Acta Botanica Brasilica, 2023, 37: e20230017
doi: https://doi.org/10.1590/1677-941X-ABB-2023-0017

Original article

Is the labellum informative to distinguish species of *Scaphyglottis* (Orchidaceae)? Insights from geometric morphometrics

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Received: January 25, 2023
Accepted: July 05, 2023

ABSTRACT

Scaphyglottis Poepp. & Endl. (Orchidaceae) is a neotropical genus with 78 species, 14 of which are found in Brazil. Some of these taxa have an intricate taxonomy, especially due to the morphological similarity among species and the existence of species complexes. Our study aims to test if the labellum shape, the main source of species diagnostic characters, can confirm the proposed circumscription of Brazilian species of *Scaphyglottis*. We analyzed 136 flowers from 12 species, to which we applied 12 landmarks, using a geometric morphometrics approach. Our results show that most species can be distinguished. Most of the variation is observed in the claw and apices of labellum lobes, confirming that the labellum is important tool in taxon distinction. Species with distinctly 3-lobed labellum showed some overlap but, for these taxa, we have found distinction specially in the lateral lobes. Our study showed new morphologically variable features in *Scaphyglottis* that can be used in future taxonomic studies. For widely distributed species we suggest a population-level approach, as local environmental factors can impact morphology, causing expected intraspecific variation.

Keywords: Amazon, multivariate analysis, species complexes, species delimitation, taxonomy.

Introduction

Standard external morphological features, traditionally used in plant taxonomy, are not always sufficient to delimit taxa (Galtier 2019; Lawley *et al.* 2022). In closely related or morphologically similar species, the use of qualitative morphological data alone will likely merge distinct taxa or segregate populations of a same taxon. Although molecular approaches can help solve some of these issues, a morphological circumscription is still needed to delimit species in a practical sense (Galtier 2019).

Distinguishing species that suffered allopatric speciation is usually simpler, but this is often not the case with young or cryptic lineages (Pinheiro *et al.* 2018; Pessoa *et al.* 2021). In the first case, these lineages can be included within one of the many species concepts (De Queiroz 2007; Pinheiro *et al.* 2018), but this is not always applicable to the second case, as other evolutionary and ecological processes,

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such as hybridization and introgression, or development homeostasis (Mayr 1963), can be at play and lead lineages to form species complexes (Pinheiro *et al*. 2018; Pessoa *et al*. 2022).

Several tools have been used to study species complexes, such as near-infrared spectroscopy (NIR) (Durgante *et al.* 2013; Lang *et al*. 2017; Prata *et al*. 2018; Paiva *et al*. 2021), cytogenetics (Assis *et al.* 2013; Medeiros-Neto *et al.* 2017; Querino *et al.* 2020; Pessoa *et al.* 2021; Camelo-Júnior *et al.* 2022), population genetics (Pessoa *et al*. 2012; Esteves & Vicentini 2013; Rodrigues *et al.* 2014), and geometric morphometrics (GM) (Pinheiro & Barros 2007; Nery & Fiaschi 2019; Aristizábal *et al.* 2020; Pessoa *et al.* 2021; Camelo-Júnior *et al.* 2022). This latter phenetic approach allows, through statistical analysis of shape variation among individuals and populations, to test taxon delimitation using highly variable structures. Besides being a low-cost application, depending only on the availability of specimens, this method has an advantage over other tools in that its results can be directly applied to build the morphological circumscription of the taxon (Camelo-Júnior *et al.* 2022), complying with the more commonly used morphological species concept (De Queiroz 2007).

In several species and genera of Orchidaceae, GM approaches have been used to test species limits (Pinheiro & Barros 2009; Menini-Neto *et al*. 2019; Lau *et al*. 2021; Pessoa *et al*. 2021; Camelo-Júnior *et al*. 2022) but this tool still have not been employed for several other genera with some difficult species delimitation cases. *Scaphyglottis* Poepp. & Endl. (Laeliinae, Orchidaceae), a monophyletic Neotropical genus (Dressler *et al*. 2004) with 78 species (Govaerts 2022), is one of these cases. The genus is recognized for presenting overlapping pseudobulbs, conduplicate leaves, flowers that are almost always resupinate, conspicuous column foot and 4–6 pollinia (Dressler *et al*. 2004; Szlachetko & Kolanowska 2014a). The species are distributed from Mexico to Bolivia and Brazil (Govaerts 2022) and Colombia is the country with the highest number of native species (33 spp.), followed by Costa Rica (21 spp.), Ecuador (20 spp.) and Peru (17 spp.) (POWO 2022).

In Brazil, the genus is represented by 14 species: *S. bidentata* (Lindl.) Dressler., *S. boliviensis* (Rolfe) B.R. Adams., *S. brasiliensis* (Schltr.) Dressler., *S. emarginata* (Garay) Dressler., *S. fusiformis* (Griseb.) R.E. Schultes., *S. graminifolia* (Ruiz & Pav.) Poepp. & Endl., *S. imbricata* (Lindl.) Dressler., *S. livida* (Lindl.) Schltr., *S. modesta* (Rchb.f.) Schltr., *S. prolifera* (Sw.) Cogn., *S. punctulata* (Rchb. f.) C. Schweinf., *S. reflexa* Lindl., *S. sickii* Pabst., and *S. stellata* Lodd. ex Lindl (Araújo *et al.* 2022, Fig. 1). Although the genus is widely distributed in Brazil, the phytogeographical domains with higher diversity are the Amazon Forest (12 spp.) and the Atlantic Forest (6 spp.) (BFG 2022). Among the species found in Brazil, some can be considered part of species complexes due to the morphological similarities and overlapping of diagnostic characters.

Although vegetative features are useful to delimit a sizeable portion of the Brazilian species of *Scaphyglottis* (Dressler 1993; Pessoa & Alves 2012; Szlachetko & Kolanowska 2013; Szlachetko & Kolanowska 2014a; b; Koch *et al*. 2018; Santos & Silva 2020; Araújo *et al*. 2022), there are some taxa in Brazil with questionable taxonomic affinities. Although these taxa have similar vegetative and floral morphology, they have been commonly identified with distinct names, examples are the species pairs: *S. stellata*–*S. boliviensis*, *S. sickii*–*S. prolifera* and *S. brasiliensis*–*S. reflexa*.

The labellum is considered a fundamental character to distinguish species in Orchidaceae (Dressler 1993; Carnevali & Ramírez-Morillo 2003; Szlachetko & Kolanowska 2014a; Araújo *et al*. 2022), and have been used as a major source of information in previous studies that applied geometric morphometrics (Menini-Neto *et al*. 2019; Lau *et al*. 2021; Pessoa *et al*. 2021; Camelo-Júnior *et al*. 2022). Thus, in this study we evaluate if geometric morphometrics of the labellum is able to distinguish the species of *Scaphyglottis* that occur in Brazil. The main goals are: i) to produce evidence of valuable diagnostic characters to delimit these species and ii) to evaluate delimitation of taxa with similar vegetative morphology.

Material and methods

Sampling

We sampled 136 flowers from 103 specimens from herbarium collections and field expeditions (Tab. S1). Our sampling is representative of the geographical distribution of *Scaphyglotttis* in Brazil (Fig. 2). Specimens from the following herbaria were analyzed: BHCB, EAC, EAN, FURB, PEUFR, HST, IAN, INPA, IPA, MG, RB, UEC, UFMT, UFP and UPCB (acronyms according to Thiers 2022, continuously updated). Three field expeditions were carried out at Reserva Florestal Adolpho Ducke (Manaus, AM), Parque Estadual Serra do Aracá (Barcelos, AM), Serra do Tepequém (Amajari, RR) and Serra Grande (Cantá, RR), where we collected several specimens of six species of *Scaphyglottis* (*S. bidentata*, *S. fusiformis*, *S. imbricata*, *S. reflexa*, *S. sickii* and *S. stellata*). All specimens collected on the field were kept in cultivation and until now, ten specimens have been deposited in herbarium collections. For each sampled specimen, we removed one to two flowers for analysis, with authorization of the herbarium curators. Some taxa had low sampling, either due to rarity and scarcity of specimens in collections or due to the available specimens being sterile or having only fruits. Specifically, *S. graminifolia* (one single flower), *S. livida* (three flowers), *S. reflexa* (three flowers) and *S. brasiliensis* (four flowers) had a low number of sampled flowers. We could not include *S. emarginata* and *S. imbricata* for the same reason. Therefore, 12 from the 14 Brazilian species were

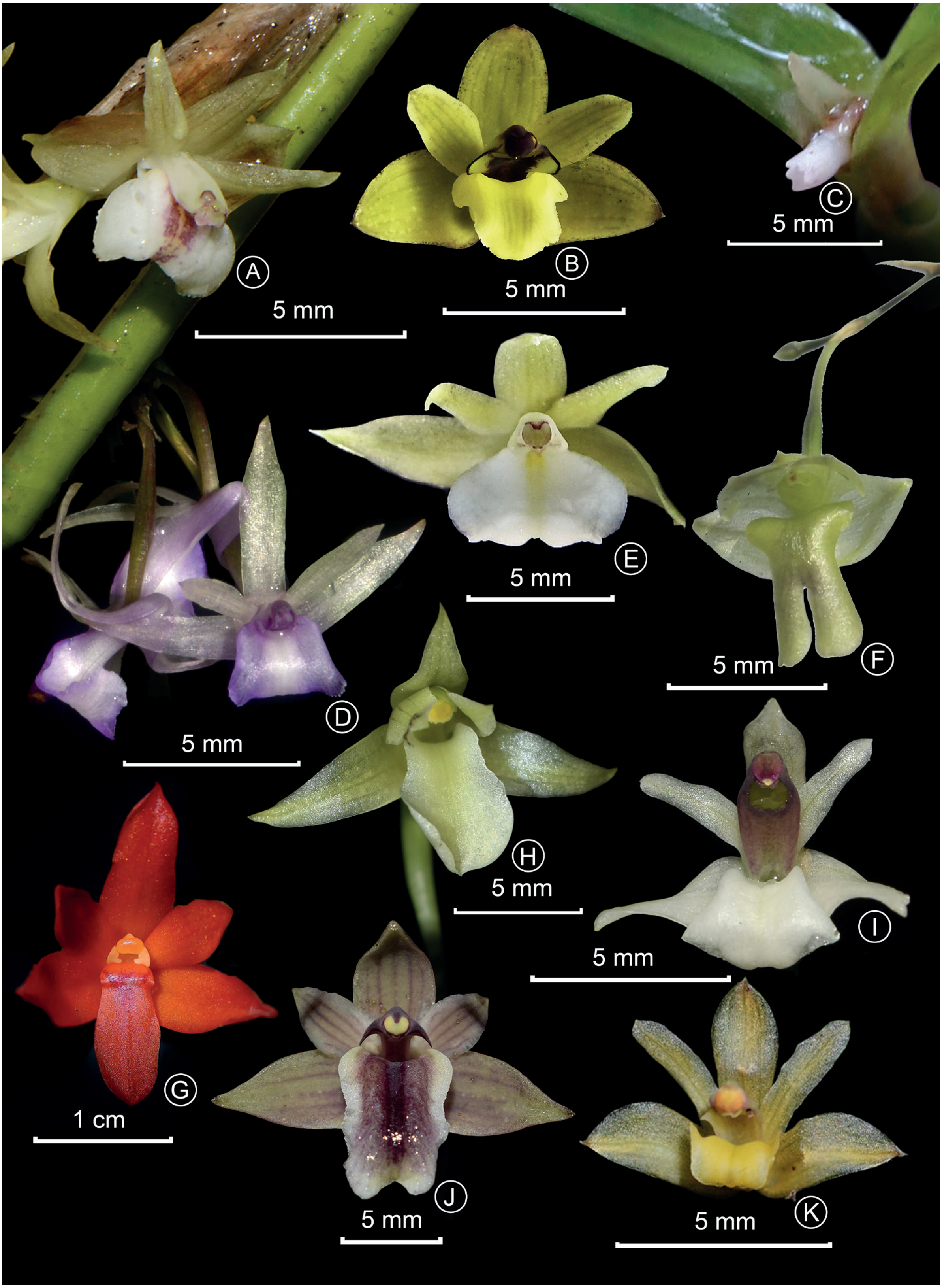

Figure 1. *Scaphyglottis* Poepp. & Endl in Brazil. A. *S. reflexa* Lindl., **B**. *S. modesta* (Rchb.f.) Schltr., **C**. *S. sickii* Pabst., **D**. *S. stellata* Lodd. ex Lindl, **E**. *S. brasiliensis* (Schltr.) Dressler., **F**. *S. livida* (Lindl.) Schltr., **G**. *S. bidentata* (Lindl.) Dressler., **H**. *S. fusiformis* (Griseb.) R.E. Schultes., **I**. *S. prolifera* (Sw.) Cogn., **J**. *S. punctulata* (Rchb. f.) C. Schweinf. **K**. *S. boliviensis* (Rolfe) B.R. Adams. Photos by Barbosa-Silva, R. (**A**), Marcusso, G. (**B**, **E**), Araújo, A. M. (**C**), Farroñay, F. (**D**, **H**, **I**, **J**), Pessoa, E. (**F**), Cordeiro, J. M. P. (**G**), Terra-Araújo, M. (**K**). Figure assembled by Miranda, G.

analyzed. Specimens identifications were verified according to Dressler (1993) and Szlachetko and Kolanowska (2014a).

Geometric Morphometrics (GM)

The flowers were rehydrated, and the labella were mounted in white cardstock paper. The labella were later scanned using an Epson Expression 10000XL scanner in 1200 dpi resolution for geometric morphometrics analyses. The mounted flower images were digitized using TPSutil 1.78 (Rohlf 2015). We added 12 landmarks to the labellum, using TPSdig2 (Rohlf 2015). Landmarks 1 and 11 represent the labellum column foot junction, landmark 12 represents the median point between landmarks 1 and 11, landmarks 2 and 10 represent the claw and lateral lobes junction, landmarks 3 and 9 correspond to the lateral lobe apices, 4 and 8 represent the lateral lobe and median lobe junction, 5 and 7 represent the median lobe apices and 6 represents the apex or central point of the labellum invagination (Fig. 3).

The files with the added landmarks were transferred and analyzed in the software MorphoJ 1.06d (Klingenberg 2011), and then submitted to Procrustes analysis, where the size variation is removed from the analysis, preserving the variation of the points from the centroid and changes in shape (Goodall 1991; Bookstein 1996). An exploratory Principal Component Analysis (PCA) (Hotelling 1933) was performed, aiming to group the specimens according to the labellum variation and to detect potential outliers. We then conducted a Canonical Variable Analysis (CVA) (Campbell & Atchley 1981) to discriminate the variation between pre-determined groups (i.e., the species) and the sequence in which they separate according to the canonical variables (CVs).

Because of the high morphological variation of the 12 analyzed species, we conducted new PCA and CVA analyses for species with 3-lobed labellum separately (*S. boliviensis*, *S. brasiliensis*, *S. prolifera* and *S. stellata*), as their similarities in the labellum led to overlapping of the ellipses when the total dataset is analyzed. We applied the methods described above to this reduced dataset.

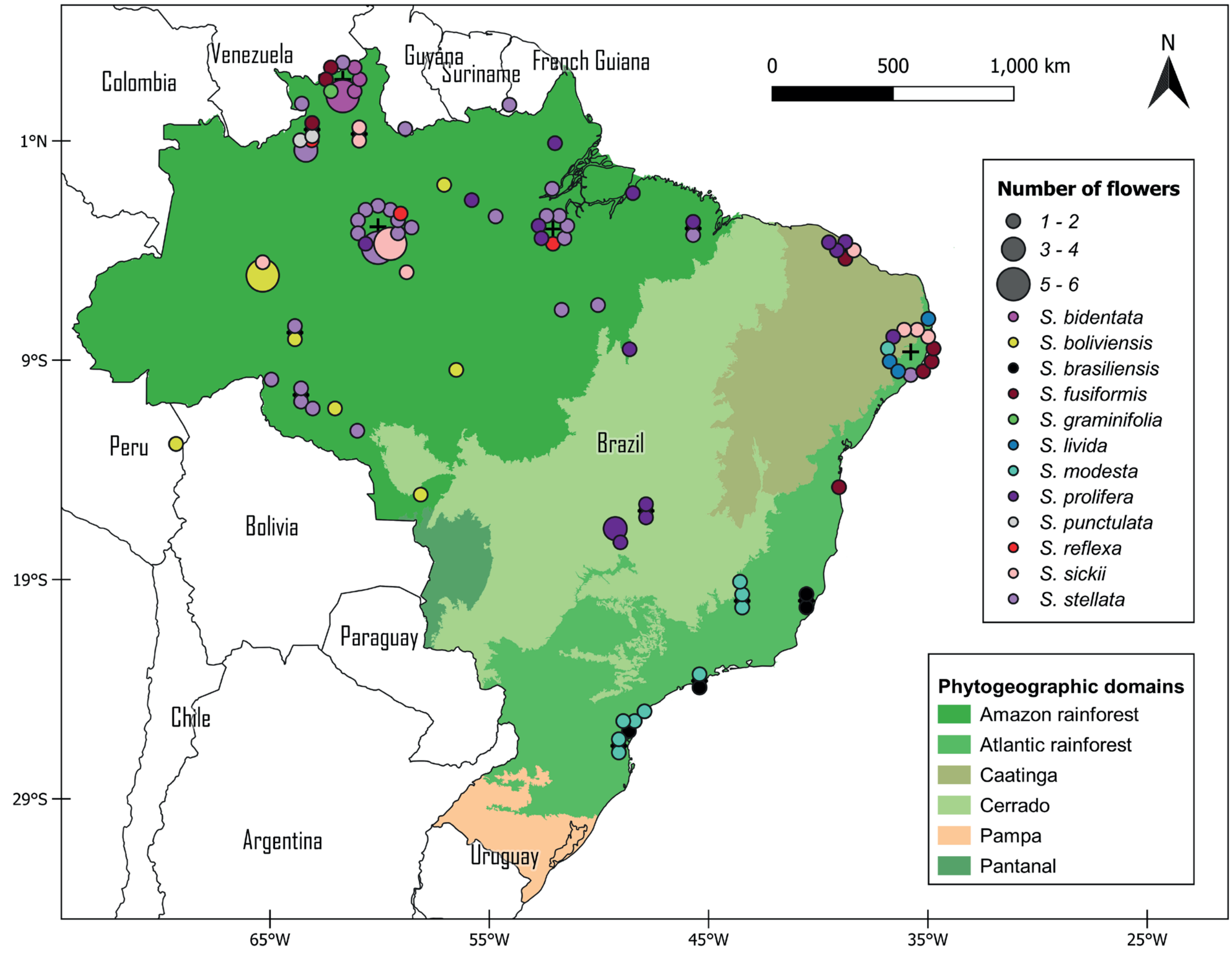

Figure 2. Distribution map of species sampled in the morphometric analysis, indicating the number of flowers analyzed by specimen. The symbol (+) indicates that specimens are from the same place, circles represent the specimens. Map prepared by Falen, L.

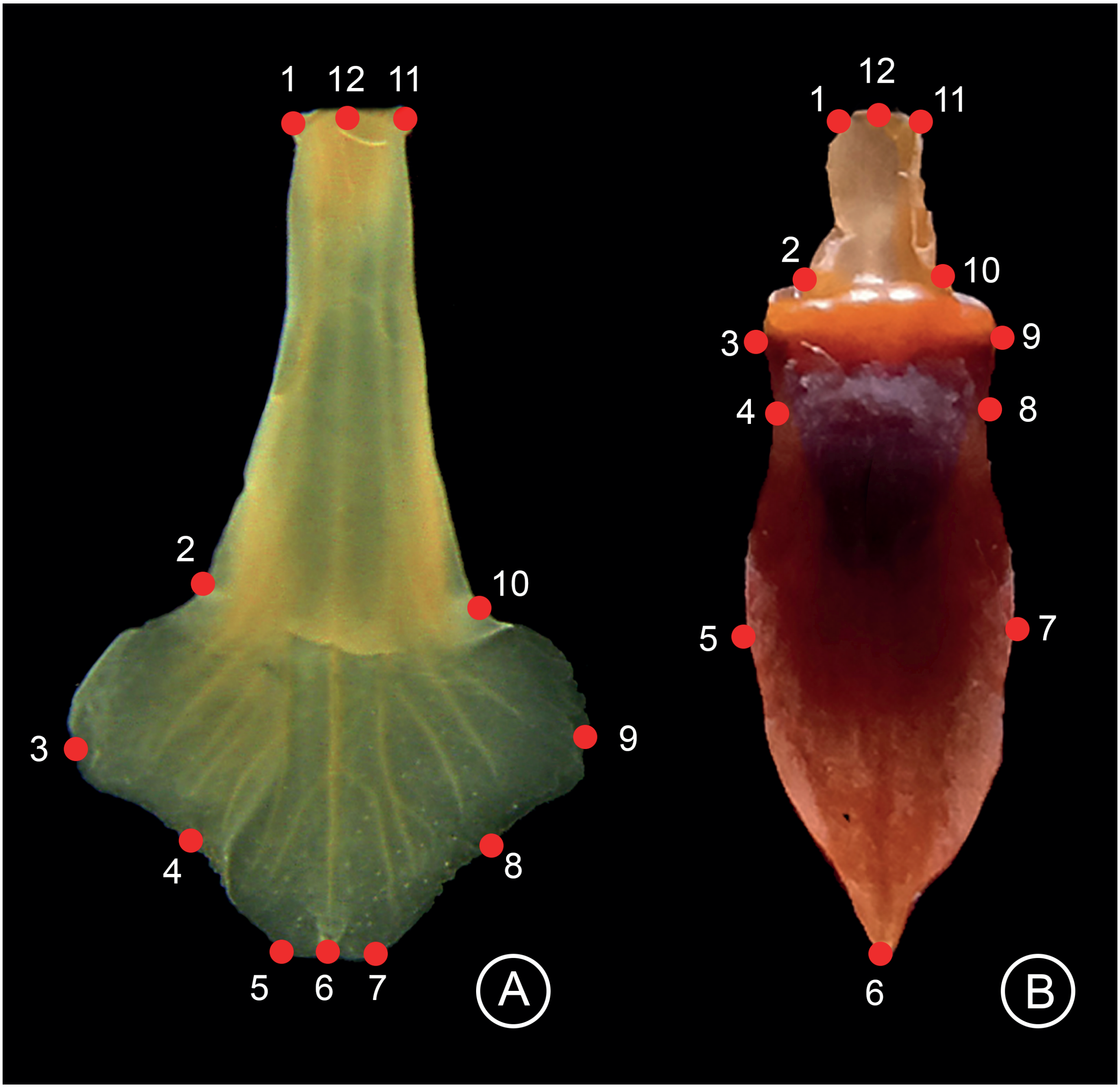

Figure 3. Location of landmarks applied to the labellum of *Scaphyglottis* for geometric morphometric analysis. **A.** *S. prolifera*, **B.** *S. bidentata*. Photos by Araújo, A. M. Plate prepared by Miranda, G.

Results

The PCA exploratory analysis shows that the variation explained by the first two axes represents 52% and 25% of the variation respectively, and the accumulated variation of the three first axes is 86% (Tab. S2). For the first axis (PC1; variance = 52%), the larger landmark variation is found between landmarks 3 and 9, which represent the lateral lobe apices, 5 and 7 representing the median lobes, and 6 representing the labellum apex. For PC2 (variance = 25%), most of the variation is also found between landmarks 3 and 9, and between landmarks 4 and 8, which represent the junction between lateral and median lobes. For PC3 (variance = 8.6%), the larger variance is found in landmark 6, representing the apex of the labellum. Overall, the ellipses that represent the species are considerably overlapped (Fig. S1), except for *S. bidentata* and *S. livida*.

The CVA discriminant analysis showed an accumulated variation of 77.8% in the first three CVs (Tab. S2). On CV1 (variance = 50.5%) most of the variation is represented by landmarks 3 and 9, which represent the lateral lobe apices, 5 and 7 representing the median lobe apices, and 6, representing the apex position or the central point of the labellum invagination (Fig. 4A). In CV2 (variance =

27.2%) and CV3 (variance = 11.8%) the larger variation is seen in landmarks 5, 6 and 7, which represent the median lobe apices and the apex or central point of the labellum invagination (Fig. 4B-C).

The variation of the landmark position of CV1 allowed to separate *S. punctulata*, *S. livida*, *S. graminifolia, S. fusiformis* and *S. bidentata* in the negative pole, and *S. prolifera* and *S. stellata* in the positive pole, while *S. boliviensis*, *S. sickii, S. brasiliensis, S. modesta,* and *S. reflexa* were little impacted (Fig. 5A). The variation found in CV2 separated *S. bidentata*, *S. modesta* and *S. reflexa* in the positive pole and *S. fusiformis, S. punctulata, S. livida, S. graminifolia,* and *S. sickii* in the negative pole, while *S. brasiliensis, S. boliviensis, S. stellata* and *S. prolifera* were little impacted (Fig. 5A). CV3 allowed to separate *S. livida, S. fusiformis, S. reflexa* and *S. brasiliensis* in the positive pole and *S. sickii* in the negative pole, while the other species were little impacted. We highlight that *S. boliviensis* was not separated from the other species in any of the CVs that had higher variance, always being in the group of species that were not impacted. This is also clear from the general observations in Fig. 5A–B, where a strong overlap of this species with the remaining taxa is observed.

In the analysis conducted with the smaller species dataset (*S. boliviensis, S. brasiliensis*, *S. prolifera* and *S. stellata*), the larger variation in PC1 (variance = 54.425%) is found in landmarks 3 and 9, which represent the lateral lobe apices. For PC2 (variance = 30.589%), the largest variation is in landmarks 2 and 10, which represent the junction of the claw with the lateral lobes. The four species remained strongly overlapped in this PCA (Fig. S3-S4). In the CVA, the accumulated variation of the first two CVs is 99.99% (CV1 with 45.35%, CV2 with 40.99%, and CV3 with 13.65%, Tab. S2). In CV1, the largest variation is found between landmarks 3 and 9, which represent the lateral lobe apices, and 2 and 10, which represent the junction of the claw with the median lobes. The same landmarks showed the largest variation in CV2, with the addition of 4 and 8, which represent the lateral and median lobes junction (Fig. 6). CV1 separated *S. prolifera* in the positive pole and *S. boliviensis* in the negative pole, while CV2 separated *S. stellata* in the positive pole and *S. brasiliensis* in the negative. In this new analysis, we observed that *S. brasiliensis* overlapped with the remaining species, but *S. boliviensis* was strongly separated from *S. stellata*, which is the more morphologically similar species (Fig. 7).

Discussion

In this study we tested if geometric morphometrics of the labellum is useful to distinguish species of *Scaphyglottis* that occur in Brazil, as it has been used to separate taxa in Orchidaceae in many recent studies (*e.g.*: Pinheiro & Barros 2007; Pinheiro & Barros 2009; Menini-Neto *et al*. 2019; Lau *et al*. 2021; Pessoa *et al*. 2021; Camelo-Júnior *et al*. 2022). Previous taxonomic studies of *Scaphyglottis* used a combination of vegetative characters (shape and size of pseudobulbs and leaves), besides flower color, size, and overall shape of the labellum to distinguish species (Dressler 1993; Carnevali & Ramírez-Morillo 2003; Szlachetko & Kolanowska 2014a; Araújo *et al*. 2022). We found evidence that the lateral lobe apices, the junction of the claw with the lateral lobes, the median lobe apices, and the apex or central point of the labellum have enough morphological variation to delimit these species, allowing their identification solely based on this structure.

The species with higher morphological variation in Brazil, *S. bidentata* and *S. livida,* were easily separated when compared to the other analyzed species in the first PCA (Fig. S1-S2), which could not separate most of the species. This pattern was also found in previous studies with the labellum in Orchidaceae, in which the PCA showed little power to

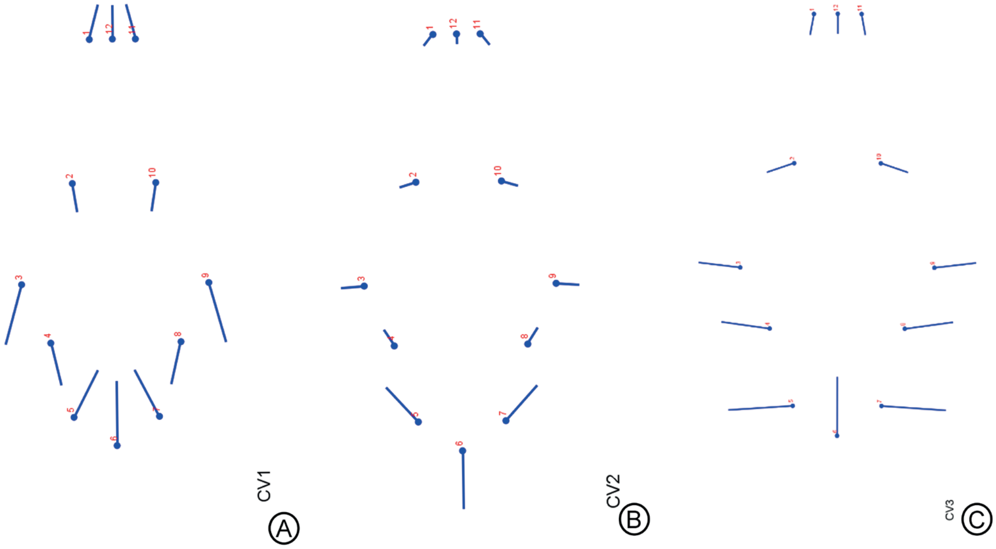

Figure 4. Variation of landmarks in **(A)** CV1, **(B)** CV2, and **(C)** CV3.

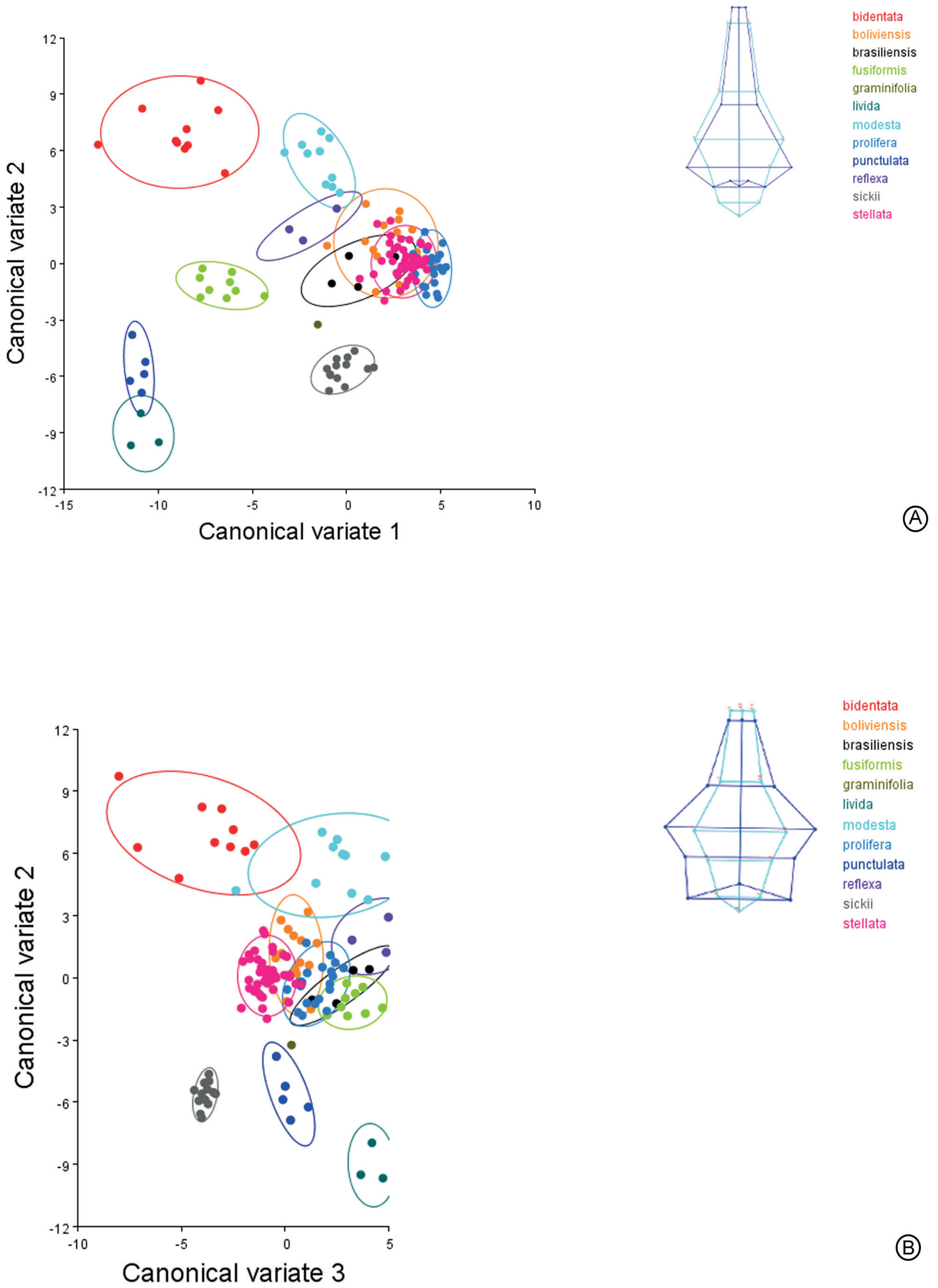

Figure 5. Canonical variable analysis (CVA) showing the CV1 + CV2 axes (**A**) and CV3 + CV2 axes (**B**) for 103 specimens of *Scaphyglottis*.

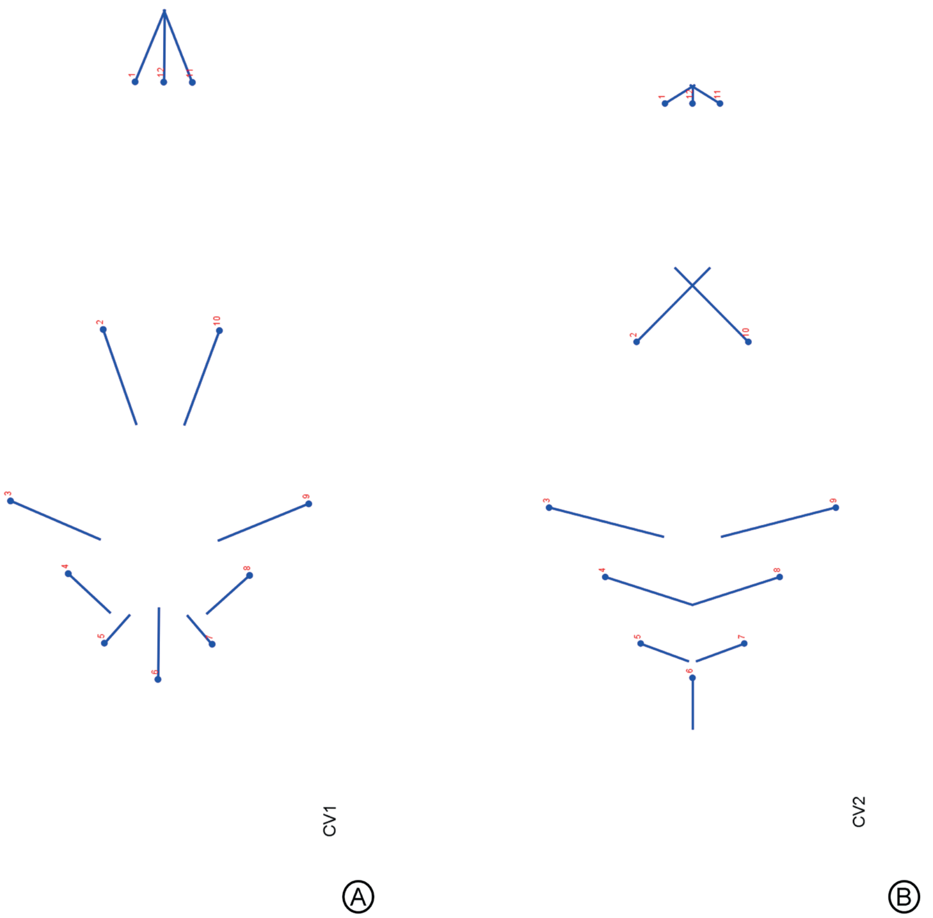

Figure 6. Variation of landmarks for the species *S. boliviensis*, *S. brasiliensis*, *S. prolifera* and *S. stellata*. **(A)** CV1, **(B)** CV2.

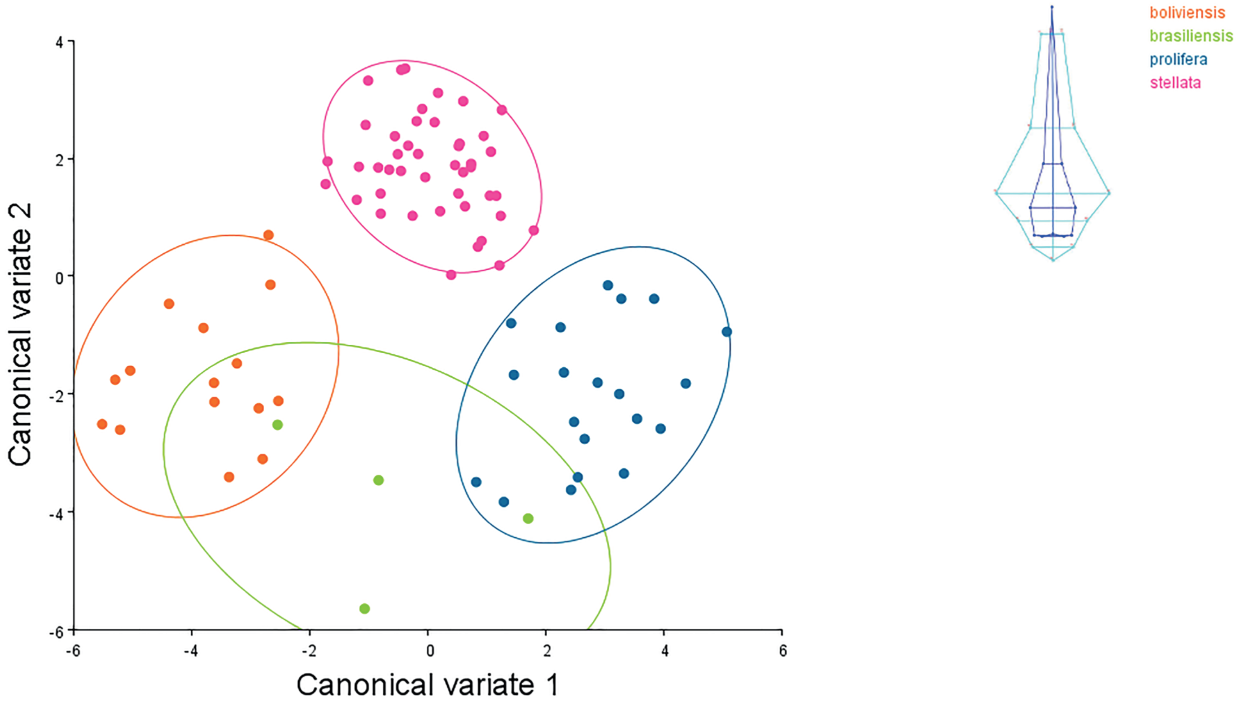

Figure 7. Canonical variable analysis (CVA) showing the CV1 + CV2 axes for the species *S. boliviensis*, *S. brasiliensis*, *S. prolifera* and *S. stellata*.

distinguish among morphologically similar species, being mostly used as a preliminary approach (Goldman *et al*. 2004; Pinheiro & Barros 2009; Menini-Neto *et al*. 2019; Lau *et al*. 2021; Pessoa *et al*. 2021; Camelo-Júnior *et al*. 2022).

The CVA allows better separation of the species, besides indicating the regions of the labellum that show higher variation, such as the median lobe apex (landmarks 5, 6 and 7), and the wider point of the labellum (landmarks 3 and 9) (Fig 3). Similar results indicating these regions as more variable were also found by Pinheiro and Barros (2007; 2009) in Maxillariinae, Pessoa *et al*. (2021) in Laeliinae and Camelo-Júnior *et al*. (2022) in Oncidiinae. However, among the species of *Scaphyglottis* with a more distinctly 3-lobed labellum, *S. boliviensis, S. brasiliensis*, *S. prolifera* and *S. stellata*, these regions showed low variation when compared to the other species, remaining overlapped in the analyses (Fig. 4).

The second CVA analysis, containing only these four species, allowed their separation with higher variation in the lateral lobe apex and claw junction (landmarks 3 and 9, 2 and 10, respectively) (Fig. 6), separating *S. boliviensis* and *S. stellata*, which are frequently misidentified in herbarium collections (CRIA 2022). Previous studies used similar strategies to try and separate species that remain overlapped in preliminary analyses (Camelo-Júnior *et al*. 2022). In the case of these four species, landmarks 2 and 10, referring to the junction of the claw with the median lobes, had an essential role in separating them. These landmarks contain the length and width of the claw (Fig. 7).

Among these four taxa, *S. stellata* and *S. boliviensis* can be vegetatively identified only by leaf width (> 0.4 cm *vs*. < 0.3 cm, respectively), while regarding floral characters, *S. boliviensis* has a more prominent callus in the labellum than *S. stellata* (Dressler 1993; Koch *et al*. 2018). Our results indicate that the claw is smaller in *S. stellata* than in *S. boliviensis*. Both species have synonyms that were described based on specimens with cream-colored/whitish or lilac flowers (Reichenbach 1869; Schweinfurth 1955; Mansfeld 1928). We believe that further investigation at population level is needed to decide either to maintain those as synonyms or reinstate them as separate species; nonetheless our results indicate that the claw size is an important feature that might help in this task.

Scaphyglottis brasiliensis, one of the taxa included in this second analysis, is morphologically related to *S. reflexa* and *S. emarginata* in Brazil, with these two latter species presenting cylindrical leaves (Lindley 1839; Schlechter 1922; Pessoa & Alves 2012) and being relatively rare in herbarium collections. Our analysis, however, did not show similarities between the labellum of *S. brasiliensis* and *S. reflexa* (Fig. 4A). Among the group of species with 3-lobed labellum, *S. brasiliensis* has the longest and narrowest claw (Hoehne 1949). However, this species overlaps with other three taxa, presenting the wider ellipse in the analysis plot, with datapoints further separated from each other, indicating either insufficient sampling or large morphological variation. This group of species with cylindrical leaves, already treated as a separate genus, *Reichenbachanthus* Barb. Rodr. (Brade 1935; Garay 1967), deserves a dedicated taxonomic study, although our results show these two taxa are distinguishable.

Lastly, we could easily distinguish *S. prolifera* from *S. sickii* based on the first CVA (Fig. 4). These two species are considered more morphologically related to each other than to other species that occur in Brazil, due to the smaller leaves and pseudobulbs, and flower color and size. *S. prolifera* has the least prominent 3-lobed labellum among the species included in the second analysis, and according to Dressler (1993), it is not a typically 3-lobed labellum as seen in other species, but a labellum where the median portion extends to the sides. However, this character (position of the landmarks) probably caused the overlapping with *S. boliviensis, S. brasiliensis* and *S. stellata* in the first analysis, due to being different than the remaining species with unlobed labellum.

Studies that highlight the morphological variation in Orchidaceae species with wide geographical ranges and varied habit (epiphytic, rupicolous or facultative terrestrial) were carried out in Epidendroideae and Maxillariinae (Pinheiro & Barros 2007; 2009) and Laeliinae (Menini-Neto *et al*. 2019). Taking into account that some species of *Scaphyglottis* have similar attributes, further studies to evaluate the geographic organization of morphological variation and its relation to environmental gradients should be considered, especially in widely distributed species like *S. stellata*, that is known from Nicaragua to central Brazil. The labellum has an essential role in pollinator attraction in Orchidaceae, mainly due to color, size, odor, and floral resources (nectar, pollen, oils) (van der Pijl & Dodson 1966). In *Scaphyglottis*, nectar usually accumulates in the labellum base (Dressler 2001), therefore, selective pressures associated with pollination might influence the variation found in this structure, especially in the size and width of the claw. It might explain differences in the labellum morphology found in similar species studied here. However, reproductive biology studies need to be carried out in *Scaphyglottis* species to elucidate evolutionary pressures related to labellum shape variation and avoid speculative conclusions.

Our study showed new morphologically variable features in *Scaphyglottis* that can be used in future taxonomic studies, or even for identification of specimens based on labellum fragments (Fig. 8). We highlight that six of the more morphologically close species did not overlap in their labellum morphology (i.e., *S. sickii* and *S. prolifera*; *S. reflexa* and *S. brasiliensis; S. punctulata* and *S. fusiformis*), possibly indicating different pollination strategies. For widely distributed species, such as *S. prolifera*, *S. stellata* and *S. reflexa*, we suggest a population-level approach, as local environmental factors can impact morphology, causing expected intraspecific variation (Jonas & Geber 1999; Blinova 2012; Menini-Neto *et al*. 2019).

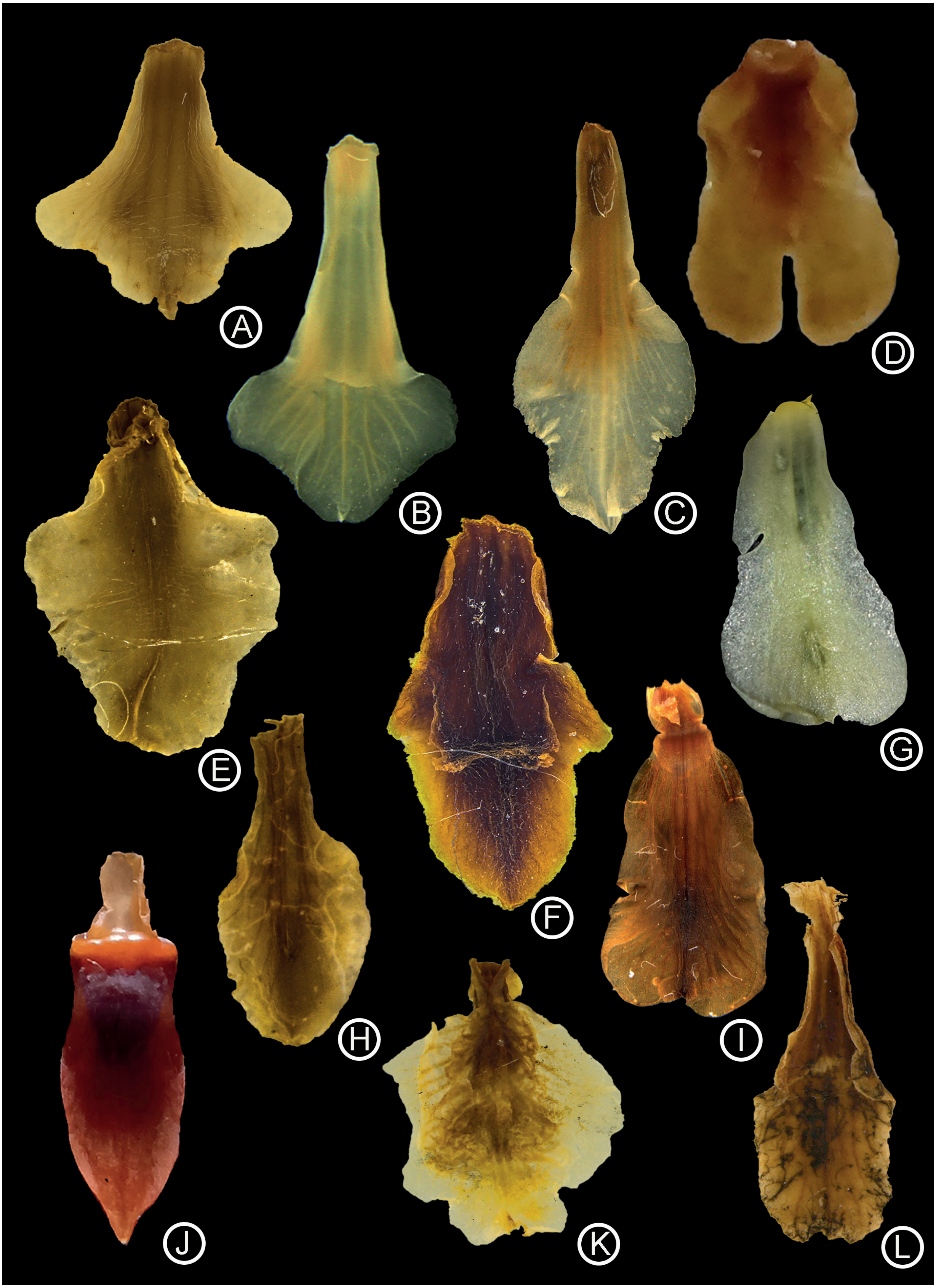

Figure 8. Labellum shape variation of *Scaphyglottis*. **A**. *S. boliviensis*, **B**. *S. prolifera*, **C**. *S. stellata*, **D**. *S. livida*, **E**. *S. brasiliensis*, **F**. *S. modesta*, **G**. *S. fusiformis*, **H**. *S. sickii*, **I**. *S. punctulata*, **J**. *S. bidentata*, **K**. *S. reflexa*, **L**. *S. graminifolia*. Photos by Araújo, A. M. (**A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, **J**, **K**, and **L**) and Farroñay, F. (**I**). Prepared by Miranda, G.

Supplementary Material

Table S1. Specimens analyzed for geometric morphometrics, and their respective locations.

Table S2. *Analysis 1*: A. First three eigenvalues, variance and accumulated variance of the principal component analysis (PCA). B. First three eigenvalues, variance and accumulated variance of the canonical variable analysis (CVA). *Analysis 2*: C. First three eigenvalues, variance and accumulated variance of the principal component analysis (PCA). D. First three eigenvalues, variance and accumulated variance of the canonical variable analysis (CVA).

Figure S1. Principal component analysis (PCA) for 103 specimens of *Scaphyglottis*. (A) PC1+PC2 axes, (B) PC1+PC3 axes.

Figure S2. Landmark variation in (A) PC1, (B) PC2, and (C) PC3.

Figure S3. Principal component analysis (PCA) for the species *S. boliviensis*, *S. brasiliensis*, *S. prolifera* and *S. stellata*, PC1+PC2 axes.

Figure S4. Landmark variation in (**A**) PC1 and (**B**) PC2 for the species *S. boliviensis*, *S. brasiliensis*, *S. prolifera* and *S. stellata*.

Acknowledgments

We thank the Graduate Program in Botany (INPA) and FAPEAM; FUNBIO and Instituto Humanize for the Preserving the Future grant (Call 07/2020), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Financing Code 001 for the master's scholarship granted to AMA. We thank herbarium curators and technicians for access to collections and loan preparation, to the curator Mike Hopkins and the technician Mariana Mesquita from the INPA herbarium for access to vouchers and the Museu da Amazônia (MUSA) for the structure to keep living specimens of *Scaphyglottis*. We thank Lourdes Falen for preparing the map; Edmilson Camelo for analysis support; Gustavo Miranda for editing photos and assembling figures; Carolina Siniscalchi for the help with English version; Francisco Farroñay, Rafael Barbosa-Silva, Mario Terra-Araújo, Joel Cordeiro and Gabriel Marcusso for the photos; Alessandro Coelho, Luiz Teixeira and Marcelo Miranda for donating specimens. EMP also acknowledges the productivity grant from CNPq (303556/2022-6). LLG received financial support from UFOPA/ICTA/Pipex 01/2021 and CNPq (422191/2021-3).

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