permanent diatom preparations were mounted in Naphrax (refraction index=1.73). LM observations were performed with a Zeiss Axio Scope.A1 microscope equipped with an oil immersion objective (×100, n.a. 1.4, differential interference contrast) and Axio Cam ERc 5s camera. Valve ultrastructure was examined using a JEOL JSM-6510LV scanning electron microscope.

Molecular investigation

Total DNA of the strain Ind391 was extracted using HelixTM (Bio-Rad Laboratories, USA) according to the manufacturer's protocol. A fragment of 18S rDNA (435 bp, including V4 domain) was amplified using primers D512 and D978 (Zimmermann et al. 2011). Amplification of the 18S rDNA fragment was carried out using the premade mix ScreenMix (Evrogen, Russia) for the polymerase chain reaction (PCR). The conditions of amplification for 18S rDNA fragment were: an initial denaturation of 5 min at 95 °C, followed by 35 cycles: denaturation at 94 °C (30 s), annealing at 52 °C (40 s), elongation at 72 °C (50 s); and a final extension of 5 min at 72 °C.

The resulting amplicons were visualized by horizontal agarose gel electrophoresis (1%), colored by SYBR Safe (Life Technologies, United States). Purification of DNA fragments was performed with the mix of FastAP, 10× FastAP Buffer, Exonuclease I (Thermo Fisher Scientific, USA) and water. 18S rDNA fragment was decoded from two sides using forward and reverse PCR primers and the Big Dye system (Applied Biosystems, USA), followed by electrophoresis using a Genetic Analyzer 3500 sequencer (Applied Biosystems).

Editing and assembling of the consensus sequences were carried out by comparing the direct and reverse chromatograms using the Ridom TraceEdit program (ver. 1.1.0) and Mega7 (Kumar et al. 2016). Newly determined sequence and DNA fragments from 32 other diatoms, which were downloaded from GenBank (taxa and Accession Numbers are given in Fig. 1), were included in the alignments. Diatom species from genera *Geissleria* Lange-Bertalot & Metzeltin and *Placoneis* Mereschkowsky were chosen as the outgroup. The nucleotide sequences of the 18S rDNA gene were aligned separately using the Mafft v7 software and the E-INS-i model (Katoh and Toh 2010). The resulting alignment had the length of 441 characters.

The dataset was analyzed using the Bayesian interference (BI) method implemented in Beast ver.1.10.1 (Drummond and Rambaut 2007) to construct phylogeny. For each of the alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.10 (Darriba et al. 2012). This BIC-based model selection procedure selected HKY+I+G model, shape parameter $\alpha = 0.5800$ and a proportion of invariable sites (pinvar) = 0.7460. A Yule process tree prior was used as a speciation model. The analysis ran for 15 million generations with chain sampling every 100 generations. The parameters - estimated convergence, effective sample size (ESS) and burn-in period were checked using the software Tracer ver. 1.7.1. (Drummond and Rambaut 2007). The initial 25% of the trees were removed, the rest remained to reconstruct a final phylogeny. The phylogenetic tree and posterior probabilities of its branching were obtained on the basis of the remaining trees, having stable estimates of the parameter models of nucleotide substitutions and likelihood. Maximum Likelihood (ML) analysis was performed using the program RA×ML (Stamatakis et al. 2008). The nonparametric bootstrap analysis with 1000 replicates was used. The statistical support values were visualized in FigTree (ver. 1.4.2) and Adobe Photoshop CC (19.0).

Results

Figure 1

The molecular analysis has established that the strain *Achnanthidium gladius* sp. nov. belongs to a group of close species that includes *A. minutissimum* and *A. digitatum* (ML 87/BI 100). A branch that includes three strains of two species (*Achnanthidium minutissimum* and *A. saprophilum*) is also close (ML 87/BI 100). Overall, the new species belongs to a large clade of monoraphid and cymbelloid taxa from such genera as *Cocconeis, Pauliella, Psammothidium, Planothidium, Geissleria, Placoneis*, and others.

The morphological description of the new species is given below.



Figure 1. Phylogenetic position of *Achnanthidium gladius* Ind391 (indicated in bold) based on Bayesian inference for the partial 18S rDNA gene. Total length of the alignment is 441 characters. Bootstrap supports from ML (constructed by RaxML) are presented above the horizontal lines (slash). Posterior probabilities from BI (constructed by Beast) are presented below the horizontal lines (slash). Only BS and PP above 50 and 0.9 are shown. All sequences have strain numbers (if available) and GenBank numbers.

Achnanthidium gladius Tseplik, Kulikovskiy, Glushchenko & Genkal, sp. nov. Figures 2, 3

Holotype. Slide no 04123 in collection of MHA, Main Botanical Garden Russian Academy of Science, Moscow, Russia, represented here by Fig. 2G.

Reference strain. Strain Ind391, isolated in sample no. I241.

Type locality. Indonesia, Lake Matano, 02°31.985'S, 121°26.279'E.

Description. *LM* (Fig. 2). Valves relatively big, linear to linear-lanceolate, with gradually narrowing, rounded apices. Length 27.7–30.4 μ m, breadth 3.3–4.0 μ m. Raphe straight, filiform. Striae on the raphe valve radiate, uniseriate, 27–28 in 10 μ m, consist of round and narrow elongated areolae. On the rapheless valve the central and axial areas are absent. Striae are almost parallel, 26–27 in 10 μ m, becoming noticeably more radiate near the valve apices. Areolae not resolved in LM.

SEM (Fig. 3). External proximal raphe ends simple, distal raphe ends straight. Internal proximal raphe ends slightly bent in opposite directions, distal raphe ends terminate is weakly developed helictoglossae. Raphe positioned in a narrow axial area that slightly widens near the centre of the valve. A distinct central area is absent, but 2 or 3 striae in the center of the valve are spaced wider than the majority of the striae. Areolae are mostly elongated.

Etymology. The specific epithet "*gladius*" refers to the similarity in valve morphology contour with a sword.

Distribution. As yet known only from the type locality.



Figure 2. A–X *Achnanthidium gladius* Tseplik, Kulikovskiy, Glushchenko & Genkal, sp. nov. LM, DIC, size diminution series. Slide no 04123. **A–L** raphe valves **M–X** rapheless valves. Holotype (**G**). Scale bar: 10 µm.

Sequence data. Partial 18S rDNA gene sequence comprising V4 domain sequence (GenBank accession number).

Discussion

The new species *Achnanthidium gladius* sp. nov. belongs to the genus *Achnanthidium* and possesses the characteristic features of the genus, including a linear-lanceolate valve shape, radiate uniseriate striae and straight external distal raphe ends. *Achnanthidium* species are divided into two morphological groups: the *A. minutissimum* species complex has straight external distal raphe ends, and the *Achnanthidium pyrenaicum* (Hustedt) Kobayasi species complex has external distal raphe ends that are distinctly curved to one side. Since *A. gladius* sp. nov. has straight external distal raphe ends, it belongs to the *A. minutissimum* species complex. The new species has quite large valves, which is characteristic only for several of the known *Achnanthidium* species (Table 1).

There are some known *Achnanthidium* species that are morphologically similar to *A. gladius* sp. nov. The most similar is *Achnanthidium initium* Karthick, Taylor & Hamilton (Karthick et al. 2017); however, it can be differentiated from the new species



Figure 3. A–H *Achnanthidium gladius* Tseplik, Kulikovskiy, Glushchenko & Genkal, sp. nov. SEM. Sample no 04123. **A–F** raphe valves **G, H** rapheless valves **A–C, G, H** external views **D–F, H** internal views. Scale bars: 2 µm (**A, D, G, H**), 1 µm (**C, F**), 0.5 µm (**B, E**).

	Outline	Valve apices	Axial area	Central area	Valve length (µm)	Valve width (µm)	Striae in raphe valve (in 10 μm)	Striae in rapheless valve (in 10 μm)	Distribution	Reference
<i>A gladius</i> sp. nov.	relatively big, linear to linear- lanceolate	gradually narrow- ing, rounded	absent	absent, but 2 or 3 striae in the center of the valve are spaced wider than the majority of the striae	27.7– 30.4	3.3-4.0	27–28	26–27	Indonesia: Lake Matano	This study
A. initium	linear- lanceolate to lanceolate	rounded to weakly rostrate rounded	slightly expanded into asymmetrical transverse fascia at central area at the raphe valve; narrow linear at the rapheless valve	asymmetrical transverse fascia at the raphe valve; slightly expanded at the rapheless valve	11.5-25.5	9.0–13.0	29–34	32–35	India: Masilla Waterfalls	Karthick et al. 2017
A. sublanceolatum	linear- lanceolate	rounded or weakly pro- tracted	narrow, linear- lanceolate	rounded at the raphe valve; slightly expanded at the rapheless valve	18–35	4.0-4.5	20–23 at the middle portion, 36–42 near the apices	21–24 in the center, and 30–36 near the apices	China: Taiping Lake	Yu et al. 2019
A. standeri	not or very slightly dorsiventral, subelliptical- lanceolate, dorsal and ventral margins moderately arched	pro- tracted, apiculate to rostrate	narrow, linear, narrowing slightly towards to the ends, almost median line of the valve	irregular, asym- metric space of different extent	8–37	2.8-4.4	28–30	24–26	South Africa	Taylor et al. 2011

Table 1. Comparison of new species with similar taxa of *Achnanthidium*.

by several features. The most obvious one is the fascia on the raphe valve of *A. initium*, while *A. gladius* sp. nov. lacks a central area; also *A. initium* has external distal raphe ends that are curved to opposite sides. Furthermore, these species differ in valve length $(27.7-30.4 \ \mu\text{m} \text{ in } A. gladius \text{ sp. nov.}, 11.5-25.5 \ \mu\text{m} \text{ in } A. initium$) and the striae density on both raphe and rapheless valves (raphe valves: 27-28 in 10 \ \mm m in *A. gladius* sp. nov., 29-34 in 10 \ \mm in *A. initium*; rapheless valves: 26-27 in 10 \ \mm m in *A. gladius* sp. nov., 32-35 in 10 \ \mm m in *A. initium*).

Two more similar species are *Achnanthidium sublanceolatum* Yu, You & Kociolek (Yu et al. 2019) and *Achnanthidium standeri* Taylor, Morales & Ector (Taylor et al. 2011). *A. sublanceolatum* is similar to the new species in valve shape and size, but has lower striae density (20–23 in 10 μ m on the raphe valve, 21–24 in 10 μ m on the rapheless valve) and curved external distal raphe ends. *A. standeri*, as well as the new

species, belongs to the *A. minutissimum* species complex. It can be differentiated by the fascia on the raphe valve and radiate striae on the rapheless valve.

On the phylogenetic tree the strain *A. gladius* sp. nov. belongs to the clade that includes a lot of *A. minutissimum* strains. The strain used in the present study forms a cluster with three *A. minutissimum* strains, but is separated from them with high statistical support, which indicates that our strain is a separate species. Although *A. minutissimum* and *A. gladius* sp. nov. are closely related, morphologically they are significantly different, which confirms the identification of *A. gladius* sp. nov. as a separate species. The group that includes the new species is sister to a cluster of *A. digitatum* strains, which was recently separated from *A. minutissimum*.

Extensive molecular investigations are required for better understanding of taxonomy in the genus *Achnanthidium* and the *A. minutissimum* species complex. Often it is impossible to separate species of the genus without molecular methods. However, molecular data is available for a very small number of *Achnanthidium* species: Gen-Bank has sequences for 13 identified species and several sequences that are labeled as *Achnanthidium* sp. Including DNA sequences in new species descriptions makes the subsequent identification of these species much easier, and also contributes to the establishment of a database of various *Achnanthidium* strains.

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RESEARCH ARTICLE



Two new species of *Rubus* L. (Rosaceae) from the western Andes of Ecuador

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Abstract

Two new species of *Rubus* (Rosaceae) from the western Andes of northern Ecuador are described. *Rubus longistipularis* D.A. Espinel-Ortiz & Romol. is a scandent or climbing shrub found in the mountain forests of Chocó Andino from northern Ecuador. *Rubus maquipucunensis* D.A. Espinel-Ortiz & Romol. is a vine or climbing shrub found in the rainforests of Chocó Andino from Pichincha and Santo Domingo de los Tsáchilas. The species mentioned here are morphologically differentiated from all the *Rubus* species from Ecuador with a detailed botanical description, illustrations and photographs. We also report, for the first time, possible hybridisation between *R. longistipularis* and *R. boliviensis* Focke, as the samples reviewed showed mixed characteristics from both species.

Keywords

Andean slopes, blackberry, Chocó, Ecuadorian, Rubeae, taxonomy

Introduction

Rubus L. is the most diverse genus of the Rosaceae family with more than 500 species classified in 14 subgenera (Focke 1910, 1911, 1914; Kalkman 1987; Jarvis 1992; Bean 1997). The genus presents a cosmopolitan distribution with most spe-

cies in Asia, Europe and North America (Lingdi and Boufford 2003). Recently, Carter et al. (2019) suggested that the genus originated in North America, and migrated repeatedly to the other continents, thus explaining that these events may have favoured the high diversity of this genus in Asia, Europe and the world. In South America, only four subgenera with fewer than 50 species have been reported, mostly throughout the Andes (Romoleroux 1996; Romoleroux et al. 2014; Espinel-Ortiz and Romoleroux 2020).

In Ecuador, the genus *Rubus* inhabits most of the Andean ecosystems from 450 to 4500 m a.s.l. However, it is most abundant from 2300 to 3500 m a.s.l., especially in disturbed areas, in hedges, clearings and on margins of cloud forests, and in "páramo" regions (Espinel-Ortiz and Romoleroux 2020). Here, 22 species of *Rubus* have been recorded and classified in three subgenera: *Idaeobatus* (Focke) Focke, *Orobatus* Focke and *Rubus* L. The introduced subgenus *Idaeobatus* comprises 3 species, while the native subgenus *Rubus* consists of 10 native species, and Andean endemic subgenus *Orobatus* entails 7 native and 2 endemic species in the country (Romoleroux 1996; Espinel-Ortiz and Romoleroux 2020). The species described here belong to the subgenus *Rubus*, and both are distributed on the western Andean slopes of northern Ecuador. Herbarium specimens representing these species showed low sample numbers and were often annotated as *Rubus boliviensis*, *Rubus glaucus* and *Rubus floribundus* because of the resemblance to these species. However, vegetative and reproductive characters of the new species differ greatly from those of the species reported by Romoleroux (1996).

Methodology

Rubus collections of Herbaria HUTI, Q, QAP, QCA and QCNE were revised, and samples not fitting with the species reported for Ecuador were studied. Additional samples from AAU, DAV, HA and MO were revised from online images.

The botanical terms used in the descriptions followed those used by Stearn (1986), and the pubescence types were based on the terms of Hickey and King (2000), and Wilhelm and Rericha (2020). Some specimens examined for the descriptions (e.g. D. Espinel-Ortiz & H.G. Abad 281) were mounted in more than one herbarium sheet, and/or have additional dry or alcohol material, and each part had its own herbarium barcode (DAV, QAP and QCA). For these samples, we wrote all the herbarium barcodes for each part in type and examined specimens when available.

The geographic coordinate from sample P. Delprete & G. Webster 6073 (QCA-240552) was misplaced, since the sample was collected in Ecological Reserve Maquipucuna (Pichincha) and the coordinate of the label was from Ecological Reserve Cotacachi-Cayapas (Esmeraldas), approximately 42 km to the north. Therefore, the coordinate was eliminated in the examined specimens.

Taxonomic treatment

Rubus longistipularis Espinel-Ortiz & Romol., sp. nov.

urn:lsid:ipni.org:names:77234524-1 Figs 1–3

Diagnosis. *Rubus longistipularis* is characterised by its villous to pannose white pubescence in branches, stipules, petioles, and leaves, and pannose and sericeous pubescence in sepals, its long (20.0-34.7 mm) stipules, 15-27 secondary veins on leaflets, flowers with deeply concave, pink petals with fuchsia borders, and fruits with up to 195 small drupelets ($1.5-3.1 \times 0.9-2.5 \text{ mm}$).

Type. ECUADOR. Pichincha: Nono-Tandayaparoad, between km 116–117,00°01.787'S, 78°38.567'W, 1950 m, 26 Jul 2021 (fl, fr), *D. Espinel-Ortiz & H.G. Abad 281* (holotype: QCA (QCA-243418 and QCA-7010714 to QCA-7010723); isotypes: HA, HUTI, QAP).

Description. Scandent or climbing shrub, growing up to more than 3 m over the vegetation, glaucous pubescence all over the plant, with all prickles, from the base $\frac{1}{3}-\frac{2}{3}$ villous, and glabrous towards the apex, rarely with subsessile glands. Branches obtuseangled, woody, light greenish-white when young to dark brown when old, villous to pannose, 3.7–7.2 mm diam., with scattered sessile and subsessile glands; sparsely prickly, unarmed or with up to 4 prickles (per total area of 5 cm long of the branch), falcate, 2.6-5.3 \times 3.1–7.9 mm. Stipules asymmetrically, anguste subulate, (14.7–) 20.0–34.7 \times 1.7–3.3 mm, margin entire, chartaceous; adaxial surface villous on the midvein and towards the margin, rarely with subsessile glands towards the margin; abaxial surface sparsely pannose to villous with scattered subsessile glands. Petioles (3.96-) 9.61-12.2 cm long, villous to pannose with scattered long hairs, especially towards the leaf blade or when young, with (1-) 3-8 (-12) prickles, falcate, 2.0-4.5 × 2.0-5.8 mm; lateral petiolules (2.5-) 6.4-14.7 (16.3-) mm long, unarmed or with up to 4 prickles, falcate, $1.2-1.9 \times 1.7-3.0$ mm; terminal petiolules (1.0–) 2.4–8.3 cm long, with (2–) 5–15 prickles, falcate, $1.5-3.1 \times 1.7-$ 5.4 mm. Leaves trifoliate, rarely 4–5-foliolate; leaflets ovate to elliptic, base rounded to obtuse, or subcordate, apex acuminate to slightly apiculate, margin serrulate, lateral leaflets (4.5-) 6.4–14.4 × (2.5–) 3.6–7.9 cm, terminal leaflet (5.9–) 8.5–17.0 × (3.2–) 4.2–9.6 cm, chartaceous, with (11–) 15–27 secondary veins; adaxial surface sparsely hirsute on the midvein, and sparsely pilose mainly on secondary veins and slightly tomentose towards the border, with scattered orange to red sessile and subsessile glands, unarmed; abaxial surface sparsely villous on the midvein and secondary veins, and pannose, with scattered orange to red subsessile glands on the veins, and 2-18 prickles on the primary vein, falcate, 0.2-2.5 × 0.5–3.6 mm. Inflorescences lax, compound, terminal and axillary cymes, 23–106-flowered, 9.6-32.5 cm long, with simple or trifoliate leaves below; peduncles terete, white to slightly brownish, (9.5-) 14.2-66.1 (-81.0) mm long, pannose, eglandular, unarmed or with up to 3 prickles, falcate, $0.5-1.7 \times 0.8-2.3$ mm; pedicels terete, white, pannose and slightly sericeous, 5.6-16.8 (-23.3) mm long, eglandular, unarmed or with up to 14 prickles, triangular to falcate, $0.2-1.5 \times 0.1-1.8$ mm. Flowers 17.5–24.0 mm diam.;



Figure I. *Rubus longistipularis* D.A. Espinel-Ortiz & Romol. **A** habit and inflorescence **B** branch and stipule **C** leaf **D** leaf adaxial surface **E** leaf abaxial surface **F** leaf border **G** pedicel **H** flower **I** sepal adaxial surface **J** sepal abaxial surface **K** fruit. (**A** based on P.M. Jørgensen & S.S. Vire 56085 (QCA) **B–K** based on D. Espinel-Ortiz & H.G. Abad 281 (QCA)). Illustrations by Carla Rodríguez.


Figure 2. *Rubus longistipularis* D.A. Espinel-Ortiz & Romol. **A** habit **B** leaf abaxial surface and stipules **C** flower **D** infructescence with immature and mature fruits. Photos by David A. Espinel-Ortiz.



Figure 3. *Rubus longistipularis* D.A. Espinel-Ortiz & Romol. Holotype collection D. Espinel-Ortiz & H.G. Abad 281 (QCA) **A** branch apex QCA243418 **B** leaf abaxial surface QCA7010715 **C** terminal inflorescence QCA7010718 **D** infructescence QCA7010719.

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sepals 5, ovate to elliptic, apex acuminate, margin involute, 8.8–11.9 × 4.0–5.8 mm, light greenish-grey to greenish-white, acrescent; adaxial surface deeply concave, sericeous, and pannose towards the apex and the margin, eglandular, unarmed; abaxial surface deeply convex, shortly lanate and slightly tomentose towards the apex, eglandular, unarmed; petals 5, broadly elliptic to broadly obovate, margin entire, 8.8–12.7 × 9.8–12.6 mm, fuchsia when opening, completely pink or pink with fuchsia borders when fully opened, glabrous, eglandular, adaxial surface deeply concave, abaxial surface deeply convex; stamens with anthers glabrous, filaments fuchsia, glabrous; pistils, stigmas and styles glabrous, ovaries densely villous. **Fruits** green to dark red when immature, and black at maturity, ovoid to oval, $11.5-23.9 \times 8.0-17.1$ mm (when fresh); drupelets 50–195 per receptacle, $1.5-3.1 \times 0.9-2.5$ mm (when fresh), sparsely villous and deeply villous towards the apex.

Additional specimens examined (Paratypes). ECUADOR. — Santo Domingo de los Tsáchilas: Chillogallo-Santo Domingo road, below Chiriboga, 00°15.000'S, 78°47.000'W, 2000 m, 13 Aug 1980 (fl), L. Holm-Nielsen, B. Øllgaard & C. Sperling 24755 (AAU). - Imbabura: Cuicocha-Apuela road, km 28, disturbed cloud forest, 00°22.000'N, 78°28.000'W, 2480-2670 m, 05 Oct 1984 (fl), P.M. Jørgensen & S.S. Vire 56085 (AAU, QCA (QCA-91776), QCNE (QCNE-12185)). - Pichincha: Quito, west side from Pelagallo, sendero Guantopungo-Chichipunta trail, 00°04.400'N, 78°34.470'W, 2432 m, 25 Sep 2021 (fl, fr), C.E. Cerón, C.I. Reyes 89354 (QAP (QAP-107614 and QAP-107574)); Quito, Nanegalito, Golán, road between Edén Mágico and Ecological Reserve San Luis, 00°04.460'N, 78°33.340'W, 2300-2500 m, 06 Feb 2021 (fr), C.E. Cerón, C.I. Reyes & C. Bacuilima 87651 (QAP (QAP-106251), QCA (QCA-243453)); Quito, Nanegalito, Golán, near Mrs Margarita Bacuilima property, 00°05.370'N, 78°33.420'W, 2281 m, 26 Apr 2021, C.E. Cerón, C.I. Reyes & J. Bacuilima 88206 (QAP (QAP-106667)); Quito, Nanegalito, Golán, road between Edén Mágico-El Alí, 00°06.260'N, 78°33.140'W, 2603 m, 18 May 2021, C.E. Cerón, C.I. Reyes & C. Bacuilima 88386 (QAP (QAP-105946)); Quito, Nanegalito, El Porvenir, near Guerrero family property, 00°06.190'N, 78°33.240'W, 2427–2500 m, 21 Aug 2021 (fr, fl), C.E. Cerón, C.I. Reyes y J. Bacuilima 89113 (QAP (QAP-107423), QCA (QCA-243441), QCNE); Orchid Reserve Pahuma, Chorrera trail, 00°01.000'N, 78°38.000'W, 2000-2500 m, 08 Sep 1996 (fl), C.E. Cerón & E. Freire 32387 (QAP (QAP-25420)); Tandayapa-Tambo-Nono road, disturbed cloud forest, 00°01.429'S, 78°38.630'W, 1974 m, 05 Mar 2021, D. Espinel-Ortiz & C. Restrepo 276 (QCA (QCA-243396, QCA-7010701 and QCA-7010702)); Nono-Tandayapa road, between km 117-118, 00°01.967'S, 78°38.491'W, 1925 m, 29 Oct 2021 (fr), D. Espinel-Ortiz & C. Restrepo 296 (QCA); Nono-Tandayapa road, between km 123-124, 00°02.533'S, 78°38.204'W, 2125 m, 02 Aug 2021 (fl, fr), D. Espinel-Ortiz, C. Restrepo & A. Sanguano 285 (QCA (QCA-243397 and QCA-7010703)); same locality as for preceding, 00°02.539'S, 78°38.215'W, 2091 m, 21 Oct 2021 (fr), D. Espinel-Ortiz & H.G. Abad 294 (HUTI, QAP, QCA (QCA-243454, QCA-7010752 and QCA-7010752), QCNE); same locality as for preceding, 00°02.525'S, 78°38.210'W, 2091 m, 21 Oct 2021 (fr), D. Espinel-Ortiz & H.G. Abad 295 (QCA (QCA-243452)); Quito-Chiriboga road, 2 km after Corazón Station of Petroecuador,



Figure 4. Distribution map of *Rubus maquipucunensis* (red triangles), and *R. longistipularis* (black circles) from the western Andes of North Ecuador. Map generated by David A. Espinel-Ortiz.

00°16.814'S, 78°42.067'W, 2399 m, 13 Dec 2020, *D. Espinel-Ortiz, C. Restrepo & C. García 262* (QCA (QCA-243398, QCA-7010704 to QCA-7010709), QUSF); same locality as for preceding, 00°16.804'S, 78°42.144'W, alt. 2377 m, 13 Dec 2020, *D. Espinel-Ortiz, C. Restrepo & C. García 264* (LOJA, QCA (QCA-243390)); same locality as for preceding, 00°16.809'S, 78°42.142'W, 2355 m, 28 Jan 2021, *D. Espinel-Ortiz & C. Restrepo 268* (QCA (QCA-243395 and QCA-7010700)). – **Cotopaxi:** Campo Alegre, ca. 20 km NE of Sigchos, 00°35.050'S, 78°47.600'W, 2614 m, 11 Jul 2003 (fl, fr), *J. Ramos, L. Ramos, A. Tigse & R. Tigse 5801* (MO, QCA (QCA-137959), QCNE (QCNE-200267)).

Distribution. *Rubus longistipularis* is distributed in the north of the Ecuadorian Western-Cordillera from 1900 to 2700 m a.s.l., in the provinces of Santo Domingo de los Tsáchilas, Imbabura, Pichincha and Cotopaxi (Fig. 4).

Ecology. This species occurs in Chocó Andino montane cloud forests dominated by trees and shrubs and also in nearby disturbed areas. *Rubus longistipularis* can be found living in sympatry with *Rubus adenotrichos* Schltdl., *R. boliviensis* Focke, *R. glaucus* Benth., *R. niveus* Thunb. and *R. urticifolius* Poir. As branches grow older, they may become glabrescent and lose prickles and stipules. In some flowers, two sepals may be united in the apex, but they separate completely when fruiting occurs. Since flower blossoming, it takes about three months for the fruits to appear and ripen. Flowering and fruiting collections dated from the months of February, July, August, September and October.

Etymology. The specific epithet refers to the long (20.0–34.7 mm) asymmetrically, anguste subulate stipules.

Preliminary assessment of conservation status. *Rubus longistipularis* is known from five localities, impacted by human activities, including regression to agriculture and road openings. Following the IUCN (2019) guidelines, based on the geographic distribution and altered land use at the localities, this species should be categorised as least concern (LC).

Notes. *Rubus longistipularis* may resemble *R. boliviensis* by its habit and big leaves, but differs from this species by its white villous to pannose branches, in contrast with the pannose, pilose or puberulent to glabrescent branches of *R. boliviensis*. Moreover, *R. longistipularis* has trifoliate leaves with ovate to elliptic leaflets while *R. boliviensis* has 5-foliolate leaves with ovate-elliptic leaflets. Furthermore, *R. longistipularis* has fruits with more (50–195) and narrower (1.5–3.1 × 0.9–2.5 mm) drupelets while *R. boliviensis* has fruits with fewer (20–50) and wider (2.0–3.0 × 2.0–3.0 mm) drupelets. *Rubus longistipularis* resembles *R. glaucus* by its habit, trifoliate leaves and fruits, but differs by its white villous to pannose branches, pannose and slightly sericeous pedicels and bigger petals (8.8–12.7 × 9.8–12.6 mm) compared to the glabrous and pruinose branches, glabrous pedicels and smaller petals (7.0–10.0 × 5.0–8.0 mm) of the latter. Moreover, *R. longistipularis* differs from both species by its longer stipule (20.0–34.7 × 1.7–3.3 mm), in contrast with the smaller stipules of *R. boliviensis* (6.0–10.0 × 1.0–2.0 mm) and *R. glaucus* (5.0–12.0 × 0.3–0.8 mm).

Rubus maquipucunensis Espinel-Ortiz & Romol., sp. nov.

urn:lsid:ipni.org:names:77234525-1 Figs 5–7

Diagnosis. *Rubus maquipucunensis* is characterised by its villous to villous-hispid branches, trifoliate leaves with broadly elliptic or broadly ovate to elliptic leaflets, long inflorescences (22.6–59.4 cm long), flowers with fuchsia or pink petals and fuchsia filaments, and fruits with big drupelets (4.0–6.1 × 3.1–5.4 mm).

Type. ECUADOR. Pichincha: cantón Quito, parroquia Nanegal, in front of the Ecological Reserve Maquipucuna entrance, 00°07.457'S, 78°37.744'W, 1278 m, 11 Feb 2021 (fl, fr), *D. Espinel-Ortiz, C. Restrepo & A. Sanguano 269* (holotype: QCA (QCA-243282 and QCA-7010670 to QCA-7010679); isotypes: HA, HUTI, LOJA, Q, QCNE).

Description. Woody vine growing up to 20 m long, or **climbing shrub. Branches** obtuse-angled to slightly terete, woody, green to brown, densely villous to villous-hirsute, 2.0–12.1 mm diam., with scattered subsessile glands; unarmed or with 3–19 prickles (per total area of 5 cm long of the branch), gradually narrowed from a broad base, curved at the apex, $1.0-3.1 \times 1.5-5.4$ mm, glabrous. **Stipules** subulate, $3.9-9.2 \times 0.1-$ 0.3 mm, chartaceous, villous, with scattered sessile and subsessile glands. **Petioles** 3.8-10.4 cm long, villous, with (1–) 11-23 (–27) curved prickles $0.5-3.0 \times 1.0-4.6$ mm; lateral petiolules (3.6-) 9.1-13.8 mm long, unarmed or with up to 9 curved prickles $0.1-0.9 \times 0.3-1.4$ mm; terminal petiolules (2.3-) 3.6-5.3 cm long, with (4–) 18-35 150



Figure 5. *Rubus maquipucunensis* D.A. Espinel-Ortiz & Romol. **A** vine **B** scandent shrub **C** vine branch **D** shrub branch and stipule **E** vine leaf adaxial surface **F** shrub leaf adaxial surface **G** vine leaf abaxial surface **H** shrub abaxial surface **I** leaf border **J** inflorescence **K** pedicel **L** flower and sepal adaxial surface **M** sepal abaxial surface **N** fruit. (**A–I** based on D. Espinel-Ortiz, C. Restrepo & A. Sanguano 275 (QCA), **J** based on D. Espinel-Ortiz, C. Restrepo & A. Sanguano 269 (HUTI) **L–N** based on D. Espinel-Ortiz, C. Restrepo & A. Sanguano 269 (QCA)). Illustrations by Carla Rodríguez.

curved prickles $1.0-2.0 \times 0.8-3.6$ mm. Leaves trifoliate; leaflets broadly elliptic or broadly ovate to elliptic, base rounded to obtuse or slightly subcordate, apex cuspidate to abruptly acute, margin serrate, lateral leaflets (5.4-) 7.5–12.5 $(-17.1) \times (3.4-)$ 4.1– 9.2 (-12.2) cm, terminal leaflet (6.5-) 9.2-14.5 (-18.5) × (2.9-) 4.7-10.1 (-14.3) cm, chartaceous, with (7-) 11-16 (-18) secondary veins; adaxial surface villous-hirsute on primary and secondary veins with scattered short strigose hairs, or villous-hirsute in the midvein and sparsely adpressed villous in the veins; with subsessile and sessile glands, unarmed; abaxial surface sparsely villous and pilose on veins, or villous on veins and leaf blade with scattered subsessile glands, and (2-) 6–18 (-22) prickles on the primary vein, gradually narrowed from a broad base, straight to curved at the apex, $0.3-1.3 \times$ 0.3–1.9 mm, glabrous. Inflorescences lax, compound, terminal cymes, 36–196-flowered, 22.6-59.4 cm long, with simple or trifoliate leaves below; peduncles terete, slightly light gold, 4.7-36.7 mm long, shortly lanate, with scattered sessile glands, unarmed or with 1-17 prickles, gradually narrowed from a broad base, straight to curved at the apex, $0.1-1.0 \times 0.1-1.4$ mm, glabrous; pedicels terete, slightly light gold, shortly lanate, 5.7–11.9 (–15.3) mm long, eglandular, unarmed. Flowers 14.2–22.6 mm diam.; sepals 5, broadly ovate to broadly elliptic, apex deeply mucronate, margin entire, $3.6-5.6 \times$ 2.9–4.7 mm, tawny brown to ochre, acrescent; adaxial surface deeply concave, pannose, eglandular, unarmed; abaxial surface deeply convex, shortly lanate, and pannose on the margins and towards the apex, eglandular, unarmed; petals 5, broadly obovate, margin entire or erose, $5.6-11.6 \times 5.2-10.1$ mm, fuchsia when opening, completely pink or white with pink borders when fully opened, glabrous, eglandular, adaxial surface straight to concave, abaxial surface straight to convex; stamens with anthers glabrous, filaments fuchsia, glabrous; pistils, stigmas and styles glabrous, ovaries pilose. Fruits green to dark red when immature, and black at maturity, ovoid-globose, 11.0-14.8 × 12.1–15.6 mm (when fresh); drupelets 14–32 per receptacle, $4.0-6.1 \times 3.1-5.4$ mm (when fresh), pilose towards the base and apex.

Additional specimens examined (Paratypes). ECUADOR. - Santo Domingo de los Tsáchilas: Old road along Chiriboga, Quito-Santo Domingo, 1275 m, 08 April 1984 (fl), C.H. Dodson & M. Thurston 14195 (MO (MO-1559904)); old road San Juan-Chiriboga, km 60-70, 00°17.000'S, 78°50.000'W, 1000-1500 m, 09 Jan 1993 (fl), K. Romoleroux & A. Freire 1514 (QCA (QCA-92036), QCNE (QCNE-77110)). - Pichincha: Near San Florencio, growing in subandes, 1889 (fl), A. Sodiro 410? (Q (Q-3613)); Ecological Reserve Maquipucuna, edge of pasture in secondary rainforest, trail from Hacienda El Carmen to Hacienda Esparragos, ca. 6 km airline SE of Nanegal, 00°07.500'N, 78°38.000'W, ca. 1300 m, 11 Sep 1989 (fl, fr), G. Webster, K. Bainard & R. Schilling 27403 (DAV (DAV-331349 and DAV-331350), QCA (QCA-91821), QCNE (QCNE-44060)); Ecological Reserve Maquipucuna, secondary rainforest, trail from Hacienda Esparragos to Cerro de Sosa, ca. 5 km airline SE of Nanegal, 00°07.000'N, 78°38.000'W, 1400-1500 m, 18 Sep 1989 (fr), G. Webster & M. Rios 27716 (DAV (DAV-331334), QCA (QCA-91761)); Ecological Reserve Maquipucuna, disturbed rainforest along Quebrada de la Cal, 4 km airline SE of Nanegal, 00°07.500'N, 78°38.000'W, 1250 m, 20 Jul 1990 (fl, fr), G. Webster & B. Castro 28351 (DAV (DAV-331347 and DAV-331348), QCNE (QCNE-44101)); Ecological Reserve Maquipucuna, Maquipucuna mountains,



Figure 6. *Rubus maquipucunensis* D.A. Espinel-Ortiz & Romol. **A** vine **B** scandent shrub and inflorescence **C** leaf abaxial surface **D** immature fruits **E** mature fruits **F** flower. Photos by Camilo Restrepo (**A–C, E–F**) and David A. Espinel-Ortiz (**D**).

Cerro Sosa, primary rainforest, 00°05.500'N, 78°37.000'W, 1725 m, 03 Jul 1991 (fl), G. Webster, B. Castro & N. McCarten 28693 (DAV (DAV-331346)); Ecological Reserve Maquipucuna, trail S from Hacienda El Carmen, secondary rainforest, 00°07.000'N, 78°39.000'W, 1300 m, 06 Jul 1992 (fl), G. Webster & UREP participants 29038 (DAV (DAV-331345), QCNE (QCNE-81119)); Ecological Reserve Maquipucuna, disturbed rainforest along trail from guava plantation to Alambí river, 00°07.300'N, 78°38.000'W, 1300-1400 m, 10 Jul 1992 (fl), G. Webster & R. Rhode 29284 (DAV (DAV-331351), QCA (QCA-92244), QCNE (QCNE-75592)); same locality as for preceding, 1200-1400 m, 12 Jul 1992 (fl), P. Delprete & G. Webster 6073 (QCA (QCA-240552)); Ecological Reserve Maquipucuna, trail to Cerro Montecristi, 00°07,070'N, 78°34,000W, 1700 m, 06 Nov 1999 (fl, fr), C.E. Cerón, R. Arcos, C. Sevilla & A. Mosquera 39731 (QAP (QAP-28345)); Ecological Reserve Maquipucuna, disturbed rainforest along "Autoguiado" trail, 00°07.341'N, 78°37.741'W, 1258 m, 01 Sep 2020, D. Espinel-Ortiz, E. Bastidas-León & C. Restrepo 239 (QCA (QCA-243392 and QCA-7010699)); same locality as for preceding, 00°07.294'N, 78°37.784'W, 1326 m, 11 Feb 2021 (fl), D. Espinel-Ortiz, C. Restrepo & A. Sanguano 273 (HUTI, QCA (QCA-243371 and QCA-7010694)); Ecological Reserve Maquipucuna, trail to the river after orchid field, 00°07.449'N, 78°37.889'W, 1280 m, 11 Feb 2021, D. Espinel-Ortiz, C. Restrepo & A. Sanguano 270 (QCA (QCA-243372 and QCA-7010695)); Ecological Reserve Maquipucuna, trail to the river, 00°07.350'N, 78°38.158'W, 1249 m, 11 Feb 2021, D. Espinel-Ortiz, C. Restrepo & A. Sanguano 271 (QCA (QCA-243374)); same locality as for preceding, 00°07.419'N, 78°38.246'W, 1273 m, 11 Feb 2021, D. Espinel-Ortiz, C. Restrepo & A. Sanguano 272 (HA, QCA (QCA-243373 and QCA-7010696)); Marianitas ca. 3 km after the bridge over river Alambí, road to Ecological Reserve Maquipucuna, 00°07.466'N, 78°38.810'W, 1239 m, 22 Feb 2021, D. Espinel-Ortiz, C. Restrepo & A. Sanguano 275 (QCA (QCA-243375, QCA-7010697 and QCA-7010698)); same collection data as for holotype, 18 May 2021 (fl), D. Espinel-Ortiz, Restrepo C. & O. Tejada 277 (QCA (QCA-243370)).

Distribution. *Rubus maquipucunensis* is distributed in the north of the Ecuadorian Western-Cordillera from 1000 to 1725 m a.s.l., in the provinces of Pichincha and Santo Domingo de los Tsáchilas (Fig. 4).

Ecology. This species occurs in Chocó Andino rainforests dominated by trees, shrubs, and vines, and also in nearby disturbed areas. *Rubus maquipucunensis* can be found living in sympatry with *Rubus urticifolius*. As branches grow older, they become glabrescent and lose prickles. Also, young leaves or leaves of juvenile individuals are significantly smaller and may seem different than the mature leaves. Flowering and fruiting branches grow at the top of the plant where more light is available, and it takes more than 15 days for the flowers to bloom. Flowering and fruiting collections dated from January, February, April, May, July, September and November.

Etymology. The specific epithet honours the Ecological Reserve Maquipucuna ("Mano amiga" or "Friendly hand" in Kichwa) where a high number of samples were collected, and where this species is protected and easily found.



Figure 7. Rubus maquipucunensis D.A. Espinel-Ortiz & Romol. Holotype collection D.A. Espinel-Ortiz, C. Restrepo & A. Sanguano 269 (QCA) A vine QCA243282 B climbing shrub QCA7010670 C inflorescence QCA7010674 **D** infructescence QCA7010672.

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Preliminary assessment of conservation status. *Rubus maquipucunensis* is known from three localities of which two are impacted by human activity, including road opening, and the other locality is an Ecological Reserve. Following the IUCN (2019) guide-lines, based on the reduced geographic distribution and altered land use, this species should be categorised as vulnerable (VU); at least until other populations are discovered.

Notes. Rubus maquipucunensis may resemble R. boliviensis by its habit and flowers, and *R. floribundus* by its habit and inflorescences, but differs from both species by its villous to villous-hirsute branches, in contrast with the pannose, pilose or puberulent to glabrescent branches of R. boliviensis, and tomentose to glabrescent branches of R. floribundus. Moreover, R. maquipucunensis has trifoliate leaves with broadly elliptic or broadly ovate to elliptic leaflets while R. boliviensis and R. floribundus have 5-foliolate leaves with ovate-elliptic leaflets. Furthermore, R. maquipucunensis has fruits with fewer (14–32) and bigger (4.0–6.1 \times 3.1–5.4 mm) drupelets while *R. boliviensis* and R. floribundus have fruits with more (20-50 in R. boliviensis, and 40-50 in R. floribundus) and smaller $(2.0-3.0 \times 2.0-3.0 \text{ mm in } R. \text{ boliviensis}$, and $2.5-4.0 \times 2.0-3.0 \text{ in}$ R. floribundus) drupelets. Rubus maquipucunensis resembles R. killipii by its habit and long inflorescences, but differs by its shortly lanate peduncles and pedicels, and fuchsia to pink petals compared to the pannose peduncles and pedicels, and white petals of the latter. In addition, R. maquipucunensis has trifoliolate leaves while R. killipii has 5-foliolate leaves. As R. killipii fruits have not been described yet, they cannot be compared with those of R. maquipucunensis. Rubus maquipucunensis resembles R. selleanus Helwig by its trifoliate leaves with broadly elliptic leaflets, but differs by its longer inflorescences (22.61-59.38 cm) compared to the shorter inflorescences (10-13 cm) of the latter. In addition, *R. maquipucunensis* has longer petioles (3.8–10.4 cm), bigger leaflets $(7.5-12.5 \times 4.1-9.2 \text{ cm})$ and sepals with mucronate apex, while *R. selleanus* has shorter petioles (1.5–3.5 cm), smaller leaflets ($6-8 \times 5.5-7$ cm) and sepals with obtuse apex. Finally, R. maquipucunensis is found in Ecuador whereas R. selleanus has been recorded only in Hispaniola Island (Haiti and Dominican Republic).

Possible hybrids

Rubus longistipularis Espinel-Ortiz & Romol. × Rubus boliviensis Focke

Specimens examined. ECUADOR. – Imbabura: Cotacachi, road Cuicocha-Apuela, Comuna Santa Rosa-Pucará, Apuela entrance, 00°21.826'N, 78°29.901'W, 1998 m, 28 Nov 2020, *D. Espinel-Ortiz, M.P. Ortiz, M.A. Espinel-Ortiz y C. Castillo 260* (QCA (QCA-243440, QCA-7010741 and QCA-7010742)). – Pichincha: Nono-Tandayapa road, between km 116–117, 00°01.787'S, 78°38.567'W, 1950 m, 02 Aug 2021, *D. Espinel-Ortiz, C. Restrepo & A. Sanguano 286* (HA, HUTI, QCA (QCA-243438 and QCA-7010738 to QCA-7010740)).

Notes. The above specimens may be hybrids between *Rubus longistipularis* and *R. boliviensis*. Both samples were collected in places where both species coexist and showed mixed characteristics from both species. For instance, the stipules of samples D. Espi-

nel-Ortiz et al. 260 and D. Espinel-Ortiz et al. 286 were shorter (10.8–18.2 mm) and narrower (0.7–1.7 mm) than those of *R. longistipularis* (20.0–34.7 × 1.7–3.3 mm), but longer and wider if compared to those of *R. boliviensis* (6.0–10.0 × 1.0–2.0 mm). Furthermore, both samples showed sparsely pilose to pilose leaf margins and deeply villous to slightly pannose leaf abaxial surface, whereas *R. longistipularis* has tomentose leaf margins and pannose leaf abaxial surface, and *R. boliviensis* has glabrous leaf margin and densely villous leaf abaxial surface. The prickles from both samples were from the base $\frac{1}{3}$ - $\frac{2}{3}$ sparsely villous and glabrous towards the apex, while that of *R. longistipularis* is villous, and that of *R. boliviensis* is from the base $\frac{1}{3}$ sparsely pilose to glabrous. Finally, sample D. Espinel-Ortiz et al. 286 showed mainly 3–5-foliolate leaves, while *R. longistipularis* has mainly trifoliate leaves and *R. boliviensis* has 3–5-foliolate leaves.

Taxonomic key for Ecuadorian species

1	Stipules linear-falcate, ovate or suborbicular; leaves simple or 3-foliolate (subg.
	<i>Orobatus</i>) 2
_	Stipules subulate or filiform; leaves 3-foliolate, palmately 5-foliolate or imparipin-
	nate11
2	Leaves simple
_	Leaves 3-foliolate7
3	Stipules linear-falcate
_	Stipules asymmetrically ovate
4	Upper leaf surface bullate
_	Upper leaf surface not bullate
5	Lower leaf surface pannose-tomentose
_	Lower leaf surface glabrous or sparsely pilose on veins
6	Flowers solitary or rarely in inflorescences 2–3 cm long, with less than 4 flowers
_	Inflorescences 5–9 cm long, with more than 5 flowers
7	Flowers solitary or in few-flowered lax inflorescences; sepals as long as or longer
	than petals
_	Flowers in simple or compound, compact inflorescences; sepals shorter than pet-
	als
8	Stipules ovate; flowers usually solitary or sometimes in inflorescences with 2-4
	flowers
_	Stipules suborbicular; inflorescences with more than 4 flowers
9	Lower leaflet surface glabrous or sparsely pilose, unarmed sepals
_	Lower leaflet surface tomentose or villous, prickly sepals
10	Leaves and inflorescences pubescent, prickly sepals
_	Leaves and inflorescences glabrous, unarmed sepals
11	Drupelets united and falling collectively from dry receptacle (subg. <i>Idaeobatus</i>)12
_	Drupelets remaining on the fleshy receptacle and falling off together with it (subg.
	<i>Rubus</i>)

12	Leaves 3-foliolate; fruit yellow
_	Leaves imparipinnate, 5 or 7-foliolate; fruit pink-purplish to black or red13
13	Lower leaf surface pannose with stipitate glands, whitish; stem pruinoseR. niveus
_	Lower leaf surface sparsely pilose with subsessile and sessile glands, greenish; stem
	not pruinose
14	Inflorescences few-flowered, usually less than 30 flowers per inflorescence (except
	for <i>R. maquipucunensis</i> and <i>R. longistipularis</i> that can have more than 40 flowers);
	basal leaves 3-foliolate, rarely 4 or 5-foliolate
-	Inflorescences many-flowered, usually more than 40 flowers per inflorescence;
	basal leaves 5-foliolate, rarely 3-foliolate
15	Stems glabrous, glaucous or puberulent
_	Stems tomentose, velutinous, villous, pilose or pannose
16	Stems glaucous; drupelets < 6 mm long, more than 50 per receptacle <i>R. glaucus</i>
_	Stems not glaucous; drupelets > / mm long, less than 30 per receptacle
17	
1/	Leanets with less than 9 pairs of secondary veins; stems pilose; petals greenish
	Wille
_	villous or pannose: petals fuchsia, reddish violet, white or pink
18	Stems glaucous: stipules > 20 mm long: drupelets < 3 mm long, more than 50 per
10	receptacle R longistinularis
_	Stems not glaucous: stipules < 14 mm long: drupelets > 4 mm long, less than 40
	per receptacle
19	Vine or climbing shrub; leaflets broadly elliptic or broadly ovate to elliptic; up to
-	16 secondary veins
_	Scandent shrub; leaflets ovate to slightly elliptic; up to 13 secondary veins20
20	Leaflet surface velutinous or tomentose, with sessile and subsessile glands
	R. bogotensis
_	Leaflet surface sparsely villous or pilose, eglandular R. peruvianus
21	Stems and branches glandular
_	Stems and branches eglandular
22	Stems densely covered with long-stipitate glands
-	Stems with scattered, short-stipitate glands
23	Petioles pulvinate; base of the leaflets asymmetrical
_	Petioles not pulvinate; base of the leaflets rounded
24	Lower leaflet surface glabrous; leaflets with 7–10 pairs of secondary veins
	<i>K. killipii</i>
-	Lower leaflet surface public public public surface
23	Stems with reddish, setose hairs
-	Leaflets with 10, 12 or rarely 1/ pairs of secondary yeing leaf marging corrects
20	<i>R Anihum Aus</i>
_	Leaflets with 14-18 pairs of secondary yeins leaf-marging service P haliniansis
	Dearets with 11 10 pairs of secondary venis, rear-margins serulate R. Outveniss

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RESEARCH ARTICLE



Two new species in the fern genus Lomariopsis (Lomariopsidaceae) from East Asia

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Abstract

Two East Asian *Lomariopsis* (Lomariopsidaceae, Polypodiales) species, *Lomariopsis moorei* and *Lomariopsis longini*, which were previously misidentified as *L. spectabilis*, are here described as new species based on evidence from morphological characters and a molecular phylogeny. The two species differ from the three other described species in East Asia by their venation, pinna shapes, and perine morphology. A phylogeny based on a combined dataset of three chloroplast regions (*rbcL+ rps4-trnS + trnL-L-F*) showed that *L. moorei* and *L. longini* each formed a well-supported monophyletic group which was distantly related to both *L. spectabilis* and the other morphologically similar East Asian species, *L. boninensis*.

Keywords

Cryptic species, independent gametophyte, *Lomariopsis boninensis*, *Lomariopsis longini*, *Lomariopsis moorei*, *Lomariopsis spectabilis*, phylogeny, systematics

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Introduction

Lomariopsis Fée is the most species rich genus in the fern family Lomariopsidaceae and contains approximately 60 spp., accounting for 85% of the family (PPG I 2016). This genus has a wide distribution in tropical and subtropical regions; there are 15 species in the Neotropics (Moran 2000), nine species in Africa (Roux 2009), 11 species in the islands of the Indian Ocean (Holttum 1939b; Roux 2009; Rakotondrainibe and Jouy 2017), and 12 species in Asia and the Oceanian region (Holttum 1932, 1939a, 1966, 1978). The latest phylogeny of *Lomariopsis* included 24 species (ca. 40% of the species diversity in *Lomariopsis*), but only two species from Asia and the Oceanian region have been sampled (Chen et al. 2017) while the vast majority (ca. 10 species) from these areas have not yet been surveyed. In addition, because gametophytes of *Lomariopsis* species are able to establish as long-lived, asexual colonies in the wild (Watkins and Moran 2019), several species are found as gametophyte-only populations, which is called independent gametophytes (Pinson et al. 2017). In Japan and Taiwan, gametophytes of unknown species have been also reported (Ebihara et al. 2013; Wu et al. in press), a finding which further points out that the efforts of systematics research for Asian Lomariopsis remains inadequate, and there might have been undocumented and cryptic species.

To investigate phylogenetically *Lomariopsis* from these poorly sampled areas, we sampled most Asian and Oceanian species, including all species in East Asia where two previously unidentified species were discovered. They both had been misidentified as *L. spectabilis* Mett. One was from Chiayi County in Taiwan and Hainan Island in China, and the other one was from northern Vietnam and west southern China. They are superficially similar to two Asian species, *L. boninensis* Nakai and *L. spectabilis* in morphology. In this study, we presented a new *Lomariopsis* phylogeny supplied with comprehensive East Asian sampling, and reevaluated diagnostic characters leading to the description of these species.

Materials and methods

Perine morphology and spore number in sporangia

Spores were taken from mature sporangia and fixed on double-sided tape, and then gold coated with a sputter-coater for 1–3 min. Spores were subsequently examined with a tabletop SEM (TM 3000; Hitachi, Ibaraki, Japan).

To examine the spore number per sporangium, at least five mature, unopened sporangia per specimen were collected. These sporangia were broken individually, and we counted the number of spores inside under a stereomicroscope.

DNA extraction and chloroplast DNA region sequencing

Twenty-nine samples were included in our molecular phylogenetic study. Voucher information is provided in Appendix 1 (i.e., those samples noted with *). Total DNA extraction was done following the modified CTAB protocol of Kuo (2015). Three chloroplast (cp) regions were amplified and sequenced: trnL-L-F (trnL gene + trnL*trnF* intergenic spacer), the gene *rbcL*, and *rps4-trnS* (*rps4* gene + intergenic spacer), which were also used in previous phylogenies of Lomariopsis and Lomariopsidaceae (Rouhan et al. 2007; Li et al. 2009; Chen et al. 2017). The primers used for PCR amplification and sequencing were: FernL 1Ir1 (Li et al. 2010) and f (Taberlet et al. 1991) for trnL-L-F; rps5 (Nadot et al. 1994) and trnS (Souza-Chies et al. 1997) for rps4-trnS; af (Hasebe et al. 1994) and 1379R (Pryer et al. 2001) for rbcL. PCR amplifications were prepared in 15 μ L reactions each containing 20 ng of genomic DNA, 1× SuperRed PCR Master Mix RED (TOOLS, Newtaipei City, Taiwan) and 0.5 µM of each primer. A typical amplification program began with one initial denaturation step for 5 min at 94 °C then 35 cycles of 1 min at 94 °C, 30 s at 55 °C, and 1 min at 72 °C followed by a final extension of 10 min at 72 °C and was performed on a SimpliAmp Thermal Cycler. PCR products were cleaned using ExoSAP-IT (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and then sequenced with the same PCR primers with an ABI 3730XL (Thermo Fisher Scientific, Waltham, Massachusetts, USA) by the Genomics BioSci. & Tech. company in Taiwan. GenBank accession numbers of the sequences are listed in Appendix 1.

Phylogenetic analyses

In total, we sampled 35 Lomariopsis species, including African/Malagasy and Neotropical members sequenced in previous studies (Rouhan et al. 2007; Lehtonen and Cárdenas 2019), and representatives from the three remaining Lomariopsidaceae genera (Chen et al. 2017) as outgroups. Importantly, our Lomariopsis sampling covered almost all Asian and Oceanian species (Holttum 1932, 1939a, 1939b, 1978, Moran 2000), including four of which were phylogenetically investigated for the first time. Before this study, three species were known to be distributed in East Asia: L. lineata (C.Presl) Holttum (syn. L. cochinchinensis Fée,), L. chinensis Ching, and L. boninensis. The materials of East Asian "L. spectabilis" belonged to either L. boninensis or one of the two new species described here. Except for L. chinensis, all East Asian species were included in our sampling. Voucher information for all samples is provided in Appendix 1. The sequences were aligned using MUSCLE (Edgar 2004) as implemented in AliView (Larsson 2014). The alignment of every coding gene was further divided into three partitions based on the codon positions. The portions of *rps4-trnS* IGS (intergenic spacer), *trnL* gene, and *trnL-FIGS* were each treated as an independent partition as well. In the phylogenetic analyses, each partition was assigned the appropriate substitution model, which was inferred by ModelFinder (Kalyaanamoorthy et al. 2017) and using the Bayesian information criterion (BIC, Schwarz 1978).

We used IQtree 1.6.8 (Nguyen et al. 2015) to infer maximum likelihood (ML) phylogenies with 1,000 standard bootstrap replicates. The Bayesian phylogenetic analysis was performed using Mr Bayes 3.2.7 (Ronquist et al. 2012). Two simultaneous runs were carried out with four chains (5×10^6 generations each). Each chain was

Characters	L. moorei	L. longini	L. boninensis	L. spectabilis	
Vein apices ending at the laminar margins	yes	no	yes	yes	
Widest part at upper pinna	<1/2	<1/3	<1/2	~1/2	
Stipe scales	dark brown, narrow-	dark brown, broadly	dark brown, broadly	light brown, narrowly	
	ly lanceolate (usually	lanceolate (usually	lanceolate (usually	lanceolate (usually <2	
	< 2 mm wide)	>3 mm wide)	>2 mm wide)	mm wide)	
Fertile pinna pairs	10-14	3–9	4–16	6-14	
Swollen ring on articulate at abaxial side,	obvious	obvious	inconspicuous	inconspicuous	
especially upper pinnae (Fig. 6)					
Spore perine (Fig. 4)	spiny	folded	spiny	cristate	
Spore number per sporangium	64	32	64	16 or 32	

Table 1. Morphological comparisons of the two new *Lomariopsis* species with their Asian morphologically similar relatives.

sampled every 1,000 generations. Log likelihoods of MCMC runs were inspected in Tracer 1.6 (Rambaut and Drummond 2013) to confirm their convergence. The first 25% of the generations were conservatively discarded as burn-in.

Results

The combined cpDNA alignment matrix included 3,817 nucleotide sites: rbcL (1,431 bp), rps4-trnS (1,233 bp), and trnL-L-F (1,153 bp) with 27.5% of variable sites. In our phylogeny (Fig. 1), the two new species, *L. moorei* and *L. longini*, each formed a monophyletic group, and were genetically distant from *L. boninensis*, *L. spectabilis*, and other Asian and Oceanian species. The line drawings of the two new species are provided in Figs 2 and 3, and their morphological comparisons with the two Asian relatives are summarized in Table 1. Perine morphology of the four species is shown in Fig. 4.

Taxonomic treatment

Lomariopsis longini L.Y.Kuo & Y.H.Wu, sp. nov. urn:lsid:ipni.org:names:77234526-1 Figs 2, 5A

Diagnosis. *Lomariopsis longini* differs from the other similar species, *L. spectabilis*, *L. boninensis*, and *L. moorei*, by its lanceolate upper sterile pinna with the widest portion occurring below the middle of the pinna, and the veins end ca. 0.5 mm before the margins.

Type. VIETNAM. Ha Tinh. Huong Son District: Son Kim Municipality, Rao Bun stream, 4 May 2004, *P.K. Loc 5095* (holotype: P [P00888363]! isotype: MO!).

Description. Rhizomes stramineous, 0.7-1.2 cm in diam., densely scaly; rhizome scales brown (but blackened at point of attachment), lanceolate, ca. $4-9 \times 1.5-3.7$ mm. Fronds 1-pinnate, leathery, mature laminae pinnate, dimorphic. Sterile fronds 30-60 cm long, stipes stramineous, 10-20 cm, grooved adaxially, base with scattered



Figure 1. Maximum likelihood (ML) tree based on the cpDNA *rbcL* + *rps4-trnS* + *trnL-L-F* dataset. Bootstrap supports (BS) and Bayesian inference posterior probabilities (BI PP) are indicated on each branch as BS/BI PP. The arrows indicate the clades consisting of Asian and Oceanian species.

scales, lateral pinnae 3–9 pairs, lanceolate, widest in the lower third, $7-16 \times 1.5-1.7$ cm, apex acuminate; pinna bases cuneate and decurrent, margins entire; lateral pinnae articulate to rachis, swollen ring on abaxial articulation, terminal pinna with a similar size as lateral pinnae, not articulate; upper part of rachis narrowly winged; veins free, simple or furcate, oblique, not extended to margin. Fertile laminae similar to sterile laminae but pinnae much contracted; pinnae linear, $10-20 \times ca$. 0.2 cm, equilateral, stalks 0.5–1.1 cm, pinna rachis articulate. Sori acrostichoid. Spores 32 per sporangium, perine with cristae.



Figure 2. Illustration of *Lomariopsis longini* L.Y.Kuo & Y.H.Wu, sp. nov., based on the holotype *P.K. Loc* et al. 5095 (P00888363).

Paratypes. VIETNAM. Nghe An Province: 28 Oct 2014, *L.B. Zhang, L. Zhang & N.T. Lu 7185* (CDBI, MO, VNMN); Quang Binh Province: Bo Trach District, Phong Nha-ke Bang National Park, 7 Dec 2004, *S.K. Wu and L.K. Phan WP897* (KUN); 13 Dec 2004,

WP1124 (KUN); Quang Tri Province: Dakrong District, Trieu Nguyen Commune, 4 Nov 2009, *Y.H. Chang 20091104-005* (TAIF); 2 Nov 2009, *C.W. Chen Wade 983* (TAIF); Vinh Phuc Province: Tam Dao District, Tam Dao National Park, 14 Dec 2010, *L.Y. Kuo 1862* (TAIF, VNMN). CHINA. Yunnan Province: 25 Aug 2014, *Q. Wei WQ243* (KUN); 2 Sep 2011, *S.Y. Dong 3597* (IBSC); 28 Mar 1987, *W.M. Ju et H.C. Yan Ju and Yan 21930* (IBK).

Distribution. Northern Vietnam, west southern China (Yunnan).

Ecology. In shaded places, understory of evergreen broad leaf forests, below 1,000 m in elevation.

Etymology. The lanceolate shape of the terminal pinnae of sterile leaves is similar to the holy lance, which is also called Lance of Longinus.

Lomariopsis moorei Y.H.Wu & L.Y.Kuo, sp. nov.

urn:lsid:ipni.org:names:77234527-1 Figs 3, 5B, 6A

Diagnosis. *Lomariopsis moorei* is most similar to *L. boninensis*, but scales on stipes are narrower (usually < 2 mm) in *L. moorei* (Fig. 5B) and broader (usually > 2 mm) in *L. boninensis*. The swollen ring at the region of articulation on the abaxial side (especially upper pinnae) is more obvious in *L. moorei* (Table 1; Fig. 6A).

Type. TAIWAN. Chiayi County: Dapu Township, Zengwen Reservoir, 9 November 2020, Y.H. Wu *YX052* (holotype: TAIF! isotype: TAIF!).

Description. Rhizomes rufous, 1.0–1.2 cm in diam., densely scaly; rhizome scales reddish brown (but blackened at point of attachment), narrowly lanceolate, ca. $4-6 \times 0.9-2.2$ mm. Fronds 1-pinnate, leathery, juvenile sterile laminae simple, shortly stalked, narrowly lanceolate, $20-25 \times 1.0-1.5$ cm, base narrowly cuneate, apex acute; mature lamina pinnate, dimorphic. Sterile fronds 30–50 cm long, stipes green, 10–20 cm, grooved adaxially, base with scattered scales, lateral pinnae 6-14 pairs, 1-5 cm apart, narrowly lanceolate, widest in the proximal half, $14-21 \times 1.5-2.2$ cm, narrowly cuneate, apex acute; base cuneate and decurrent, margin entire or slightly undulate; lateral pinnae articulate to rachis, swollen ring on abaxial articulation, terminal pinna $16-27 \times 1.5-2.2$ cm, not articulate; upper part of rachis narrowly winged; veins free, simple or furcate, oblique. Fertile laminae similar to sterile laminae but pinnae much contracted; pinnae linear, $10-20 \times ca$. 0.3 cm, equilateral, pinna rachis 0.3-0.8 cm wide, rachis articulate. Sori acrostichoid; perine consisting with glandular projections. Spores green (= chlorophyllous) and spiny, 64 per sporangium.

Paratypes. TAIWAN. Chiayi County: Dapu Township, Zengwen Reservoir, 9 Nov 2020, *Y.H. Wu YX053* (TAIF). CHINA. Hainan Province: Wuzhishan City, Mt. Wuzhi National Nature Reserve, 16 July 2007, *Y.S. Chao 1211* (TAIF); Mt. Diaoluo, 27 Feb 2012, *W.H. Wu 1062* (TAIF); 21 Nov 2000, *G.M. Zhang et D. Li 117* (PE); 14 Dec 2003, *S.Y. Dong 1045* (PE).

Distribution. Taiwan (Chiayi County) and China (Hainan Is.).

Ecology. In shaded places, understory of evergreen broadleaf forests, below 1,000m in elevation.



Figure 3. Illustration of *Lomariopsis moorei* Y.H.Wu & L.Y.Kuo, sp. nov., based on the holotype Y.H. Wu *YX052* (TAIF). A fallen fertile pinna is at the left bottom.

Etymology. The name moorei is dedicated to Dr./Mr. Shann-Jye Moore (1966–2010), an enthusiastic fern taxonomist and knowledgeable pteridologist from Taiwan. The Mr. Shann-Jye Moore Memorial Scholarship has been established by the Taiwan



Figure 4. Spore perine morphology by SEM **A** *Lomariopsis moorei* **B** *L. longini* **C** *L. boninensis* **D** *L. spectabilis.* Scale bars: 15 µm.

Society of Plant Systematics to commemorate his passions, and to support Taiwanese students studying the systematics of ferns and lycophytes.

Note. We have not yet found entire sporophyll from the type locality, but fallen fertile pinnae on 14 Aug 2020 (Fig. 3), which contained intact sporangia with green spores. Although mature sporophytes were found to have a restricted distribution in Taiwan, independent gametophytes of this species were found throughout Taiwan Main Is using a DNA-identification approach to survey gametophyte populations (Wu et al. in press).

Discussion

In previous molecular phylogenies of *Lomariopsis*, none of Oceanian species were included, and *L. lineata* and *L. spectabilis* (including the misidentified *L. boninensis* and *L. longini*) were the only Asian species (Rouhan et al. 2007; Li et al. 2009; Chen et al. 2017). Here, with a comprehensive sampling in these areas, the present phylogeny (Fig. 1) provides new insights into the evolutionary relationships and systematics for *Lomariopsis* species from these areas. In the present tree, the nine Asian/Oceanian species are retrieved into two well-supported clades. The first clade consists of *L. boninensis* only, while the second clade accommodates the remaining



Figure 5. Stipe scales A Lomariopsis longini (L.Y. Kuo1862, TAIF) B Lomariopsis moorei (Y.H. Wu *YX052*, TAIF). Scale bar: 1 mm.

eight species. These Asian and Oceanian clades are either nested within, or closely related to other paleotropical species (Africa and Madagascar), but their inter-clade relationships remain unclear (Fig. 1). Data from additional genetic regions will be necessary to better resolve the uncertainties of these nodes, and hence to confirm biogeographical origin(s) of Asian/Oceanian taxa. Among all six described East Asian species, *L. chinensis* is the only one missing from the current phylogeny. To the best of our knowledge, this species has only been collected once, as the type collections. Despite the lack of phylogenetic information, *L. chinensis* is morphologically unique in the genus and easily distinguished from other *Lomariopsis* species because of its reticulate leaf venation.



Figure 6. Articulation of upper pinnae (abaxial surface) to the rachis **A** *Lomariopsis moorei* (Y.H. Wu YX052, TAIF) **B** *Lomariopsis boninensis* (TNS790636).

Lomariopsis species diversity in Asia and Oceania could still be underestimated, and more undocumented species could be eventually revealed by phylogenetic analyses using multiple specimens in each morphologically-defined species, similar to the case of discovering the two new species here described. Indeed, L. moorei and L. longini, together with L. boninensis, are genetically distant taxa in East Asia even if all three have been long misidentified and confused under a single name of the South East Asian species, L. spectabilis, due to their overall similar morphology (DeVol and Kuo 1975; Tsai and Shieh 1994; Iwatsuki et al. 1995; Yang and Liu 2002; Li et al. 2009; Phan 2010; Xing et al. 2013; Knapp 2014; Chen et al. 2017; Ebihara 2017; TPG 2019). However, clear molecular phylogenetic results spurred us to seek other characters supporting the distinction between these lineages, and these actual species now can be identified based on microscopic characters (Table 1). These characters include perine ornamentation, which has been revealed to have highly diversified forms in Lomariopsis (Rouhan et al. 2007). Additionally, we found that the spore number per sporangium varies among these species, which can also help in distinguishing species. However, unlike most cases in ferns of the Polypodiales, such a reduction in the number of spores in sporangia (e.g., 64 to 32) may not represent a reproductive switch to apomixis for Lomariopsidaceae (Chen et al. 2017). Further cytological investigations, e.g., through flow cytometry to infer both spore vs. leaf genome sizes (Kuo et al. 2017), are necessary to clarify whether changes in the two phenomena (i.e., spore number per sporangium and reproductive mode) are linked in these *Lomariopsis* species.

Key to *Lomariopsis longini*, *L. moorei*, *L. spectabilis*, and other morphologically close species in East Asia

1	Sterile lateral pinnae with lateral veins spreading (borne at nearly right angles
	to the pinna rachis), free, occasionally anastomosing
_	Sterile lateral pinnae with veins oblique, free
2	Sterile lateral pinnae, abruptly narrowed to a caudate apex (2–3 cm long)
	L. lineata
_	Sterile lateral pinnae with acuminate apex

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Appendix I

Voucher information and GenBank accession numbers. –, sequences not available. *sequences obtained in this study.

Species name	Locality	Voucher (Herbarium)	GenBank accession number		
		_	rbcL	rps4-trnS	trnL-L-F
Cyclopeltis crenata	China, Hainan	Wu935 (TAIF)	*	*	*
Cyclopeltis novoguineensis	Solomon Islands	SITW068 (TAIF)			
Cyclopeltis semicordata	Costa Rica	CJR et al. 08-195 (DUKE)			
Dracoglossum plantagineum	Guadeloupe	Christenhusz 4065 (TUR)			
Dryopolystichum phaeostigma	Solomon Islands	SITW10443 (BSIP, TAIF, TNM)			
Lomariopsis aff. brackenridgei	VANUATU, Espiritu Santo Is.	Matsumoto 01-857 (TNS)	-	-	*
Lomariopsis boninensis	Japan, Bonin Is.	TNS 763923 (TNS)		*	*
Lomariopsis boninensis	Japan, Ishigaki Is.	Ebihara 2957 (TNS)	*	*	*
Lomariopsis boninensis	TAIWAN, Orchid Is.	CYH20091123_03 (TAIF)	*	*	*
Lomariopsis boninensis	TAIWAN, Orchid Is.	CYH20110918_25 (TAIF)	-	*	*
Lomariopsis boninensis	Taiwan	Kuo 2423 (TAIF)	*	*	*
Lomariopsis boninensis	Taiwan	Kuo 976 (TAIF)	*	*	*
Lomariopsis brackenridgei	Fiji	FJ_2011_207 (WELT)	*	*	*
Lomariopsis brackenridgei	FRENCH POLYNESIA, Moorea	JNG3191 (UC)	-	-	*
Lomariopsis cf. lineata	Unknown Origin	F042 (SING)	*		
Lomariopsis japurensis	Unknown Origin	Lehtonen 989 (TUR)			
Lomariopsis kingii	Indonesia, Bacan	DK1398 (UC)	*	*	*
Lomariopsis kingii	New Guinea	DK2800 (UC)	*	*	*
Lomariopsis leptocarpa	Micronesia	Masuda 6919 (TNS)	*	*	*
Lomariopsis lineata	Indonesia, Sulawesi	DK925 (UC)	*	*	*
Lomariopsis lineata	Philippines	Kuo 2052 (TAIF)	*	*	*
Lomariopsis lineata	PHILIPPINES	Wade 3946 (TAIF)	-	-	*
Lomariopsis novaecaledoniae	New Caledonia	Nakamura 2157 (TNS)	-	*	*
Lomariopsis sorbifolia	Guadeloupe	Christenhusz 4070 (TUR)		*	*
Lomariopsis longini	China, Yunnan	Dong 3597 (IBSC)	*	*	*
Lomariopsis longini	China, Yunnan	WQ243 (KUN)	*	*	*
Lomariopsis longini	Vietnam	Wade983 (TAIF)	*	*	*
Lomariopsis longini	Vietnam	Zhang et al.7185			
Lomariopsis longini	Vietnam	WP897 (KUN)	-	-	*
Lomariopsis longini	Vietnam	WP1124 (KUN)	-	-	*
Lomariopsis moorei	Taiwan	YX052 (TAIF)	*	*	*
Lomariopsis moorei	CHINA, Hainan	HN197 (PE)	*	*	*
Lomariopsis moorei	Taiwan	Ha 10**	-	-	*
Lomariopsis moorei	CHINA, Hainan	Wu 1062 (TAIF)	*	*	*
Lomariopsis spectabilis	Indonesia, Java	Wade 1100 (TAIF)	*	*	*

Species name	Locality	Voucher (Herbarium)	GenBa	nk accession r	number
		_	rbcL	rps4-trnS	trnL-L-F
Lomariopsis spectabilis	Indonesia, Java	Wade 1838 (TAIF)	*	*	*
Lomariopsis cordata	Madagascar	Rakotondrainibe 1771 (P)	-	-	
Lomariopsis crassifolia	Madagascar	Janssen et al. 2527 (P)	-	-	
Lomariopsis crassifolia	Madagascar	Humblot 442 (P)	-	-	
Lomariopsis guineensis	Sierra Leone	Fay & Fay s.n., in 1985 (NY)	-	-	
Lomariopsis hederacea	Cameroon	Raynal 9954 (P)	-	-	
Lomariopsis jamaicensis	Jamaica	Maxon & Killip 1463 (NY)	-	-	
Lomariopsis japurensis	Ecuador	Moran 6021 (NY, QCA, QCNE)	-	-	
Lomariopsis japurensis	Ecuador	Moran 6061 (NY, QCA, QCNE)	-	-	
Lomariopsis japurensis	Costa Rica	Moran 6381(CR, INB, NY)	-	-	
Lomariopsis japurensis	Bolivia	Sundue 708 (LPB, NY, USZ)	-	-	
Lomariopsis japurensis	Peru	Bell 88180 (NY)	-	-	
Lomariopsis japurensis	Bolivia	Jimenez 2016 (LPB, NY)	-	-	
Lomariopsis kunzeana	Haiti	Zanoni 28649 (EHH, NY)	-	-	
Lomariopsis kunzeana	United States	Peck s.n. (NY)	-	-	
Lomariopsis latipinna	Ecuador	Moran 6027 (NY, QCA, QCNE, TUR)	-	-	
Lomariopsis lineata	THAILAND	Larsen 45851 (AAU, NY)	-	-	
Lomariopsis longicaudata	Madagascar	Janssen 2493 (P)	-	-	
Lomariopsis longicaudata	Madagascar	Rakotondrainibe 6191 (P)	-	-	
Lomariopsis madagascarica	Madagascar	Kessler 12786 (NY)	-	-	
Lomariopsis madagascarica	Madagascar	Decary 18186 (P)	-	-	
Lomariopsis mannii	Democratic Republic Of Congo	Kassner s.n., in 1914 (P)	-	-	
Lomariopsis marginata	Brazil	Pires et al. 50316 (NY)	-	-	
Lomariopsis marginata	Brazil	Amorim 1920 (CEPEC, NY)	-	-	
Lomariopsis marginata	Brazil	Labiak 104 (NY, UPCB)	-	-	
Lomariopsis maxonii	Costa Rica	Moran 4172 (CR, NY, UC)	-	-	
Lomariopsis maxonii	Costa Rica	Smith 1660 (CR, NY, UC)	-	-	
Lomariopsis nigropaleata	Ecuador	Moran 6053 (NY)	-	-	
Lomariopsis nigropaleata	Bolivia	Jimenez 1949 (LPB, NY)	-	-	
Lomariopsis palustris	Sierra Leone	Fay 1124 (NY)	-	-	
Lomariopsis pervillei	Madagascar	Rakotondrainibe et al. 6626 (P)	-	-	
Lomariopsis pervillei	Madagascar	Rakotondrainibe et al. 6623 (P)	-	-	
Lomariopsis warneckei	Comoros	Rakotondrainibe et al. 6707 (P)	-	-	
Lomariopsis pollicina	Madagascar	Kessler 12785 (GOET, NY)	-	-	
Lomariopsis prieuriana	Venezuela	Cortez 475 (NY, VEN)	-	-	
Lomariopsis prieuriana	Panama	Moran 5080 (MO, NY, PMA)	-	-	
Lomariopsis recurvata	Mexico	Rivera 1343 (NY)	-	-	
Lomariopsis recurvata	Mexico	Hernandez 2286 (NY)	-	-	
Lomariopsis rossii	Liberia	Fav 1237 (NY)	-	-	
Lomariopsis salicifolia	Ecuador	Moran 6022 (AAU, NY, QCA, OCNE, TUR)	-	-	
Lomariopsis salicifolia	Ecuador	Moran 6129 (NY, OCA, OCNE)	-	-	
Lomariopsis salicifolia	Ecuador	Moran 6956 (NY, OCA, OCNE)	-	-	
Lonian ropois surregoina	Doolibox				
Lomariopsis warneckei	Madagascar	Janssen et al. 2444 (P)	-	-	
Lomariopsis warneckei	Madagascar	Rouhan et al. 318 (P)	-	-	
Lomariopsis vestita	Costa Rica	Moran 6382 (CR, INB, NY, USJ)	-	-	
Lomariopsis vestita	Costa Rica	Folsom 9011 (NY)	-	-	
Lomariopsis wrightii	Сива	Underwood 948 (NY)	-	-	

RESEARCH ARTICLE



A new subspecies of Stellaria alsine (Caryophyllaceae) from Yakushima, Japan

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Abstract

An unknown taxon of *Stellaria* was discovered in Yakushima, a Japanese island known to harbor several endemic species. To determine the identity of this taxon, this study employed MIG-seq for the reconstruction of a finely resolved phylogenetic tree of the newly discovered taxon, along with some related species of *Stellaria*. The results showed that the newly discovered taxon is a relative of *S. alsine*. Based on this result, *Stellaria alsine* subsp. *nana* **subsp. nov.** was published.

Keywords

cpDNA, DNA barcoding, island, ITS, MIG-seq, next generation sequencing, threatened plants

Introduction

Stellaria L. is a diverse genus belonging to the family Caryophyllaceae (tribe Arenarieae), and comprises approximately 190 species distributed primarily in the temperate areas of the Northern Hemisphere (Chen and Rabeler 2001). In Japan, 19 species have been recorded to date by Kadota (2017), including a recently discovered species, *S. hibinoi*, as described by Serizawa (2015).

This study describes an additional new taxon that inhabits the mountainous area of Yakushima, an island where a variety of endemic plant taxa have been previously recorded (Yahara et al. 1987). This taxon has a dwarf stem, 2.5-6 cm long. Morphologically, this taxon is similar to *S. alsine* Grimm, but it is difficult to determine the taxonomic relationship of this dwarf plant based solely on morphological observations. Thus, we carried out a phylogenetic analysis of *S. alsine* and the newly discovered taxon, as well as several other species of Stellaria using MIG-seq (multiplexed ISSR genotyping by sequencing; Suyama and Matsuki 2015). MIG-seq is capable of efficiently detecting genome-wide SNPs using inter-simple sequence repeats (ISSRs) as multiplex PCR primers. This method has been applied successfully to taxonomically differentiate between groups that are difficult to classify, such as Fagaceae (Binh et al. 2018; Strijk et al. 2020), Lauraceae (Zhang et al. 2020) and Asparagaceae (Yahara et al. 2021), in order to reconstruct highly resolved phylogenetic relationships among closely related species and infraspecific taxa. The MIG-seq tree obtained in this study demonstrates that the newly discovered taxon is sister to, but highly diverged from, S. alsine. Based on this finding, we provide a formal description of S. alsine subsp. nana subsp. nov. and discuss the implications of this discovery.

Methods

Field survey

Yakushima (Yaku Island) is a roughly circular island with a circumference of approximately 130 km and is located approximately 60 km south of the main island of Kyushu. Since the initial taxonomic review of 45 species of vascular plants endemic to Yakushima (Yahara et al. 1987), six additional new species have been described in the same region (Yahara and Tsukaya 2008; Katsuyama 2009; Chen et al. 2014; Hori et al. 2015; Suetsugu and Fukunaga 2016; Suetsugu et al. 2016). Furthermore, an unknown taxon of *Cardamine* was also discovered in the mountainous area of Yakushima (Kudoh 2017). Between July 19 and July 24, 2020, a field trip was made to the mountainous area of Yakushima in order to collect this unknown species of *Cardamine*, whereby we serendipitously discovered a plant with cleistogamous flowers of the unknown taxon of *Stellaria*. Later, fruiting specimens of this taxon were collected on September 4, 2020, and a specimen with a chasmogamous flower was collected on May 3, 2021 in the same area by K. Fuse. In addition, we collected samples of *S. alsine* subsp. *alsine*, *S. aquatica* (L.) Scop., *S. diversiflora* Maxim., *S. media* (L.) Vill., *S. monosperma* Buch.-Ham. ex D. Don, and *S. neglecta* Weihe (Table 1), and examined phylogenetic relationships.

DNA isolation, sequencing, and construction of SNP-based phylogenetic trees

Total DNA was extracted from dried leaves using the CTAB method (Doyle and Doyle 1990). *De novo* SNP discovery was performed using MIG-seq (Suyama and Matsuki 2015). Based on the methodology described by Suyama and Matsuki (2015), a MIG-

Scientific name	Voucher ID	Locality	Latitude, Longitude
Stellaria alsine subsp. alsine	JPN0029	Itoshima, Fukuoka	33.48162500, 130.2413472
Stellaria alsine subsp. alsine	JPN3306	Itoshima, Fukuoka	33.57076666, 130.2034750
Stellaria alsine subsp. nana	JPN0573	Yakushima, Kagoshima	30.34137777, 130.4770056
Stellaria alsine subsp. nana	JPN1791	Yakushima, Kagoshima	30.34171944, 130.4753583
Stellaria aquatica	JPN3046	Mitsukaido, Ibaraki	36.01760555, 139.9974056
Stellaria aquatica	JPN3106	Itoshima, Fukuoka	33.49042500, 130.2582250
Stellaria diversiflora	JPN1352	Mt. Yokogura, Kochi	33.53525555, 133.2013083
Stellaria media	JPN0008	Itoshima, Fukuoka	33.48307500, 130.2636556
Stellaria media	JPN0069	Itoshima, Fukuoka	33.47711944, 130.2409528
Stellaria media	JPN0816	Itoshima, Fukuoka	33.47456666, 130.2512972
Stellaria monosperma	JPN2119	Mt. Shiraiwa, Miyazaki	32.57839166, 131.1143333
Stellaria monosperma	JPN2998	Osugi-dani, Mie	34.21359722, 136.1698139
Stellaria neglecta	JPN0006	Itoshima, Fukuoka	33.48307500, 130.2636556

Table 1. A list of samples used in the molecular phylogenetic analyses.

seq library was prepared via a two-step PCR amplification process with minor modifications, namely the annealing temperature of the first PCR was altered from 48 °C to 38 °C. Subsequently, the second PCR products were purified in the size range of 300-800 bp and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a MiSeq Reagent Kit v3 (150 cycle, Illumina). Sequencing of the first 17 bases of reads 1 and 2 (SSR primer regions and anchors) was bypassed using the 'DarkCycle' function of the MiSeq platform. Additionally, low-quality reads and extremely short reads containing adapter sequences were removed using the Trimmomatic 0.39 software (Bolger et al. 2014). The Stacks 2.41 pipeline (Catchen et al. 2013; Rochette et al. 2019) was used to obtain individual genotypes with the following parameters: minimum depth of coverage required to create a stack (m) = 3, maximum distance between stacks (M)= 2, and maximum mismatches between loci when building the catalog (n) = 2. Three different filtering criteria were applied for quality control of the SNP data. First, any SNP site where one of two alleles had less than three counts was filtered out due to the difficulty in distinguishing polymorphisms from sequencing errors that arise when the minor allele count of SNPs is too low (Roesti et al. 2012). Second, loci containing SNPs with high heterozygosity ($Ho \ge 0.6$) were removed as the excess heterozygosity may have resulted from artifactual loci constructed from several paralogous genomic regions. Third, the SNPs retained by three or more samples were included in the SNP dataset.

Maximum likelihood phylogeny based on SNPs was inferred using the RAxML 8.2.10 software (Stamatakis 2014). A GTRCAT model was applied during this process and 1,000 replicates of parallel tree search bootstrapping were performed.

Phylogenetic analysis using chloroplast and nuclear genomic sequences

Three chloroplast and two nuclear genomic regions were sequenced using next-generation DNA sequencing. In this regard, *rbcL*, *trnL* intron, *psbA-trn*H, ITS1, and ITS2 were initially simultaneously amplified using the Multiplex PCR Assay Kit Ver. 2 (Takara Bio, Kusatsu, Japan) (first PCR). The first set of primers consisted of tail sequences and locus-specific primers (Suyama et al. 2022). Subsequently, the first PCR products were purified and used for the second PCR. The second PCR was conducted using primer pairs, including tail sequences, adapter sequences for Illumina sequencing, and the index sequence to identify each individual sample. In this step, the second PCR product from each sample was mixed and sequenced on the Illumina MiSeq platform using a MiSeq Reagent Nano Kit v2 (500 cycle, Illumina). The sequencing of the first three bases of reads 1 and 2 (anchor region for the 2nd PCR primer) was bypassed using the 'DarkCycle' option of the MiSeq system. Both ends of the fragments and index sequences were read by paired-end sequences (reads 1 and 2) and index sequencing. The number of bases per read was 251 bases for read 1 and 251 bases for read 2.

The sequences of the five regions were determined using the Claident pipeline (Tanabe and Toju 2013, http://www.claident.org/, Tanabe, A.S., Claident, Date of access: 05/01/2021). The raw MiSeq BCL data were first converted into FASTQ data using the BCL2FASTQ program provided by Illumina, followed by demultiplexing of the raw FASTQ data based on index and primer sequences, using the clsplitseq program in Claident. Subsequent analysis of the data was performed per region per individual. In ITS1 and ITS2, the pair-end reads were merged since reads 1 and 2 were overlapping. In *rbcL*, *trnL* intron, and *psbA-trn*H, reads 1 and 2 were independently analyzed because the length of the sequenced reads was too short to allow for merging. Additionally, the low-quality 3' tail was trimmed, and the low-quality sequences were filtered out using the clflterseq program. The noisy sequences were removed using the clcleanseqv program. Finally, the remaining reads were clustered with a cut-off sequence similarity of 99%. An OTU that had the most observed reads within the individual was treated as a representative OTU sequence.

Multiple alignments were performed using the MAFFT 7.313 program (Katoh and Standley 2013), and alignment columns containing gaps were trimmed using a heuristic selection method based on the similarity statistics of trimAl 1.4.rev15 (Capella-Gutierrez et al. 2009). The Kakusan 4.0 software (Tanabe 2011) was used to find suitable nucleotide substitution models and partitioning strategies for the nucleotide datasets by independently running the chloroplast and nuclear genomic regions through this program. The AICc criterion (Sugiura 1978) was used to compare the Nonpartitioned, the Partitioned_ Equal_Mean_Rate, as well as the Separate models. For chloroplast genomic regions, the Partitioned_Equal_Mean_Rate model (GTR + Γ), which assumes an equal rate of nucleotide substitutions across arbitrarily specified partitions, proved optimal. Contrastingly, for nuclear genomic regions, the Nonpartitioned model (GTR + Γ) proved optimal. Maximum likelihood phylogenies were further inferred using RAxML 8.2.10 (Stamatakis 2014), whereby 1,000 replicates of parallel tree search bootstrapping were conducted.

Data resources

All raw MIG-seq data were deposited at the DDBJ Sequence Read Archive (DRA) with accession number DRA011466. The demultiplexed raw reads of ITS and cpDNA regions were deposited at the DDBJ Sequence Read Archive (DRA) and assigned Accession no. DRA011467.


Figure 1. A phylogenetic tree of *Stellaria alsine* and its relatives reconstructed using MIG-seq data. Stals: *S. alsine* subsp. *alsine*. Staqu: *S. aquatica*. Stmed: *S. media*. Stnan: *S. alsine* subsp. *nana*. Stneg: *S. neglecta*. Bootstrap values are shown on the nodes. Branch length represents the average number of substitutions per SNP site.

Results

MIG-seq tree

A total of 15,551,282 raw reads $(1,196,252 \pm 87,288 \text{ reads per sample})$ were obtained via MIG-seq, of which 14,923,278 reads $(1,147,944 \pm 84,758 \text{ reads per sample})$ remained after quality control. Following the *de novo* SNP detection and filtering, a dataset comprised of 881 SNPs from 703 loci was obtained. Three samples – Stmon-JPN2119, Stmon-JPN2998, and Stdiv-JPN1352 – contained more than 90% of the miscalled SNPs; thus, these samples were eliminated from further SNP analysis.

Fewer shared loci were observed between *S. alsine* and *S. diversiflora*, as well as between *S. alsine* and *S. monosperma*, resulting in the exclusion of *S. diversiflora* and *S. monosperma* from the phylogenetic reconstruction. In the tree obtained (Fig. 1), the sister relationship between *S. alsine* subsp. *alsine* and subsp. *nana* was



Figure 2. A phylogenetic tree of *Stellaria alsine* and its relatives reconstructed using ITS sequences. Stals: *S. alsine* subsp. *alsine*. Staqu: *S. aquatica*. Stdiv: *S. diversiflora*. Stmed: *S. media*. Stmon: *S. monosperma*. Stnan: *S. alsine* subsp. *nana*. Stneg: *S. neglecta*. Bootstrap values are shown on the nodes. Branch length represents the average number of substitutions per site.

supported by a 100% bootstrap value. None of the three species included in the phylogenetic reconstruction, i.e., *S. aquatica*, *S. media*, and *S. neglecta*, are directly related to *S. alsine*.

ITS tree

A total of 64,378 reads $(4,952 \pm 387 \text{ reads per sample, ITS1})$ and 68,362 reads $(5,258 \pm 357 \text{ reads per sample, ITS2})$ were obtained. After the gaps were trimmed, the total length of the remaining sequences was 662 bp. The monophyly of S. *alsine* subsp. *alsine* and subsp. *nana* was supported by a 100% bootstrap value (Fig. 2). Moreover, none of the five other species were directly related to S. *alsine*. Additionally, the sister relationship between *S. diversiflora* and *S. monosperma* was supported by a 92% bootstrap value.

cpDNA tree

A total of 23,206 reads $(1,785 \pm 129 \text{ reads per sample}, rbcL)$, 2,142 reads $(165 \pm 24 \text{ reads per sample}, trnL \text{ intron})$, and 55,274 reads $(4,252 \pm 569 \text{ reads per sample}, psbA-trnH)$ were obtained. After the gaps were trimmed, the total length of the remaining



Figure 3. A phylogenetic tree of *Stellaria alsine* and its relatives reconstructed using cpDNA sequences. Bootstrap values are shown on the nodes.

sequences was 1,467 bp. The monophyly of S. *alsine* subsp. *alsine* and subsp. *nana* was supported by a 100% bootstrap value. As observed in the case of ITS, none of the five other species was directly related to S. *alsine* (Fig. 3). Furthermore, the sister relationship between *S. diversiflora* and *S. monosperma* was supported by a 100% bootstrap value.

Discussion

Phylogenetic trees obtained using MIG-seq, ITS sequences, and cpDNA sequences supported the monophyly of *Stellaria alsine* subsp. *alsine* and subsp. *nana*; the boot-strap supports for the monophyly were 100% in all trees. While *S. alsine* subsp. *nana* is a much smaller plant compared to *S. alsine* subsp. *alsine*, both subspecies are similar in their presence of oblong-lanceolate leaves that are glabrous and sessile. Both molecular and morphological evidence supports the deduction that subsp. *nana* is derived from subsp. *alsine* by adapting to the mountaintop habitats of Yakushima, where many dwarf endemics are found (Yahara et al. 1987).

In Japan, *S. alsine* ssp. *alsine* has been identified as *S. uliginosa* Murray var. *undulata* (Thunb.) Fewnzl. (Kadota 2017), but *S. uliginosa* is generally treated as a synonym of *S. alsine*, which is widely distributed in Europe, Asia, and eastern North America (Chen and Rabeler 2001). A recent molecular phylogenetic study (Sharples 2019) proposed that *S. alsine* is polyphyletic and included two unrelated lineages: one lineage from European Russia belongs to the Nitentes clade, and another one from eastern Asia and

eastern North America form the Uliginosae clade. *Stellaria alsine* was described from Europe (Grimm 1767), but Sharples (2019) cited the material from European Russia as 'S. cf. *alsine*', while those from N America and E Asia as 'S. *alsine*'. In this paper, we followed this treatment for the E Asian lineage as S. *alsine s. lat*.

In Japan, *S. alsine* ssp. *alsine* is a weedy species common in disturbed habitats near farmlands, including paddy fields, and along mountain paths. On the other hand, *Stellaria alsine* subsp. *nana* grows in natural habitats on rocks along streams in the mountainous area of Yakushima, at high elevations of 1500–1700 m. Another example of a weed-derived lineage dwarfed in the mountainous area of Yakushima is *Plantago asiatica* L. var. *yakusimensis* (Masam.) Ohwi (Plantaginaceae) (Yahara et al. 1987). These examples suggest that dwarfed endemics in Yakushima include lineages not only of ancient origin, such as *Mitella doiana* Ohwi (Saxifragaceae) (Okuyama et al. 2005), but also those of more recent origin. The discovery of *S. alsine* subsp. *nana* provides new materials for studying the origin and evolution of dwarfed endemic plants in Yakushima.

Taxonomy

Stellaria alsine Grimm subsp. nana K. Fuse & Yahara, subsp. nov.

urn:lsid:ipni.org:names:77234845-1 Fig. 4

Diagnosis. Stellaria alsine subsp. nana differs from the typical subspecies in its shorter stem (2.5–6 cm vs. 15–30 cm), smaller lamina (0.5–1 cm \times 1–3 mm vs. 0.5–2 cm \times 2–4 mm), shorter pedicels (0.1–0.6 cm long vs. 0.5–2 cm long), as well as chasmogamous flowers with 3 stamens and 2 styles (vs. 5 stamens and 3 styles) that are usually solitary (vs. usually 3–5 on a terminal or axillary cyme).

Type. JAPAN. Kagoshima Prefecture: Yakushima, along a path to Mt. Nagata, on rocks along streams, 30°20'28.96"N, 130°28'37.22"E, 1500 m elevation, 21 July 2020, with cleistogamous flowers, *T. Yahara*, *H. Sato*, *K. Fuse*, *Y. Higashi JPN0573* (*holotype*: FU!, *isotype*: KYO!).

Description. Herbs possibly biennial. Stems caespitose, 2.5–6 cm long, glabrous, erect in upper and middle parts, prostrate in lower parts. Leaves deciduous, sessile; blade oblong-obovate or narrowly obovate, 0.5–1 cm × 1–3 mm, glabrous, single-veined, base attenuate, apex acute, margin entire. Flowers solitary or 2–3 flowers in an axillary or terminal cyme with a ca. 6 mm scape. Pedicel 1–6 mm long, slender, glabrous. Sepals ovate-lanceolate, ca. 2.5 mm long in chasmogamous flowers, ca. 2.0 mm long in cleistogamous flowers, glabrous, apex acute. Petals of chasmogamous flowers 5, ca. 2.6 mm long, 2-cleft nearly to base; lobes oblanceolate, apex obtuse; petals absent in cleistogamous flowers. Stamens of chasmogamous flowers 3, 0.8 mm long; filaments ca. 0.4 mm long, glabrous, anthers globular, 0.4 mm in diam. Styles 2, glabrous. Capsule obovoid, 2.5 mm long, as long as sepals when mature, 2-loculated. Seeds dark brown, reniform, ca. 0.7 mm long, slightly flattened, tuberculate with raised papillae, without an appendage.



Figure 4. *Stellaria alsine* subsp. *nana* K. Fuse & Yahara **A** a living stem of *K. Fuse & T. Saito JPN4891* bearing a chasmogamous flower **B** the chasmogamous flower of A **C** Living stems of the holotype, *T. Yahara* et al. *JPN0573*, bearing cleistogamous flowers **D** a fruit of *K. Fuse & T. Saito JPN1791*. Scale bars: 1 cm (**A**, **C**); 1 mm (**B**); 2 mm (**D**).

Phenology. Chasmogamous flowers were observed in May, cleistogamous flowers and immature fruits were observed in July, and mature fruits and seeds were observed in September.

Distribution and habitat. Yakushima, Japan (endemic). At present, this subspecies has been identified in two populations growing on rocks along streams at 1500 m elevation in the vicinity of Mt. Nagata.

Etymology. The subspecific epithet is derived from its dwarf habit.

Conservation status. Vulnerable (VU). The population size was estimated to be between 250 and 1000 mature individuals. The habitat is located within the protected area of Yakushima (Island) National Park and no threats are detected at present.

Additional specimens examined. JAPAN. Kagoshima Prefecture: Yakushima, along a path to Mt. Nagata, 30°20'28.96"N, 130°28'37.22"E, 1500 m elevation, 4 September 2020, with fruits, *K. Fuse*, *T. Saito JPN1791* (FU!); a gorge N of Mt. Nagata, 30°20'39.7"N, 130°29'28.5"E, 1700 m elevation, 3 May 2021, with chasmogamous flowers, *K. Fuse*, *T. Saito JPN4891* (FU!).

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RESEARCH ARTICLE



A new species (Begonia giganticaulis) of Begoniaceae from southern Xizang (Tibet) of China

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Abstract

Begonia giganticaulis, a huge new species in *Begonia* sect. *Platycentrum* of Begoniaceae from southern Xizang (Tibet) of China, is described. Morphologically, it is mostly similar to *B. longifolia* and *B. aceto-sella*, but clearly differs from the former mainly by its dioecious and taller plants, sparse hairs on abaxial veins, longer inflorescence, unique shape of fruits, and differs from the latter mainly by its late and longer flowering time, 6-tepals of female flower and 3-loculed ovary. The phylogenetic analyses also support the separation of the new species from other taxa. Based on the current data, its conservation status is assigned to Endangered (B2a) according to the IUCN Red List Categories and Criteria.

Keywords

Conservation status, molecular evidence, morphology, southern Tibet, taxonomy

^{*} The authors contributed equally.

Introduction

Zangnan (southern Tibet) of China is located to the south of the Himalayas, including most parts of Cona, Lhünzê, Mêdog and Zayü counties, and some smaller parts of Nang and Mainling counties (Liu 2019). This region is very warm and rainy because of the southwest monsoon carrying heavy water and heat from the Indian Ocean. Owing to high average annual precipitation and high-proportion of forest coverage (Hao et al. 2010), the plant diversity is very high in Zangnan. However, this area still remains under-explored and needs more study in the future.

After a series of plant surveys recently, the authors have a better understanding of the diversity of *Begonia* in Tibet, particularly in its southern part (namely Zangnan) including Mêdog county. Up until now, 39 species and 4 varieties had been found in Tibet (Gu et al. 2007; Camfield and Hughes 2018; Tian et al. 2020) (Table 1). In addition, *Begonia limprichtii* Irmsch. (Irmscher 1922) was newly reported by Borah et al. (2021a) in southern Tibet, but this record is likely based on a wrong identification and further study is needed. Of these, 31 species and 3 varieties are distributed in Mêdog. Recently, after several field surveys in Mêdog, we found several new species and at least three natural hybrids. Here we described *Begonia giganticaulis* D.K.Tian & W.G.Wang sp. nov. from Mêdog, a new species of huge plant size, which is morphologically similar to both *B. longifolia* Blume (Blume 1827) and *B. acetosella* Craib (Craib 1912). The morphological differences of the three species are compared, and the new species is also supported by molecular evidence.

Material and methods

Morphological analysis

The field surveys were conducted on habitat, distribution, population size, morphology and specimen collection of the new species. Diagnosis of the morphological difference between the new species and its similar species was based on literature review, examination of herbarium specimens, and observation of both wild and cultivated plants.

Phylogenetic analysis

The treatment on sections of *Begonia* follows Shui et al. (2019). To ascertain the relationship of the new species within sect. *Platycentrum* (Klotzsch) A.DC. (de Candolle 1859), two female and three male individuals were sampled, and three individuals of *B. longifolia*, two individuals of *B. acetosella*, and three individuals of *B. acetosella* var. *hirtifolia* Irmsch. (Irmscher 1939) were sampled and sequenced. 13 taxa within sect. *Platycentrum* were selected based on Moonlight et al. (2018) to ascertain the phylogenetic relationship of the new species. *Begonia cavaleriei* H.Lév. (Léveille 1909) from sect. *Coelocentrum* Irmsch. (Irmscher 1939) was used as outgroup. All

ibet.

Species	Reference	County
Begonia aborensis Dunn	Dunn 1920	Mêdog
<i>Begonia acetosella</i> Craib	Craib 1912	Mêdog
Begonia annulata K.Koch	Koch 1857	Mêdog
Begonia asperifolia Irmsch.	Irmscher 1927	Bomê, Zayü, Lhünzê,
		Mêdog
Begonia burkillii Dunn	Dunn 1920	Mêdog
Begonia cathcartii Hook.f. & Thomson	Hooker 1855	Zayü
Begonia dioica BuchHam. ex D.Don	Don 1825	Dinggyê
Begonia difformis (Irmsch.) W.C.Leong, C.I Peng & K.F.Chung	Leong et al. 2015	Mêdog
<i>Begonia flagellaris</i> Hara	Hara 1973	Gyirong, Nyalam
<i>Begonia flaviflora</i> var. <i>flaviflora</i> Hara	Hara 1970	Mêdog
Begonia flaviflora var. gamblei (Irmscher) Golding & Karegeannes	Golding and Karegeannes 1984	Mêdog
Begonia giganticaulis D.K.Tian & W.G. Wang sp. nov.	In this study	Mêdog
Begonia grandis Dryand.	Dryander 1791	Zayü
Begonia griffithiana (A.DC.) Warb.	Warburg 1894	Mêdog
Begonia handelii Irmsch.	Irmscher 1921	Mêdog
Begonia hatacoa BuchHam. ex D.Don	Don 1825	Mêdog, Cona
Begonia iridescens Dunn	Dunn 1920	Mêdog, Zayü
Begonia josephii A.DC.	de Candolle 1859	Cona, Dinggyê, Lhün-
0 7 1		zê, Mêdog, Yadong
Begonia kekarmonyingensis Taram, D.Borah & M.Hughes	Taram et al. 2021	Mêdog
Begonia labordei H.Lév.	Léveillé 1904	Zayü
Begonia limprichtii Irmsch. *(wrong identification, based on the distribu-	Borah et al. 2021a	Mêdog
tion and morphological characteristics)		-
Begonia longifolia Blume	Blume 1827	Mêdog
Begonia medogensis J.W.Li, Y.H.Tan & X.H.Jin	Li et al. 2018	Mêdog
Begonia megaptera A.DC.	de Candolle 1859	Zayü
Begonia nepalensis (A.DC.) Warb.	Warburg 1894	Cona
Begonia ovatifolia A.DC.	de Candolle 1859	Mêdog
Begonia oyuniae M.Taram & N.Krishna	Taram et al. 2020	Mêdog
<i>Begonia plamata</i> var. <i>plamata</i> D.Don	Don 1825	Mêdog
Begonia palmata var. bowringiana (Champion ex Bentham) Golding &	Golding and Karegeannes 1984	Mêdog
Karegeannes		
Begonia palmata var. khasiana (Irmsch.) Golding & Kareg	Golding and Karegeannes 1984	Mêdog
Begonia pasighatensis D.Borah, Taram & Wahlsteen	Borah et al. 2021b	Mêdog
Begonia picta Sm.	Smith 1805	Gyirong, Mêdog,
		Nyalam
Begonia pseudoheydei Y.M.Shui & W.H.Chen	Chen et al. 2019	Mêdog
Begonia rex Putz.	Putzey 1857	Mêdog
Begonia roxburghii (Miq.) A.DC.	de Candolle 1864	Mêdog
Begonia scintillans Dunn	Dunn 1920	Mêdog
Begonia shilendrae Rekha Morris & P.D.McMillan	Morris and McMillan 2012	Cona
Begonia sikkimensis var. sikkimensisA.DC.	de Candolle 1859	Mêdog
Begonia sikkimensis var. kamengensis Rekha Morris, P.D.McMillan &	Golding 2009	Cona
Golding ex Golding		
Begonia silletensis Clarke	Clarke 1879	Mêdog
Begonia tessaricarpa C.B.Clarke	Clarke 1879	Mêdog
Begonia thomsonii A.DC.	de Candolle 1859	Mêdog
Begonia xanthina Hook.f.	Hooker 1852	Mêdog
Begonia zhongyangiana W.G.Wang et S.Z.Zhang	Wang et al. 2019	Mêdog

the voucher specimens were deposited in the herbarium of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (HITBC). For DNA sequencing, the total genomic DNA was extracted from silica-dried leaves by a modified CTAB

Taxa	Collector, Voucher (Her- barium)	Origin	ITS	rpL16	ndhA	References
<i>Begonia aceto-</i> <i>sella</i> Craib	Wang, W.G., WWG004 (HITBC)	Mengla, Yun- nan, China				In this study
<i>B. acetosella</i> Craib	Wang, W.G., WWG005 (HITBC)	Mengla, Yun- nan, China				In this study
<i>B. acetosella</i> var. <i>hirtifolia</i> Irmsch.	Wang, W.G., WWG0261 (HITBC)	Ruili, Yunnan, China			—	In this study
<i>B. acetosella</i> var. <i>hirtifolia</i> Irmsch.	Wang, W.G., WWG0262 (HITBC)	Ruili, Yunnan, China				In this study
<i>B. acetosella</i> var. <i>hirtifolia</i> Irmsch.	Wang, W.G., WWG0300 (HITBC)	Ruili, Yunnan, China	_			In this study
<i>B. aptera</i> Blume	—	—		—		Chiang (unpublised); Thomas et al. (2011)
<i>B. balansana</i> Gagnep.	_	_				Forrest and Holling- sworth (2003); Chung et al. (2014); Moonlight et al. (2018)
<i>B. cathayana</i> Hemsl.	_	_				Yang et al. (unpub- lished); Chung et al. (2014); Zhao (unpub- lished)
<i>B. cavaleriei</i> H.Lév.	_	—				Chung et al. (2014); Tong et al. (2019)
<i>B. decora</i> Stapf	—	—				Chung et al. (2014); Thomas et al. (2011)
<i>B. giganticau- lis</i> D.K.Tian & W.G.Wang	Wang, W.G., Li, Y.Y., Ma, X.D. & Shen, J.Y., WWG2014–1 (HITBC)	Mêdog, Tibet, China				In this study
<i>B. giganticau- lis</i> D.K.Tian & W.G.Wang	Wang, W.G., Li, Y.Y., Ma, X.D. & Shen, J.Y., WWG2015–1 (HITBC)	Mêdog, Tibet, China				In this study
<i>B. giganticau- lis</i> D.K.Tian & W.G.Wang	Wang, W.G., Li, Y.Y., Ma, X.D. & Shen, J.Y., WWG2015–2 (HITBC)	Mêdog, Tibet, China				In this study
B. giganticau- lis D.K.Tian & W.G.Wang	Wang, W.G., Li, Y.Y., Ma, X.D. & Shen, J.Y., WWG2014–3 (HITBC)	Mêdog, Tibet, China				In this study
<i>B. giganticau- lis</i> D.K.Tian & W.G.Wang	Wang, W.G., Li, Y.Y., Ma, X.D. & Shen, J.Y., WWG2014–2 (HITBC)	Mêdog, Tibet, China				In this study
<i>B. handelii</i> Irmsch.	_	_				Forrest and Holling- sworth (unpublished); Chung et al. (2014); Moonlight et al. (2018)
<i>B. hatacoa</i> BuchHam. ex D.Don	_	_				Forrest and Holling- sworth (unpublished); Chung et al. (2014); Thomas et al. (2011)
<i>B. longifolia</i> Blume	Wang, W.G., WWG001 (HITBC)	Mengla, Yun- nan, China				In this study
<i>B. longifolia</i> Blume	Wang, W.G., WWG002 (HITBC)	Mengla, Yun- nan, China				In this study

Table 2. Sampled taxa and GenBank accession numbers of *Begonia giganticaulis* and the related taxa used for phylogenetic analysis.

Taxa	Collector, Voucher (Her-	Origin	ITS	rpL16	ndhA	References
	barium)					
B. longifolia	Wang, W.G., WWG003	Mengla, Yun-				In this study
Blume	(HITBC)	nan, China				
B. nepalensis	_	_		_		Tebbitt et al. (2006);
(A.DC.)						Moonlight et al. (2018)
Warb.						
B. obovoidea	_	_	—	—		Thomas et al. (2011)
Craib						
B. pavonina	—	—				Chung et al. (2014);
Ridl.						Thomas et al. (2011)
B. pedatifida	_	_				Tong et al. (2019)
H.Lév.						
B. roxburghii	—	_		_		Forrest and Holling-
A.DC.						sworth (2003); Thomas
						et al. (2011)
B. versicolor	—	—				Forrest and Holling-
Irmsch.						sworth (unpublished);
						Chung et al. (2014);
						Thomas et al. (2011)

protocol (Doyle and Doyle 1987). The chloroplast DNA *rpL16* intron, *ndhA* intron and the nuclear ribosomal DNA internal transcribed spacer (nrITS) region were used to infer the phylogenetic relationship of the new species. The *rpL16* intron were amplified by the primer rpL16-F and rpL16-R and sequenced by the primer rpL16-R and Beg-rpL16 (Chung et al. 2014). For the amplification of the *ndhA* intron the primer *ndhAX*1 and *ndhAX*2 (Thomas et al. 2011) were used. The nrITS region was amplified and sequenced by the primer 51NT and 26S1Rev (Clement et al. 2004). The sampled sequences were downloaded from NCBI and accession numbers were listed in Table 2.

Sequences of each DNA region were aligned by MUSCLE online (https://www. ebi.ac.uk/Tools/msa/muscle/, Madeira et al. 2019) and adjusted manually when necessary. Indels were treated as gap. For testing the congruence within *rpL16* intron, *ndhA* intron and nrITS, the analysis of the incongruence length difference (ILD) was performed with 100 replicates under default heuristic search using PAUP v.4.0a (Swofford 2002) and the phylogenetic trees were constructed based on each dataset. The p value was 0.40 and no conflict among each phylogenetic trees, indicating the congruence among these datasets (Farris et al. 1994).

The parsimony analysis was conducted using PAUP v.4.0 b10 (Swofford 2002). The Maximum Parsimony (MP) analysis was run using a heuristic search with 1, 000 replicates and tree-bisection-reconnection (TBR) with no reconnection limit. Bootstrap was used to assess the node support by 1000 replicates using TBR branch swapping. The Bayesian analysis was conducted using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003) with 1, 000, 000 generations under the Markov chain Monte Carlo (MCMC) chains. The average standard deviation of split frequencies was 0.004210 after 1, 000, 000 generations. The consensus tree was constructed after burn-in 25% of the trees. The Posterior Probability (PP) was used to assess the branch supports.

Results

Taxonomic treatment

Begonia giganticaulis D.K.Tian & W.G.Wang, sp. nov.

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Type. CHINA. Xizang (Tibet) Autonomous Region: Mêdog county (墨脱县), Beibeng town (背崩乡), Baimu Xiri river (白母西日河), forest slope of river valley or water's edge along stream, 29°21'9"N, 95°11'21"E, elev. 1320 m, 10 September 2020, *Dai-Ke Tian, Fang Wen, Qing-Gong Mao, & Zhu Lu, TDK4773-A* (holotype CSH! Barcode number: 0180561, ♀)

Diagnosis. The new species is mostly similar to *B. longifolia* and *B. acetosella*, but clearly differs from the former mainly by its dioecious (vs. monoecious), taller (to 4 m vs. less than 2 m) plants, longer (vs. shorter) inflorescence, and unique shape of fruits, and differs from the latter mainly by its taller (to 4 m vs. less than 2 m) plants, late and longer (Jun. to Oct. vs. Mar. to Apr.) flowering time, longer (6–20 mm vs. 5–12 mm) pedicel, 6 (vs. 4) tepals of pistillate flower and 3 (vs. 4)-loculed ovary (Table 3, Fig. 3).

Description. Herb perennial, evergreen, to 4 m tall, dioecious. Rhizome short, stout, nearly unbranched, reddish brown, to 12 cm thick. Stem erect, reddish brown or green, glabrous, internodes to 5 cm thick, with many longitudinally fusiform whitish spots, cross section of stem often reddish brown, nodes notably enlarged, to 7 cm thick, with unequally oval to round whitish spots, many shrubby branches on the upper part of main stem. Stipule long-triangular, light green or pinkish green, $9-25 \times 2-8$ mm, glabrous, margin entire, dorsal ridge pinkish, apex acuminate with arista 4–6 mm long. Petioles green, pink to red, glabrous, 7–22 cm long, 1–3 mm thick. Leaf blade ovate-lanceolate to lanceolate, $4-19 \times 0.8-8$ cm, adaxial green, muriculate to nearly glabrous, adaxial veins slightly concave; abaxial greyish green, veins usually red, convex, main veins sparsely and obliquely strigose; base obliquely cordate, margin shallowly and remotely denticulate, apex long caudate; Inflorescence dichasial cyme, axillary, short, 3-5 cm long, unbranched to branched once, rachis glabrous, green, pinkish green to red, base usually red-brown, 7-15 mm long, 1-1.5 mm thick, 3-11 male flowers or 1–5 female per inflorescence. Bract often caducous, pinkish green, long triangular, glabrous, ca. 6 × 3 mm, apex acuminate; bracteoles smaller. Staminate flower: pedicel glabrous, white, whitish or pinkish green, 10–14 mm long, ca.1 mm thick; corolla 18–24 mm in diameter; tepals 4, subequal, glabrous, outer 2, obovate, $9-14 \times 6-9$ mm, apex obtuse, adaxially white and middle-upper part abaxially pink, or pure white for some individuals, longitudinal veins unapparent; inner 2, pure white, obovate to obovatelanceolate, $8-13 \times 5-7$ mm, apex obtuse; and roccium nearly actinomorphic, ca. 5 mm long, 6-7 mm in diam; stamens 48-60, filaments free, 1-2 mm long; anther yellow, 2-3 mm long, apex obtuse or nearly so. Pistillate flower: pedicel white or green-white,



Figure 1. Habitat and large-sized plant of *Begonia giganticaulis* D.K.Tian & W.G.Wang, sp. nov. **A** habitat showing plants (arrows indicate) growing along stream bank **B** flowering plant growing along slope of valley **C** one of the tallest individuals with Dr. Dai-Ke Tian. (Photos **A** by Dai-Ke Tian **B** by Shi-Wei Guo **C** by Qing-Gong Mao).



Figure 2. Morphology of *Begonia giganticaulis* D.K.Tian & W.G.Wang, sp. nov. **A** one of the single tallest plants cut into four sections **B** main stem base **C** stems showing colour of nodal cross-sections **D** main stem with much expanded node and whitish-green lines or spots **E** expanded node on terminal branch **F**, **G** male plant branches showing inflorescences and different colours **H** female branches **I** adaxially (left) nearly glabrous and abaxially sparse hairs on veins (right, arrows indicate) on blade surfaces **J** stipules showing shape and colour. (Photo **F** by Wen-Guang Wang; others by Dai-Ke Tian).



Figure 3. Flower and fruit morphology of *B. giganticaulis* compared with its close species *B. longifolia* and *B. acetosella* **A–H** *Begonia giganticaulis* **A** staminate flowers with pinkish outer tepals **B** staminate flowers with white tepals **C**, **D** pistillate flower **E** ovary sections showing different colour **F** fruits on branch **G**, **H** dorsal and front views of fruits **I–K** *B. longifolia* **I** flowering and fruiting branch **J** fruits showing short horns **K** ovary dissection **L–O** *B. acetosella* **L** staminate flower **M** Pistillate flower **N**, **O** fruits with short horns or wings. (Photos **C** by Shi-Wei Guo **E** (left) **L**, **M** & **O** by Wen-Guang Wang; others by Dai-Ke Tian).



Figure 4. Illustration of *Begonia giganticaulis* D.K.Tian & W.G.Wang, sp. nov. (Drawn by Mr. Zhi-Min Li) **A** male flowering branches **B** female flowering branches **C** main stem line spots, much expanded node and internode base **D** expanded node and internode base on small upper branches **E** leaf blade **F** leaf (abaxial), showing sparse hairs on veins **G** stipule **H** staminate flowers **I**, **J** pistillate flower **K** side view of androecium **L** stamens **M** ovary and stigmas **N** fruit **O** dissection of ovary showing placentae.

Character	B. giganticaulis	B. longifolia	B. acetosella		
Plant					
sexuality	dioecious	monoecious	dioecious		
height (m)	up to 4	less than 2	less than 2		
Petiole length (cm)	0.7-7	1-12	1-10		
Leaf blade surface	muriculate	glabrous to less muriculate	muriculate to hirsutulous		
Inflorescence					
peduncle length (mm)	7-15	4-10	2-10		
flower number	1-11	1-11(15)	1-3(5)		
Tepal number of pistillate flower	6	6 6			
Tepal colour	pinkish to white	white	pinkish to white		
Ovary	3-loculed	3-loculed	4-loculed		
Pedicel length (mm)					
male flower	10-20	5-12	8-12		
female flower	6-12	5-12	5-10		
Fruit horn or wing	none to rarely short crest	none to short crest or horns	short to long horns or wings		
Flowering time	June-October	June-December	March-April		

Table 3. Morphological comparison of Begonia giganticaulis, B. longifolia and B. acetosella.

6–12 mm long, 0.8–1 mm thick; corolla 20–25 mm, tepals 6, rarely 4, glabrous, outer 3 (rarely 2), obovate or long obovate, thick and rigid, $12-18 \times 7-10$ mm, adaxial surface nearly white, distinctly concave, abaxially pink on middle-upper part, inner 3 (rarely 2), obovate-lanceolate to oblanceolate or long elliptical, slightly narrower than outer tepals, $10-19 \times 6-8$ mm, white, glabrous, apex obtuse; styles + stigmas 5 mm long, 7–8 mm wide; styles 3, free; stigmas yellow, nearly U-shaped, each side spirally twisted 1.5 circles; ovary pink or green, with white convex spots; placentation axile, 3-loculed, each placenta 2-branched. **Peduncle** green to pinkish green, glabrous, 8-12 mm long, ca. 1 mm thick. **Fruit** red, pink or green, glabrous, triangular-gyroscopic, $8-11 \times 1-12$ mm wide, concave between two placentas, wingless to occasionally short ridged, apex with beak 3–4 mm long. Flowering Jun.–Oct., fruiting Jul.–Dec.

Additional specimen examined. CHINA. Xizang: Mêdog County (墨脱县), Beibeng Town (背崩乡), Baimu Xiri River (白母西日河), forest slope of river valley or water's edge along stream, 29°21'9"N, 95°11'21"E, elev. 1320 m, 10 September 2020, Dai-Ke Tian, Fang Wen, Qing-Gong Mao, & Zhu Lu TDK4773-B (paratype CSH!, ♂); 29°20'0"N, 95°10'49"E, elev. 1110 m, 10 September 2020, Dai-Ke Tian, Fang Wen, Qing-Gong Mao, & Zhu Lu TDK4765-A, TDK4765-B, (paratype CSH!); 29°18'32"N, 95°10'38"E, elev. 980 m, 10 September 2020, Dai-Ke Tian, Fang Wen, Qing-Gong Mao, & Zhu Lu TDK4777 (paratype CSH!); near Ani Bridge (阿尼桥), 29°17'8.41"N, 95°10'3.23"E, elev. 810 m, 3 July 2020, Wen-Guang Wang, You-Yun Li, Xing-Da Ma, & Jian-Yong Shen, WWG 2014 (paratype, HITBC!), WWG 2015 (paratype HITBC!); elev. 1100 m, 16 September 1974, anonymous 2608 (paratype PE!); elev. 800-1400 m, 30 June 1980, Wei-Lie Chen 10809 (PE!); near No. 2 Bridge, 29°16'42"N, 95°10'49"E, elev. 810 m, 1 October 2017, Dai-Ke Tian, Yan Xiao, Xin Zhong, Li-Zhi Tian & Zhu Lu TDK3429 (paratype CSH!); Beibeng to Hanmi (汗密), elev. 840 m, 7 August 2010, South Tibet Expedition Team (藏南队), Xiao-Hua Jin, Shu-Dong Zhang, Zhong-Yang Li, Bao-Cheng Wu, Xian-Yun Mu, Jing Li & Wei-Tao



Figure 5. Distribution of *B. giganticaulis* (triangles) from southern Xizang, China.

Jin, STET2304 (paratype PE!); Hanmi to Maniweng (马尼翁), elev. 800–1000 m, 6 August 1974, Qingzang Team 74-4114 (paratype PE!); elev. 1200 m, 24 June 1983, Bo-Sheng Li & Shu-Zhi Chen 05229 (paratype PE!); Maniweng to Ani Bridge, elev. 700–1000 m, 3 August 1972, Tibet Expedition Team, Institute of Biology 1631 (paratype HNWP!).

Distribution and habitat. Currently known from at least two localities in Mêdog, southern Xizang (Tibet), China (Fig. 5). It grows on the slopes under forest along streams, elevation 450–1400 m.

Conservation status. *Begonia giganticaulis* is currently found in at least two localities in Mêdog of Tibet. Additional populations might be discovered when more surveys are conducted in China-India border region. However, based on current data, it should be categorised as Endangered: B2a (IUCN 2019) due to < 500 km² area of occupancy with severely fragmented habitat consisting of < 5 known populations to-tally under 1000 individuals by estimation.



Figure 6. Phylogenetic tree inferred by MP **A** and BI **B** analyses based on the combined matrix of two plastid loci (*rpL16* intron and *ndhA* intron) and nuclear ITS. Maximum parsimony bootstrap **A** and Bayesian inference posterior probability values **B** are labelled on the branches; when the number is below 80 and 0.80 in maximum parsimony bootstrap and Bayesian inference posterior probability, respectively, the branches are labelled—.

Etymology. The specific epithet refers to the huge (very tall and thick stem) plant size of the new species, which is the tallest begonia in Asia.

Molecular systematic relationship

We obtained 12 nrITS, 13 *rpL16* intron, and 13 *ndhA* intron of the new species and related *Begonia* taxa. In order to reconstruct the phylogenetic relationship of the new species, 13 taxa within sect. *Platycentrum* were included and *B. cavaleriei* from sect. *Coelocentrum* was selected as outgroup. In total, the matrix was composed of 26 accessions and contained the 962 bp *rpL16* intron, the 1109 bp *ndhA* intron and the 672 bp nrITS sequence. Of the total 2743 characters, 132 were parsimony informative.

Based on MP analysis, the new species was clustered with *B. acetosella* and *B. acetosella* var. *hirtifolia* (Fig. 6A), while it was clustered with *B. longifolia* under BI analysis (Fig. 6B). Both MP and BI analyses showed that all five individuals of the new species were clustered together and separated from other taxa (Fig. 6A, BS: 100%; Fig. 6B, PP:1.00).

Notes. – The earliest specimen of *Begonia giganticaulis* was collected in 1972 between Maliweng and Ani Bridge, Mêdog, Tibet, China. This species is similar to *B. acetosella* in appearance when its flowers are unavailable for observation, therefore, it was misidentified (24 June 1983, *Bo-Sheng Li & Shu-Zhi Chen 05229*, PE! was wrongly identified as *B. acetosella* by C.Z. Gu in March 2004). Also, due to its high similarity to *B. longifolia* particularly in morphology of flowers and fruits, *B. giganticaulis* was wrongly labelled as *B. longifolia* by Morris (2010) who found this species in southern Mêdog county.

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RESEARCH ARTICLE



Karyotype and genome size variation in white-flowered Eranthis sect. Shibateranthis (Ranunculaceae)

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Abstract

Comparative karyomorphological analyses of six out of the eight white-flowered species of Eranthis sect. Shibateranthis have been carried out. All studied specimens of E. byunsanensis, E. lobulata, E. pinnatifida, and *E. stellata* had a somatic chromosome number 2n = 16 with basic chromosome number x = 8. On the contrary, *E. tanhoensis* and *E. sibirica* had a basic chromosome number x = 7. The specimens of *E. tanhoensis* were diploid with 2n = 14, while the specimens of *E. sibirica* were polyploid with 2n = 42. Monoploid chromosome sets of the investigated diploid species had 4-5 metacentric chromosomes and 2-4 submetacentric/subtelocentric/acrocentric chromosomes. The highest level of interchromosomal asymmetry, estimated via CV_{CL}, was found in *E. byunsanensis* and *E. pinnatifida*. The highest levels of intrachromosomal asymmetry (M_{CA}) and heterogeneity in centromere position (CV_{CI}) were found in *E. lobulata* and E. byunsanensis, while E. sibirica had the most symmetric karyotype. A multivariate PCoA analysis of basic karyotype parameters (2n, x, THL, CV_{CL}, M_{CA}, and CV_{Cl}) highlighted no overlap among species accessions, which was also confirmed by LDA. The average absolute monoploid DNA content (1Cx) of the 23 investigated samples of six *Eranthis* species varied from 9.26 \pm 0.25 pg in *E. sibirica* to 15.93 \pm 0.32 pg in E. stellata. Overall karyological affinity was highlighted between E. lobulata and E. stellata, on one side, and between E. byunsanensis and E. pinnatifida, on the other side. Interestingly, there was no significant correlation between total haploid (monoploid) chromosome length (THL) and 1Cx values in these species.

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Keywords

Asia, chromosomes, Eranthis, genome size, karyotype, Ranunculaceae

Introduction

Chromosomal analysis is widely used in systematic and evolutionary studies of plants (Yuan and Yang 2006; Guerra 2012; Ilnicki 2014; Baltisberger and Hörandl 2016; Peruzzi et al. 2017). The main features of a karyotype are chromosome number, size and morphology of chromosomes (Astuti et al. 2017). Differences and similarities in karyotypes between taxa may reflect their evolutionary relationship (Shubert 2007; Peruzzi et al. 2009; Escudero et al. 2014; Baltisberger and Hörandl 2016). At present, it is appropriate to use the comparative analysis of karyotypes as part of an integrative approach to solving the issues of systematics and phylogeny (Astuti et al. 2017; Mráz et al. 2019; Erst et al. 2020b).

The genus *Eranthis* Salisb. belongs to Ranunculaceae Juss. tribe Cimicifugeae Torr. & A.Grey (Wang et al. 2009). This genus consists of ten to thirteen early flowering herbaceous perennial species distributed across Southern Europe, Western, Central and temperate Asia (Stefanoff 1963; Rukšāns and Zetterlund 2018; Park et al. 2019; Erst et al. 2020b). This genus generally exhibits a high level of endemism and it is distributed in both mainland and islands. Eranthis species seldom co-occur and the size of their distribution range usually varies significantly (Oh and Oh 2019). Eranthis is divided into two sections: E. sect. Eranthis and E. sect. Shibateranthis (Nakai) Tamura (Tamura 1987). The species belonging to the first section exhibit perennial tubers or tuberous rhizomes, yellow to orange sepals and yellow petals without pseudonectaries, whereas species of the second section have perennial tubers, white or slightly pink sepals and white petals with pseudonectaries (Tamura 1995; Zetterlund 2018; Park et al. 2019; Rukšāns and Erst et al. 2020b; Huang et al. 2021). The yellow-flowered E. sect. Eranthis includes five species distributed in Southern Europe (Eranthis bulgarica (Stef.) Stef., E. hyemalis (L.) Salisb.), Western Asia (E. cilicica Scott & Kotschy, E. iranica Rukšans & Zetterl) and Central Asia (E. longistipitata Regel). The whiteflowered E. sect. Shibateranthis includes eight species distributed in temperate Asia. Two species occur in Siberia (E. sibirica DC. and E. tanhoensis Erst), two in Tibet (E. albiflora Franch. and E. lobulata W.T.Wang), two in Korea (E. byunsanensis B.Y.Sun and E. pungdoensis B.U.Oh), one in Japan (E. pinnatifida Maxim.), and one is widespread and grows in China, Korea and the Far East of Russia (E. stellata Maxim.) (Oh and Oh 2019; Park et al. 2019; Erst et al. 2020b).

The somatic chromosome number 2n = 2x = 16 has been reported in *Eranthis* in eight species from both sections: *E. byunsanensis* (Kim et al. 2011), *E. cilicica* (Langlet 1932), *E. hyemalis* (Colasante and Ricci 1974; Tak and Wafai 1996; Gömürgen 1998; Caparelli et al. 2007; Erst et al. 2020a), *E. lobulata* (Erst et al. 2019), *E. longistipitata* (Erst et al. 2019), *E. pinnatifida* (Kurita 1955), *E. sibirica* (Gnutikov et al. 2016, 2017), and *E. stellata* (Yuan and Yang 2006). According to another study, *E. stellata*

from Russian Far East would have somatic chromosome number 2n = 14 (Starodubtsev 1985), and this number was recently found in *E. tanhoensis* (Erst et al. 2020b). Additionally, polyploid cytotypes have been revealed in the genus *Eranthis*, e. g., triploid *E. hyemalis* with 2n = 24 (Colasante and Ricci 1974), tetraploid *E. sibirica* that had 2n = 32 (Krogulevich 1976) chromosomes, and the same species was recently found actually hexaploid by Erst et al. (2020b). The karyotype has been analyzed for five species: *E. pinnatifida* (Kurita 1955), *E. hyemalis* (Gömürgen 1998), *E. stellata* (Yuan and Yang 2006), *E. sibirica* and *E. tanhoensis* (Erst et al. 2020b).

The genome size (absolute nuclear DNA content), estimated by flow cytometry, is an essential genome feature together with the chromosome number and karyomorphological parameters (Doležel and Bartoš 2005). Flow cytometry can be considered a quick and useful method for understanding taxonomic relationships (Mabuchi et al. 2005; Zonneveld 2010). However, the Plants DNA C-value DataBase (https://cvalues. science.kew.org) contains data on *E. cilicica, E. hyemalis*, and *E. pinnatifida* only. This study reports data on comparative karyotype analysis and genome size of six out of eight white-flowered species of *Eranthis* sect. *Shibateranthis* (Fig. 1): *E. byunsanensis, E. lobulata, E. pinnatifida, E. sibirica, E. stellata*, and *E. tanhoensis*.

Methods

Plant samples

Plant material (tubers) of *E. byunsanensis, E. lobulata, E. pinnatifida, E. sibirica, E. stellata,* and *E. tanhoensis* was collected during field investigations in Russia, China, Japan and South Korea during 2018–2020. The list of the samples examined is presented in Table 1. Herbarium specimens were deposited in the E and NS herbaria (herbarium acronyms according to Thiers 2019, continuously updated).

Karyotype analysis

The comparative karyotype analysis was conducted for 22 populations: one of *E. byun-sanensis* and *E. lobulata*, four of *E. pinnatifida*, three of *E. sibirica*, five of *E. stellata*, and eight of *E. tanhoensis* (Table 1). Somatic chromosomes of *Eranthis* were studied from root tip cells. Tubers were germinated in wet moss at ~ 15 °C for 2–4 weeks. Newly formed 1–2 cm long roots were excised and pretreated in 0.5% colchicine solution at 15 °C for 3–4 h. Roots were fixed in a mixture of 96% ethanol and glacial acetic acid (3:1). Root tips were stained with 1% aceto-haematoxylin, and the karyotypes were investigated by the squash method (Smirnov 1968). Chromosomes were counted in 30–100 mitotic cells for each population (a more detailed study was conducted for *E. sibirica* and *E. tanhoensis*). Mitotic metaphase chromosome plates were studied using an Axio Star microscope (Carl Zeiss, Munich, Germany) and photographed using an Axio Imager A.1 microscope (Carl Zeiss, Munich, Germany) with AxioVi-



Figure 1. The studied species of white-flowered *Eranthis* sect. *Shibateranthis* **A** *E. stellata* (photo by V.V. Yakubov) **B** *E. sibirica* (photo by A.S. Erst); **C** *E. tanhoensis* (photo by A.S. Erst) **D** *E. lobulata* (photo by K.-L. Xiang) **E** *E. pinnatifida* (photo by A.S. Erst) **F** *E. byunsanensis* (photo by H.J. Choi).

sion 4.7 software (Carl Zeiss, Munich, Germany) and AxioCam MRc5 CCD-camera (Carl Zeiss, Munich, Germany) at 1000× magnification in the Laboratory for Ecology, Genetics and Environmental Protection (Ecogene), National Research Tomsk State University (Tomsk, Russia). KaryoType software (Altinordu et al. 2016) was used for karyotyping, and Adobe Photoshop CS5 (Adobe Systems, USA) and Inkscape 0.92 (USA) were used for image editing.

Karyotype formulas were derived, based on measurements of the photographed mitotic metaphase chromosomes. The measurements were performed on 4–12 metaphase plates per population. We used 2–6 metaphase plates per population with the most condensed chromosomes to calculate mean karyomorphological parameters.

N°	Species	Voucher information	2 <i>n</i>	Ploidy	$1Cx \pm SD (pg)$
1	E. lobulata	China, Sichuan Province, Jiuding Shan Mountain, 31°32'36.0"N, 103°51'12.0"E, 14 May 2018, L. Zhang	16	2x	13.87 ± 0.29
2	E. stellata*	Russia, Primorsky Krai, Vladivostok City, Akademicheskaya Station, 43°11'25.9"N 131°55'31.7"E, 12 Apr 2018, V.V. Yakubov	16	2 <i>x</i>	15.88 ± 0.31
3	E. stellata*	Russia, Primorsky Krai, Vladivostok City, Malaya Sedanka River, 43°12'36"N, 131°59'24"E, 16 Apr 2019, V.Yu. Nikulin & A.Yu. Nikulin	16	2 <i>x</i>	15.94 ± 0.34
4	E. stellata	Russia, Primorsky Krai, Vladivostok City, forest in the vicinity of "13 th km" railway station, 43°11'32"N, 131°55'49"E, 11 Apr 2019, V.Yu. Nikulin & A.Yu. Nikulin	16	2 <i>x</i>	15.97 ± 0.31
5	E. stellata*	Russia, Primorsky Krai, Vladivostok City, Russkiy Island, 42°59'05.0"N 131°51'51.5"E, 14 May 2019, V.Yu. Nikulin & A.Yu. Nikulin	16	2 <i>x</i>	14.23 ± 0.23
6	E. stellata	China, Jilin Province, Fusong County, Baishan City, Changbai Mt., 852 m alt., 42°06'55.5"N, 127°30'29.0"E, 29 Apr 2019, K. Xiang	16	2 <i>x</i>	15.99 ± 0.91
7	E. tanhoensis*	Russia, Republic of Buryatiya, Kabansky Raion, Bolshoi Mamai River, mixed for- est, 51°23'30.1"N, 104°52'00.8"E, 20 Jun 2019, A.S. Erst, E.Yu. Mitrenina, D.A. Krivenko & O.A. Chernysheva	14	2 <i>x</i>	12.44 ± 0.27
8	E. tanhoensis*	Russia, Republic of Buryatia, Dulikha River, 51°32'04.9"N, 105°01'43.2"E, 1 May 2019, A.S. Erst, D.A. Krivenko, O.A. Chernysheva	14	2 <i>x</i>	12.49 ± 0.22
9	E. tanhoensis*	Russia, Buryatia Republic, Kabansky Raion, Tolbazikha River, 51°26'21.06"N, 104°41'09.82"E, 2 May 2019, A.S. Erst, D.A. Krivenko, O.A. Chernysheva	14	2 <i>x</i>	12.38 ± 0.26
10	E. tanhoensis*	Russia, Irkutsk Oblast, Slyudyansky Raion, Malye Mangaly River, 51°26'48.17"N, 104°34'16.62"E, 02 May 2019, A.S. Erst, D.A. Krivenko, O.A. Chernysheva	14	2 <i>x</i>	12.07 ± 0.06
11	E. tanhoensis*	Russia, Irkutsk Oblast, Slyudyansky Raion, Semirechka River, 51°28'56.92"N, 104°19'43.47"E, 02 May 2019, A.S. Erst, D.A. Krivenko, O.A. Chernysheva	14	2 <i>x</i>	12.41 ± 0.29
12	E. tanhoensis*	Russia, Buryatia Republic, Kabansky Raion, Osinovka River (Tankhoi Village), 51°33'06.2"N, 105°05'34.7"E, 01 May 2019, A.S. Erst, D.A. Krivenko, O.A. Chernysheva	14	2 <i>x</i>	12.56 ± 0.16
13	E. tanhoensis*	Russia, Buryatia Republic, Kabansky Raion, Mishikha River, 51°37'32.6"N, 105°32'03.4"E, 01 May 2019, A.S. Erst, D.A. Krivenko, O.A. Chernysheva	14	2 <i>x</i>	12.07 ± 0.07
14	E. tanhoensis*	Russia, Buryatia Republic, Kabansky Raion, Shestipalikha River, 51°32'46.4"N, 105°04'28.9"E, 01 May 2019, A.S. Erst, D.A. Krivenko, O.A. Chernysheva	14	2 <i>x</i>	12.77 ± 0.09
15	E. sibirica*	Russia, Irkutskaya Oblast', Slyudyanksky Raion, vicinity of Slyudyanka Town, mixed forest, 51°38'02.94"N, 103°41'13.90"E, 531 m alt., 02 May 2019, A.S. Erst, D.A. Krivenko & O.A. Chernysheva	42	6x	9.23 ± 0.14
16	E. sibirica*	Irkutskaya Oblast', Slyudyanksky Raion, Burovschina River, 51°37'06.00"N, 103°49'16.17"E, 475 m, 20 Jun 2019, A.S. Erst, D.A. Krivenko, E.Yu. Mitrenina & O.A. Chernysheva	42	6x	9.27 ± 0.23
17	E. sibirica*	Irkutskaya Oblast', Slyudyanksky Raion, Utulik River, 51°32'50"N, 104°02'45"E, 464 m alt., 20 Jun 2019, A.S. Erst, D.A. Krivenko, E.Yu. Mitrenina & O.A. Cher- nysheva	42	6x	9.22 ± 0.25
18	E. byunsanensis	South Korea, Gyeonggi-do, Anyang-si, Suli-san, 37°21'42.8"N, 126°54'01.9"E, 190 m alt., 24 Mar 2019, H. Ikeda, HT. Im, KS. Chung, M. Fujii, M. Sakamoto & C. Hasekura, N°19032401	16	2 <i>x</i>	10.75 ± 0.26
19	E. pinnatifida	Japan, Saitama Prefecture, Chichibu-shi, Shiroku, near village, 35°57'24"N, 138°59'16"E, 340 m alt., 01 Apr 2019, A.S. Erst, T.V. Erst, H. Ikeda et al., N° 1	16	2 <i>x</i>	9.87 ± 0.29
20	E. pinnatifida	Japan, Mie Prefecture, Inabe-shi, Fujiwara-cho, Ogaito, forest, 35°10'11"N, 136°28'35"E, 180 m alt., 03 Apr 2019, A.S. Erst, T.V. Erst, H. Ikeda et al., N° 2	16	2 <i>x</i>	9.80 ± 0.46
21	E. pinnatifida	Japan, Mie Prefecture, Inabe-shi, Hokusei-cho, Betsumyo, 35°8'23"N, 136°28'20"E, 640 m alt., 04 Apr 2019, A.S. Erst, T.V. Erst, H. Ikeda et al., N° 5	16	2 <i>x</i>	9.81 ± 0.10
22	E. pinnatifida	Japan, Nagano Prefecture, Shiojiri-shi, Hideshio, near station, 36°2'58"N, 137°53'45"E, 825 m alt., 04 Apr 2019, A.S. Erst, T.V. Erst, H. Ikeda et al., N° 6	16	2 <i>x</i>	9.80 ± 0.43
23	E. pinnatifida	Japan, Nagano Prefecture, Shiojiri-shi, Motoyama, pine forest, 36°3'40"N, 137°53'50"E, 800 m alt., 04 Apr 2019, A.S. Erst, T.V. Erst, H. Ikeda et al., N° 7	16	2 <i>x</i>	9.85 ± 0.27

Table 1. Chromosome number, ploidy and genome size in white-flowered Eranthis sect. Shibateranthis.

* population already studied by Erst et al. (2020b) concerning chromosome number and genome size.

The degree of chromosome condensation was estimated from the total haploid length of the chromosome set. The symbols used to describe the karyotypes corresponded to those coined by Levan et al. (1964): m = median centromeric chromosome with arm ratio (r) of 1.0–1.7 (metacentric chromosome); sm = submedian centromeric chromosome with arm ratio of 1.7–3.0 (submetacentric chromosome); st = subterminal centromeric chromosome with arm ratio of 3.0–7.0 (subtelocentric chromosome); t = terminal centromeric chromosome with arm ratio of 7.0 and more (acrocentric chromosome); T = chromosome without obvious short arm (telocentric chromosome). Mean values of arm ratio (r), centromeric indices (CI), mean chromosome length (CL), and relative chromosome length (RL) for each chromosome pair and total haploid length (THL) were determined. In addition, we calculated the Coefficient of Variation of Chromosome Length (CV_{CL}; Paszko 2006), Coefficient of Variation of Centromeric Index (CV_{CI}; Paszko 2006), and Mean Centromeric Asymmetry (M_{CA}; Peruzzi and Eroğlu 2013).

To determine the karyological relationships among taxa, we carried out a multivariate PCoA (Principal Coordinate Analysis) using Gower's general coefficient of similarity, including six basic karyomorphological parameters (2n, x, THL, M_{CA}, CV_{CL} , and CV_{CI}) in the data matrix (Peruzzi and Altınordu 2014), by plotting every single metaphase. Then, we also subjected the same data matrix to LDA (Linear Discriminant Analysis) to test the diagnosability of the six species on karyomorphological grounds. Finally, we tested the Spearman correlation between THL and 1Cx for each species, using mean data. To perform PCoA, LDA and correlation tests, the software Past 4.06b (Hammer et al. 2001; Hammer 2021), freely available online, was used.

Flow cytometry

Flow cytometry with propidium iodide (PI) staining was used to determine the absolute DNA content. In this study, we have determined this parameter in representatives of four Eranthis species: E. byunsanensis, E. lobulata, E. pinnatifida and E. stellata from 10 different populations (Table 1). Silica-gel-dried leaf material (0.5–1.0 cm²) was chopped with a sharp razor blade in a 1 ml cold nuclei extraction buffer composed of 50 mM Hepes, 10 mM sodium metabisulphite, 10 mM MgCl., 0.5% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.3% Tween 20, 0.2% Triton X-100, 50 μ g/ml RNase, 1 μ g/ml β -mercaptoethanol and 50 μ g/ml propidium iodide (PI). The samples were filtered through 50 μ m nylon membranes into sample tubes and incubated in the dark at 4 °C for 15 min. The samples were measured using a Partec CyFlow PA flow cytometer equipped with a green laser at 532 nm wavelength. The absolute nuclear DNA content, the 2C-value according to Greilhuber et al. (2005), was calculated as the ratio of the mean fluorescence intensity of the sample nuclei to that of the external standard multiplied by the total nuclear DNA content of the standard. The possible effect of secondary metabolites on the binding of the intercalating dye was evaluated by measuring the fluorescence of Allium fistulosum L. leaf

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samples prepared as described above, but with the addition of the supernatant from *Eranthis* samples centrifuged without PI. The samples were measured three times at 10 min intervals. If no variation in the average values of the detection channels was observed for the *A. fistulosum* peak, the effect of secondary metabolites was considered negligible. The 1Cx-value (monoploid DNA content *sensu* Greilhuber et al. 2005) was calculated by dividing the 2C-value by the ploidy level of the species. The species, used as external standards, were *Zamioculcas zamiifolia* Engl., 2C = 48.35 pg and *Vicia faba* L. "Inovec" 2C = 26.90 pg (Doležel et al. 1992; Skaptsov et al. 2016). We used the Statistica 8.0 software (StatSoft, Inc.), Flowing Software 2.5.1 (Turku Centre for Biotechnology) and CyView software (Partec, GmbH) for data analyses. Flow cytometry was performed at the Laboratory for Bioengineering of the South-Siberian Botanical Garden, Altai State University (Barnaul, Russia).

Results

Karyotypes

Karyomorphometric data, microphotographs of metaphase plates and idiograms for the studied species are presented in Tables 2, 3 and Figs 2, 3.

Eranthis lobulata

Notes. The somatic and basic chromosome numbers in *E. lobulata*, endemic to China, are 2n = 16 and x = 8, respectively (Table 1; Fig. 2A). Five pairs of chromosomes (I–V) are metacentric, and three pairs (VI–VIII) are submetacentric, subtelocentric and acrocentric (Tables 2 and 3; Fig. 3). A pair of submetacentric chromosomes exhibits a secondary constriction. We also found a single small B chromosome in some cells. These Bs are metacentric, about 2.5 µm long. The karyotype formula of *E. lobulata* is $2n = 2x = 16 = 10m + 2\text{sm}^{\text{sat}} + 2\text{st} + 2\text{t} + 0-1\text{B}.$

Eranthis stellata

Notes. In all five studied populations of *E. stellata* from Primorsky Krai of Russia and Jilin Province of China, the somatic and basic chromosome numbers are 2n = 16 and x = 8, respectively (Table 1; Fig. 2B–C). Five pairs of chromosomes (I–V) are metacentric, two pairs (VI–VII) are submetacentric, and one pair (VIII) is acrocentric (Tables 2 and 3; Fig. 3). A pair of submetacentric chromosomes (VII) exhibits a secondary constriction. The karyotype formula of *E. stellata* is $2n = 2x = 16 = 10m + 2sm + 2sm^{sat} + 2t$. No B was observed in this species. Here, we present the results of the karyomorphological analysis of *E. stellata* from the "Academicheskaya Station" population (pop. 2).

Species	Chromosome pair	CL (µm)	r	CI	RL (%)	Туре
E. lohulata	I	8 46 + 0 42	1 07 + 0 04	0.48	7.80	m
	I	8.19 ± 0.31	1.16 ± 0.09	0.46	7.55	m
	III	7.43 ± 0.30	1.17 ± 0.07	0.46	6.85	m
	IV	7.38 ± 0.16	1.36 ± 0.10	0.42	6.80	m
	V	7.00 ± 0.29	1.28 ± 0.05	0.44	6.45	m
	VI	6.11 ± 0.15	2.05 ± 0.09	0.33	5.63	sm ^{sat}
	VII	5.05 ± 0.21	5.04 ± 0.51	0.17	4.66	st
	VIII	4.62 ± 0.24	8.35 ± 0.84	0.11	4.26	t
E. stellata (pop. 2)	Ι	9.61 ± 0.34	1.07 ± 0.04	0.48	7.84	m
	II	9.29 ± 0.31	1.07 ± 0.04	0.48	7.58	m
	III	8.85 ± 0.39	1.06 ± 0.03	0.49	7.22	m
	IV	8.31 ± 0.42	1.06 ± 0.04	0.49	6.78	m
	V	7.89 ± 0.16	1.33 ± 0.07	0.43	6.44	m
	VI	6.21 ± 0.25	2.00 ± 0.19	0.33	5.06	sm
	VII	6.13 ± 0.40	2.14 ± 0.18	0.32	5.00	sm ^{sat}
	VIII	5.01 ± 0.34	7.86 ± 0.38	0.11	4.08	t
E. tanhoensis (pop. 12)	Ι	8.68 ± 0.36	1.09 ± 0.05	0.48	8.74	m
· · · · · · · · · · · · · · · · · · ·	II	8.56 ± 0.41	1.23 ± 0.06	0.45	8.62	m ^{sat}
	III	8.16 ± 0.29	1.07 ± 0.05	0.48	8.21	m
	IV	7.73 ± 0.35	1.07 ± 0.05	0.48	7.78	m
	V	6.63 ± 0.46	1.37 ± 0.11	0.42	6.67	m
	VI	5.72 ± 0.46	1.92 ± 0.14	0.34	5.76	sm
	VII	4.19 ± 0.38	2.34 ± 0.15	0.30	4.22	sm
E. sibirica (pop. 15)	Ι	9.51 ± 0.24	1.08 ± 0.04	0.48	2.88	m
4.1.2)	II	9.47 ± 0.29	1.03 ± 0.02	0.49	2.87	m
	III	9.20 ± 0.06	1.17 ± 0.03	0.46	2.78	m
	IV	9.13 ± 0.13	1.10 ± 0.06	0.48	2.76	m
	V	9.00 ± 0.11	1.05 ± 0.02	0.49	2.72	m
	VI	8.91 ± 0.14	1.39 ± 0.12	0.42	2.70	m
	VII	8.88 ± 0.07	1.20 ± 0.03	0.45	2.69	m
	VIII	8.87 ± 0.16	1.05 ± 0.03	0.49	2.68	m
	IX	8.67 ± 0.10	1.08 ± 0.05	0.48	2.62	m
	Х	8.47 ± 0.09	1.27 ± 0.09	0.44	2.56	m
	XI	8.44 ± 0.15	1.07 ± 0.03	0.48	2.55	m
	XII	8.14 ± 0.13	1.16 ± 0.02	0.46	2.46	m
	XIII	7.71 ± 0.04	1.18 ± 0.09	0.46	2.33	m
	XIV	7.46 ± 0.15	1.35 ± 0.15	0.43	2.26	m
	XV	7.26 ± 0.21	1.70 ± 0.06	0.37	2.20	sm
	XVI	7.10 ± 0.04	1.28 ± 0.03	0.44	2.15	m
	XVII	6.89 ± 0.05	1.61 ± 0.05	0.38	2.08	m
	XVIII	6.45 ± 0.31	1.70 ± 0.08	0.37	1.95	sm
	XIX	5.36 ± 0.23	1.97 ± 0.09	0.34	1.62	sm
	XX	5.24 ± 0.25	1.74 ± 0.03	0.37	1.59	sm ^{sat}
	XXI	5.08 ± 0.34	2.29 ± 0.14	0.30	1.54	sm
E. byunsanensis	Ι	8.59 ± 0.19	1.05 ± 0.03	0.49	8.55	m
•	II	8.13 ± 0.31	1.06 ± 0.04	0.49	8.09	m
	III	7.65 ± 0.13	1.07 ± 0.04	0.48	7.61	m
	IV	6.18 ± 0.09	1.40 ± 0.05	0.42	6.15	m
	V	5.68 ± 0.21	1.19 ± 0.05	0.46	5.65	m
	VI	5.44 ± 0.19	5.22 ± 0.30	0.16	5.41	st
		5.19 ± 0.08	1.74 ± 0.05	0.37	5.17	sm
	VII	5.20 ± 0.13	5.64 ± 0.19	0.15	5.17	st
	VIII	3.52 ± 0.07	4.06 ± 0.37	0.20	3.50	st

Table 2. Karyomorphological parameters in white-flowered *Eranthis* sect. *Shibateranthis*.

Species	Chromosome pair	CL (µm)	r	CI	RL (%)	Туре
E. pinnatifida (pop. 21)	Ι	9.24 ± 0.18	1.12 ± 0.02	0.47	8.58	m
	II	8.63 ± 0.24	1.08 ± 0.06	0.48	8.02	m
	III	8.25 ± 0.31	1.13 ± 0.03	0.47	7.66	m
	IV	6.62 ± 0.12	1.37 ± 0.07	0.42	6.15	m
	V	6.24 ± 0.26	2.77 ± 0.14	0.27	5.80	sm
	VI	5.88 ± 0.18	2.38 ± 0.07	0.30	5.46	sm
	VII	5.04 ± 0.11	1.95 ± 0.13	0.34	4.68	sm
	VIII	3.92 ± 0.09	3.10 ± 0.29	0.24	3.64	st ^{sat}

Notes: CL – chromosome length, mean value \pm standard deviation; r – arm ratio, mean value \pm standard deviation; CI – centromeric index; RL – relative chromosome length; m – metacentric chromosome; sm – submetacentric chromosome; st – subtelocentric chromosome; t – acrocentric chromosome; st – chromosome showing secondary constriction.

Eranthis tanhoensis

Notes. In all eight studied populations of *E. tanhoensis*, Siberian endemic species, the somatic and basic chromosome numbers are 2n = 14 and x = 7, respectively (Table 1; Fig. 2D–E). Five pairs of chromosomes (I–V) are metacentric, two pairs (VI–VII) are submetacentric (Tables 2 and 3; Fig. 3). A pair of metacentric chromosomes (II) exhibited a secondary constriction. We found small B chromosomes in plants from two populations (pops 10 and 13). The maximum number of Bs in root tip cells appeared to be 8. They were represented by small metacentric and dot-shaped chromosomes, which are obviously telocentric. The karyotype formula of *E. tanhoensis* is $2n = 2x = 14 = 8m + 2m^{sat} + 4sm + 0-8B$. Here, we present the results of the karyomorphological analysis of *E. tanhoensis* from the "Tanhoi Village" population (pop. 12).

Eranthis sibirica

Notes. The somatic chromosome number of *E. sibirica*, another endemic species from Siberia, is 2n = 42. The chromosome set of the species includes metacentric and submetacentric types of chromosomes. The karyotype formula of *E. sibirica* is $2n = 6x = 42 = 32m + 8sm + 2sm^{sat}$. Here, we present the results of the karyomorphological analysis of *E. sibirica* from the "Slyudyanka Town" population (pop. 15) (Tables 2 and 3; Figs 2F and 3).

Eranthis byunsanensis

Notes. The chromosome set of the Korean endemic *E. byunsanensis* includes five pairs of metacentric chromosomes (I–V), one submetacentric (in the "pair" VI) and five subtelocentric chromosomes (in the "pair" VI and pairs VII–VIII) (Tables 2 and 3; Figs 2G and 3). The karyotype formula of *E. byunsanensis* is 2n = 2x = 16 = 10m + 1 sm + 5 st.



Figure 2. Mitotic metaphase plates of white-flowered *Eranthis* sect. *Shibateranthis* A *E. lobulata*, 2n = 16 **B** *E. stellata* (pop. 2), 2n = 16 **C** *E. stellata* (pop. 6), 2n = 16 **D** *E. tanhoensis* (pop. 12), 2n = 14 **E** *E. tanhoensis* (pop. 10), 2n = 14+0-8B (arrows point at **B** chromosomes) **F** *E. sibirica* (pop. 15), 2n = 42 **G** *E. byunsanensis*, 2n = 16 (arrows point at the heteromorphic chromosome pair) **H** *E. pinnatifida* (pop. 21), 2n = 16 **L** *carrows point* at heteromorphic chromosome pair). Scale bars: 10 µm. Microphotographs by E.Yu. Mitrenina.

Eranthis pinnatifida

Notes. The Japanese endemic *E. pinnatifida*, unlike other related species, has four rather than five pairs of metacentric chromosomes (I–IV) and four rather than three pairs of submetacentric (V–VII) and subtelocentric chromosomes (VIII). The karyotype formula of the plants from three studied populations (pops 19, 21 and 22) is $2n = 2x = 16 = 8m + 6sm + 2st^{sat}$. These plants have secondary constric-






Figure 3. Haploid idiograms of white-flowered *Eranthis* sect. *Shibateranthis* species. I–VIII – chromosome pairs; m – metacentric chromosome; sm – submetacentric chromosome; st – subtelocentric chromosome; t – acrocentric chromosome; T – telocentric chromosome; B – B chromosome.

tions and small satellites at terminal regions of short arms of the pair VIII (Table 2; Figs 2H and 3). Specimens from the fourth population (pop. 20) have a pair of heteromorphic chromosomes (VIII) represented by one metacentric and one subtelocentric chromosome (Fig. 2I). The karyotype formula of these plants is $2n = 2x = 16 = 8m + 1m^{sat} + 6sm + 1st^{sat}$. In these plants, the secondary constriction in the metacentric homologue to the VIII pair is located in the pericentromeric region. Here we present the results of the karyomorphological analysis of *E. pinnatifida* from the "Inabe-shi" population (pop. 21).

Species	Ν	Ploidy	2n	Karyotype formula	THL	CV _{CL}	M _{CA}	CV _{CI}
		level						
E. lobulata	6	2x	16	$10m + 2sm^{sat} + 2st + 2t$	54.24 (0.92)	19.68 (0.36)	28.15 (0.80)	38.05 (1.09)
				+ 0–1B				
E. stellata (pop. 2)	5	2x	16	$10m + 2sm + 2sm^{sat} + 2t$	61.30 (1.91)	20.77(0.84)	21.63(0.51)	31.69(0.83)
E. tanhoensis (pop. 12)	5	2x	14	$8m + 2m^{sat} + 4sm + 0 - 8B$	49.67 (2.02)	22.11 (1.10)	15.46 (0.76)	16.32 (1.24)
E. sibirica (pop. 15)	2	6x	42	$32m + 8sm + 2sm^{sat}$	165.24 (0.85)	17.55(0.87)	13.41(0.65)	12.87(0.18)
E. byunsanensis	4	2x	16	10m + 1sm + 5 st	50.26 (0.83)	25.69 (0.66)	26.43 (0.23)	37.18 (1.11)
E. pinnatifida (pop. 21)	5	2x	16	$8m + 6sm + 2 st^{sat}$	53.82 (0.81)	25.81 (0.89)	25.16 (0.72)	24.90 (0.88)

Table 3. Karyotype parameters in white-flowered *Eranthis* sect. *Shibateranthis*.

Notes: THL – total haploid length, CV_{CL} – Coefficient of Variation of Chromosome Length, M_{CA} – Mean Centromeric Asymmetry, CV_{CL} – Coefficient of Variation of Centromeric Index; mean value (standard deviation). m – metacentric chromosome; sm – submetacentric chromosome; st – subtelocentric chromosome; t – acrocentric chromosome; s^{ast} – satellite chromosome; B – B chromosome.



Figure 4. PCoA (Coordinate 1, 65.31% of variance explained vs. Coordinate 2, 16% of variance explained) based on six karyological parameters of white-flowered *Eranthis* sect. *Shibateranthis* species.

Karyotype structure

The highest level of interchromosomal asymmetry, estimated via CV_{CL} , was found in *E. byunsanensis* and *E. pinnatifida*. The highest levels of intrachromosomal asymmetry (M_{CA}) and heterogeneity in centromere position (CV_{CL}) were found in *E. lobulata* and *E. byunsanensis*, while *E. sibirica* had the most symmetric karyotype (Table 3). We analyzed 27 accessions (metaphase plates) by PCoA (cumulative variance explained by the first two axes: 81.31%). No overlap among species was evident (Fig. 4). Indeed, LDA correctly attributed objects (accessions) to the six species in 100% of cases (jackknifed).

Genome size

The absolute nuclear DNA content for 23 studied populations of six species of *Eranthis* is presented in Table 1. There was no significant correlation (r = 0.51, p = 0.29) be-

tween mean 1Cx values and total haploid (monoploid) chromosome length (THL) in these species. Indeed, for instance, while the 1Cx value is the smallest in the hexaploid *E. sibirica*, THL in this species is higher than in the diploid *E. tanhoensis*, sharing the same basic chromosome number x = 7 (Table 3).

Discussion

Karyotype structure in Eranthis

According to our results and other data (Kurita 1955; Tak and Wafai 1996; Gömürgen 1998; Yuan and Yang 2006), chromosome sets of different species of *Eranthis* share some common features, albeit showing some species-specific peculiarities, which allow a clear-cut distinction among species based on karyo-morphological features according to LDA (see also Fig. 4). The traits of the karyotype within each species are sufficiently stable. However, in some cases, polymorphism was observed in the chromosome morphology, for instance, in *E. pinnatifida*. The karyotypes of *E. byunsanensis* and *E. lobulata* were described here for the first time.

The chromosomes of *Eranthis* belong to the *Ranunculus*-type (Langlet 1932). The karyotypes of *E. lobulata* and *E. stellata* are similar. Both species show a chromosome pair with a very small, not always visible, short arm. In the other four studied species of *Eranthis*, no chromosome of this type was found. The secondary constrictions in *E. lobulata* and *E. stellata* are localized in the short arms of submetacentric chromosome pairs. Different localization of secondary constrictions in these species (Fig. 3) is possibly due to a paracentric inversion. Previously, the karyotype of *E. stellata* from China (Jilin Province) was studied by Yuan and Yang (2006). These authors described its formula as 2n = 2x = 16 = 10m + 2sm + 2st + 2T. In contrast to our data, they assigned pair VII to subtelocentric rather than submetacentric chromosome type. Our data show that the arm ratio of this chromosome pair is 2.14 ± 0.18 (Table 2), congruent with a sm chromosome-type. They also did not find a short arm in pair VIII and referred it to T-type (telocentric chromosomes). We found short arms in this VIII chromosome pair, which led us to classify it as chromosomes of t-type (acroemetic chromosomes).

Two species, endemic to Siberia, *E. sibirica* and *E. tanhoensis*, show atypical dysploid basic chromosome number for *Eranthis* (x = 7) and exhibit hexaploid (2n = 42) and diploid (2n = 14) cytotypes, respectively (Erst et al. 2020b). Since there are different definitions of the term "basic chromosome number (x)" concerning polyploids (Peruzzi 2013), we clarify that, in the study, we mean, "x" as "chromosome number found in the gametes of their diploid relatives", according to Darlington (1958). A recent phylogenetic study (Xiang et al. 2021) found that *E. tanhoensis* and *E. sibirica* are closely related species that formed separate groups with basic chromosome number x = 7 within the North Asian clade of *Eranthis*. The same basic chromosome number x = 7 with 2n = 14 was previously reported in the genus *Eranthis* for *E. stellata* (Starodubtsev 1985), albeit this author does not provide any microphotograph of the

metaphase plate. We re-analyzed plants from the same area (pop. 3; Russia, Primorsky Krai, Malaya Sedanka River), but we found a somatic chromosome number 2n =16. At the same time, previous studies on *E. sibirica* reported 2n = 16 (Gnutikov et al. 2016, 2017) and 2n = 32 (Krogulevich 1976) chromosomes. However, the diploid plants described in these studies apparently refer to the recently described species *E. tanhoensis*. Some populations of this species show B chromosomes that researchers may have identified as regular chromosomes. In addition, a pair of metacentric chromosomes show large satellites, which, when using the squash method, are sometimes detached and can be misidentified as small telocentric chromosomes. Based on the large amount of material analyzed and on careful analysis of chromosome morphology, we conclude that the basic chromosome number of the studied populations of *E. tanhoensis* and *E. sibirica* is x = 7. However, we do not rule out the possible occurrence of different cytotypes in plants from Siberia.

The karyotypes of the two related species, endemic to Korea and Japan, also show peculiar features. *Eranthis byunsanensis* has a heteromorphic pair of chromosomes (VI). Unfortunately, we had material from a single population of this species. Therefore, we cannot conclude whether this feature is characteristic of the whole species or just a heterozygous chromosomal mutation. *Eranthis pinnatifida* has another feature that distinguishes it from other diploid species: four pairs of isobrachial chromosomes and four pairs of heterobrachial chromosomes. Our results concerning this species are consistent with the data published by Kurita (1955). Among the four *E. pinnatifida* populations studied, one population (pop. 20) shows a heteromorphic pair of chromosomes. In this case, we are sure that this mutation is just a polymorphic variant.

Carta et al. (2020) estimated x = 7 as the most likely ancestral basic chromosome number in Ranunculaceae. However, we hypothesize that, in Siberian species, *E. sibirica* and *E. tanhoensis*, the basic chromosome number evolutionarily reduced from x = 8 to x = 7 and not vice versa. This hypothesis is because most of the tribe Cimicifugeae members (i.e., *Actaea, Anemonopsis, Beesia, Cimicifuga, Souliea* and closely related *Helleborus*; Wang et al. 2009) have x = 8 (Rice et al. 2015). In addition, it has been established that *Eranthis* originated in East Asia and then dispersed to the west Qinghai-Tibetan Plateau and Mediterranean regions (Xiang et al. 2021). East Asian *Eranthis* species (i.e., *E. byunsanensis, E. lobulata, E. pinnatifida*, and *E. stellata*) have x = 8. According to a recent phylogenetic study (Xiang et al. 2021), *E. sibirica* and *E. tanhoensis* are a derived group within the North Asian clade of *Eranthis* with noncanonical basic chromosome number x = 7 for the tribe Cimicifugeae.

The karyotypes of the two related species *E. stellata* and *E. tanhoensis*, with 2n = 16 and 2n = 14 chromosomes, respectively, are similar concerning five metacentric (I–V) and two submetacentric (VI–VII) chromosome pairs and differ by the presence of acrocentric pair (VIII) in *E. stellata*. It is well known that the basic chromosome number can change (dysploidy) due to chromosome rearrangements, fusion or fission of some chromosomes of the set and chromosome loss (Shubert 2007; Guerra 2008; Escudero et al. 2014). Dysploidy can establish powerful crossing barriers between sympatric taxa, as it disturbs regular chromosome pairing and bivalent formation at meiosis, drastically reducing hybrid fertility. These processes can result in the formation of new

species (Grant 1981; Levin 2002; Baltisberger and Hörandl 2016). Such restructuring is known, for example, in the evolution of *Arabidopsis thaliana* (2n = 10) from *A. lyrata* (2n = 16) (Koch and Kiefer 2005). A similar case of descendant dysploidy was revealed for other Brassicaceae (Lysak et al. 2006) and plants from other families (Levin 2002). For Ranunculaceae, a decrease in the basic chromosome number from x = 8 to x = 7, caused by chromosome rearrangements, is known within *Ranunculus* (Baltisberger and Hörandl 2016) and *Anemone* (Mlinarec et al. 2012).

The shift to x = 7 in *Eranthis* possibly led to reproductive isolation of the populations with a new cytotype and, ultimately, speciation. We assume that further isolation of *E. tanhoensis* and *E. sibirica* was associated with polyploidization of the latter species. However, the type of polyploidy (i.e., autopolyploidy or allopolyploidy) has to be determined for this species. The karyotype of *E. sibirica* is similar to that of *E. tanhoensis* in chromosome morphology (metacentric and submetacentric chromosomes only), and they differ from the karyotypes of other related species. The organization of *E. sibirica* karyotype with 2n = 42 seems functionally diploid. The chromosomes are grouped in pairs (Fig. 3) and not in groups of 6. It is known that the size and shape of homologous chromosomes may change in the course of the diploidization process following polyploidization, i.e., due to the genome downsizing. Repetitive DNA sequences, both non-coding and coding, gene duplicates may be eliminated from the genome, resulting in changes in the karyotype parameters (Leitch and Bennett 2004; Mandáková and Lysak 2018; Wang et al. 2021).

A distinguishing feature of *E. tanhoensis* is the presence of small Bs in some of its populations (pops 11 and 14). Sporadic Bs were previously detected in individual cells only in *E. lobulata* (Erst et al. 2019). In some representatives of *E. tanhoensis*, up to 8 Bs could be observed in many cells. Bs are often found in representatives of Ranunculaceae and other families (Rice et al. 2015). However, their origin and possible adaptive and/or evolutionary roles are still poorly understood (Datta et al. 2016; Dhar et al. 2019). It is generally accepted that Bs are formed from A chromosomes in different ways. The most convincing case was the fully documented origin of a nascent B in trisomic *Plantago lagopus* L. from a supernumerary. This origin was associated with chromosome fragmentation, specific DNA sequence amplification, the addition of telomeric repeats and centromeric misdivision (Dhar et al. 2002). Bs could also escape as small centric fragments following unequal translocation and a reduction in chromosome number (Jones et al. 2008). Bs in E. tanhoensis may be preserved fragments of the lost ancestral pair VIII. The presence of Bs in the genome increases the adaptive capabilities of the population to adverse environmental conditions (Datta et al. 2016), which can be quite relevant for plants growing in this climatic zone.

Genome size of Eranthis

The Kew list of DNA C-values contains only one C-value for white-flowered *Eranthis* (i.e., 1C = 8.20 pg) determined by Zonneveld et al. (2005) for *E. pinnatifida*. In the present study, we determined the genome size for six white-flowered *Eranthis* species. According to our data, the Japanese *E. pinnatifida* has an average $1Cx = 9.80 \pm 0.33$

pg. It is the lowest absolute nuclear DNA content among the studied diploids. A lower 1Cx value (9.26 \pm 0.25 pg) was found only in the polyploid *E. sibirica*. Remarkably, a closely related diploid species, such as *E. tanhoensis*, shows 1Cx = 12.48 \pm 0.25 pg. According to the genome downsizing theory, an increase in the ploidy level leads to a decrease in the size of the monoploid genome. The loss of DNA in polyploids is a widespread phenomenon occurring in many plant groups (Shaked et al. 2001; Leitch and Bennett 2004; Adams and Wendel 2005). In the present study, *Eranthis stellata* exhibited the highest 1Cx-value of 15.93 \pm 0.32 pg and the highest total haploid length as well. However, it is interesting to note that, in this system, we found no significant correlation between 1Cx and THL, as otherwise commonly found in plants (Levin 2002; Peruzzi et al. 2009), where this correlation typically exceeds r = 0.8. This inconsistency could be explained by different condensation degrees of the studied chromosomes. Nonetheless, it also may suggest differences in chromosomes width and volume (Kramer et al. 2021), not addressed in this study.

Conclusions

In this study, the comparative karyomorphological analyses and genome size determination of six white-flowered species of *Eranthis* sect. *Shibateranthis* from different populations have been carried out. The chromosome complements of *E. lobulata* and *E. byunsanensis* were determined for the first time. Karyotypes of studied *Eranthis* are shown to have both common features and species-specific features related to chromosome number, size and morphology. All the studied species can be distinguished based on their karyotype structure. They have the basic chromosome numbers x = 8 and x = 7, diploid and polyploid cytotypes. Additionally, *E. tanhoensis* and *E. lobulata* have small supernumerary chromosomes in the root tip cells. The monoploid genome size (Cvalue) determined by flow cytometry varies more than 1.5 times in the studied species.

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