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Morphological overlap and species boundaries *in the Microgramma vacciniifolia* Clade

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ABSTRACT

Species delimitation in ferns is often challenging due to limited reproductive information, frequent morphological overlap, and high phenotypic plasticity. Here, we investigated morphological variation within the Vacciniifolia clade of *Microgramma* (Polypodiaceae), focusing on the closely related and sympatric *M. crispata* and *M. vacciniifolia*. We analyzed 19 linear morphometric characters from 182 specimens of the four species, using Principal Component Analysis (PCA), Kruskal–Wallis tests, and Discriminant Analysis of Principal Components (DAPC). Morphometric analyses revealed broad overlap among species despite significant differences in 15 of 19 variables. Leaf size traits were the main drivers of variation, and shape-related characters showed limited discrimination. *Microgramma geminata* was the most morphologically distinct species, while the afrotropical *M. mauritiana* presented a broad morphological variation. *Microgramma crispata* and *M. vacciniifolia* showed extensive overlap in morphospace and low discrimination in DAPC analyses. Morphological similarity between these species was not associated with geographic proximity, suggesting that sympatry alone does not explain the observed morphological overlap. Our results indicate that leaf morphology alone is insufficient to consistently delimit species boundaries within this complex and highlight the need for integrative approaches combining genomic, spectral, and morphometric data, among others, to better understand evolutionary relationships and species limits in *Microgramma*.

Keywords: fern systematics; morphometrics; phenotypic variation; Polypodiaceae; species delimitation.

RESUMO

A delimitação de espécies em samambaias frequentemente é difícil devido à escassez de informação reprodutiva, frequente sobreposição morfológica e elevada plasticidade

fenotípica. Neste estudo, investigamos a variação morfológica dentro do clado Vacciniifolia de *Microgramma* (Polypodiaceae), com foco nas espécies proximamente relacionadas e simpátricas *M. crispata* e *M. vacciniifolia*. Foram analisados 19 caracteres morfométricos lineares de 182 espécimes das quatro espécies, utilizando Análise de Componentes Principais (PCA), testes de Kruskal–Wallis e Análise Discriminante de Componentes Principais (DAPC). As análises morfométricas revelaram ampla sobreposição entre as espécies, apesar de diferenças significativas em 15 das 19 variáveis analisadas. Caracteres relacionados ao tamanho das folhas foram os principais responsáveis pela variação observada, enquanto caracteres relacionados à forma apresentaram baixo poder discriminatório. *Microgramma geminata* foi a espécie morfologicamente mais distinta, enquanto a espécie afrotropical *M. mauritiana* apresentou ampla variação morfológica. *Microgramma crispata* e *M. vacciniifolia* exibiram extensa sobreposição no morfoespaço e baixa discriminação nas análises de DAPC. A similaridade morfológica entre essas espécies não esteve associada à proximidade geográfica, sugerindo que a simpatria, isoladamente, não explica a sobreposição morfológica observada. Nossos resultados indicam que a morfologia foliar, isoladamente, é insuficiente para delimitar consistentemente os limites entre espécies dentro desse complexo, e destacam a necessidade de abordagens integrativas que combinem dados genômicos, espectrais e morfométricos, entre outros, para melhor compreender as relações evolutivas e os limites específicos em *Microgramma*.

Palavras-chave: delimitação de espécies; morfometria; Polypodiaceae; sistemática de samambaias; variação fenotípica.

1. INTRODUCTION

Traditional plant species delimitation heavily relies on macromorphological characters. In typological taxonomy, species hypotheses are created using qualitative traits and measurement ranges of morphological variation, relying on overlapping or non-overlapping value ranges and categorical character states (Dayrat 2005; André & Almeida 2025). This method becomes challenging within species complexes, which are lineages still incompletely separated, recently diverged, or with lax reproductive barriers (Pinheiro et al. 2018). Similarly, taxonomic challenges arise when dealing with cryptic species, distinct lineages that are reproductively isolated but lack noticeable morphological differences (Bickford et al. 2007). In addition, in plants, issues such as hybridization, introgression, and broad phenotypic plasticity can complicate species definition, posing challenges to both taxonomic classification and species conservation efforts (Alix et al. 2017; Schmickl & Yant 2021). Ferns, the sister lineage to seed plants, are vascular, seed-free vascular plants, displaying a distinctive life cycle (Haufler et al. 2016). They exhibit two distinct, independent phases: a perennial, diploid sporophyte, and a usually ephemeral, haploid gametophyte (Haufler et al. 2016). Unlike flowering plants, fern morphology lacks the informativeness of characteristics from flowers and seeds. Species descriptions and taxonomic revisions of ferns generally do not include reproductive features or other characters from the gametophytic phase (*e.g.*, Prado & Moran 2008; Mickel 2016; Bohn et al. 2020; Fawcett and Smith 2021; Sanín et al. 2023), as the gametophytes are typically absent in herbarium specimens. In nearly all fern families, species circumscriptions and identification rely solely on sporophyte traits. Additionally, ferns lack the intricate breeding systems and pre-zygotic barriers found in seed plants and can be more prone to hybridization (Haufler 2008).

Microgramma C.Presl (Polypodiaceae) is a monophyletic tropical fern genus comprising approximately 30 species (Almeida 2014). Typically, these ferns occur as epiphytes in

rainforests but can also be saxicolous or terrestrial and may inhabit dry forests and savannas (Almeida 2014). One of the clades identified in the phylogenetic analysis by Almeida et al. (2021), known as the Vacciniifolia clade, comprises four species: tropical American *Microgramma crispata* (Fée) R.M.Tryon & A.F.Tryon, *M. geminata* (Schrad.) R.M.Tryon & A.F.Tryon, and *M. vacciniifolia* (Langsd. & Fisch.) Copel., and the afrotropical *M. mauritiana* (Willd.) Tardieu (Figure 1). The three tropical American species are sympatric, with *M. crispata* and *M. geminata* endemic to Eastern South America (Figure 1), within the Brazilian Atlantic Forest domain. *Microgramma vacciniifolia* occurs additionally in the Andes (Bolivia, Colombia, Peru), extending from Northeastern Brazil southward to Argentina, Paraguay, and Uruguay, with disjunct populations in Trinidad and Tobago and Venezuela. All three species cooccur in Eastern Brazil's Atlantic Forest.

All African populations are subsumed into *M. mauritiana* (sensu Almeida 2014), a broadly circumscribed taxon that includes a wide morphological variation and is considered a species complex (TE Almeida, pers. comm.). Individuals range from monomorphic to dimorphic plants, with narrow and small to wide and large fronds, with paraphyses present or absent in the sori. Variation in the same features has been used to split taxa in South America (*e.g.*, Smith et al. 2018).

The phylogenetic relationships among the species, as recovered by Almeida et al. (2021), show *M. mauritiana* as sister to the clade composed of *M. geminata*, which is in turn sister to *M. crispata* + *M. vacciniifolia*. This study suggested the African clade originated from a long-distance dispersal event from a tropical American ancestor to Africa, followed by speciation (Almeida et al. 2021).

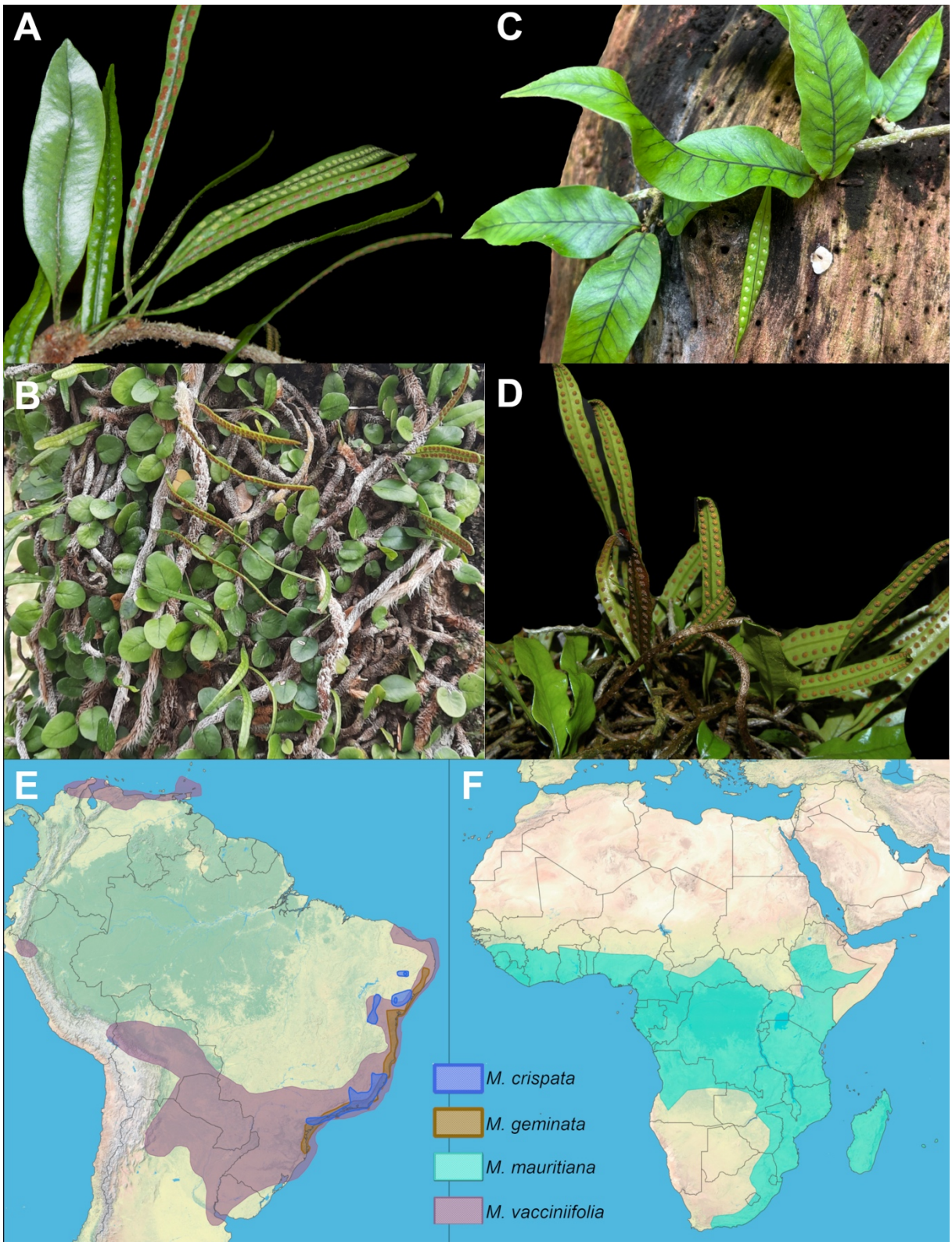


Figure 1 - Representative morphology and geographic distribution of the studied *Microgramma* species. (A) *M. crispata*. (B) *M. vacciniifolia*. (C) *M. geminata*. (D) *M. mauritiana*. (E) Geographic distribution map of the tropical American species. (F) Geographic distribution map of the afrotropical species. Color-coded ranges corresponding to each taxon.

Among the tropical American taxa, morphologically, *M. geminata* is easily distinguished, as it is monomorphic, has sori imprinted on the adaxial surface, and has leaves emerging from short lateral branches of the main rhizome (Figure 1B). *Microgramma crispata* and *M. vacciniifolia*, however, are very similar. Both species are dimorphic and are usually distinguished by the patent scales of the rhizome in *M. crispata* vs. appressed rhizome scales in *M. vacciniifolia* (Almeida 2026). The sterile fronds in *M. crispata* are the same length as the fertile fronds, but in *M. vacciniifolia*, sterile fronds vary broadly in length (from 1/5 of to almost the same size as the fertile frond). Although both were recovered as monophyletic, the phylogenetic inference of Almeida et al. (2021) was based on only two specimens per species, all from eastern South America. The relatively recent estimated divergence time of 4.87 Ma (95% HPD 7.82–0 Mya) suggests that these lineages may not be fully independent. Furthermore, field observations indicate that the sterile frond size in *M. vacciniifolia* varies throughout rhizome development, and the presence of fertile leaves on small individuals suggests a potential case of neoteny or phenotypic plasticity (TE Almeida pers. obser.). This, and their sympatric occurrence in Eastern Brazil, raises the question whether *M. crispata* may represent a morphological extreme or even an ecotype within the broad variation of *M. vacciniifolia*.

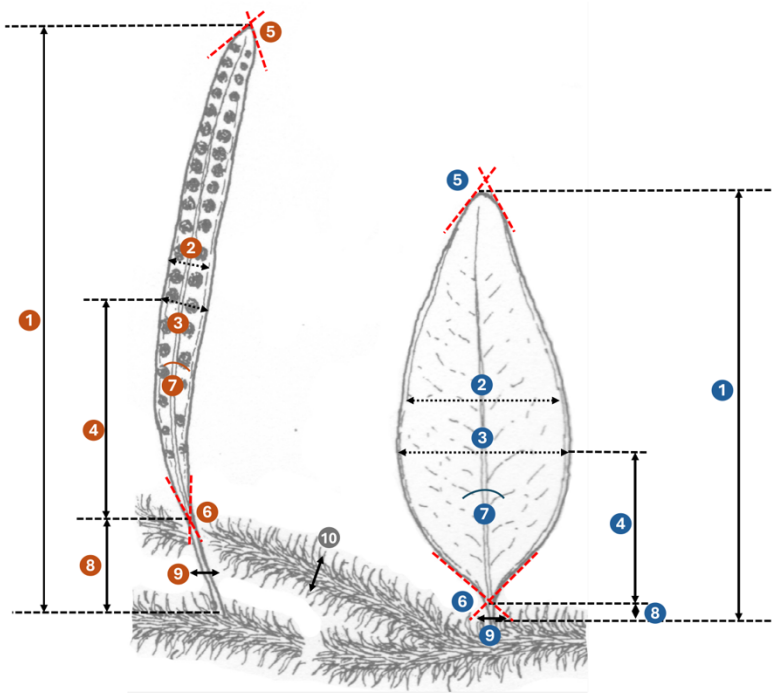
The morphological variation case of the Vacciniifolia clade hints at a potential relationship between divergence, geographic isolation, and hybridization shaping species boundaries (Friis & Balslev 2005). In this study, we used morphometrics to quantitatively assess frond morphological variations within the Vacciniifolia clade and test whether it supports current species delimitation, with a particular focus on the closely related pair *Microgramma crispata* and *M. vacciniifolia*.

2. MATERIALS AND METHODS

Specimens analyzed– Initially, we selected images from 300 specimens selected from physical herbaria and the online repositories speciesLink (https://specieslink.net/) and REFLORA (https://reflora.jbrj.gov.br/reflora/herbarioVirtual/). However, 118 specimens were excluded due to issues such as poor image quality, overlapping fronds, or incomplete material. Morphometric analyses are based on measurements from 182 digitized herbarium specimens of the four species of the Vacciniifolia clade (*Microgramma crispata*, *M. geminata*, *M. mauritiana*, and *M. vacciniifolia*). These specimens are deposited in 29 herbaria: ASE, ASU, B, BHCB, BM, C, CESJ, CVRD, EAC, ESA, F, G, GH, HST, HUEFS, IAC, JPB, MBM, MBML, MO, NY, P, RB, RBR, RFA, UEC, UFP, UPCB, and US herbaria (Thiers 2026 [continuously updated]) (Table S1).

The final dataset comprises 101 specimens of *M. vacciniifolia* and 14 of *M. crispata* as the ingroup, with 31 of *M. geminata* and 36 specimens of *M. mauritiana* serving as the outgroup.

Morphological characters and measurements– We measured nineteen quantitative characters or linear morphometric analysis: nine from sterile leaves, nine from fertile leaves, and one from the rhizome (Table 1, Figure 2). Linear measurements were obtained from specimen images using the ImageJ® software (Schneider et al. 2012). Each image was calibrated once based on its included scale. Landmarks were placed before measurement and standardized whenever necessary (Figure 2). For each character, we took at least two measurements. The resulting morphological data matrix was then standardized with the mean of each variable and scaled by its standard deviation.



#		Sterile leaf (abbrev.)	Fertile leaf (abbrev.)
1	Length	LSL	LFL
2	Width at half length	WHSL	WHFL
3	Max width	MWSL	MWFL
4	Distance base to max width	DBSL	DBFL
5	Blade apex angle	BASL	BAFL
6	Blade base angle	BBSL	BBFL
7	Symmetry angle	SASL	SAFL
8	Petiole length	PLSL	PLFL
9	Petiole diameter	PDSL	PDFL
Rizome			
10	Rhizome diameter	RD	

Figure 2 – Drawing of fertile (left) and sterile (right) leaves showing the nineteen morphological characters measured. Fertile leaf (red circles): 1 - LFL = Length; 2 - WHFL = Leaves width at half its length; 3 - MWFL = Maximum leaves width; 4 - DBFL = Distance between blade base and maximum width; 5 - BAFL = Blade apex angle; 6 - BBFL = Blade base angle; 7 - SAFL = Symmetry angle; 8 - PLFL = Petiole length; and 9 - PDFL = Petiole diameter. Sterile leaf (blue circles): 1 - LSL = Length; 2 - WHSL = Leaves width at half its length; 3 - MWSL = Maximum leaves width; 4 - DBSL = Distance between the blade base and the maximum width; 5 - BASL = Blade apex angle; 6 - BBSL = Blade base angle; 7 - SASL = Symmetry angle; 8 - PLSL = Petiole length; and 9 - PDSL = Petiole diameter. Rhizome (grey circle): 10 - RD = Diameter.

***Statistical analyses*–** The analyses were performed using the programming language R in the RStudio v.4.2.3 environment (Posit Team 2026). The 'fviz()' function of the *Factoextra* package (Kassambara & Mundt 2020) was used to perform the Principal Component Analysis (PCA) as an exploratory data analysis. To further explore morphological variation, a three-dimensional PCA was performed using the package *plotly* (Sievert 2020). We performed PCA on two datasets: one with all four species of the Vacciniifolia clade, and another with only *M. crispata* and *M. vacciniifolia*. For these two species, we further investigated the relationship between morphological variation (Euclidean distance on raw morphological variables) and

geographic distance (Haversine distance) using a Mantel test with Pearson's correlation and 999 permutations, using the *vegan* package (Oksanen et al. 2026).

Table 1 – Characters used in the morphometric analysis. Sterile leaf length (LSL), Leaf width at half length (WHSL), Maximum leaf width (MWSL), Distance between the blade base and the maximum width (DBSL), Blade apex angle (BASL), Blade base angle (BBSL), Symmetry angle (SASL), Petiole length (PLSL), and Petiole diameter (PDSL).

Character	Sterile leaf (code)	Fertile leaf (code)	Measurement unit
1. Length	LSL	LFL	cm
2. Leaf width at half its length	WHSL	WHFL	cm
3. Maximum leaf width	MWSL	MWFL	cm
4. Distance between blade base and the maximum width	DBSL	DBFL	cm
5. Blade apex angle	BASL	BAFL	Degrees
6. Blade base angle	BBSL	BBFL	Degrees
7. Symmetry angle	SASL	SAFL	Degrees
8. Petiole length	PLSL	PLFL	cm
9. Petiole diameter	PDSL	PDFL	cm
	Rhizome		
10. Diameter	RD		cm

Following exploratory data analysis, a nonparametric Kruskal-Wallis test (Conover 1999) was used to assess differences in the distribution of the quantitative variables among species. Statistical differences in measured characters among the morphotypes of the Vacciniifolia clade were taken as evidence of discontinuity in their morphological variation. We then used a Discriminant Analysis of Principal Components (DAPC) to classify individuals using our four-species hypotheses as priors, and to visualize group separation based on the nineteen morphological characters, using the *adegenet* package, v. 2.1.11 (Jombart 2008). To

statistically evaluate the degree of morphological divergence between the species, the scores of the first two discriminant axes (LD1 and LD2) were compared using a One-way Analysis of Variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) post-hoc test to identify specific pairwise differences between taxa ($p < 0.05$).

The raw data, reproducible R scripts, and complete analysis outputs are available at https://github.com/labevofern/research/Paiva et al._Morphometrics_Vacciniifolia_clade

3. RESULTS

In the analysis including all four species, the first two axes of Principal Component Analysis explained 43.3% of the total variance (PC1 30.5% and PC2 12.8%; Figure 3A–B; Table S2). PC1 showed a gradient of separation among species, with *M. geminata* and *M. mauritiana* positioned mostly on the right side and *M. crispata* and *M. vacciniifolia* on the left side of the first axis (Figure 2A). This separation was driven by leaf dimensions of both fertile and sterile leaves, including lengths and widths (LSL, LFL, MWFL, MWSL, WHFL, and WHSL), as well as petiole length (PFLF and PLSL; Figure 3B). These traits were positively correlated with PC1 and were more pronounced in *M. geminata*. PC2 further separated the species, distinguishing *M. vacciniifolia*, which extended toward the negative end of the axis. This separation was strongly associated with the apex and base angles of both fertile and sterile leaves (BBFL, BBSL, BAFL, and BASL; Figure 3B). Although *M. geminata* and *M. mauritiana* exhibited considerable overlap in the morphospace, *M. vacciniifolia* showed the greatest intraspecific variation, as indicated by the larger area of its 95% confidence ellipse (Figure 3A–B). PC3 contributed an additional 7.2%, increasing the cumulative variance to 50.5%, and confirmed the overlap pattern of *M. geminata* + *M. mauritiana* and *M. crispata* + *M. vacciniifolia* (Table S2; interactive figure available at https://github.com/labevofern/reserarch/Paiva et al._Morphometrics_Vacciniifolia_clade).

In the PCA restricted to the two sister and morphologically close species, *M. crispata* and *M. vacciniifolia*, the first two principal components accounted for 43.2% of the total morphological variance (PC1 30.4%; PC2 12.8%; Figure 3C–D). The PCA biplot revealed broad overlap in the morphospace occupied by both species, indicating that they share a similar phenotypic range for the measured traits. Except for base and apex angles of fertile and sterile leaves, and the symmetry angle of fertile leaves, all other variables were drivers of variation along the first axis (Figure 3D). PC3 contributed an additional 10.8%, increasing the total explained variance to 54% (Table S2). The three-dimensional morphospace confirmed that even with the inclusion of a third axis, the two taxa maintain a high degree of overlap (Table S2, interactive figures available at https://github.com/labevofern/research/Paiva et al._Morphometrics_Vacciniifolia_clade).

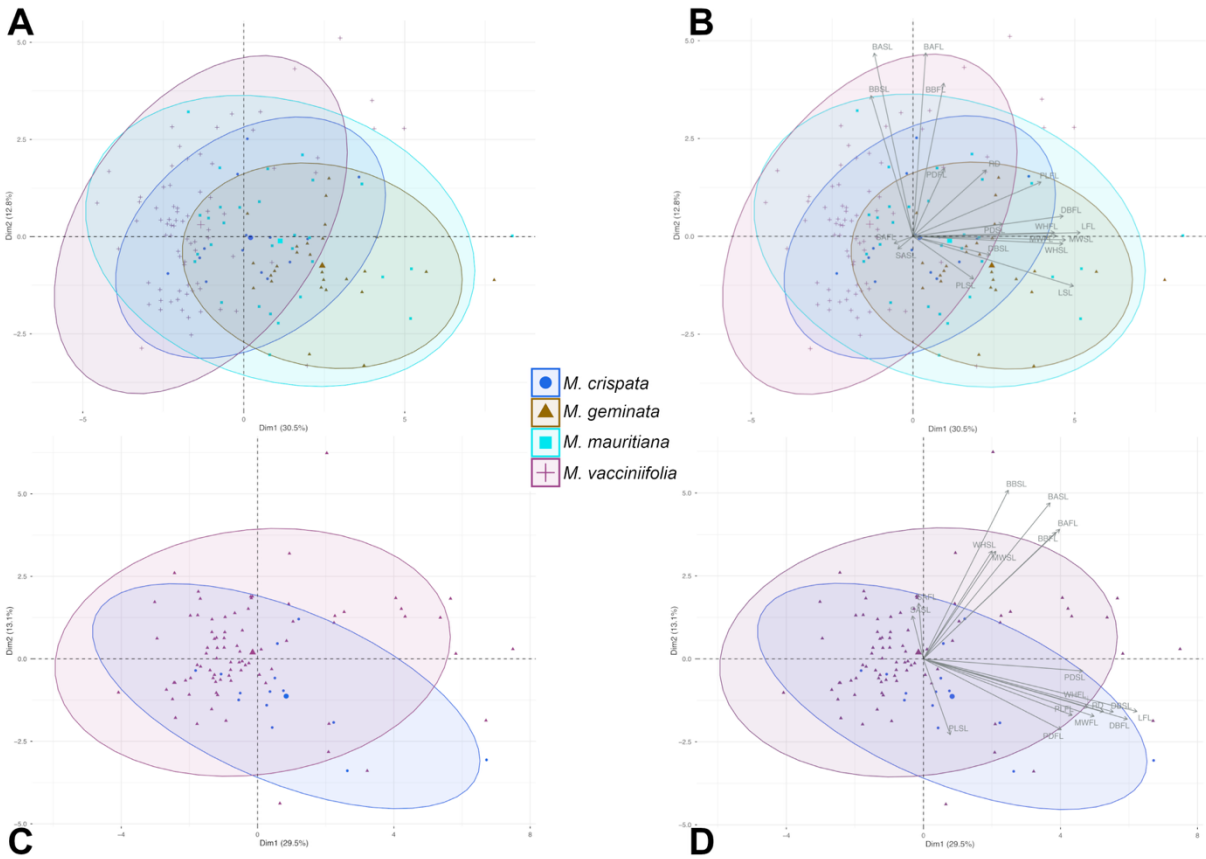


Figure 3. Principal Component Analysis (PCA) of leaf morphometric traits among *M. vacciniifolia* clade species. A–B. Analysis including the four species. A. Individual specimen

distribution. B. Individual specimen distribution showing a variable biplot. C–D. Analysis including only *M. crispata* and *M. vacciniifolia*. (C) Individual specimen distribution. D. Individual specimen distribution showing a variable biplot. Ellipses represent the 95% confidence interval for each group. Grey vectors indicate the direction and magnitude of each morphometric variable's contribution to Dimensions 1 (Dim1) and 2 (Dim2). Percentages on the axes represent the proportion of total variance explained by each principal component. *Microgramma crispata* (blue circles), *M. geminata* (brown triangles), *M. mauritiana* (cyan squares), and *M. vacciniifolia* (purple crosses).

The Mantel test revealed a weak and non-significant correlation between morphological and geographic distances ($r = 0.081$, $P = 0.099$). To investigate whether the observed phenotypic overlap between *M.* crispata and *M. vacciniifolia* corresponds to areas where they occur in sympatry (Figure 1D), we mapped the individuals that overlapped in the PCA (Figure 3C–D; Table S3–4). The results revealed no clear spatial structure; individuals with overlapping phenotypes were not restricted to sympatric zones.

The non-parametric Kruskal-Wallis test confirmed significant differences ($p < 0.05$) in 15 out of the 19 quantitative variables analyzed (Table S5; Figure S1–S2). Significant morphological differences were observed among the four species, though the patterns varied between sterile and fertile leaves (Table S5). For sterile leaves, highly significant differences ($p < 0.001$) were found in all measured variables, except petiole diameter (PDSL) and symmetry angle (SASL), which were still significant ($p < 0.05$; Table S5). A similar pattern was found for the fertile leaves, where blade size traits, including lengths and widths, were highly significantly different among species ($p < 0.001$). However, other fertile leaf traits showed less variation: petiole diameter (PDFL) was significant ($p < 0.05$), while the apex angle (BAFL), base angle (BBFL), symmetry angle (SAFL), and rhizome diameter (RD) did not differ significantly ($p > 0.05$). The lack of significant variation in these traits suggests they may be conserved across the clade.

In the Discriminant Analysis of Principal Components (DAPC; Figure 4), of the 19 principal component (PC) axes, the first fifteen axes and three discriminant functions were retained,

cumulatively accounting for nearly all (>99%) of the total morphological variance. ANOVA's performed on the discriminant function scores revealed significant morphometric differences among groups for both Axis 1 ($F = 110.5$; $p < 0.001$) and Axis 2 ($F = 26.62$; $p < 0.001$) (Table 2). Despite the spatial clustering in the scatterplot, the overall assignment success was 62.25%. *Microgramma vacciniifolia* exhibited the highest diagnostic stability with a 96.1% correct reassignment rate, followed by *M. geminata* (74.2%). In contrast, *M. mauritiana* showed significant morphometric overlap (55% correct reassignment), while *M. crispata* was not recovered as a morphologically cohesive group (35.7% reassignment).

Table 2 – Summary of DAPC (Discriminant Analysis of Principal Components) classification and assignment success for the four studied *Microgramma* species. Columns indicate the original taxonomic identification (N Prior), the number of individuals assigned to each cluster by the DAPC model (N Post), and the percentage of individuals correctly assigned.

Species	**N (Prior)**	**N (Post)**	**Assignment Success (%)**
M. crispata	14	8	35.7%
M. geminata	31	28	74.2%
M. mauritiana	36	27	55%
M. vacciniifolia	102	120	96.1%
Total	183	183	-

Tukey's HSD test (Table 3) for Axis 1 highlighted that *M. geminata* is significantly distinct from all other species ($p < 0.001$), and the only pair not showing significant separation along this axis was *M. crispata* vs. *M. mauritiana* ($p = 0.405$). For Axis 2, the primary differentiation occurred between *M. mauritiana* and *M. vacciniifolia* ($p < 0.001$), *M. mauritiana* and *M. geminata* ($p < 0.001$), and *M. mauritiana* and *M.crispata* ($p = 0.003$), while all other pairwise comparisons were non-significant. These results statistically support the observed overlap in the scatterplot, particularly between the *M. crispata* and *M.*

vacciniifolia (Table 3). Posterior membership probabilities confirmed this trend, showing that individuals morphologically identified as *M. geminata* had higher correct classification, with *M. crispata* and *M. mauritiana* displaying high levels of conflicting classification or were predominantly assigned to the *M. vacciniifolia* cluster (Figure 5).

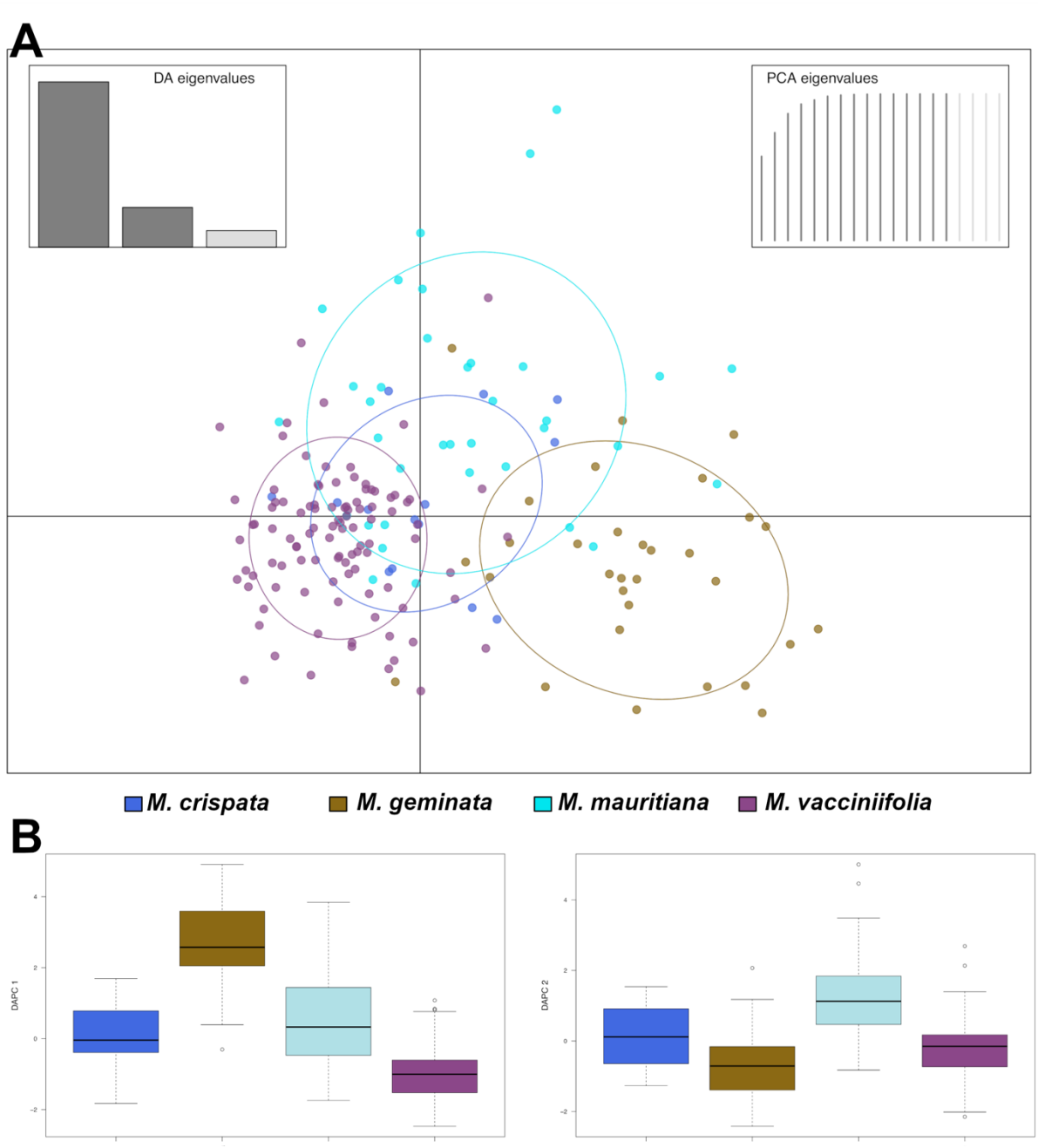


Figure 4 - Discriminant Analysis of Principal Components (DAPC) of the four *Microgramma* species. **A.** scatterplot of the first two DAPC discriminant functions, with points representing individuals and clusters color-coded by species. B. Boxplots illustrating the distribution of DAPC scores for discriminant functions 1 (left) and 2 (right) across the four *Microgramma* species. Blue: *M. crispata*; brown: *M. geminata*; cyan: *M. mauritiana*; purple: *M. vacciniifolia*.

Table 3 – Post-hoc Tukey's HSD pairwise comparisons of DAPC scores across the first two discriminant axes. Axis 1 represents the primary morphological differentiation (horizontal), while Axis 2 represents secondary differentiation (vertical). Significant values ($p < 0.05$) are indicated in bold.

Pairwise comparison	Difference (Axis 1)	p-adj (Axis 1)	Difference (Axis 2)	p-adj (Axis 2)
M. geminata vs. *M. crispata*	2.557	**< 0.001**	-0.818	0.057
M. mauritiana vs. *M. crispata*	0.491	0.405	1.119	**0.003**
M. vacciniifolia vs. *M. crispata*	-1.090	**0.001**	-0.425	0.444
M. mauritiana vs. *M. geminata*	-2.066	**< 0.001**	1.937	**< 0.001**
M. vacciniifolia vs. *M. geminata*	-3.647	**< 0.001**	0.393	0.225
M. vacciniifolia vs. *M. mauritiana*	-1.581	**< 0.001**	-1.544	**< 0.001**

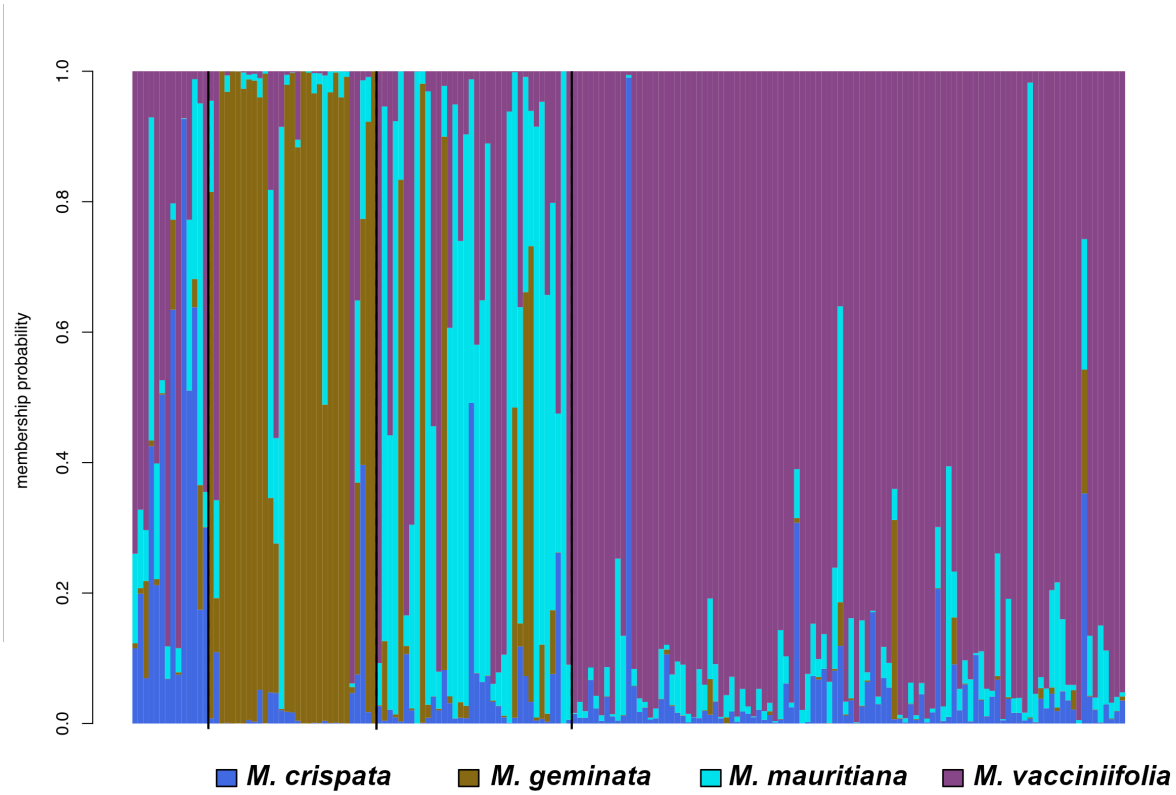


Figure 5 - DAPC membership probability. Each vertical bar represents a single individual, and the colors indicate the probability of assignment to each of the four *a priori* groups (species).

Black vertical lines separate individuals according to their a priori taxonomic classification, ordered from left to right: *M. crispata*, *M. geminata*, *M. mauritiana*, and *M. vacciniifolia*.

4. DISCUSSION

We confirm the high morphological complexity within the Vacciniifolia clade of *Microgramma*, as well as intraspecific variation within each taxon. Our results show that the four studied species tend to occupy different regions of the morphospace based on leaf proportions, although considerable morphological overlap persists among them (Figure 3). The sister species *M. crispata* and *M. vacciniifolia* show a high degree of morphological similarity, which was not associated with geographic sympatry. Significant differences among species were found in 15 of the 19 quantitative variables, indicating partial morphological differentiation among taxa. However, the DAPC showed contrasting levels of discrimination, with *M. geminata* and *M. vacciniifolia* forming more distinct morphological clusters, while *M. crispata* and *M. mauritiana* showed high levels of overlap and frequent misclassification. The exploratory PCA captured nearly half the morphological variation, suggesting that leaf morphology is complex and likely influenced by other factors not fully captured in a two-dimensional morphospace. It showed that the distribution of the studied species in the morphospace is primarily organized by a gradient in leaf size, or allometric scaling (PC1, 30.5%; Figure 3). Length and width of both fertile and sterile leaves, together with petiole length, make size a major distinguishing factor among the species. *Microgramma geminata* stands out in this axis due to its longer petioles and larger laminar area (Figure 3). However, size alone does not account for total morphometric diversity found in the clade; the second axis (PC2, 12.8%; Figure 3) reveals more geometric variation. This axis is defined mainly by leaf base and apex angles. *Microgramma vacciniifolia* shows the broadest morphospace occupation in this dimension, with a defined morphological distinction and the highest intraspecific shape variation. This broad morphological variation may be associated with

genetic variation or phenotypic plasticity (Duminil & DiMichele 2009, Bünger et al. 2015, Gaem et al. 2022) and might correlate with the species' wide distribution across the entire Atlantic Forest, as well as areas of the Chaco, the Andes, and northern South America (Almeida 2014, 2026).

Microgramma geminata is, morphologically, the most distinct species in the Vacciniifolia clade (Table 3; Figure 4B). In addition to its distinct size, the species can be recognized by several categorical traits, including its monomorphic leaves, commonly born in short, lateral rhizome branches, and its impressed sori, which form circular projections on the adaxial laminar surface (Almeida 2014, 2026). Within the clade, it overlaps more in the morphospace with the afrotropical *M. mauritiana* (Figure 3), but those species occur in allopatry.

As currently circumscribed (Almeida 2014), *M. mauritiana* displays considerable intraspecific morphological variation. This variation, captured by its broad morphospace occupation, both in the shape and size dimensions (Figure 3), may explain both its poor classification performance (Table 3) and the overall lower distinction from the other species in the clade (Figures 3-4). Our results here further support the recognition of *M. mauritiana* as a species complex. For the afrotropical populations of *Microgramma*, further phylogenetic studies with expanded sampling and additional tools are required to understand its circumscription and its morphological relationships.

Our analysis also confirmed that *Microgramma crispata* is morphologically very close to *M. vacciniifolia* and that morphological features such as total length of sterile leaves (LSL), angle of the base of fertile leaves (BBFL), angle of symmetry of fertile leaves (SASL), and other angle measurements are not informative enough to discriminate between them. The spatial proximity observed even in a higher-dimensional space (3D-PCA, interactive figure available at https://github.com/labevofern/research/Paiva et al._Morphometrics_Vacciniifolia_clade), further suggests that the morphological boundaries between *M. crispata* and *M. vacciniifolia*

are not discrete, given the data sampled in this work. Our results also showed no correlation between geographical proximity and observed morphological overlap between the specimens of both species.

According to Almeida (2014), *M. crispata* and *M. vacciniifolia* are usually distinguished by differences in the length proportions of fertile versus sterile leaves, and by the shape of the leaf base and apex. However, our results show substantial overlap in those traits, in addition to broad shape variation in *M. vacciniifolia*. The only other constant trait used to separate the species is scale position on the rhizome (patent in *M. crispata* and usually appressed in *M. vacciniifolia*). Considering issues in taxonomy to delimit species in different plant groups, morphometric analysis acts as an essential tool in the discrimination between species through the determination of morphological characters that contribute to their delimitation (Pinheiro & Barros 2007; Melo & Borba 2011; Kaplan and Marhold 2012; Baleeiro et al. 2016; Leal et al. 2016; Ramírez-Valencia and Sanín 2017; Sanín et al. 2019). Furthermore, as added by Dayrat (2005), species with wide morphological variation must be analyzed to avoid distorted interpretations, such as the recognition of new species that can result in larger taxonomic problems within the group.

In the study of spectral signatures in *Microgramma* of Paiva et al. (2021), *M. crispata* and *M. vacciniifolia* were found to be distinct. The confusion matrices from all discriminant analyses and cross-validation methods applied to the spectral data showed no misidentification errors between these species (Paiva et al. 2021). This may indicate structuring in the spectral characteristics of these species, but the result should be interpreted with caution: the study investigated 13 species from distinct clades within the genus, and sampling was not sufficiently broad or geographically representative for *M. crispata* and *M. vacciniifolia* (Paiva et al. 2021). Ideally, a wide spectral sampling with a larger number of specimens is needed to understand the spectral differentiation between them.

Sympatry, together with ecological aspects related to species distribution, can explain the observed morphological overlap (*e.g.*, Baleeiro et al. 2016). *Microgramma vacciniifolia* has a wide geographical distribution (Figure 1), while *M. crispata* shows a more restricted distribution overlapping with the extent of occurrence of *M. vacciniifolia* (Figure 1E). The overlap observed in the analysis can indicate the clinal nature of the morphological traits in question. Widespread species can display phenotypic plasticity to adapt and survive across broad geographic and ecological gradients (Sultan 2000), and other morphometric studies have shown that widespread plants can exhibit extensive morphological variation (*e.g.*, Pinheiro & Barros 2007, Ribeiro et al. 2007, Bünger et al. 2015).

Furthermore, as sister species occurring sympatrically, the occurrence of phenomena like hybridization and introgression cannot be dismissed (Pinheiro & Barros 2007, Zhang et al. 2013, Mendez-Reneau et al. 2024), and should be investigated. The existence of hybrids in the genus has already been suggested. *Microgramma mortoniana* de la Sota, for example, has been described as a hybrid between *M. squamulosa* and *M. vacciniifolia* (Sota 1973). Although *M. mortoniana* has been reported as an allotetraploid (Sota & Pazos 1980), this hypothesis is yet to be tested genetically. Its recognition is mainly based on distribution overlap and intermediate states of morphological traits (Sota 1973). Like *M. crispata* and *M. vacciniifolia*, other *Microgramma* species also exhibit different levels of overlap, such as *M. reptans* and *M. tobagensis* (Smith et al. 2018; Mendonça et al. 2026).

The extensive overlap observed between *M. crispata* and *M. vacciniifolia* indicates that leaf morphology alone may be insufficient to discriminate these taxa. This pattern may reflect recent divergence, retention of ancestral traits, or elevated phenotypic plasticity within the clade. In the absence of information on reproductive aspects such as pre- and post-zygotic incompatibility or reproductive isolation, one of the most reliable approaches to understanding real discontinuities between lineages is through molecular phylogenetic

hypotheses (de Queiroz 2007). Subsequently, based on these lineages, one can search for correlated morphological discontinuities to obtain informative diagnostic characters.

Given that the morphometric approach was not fully capable of establishing clear limits for *M. crispata* and *M. vacciniifolia* and the sympatric condition involved, we encourage the relationships between both and their delimitation to be explored with the association of different tools. Future studies should incorporate broader infraspecific and population-level sampling combined with genomic-scale approaches. Such strategies have proven effective in revealing evolutionary patterns and lineage boundaries that may remain undetected using morphology alone (*e.g.*, Kao et al. 2020; Mendez-Reneau et al. 2023; 2024). In addition, integrating genomic data with other sources of evidence at comparable scales, such as spectral, morphometrics, and cytogenetic data, may provide a more comprehensive understanding of species limits and evolutionary relationships (Prata et al. 2018; Damasco et al. 2019; Riina et al. 2020; Damasco et al. 2021). Near-infrared spectroscopy (NIR), for example, has emerged as a reliable and cost-effective tool for rapid species identification and discrimination (Lang et al. 2017; Prata et al. 2018; Damasco et al. 2019; Paiva et al. 2021; Mendonça et al. 2026).

5. CONCLUSION

Our results indicate that, within the Vacciniifolia clade, *M. geminata* and *M. mauritiana* can be recognized as morphologically distinct species, and that *M. mauritiana*'s broad variation should be investigated in depth. Although *M. crispata* and *M. vacciniifolia* can be recognized as distinct taxa with the support of some morphological traits, there is a high degree of morphological overlapping between those species. We suggest the use of integrative systematic techniques as an aid in delimiting this and other taxonomically complex groups.

6. AUTHORS CONTRIBUTIONS

Conceptualization: TEA and DNAP; Methodology: DNAP, JVA, and TEA; Formal analysis and investigation: DNAP, JVA, and TEA; Writing - original draft preparation: DNAP, JVA, and TEA; Writing - review and editing: DNAP, JVA, and TEA; Supervision: TEA.

7. DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study, including raw data, processed data, and R analysis code, are publicly available on GitHub: https://github.com/labevofern/research/Paiva_et_al._Morphometrics_Vacciniifolia_clade.

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9. CONFLICTS OF INTERESTS

The authors declare no conflicts of interests no conflicts of interest (personal, scientific, commercial, political, or financial).

10. FUNDING INFORMATION

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11. SUPPLEMENTARY MATERIAL

The following online material is available for this article:

Table S1 –Voucher specimens used in this study.

Table S2 – Standard deviation, proportion of variance, and cumulative proportion of variance of PCA components, for all four species, and for *M. crispata* vs. *M. vacciniifolia* only.

Table S3 – List of specimens of *M. crispata* and *M. vacciniifolia* that overlapped in the PCA, with their respective country of origin and geographic coordinates.

Tables S4 – List of specimens of *M. crispata* and *M. vacciniifolia* that did not overlap in the PCA, with their respective country of origin and geographic coordinates.

Table S5 – Kruskal–Wallis test results for 19 morphological characters (sterile leaves, fertile leaves, and rhizome), showing chi-square statistics, degrees of freedom, p-values, and significance levels for each character.

Figure S1 - Variation in sterile leaves among *M. crispata*, *M. geminata*, *M. mauritiana*, and *M. vacciniifolia*: Sterile leaf length (LSL), Leaf width at half length (WHSL), Maximum leaf width (MWSL), Distance between the blade base and the maximum width (DBSL), Blade apex angle (BASL), Blade base angle (BBSL), Symmetry angle (SASL), Petiole length (PLSL), and Petiole diameter (PDSL). The boxplots illustrate the distribution, with the central line within each box representing the median, while the upper and lower boundaries of the box indicate the first and third quartiles, respectively. Whiskers extend to 1.5 times the interquartile range, and individual points represent outliers. *Microgramma crispata* (blue), *M. geminata* (yellow), *M. mauritiana* (green), and *M. vacciniifolia* (purple).

Figure S2 - Variation in sterile leaves among *M. crispata*, *M. geminata*, *M. mauritiana*, and *M. vacciniifolia*: Length (LFL), Leaf width at half length (WHFL), Maximum leaf width (MWFL), Distance between blade base and maximum width (DBFL), Blade apex angle (BAFL), Blade base angle (BBFL), Symmetry angle (SAFL), Petiole length (PLFL), Petiole diameter (PDFL), and Rhizome diameter (RD). The boxplots illustrate the distribution, with the central line within each box representing the median, while the upper and lower

boundaries of the box indicate the first and third quartiles, respectively. Whiskers extend to 1.5 times the interquartile range, and individual points represent outliers. *Microgramma crispata* (blue), *M. geminata* (yellow), *M. mauritiana* (green), and *M. vacciniifolia* (purple).

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