Plant Systematics and Evolution (2020) 306:57
https://doi.org/10.1007/s00606-020-01682-8

ORIGINAL ARTICLE

Delimiting a constellation: integrative taxonomy of a star-shaped *Hydrocotyle* species complex (Araliaceae) from the Brazilian Atlantic forest

Eduardo K. Nery[1] · Maria E. Matchin-Viera[2] · Olga Camacho[1] · Mayara K. Caddah[2] · Pedro Fiaschi[2]

Received: 16 November 2019 / Accepted: 8 April 2020 / Published online: 7 May 2020

Abstract
Species delimitation must overcome the inconsistency among the different delimitation criteria to maintain species as reliable units for biological research and conservation. The general lineage species (GLS) concept embraces different methods of species delimitation and thus provides an integrative framework for taxonomy. The *Hydrocotyle stella* complex contains six morphotypes that occur along mountain ranges within the Brazilian Atlantic forest, possibly representing undescribed species. In this study, we adopted the GLS concept to assess whether *H. stella* would be a single species or not, in which we considered genetic structure and morphological discontinuity as evidences for species delimitation. We applied ISSR-based population genetics and morphometrics to 12 populations of *H. stella*, representing all morphotypes across their main geographical range. Population genetics revealed three genetically structured groups ($\Phi_{ST} > 0.22$), each associated with different mountain range regions. Morphometrics indicated significant ($p < 0.001$) morphological divergence among genetically structured groups, but not for all of the traditional leaf morphotypes. Based on congruency between genetic structure and morphological discontinuity, we recognized three species within the complex, *H. alpina*, *H. palacea*, and *H. quinqueradiata,* and provided their due taxonomic treatment.

Keywords *Hydrocotyle* · ISSR markers · Morphometrics · Population genetics · Species delimitation · Taxonomy

Introduction

Species are at the core of evolutionary theory (Hull 1977), and beyond that they are a main reference for resource exploitation, environmental management, and technological development. Therefore, the recognition and circumscription of species (i.e., species delimitation) is a major goal within systematics (Wiens 2007). However, species delimitation can be a challenging task since it can follow different criteria that comply with a wide range of species concepts (Mayden 1997). Each species concept bears either theoretical or operational limitations (Luckow 1995; Balakrishnan 2005), so their respective delimitation criteria can lead to different taxonomic proposals for a same group of organisms (Peterson and Navarro-Sigüenza 1999; Rheindt and Eaton 2009). Such inconsistency among delimitation criteria has casted doubts on species as the basis for conservation efforts (Hey et al. 2003; Agapow et al. 2004; Isaac et al. 2004; Zachos 2018). Moreover, due to their potential impact on the number of endangered species, species delimitation has been subjected to harsh critics that demand judicialization of species recognition (Garnett and Christidis 2017). Hence, overcoming the inconsistency among delimitation criteria is necessary to maintain species as reliable units for biological research and conservation.

The general lineage species (GLS) concept (de Queiroz 2007) can conciliate the different delimitation criteria. In

Handling Editor: Thais N. C. Vasconcelos.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00606-020-01682-8) contains supplementary material, which is available to authorized users.

✉ Eduardo K. Nery
eduardo.k.nery@gmail.com

[1] Programa de Pós-Graduação em Biologia de Fungos, Algas e Plantas, Centro de Ciências Biológicas, Campus Universitário, Trindade, Florianópolis, Santa Catarina 88040-900, Brazil

[2] Departamento de Botânica, Centro de Ciências Biológicas, Campus Universitário, Trindade, Florianópolis, Santa Catarina 88040-900, Brazil

short, de Queiroz (2007) proposes that all contemporary species concepts agree in considering species as separately evolving metapopulation lineages, but they disagree in their delimitation criteria by assessing different properties that lineages may or may not have evolved along speciation. Hence, each delimitation criterion could provide an evidence of lineage separation, and the amount of evidence, rather than a particular sort of evidence, would define whether a species should be recognized or not (de Queiroz 2007). Under this comprehensive perspective, integrative taxonomy by congruence is a sounding approach for species delimitation (Padial et al. 2010). This approach consists of an initial application of different operational criteria (e.g., molecular, morphological, or ecological methods) and a posterior search for concordant patterns across their results (Padial et al. 2010). By doing so, species delimitation becomes more accurate (i.e., reduced systematic error) (Carstens et al. 2013), as single-method approaches are inherently biased (Miralles and Vences 2013). Foremost, like other integrative approaches, integration by congruence can cover different moments of the speciation *continuum* (de Queiroz 2007).

Population genetics based on inter-simple sequence repeats (ISSR) can recognize species by their genetic structure. ISSR are derived from DNA amplification of genomic regions between near, inversely oriented, and same-motif microsatellites (SSR) (Zietkiewicz et al. 1994), and each ISSR amplicon is assumed as an allele of an independent neutral locus whose homology among samples is inferred by molecular weight (Bussell et al. 2005). Considering that gene flow reduces or ceases among separating lineages, species would be groups of organisms displaying few or none heterozygotes among each other [genetic-cluster species (Mallet 1995)]. This lack of heterozygotes among species makes allele (or genotype) frequencies of the set of species to deviate from expectations for a single panmictic group (i.e., genetic structure) (Wright 1949). Such deviation also occurs at ISSR frequencies, becoming detectable by population genetics approaches (Hausdorf and Hennig 2010).

In turn, morphometrics can validate species boundaries by the detection of morphological discontinuity. Morphometric variables result from either linear and angular measurements (traditional morphometrics), or landmarks and outlines of structures (geometric morphometrics) (Rohlf and Marcus 1993; Adams et al. 2004, 2013), and both kinds of morphometric variables quantitatively describe morphological variation and covariation. Under the assumption that morphological variation increases among separating lineages, species would be groups of organisms displaying non-overlapping morphological variation (i.e., discontinuity) [morphological species (Du Rietz 1930)]. This discontinuity produces low-frequency intervals on the distribution of morphometric variables, which can be addressed by different statistical procedures (Zapata and Jiménez 2012; Cadena et al. 2018).

Species delimitation within *Hydrocotyle* L. is currently problematic and possibly underestimates species diversity. *Hydrocotyle* comprises both annual and perennial herbs that occur in seasonally dry and arid environments (Perkins 2017) or mesic and aquatic environments (Mendoza and Fuentes 2010), respectively. Some perennial species display fast growth rates and are potential invasive weeds (Ruiz-Avila and Klemm 1996; Liu et al. 2016), while others are known constituents of traditional folk medicine (Rocha et al. 2011; Huang et al. 2013). The Neotropics and Australia harbor the highest diversity of *Hydrocotyle* species (Nicolas and Plunkett 2014), but persisting taxonomic issues are an impediment for more accurate estimates of diversity in the former region. Traditionally, *Hydrocotyle* species are recognized based on leaf morphology (Constance and Dillon 1990; Mendoza and Fuentes 2010; Henwood 2014), but the wide variation in leaf characters can prevent inference of species boundaries by traditional taxonomic means. As a consequence, taxonomic treatments to date have proposed different polymorphic species with questionable status (i.e., species complexes). Most of these complexes remain unverified (Eichler 1987a, b, c), as the last comprehensive revision of the genus dates back to the nineteenth century (Richard 1820).

However, the application of different methods has changed *Hydrocotyle* taxonomy. Large-scale sampling and sequencing of few *Hydrocotyle* species have indicated a possible DNA barcode region (plastidial *trn*H-*psb*A) (Van De Wiel et al. 2009), which may aid the recognition of other congeneric species. Most phylogenetic studies have been restricted to local groups of *Hydrocotyle* species (Choi and Park 2012; Karuppusamy et al. 2014), but they have pave the way for future integrative taxonomy. Nonetheless, molecular phylogenetic inference combined with morphological analyses has set the boundaries among annual *Hydrocotyle* species in Australia (Perkins 2019). Such study also has allowed a reinterpretation of morphological characters that have been traditionally applied to species delimitation in *Hydrocotyle* from Australia (Perkins 2019). Based on that, integrative taxonomy can be a promising approach to solve the persistent taxonomic issues within *Hydrocotyle* from the Neotropics.

Hydrocotyle stella Pohl ex DC. sensu Nery and Fiaschi (2019) is a species complex with a wide distribution within the Brazilian Atlantic forest domain. *Hydrocotyle stella* was first proposed to designate villous herbs with five-lobed leaves in Brazil (De Candolle 1830). Later, Urban (1879) synonymized *H. stella* under *Hydrocotyle quinqueloba* var. *stella* Urb., one of the ten infraspecific taxa proposed under *Hydrocotyle quinqueloba* Ruiz & Pav.

Based on morphometric analyses, Nery and Fiaschi (2019) restricted *H. quinqueloba* circumscription to plants found in the Peruvian Andes and elevated *H. quinqueloba* var. *stella* along with other five infraspecific taxa to the specie status. After this taxonomic treatment, *Hydrocotyle stella* came to comprise six star-shape leaf morphologies: the 'palacea' morphotype (PALAC) (Fig. 1a), with five-lobed leaves, lanceolate lobes, and a deeper basal sinus; the 'quadrata' morphotype (QADRA) (Fig. 1b), with four-lobed leaves and widely ovate-triangular lobes; the 'quadriloba' morphotype (QADRI) (Fig. 1c), with four-lobed leaves and ovate-lanceolate lobes; the 'quinqueradiata' morphotype (QINQE) (Fig. 1d), with five-lobed leaves and lanceolate lobes; the 'stella' morphotype (STELL) (Fig. 1e), with five-lobed leaves and widely triangular lobes; and the 'subglabra' morphotype (SUBGL) (Fig. 1f), with five-lobed leaves and ovate-lanceolate lobes. This species complex occurs mainly at the Espinhaço, Serra da Mantiqueira, Serra do Mar, and Serra Geral mountain ranges in Eastern Brazil. The geographic distribution along mountain ranges suggests a possible lack of genetic connectivity within the complex, which could contribute to lineage separation (Nery and Fiaschi 2019). On the other hand, intermediate morphologies suggest some gene flow among morphotypes (Nery and Fiaschi 2019), leaving morphological variation to be mainly due to local environmental conditions.

This study aimed to assess whether the *H. stella* complex comprises a single species or not. To infer species boundaries, we adopted the GLS concept (de Queiroz 2007) and evaluated genetic structure and morphological discontinuity within the species complex, considering congruence between these two properties as evidence for species delimitation.

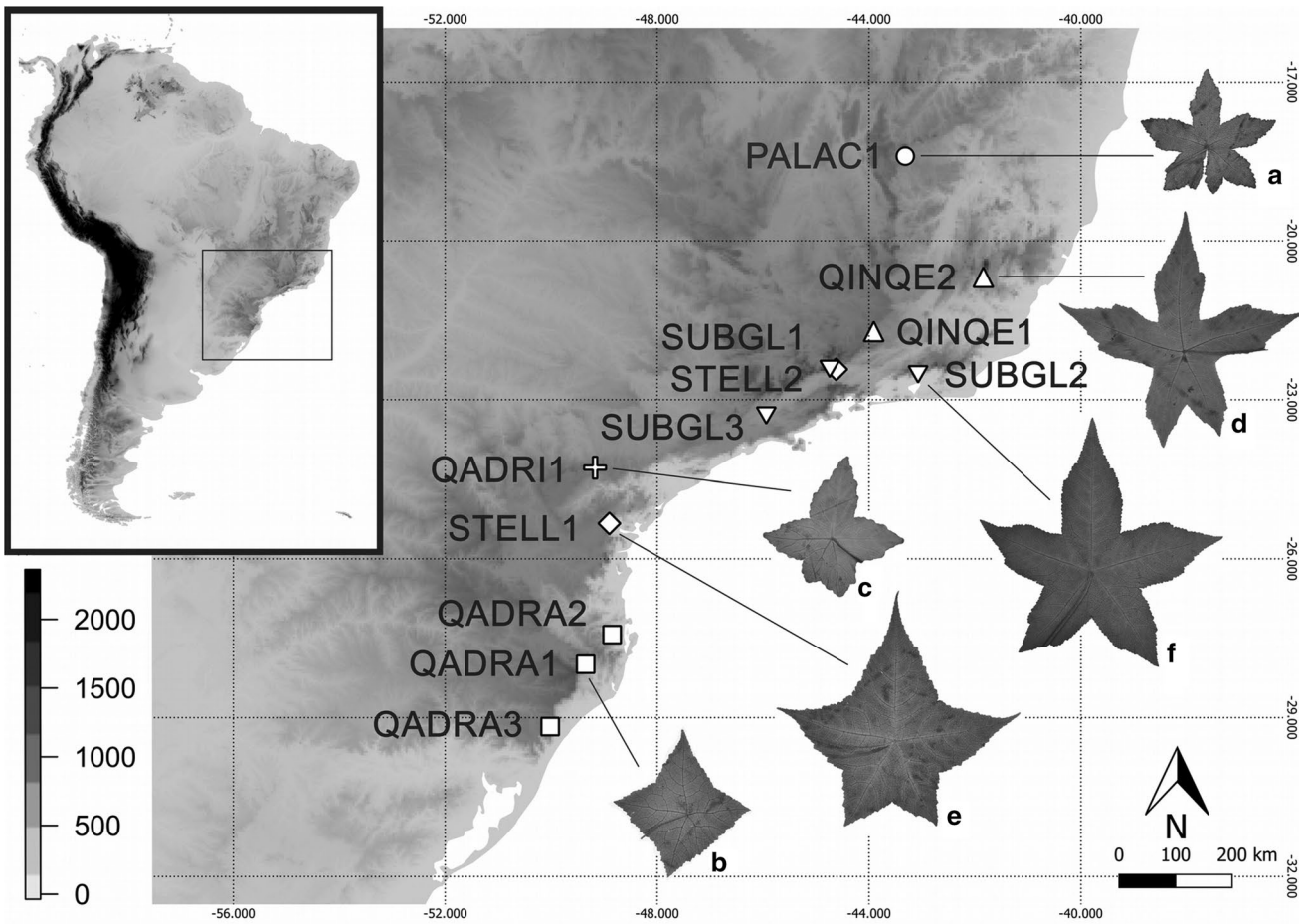

Fig. 1 Sampled populations of *Hydrocotyle stella* within the Brazilian Atlantic forest in South America. Populations of a same morphotype are represented by a same symbol. Morphotypes are shown at the right. Gray scale at the left indicates altitude in meters

Materials and methods

Sampling and DNA extraction

We sampled 12 populations (186 specimens) of the *H. stella* complex within the Brazilian Atlantic forest domain (Fig. 1), including the six morphotypes and the four mountain ranges where the species complex is currently found (Table 1). We sampled at least two populations for each morphotype, except for PALAC and QADRI. Herbarium records for these two morphotypes are rare and often indicate localities that have been disturbed (habitat loss). Each population comprised 11 to 20 specimens, stored under a same voucher number (Table 1). We defined as different specimens those samples that were at least ten meters apart in order to avoid clones. Leaf tissue samples from all specimens were stored in silica gel for DNA extraction, which followed a CTAB protocol (Doyle and Doyle 1987). We dried and stored specimens at FLOR herbarium [acronym according to Thiers (2019)].

ISSR amplification and genotyping

We amplified ISSR loci from all *H. stella* specimens ($n = 186$) based on four primers: (AC)7RG, (AG)8TG, (CA)6RY, and (CTC)6T. These primers were chosen as they returned the highest number of polymorphic loci in prior essays carried out with other 14 primers. To ensure band reproducibility, we performed three amplification essays with each primer on a subsample containing one specimen from each population. Moreover, we applied relatively high annealing temperatures (see below) for multi-locus markers to minimize amplification of non-repeatable fragments.

Amplification reactions had a total volume of 10 µl, containing 200 µM of each dNTP, 1.5 mM of $MgCl_2$, 1.25 U of Taq DNA polymerase (TopTaq Master Mix®, Qiagen), 1 × Coral Load (TopTaq Master Mix®, Qiagen), 5 µM of primer, and 20-50 ng of genomic DNA. Thermocycling had an initial denaturation of 95 °C for 3 min, 32 cycles of 95 °C for 15 s, primer-specific annealing temperature for 40 s, 72 °C for 2 min, and a final extension of 72 °C for 7 min. Annealing temperatures were 52 °C for (AC)7RG and 48 °C for the remaining primers. We stained amplicons with 2 × Gel Loading Dye Blue (Sinapse Inc) and 1 × Gel Red (Biotium), and then, we separated amplicons via electrophoresis along a 100 bp molecular ladder in 1.5% agarose gels in 1 × TBE buffer, at 100 V, for 3 h. We photographed electrophoretic gels under UV light (Online Resource 1). Based on photographs, we scored the presence (1) and absence (0) of each band (allele) (Online Resource 2), assuming homology among same-weight bands (Wolfe et al. 2001).

Population genetic analyses

We evaluated genetic structure within the *H. stella* complex via population genetic analyses of ISSR data. We analyzed ISSR data with the packages 'adegenet' (Jombart et al. 2008; Jombart and Ahmed 2011) and 'poppr' (Kamvar et al. 2014, 2015) at the R environment (R Core Team 2020). In order to summarize genetic variation and visually assess genetic structure, we performed a principal component analysis (PCA) (Pearson 1901; Hotelling 1933) of ISSR data. To identify an optimal number of genetically structured groups and to assign specimens to such groups, we performed a k-means clustering analysis of PC scores (Jombart et al. 2010), considering different grouping scenarios ($1 \leq K \leq 12$)

Table 1 Sampled populations of *Hydrocotyle stella* and their respective mountain range, geographic coordinates, associated herbarium voucher, and number of collected specimens

Population	Mountain range	Geographic coordinates	Voucher	*n*
PALAC1	Espinhaço	18°24′08.0″S; 43°18′53.1″W	*E.K. Nery 75*	16 (11)
QADRA1	Serra Geral	28°01′11.2″S; 49°17′56.0″W	*E.K. Nery 57*	20 (0)
QADRA2	Serra Geral	27°25′42.6″S; 48°50′54.9″W	*E.K. Nery 92*	18 (9)
QADRA3	Serra Geral	29°10′63.2″S; 50°01′64,3″W	*E.K. Nery 100*	11 (0)
QADRI1	Serra do Mar	24°17′03.0″S; 49°10′17.2″W	*E.K. Nery 90*	13 (8)
QINQE1	Mantiqueira	21°42′41.3″S; 43°54′33.9″W	*E.K. Nery 60*	12 (4)
QINQE2	Mantiqueira	20°41′12.0″S; 41°50′28.0″ W	*E.K. Nery 81*	19 (3)
STELL1	Serra do Mar	25°19′54.0″S; 48°54′01.0″W	*E.K. Nery 29*	18 (13)
STELL2	Mantiqueira	22°26′07.9″S 44°36′47.2″W	*E.K. Nery 42*	17 (10)
SUBGL1	Mantiqueira	22°22′24.6″S; 44°45′20.7″W	*E.K. Nery 36*	15 (5)
SUBGL2	Serra do Mar	22°31′47.7″S; 43°03′59.9″W	*E.K. Nery 44*	15 (8)
SUBGL3	Serra do Mar	23°14′47.0″S; 45°56′17.4″W	*E.K. Nery 89*	12 (6)
			Total	186 (77)

Within parentheses, the number of fertile specimens

with 1000 iterations each. Comparing successive *K*-values, we chose the optimal grouping scenario as the one imposing the greatest decrease on the Bayesian information criterion (BIC) (i.e., highest increase in model fit) (Jombart et al. 2010).

In order to estimate genetic divergence (fixation index Φ_{ST}) among genetic groups identified by k-means clustering, we performed an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) of ISSR data, considering population as a nested factor within genetic group. We tested the significance ($\alpha = 0.05$) of genetic divergence by a permutation procedure with 10,000 iterations.

In order to evaluate whether genetic divergence among populations could be due to geographic isolation, we performed a Mantel test (Mantel 1967) over genetic and geographic distances among *H. stella* populations. For such, we estimated genetic divergence as Nei's genetic distance (Nei 1972) and assumed geographic distance (km) as a proxy of geographic isolation. We tested the significance ($\alpha = 0.05$) of estimated correlation coefficient (Pearson's *r*) with a permutation procedure with 10,000 iterations.

Morphometric analyses

We evaluated morphological discontinuity within the *H. stella* complex via morphometric analyses of different morphological structures. We applied geometric morphometrics to leaf blade in order to independently quantify leaf shape and size, and we applied traditional morphometrics to the other structures as their variation was predominantly size-related.

For geometric morphometrics, we measured all sampled specimens ($n = 186$) (Table 1), each specimen represented by three leaves. We sampled the leaves before the second most distal node of stems in order to minimize variation due to growth. We photographed the abaxial surface of leaf blades next a ruler for scale with a Nikon 5100® and then converted images to the '.TPS' file extension with the 'tpsUtil' software (Rohlf 2015). Using the software 'tpsDig2' (Rohlf 2015), we obtained raw coordinates of leaf blade by positioning seven landmarks (Fig. 2): (1) petiole insertion on leaf blade; (2) median lobe apex; (3) median lobe sinus; (4) lateral lobe apex; (5) lateral lobe sinus; (6) basal lobe apex; and (7) basal sinus. Landmarks covered only the right side of leaves to avoid the quantification of leaf asymmetrical variation.

We analyzed the raw coordinates and depicted leaf shape changes with the package 'geomorph' (Adams and Otarola-Castillo 2013) at the R environment (R Core Team 2020). First, we performed a Procrustes superimposition (Rohlf and Slice 1990) of the raw coordinates to separate leaf blade shape (aligned coordinates) from leaf blade size (centroid size), and then, we performed a relative warp analysis (RWA) (Bookstein 1989) of the aligned coordinates to summarize leaf shape variation into a few variables (relative warps). We retained the relative warps and the centroid size as variables describing leaf shape and size, respectively. We depicted changes of leaf shape by wireframe diagrams, which contrast the consensus configuration (the great mean) with a target configuration (Klingenberg 2013).

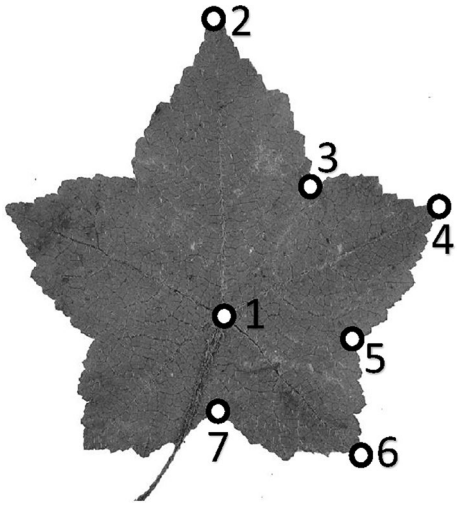

Fig. 2 Landmarks on the abaxial surface of leaf blades of *Hydrocotyle stella*

For traditional morphometrics, we measured only fertile specimens ($n = 77$) (Table 1), each specimen measured three times per character when feasible. We measured only structures before the second most distal node along stems. We measured seven morphological characters: internode length, petiole length, stipule length, stipule width, peduncle length, pedicel length, and the number of flowers per umbel (Online Resource 3).

We analyzed morphometric variables with the package 'Morpho' (Schlager 2017) at the R environment (R Core Team 2020). First, we averaged values to specimen to avoid pseudoreplication and to minimize residual variation, and we standardized variables to avoid overweighting due to measurement scale.

In order to assess whether genetic groups displayed morphological discontinuities or not, we performed a canonical variate analysis (CVA) (Campbell and Atchley 1981) of morphometric variables. Additionally, we compared the consistency of morphological circumscriptions in two alternative scenarios: genetic groups by k-means and traditional leaf morphotypes by Nery and Fiaschi (2019). For such, we first estimated morphological divergence among circumscriptions in each scenario by Mahalanobis distance (D^2) (Mahalanobis 1936), and then, we tested their statistical significance ($\alpha = 0.05$) by a permutation procedure with 10,000 iterations, applying the Bonferroni correction for multiple comparisons.

In order to evaluate whether morphological characters would reflect genetic divergence and would be taxonomically reliable, we performed a multivariate analysis of variance (MANOVA) of morphometric variables, considering population as a nested factor within genetic group. Morphological characters displaying higher percentage of variation (sum of squares, SS) at the genetic group than at other levels were considered taxonomically reliable.

Results

Population genetic analyses

The four SSR-targeting primers amplified 94 loci, of which 84 (92%) were polymorphic (Table 2). PCA of ISSR data described 33% of the genetic variation with its first two axes (Fig. 3). PC1 (17.3% of variation) separated morphotypes into three groups: (1) PALAC; (2) QINQE + SUBGL; and (3) QADRA + QADRI + STELL. PC2 (16.1% of variation) separated morphotypes similarly to PC1. Additionally, PC2 also displayed a south-to-north-oriented geographic pattern, where the southernmost populations (e.g., QADRA1 and QADRA3) occupied the lower PC2 scores and the northernmost populations (e.g., PALAC1 and QINQE2) occupied the higher PC2 scores (Fig. 3). Clustering by k-means optimally assigned specimens into three genetic groups (Fig. 3).

AMOVA of ISSR data indicated significant ($p < 0.001$) genetic divergence at both genetic group and population levels. Genetic groups identified by k-means displayed high genetic divergence ($\Phi_{ST} = 0.22$), accounting for almost 23% of total genetic variance (Table 3). Populations within each genetic group displayed extremely high genetic divergence ($\Phi_{ST} = 0.85$), accounting for 66% of total genetic variance (Table 3).

The Mantel test indicated a significant ($p < 0.005$) but moderate ($r = 0.41$) association between genetic and geographic distances among *H. stella* populations. By contrasting distances visually, genetically divergent populations, such as STELL1 and SUBGL1, are geographically close, and the opposite also holds, such as for STELL1 and STELL2 (Online Resources 4).

Table 2 SSR-targeting primers and their respective number of amplified loci, polymorphic loci, and percentage (%) of polymorphic loci

Primer	Amplified	Polymorphic	% polymorphic
$(AC)_7RG$	34	34	100
$(AG)_8TG$	20	19	95
$(CA)_6RY$	16	15	93
$(CTC)_6T$	24	19	79
Total	94	87	92

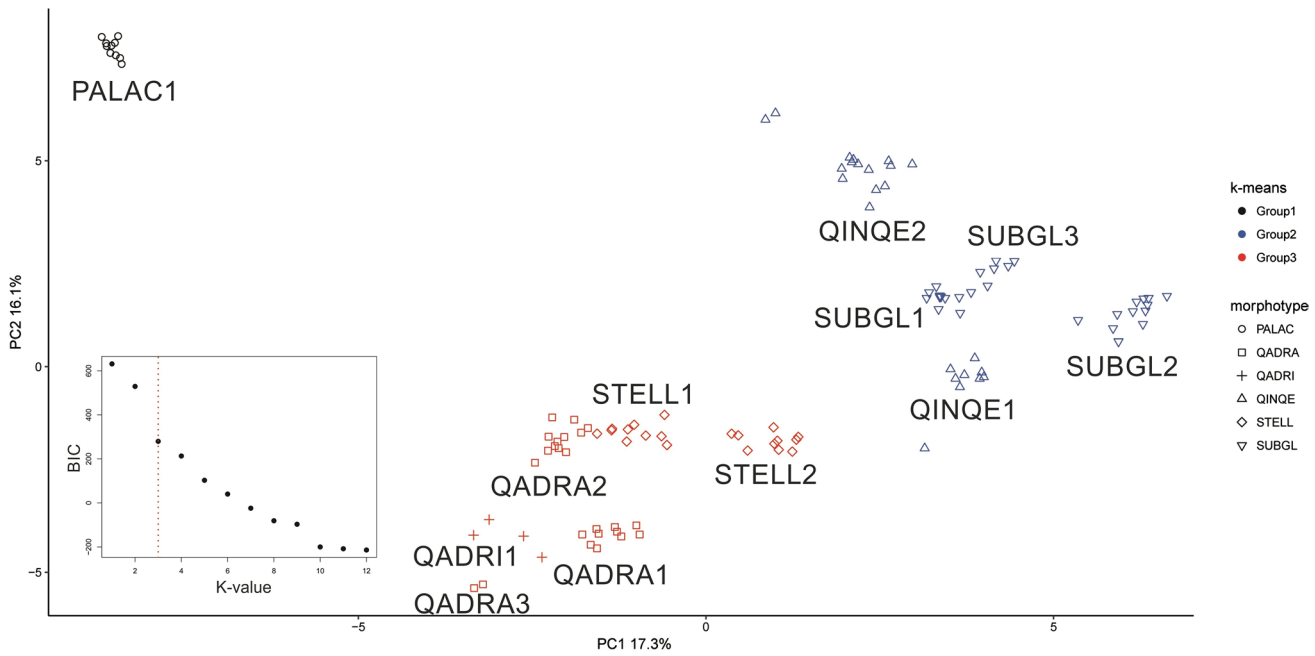

Fig. 3 PCA and k-means clustering of ISSR data from *Hydrocotyle stella* populations. At the wider plot, each principal component (PC) displays a percentage of genetic variation. Symbols represent morphotypes, and colors represent genetic groups by k-means clustering. Identification labels near each population. At the lower left plot, Bayesian information criterion (BIC) for each genetic grouping scenario (*K*), dashed line indicates the optimal *K* value

Table 3 AMOVA of ISSR data from *Hydrocotyle stella*, considering two nested factors: genetic groups by k-means, and populations within each genetic group

	df	SS	MS	%	Φ_{ST}	*p* value
Among genetic groups	2	534	267	22.8	**0.22**	< 0.001
Among populations	9	1011	112	66.0	**0.85**	< 0.001
Within populations	99	209	2	11.2	–	–

Each factor is followed by its respective degrees of freedom (*df*), sum of squares (SS), mean sum of squares (MS), percentage of explained variation (%), fixation index value (Φ_{ST}), and *p* value from permutation procedure. Significant fixation values in bold

Morphometric analyses

RWA of the aligned coordinates summarized 76% of leaf shape variation, which occurred mainly at lobe length, angle between sinuses, and basal sinus depth (Online Resource 5). RW1 (55.1% of variation) displayed an elongation of median and basal lobes, a narrowing of median and lateral sinus, and a deepening of the basal sinus. RW2 (20.9% of variation) displayed a reduction in the lateral lobe and a narrowing of median and lateral sinuses.

CVA of morphometric variables indicated morphological discontinuities among the three genetic groups identified by ISSR data (Fig. 4). CV1 (91.1% of between-group variation) represented decreasing leaf shape RW1 scores, while CV2 (8.8% of between-group variation) represented increasing values for all other variables (Online Resource 6). CV1 clearly separated Group1 from Group2 and Group3 and displayed a mild overlapping between the former two groups (Fig. 4). CV2 did not separate groups (Fig. 4).

Permutation test of D^2 indicated significant ($p < 0.001$) morphological divergence among all three genetic groups, with D^2 of 3.9, 6.7, and 3.6 for comparisons between Group 1–2, Group 1–3, and Group 2–3, respectively. On the other hand, morphological divergence was mostly not significant among traditional leaf morphotypes (Table 4). PALAC was the only morphotype to significantly diverge from the others. SUBGL diverged from QADRA and STELL, while QADRI diverged only from STELL.

MANOVA indicated significant ($p < 0.001$) morphological variation at both genetic group and population levels. Genetic groups accounted for 24% of total morphological variation, while populations accounted for 26% (Table 5). Regarding each character, RW1 of leaf shape varied the most (82%) at the genetic group level, followed by peduncle length (31%) (Online Resource 7). The majority of other

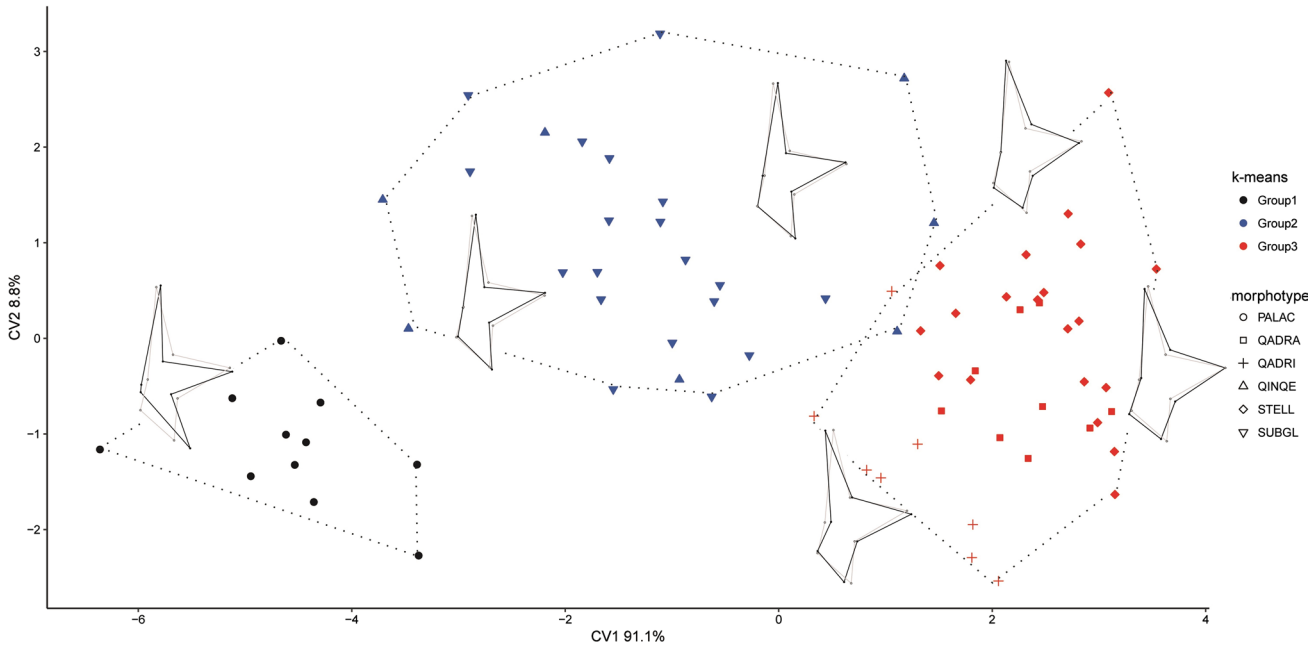

Fig. 4 CVA of morphometric variables measured on *Hydrocotyle stella* populations. Each canonical variate (CV) displays a percentage of between-group variation. Symbols represent morphotypes, and colors represent genetic groups recognized by k-means clustering of ISSR data. Convex hulls encompass the morphological variation within genetic groups. Wireframe diagrams represent mean leaf shapes of morphotypes

Table 4 Permutation test of D^2 based on morphological characters measured on *Hydrocotyle stella*

	PALAC	QADRA	QADRI	QINQE	STELL	SUBGL
PALAC		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
QADRA	**8.3**		0.60	0.40	1.00	< 0.001
QADRI	**6.2**	3.6		0.10	< 0.001	0.10
QINQE	**9.5**	3.9	4.7		0.10	1.00
STELL	**9.5**	2.5	**5.3**	4.1		< 0.001
SUBGL	**5.2**	**4.2**	4.0	2.1	**4.8**	

Lower triangle displays D^2 among currently recognized morphotypes, while the upper triangle displays p values from the permutation test. Significant values in bold

Table 5 MANOVA of morphometric variables measured on *Hydrocotyle stella*, considering two nested factors: genetic groups by k-means, and populations within each genetic group

	df	SS	MS	%	*F*	*p* value
Among genetic groups	2	177	88.5	**24.3**	15.3	< 0.001
Among populations	7	191	27.2	**26.3**	3.18	< 0.001
Within populations	64	358	5.5	49.4	–	–

Each factor is followed by its respective degrees of freedom (*df*), sum of squares (SS), mean sum of squares (MS), percentage of explained variation (%), approximated F-statistics (*F*), and *p* value. Significant percentages in bold

characters varied significantly at both genetic group and population levels, with different degrees of variation at each level (Online Resource 7). Internode length varied marginally (0.1%) at both genetic group and population level.

Discussion

In this study, we assessed species boundaries within the *H. stella* complex, which comprises six leaf morphotypes occurring at mountain ranges of the Brazilian Atlantic forest. In order to infer species boundaries, we adopted the GLS concept (de Queiroz 2007) and applied ISSR-based population genetics and morphometrics to 12 populations of *H. stella,* considering genetic structure and morphological discontinuity as evidences of lineage separation. These two lines of evidence congruently delimited three groups within the *H. stella* complex and indicated that traditional leaf morphotypes do not represent lineages.

Population genetic analyses based on ISSR revealed that the *H. stella* complex comprises three genetically structured groups [species sensu Mallet (1995)]. These genetic groups included different leaf morphotypes (Fig. 3): Group1 = PALAC; Group2 = QINQE + SUBGL; Group3 = QADRA + QADRI + STELL. Group1 was the northernmost group, occupying the Espinhaço range (Fig. 1). Group2 occupies the Serra da Mantiqueira and northern Serra do Mar (Fig. 1). Group3 was the southernmost group, occupying the Serra Geral and southern Serra do Mar (Fig. 1). Such geographic groups displayed high genetic divergence ($\Phi_{ST} = 0.22$, $p < 0.001$) (Table 3), suggesting that mountain ranges could have fostered genetic isolation among them. Mountains and their associated elevational gradient can restrict either pollination or seed dispersal (Zelikova et al. 2008; Zhu and Lou 2010), which then can lead to genetic divergence among populations occupying different mountains (Reis et al. 2015; Konečná et al. 2019; Li et al. 2019). However, genetic distance among *H. stella* population was only moderately associated with geographic distance ($r = 0.41$, $p < 0.005$), suggesting that geographic isolation only partially explains genetic divergence. Indeed, genetic groups displayed high genetic divergence despite some geographic overlap (e.g., STELL2 and SUBGL1 populations are geographically close but genetically divergent) (Online Resource 4), which may reflect the establishment of intrinsic reproductive barriers among such groups (Widmer et al. 2009).

Furthermore, the reproductive biology of *Hydrocotyle* may account for the majority of genetic divergence among populations. Populations of a same geographic group displayed extremely high genetic divergence ($\Phi_{ST} = 0.85$, $p < 0.001$) (Table 3), suggesting that gene flow is restricted despite of geographic proximity. As self-compatibility is frequent among *Hydrocotyle* species (Keighery 1982), individuals may favor self-pollination over cross-pollination, producing mainly an endogamic offspring. By doing so, *Hydrocotyle* populations separated by meters become reproductively isolated and genetically distinct, allowing local adapted forms to evolve (Knight and Miller 2004). Moreover, the dry fruits of *Hydrocotyle* apparently are

not dispersed by animal vectors (pers. obs.), so their seed dispersal may have a short range, reducing the exchange of genotypes among populations. Consequently, genetic similarity among geographically separated populations is unlikely to be due to recent gene flow (e.g., STELL1 and STELL2 populations) (Online Resource 4), so it may better reflect shared ancestral polymorphisms (Muir and Schlötterer 2005). Although studies on the reproductive biology of the *H. stella* complex are required, these general reproductive traits of *Hydrocotyle* could account for the extremely high genetic structure among populations, which persists regardless of geographic distribution.

Interestingly, populations displayed a higher fixation value than more comprehensive geographic groups (species) within the *H. stella* complex (Table 3), a pattern worth of note. Based on intuition, one may expect species to display higher fixation values than their populations. However, such an expectation lacks support from empirical data retrieved from different taxonomic groups, showing that both species and population levels can display either high or low fixation values (Hey and Pinho 2012). This holds because fixation indexes are *relative* measures of genetic divergence, varying from 0 to 1 accordingly to the proportion of shared alleles among the units being analyzed (Bird et al. 2011). For instance, if one compares two conspecific populations that are geographically isolated from each other, the fixation value will be high (near 1) because these two populations rarely exchange alleles; on the other hand, if one compares two hybridizing species, one of which containing the former two isolated populations, the fixation value will be low (near 0) because these species often exchange alleles, despite of a pair of populations that do not admix. Hence, the interpretation of fixation indexes are solely restricted to units at the same level, as each level comprises a different number of alleles and gene exchange dynamics (Bird et al. 2011). Unfortunately, this interpretation has been overlooked, and plausible species have been disregarded because of their lower fixation values when compared to populations (e.g., as in Lima et al. 2015).

Additionally, this study has shown that ISSR markers are suitable tools for species delimitation. ISSR and other dominant markers have been disregarded in species delimitation studies on the basis of uncertain homology, lower reproducibility, and heterozygote undetectability, but such drawbacks can be minimized (Bussell et al. 2005). First, cloning and sequencing of same-weight ISSR bands have revealed that homology holds at lower taxonomic levels (i.e., congeneric species and below) (Wolfe et al. 2001). Second, reproducibility of ISSR amplification is not a concern within a same system once protocols have been standardized (Ng and Tan 2015). Third, although dominant markers cannot detect heterozygotes, they still provide reliable estimates of admixture, which converge with those based on codominant markers (Sanz et al. 2009). Even so, statistical models have been developed to handle the uncertainty of inferring heterozygotes from dominant markers, reducing their bias to the degree of codominant markers (Zhivotovsky 1999; Falush et al. 2007). Thus, ISSR markers are a useful source of genetic data for species delimitation, being especially suitable for primary assessment of systems that are poorly known, such as *Hydrocotyle*.

Morphometric analyses of the *H. stella* complex indicated that the three genetic groups recognized by ISSR data were separated by morphological discontinuities [species sensu Du Rietz (1930)]. Group1 clearly separated from the others, while Group2 and Group3 mildly overlapped (Fig. 4). Morphological divergence among such groups was highly significant ($p < 0.001$), indicating consistent morphological boundaries. In contrast, morphological divergence among traditional leaf morphotypes was mostly not significant (Table 4), suggesting that distinction among such morphotypes was somehow arbitrary. These morphotypes correspond to infraspecific taxa formerly proposed by Urban (1879), who probably followed a typological perspective in which every morphological variation was worthy of taxonomic classification (Nery and Fiaschi 2019). Following this standpoint, taxa may not always be separated by morphological discontinuities and thus may not reflect evolutionary independent lineages (species). Hence, genetically structured groups recognized by this study would represent a better morphological circumscription for the *H. stella* complex, since they displayed consistent morphological boundaries.

Overall, morphological characters varied significantly ($p < 0.001$) at both genetic group and population levels, but with different degrees (SS) at each level (Table 5). Leaf shape (RW1) and peduncle length showed the highest percentage of variation among genetic groups (Online Resource 7), suggesting a major influence of genetic factors over their variation. Such pattern is unexpected for leaf characters, which are traditionally thought to vary mostly due to environmental factors (Gosler et al. 1994). Nonetheless, leaf shape varied mainly among genetic groups and thus is a reliable taxonomic character for species delimitation within the *H. stella* complex and possibly within other *Hydrocotyle* systems in the Neotropics. In contrast, internode length showed the lowest percentage of variation among genetic groups and populations (Online Resource 7), suggesting a major influence of non-genetic factors over its variation. Indeed, internodes can display phenotypic plasticity in response to light foraging (Evans and Cain 1995), so their variation due to local environmental conditions is expected. Consequently, internode length cannot be considered a reliable taxonomic character for delimiting species within the *H. stella* complex. The remaining morphological characters (leaf size, petiole length, pedicel length, and the number of flowers per umbel) varied significantly at both genetic group and population

levels, but with different percentages of variation at each level (Online Resource 7). Hence, these characters should be applied with caution to taxonomic decisions within the *H. stella* complex and likely within other *Hydrocotyle* complexes in the Neotropics.

Considering the lines of evidence assessed, we here recognize three species within the *H. stella* complex, whose circumscriptions correspond to Group1, Group2, and Group3. We provide their taxonomic treatment, with the due nomenclatural arrangements, as follows.

Conclusion

Under an integrative framework, we infer species boundaries within the *Hydrocotyle stella* complex, a puzzling group with a wide distribution within the Brazilian Atlantic forest. The species complex is genetically structured into three groups that displayed consistent morphological boundaries and occupy different mountain range regions. Based on that, we propose an updated taxonomic treatment that recognizes three *Hydrocotyle* species in Brazil. Moreover, we exemplified a theoretically and operationally robust approach of integrative taxonomy and provided the first molecular assessment of *Hydrocotyle* from the Neotropics.

Taxonomic treatment

Key to the star-shaped *Hydrocotyle* species in Brazil

1a Leaf blades marginally attached to petioles 2
1b Leaf blades peltately attached to petioles 3
2a Leaf lobes deltate-triangular, leaf margins entire or crenate, flowers subsessile (pedicels < 0.5 mm long) ..... ***H. bowlesioides***
2b Leaf lobes ovate-lanceolate, leaf margins double-serrate, flowers pedicellate (pedicels > 0.5 mm long) ..... ***H. palacea***
3a Median leaf lobes shorter than wide (depressed) 4
3b Median leaf lobes longer than wide (elongated) 5
4a tems, petioles, and peduncles villously covered by reddish trichomes, leaf blades 7–9-lobed ..... ***H. barbarossa***
4b Stems, petioles, and peduncles glabrous or pubescent with whitish trichomes, leaf blades 5–6-lobed ..... ***H. langsdorffii***
5a Umbels 8–16-flowered, pedicels 1–5 mm long ... ***H. asterias***
5b Umbels 15–100-flowered, pedicels 5–13 mm 6
6a Stems, petioles, and peduncles glabrous, leaf blades 6–10-lobed ..... ***H. macrophylla***
6b Stems, petioles, and peduncles glabrescent to villous, leaf blades 4–6-lobed 7
7a Leaf blade constantly 5-lobed, basal sinus deeper than the others ..... ***H. palacea***
7b Leaf blade 4–5(–6)-lobed, basal sinus as deep as or shallower than the others 8
8a Leaf lobes ovate-lanceolate, sinuses between median and lateral lobes ≤ 90° ..... ***H. quinqueradiata***
8b Leaf lobes deltate-triangular, sinuses between median and lateral lobes > 90° ..... ***H. alpina***

Hydrocotyle alpina Vell., Fl. Flumin.: 123. 1829 ('1825').—LECTOTYPE (**designated here**): [illustration] Plate 89 on "Flora fluminensis" parchment stored in the Manuscript Section of the Biblioteca Nacional of Rio de Janeiro [mss1198652_092], later published by Vellozo (Fl. flumin. Icon. 3: t. 89. 1831). —EPITYPE (**designated here**): Brazil, Rio de Janeiro: Itatiaia, Parque Nacional do Itatiaia, parte baixa, trilha para os Três Picos, 7 Apr 2017, *E.K. Nery 42* (FLOR barcode FLOR0065675!) (Fig. 5).
= *Hydrocotyle stella* Pohl. ex DC., Prodr. 4: 61. 1830. ≡ *Hydrocotyle quinqueloba* var. *stella* Urb., Fl. Bras. 11: 275. 1879.—NEOTYPE [designated by Nery and Fiaschi (2019)]: Brazil, Rio de Janeiro: Serra da Estrela, *s.d.*, *H.A. Weddel 867* (P barcode P03259033 [web!]).
= *Hydrocotyle quinqueloba* var. *quadrata* Urb., Fl. Bras. 11: 275. 1879.— SYNTYPES: Brazil, Rio de Janeiro: "*in umbrosis humidis prope Rio de Janeiro*", *s.d.*, *L. Riedel 324*; Minas Gerais: *s.loc.*, *s.d.*, *L. Riedel 370*; *s.loc.*, *s.d.*, *H.W. Schott 5354*.
= *Hydrocotyle quinqueloba* var. *quadriloba* Urb., Fl. Bras. 11: 275. 1879.—LECTOTYPE (**designated here**): Brazil, São Paulo: *s.loc.*, *s.d.*, *A. Saint-Hilaire C2 1499* (P barcode P00115389 [web!]; isolectotype: P barcode P00115390 [web!]).

Etymology: The epithet possibly refers to the "*Alpes Fluminenses,*" denomination given by Fra. José Vellozo to different montane localities in the former province of Rio de Janeiro.

Description: Perennial herbs with prostrate to ascending stems and fibrous roots at the nodes. Stems cylindrical, internodes 2.5–26 cm long, glabrescent, pubescent or villous. Leaves simple, blade peltate, 2–13 × 2–16 cm, 4–5(–6)-lobed, sinuses between median and lateral lobes wider than 90 degrees, basal sinus as deep as or shallower than the other sinuses, median lobe deltate to triangular, 0.8–6(–7) × 0.7–5(–6) cm, apex acuminate, margins double-serrate, proximal tooth margins convex at the lobe base, adaxially glabrescent, pubescent, or villous, abaxially pubescent or villous; petioles cylindrical, 1.5–19 cm long, pubescent, villous or lanate; stipules palmately lobed, 1.3–4.5 × 1.4–7.5 mm. Umbels simple, 15–70-flowered, peduncles 3–18.5 cm long, villous

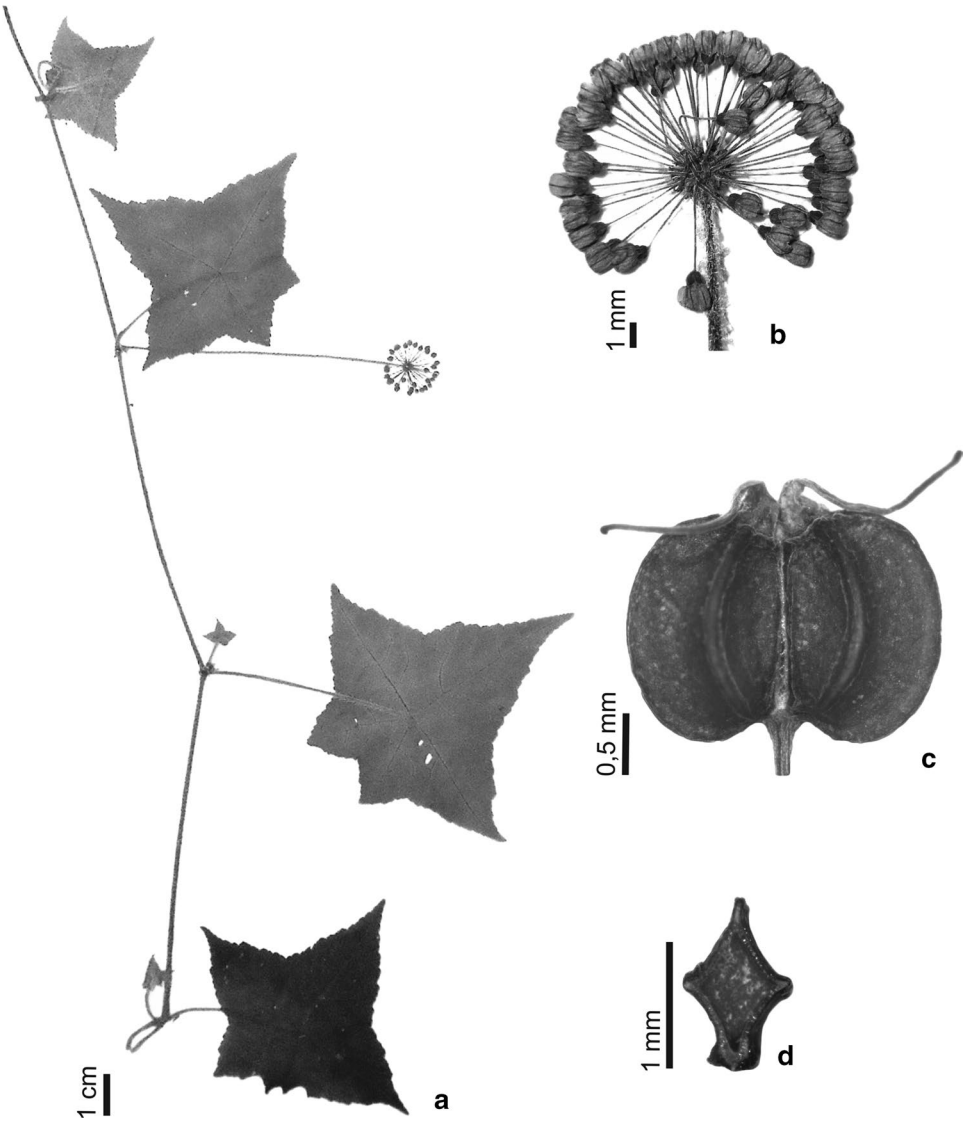

Fig. 5 *Hydrocotyle alpina:* **a** habit; **b** umbel; **c** schizocarp, lateral view; **d** mericarp, transversal section. a–d *E.K. Nery 42* (FLOR)

or lanate. Flowers bisexual, pedicellate; pedicels 2–10 mm long, glabrous or rarely puberulent; sepals absent; petals ovate, elliptic, or oblong, 1–2 × 0.3–0.6 mm, whitish, often red-dotted when dried. Schizocarps oblate to transversely elliptic, 1–3 × 1.2–4 mm, base cordate, apex round, ribs conspicuous, mericarps rhombic to trullate in transversal section; stylopodia conical, fruiting styles longer than ½ of mature fruit width.

Notes: *Hydrocotyle alpina* (Fig. 5) corresponds to Group3 in our study. This group includes the QADRA, QADRI, and STELL morphotypes that were once named as *Hydrocotyle quinqueloba* var. *quadrata* Urb., *Hydrocotyle quinqueloba* var. *quadriloba* Urb., and *Hydrocotyle quinqueloba* var. *stella* Urb., respectively (Nery and Fiaschi 2019). Regarding the first name, it was described based on three collects (Urban 1879), but we did not succeed to find them for typification. Regarding the second name, it was described based on a single collect, *A. Saint-Hilaire C2 1499* (Urban 1879), and three vouchers at P held this collector's numbering, P00115389, P00115390, and P00757665. We chose the first one as the lectotype since it has a label annotated by the describing author, and we disregard the third one since it was not available for our evaluation. Regarding the last name, it was proposed based on *Hydrocotyle quinqueloba* Ruiz & Pav., *Hydrocotyle alpina* Vell., *Hydrocotyle stella* Pohl ex DC. As discussed by Nery and Fiaschi (2019), *H. quinqueloba* does not apply to plants found in Brazil, being thus an unsuitable name for the species circumscribed. However, Nery and Fiaschi (2019) wrongly granted the priority to *H. stella* over *H. alpina*, as they were not aware of the effective publication dates of Vellozo's *Flora Fluminensis* (Carauta 1973). In 1829, an incomplete version of *Flora Fluminensis* was published (Carauta 1973), and such version already included a diagnosis of *H. alpina* (Vellozo 1829), which was later illustrated on a plate (Vellozo 1831). Hence, *H. alpina*

(Vellozo 1829) was validly published before *H. stella* (De Candolle 1830), holding priority as species name. Since the protologue of *H. alpina* does not cite any collect (Vellozo 1829), we have chosen the plate by Velloso (1831) as the lectotype. Nonetheless, this plate does not represent reproductive structures and poorly represents other morphological details (e.g., leaf margins), providing incomplete information for species identification. Hence, we have chosen an epitype, *E.K. Nery 42* (FLOR0065675), to provide additional reference over species morphology.

Additional specimens examined: Brazil, ESPÍRITO SANTO, São Roque do Canaã, Alto Misterioso, 19 Jul 2005, *A.P. Fontana 1604* (MBML); Santa Teresa, Valsugana Velha, Estação Santa Lúcia, bacia do Rio Timbuí, 12 Nov 1990, *H.Q. Boudet Fernandes 3032* (MBML); PARANÁ, Campo Largo, Serra do Purunã, 1 Feb 1983, *R. Kummrow 2212* (BOTU); Colombo, 27 Jan 1985, *D.B. Falkenberg 2213* (FLOR); Jaguariaíva, 11 Jan 1973, *G. Hatschbach 31116* (MBM); Morretes, Estrada da Graciosa, 13 Feb 2017, *E.K. Nery 29* (FLOR); Tibagi, estrada Castro-Tibagi, Fazenda Palmito, 30 Jan 1959, *G. Hatschbach 5491* (MBM); RIO DE JANEIRO, Itatiaia, Taquaral, 19 May 1935, *A.C. Brade 14669* (ESA); 23 Mar 1945, *A.C. Brade 17493* (RB); Parque Nacional do Itatiaia, trilha para cachoeira Itaporani, 18 Feb 2003, *S.J. Silva Neto 1800* (RB); entre via Duarte e Itamonte, *J. Paula-Souza 5816* (ESA); RIO GRANDE DO SUL, Cambará do Sul, Faxinal, 1 Mar 1986, *M. Sobral 5040* (ICN); Parque Nacional de Aparados da Serra, RS427, 7 Aug 2018, *E.K. Nery 99* (FLOR); SANTA CATARINA, Antônio Carlos, RPPN Caraguatá, 27 Apr 2018, *E.K. Nery 92* (FLOR); Ascurra, 19 Feb 2015, *L.A. Funez 4253* (FURB); Corupá, RPPN - Emilio Fiorentino Battistella, 17 Jan 2015, *L.A. Funez 3328* (FURB); Urubici, Serra do Corvo Branco, estrada Urubici-Grão Pará, 28 Mar 2011, *P. Fiaschi 3654* (SPF); Cânion do Espraiados, estrada em direção ao alto dos

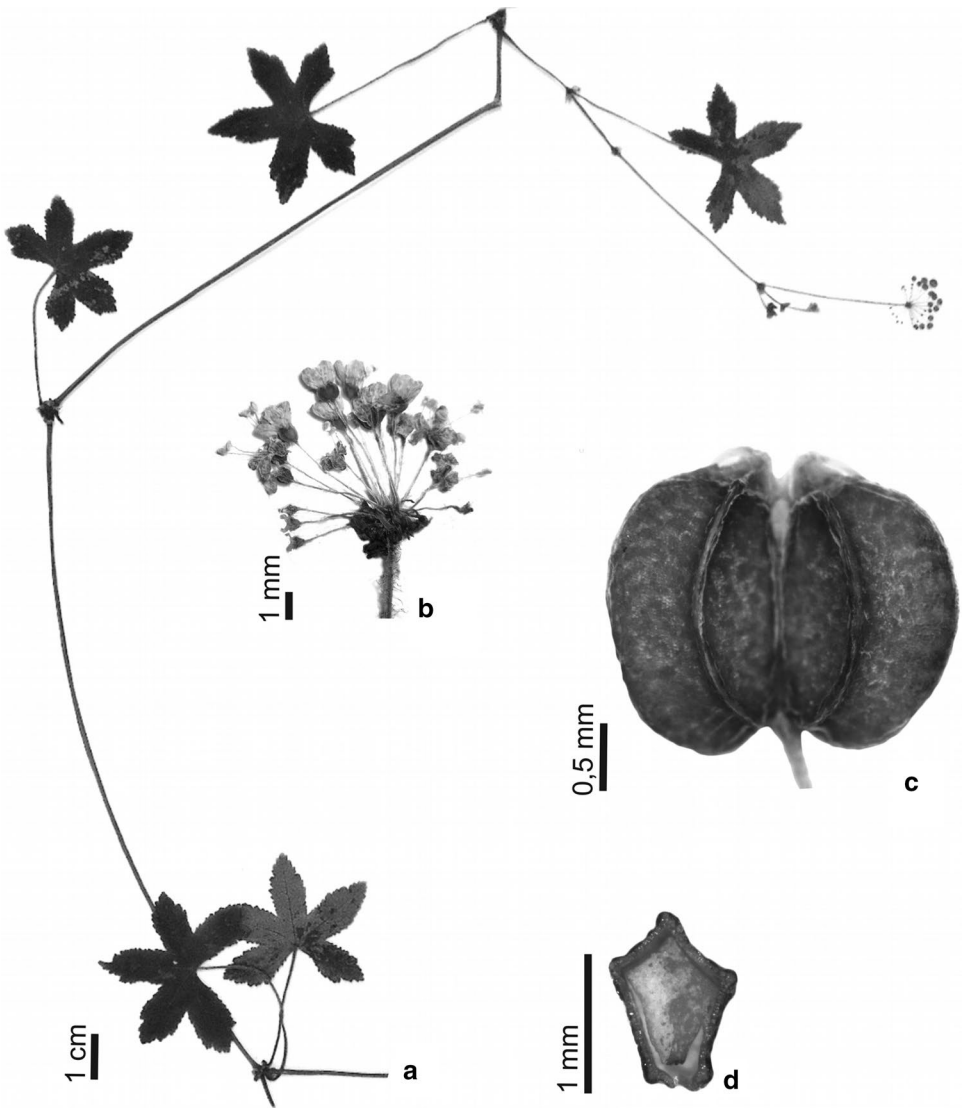

Fig. 6 *Hydrocotyle palacea:* **a** habit; **b** umbel; **c** schizocarp, lateral view; **d** mericarp, transversal section. a–d *E.K. Nery 75* (FLOR)

cânions, 12 Nov 2017, *E.K. Nery 57* (FLOR); SÃO PAULO, Itararé, antiga estrada para a Fazenda Experimental de Itararé, 13 Apr 2018, *E.K. Nery 90* (FLOR); Eldorado, Parque Estadual Intervales, trilha de acesso à Estação Ecológica Xituê, 22 Apr 2003, *R.A.G. Viani 196* (ESA).

Hydrocotyle palacea (Urb.) Nery & Fiaschi stat. nov. ≡ *Hydrocotyle quinqueloba* var. *quinqueradiata* f. *palacea* Urb., Fl. Bras. 11: 275. 1879.—HOLOTYPE: Brazil: *s.loc.*, *s.d.*, *A. Saint-Hilaire s.n.* (P barcode P00115391 [web!]) (Fig. 6).

Etymology: The epithet derives from the latin term *palaceous*, meaning "margin-attached" (Stearn 2004), a reference to the frequent expression of non-peltate leaves.

Description: Perennial herbs with prostrate to ascending stems and fibrous roots at the nodes. Stems cylindrical, internodes 2.5–19 cm long, villous, lanate, or rarely glabrescent. Leaves simple, blade often non-peltate or rarely peltate, 3–6.5 × 3.5–7 cm, 5-lobed, sinuses between median and lateral lobes narrower than 90 degrees, basal sinus deeper than the other sinuses, median lobe ovate to lanceolate, 1.5–3.5 × 0.7–2 cm, apex acuminate, margins double-serrate, proximal tooth margins straight or concave at the lobe base, adaxially puberulent or pubescent, abaxially pubescent or villous; petioles cylindrical, 1.5–8.5 cm long, villous or lanate; stipules palmately lobed, 1.5–4.5 × 2–3.5 mm. Umbels simple, 15–40-flowered, peduncles 3–7.5(–9.5) cm long, lanate. Flowers bisexual, pedicellate; pedicels 2–7.5 mm long, glabrous; sepals absent; petals ovate, elliptic, or oblong, 1.3–1.7 × 0.3–0.5 mm, whitish, often red-dotted when dried. Schizocarps oblate to transversely elliptic,

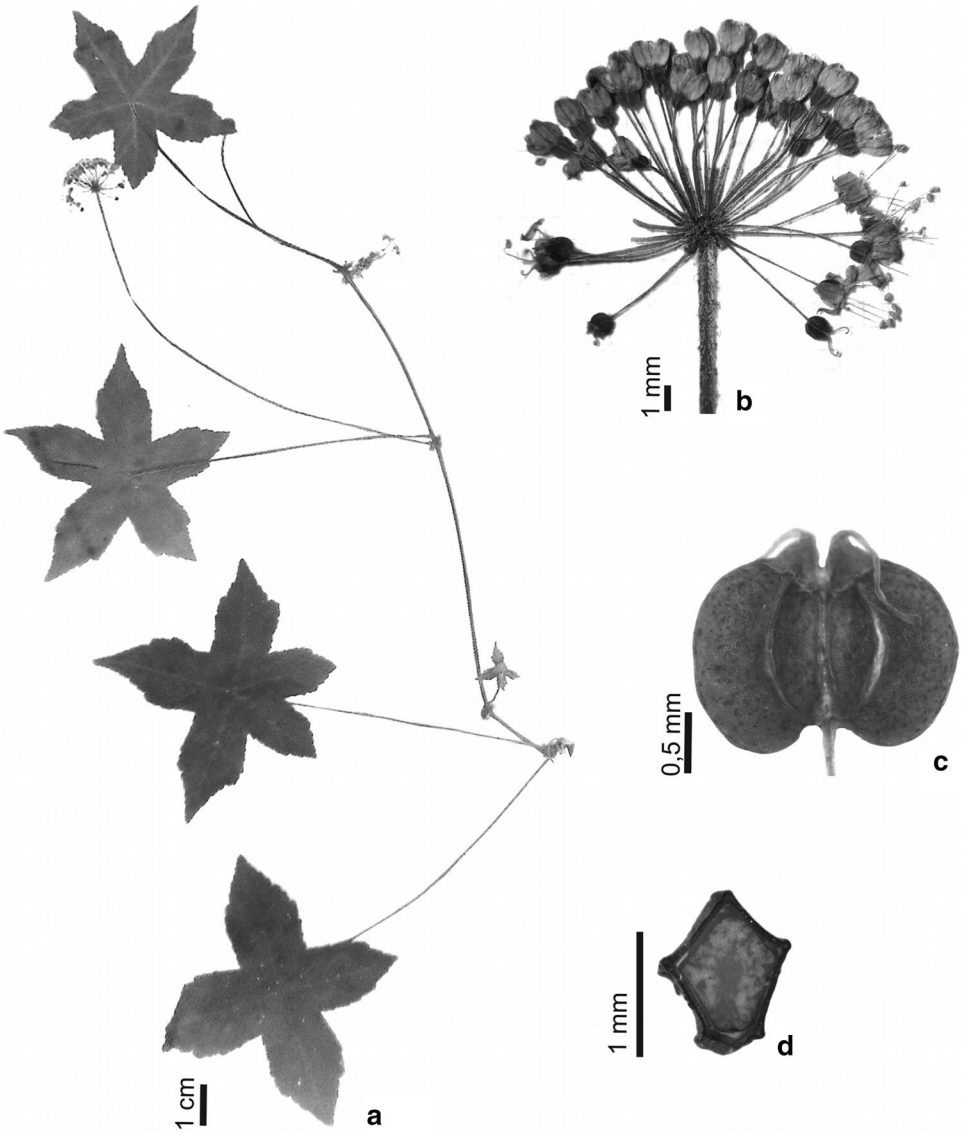

Fig. 7 *Hydrocotyle quinqueradiata:* **a** habit; **b** umbel; **c** schizocarp, lateral view; **d** mericarp, transversal section. a–b *E.K. Nery 36* (FLOR); c–d *E.K. Nery 89* (FLOR)

1–2 × 1.8–2.4 mm, base cordate, apex round, ribs conspicuous, mericarps trullate in transversal section; stylopodia conical, fruiting styles longer than ½ of mature fruit width.

Notes: Hydrocotyle palacea (Fig. 6) corresponds to Group1 in our study. This group comprehends only the PALAC morphotype, which was once named *Hydrocotyle quinqueloba* var. *quinqueradiata* f. *palacea* Urb. (Nery and Fiaschi 2019). Here, we have decided to preserve this name, since it aptly describes the leaf morphology of the species being circumscribed.

Additional specimens examined: Brazil, MINAS GERAIS, Diamantina, BR269 km10, 24 Feb 1975, *G. Hatschbach 36457* (NY [web]); Santo Antônio do Itambé, eastern slope of Pico do Itambé, 11 Feb 1972, *W.R. Anderson 35826* (NY [web]); caminho para o Pico do Itambé, 26 Feb 2002, *V.C. Souza 28472* (ESA); trilha do Pico do Itambé, 11 Oct 2006, *L.M. Versieux 313* (SPF); trilha para a Lapa do Morcego, 22 Jan 2018, *E.K. Nery 75* (FLOR); São Gonçalo do Rio Preto, Pico Dois Irmãos, 2 Apr 2004, *P.L. Viana 1525* (BHCB).

Hydrocotyle quinqueradiata (Urb.) Nery & Fiaschi, **comb & stat. nov.** ≡ *Hydrocotyle quinqueloba* var. *quinqueradiata* Urb., Fl. Bras. 11: 275. 1879.—LECTOTYPE (**designated here**): Brazil, Rio de Janeiro: Serra dos Órgãos, *s.d.*, *G. Gardner 431* (P barcode P00115384 [web!]) (Fig. 7).
= *Hydrocotyle quinqueloba* var. *quinqueradiata* f. *subglabra* Urb., Fl. Bras. 11: 275. 1879. ≡ *Hydrocotyle quinqueloba* var. *glabra* Cham., Linnaea 8: 329. 1833.—LECTOTYPE (**designated here**): Brazil, Rio de Janeiro: *s.loc.*, *s.d.*, *F. Sellow s.n.* (HAL barcode HAL0025126 [web!]).

Etymology: The epithet refers to the frequent expression of 5-lobed leaves.

Description: Perennial herbs with prostrate or ascending stems and fibrous roots at the nodes. Stems cylindrical, internodes 2.5–20 cm long, glabrescent, pubescent, or villous. Leaves simple, blade peltate, 4–14 × 4–14 cm, 4–5(–6)-lobed, sinuses between median and lateral lobes equal to or narrower than 90 degrees, basal sinus as deep as or shallower than the other sinuses, median lobe ovate to lanceolate, 1.5–7 × 1–4 cm, apex acuminate, margins double-serrate, proximal tooth margins straight or concave at the lobe base, adaxially glabrescent, pubescent or villous, abaxially puberulent, pubescent or villous; petioles cylindrical, 3–20 cm long, pubescent, villous, or rarely lanate; stipules palmately lobed, 1.5–4.5 × 2.5–5 mm. Umbels simple, 20–95-flowered, peduncles 6–14.5(–19) cm long, villous or lanate. Flowers bisexual, pedicellate; pedicels 2–10 mm long, glabrous; sepals absent; petals ovate, elliptic or oblong, 1–1.5 × 0.3–0.5 mm, whitish, often red-dotted when dried. Schizocarps oblate to transversely elliptic, 1–2 × 1.4–2.8 mm, base emarginate to cordate, apex round, ribs conspicuous, mericarps trullate in transversal section; stylopodia conical, fruiting styles longer than ½ of mature fruit width.

Notes: Hydrocotyle quinqueradiata (Fig. 7) corresponds to Group2 in our study. This group includes the QINQE and SUBGL morphotypes that were once named as *Hydrocotyle quinqueloba* var. *quinqueradiata* Urb. and *Hydrocotyle quinqueloba* var. *quinqueradiata* f. *subglabra* Urb., respectively (Nery and Fiaschi 2019). Regarding the first name, it was described based on *Hydrocotyle quinqueloba* Ruiz & Pavón (Urban 1879). Nonetheless, *H. quinqueloba* applies only to plants found in Peru (Nery and Fiaschi 2019), being an unsuitable name for the species circumscribed here. Regarding the second name, it was described based on *Hydrocotyle quinqueloba* var. *glabra* Cham. (Urban 1879), another infraspecific name which does not hold priority. Since the species being circumscribed encompasses two taxa proposed by Urban (1879), we have decided to acknowledge the author's work and use one of the names proposed by him. *Hydrocotyle quinqueloba* var. *quinqueradiata* f. *subglabra* was considered a misleading epithet since it suggests glabrescent indument, which is not a diagnostic feature for the species. Hence, we chose to transfer *Hydrocotyle quinqueloba* var. *quinqueradiata* to the species status. The transferring does not imply in a homonym with the invalidly published *Hydrocotyle quinqueradiata* Thouars ex DC., which was proposed only as synonym of *Hydrocotyle petiolares* DC. The lectotype designated here, *Gardner 431* (P00115384), is a representative specimen cited by Urban (1879) when proposing *H. quinqueloba* var. *quinqueradiata.* However, other vouchers labeled as *Gardner 431* at P and TCD (P00115385 and TCD0017838, respectively) are a misleading reference, as their specimens do not display the species morphology. These vouchers indeed hold specimens of *Hydrocotyle macrophylla* Pohl ex DC. and should be disregarded.

Additional specimens examined: Brazil, ESPÍRITIO SANTO, Dores do Rio Preto, Parque Nacional do Caparaó, estrada para a Tronqueira, 26 Jan 2018, *E.K. Nery 81* (FLOR); GOIÁS, Alto Paraíso de Goiás, camping Portal da Chapada, 11 jan 2002, *L.H. Soares-Silva 1183* (RB); Formosa, córrego Itaquara, 2 May 1966, *H.S. Irwin 15579* (RB); MINAS GERAIS, Caparaó, Parque Nacional do Caparaó, estrada para Tronqueira, 29 Sep 1995, *J.A. Lombardi 950* (BHCB); Ouro Preto, Itacolomi, 28 Dec 1950, *A. Macedo 2793* (NY [web]); Serra de Capanema, 28 Feb 2008, *F.F. Carmo 2342* (BHCB); RIO DE JANEIRO, Nova Friburgo, Rio Bonito de Lumiar, Pousada dos Cristais, 1 Mar 2004, *R.C. Forzza 2748* (RB); Teresópolis, Parque Nacional da Serra dos Órgãos, 28 Apr 2009, *M. Nadruz 2315* (RB), na Trilha Suspensa, 8 Apr 2017, *E.K. Nery 44* (FLOR); SÃO PAULO, Campos dos Jordão, 17 May 1985, *A. Amaral 65* (BOTU); Jundiaí, Reserva Biológica Municipal da Serra do

Japí, 23 Oct 2007, *J.A. Lombardi 6980* (HRCB); São José dos Campos, Reserva Biológica da Serra do Japí, trilha do portão 6, em direção ao viveiro, 9 Apr 2018, *E.K. Nery 89* (FLOR).

Acknowledgements We thank C.F.D. Gurgel and S. Koehler who read the first version of the manuscript and provided valuable advices, as well as for the comments of three anonymous reviewers that greatly improved the manuscript. We thank FLOR herbarium staff, S. Venturi, for arranging loans and preserving our samples. EKN thanks A. Reichert and L. Redivo for photographs used in figures and G. Goebel for assisting laboratory work. EKN thanks the Brazilian Federal Agency for Graduate Education (CAPES) for the Graduate Student Fellowship, and the American Society of Plant Taxonomists for the 2017 Research Grant for Graduate Students. OC thanks CAPES for the Post-Doctoral Fellowship. PF thanks Brazil's National Council for Scientific and Technological Development (CNPq) for the Research Productivity Fellowship (#306228/2016-5).

Funding Funding was provided by Brazil's National Council for Scientific and Technological Development (CNPq) (Grant #306228/2016-5).

Compliance with ethical standards

Conflict of interest The authors declare that the manuscript does not present any kind of conflict of interest. The purpose and content of the work are original and not previously published.

Information on Electronic Supplementary Material

Online Resource 1. Electrophoretic profiles for selected ISSR primers.
Online Resource 2. ISSR data matrix.
Online Resource 3. Morphometric data matrix.
Online Resource 4. Genetic and geographic distance among populations.
Online Resource 5. Relative warp analyses of leaf shape.
Online Resource 6. Canonical loadings for morphological characters
Online Resource 7. ANOVAs of morphometric characters, considering genetic group and population levels.

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