Botanical Journal of the Linnean Society, 2020, **194**, 469–479. With 5 figures.

Accommodating trait overlap and individual variability in species diagnosis of *Ischnosiphon* (Marantaceae)

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Received 30 September 2019; revised 24 March 2020; accepted for publication 29 May 2020

Recognition and delimitation of taxonomic categories of biological organisms are still challenging and full of controversy. We used *Ischnosiphon* as a model to unravel the importance of morphometrics as individual-based variables to disentangle the morphological variability of plant species. *Ischnosiphon* spp. continue to be problematic for users, taxonomists and ecologists, due mainly to the huge morphological variability, the species criteria and circumscription proposed for many taxa and the many habitat and vegetative macro-morphological characters lacking in most currently available exsiccates. Twenty-three morphometric variables were sampled from 228 individuals, belonging to 22 *Ischnosiphon* spp. Principal components and discriminant multivariate analyses were used to describe and identify patterns of morphological variation in *Ischnosiphon*. Individual-landmark assessment analysed with multivariate methods captured morphometric intraspecific diversity and morphological variability in *Ischnosiphon* spp., along with the continuous variation of important morphological traits. By examining the morphology of *Ischnosiphon* spp. through individual-landmark assessment, we demonstrate that different morphological species concepts used today in the identification of the species are difficult to apply. We propose a replicable and analytical framework to accommodate individual variability in species diagnosis in morphologically diverse plant groups.

ADDITIONAL KEYWORDS: morphometrics – neotropics – shape – size – South America – Zingiberales.

INTRODUCTION

Recognition and delimitation of taxonomic categories of biological organisms are still challenging and full of controversy. Despite the many advances in the last decades in biological systematics, many practices in the field have not changed for centuries (Mishler, 2009). In particular, the main criteria used to diagnose life forms continues to be based mainly on macro-morphological abstract characters, which are often categorical and derived from cognitive analysis of a few samples or single type individuals (Scott & Hallam, 2002; Ahrends *et al.*, 2011; Gomes *et al.*, 2013). In particular, taxonomic units, such as species, are interpretations of phenomena taking place among variable individuals and could, therefore, be thought as metadata, whereas the data are gathered from specimens of an organism (Dupré, 1981; Ghiselin, 1987; Brogaard, 2004; Assis, 2011). Correspondingly, integrative approaches considering robust evidence taken from organisms and their environment may yield deeper insights on biological traits, in turn benefitting our understanding of diversity and improving our ability to propose predictive and natural classifications (Stuessy, 2009). In particular, many quantitative and qualitative biological variables can be easily measured in specimens already in biological collections (Lane, 1996; Stern & Eriksson, 1996), which in turn can be used to analyse phenotypic differentiation (Henderson, 2006; Chalcoff, Ezcurra & Aizen, 2008).

The quantification of individual morphological variability enables the use of objective and replicable methods that impact the accuracy of species diagnoses. Multidisciplinary approaches exploring species boundaries have gained followers in recent years, mainly with the rise of genomic data

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(e.g. de Abreu *et al*., 2018; Gutiérrez-Larruscain *et al.,* 2018; Niu *et al*., 2018; Prata *et al*., 2018). Additionally, many other approaches can be used to record and analyse differences between organisms (e.g. Fan *et al*., 2010; Durgante *et al*., 2013). Morphometrics allows the quantification of morphological differences between organisms (Duncan & Baum, 1981; Bookstein, 1998) and underlying patterns of variation at the level of individuals and populations (Henderson, 2006; Jensen, 2006). Morphometric variables measured in individuals can be used to promote solutions to taxonomic challenges such as registering and diagnosing infraspecific variation and species complexes and delimiting species (Borba *et al*., 2002,Pinheiro & Barros, 2007; Chalcoff *et al*., 2008;Pinheiro & Barros, 2009; Laphitz & Semple, 2015). Furthermore, the use of landmarks, i.e. fixed reference points that define the locations of particular traits, enables capturing size and shape variability, which can reveal evolutionary trends of speciation such as morphological adaptation (e.g. Rossoni *et al*., 2017) and niche specialization (e.g. Funk, Nosil & Etges, 2006; Liu *et al.*, 2013; Cornils & Held, 2014).

Ischnosiphon Körn. (Marantaceae) provides an ideal study group for examining morphological characters, because it has few diagnostic characters despite a great morphological variation. This genus comprises *c*. 36 species (Andersson, 1977, 1984). These are terrestrial rhizomatous herbs of forest understories of tropical America, distributed from Nicaragua to southern Bolivia and Brazil. Due to the great morphological diversity in Marantaceae, generic delimitation has been troublesome. Phylogenetic analyses of Marantaceae have found *Ischnosiphon* to be monophyletic (Andersson & Chase, 2001; Prince & Kress, 2006; Suksathan, Gustafsson & Borchsenius, 2009; Borchsenius, Suarez & Prince, 2012), but it may include *Pleiostachya* K.Schum., and further phylogenetic work should improve taxon and gene sampling to test species monophyly. Andersson, (1977) took into account numerous morphological and cytological characters, including chromosome counts and anatomical traits to delimit *Ischnosiphon* spp. Nevertheless, his extensive work is, in many aspects, hard to use due to the low accessibility of many characters, particularly flowers in herbaria material, and the time-consuming and relatively expensive techniques of anatomy and cytogenetics. Andersson proposed a generic subdivision of six sections, many of which are based on a single character, frequently microscopic, as in section *Papilloderma* L.Andersson, defined by the presence of papillae in the abaxial epidermis, or in section *Longiflori* L.Andersson, circumscribed by the thickness of the adaxial hypodermis walls.

Besides Andersson's revision, there are few morphological studies on *Ischnosiphon* spp. (see Suárez & Galeano, 1996; Costa, Espinelli & Figueiredo, 2008), and none of them thoroughly discusses intraspecific differences of quantitative morphological traits and how morphological variation is reflected in the different taxonomic entities proposed to date. Identification of *Ischnosiphon* spp. continues to be problematic for taxonomists and ecologists alike, due to the huge morphological variability under accepted species names, the inexplicit species criteria and circumscription proposed for many taxa and the absence of many habitat and vegetative macro-morphological characters available for most herbarium specimens.

The identification of *Ischnosiphon* spp. is often subjective and troublesome, mainly due to overlap in diagnostic traits between species and broad individual variability within species. To test if replicable and objective identification methods can be applied to dried leaves of herbarium specimens, we investigated whether morphometric data and multivariate discriminant analysis can be used for species diagnosis. We examine the diverse morphological variation present in leaf and inflorescence traits of *Ischnosiphon* using 23 quantitative characters.

MATERIAL AND METHODS

Morphometric analysis

Two hundred and twenty-eight herbarium specimens were studied (Supporting Information, Table S1). Anderson's (1977, 1984) circumscriptions of species and sections were adopted to assign species *a priori*. The following species are scarcely represented in herbaria material and are not considered here: *I. bahiensis* L.Andersson, *I. enigmaticus* L.Andersson, *I. fusiformis* L.Andersson, *I. helenae* L.Andersson, *I. grandibracteatus* Loes., *I. inflatus* L.Andersson, *I. rotundifolius* Körn. and *I. ursinus* L.Andersson. All *Ischnosiphon* specimens at INPA, BHCB and RB herbaria were photographed, and available digital images from NY, MO and COAH virtual herbarium collections were downloaded (herbaria acronyms follow Thiers, 2019, continuously updated).

Many traits previously considered by specialists in species descriptions in *Ischnosiphon* are not available in dried herbarium specimens. To incorporate replicability and objectivity to *Ischnosiphon* diagnosis, besides including many characters from Andersson's (1977) species descriptions, we also included several original leaf and inflorescence traits and tested their contribution to assign species membership. Sixty continuous morphological characters were measured

(Supporting Information, Table S2), 24 of which are vegetative and 36 reproductive (Fig. 1). Given that many of the morphological characters are missing in herbarium specimens, especially those concerning floral characters, morphometric variables with > 50% of unavailable data were removed from further analyses. After this quality control step, 23 characters were analysed, 18 vegetative and five reproductive (Table 1). In addition, the median of the character values for each species was calculated to replace missing information for downstream multivariate analyses (Supporting Information, Table S2). In general, the most remarkable morphometric characters of *Ischnosiphon*, which are also available in almost all dried herbarium specimens, are those related with size and shape of leaves. Indeed, of the total morphometric characters successfully measured, 11 are related with features of the lamina.

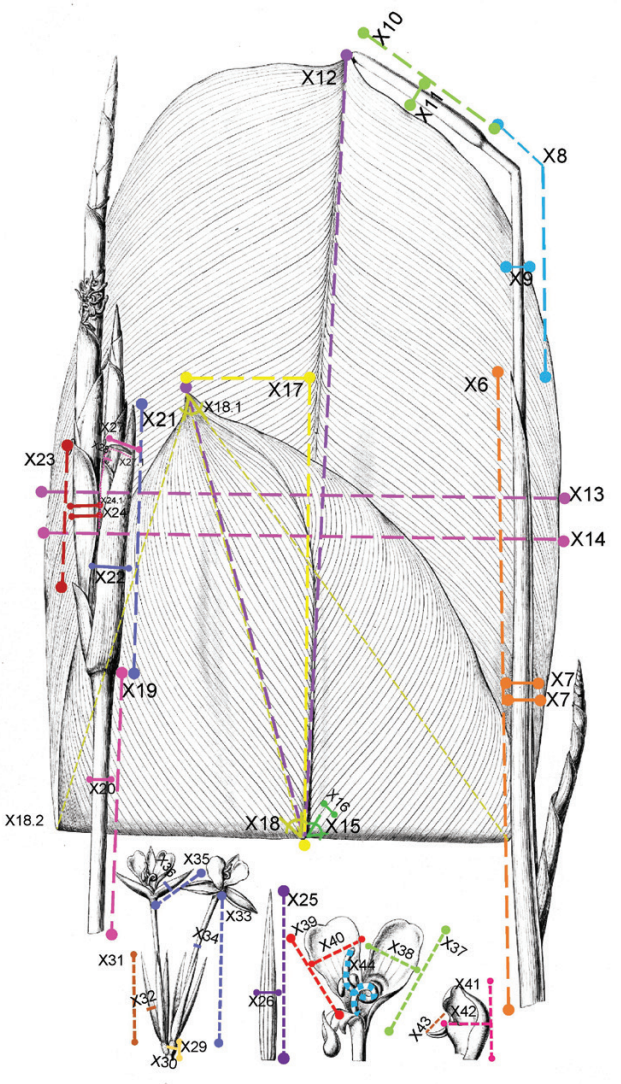

Figure 1. Characters assessed to morphometric analysis of *Ischnosiphon* species with respective landmark positions.

Traits were measured in digitized herbarium specimens using ImageJ (downloaded from https://imagej.nih.gov/ij/, ImageJ 1.52h, Java 1.8.0_112). We performed a calibration for each image based on the length scale present within images. Landmarks were assigned to each of the considered morphological characters. The oldest structures of each specimen were preferentially measured, however, when the specimen only had one leaf or when landmarks were inaccessible in the specimens, better preserved structures were prioritized for measurements. Figure 1 presents details of morphological characters. To visualize the main morphometric differences between species and its variation, density plots were created from the original morphometric data set (Supporting Information, Fig. S1).

Ordination of morphometric variability

Intraspecific differences of morphometric traits were analysed using the multivariate method of principal component analysis (PCA) based on measurements of 228 specimens and 23 morphometric variables. Before PCA analyses, all characters were scaled to have unit variance. Since PCA analysis mainly describes the global variation of data, discriminant analysis of principal components (DAPC) was performed using the *adegenet* v.3.5.3 package for the R software (Jombart, Devillard & Balloux, 2010) to calculate membership probabilities. These probabilities can be interpreted as morphometric proximities of individuals to distinct clusters. All analyses were performed in the R environment (v.3.3.2; R Core Team, 2019).

RESULTS

Shape and size variability

Descriptive statistical analysis shows that the values of morphometric characters greatly overlap between species (Supporting Information, Table S3). All *Ischnosiphon* spp. have laminas with some degree of eccentricity, i.e. the deviation of a form from circularity. *Ischnosiphon cannoideus* L.Andersson, *I. hirsutus* Petersen, *I. killipii* J.F.Macbr., *I. lasiocoleus* K.Schum. ex Ule, *I. longiflorus* K.Schum., *I. martianus* Eichler ex Petersen and *I. petiolatus* (Rudge) L.Andersson present the most eccentric (values varying between 0.94 and 0.98) and the most symmetric laminas (values of apex displacement angles varying from 1.50° to 3.40°). In contrast, less eccentric (0.74–0.88) and asymmetrical laminas (9.32°, 17.85° and 14.27°, respectively) were found in *I. arouma* Körn., *I. crassispicus* L.Andersson

Table 1. Morphological characters used in morphometric analyses of *Ischnosiphon*. PC1, PC2 and PC3: correlation between the original variables and the first, second and third principal components, respectively

Morphometrical characters	Code	PC1	PC2	PC3
Sheath length (cm)	X6	-0.233849	-0.160634	-0.120359
Sheath width (at base) (cm)	X7	-0.053151	-0.063761	0.027638
Sheath width (at widest) (cm)	X7.1	-0.261139	0.028383	-0.063057
Petiole length (cm)	X8	-0.148005	-0.209063	-0.458522
Petiole width (cm)	X9	-0.233046	-0.186455	-0.080306
Pulvinulus length (cm)	X10	-0.249479	-0.204269	-0.013025
Pulvinulus width (cm)	X11	-0.237058	-0.023931	-0.014251
Lamina length (cm)	X12	-0.232655	-0.251385	0.221796
Lamina width (at middle) (cm)	X13	-0.276539	-0.102919	0.046461
Lamina width (at widest) (cm)	X14	-0.274630	-0.079253	0.050929
Lamina secondary venation angle (°)	X15	-0.105066	0.141035	-0.523977
Lamina secondary venation distance (at widest) (cm)	X16	-0.039911	0.007595	-0.177952
Lamina apex displacement (from the center) (cm)	X17	-0.222252	0.342081	-0.050931
Lamina apex displacement angle (°)	X18	-0.150102	0.467773	-0.118873
Lamina apex angle (°)	X18.1	-0.139758	0.389163	-0.119034
Peduncle width (cm)	X20	-0.264655	0.017447	-0.011544
Pedicel width (cm)	X20.1	-0.238006	0.066293	0.128302
Spathe length (cm)	X23	-0.107439	0.400733	0.259973
Spathe width (at base) (cm)	X24	-0.118811	0.176393	0.283679
Spathe width (at widest) (cm)	X24.1	-0.212243	0.179923	0.128582
Eccentricity	X51	0.177299	-0.013305	0.329693
Leaf area (cm^2)	X52	-0.265235	-0.127100	0.203429
Leaf radius circle (cm)	X53	-0.265235	-0.127100	0.203429

and *I. obliquus* (Rudge) Körn. Other species, including *I. puberulus* Loes., *I. polyphyllus* Körn., *I. gracilis* Körn. and *I. surumuensis* Loes., present intermediate values of eccentricity and symmetry of laminas, displaying a continuous morphological variation between individuals (Supporting Information, Table S3).

Although most of the species analysed have asymmetric leaves, we observed that the species with the most symmetrical leaves are *I. cerotus* Loes., *I. petiolatus* and *I. paryrizinho* L.Andersson, in contrast to the strikingly asymmetrical leaves of *I. puberulus*, *I. obliquus* and *I. crassispicus*. Between these two extremes, the remaining species show a continuous gradient of leaf symmetry (Fig. 2). *Ischnosiphon annulatus* Loes. and *I. foliosus* Gleason were not plotted because there were fewer than three samples of each of these species. In addition to shape and size, individual-landmark assessment captured the morphometric intraspecific trait variability in *Ischnosiphon*, revealing the presence of morphological complexes such as the one formed by *I. gracilis* and *I. puberulus*, explored in more detail next.

Multivariate analysis in individual-based morphometrics

Most *Ischnosiphon* spp. show wide morphological gradients in the PCA, including a vast amount of overlap on both principal components, with the exception of *I. obliquus*, with individuals that stand apart from the remainder (Fig. 3). Individuals of *I. crassispicus* and *I. macarenae* L.Andersson appear to be at both extremes of the ordination plot, but the sample sizes for these species are low (equal or less than three specimens sampled). The first two axes of this morphometric PCA accounted for 60.12% of the total observed variation (Fig. 3). Proportions explained by second and third axes were also plotted and are available in the Supporting Information (Fig. S2). The first axis by itself explains half of the total variation, despite not having any correlation > 30% with any particular morphometric variable, a set of variables jointly defining this first axis. The second axis explains 9.9% of the total variation and is mainly defined by three lamina variables related with shape variability: lamina apex displacement (X17, $r = 0.34$), lamina apex displacement angle (X18, $r = 0.48$) and lamina apex angle (X18.1, $r = 0.39$); and one reproductive character related with size variation: spathe length (X23, $r = 0.40$) (Table 1).

When Andersson's sections were considered in the two-dimensional morphospace, we detected an inconspicuous pattern: three groups of overlapping specimens. The first group is formed by section *Bambusastrum* L.Andersson and section *Longiflori*,

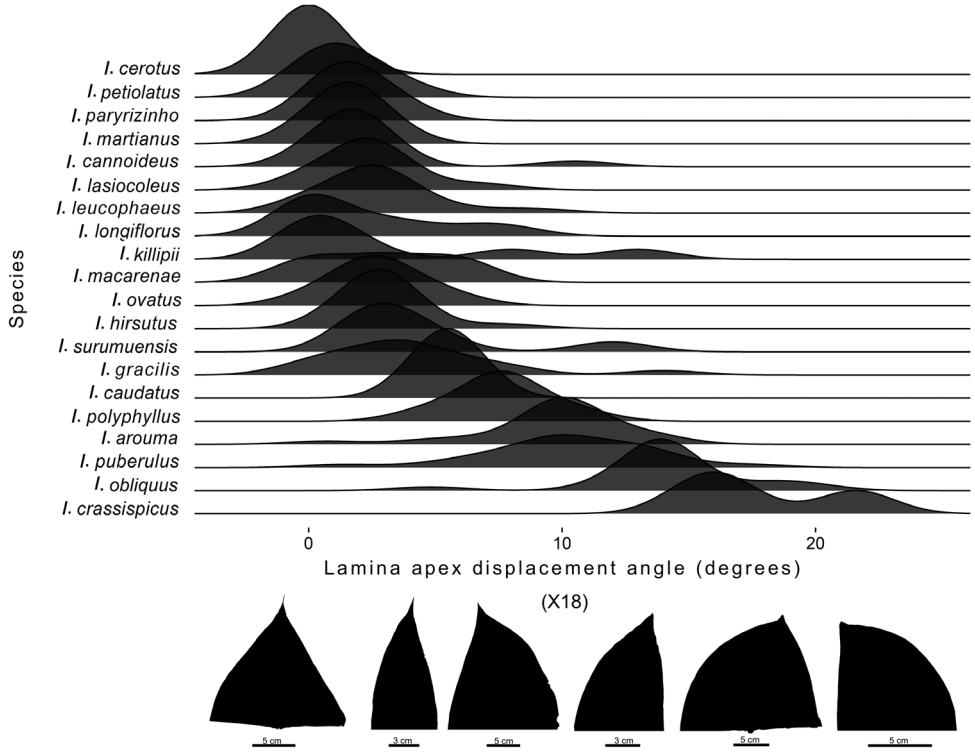

Figure 2. Density plot of lamina symmetry along 21 *Ischnosiphon* species. From left to right: silhouettes representing leaf apices from *I. cerotus* (*Schunke-Vigo 3216*), *I. cannoideus* (*Prance 14254*), *I. ovatus* (*Kuhlmann s.n.*), *I. puberulus* (*Andersson 1776*), *I. obliquus* (*Plowman 2584*) and *I. crassispicus* (*Plowman 2584*).

the second by section *Hirsuti* L.Andersson and section *Papilloderma* and the last by most individuals previously assigned to section *Ischnosiphon* L.Andersson.

THE VALUE OF INDIVIDUAL-BASED MORPHOMETRICS AT PREDICTING SPECIES

Posterior membership probabilities were calculated with the DAPC, retaining the first 20 PCs, explaining > 95% of the total variance of the morphological data set. Most individuals are confirmed into their original assigned species with this set of morphometric variables. The overall proportion of individual correct assignments is 62% when species are provided as the a priori groups and 81% when sections are analysed (Supporting Information, Table S4). In addition, 9% of individuals are morphologically more likely to be assigned to another species, whereas 7% of individuals have higher probabilities to be assigned to a different section than the one originally assigned (Supporting Information, Table S4). To visualize how well-defined the morphological clusters are, and to analyse the morphological affinity of each specimen, bar plots with probabilities of assignment to prior set groups were constructed. We detected the same number of prior clusters, corresponding to the 22 species and five sections initially proposed (Fig. 4A, B). However, these clusters present morphometric admixture, especially of individuals of *I. cannoideus*, *I. gracilis*, *I. longiflorus*, *I. puberulus* and *I. surumuensis*.

The I. gracilis *and* I. puberulus *case*

We initially detected a relative narrow morphometric variation among samples assigned to *I. gracilis* and *I. puberulus* (Fig. 3). Further exploration exclusively with individuals of this complex showed, that despite the differentiation between some individuals of *I. gracilis* and *I. puberulus*, there are intermediate phenotypes between them. The first two axes of the PCA based only on these two species explained 61.92% (52.42 and 9.5%) of all morphometric variation (Fig. 5A). The first axis again showed no correlation > 30% with any particular morphometric variable, whereas the second axis was defined by four vegetative and two reproductive variables [sheath width (X7), petiole length (X8), petiole width (X9) and lamina secondary venation angle (X15) for the former, and spathe width (X24 and X24.1), for the latter].

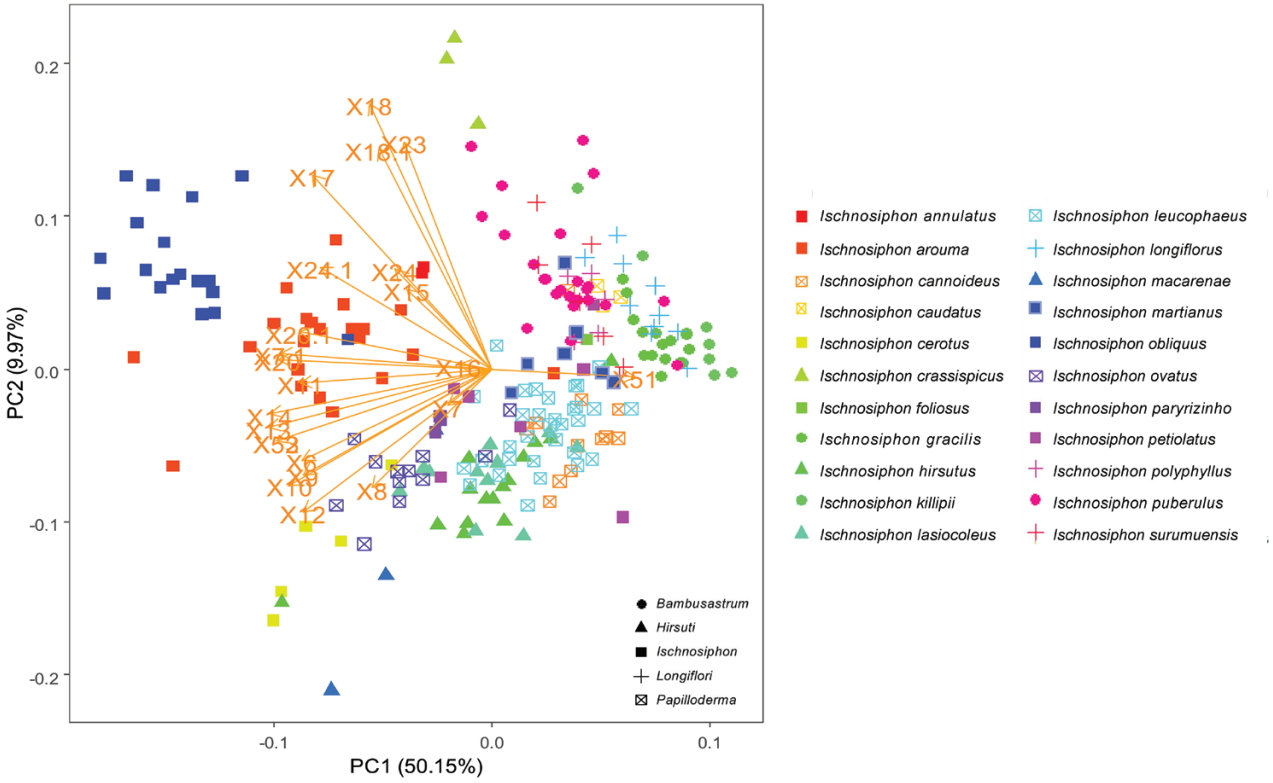

Figure 3. Principal component analysis (PCA) plot of the first two principal component axes for all individuals of *Ischnosiphon* based on morphometric variation. Shaped points refer to sections, coloured shapes refer to species and vectors to size and shape variables (arrows in orange: abbreviations are provided in Table 1).

Visualization of the distribution of all morphometric variables revealed that, despite the great overlap in morpho-space, lamina length and width (from the middle and for the widest point) and the radius of the circle with leaf area and total leaf area, are variables that can discriminate individuals of both species (Fig. 5B).

DISCUSSION

Intraspecific morphological variability

The size and shape of plant organs can change with age (e.g. England & Attiwill, 2006) and may respond to environmental gradients (e.g. Alpert & Simms, 2002; Dwyer, Hobbs & Mayfield, 2014) and population density (e.g. Sekimura *et al.*, 2000). Trait variability within species is commonly the source of morphological variation present in specimens deposited in botanical collections, specimens that need to be identified for subsequent use in taxonomy, ecology and other disciplines. Such variability in traits within species is generally ignored in traditional diagnosis and in many identification tools, which are commonly binary and categorical. Here, we successfully accommodated natural quantitative variability in species diagnosis through multivariate analyses. We have shown here that individual-based morphometrics can be used effectively to interpret interspecific complex variation. Moreover, the assessment of morphological variability through landmarks allows the capture not only of variation in size, but also the shape of jointly important discriminant features.

Our results confirmed the morphological variability of *Ischnosiphon* spp. and explicitly presented its continuous nature both within and between species. The fact that the first axis of the ordination is not strongly correlated with any particular variable (Fig. 3), where all characters contributed almost equally in the definition of this component, emphasizes that most of the morphometric variation is not enough to differentiate *Ischnosiphon* spp. Instead, we need to explore a robust set of explicit and replicable variables to discriminate taxonomic entities.

Several sympatric species with similar morphologies, such as *I. gracilis*, *I. killipii*, *I. longiflorus*, *I. polyphyllus*, *I. puberulus* and *I. surumuensis*, cannot be clearly differentiated using single categorical morphological

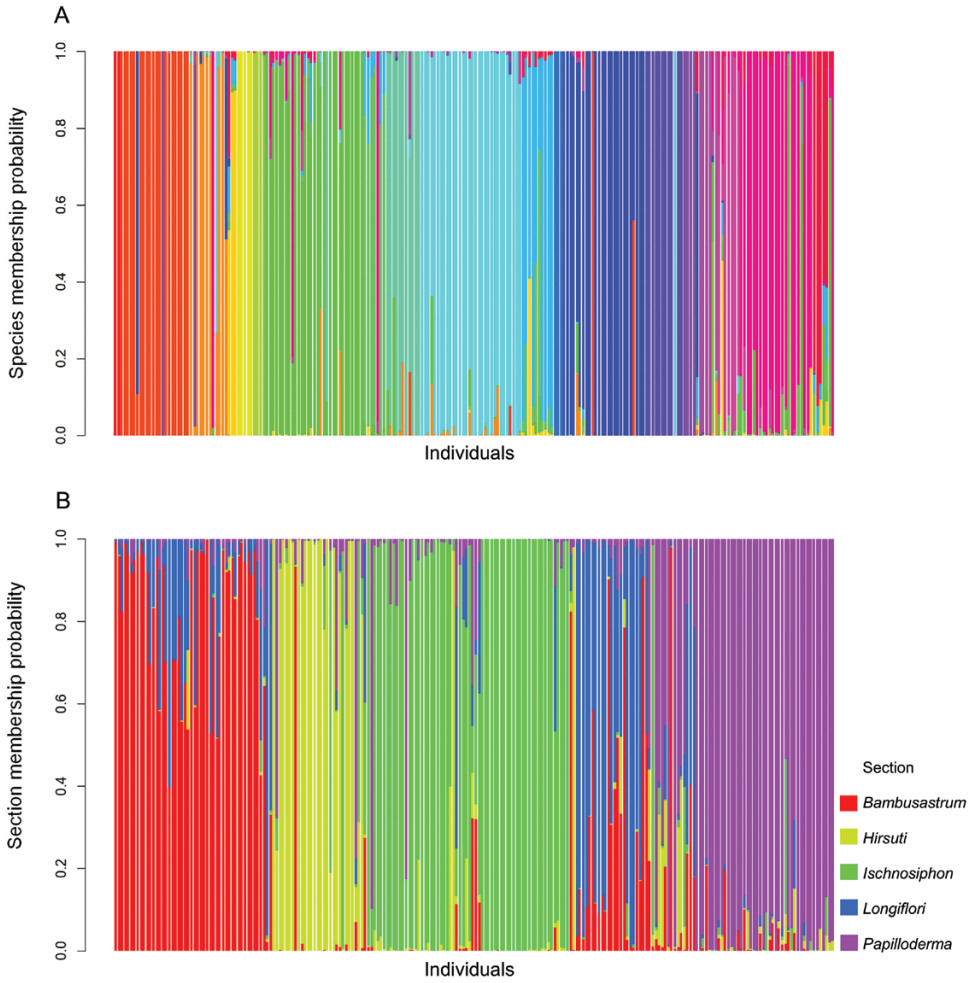

Figure 4. Membership probability obtained from DAPC analyses for each of the 228 individuals of *Ischnosiphon*. A, Species posterior membership probabilities. B, Section posterior membership probabilities. Each vertical coloured line represents an individual. Mixture clusters refers to incongruity between membership posterior assignments. See Figure 3 for species colours.

concepts. On the other hand, our approach presents high rates of positive identification based on morphometric discriminant functions in species such as *I. arouma*, *I. cannoideus*, *I. hirsutus*, *I. leucophaeus* (Poepp. & Endl.) Körn., *I. obliquus* and *I. ovatus* Körn (Fig. 4). Additionally, qualitative characters, including leaf texture and presence of hairs and waxes, allow a clearer morphological delimitation of species with great morphological overlap as *I. hirsutus*, *I. lasiocoleus*, *I. cannoideus and I. leucophaeus*. Such a combination of evidence highlights the importance of a global and integrative analysis of morphology in complex taxonomic groups. The discriminant analysis used here is shown to be an accurate method to infer membership probabilities (Jombart *et al.*, 2010; Pometti *et al.*, 2014) and it is especially recommended when large datasets are being used (Excoffier & Heckel, 2006).

THE MORPHOLOGICAL CONTINUUM OF THE *I. GRACILIS–I. PUBERULUS* COMPLEX

The case of the *I. gracilis–I. puberulus* complex illustrates how multivariate morphometrics can be used to verify in an objective and explicit way that apparently continuous variation of morphological characters exists. These two species are historically hard to differentiate using herbarium specimens due to the great morphological overlap between them. In fact, in his treatment, Andersson, (1977) considered the organization of the aerial shoot system as the main, if not the only, discontinuous character to separate morphotypes and, eventually, to propose section *Bambusastrum*, which also includes *I. enigmaticus* and *I. killipii*. However, the organization of shoot systems seems to be equally variable and is not always represented or noted in herbarium specimens, resulting in an impractical

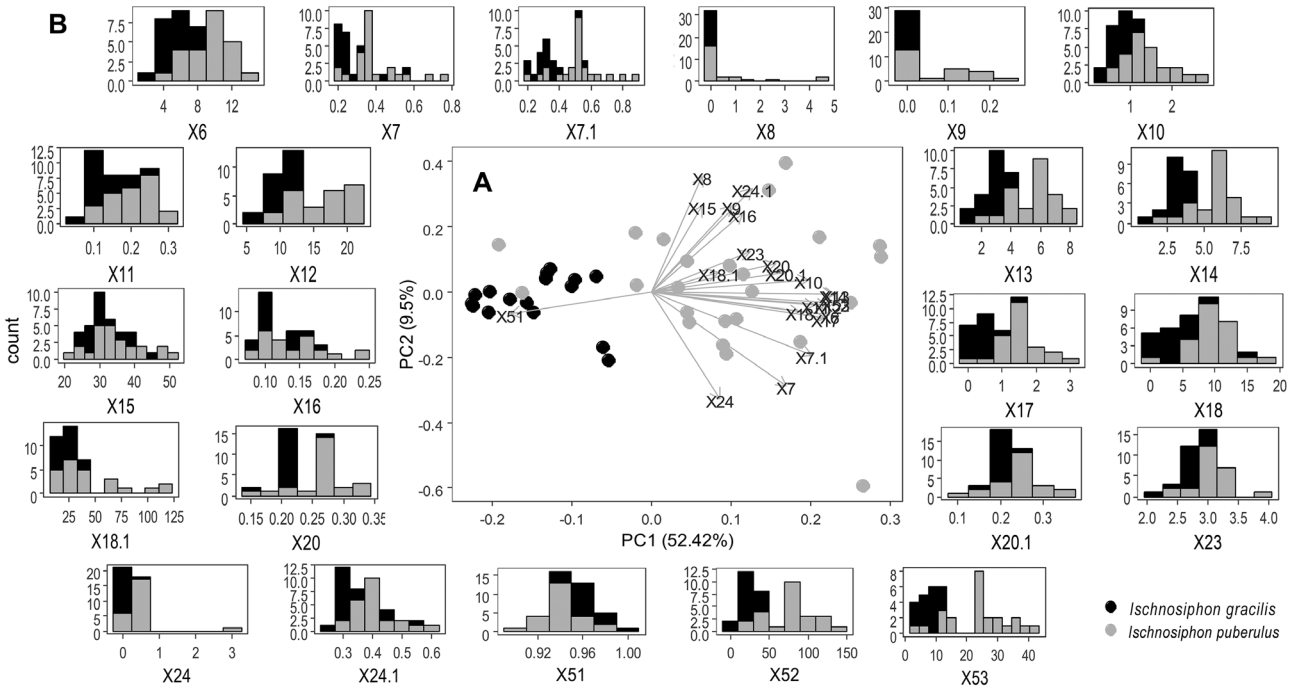

Figure 5. Comparative morphology in the *I. gracilis–I. puberulus* complex. A, Principal component analysis (PCA) plot of the first two principal component axes. Shaded points refer to species, and vectors to size and shape variables. B, Histograms for each morphometric variable. Shaded bins refer to species. See Table 1 for character codes.

criterion when working with preserved material. The morphological variation and almost complete overlap in morphology among species within this complex suggests that *I. gracilis* and *I. puberulus* may be two extremes in the continuous morphological variation of individuals that have been historically recognized as two different species. In fact, these differences are basically given by the size of the leaves (Andersson, 1984), as evidenced by Fig. 4B. Since both species are found in sympatry, it is imperative to conduct studies assessing molecular variation and life history of the species, along with more evidence to determine the limits of this morphological variation.

We do not propose that morphometrics should be used alone to recognize species, but rather should be used as a tool to delve into continuous morphological characters. A multidisciplinary and integrative approach to biodiversity description and diagnosis is achievable through the explicit consideration of data measured at the individual level (specimens in the case of herbarium material), particularly from open-access integrated repositories (e.g. try-db.org, 2020; gbif.org, 2020; virtual images of herbarium specimens). Likewise, integration of individual-based biological data can improve and stabilize species circumscription. We join others (e.g. Gomes *et al.*, 2013) in recommending that species delimitation and identification be treated as separate processes.

We also showed that different morphological species concepts used today in the identification of *Ischnosiphon* spp. are tremendously difficult to apply, as highlighted by the continuous variation on lamina apex symmetry (Fig. 2), and that macro-morphological characters such as the organization of aerial shoot systems and categorical shapes show no apparent discontinuities in dried specimens in *Ischnosiphon*. In this case, the use of other sources of factual evidence is fundamental to diagnose groups. In addition, our results emphasize the importance of pragmatic recognition and subsequent identification of species, through the generation of objective evidence, and explicit and repeatable methods based on data from individuals.

Data quality implications

The wider use of this approach in taxonomy and plant diagnosis is currently limited by the lack of sufficient character-rich specimens in botanical collections. Processing of plant material into herbarium sheets often overlooks the preservation and optimization of important morphological evidence, which clearly interferes with the quality of taxonomic work. In the case of *Ischnosiphon*, this is the greatest limitation to the use of diagnostic morphological characters in herbarium specimens, since many vegetative and

floral structures are often lacking or are not suitably preserved or recorded, making standardized and comparative analysis more difficult.

CONCLUSIONS

By examining morphometric traits of *Ischnosiphon* spp. through individual-landmark assessment, we demonstrated that, despite the elevated trait overlap between species, individual variability can be accommodated in species diagnosis by concomitantly accessing multiple quantitative traits to calculate posterior membership probabilities. We further explored individual trait variability to conclude that a species complex within the genus is formed by a morphological size gradient. The replicable analytical framework shown here successfully accommodates individual variability in species diagnosis of morphologically diverse plant groups.

ACKNOWLEDGEMENTS

We thank the curators of INPA, RB and BHCB who provided fully access to botanical collections and the global plant image repositories of JSTOR, FMNM-Botany and REFLORA for making digitized herbarium material available. We thank John MacDougal for English review. We are also thankful to Flora Brasiliensis (Centro de Referência em Informação Ambiental, CRIA) for providing high resolution image of the digitized plates.

FUNDING

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (grant to M.A.B.) and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grant number 441481/2017-5 to T.A.). The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Density plots of all morphometric variables.

Figure S2. Principal component analysis (PCA) plot of the second and third principal component axes for all individuals of *Ischnosiphon* based on morphometric variation.
Table S1. Specimens selected for morphometric analyses with collection codes and voucher information.
Table S2. Morphometric matrix with proportion of unavailable data and final selected specimens.
Table S3. Summary of descriptive statistics of morphological characters of species in analyses.
Table S4. Individual membership probabilities obtained from DAPC analyses.