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Original Article

Hiding in the Atlantic Forest: Leaf geometric morphometrics redefines endangered *Aristolochia* (Aristolochiaceae) sibling species and allows conservation strategies

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ABSTRACT

Aristolochia hypoglauca and *A. paulistana* (Aristolochiaceae) are two species that inhabit the Brazilian Atlantic Forest. They highly resemble each other especially on the size and overall shape of the caudate perianth, which causes confusion in herbaria determinations. We applied geometric morphometrics (GM) to overcome this taxonomic uncertainty. GM was based on the landmark method applied to leaves of all specimens available mostly in Brazilian herbaria. The GM results supported the recognition of the two species, as the two principal components were responsible for 94.97% of the variation assessed through the principal component analysis (PCA). The Discriminant Function and the Cross-validation tests resulted in the maximum percentage of correctly classified cases (100%). The Procrustes distance (0.2252; $p< 0.0001$), and the Mahalanobis Distance (8.4473; $p< 0.0001$) provide statistical support for leaf shape differences with taxonomic significance. Thus, we revisit the taxonomy and comparative morphology of both species, and compare them with other *Aristolochia* species with caudate floral limb native to Brazil, and commented the phenology, distribution and habitat, and conservation status. Additionally, we proposed the epitypification of *A. hypoglauca*, given that the holotype does not fulfill the purpose of precise application of the name.

Keywords: Flora of Brazil, geometric morphometrics, leaf morphometrics, Piperales, threatened species.

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Introduction

The family Aristolochiaceae is represented in Brazil by 84 species of *Aristolochia*, 39 of them occurring in the Atlantic Forest, and 18 are endemic to this biome (Freitas *et al*. 2020a; 2022). Several new species of the genus have been recently discovered and described (González 2011; Freitas *et al*. 2013a; b, 2014; 2016; 2017), and all of them face some degree of vulnerability.

Two species from the Atlantic Forest, *Aristolochia hypoglauca* Kuhlm. and *A. paulistana* Hoehne were originally described from the states of Espírito Santo and São Paulo, respectively. They are difficult to tell apart mainly due to the presence of pseudostipules, unlobed leaf blades and caudate floral limb with non-cymbiform base, being frequently misidentified in herbaria (Freitas pers. obs.) and regional taxonomic treatments (*e.g.* Araújo 2013; Abreu & Giulietti 2016).

Flowers in *Aristolochia* are remarkably diverse in terms of size, color patterning, epidermal specialization, and shape of the perianth (Freitas *et al.* 2020b). Often, these traits are crucial as diagnostic traits at a species level (González 1990; González & Stevenson 2000); thus, the absence of flowers often precludes the identification.

Plant morphometrics allows the assessment of pattern variation at low cost, provides consistent results, and helps solving taxonomic uncertainties at both species and population levels (Marhold 2011; Turco *et al*. 2022). Geometric Morphometrics (GM) has been applied to assess leaf (Cheng *et al.* 2021; Danila & Alejandro 2021; Guamba *et al.* 2021) and/or flower quantitative variation to distinguish species (Fragoso-Martínez *et al*. 2015; Menini-Neto *et al.* 2019; Freitas *et al*. 2020b; Guamba *et al*. 2021; Pessoa *et al*. 2020; Araújo et al. 2023), by identifying homologous points across samples, called landmarks, and comparing the format of each one of the structures in its entirety (Christodoulou *et al*. 2020). Linear morphometrics, on the other hand, uses only measurable values, such as length and width, not considering the shape (Christodoulou *et al*. 2020).

In Aristolochiaceae, species-level similarities on the shape and size of leaves and flowers often mislead identifications (González 1990). In such cases, GM become a powerful tool to distinguish and describe species, and to arrive at a correct identification (Freitas pers. obs.). To date, Geometric Morphometrics in Aristolochiaceae was successfully applied to examine floral traits as a proxy to disentangle the *Aristolochia cornuta* Mast./*A. iquitensis* O.C.Schmidt complex and to discover a new species, *A. wankeana* J.Freitas, F.González & Poncy (Freitas *et al.* 2020b) and to distinguish *A. trilabiata* Glaz. from the new species, *A. franzii* Frank, based on leaves and flowers (Frank 2023).

The present work aims (a) to test whether leaf morphometry corroborates the recognition of *Aristolochia paulistana* and *A. hypoglauca* as two distinct species; (b) to reassess the conservation status of the resulting entity or entities; and (3) to provide an updated key to tell apart these two species by using through the diagnostic landmarks found in the present research, and to compare them with the remaining species with caudate floral limb native to Brazil.

Material and methods

Morphometric analysis – Samples: Despite floral GM having been successfully used before to solve a species complex in *Aristolochia* (Freitas *et al.* 2020b), we choose to employ leaf GM in this study. This decision was influenced by the limited availability of flowers for *A. hypoglauca* and *A. paulistana*, which were herborized in various positions, making GM standardization challenging.

Samples of the following herbaria were studied: CEPEC, COAH, COL, CONN, CVRD, ESA, HRCB, HSTM, HUA, HUEFS, IAN, INPA, JAUM, K, MBML, MEDEL, MG, MO, NY, P, R, RB, SP, SPF, UEC, UFACPZ, UPCB, US and VIES. A total of 26 vouchers were used, seven corresponding to *A. hypoglauca* and 19 to *A. paulistana*. These specimens were marked with asterisks (*) in the list of specimens examined (see below). Additionally, we used digital photographs of specimens from the virtual herbaria CEPEC, ESA, IAC, K, MBML, RB, SP, and UEC (INCT 2023; REFLORA 2023) (acronyms after Thiers 2023) only with complete leaves accompanied by metric scales. A total of 79 leaves were examined, 30 of them corresponding to *A. hypoglauca* and 49 to *A. paulistana*.

Morphometric analysis: Landmark-based method: The shape of the leaf blades was outlined with strings of semi-landmark points. It was sampled with a configuration of 26 points, including two landmarks (base and apex) and 24 semi-landmarks, 22 of these representing homologous lines, and the remaining two (between points 23-2-24; Fig. 1) corresponding to the blade base. We used a comb of 12 rays as a graphical tool to place the same number of points along a curve segment. Combs were added to the photographs using MakeFan8 from the IMP series (Sheets 2014).

We used TpsDig2 ver. 2.31 to digitize landmarks (Rohlf 2007a), and MorphoJ to perform the statistical analysis (Klingenberg 2011). After digitizing the landmarks, we assembled a matrix with landmark coordinates through TpsRelw 1.42 (Rohlf 2007b). Then, we performed a Principal Component Analysis (PCA) to analyze the variation of the blade shape among specimens. After this PCA based on the incorrectly identified samples, an adjusted PCA was performed by considering the revised identifications made by us. To maximize individual differences according to species, we performed a canonical variate analysis (CVA) followed by a discriminant function and cross-validation analysis among both species. All analyses were implemented in MorphoJ software version 1.07a (Klingenberg 2011).

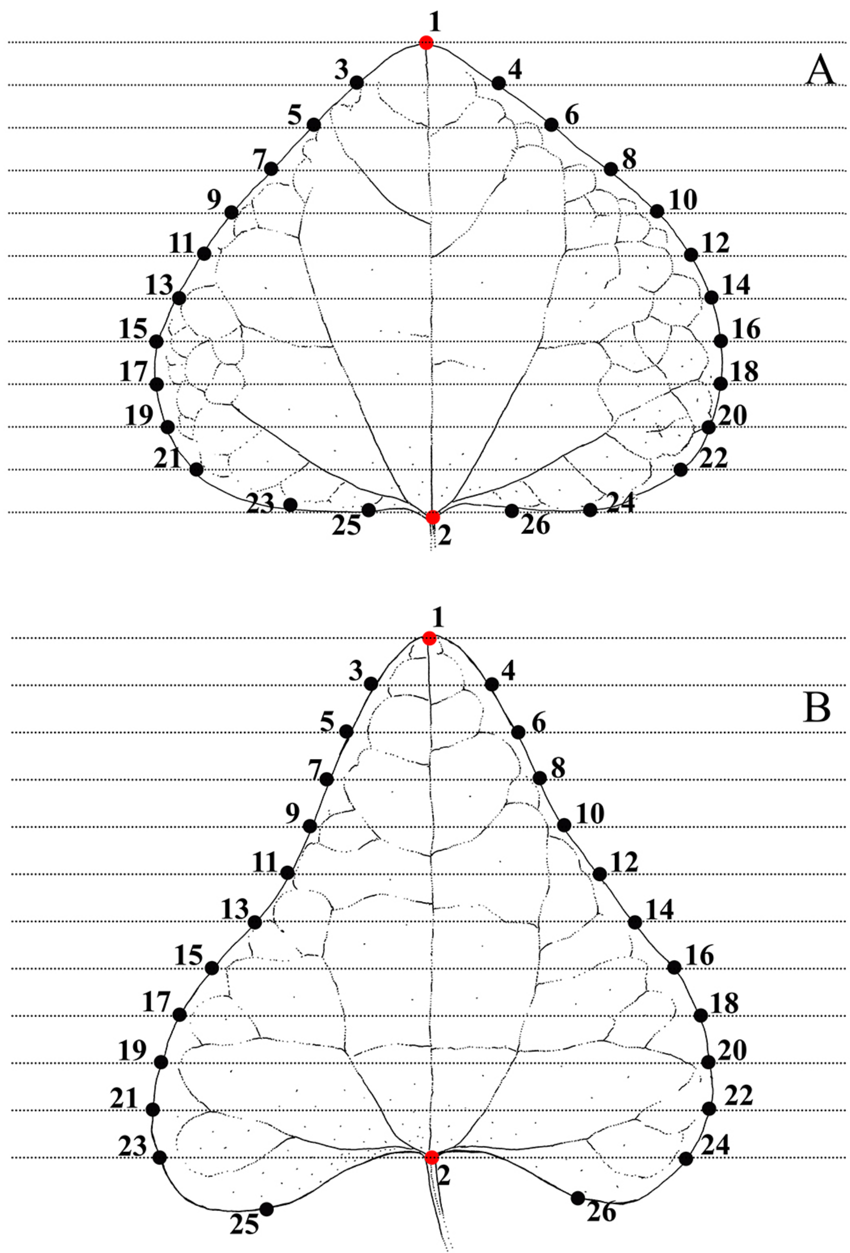

Figure 1. Landmarks (red dots) and semi-landmarks (black dots) selected to describe the homologous points of the leaf of *Aristolochia hypoglauca* (A) and *A. paulistana* (B).

We also conducted a pairwise Euclidean distance matrix, employing the unweighted pair group method with arithmetic mean (UPGMA) algorithm (Sokal and Michener 1958) in the PAST software version 2.04 (Hammer *et al*. 2001). The "Tps" software series (*e.g.* Nery & Fiaschi 2019; Karbstein *et al.* 2020; Pessoa *et al.* 2020; Cheng *et al.* 2021; Danila & Alejandro 2021; Araújo *et al.* 2023) and the MorphoJ (*e.g.* Pessoa *et al.* 2020; Guamba *et al.* 2021; Danila & Alejandro 2021; Araújo *et al.* 2023) have been used in several GM studies with plants.

Taxonomic treatment: The taxonomic framework to distinguish the two species here studied, and the remaining pseudostipule-bearing species with caudate perianth limb included in the key below followed Freitas *et al.* (2020a). Morphological terms follow Harris and Harris (2001), except for those specific traits of *Aristolochia* flowers and fruits, which were described according to González (1990), and nomenclature follows the latest International Code of Nomenclature for algae, fungi, and plants (ICBN) (Turland *et al.* 2018).

Conservation status: IUCN criteria and guidelines (IUCN 2012; 2022) were applied to assess the conservation status of the two species examined, with extent of occurrence (EOO) and area of occupation (AOO) calculated using the Geospatial Conservation Assessment Tool (GeoCat) (Bachman *et al*. 2011). The Quantum-GIS 3.24.0 software was used to draw the map of geographic distribution.

Results

Morphometric analysis

The first Principal Components Analysis (PCA) (Fig. 2A) shows a clear overlap in shape between some leaves of the two species, as a result of previous misidentifications. After revising these identifications, the adjusted PCA (Fig. 2B) shows a clear-cut separation of both species, with the two first axes explaining 94.97% of the variance, with 83.16% for PC1 and 11.81% for PC2, respectively. The comparisons between the resulting groups were performed using the Canonical Variate Analysis (CVA), wherein the first canonical variate (CV1) accounted for 100% of the total variance (Table 1; Figure S1).

The wireframes projected from the average shape of leaf blade in both species show a clear-cut variation in the movement of landmarks 9–10, 11–12 and 13–14 corresponding to the leaf blade constricted at its mid-region in *A. paulistana* as compared to the non-constricted blades in *A. hypoglauca* (Fig. 3). Similarly, landmarks 23, 24, 25 and 26 show significant variation of the leaf blade base which exhibits a more pronounced sinus in *A. paulistana* (Fig. 3).

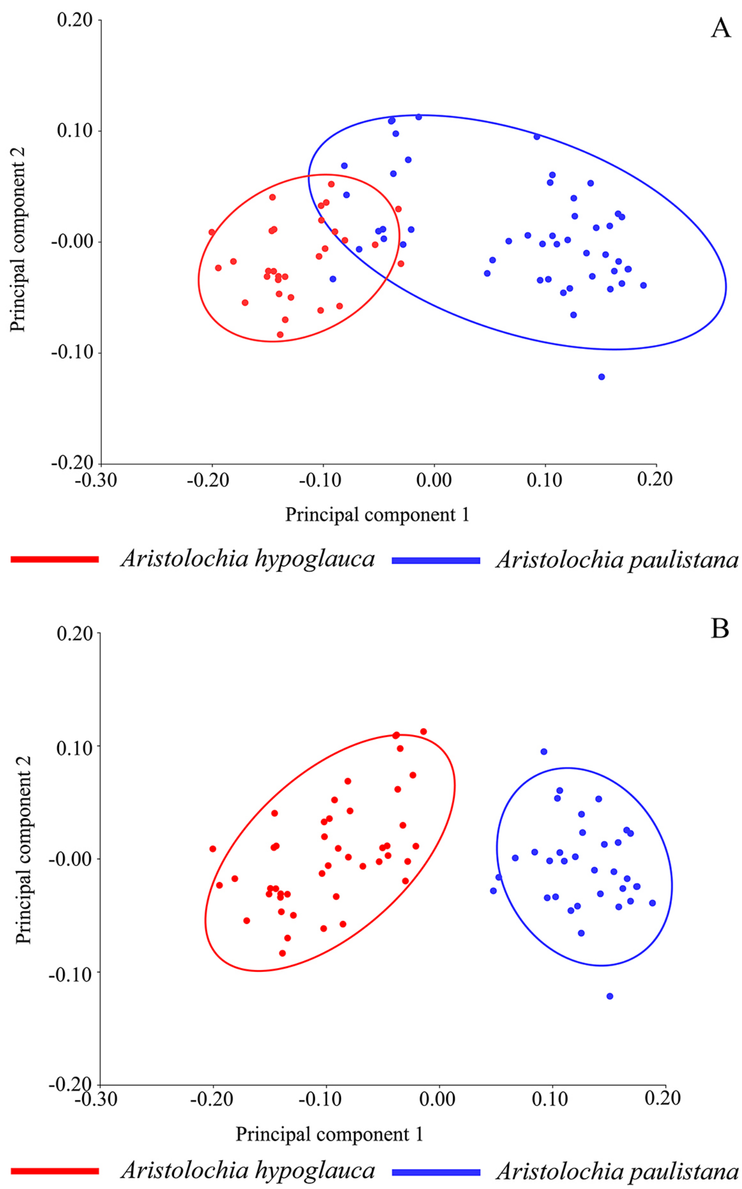

Figure 2. Principal Component Analysis (PCA) from landmarks of leaves of *Aristolochia hypoglauca* and *A. paulistana* based on the identifications of examined specimens (**A**) and after PCA analysis and revised identifications (**B**).

Discriminant function and the cross-validation test in leaves resulted in the maximum percentage (100%) of correctly classified cases (Table 1; Figure S2). PCA results are supported by CVA (Table S1). The Procrustes distance among the two species was 0.2252 ($p < 0.0001$), and the Mahalanobis Distance was 8.4473 ($p < 0.0001$), which provides statistical support to distinguish *A. paulistana* from *A. hypoglauca*, mainly by the leaf blade medially constricted in the former. The dendrogram resulting from the cluster analysis of Euclidean square distances also indicated that *A. hypoglauca* can be consistently distinguished from *A. paulistana* by using leaf morphometrics (Figure S3).

Taxonomy

Aristolochia hypoglauca Kuhlm., Arq. Inst. Biol. Veg. 3: 45. 1936. Type: Brazil. Espírito Santo, Três Ilhas, margens do Rio Doce [margins of the Rio Doce], 20 Apr 1934 (st), *J. G. Kuhlmann 238* (Holotype: RB[RB00534379!]. Epitype here designated: Rio de Janeiro. Cultivada no Jardim Botânico do Rio de Janeiro, proveniente do Rio Doce (ES) 14 May 1936 (fl) *J. G. Kuhlmann* s.n. (RB00534381! and RB00535170!). Fig. 4.

Glabrous vines. Twigs cylindrical; internodes 5.4–16 cm long. Petiole 3.9–9.5 cm long; leaf blade very wide ovate, 6.6–15 × 7.7–16 cm, not medially constricted, base truncate to slightly cordate (with sinuses to 3 mm deep), not peltate, apex obtuse to rounded, papery to chartaceous, glabrous above, puberulous and pruinose below, basal primary veins 3(5). Pseudostipules orbiculate, 1.5–2 cm, initially chartaceous, later scariose. Flowers solitary, axillary; peduncle plus ovary 8.3–23 cm long. Perianth slightly curved

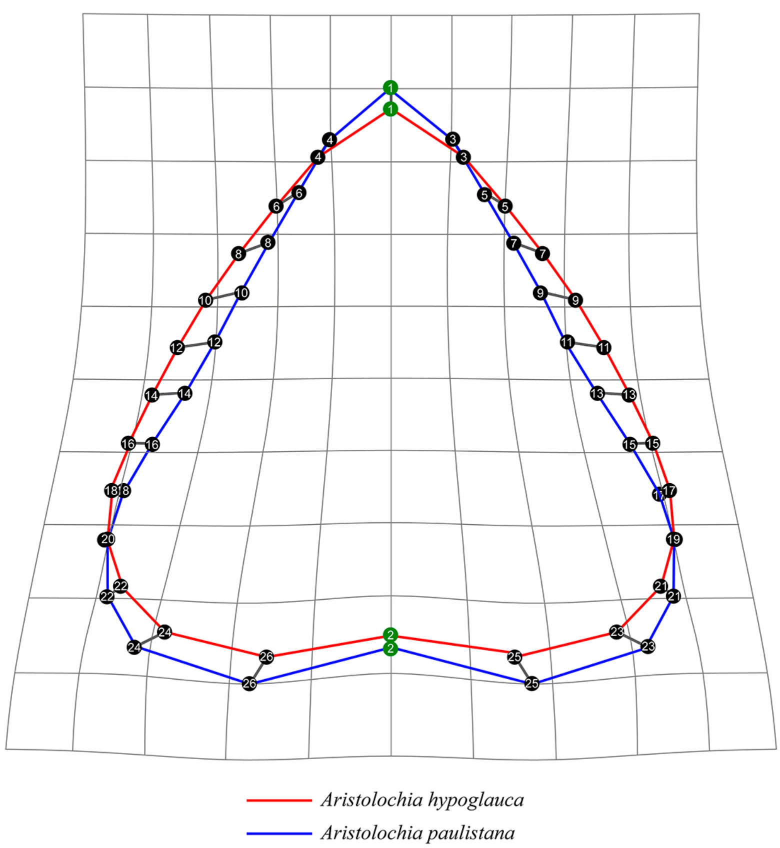

Figure 3. Wireframe representation of the leaf shape variation of *Aristolochia hypoglauca* and *A. paulistana*. The diagrams show the shape that corresponds to the average shape with the principal leaf shape variation. Numbered, green circles indicate landmarks; numbered, black circles indicate semi-landmarks.

Table 1. Differences in leaf shape between groups of *Aristolochia hypoglauca* and *A. paulistana* analyzed with Canonical Variate Analysis (CVA), percentage of correctly classified specimens, and variation among groups, scaled by the inverse of the within-group variation

	% of correctly classified	Mahalanobis Distance	P values Mahalanobis Distance	Procrustes Distance	P values Procrustes Distance	Eigenvalues	Variance %	Cumulative %
A. hypoglauca	100% (45/45)	8.4473	<0.0001	0,2252	<0.0001	17.94769786	100	100
A. paulistana	100% (34/34)							

Note: *P*-values based on permutation 10,000 rounds in MorphoJ: $P < 0.0001$)

Figure 4. *Aristolochia hypoglauca* (A-B) and *A. paulistana* (C). Photographs by J. Freitas (A-B) and Mauro Peixoto (C).

at preanthesis, later markedly curved between the utricle and the tube, outer surface glabrous and beige with thick vinaceous grooves, pruinose, inner surface with thin hairs, white in the limb and tube, and yellowish in the utricle; utricle obovoid, 4.1–8 × 2.2–4 cm; syrinx inequilateral, to 5 mm long; tube funnel-shaped, 2.1–3.6 cm long, 0.9 cm proximal diameter, 2 cm distal diameter; limb unilabiate, lanceolate, 20–35 cm long (including an apical ribbon-like, thoroughly twisted cauda), 2–3 cm wide, base cordate, not peltate. Gynostemium stipitate, 8–12 mm, stipite ca. 2 mm long, anthers oblong, 5–7 mm long, stigmatic lobes six. Ovary 6-carpellate. Capsule cylindrical to narrowly cylindrical, 4.6–6.1 × 1.5–2.5 cm, midvein of carpels prominent, ca. 1 mm thick, apex rostrate, rostrum 3–4.5 mm long; seeds ovoid, 7.5 × 7 cm, flat, warty, 1-winged, raphe lineariform.

***Additional specimens examined*: BRAZIL, Bahia**: Una, Estrada São José-Una, km 9, Ramal à direita a partir de São José, 07 Apr 1995, fl., *A. M. Amorim 1677* (CEPEC*; NY*). **Espírito Santo**: RPPN Vale do Sol, 17 Oct 2014, fl., *P. J. Coelho et al. 38* (MBML); Santa Teresa, distrito de 25 de Julho, localidade Bela Vista, 20 Apr 2005, fl., *A. P. Fontana & C. Esgario 1345* (MBML*); Santa Maria de Jetibá, São José do Rio Claro, propriedade Alfredo Renok, 21 Jan 2009, fl., *J. Freitas 02* (MBML); idem 17 Feb 2013, fr., *J. Freitas & I. G. V. Freitas 188* (MBML*); idem 23 Dec 2013, fl., *J. Freitas & L. Tonini 197* (MBML*); Rio Saltinho, terreno de Tranhago, 04 Sep 2001, fl., *L. Kollmann et al. 4495* (MBML*); Rio Saltinho, a 10 km do centro da cidade, 01 Jul 2015, fr., J. Freitas 413 (MBML*); Fundão, Goiapabaçu, 18 Mar 2005, fl., fr., *L. Kollmann et al. 7468* (MBML*); 16 Mar 2006, fl., *L. Kollmann et al. 8749* (MBML*). João Neiva, Alto Bergano, 04 Oct 2007, fl., *L. Kollmann et al. 10162* (MBML); antes do terreno do Tranhago, na rodovia sentido Santa Teresa-Fundão, 09 Jan 2013, fl., *E. J. Lírio et al. 683* (MBML); Fazenda Caioaba, trilha do córrego Caioaba, 08 Aug 2006, fl., fr., *L. F. S. Magnago 1221* (MBML). Santa Leopoldina, Encantado, propriedade da Sra. Zenith Zani, 17 Jan 2007, fl., *J. Rossini & L. C. Rossini 584* (MBML); Norte Rio Doce, Matas Rio S. José, Sep 1950, fl., fr., *J.M. Vieira 16* (RB*); Norte Rio Doce, Matas Rio S. Gabriel, Sep 1950, fl., fr., J.M. Vieira 17 (RB*). **Rio de Janeiro**: Córrego do Ouro, Estrada RJ-162, Km 45, 28 Jun 2016, fr., *J. M. A. Braga 16-008* (RB); Macaé, Frade de Macaé, 26 Sep 2013, fl., *L. Kollmann 12829* (MBML); Santa Maria Madalena. Estrada para Sossego, via Cachoeirão, 20 Sep 2017, fr., *C. D. M. Ferreira et al. 517* (RB); Sossego do Imbé, estrada da Cascata, 20 Oct 2020, fr., *M. S. Wängler et al. 2662* (RB); Parque Nacional Serra dos Órgãos, subsede próx. ao Museu Martius, 300-500 m, 20 Oct 1977, fl., *G. Martinelli et al. 3324* (RB); Organ Mon., sin date, fl., J. Miers 4041 (K*).

***Taxonomic remarks*:** Kuhlmann (1936) described *A. hypoglauca* based on his own collection (*J. G. Kuhlmann n. 238*; RB, barcode [RB00534379]) from the margin of Rio Doce (Doce River), Três Ilhas, in the state of Espírito Santo. We found three sheets deposited at the herbarium RB, the original sterile voucher cited in the protologue, and two additional gatherings (*J. G. Kuhlmann s.n.*, barcodes [RB00534381] and [RB00535170]) cultivated by Kuhlmann at the Rio de Janeiro Botanical Garden and vouchered upon flowering. Although all these specimens are stored as types at RB, only the sterile one can be recognized as the original material. Thus, we proceed to designate here the second (flower-bearing) gathering made by Kuhlmann as the epitype of the species, for interpretative purpose, in accordance with the provisions of the current ICNAFP (Turland *et al.* 2018).

***Phenology*:** *Aristolochia hypoglauca* sets flowers in January through April, and in August through December, and fruits in February, March, June, August, and September.

***Distribution and habitat*:** *Aristolochia hypoglauca* occurs in the states of Bahia, Espírito Santo and Rio de Janeiro in dense montane or submontane Atlantic Forest (Fig. 5) at elevations between 30 and 806 m, on forest edges or roadsides, sometimes associated with humid conditions.

Aristolochia paulistana Hoehne 1925, Arch. Bot. São Paulo 1: 13. Type: BRAZIL. São Paulo: Alto da Serra, Mata da Estação Biológica, Serra do Cubatão, 2 September 1921 (fl.), *F. C. Hoehne s.n.* (Lectotype designated by Freitas *et al.* 2017): SP, barcode [SP000409!]; isolectotypes: A[A00036010!], MO[MO-022305!], NY[NY00285556], SI [SI000753!], SP* [SP000410!], US[US00921452!, and US00105866!]). Fig. 4.

Glabrous vines. Twigs cylindrical, internodes 3.2–17 cm long. Petiole 2–6.5 cm long; leaf blade deltoid, 4.6–10.3 × 5–10.5 cm, medially constricted, base slightly cordate (with sinuses to 1cm deep), not peltate, apex rounded, chartaceous to coriaceous, glabrous above, puberulous and not pruinose below, basal primary veins 3(5). Pseudostipules orbiculate, chartaceous when juvenile, later scariose, 1–2 cm. Flowers solitary, axillary; peduncle plus ovary 5.2–10.1 cm long. Perianth slightly curved at preanthesis, later markedly curved between the utricle and the tube, outer surface glabrous and beige with thick reddish grooves, not pruinose, inner surface with thin hairs, white in the limb and tube, and yellowish in the utricle; utricle ovoid to globose, 2–3.9 × 1.2–2.6 cm; syrinx inaequilateral, to 5 mm long; tube funnel-shaped, 1.3–4.1 cm, 1.2–1.8 cm in proximal diameter, 1–2.5 cm in distal diameter; limb unilabiate, lanceolate, 9–19.3 cm long (including an apical ribbon-like cauda twisted except at its base), 2–3 cm wide, base cordate, not peltate. Gynostemium stipitate, 6–8 mm long, stipite ca. 3 mm long, anthers oblong, 4–5 mm long; stigmatic lobes six. Capsule cylindrical to narrowly cylindrical, 4–6.1 × 2–2.5 cm, midvein of carpels prominent, ca. 1 mm thick, apex rostrate, rostrum 6–7 mm long; seeds ovoid, 0.5–1.0 × 0.5–0.9 cm, flat, warty, 1-winged, raphe lineariform.

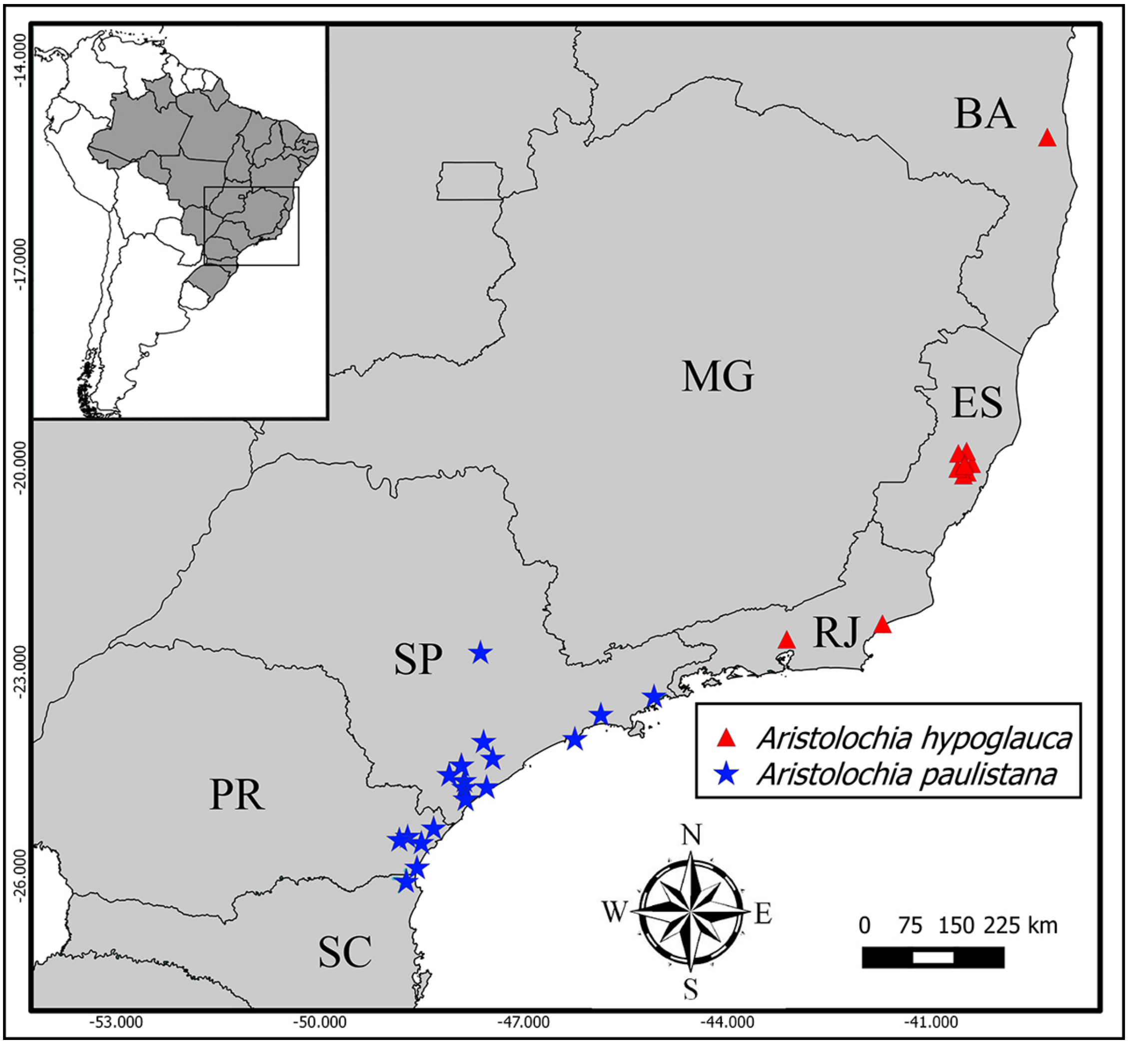

Figure 5. Map of distribution of *Aristolochia hypoglauca* (triangles) and *A. paulistana* (stars) in Brazil. Abbreviations: BA = Bahia, ES = Espírito Santo, MG = Minas Gerais, PR = Paraná, RJ = Rio de Janeiro, SC = Santa Catarina; SP = São Paulo.

***Additional specimens examined*: BRAZIL, Paraná:** [Lapa] Volta Grande, 7 Aug 1911, ster., *P. Dusén 12020* (GH); [Morretes] Jacarehy?, 18 Jul 1914, fl., *P. Dusén 15310* (BM, K, MO) "Jacarehy", 9 Jun 1915, fl., *P. Dusén 17066* (GH); "Jacarehy", 01 Apr 1915, fr., *P. Dusén s.n.* (GH, MO); Saquarema, Morretes, 40 km W of Paranagua, BR-277, c 40 m, 25º30´S, 48º40´W, 25 Jan 1985, fr., *A.H. Gentry & E. Zardini 49838* (MO, NY); Cadeado, Morretes, 30 Nov 1966, fl., *G. Hatschbach 15315* (NY, US); Antonina, Rio Capiuva, 20 m, 30 Jan 1968, fl., *G. Hatschbach 18522* (CTES, F, MO, NY, RB, UC, US); Tagaçaba, Guaraqueçaba, 29 Oct 1971, fl., *G. Hatschbach 27603* (UC); Guaraqueçaba, Tagaçaba-de-cima, 19 Nov 1993, fl., *Lima 216* (UPCB); Morro do Rio das Pacas, Guaraqueçaba, 25º16´S, 48º19´W, 20 Ene 1993, fl., *J. Prado et al. 441* (COL, NY); Paranaguá, Morro do Meio, Ilha do Mel, 14 Mar 1987, fl., *W.S. Souza & R.M. Britez 24625* (UEC*). **Santa Catarina:** Garuva, Três Barras, São Francisco do Sul, 23 Jan 1958, fl., *Reitz 6291* (US). **São Paulo:** Alto da Serra, Mata da Estação Biológica, 2 Sep 1921, fl., *F.C. Hoehne s.n.* (MO, SP); Biritiba-Mirim, Estação Biológica de Boracéia, 8 Dec 1983, fr., *Custodio Filho 1976* (SP*); Cananéia, Estrada Pariquera Açu-Cananéia, 7 Feb 1995, fl., *Leitão Filho 32738* (ESA*, SP*, SPF, UEC*); Eldorado, 23 Mar 2005, st., *Meireles 216* (ESA*); 25 Mar 2005, ster., *Oriani 595* (ESA*); Guarujá, Praia do Perequê, 10 Oct 1981, st., *K. Brown Jr 13158* (UEC*); Iguape, A 5 km a Leste, restinga próxima ao morro, 2 May 1981, st., *Brown Jr 12517* (UEC*); Pariquera-Açu, 24 Mar 1995, fr., *Ivanauskas 87* (ESA*, UEC*); Pariquera-açu, 12 Jan 1995, fl., *Bernacci 1147* (ESA*, SP*); Piracicaba, ESALQ, 28 Jan 2004, st., *Silva s.n.* (UEC*); Sete Barras, Parque Estadual de Carlos Botelho, Núcleo Sete Barras, Trilha da Figueira. Floresta Ombrófila Densa Submontana, 23 Apr 2002, fr., *Gomes 445* (ESA*); Sete Barras, Parque Estadual Carlos Botelho, Núcleo Sete Barras, 27 Sep 2003, fr., *Udulutsch 2429* (HRCB, UEC*).

***Taxonomic remarks*:** *Aristolochia hypoglauca* (Fig. 4A-B) and *A. paulistana* (Fig. 4C) exhibit caudate flowers, a trait found in four additional species naturally growing in Brazil (*A. mishuyacensis* O.C.Schmidt, *A. pohliana* Duch., *A. trilobata* L. and *A. weddellii* Duch.). These species differ in several vegetative and reproductive traits, which are summarized in the key below. Along with many other flowering plant species (cf. Carnaval *et al*. 2009; Batalha-Filho & Miyaki 2011; Werneck *et al*. 2011), *A. hypoglauca* and *A. paulistana* support the Atlantic Forest as a pivotal area of endemism in South America.

***Phenology*:** This species sets flowers in January through March, and in September and November, and fruits in March, April, September and December.

***Distribution and habitat*:** *Aristolochia paulistana* occurs in the states of Paraná, Santa Catarina and São Paulo (Fig. 5), at the edges of ombrophilous, dense montane forests, especially in pluvial forests, at elevations between 10 and 485 m.

Key to the species of Aristolochia with caudate limb perianth in Brazil

1. Leaves deeply trilobed .... *A. trilobata*

1. Leaves ovate, deltoid or sagittate 2

2. Perianth limb with a cymbiform base, and an apical cauda to 45 cm long. Amazonian forests of Colombia, Ecuador, Peru, Brazil and Bolivia .... *A. mishuyacensis*

2. Perianth limb not cymbiform at base, and an apical cauda to 30 cm long. Atlantic Forests of Brazil (except *A. weddellii* from the Amazon basin of Brazil, Guyana, Surinam and French Guiana 3

3. Leaf blade with sinuses <1cm; perianth limb unilabiate 4

3. Leaf blade with sinuses >1cm; perianth limb bilabiate 5

4. Leaf blade deltoid, medially constricted. Perianth with utricle to 4 cm long and limb to 15 cm long, base of the cauda not twisted .... *A. paulistana*

4. Leaf blade very widely ovate, medially not constricted. Perianth with utricle 4.1–8 cm long and limb 20–35 cm long, base of the cauda twisted .... *A. hypoglauca*

5. Lower perianth lip ovate, 3.5–4.5 × 1.5–2 cm. Seeds winged .... *A. pohliana*

5. Lower perianth lip widely ovate, 2–2.5 × 2–3 cm. Seeds not winged .... *A. weddellii*

Discussion

Geometric Morphometrics

In this study we tested the application of GM as a tool for distinguishing the species *A. hypoglauca* and *A. paulistana*. In addition to herbarium specimens, we also found misidentifications of these species in regional taxonomic treatments for the family (*e.g.* Araújo 2013; Abreu & Giulietti 2016). The lack of information regarding the circumscription of *A. hypoglauca* and *A. paulistana* is mainly explained by the low number of specimens unequivocally corresponding to *A. hypoglauca*. The taxonomic status of this species as distinct from *A. paulistana* was clarified by Freitas and Alves-Araújo (2017), 81 years after its original description. This circumscription, maintained by Freitas *et al*. (2020a) is here corroborated and five specimens previously treated as *A. paulistana* are identified here as *A. hypoglauca*. Until now, only *A. paulistana* has been included in a phylogenetic study, however, its position concerning other South American species used in this study is inconclusive (Ohi-Toma *et al.* 2006).

This is the first study that performs GM analysis to successfully distinguish closely related *Aristolochia* species using leaf traits (Fig. 2). Recently, Frank (2023) also successfully applied GM, using leaves and flowers, to separate two South American species, one of which was described as a new species. In this study, Frank (2023) applied 8 landmarks at specific points on the leaves, while here, we used 26, arranged with comb rays along the leaf margin (Fig. 1). The use of comb rays can prove useful in detecting small variations in leaf shape, as observed in landmarks 9–10, 11–12, and 13–14 (Fig. 3). Comb rays have been used in various studies with other plant families as a standardization of markings, thus minimizing potential errors and increasing reproducibility (Fragoso-Martínez *et al*. 2015; Vujić *et al*. 2016; Miljković *et al*. 2019; Chávez-Hernández *et al.* 2021).

Leaf GM has been successfully employed in other plant groups to differentiate species (Karbstein *et al.* 2020; Cheng *et al*. 2021; Guamba *et al*. 2021). On the other hand, some groups have not responded to leaf GM (Nery & Fiaschi 2019; Danila & Alejandro 2021), necessitating the use of other structures, such as flowers (Menini-Neto *et al.* 2019; Pessoa *et al*. 2020; Frank (2023) or fruits (Márquez *et al.* 2022), for instance. In this study, it was not possible to use flowers of *A. hypoglauca* and *A. paulistana* for floral GM analysis. This is because, for *Aristolochia*, the floral limb has proven to be the structure that provides more useful characters for GM compared to the utricle and floral tube (Freitas *et al.* 2020b; Frank 2023). This limitation is primarily due to the fact that both species studied here have caudate limbs, which are positioned variably when herborized, making their correct application challenging.

Freitas *et al.* (2020b) carried out the first GM approach based on floral traits to *Aristolochia*, aiming to untangle the *Aristolochia cornuta*/*A. iquitensis* complex, while discovering a new species. Notably, leaf morphometry was inconclusive in the latter species complex, unlike the case study presented here. The lack of clear-cut leaf shapes detected through a GM could be the result of similar leaf shapes in species growing under the same environmental conditions, i.e. primary Amazonian forests, as is the case with *A. cornuta*, *A. iquitensis* and *A. wankeana*. These cases would support the postulate that leaf shape similarity could be the result of environmental conditions (cf. Adebowale *et al.* 2012; Nery & Fiaschi 2019; Danila & Alejandro 2021).

Conservation

Aristolochia hypoglauca: The subpopulations of *A. hypoglauca* are located mostly in private, agricultural lands (Menini-Neto *et al.* 2013). This species is a heliophile plant and several individuals are often found associated with crops, including coffee (*Coffea arabica* L.) fields. Most individuals occur along roadsides or near houses, which might result in the loss of individuals due to deforestation or road expansion (Freitas & Alves-Araújo 2017; Freitas *et al.* 2019). Following the IUCN Red List criteria (IUCN 2012; 2022), *A. hypoglauca* qualifies as EN (Endangered) B1ab(iii)+2ab(iii), due to its small Extent of Occurrence (591,849 km^2) and Area of Occupancy (36, km^2); the low number of locations (only eight known locations of occurrence); and the severely fragmented and declined habitat.

The subpopulations of *A. hypoglauca* with higher number of individuals are in the state of Espírito Santo (Brazil), in the municipalities of Santa Teresa, Santa Maria de Jetibá, Santa Leopoldina, Fundão and João Neiva. Only two subpopulations were discovered occurring in protected areas, the Parque Municipal de Goiapaba-Açu, and the Reserva Particular do Patrimônio Natural Vale do Sol, both in Santa Teresa (Freitas & Alves-Araújo 2017). Although these areas are considered protected under the current Brazilian environmental regulations, these policies allow large scale vegetation intervention, which does not guarantee the survival and conservation of these subpopulations.

Ex situ conservation of *A. hypoglauca* relies on seeds from two subpopulations that were included in the germplasm bank of Rio de Janeiro Botanical Garden and seeds from one subpopulation germinated in Instituto Nacional da Mata Atlântica. Additional studies on germination and viability of seeds are urgently needed. Currently, this is the first ex situ conservation effort for *A. hypoglauca* after the historical collection cultivated at Rio de Janeiro Botanical Garden by Kuhlmann (1936). Unfortunately, the wild specimen from which Kuhlmann took the original material to describe the species no longer exists.

We call here for urgent in situ monitoring and conservation studies of wild subpopulations, and ex situ propagation since the subpopulations from conservation areas are not fully protected. For in situ conservation, projects focused on local environmental education are highly recommended. Also, it is necessary to expand the protected areas aiming to include the wild subpopulations of *A. hypoglauca*. For ex situ conservation, seeds from wild individuals and distant subpopulations are needed to increase genetic diversity in germplasm banks and living collections as sources to successfully reintroduce the species in the wild.

Aristolochia paulistana: The species has 56,269.237 km^2 of Extent of Occurrence and 68 km^2 of Area of Occupancy in eleven locations. It grows along roadsides or near houses, which can result in the loss of individuals due to deforestation and road construction. Seed dispersal limitation associated with the loss of habitat quality might also have a negative effect on the conservation status of the species. The species occurs in the Parque Estadual de Carlos Botelho, Parque Estadual de Jacupiranga and Estação Biológica de Boracéia. By applying the IUCN criteria and guidelines (IUCN 2012; 2022), *A. paulistana* qualifies as an Endangered species (EN; criteria B1ab(iii)+2ab(iii), due to its small Area of Occupancy (68 km^2), its low number of known locations of occurrence (eight), and its highly fragmented habitat, which leads to its decrease.

Conclusion

This GM study supported the recognition of the two species, which made it possible to revisit the circumscription of both species, as well as compare them with other *Aristolochia* species with caudate floral limb native to Brazil. As the holotype of *A. hypoglauca* does not fulfill the purpose of precise application of the name, according to the ICNAFP, it was necessary to propose an epitypification. The circumscription of the two species allowed us to delimitate their geographic distribution and assess its risk of extinction. *Aristolochia hypoglauca* occurs in the states of Bahia, Espírito Santo and Rio de Janeiro, while *A. paulistana* occurs in Paraná, Santa Catarina and São Paulo and both species were assessed as Endangered. This work raises the possibility of using GM in other *Aristolochia* species complexes, bringing to the group a possibility to assist and accelerate taxonomic decisions.

Supplementary Materials

Figure S1. Canonical Variate Analysis (CVA) from landmarks of leaves of *Aristolochia hypoglauca* and *A. paulistana*.

Figure S2. Discriminant function of leaves of *Aristolochia hypoglauca* and *A. paulistana*.

Figure S3. Pairwise Euclidean distance matrix, employing the unweighted pair group method with arithmetic mean (UPGMA) algorithm from landmarks and semi-landmarks of leaves of *Aristolochia hypoglauca* and *A. paulistana*.

Table S1. Discriminant analysis results for leaves for Aristolochia hypoglauca and Aristolochia paulistana.

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