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DARLEM NIKERLLY AMARAL PAIVA

DISCRIMINAÇÃO DE ESPÉCIES DE SAMAMBAIAS NEOTROPICAIS (POLYPODIACEAE): UMA ABORDAGEM INTEGRATIVA

SANTARÉM-PA
2021

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Dissertação apresentada ao Programa de Pós graduação em Biodiversidade – PPGBEES, da Universidade Federal do Oeste do Pará – UFOPA, como parte dos requisitos para obtenção do título de mestre.

Orientadora: Thaís Elias Almeida

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Para meus pais: Paulo &
Domingas

Dedico

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“Se consegui enxergar mais longe é porque estava apoiado sobre ombros de gigantes”
Isaac Newton

RESUMO

A combinação de técnicas capazes de discriminar espécies de plantas de difícil circunscrição é essencial para a compreensão da biodiversidade. O gênero *Microgramma* C.Presl (Polypodiaceae), por exemplo, devido à grande complexidade de formas associada à ampla área de ocorrência, torna-se um grupo altamente sugestivo à investigação taxonômica. Assim, temos por objetivo propor processos de discriminação em espécies de samambaias neotropicais por meio de ferramentas integrativas de grande interesse no campo científico taxonômico. Utilizamos a Espectroscopia no Infravermelho Próximo (FT-NIR) em 13 espécies (*M. baldwinii*, *M. crispata*, *M. dictyophylla*, *M. geminata*, *M. lindbergii*, *M. lycopodioides*, *M. megalophylla*, *M. percussa*, *M. persicariifolia*, *M. reptans*, *M. squamulosa*, *M. thurnii* e *M. vacciniifolia*) e análises morfométricas em quatro espécies (*M. crispata*, *M. geminata*, *M. mauritiana* e *M. vacciniifolia*). Estes são métodos importantes para a discriminação de espécies botânicas em associação com análises multivariadas, como Análise Discriminante, validações cruzadas, Análise Discriminante Canônica (ADC) e Análise de Cluster (AC). Com base em análises multivariadas, a técnica morfométrica suportou a existência de todos os táxons previamente reconhecidos, bem como a Espectroscopia no Infravermelho Próximo (FT-NIR), com a qual recuperamos mais de 95% da previsão correta. Ambas as técnicas aplicadas demonstraram um alto potencial de discriminação entre as espécies propostas, demonstrando que o uso de diferentes abordagens pode favorecer no esclarecimento e discriminação entre grupos controversos.

Palavras-Chave: Discriminação de Espécies Botânicas. FT-NIR. Morfometria. Samambaias Neotropicais. Biologia Integrativa.

ABSTRACT

The combination of techniques capable of discriminating plant species that are difficult to circumscribe is essential to the proper understanding of biodiversity. The genus *Microgramma* C.Presl (Polypodiaceae), for example, due to the great complexity of forms associated with the wide area of occurrence, becomes a group highly suggestive to the taxonomic investigation. Thus, we aim to propose processes of discrimination in species of neotropical ferns through integrative tools of great interest in the taxonomic field. We used near-infrared spectroscopy (FT-NIR) in thirteen species (*M. baldwinii*, *M. crispata*, *M. dictyophylla*, *M. geminata*, *M. lindbergii*, *M. lycopodioides*, *M. megalophylla*, *M. percussa*, *M. persicariifolia*, *M. reptans*, *M. squamulosa*, *M. thurnii* and *M. vacciniifolia*) and morphometric analysis with four species (*M. crispata*, *M. geminata*, *M. mauritiana*, and *M. vacciniifolia*). These are important methods for the discrimination of botanical species in association with multivariate analyzes, such as Discriminant Analysis, cross-validations, Canonical Discriminant Analysis (CDA), and Cluster Analysis (CA). Based on multivariate analyzes, the morphometric technique supported the existence of all previously recognized taxa, as well as the Near-Infrared Spectroscopy (FT-NIR), with which we recovered more than 95% of correct prediction. Both applied techniques demonstrated a high potential for discrimination between the proposed species, demonstrating that the use of different approaches can favor clarification and discrimination between controversial groups.

Keywords: FT-NIR. Discrimination of Plant Species. Integrative Biology. Morphometrics. Neotropical Ferns.

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INTRODUÇÃO GERAL

Discriminação de espécies de Samambaias Neotropicais[1]

O que é a pesquisa?

De acordo com o conceito biológico, espécies constituem populações que são capazes de se reproduzir e originar descendentes férteis. Apesar de muitos debates conceituais sobre a temática, ainda imperam como principais unidades de pesquisa para a classificação da biodiversidade e estudos evolutivos.

Para muitos grupos de organismos, a delimitação de espécies é um desafio. Complexos de espécies de difícil diferenciação, que apresentam variação intraespecífica e introgressão interespecífica (processo responsável pelo fluxo gênico entre indivíduos diferentes), são cenários observados em plantas, agravando a delimitação em grupos distintos.

A taxonomia é um ramo da ciência que necessariamente reúne diferentes evidências para a classificação das espécies, resultado da associação de múltiplas áreas da ciência. Nesse sentido, inúmeros estudos interessados na identificação e esclarecimento das relações dentro e entre espécies têm empregado a biologia integrativa, o que inclui o emprego de diferentes ferramentas na avaliação de limites de espécies.

Neste trabalho, utilizamos um conjunto de espécies do grupo das samambaias, do gênero *Microgramma* (Figuras 1-14), notavelmente caracterizadas pela variação morfológica e ampla distribuição geográfica de suas espécies. O gênero é considerado monofilético, ou seja, todas as espécies desse gênero descendem de um mesmo ancestral comum, porém, várias espécies são controversas no que concerne à classificação genérica, apresentando uma série de problemas taxonômicos, além do reconhecimento de novas espécies. Logo, as espécies selecionadas podem representar linhagens que ainda não estão completamente separadas, ou com barreiras reprodutivas ainda incipientes, os chamados complexos de espécies. Nesse contexto, temos por objetivo investigar se há diferenças a ponto de indicar a presença de novas espécies entre os indivíduos estudados, ou alternativamente tratar-se de espécies altamente variáveis quanto a morfologia.

Como a pesquisa foi realizada ?

Com o intuito de integrar ferramentas que auxiliem no esclarecimento de relações entre as espécies, usamos abordagens como o estudo morfométrico, método numérico amplamente utilizado na avaliação da variação morfológica, e a Espectroscopia no Infravermelho Próximo (NIR), técnica conhecida como impressão digital das folhas e atualmente muito usada na identificação de diferentes grupos de organismos, como é o

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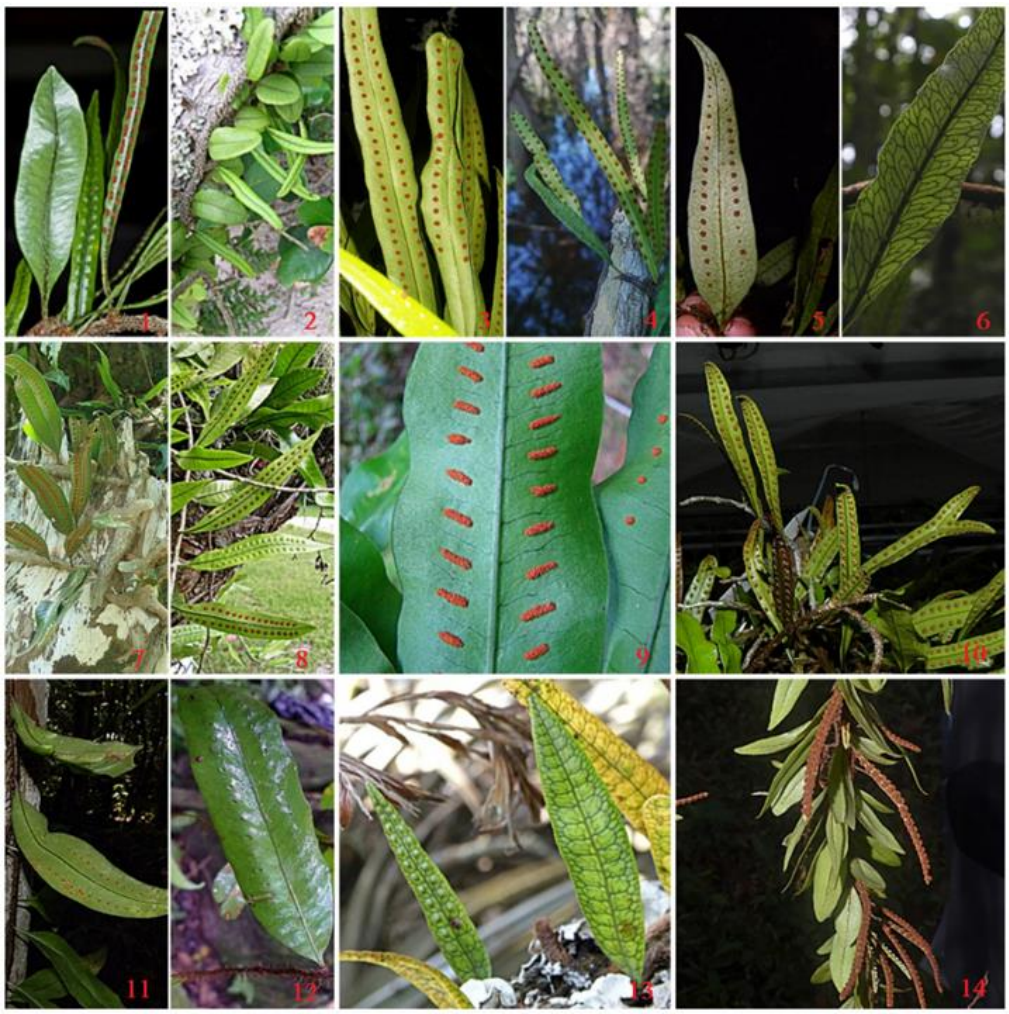
[1] Texto de comunicação científica formatado de acordo com as normas do canal ciência – Portal de Divulgação Científica e Tecnológica, do Instituto Brasileiro de Informação em Ciência e Tecnologia (Ibict).

caso das plantas. Para a coleta de dados morfométricos, realizamos medidas em caracteres morfológicos de exsicatas digitalizadas com o auxílio do Software ImageJ® Powerful Image Analysis, de domínio público. Essas medidas foram efetuadas em folhas férteis e estéreis (comprimento, largura, medidas de ângulo e medidas associadas a estruturas reprodutivas e nervuras).

Por meio da Espectroscopia no Infravermelho Próximo (NIR), obtivemos leituras (espectros foliares) das espécies selecionadas a partir de exsicatas depositadas nos herbários HSTM e INPA. Após a definição do número de leituras a serem coletadas, conduzimos a obtenção dessas leituras nas faces da folha tanto superior quanto inferior através do aparelho denominado espectrofotômetro. Os dados resultantes da morfometria e NIR foram posteriormente testados por análises estatísticas.



Figuras 1-14. Representantes do gênero *Microgramma*. 1. *Microgramma crispata* (T. E. Almeida); 2. *Microgramma vacciniifolia* (T. E. Almeida); 3. *Microgramma percussa* (C. N. Fraga); 4. *Microgramma baldwinii* (W. Milliken); 5. *Microgramma dictyophylla* (T. E. Almeida); 6. *Microgramma squamulosa* (T. E. Almeida); 7. *Microgramma megalophylla* (T. E. Almeida); 8. *Microgramma geminata* (T. E. Almeida); 9. *Microgramma persicariifolia* (T. E. Almeida); 10. *Microgramma mauritiana* (T. E. Almeida); 11. *Microgramma thurnii* (T. E. Almeida); 12. *Microgramma lindbergii* (W. Milliken); 13. *Microgramma lycopodioides* (T. E. Almeida); 14. *Microgramma reptans* (T. E. Almeida).

Qual a importância da pesquisa?

A determinação e classificação correta das espécies é de suma importância para entender como a biodiversidade se distribui. As samambaias, por exemplo, constituem um dos grupos chave na dinâmica florestal e nos processos ao longo da evolução em plantas. Deste modo, os resultados aqui apresentados, ao utilizar novas abordagens de discriminação entre espécies, não testadas até o desenvolvimento desta pesquisa para espécies específicas de samambaias, contribuem para o esclarecimento de relações entre plantas evolutivamente próximas deste e de outros grupos futuramente estudados, além de contribuir para o aumento no nível de conhecimento da flora neotropical.

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2. Capítulo 1

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Using near-infrared spectroscopy to discriminate closely related species: A case study of neotropical ferns

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ABSTRACT

Identifying plant species requires considerable knowledge and can be difficult without complete specimens. Fourier-transform near-infrared spectroscopy (FT-NIR) is an effective technique for discriminating plant species, especially angiosperms. However, its efficacy has never been tested on ferns. Here we tested the accuracy of FT-NIR at discriminating species of the genus *Microgramma*. We obtained 16 spectral readings per individual from the adaxial and abaxial surfaces of 100 specimens belonging to 13 species. The analyses included all 1557 spectral variables. We tested different datasets (adaxial+abaxial, adaxial, and abaxial) to compare the correct identification of species through the construction of discriminant models (LDA, PLS) and cross-validation techniques (leave-one-out, K-fold). All analyses recovered an overall high percentage (>90 %) of correct predictions of specimen identifications for all datasets, regardless of the model or cross-validation used. On average, there was > 95 % accuracy when using PLS-DA and both cross-validations. Our results show the high predictive power of FT-NIR at correctly discriminating fern species when using leaves of dried herbarium specimens. The technique is sensitive enough to reflect species delimitation problems and possible hybridization, and it has the potential of helping better delimit and identify fern species.

Keywords: Barcoding; Discrimination of plant species; FT-NIR; Integrative taxonomy; Metabolomics; *Microgramma*.

INTRODUCTION

Defining and identifying species using qualitative morphological traits can be challenging even though species identification is fundamental to some areas of science and sustainable dynamics (Galtier 2018; Pinheiro et al. 2018). Correct identifications also contribute significantly to understanding the evolutionary history of many species and the diversity of biological groups in rich and threatened areas, such as tropical forests (Costello 2015). Considering the biological and historical diversity of polymorphisms in plants, allied with centuries of describing species using alpha taxonomy tools, the correct identification of a specimen requires experts with considerable knowledge (Ahrends et al. 2011; Lacerda and Nimmo 2010; Richard and Evans 2006).

A problem when identifying plant species is the absence of complete specimens, including both sterile and fertile material, such as flowers or fruits of seed plants (Gomes et al. 2013). Difficult to access and insufficient or unrepresentative collections of species widely distributed in highly diverse areas can also pose a problem when identifying specimens (Lacerda and Nimmo 2010). Among the traditional identification methods used for plants, keys stand out and are widely employed (Smith 2017). However, polymorphisms and the complexity of shapes, associated with homoplasies and cryptic taxa, for example, create the need for more elaborate tools aimed primarily at the identification, conservation, and elucidation of unclear relationships of plants (Durgante et al. 2013; Pinheiro et al. 2018).

In addition to the use of macromorphology, DNA barcoding is an internationally recognized tool and widely used in species identification, ecological studies, and forensic analyses (Li et al. 2015; Shokralla et al. 2014). In studies of animal groups that used this molecular approach, the technique proved to be highly efficient

(e.g., Ohira et al. 2018; Pérez-Losada et al. 2012; Porco et al. 2012). Using DNA barcoding has been less successful at identifying plants compared to animals (Li et al. 2015). According to Fazekas et al. (2012), this is partially due to hybridization, polyploidy, and speciation related to reproductive systems. However, these are not problems common to all plant groups; the success in using DNA barcoding is lineage-dependent (Li et al. 2015). Identifying herbarium specimens using this method is also more difficult compared to fresh material, since DNA extracted from dried specimens is usually fragmented, requiring a greater combination of primers, which increases the chances of incorrect sequencing (Li et al. 2015; Vere et al. 2012). Furthermore, the widespread use of this technique is still limited because of the high cost (Stein et al. 2014).

One of the most promising alternative tools currently used in botanical identification is Fourier transformed near-infrared spectroscopy (FT-NIR) (e.g., Lang et al. 2017; Rodríguez-Fernandez et al. 2011). The principle of the technique is to irradiate fractions of biological material (e.g., a dry leaf) in the infrared region. As a result, a set of absorbance values at different wavelengths (the spectra) is defined for the material (Workman and Weyer 2007). The spectra reflect molecular bonds, such as C-H, N-H, S-H or O-H, and are therefore related to biological molecules and the metabolome of the irradiated tissue (Stuart 2005; Asner et al. 2014).

Research using near-infrared spectroscopy to discriminate plant species is gaining more and more attention in plant taxonomy, especially for angiosperms (Durgante et al. 2013; Kim et al. 2004; Krajšek et al. 2008; Lang et al. 2017). The tool has been shown to be more practical and accurate than genetic or morphological methods (Castillo et al. 2008), is capable of consistently discriminating phylogenetic relationships of flowering plant species (Kim et al. 2004) and has been used in different

works to aid in species circumscription and identification of several plant groups (Damasco et al. 2019; Durgante et al. 2013; Lang et al. 2017; Meireles et al. 2020; Prata et al. 2018; Shen et al. 2020). However, FT-NIR has not been tested to identify other groups of embryophytes, such as ferns and lycophytes, or bryophytes (Guzmán et al. 2020).

Ferns are the second most diverse group of vascular plants, occur from the tundra to tropical forests, and occupy niches from the ground to the canopy (Moran 2008). Due to the absence of flowers, fruits, and seeds, fern identification relies mainly on rhizome, frond, and sorus morphology (Tryon and Tryon 1982). Sporophyte characters such as indument (trichomes and scales), leaf shape, and the structure and arrangement of sori are fundamental elements in the differentiation between species (Christenhusz and Chase 2014; Tryon and Tryon 1982).

Microgramma (Polypodiaceae) comprises ca. 30 species, occurs in the Neotropics and tropical Africa (Almeida 2014), and is monophyletic according to the most recent circumscriptions (Almeida et al. in press; Salino et al. 2008). The genus exhibits wide morphological variation, especially in the leaves (e.g., it has both monomorphic and dimorphic species), leaf indument, and sorus arrangement (Almeida 2014). Additionally, intraspecific phenotypic variation and interspecific morphological overlap are found in closely related species, and there are species complexes, which may result in misidentifications in the genus (Almeida et al. in press). Using *Microgramma* as a model, our goal was to test the effectiveness of Fourier-transform near-infrared spectroscopy (FT-NIR) at discriminating and identifying closely related species in a fern lineage.

METHODS

Sampling–Dried leaves were selected from specimens at the BHCB, HSTM, and INPA herbaria (acronyms according to Thiers 2020 onwards: http://sweetgum.nybg.org/science/ih/). One hundred specimens belonging to thirteen species of *Microgramma* were analyzed (Table S1): *M. baldwinii* Brade, *M. crispata* (Fée) R.M.Tryon & A.F.Tryon, *M. dictyophylla* (Kunze ex Mett.) de la Sota, *M. geminata* (Schrad.) R.M.Tryon & A.F.Tryon, *M. lindbergii* (Mett. ex Kuhn) de la Sota, *M. lycopodioides* (L.) Copel., *M. megalophylla* (Desv.) de la Sota, *M. percussa* (Cav.) de la Sota, *M. persicariifolia* (Schrad.) C.Presl, *M. reptans* (Cav.) A.R.Sm., *M. squamulosa* (Kaulf.) de la Sota, *M. thurnii* (Baker) R.M.Tryon & Stolze, and *M. vacciniifolia* (Langsd. & Fisch.) Copel. All specimens had their identification confirmed by an expert (senior author). Only species with a minimum of five available specimens, with both fertile and sterile leaves, were selected. When possible, samples with fronds that were severly damaged by insects or with signs of fungi or other epiphylic organisms were avoided. Sixteen spectral readings were obtained for each specimen (when possible), which included four readings, two on the adaxial surface and two on the abaxial surface, of four different leaves. No distinction between fertile and sterile leaves was made. The acquisition of the spectra lasted 30 seconds per reading and was taken using a Thermo Nicolet spectrophotometer, FT-NIR Antaris II Method Development System (MDS). The spectral readings consisted of 1,557 leaf absorbance values in the region of 4,000 to 10,000 cm^{-1} (1000 to 2500 nm). Each measurement produced by the equipment was an average of 16 readings with a wavelength resolution of 8 cm^{-1}. The equipment was calibrated every 4 hours of use. A black body was placed over the frond to prevent light scattering.

Analyses–All analyses were implemented in the statistical program R version 4.0.2 (R Core Team 2020), under the framework provided by R package caret (Kuhn 2008), using the R packages MASS (Venables and Ripley 2002) and pls (Mevik et al. 2019). Three datasets using all FT-NIR spectrum wavelengths were tested to construct the spectral models: data of (i) adaxial+abaxial surfaces, (ii) adaxial surface only, and (iii) abaxial surface only. The datasets were explored using a principal component analysis (PCA). This technique allows the visualization of data of a smaller set of variables but still preserves the maximum information from the original variable set (Hongyu et al. 2016), thus allowing an exploratory analysis of the behavior of the spectra. The results of the PCA were represented in two-dimensional graphs using the first two main components.

To predict species based on spectral data, we used two supervised pattern recognition techniques: linear discriminant analysis (LDA) and partial least squares discriminant analysis (PLS-DA) (Berrueta and Héberger 2007). The LDA is a technique that discriminates and classifies objects based on previously defined groups (Sharma and Paliwal 2015), where the dependent variables corresponded to the species (categories) and the independent variables represent the absorbance values in the near-infrared. The PLS-DA, which also classifies the samples according to defined categories, is based on finding components that better explain the variations of the variables between classes, giving less weight to the noise and uncorrelated variations (Mevik and Cederkvist 2004). Both models were tested using the three different datasets.

Cross-validation techniques were used to assess model performance and species discrimination. The K-fold validation technique (Burman 1989) is where the set of calibration samples is divided into K subsets, with a subset taken out for validation

and the remaining K-1 subsets used to build the model. Thus, at the end of K steps, the data is used in both test subsets and validation (Yadav and Shukla 2016). Here we use K = 10, described as the value that presents the best performance in the sampling, with the least bias in the error rate estimates (Kohavi 1995). The leave-one-out (LOO) technique uses k-1 samples to generate the discriminant function and the sample not included in the model serves to validate it, obtaining the percentage of the model's prediction (Kohavi 1995). Thus, we compared the predictions of individual identities for each species in each of the datasets.

RESULTS

We found considerable variation in the near-infrared spectral data among the sampled species (Fig. 1). Among the three datasets tested, the adaxial+abaxial (i) dataset showed 97.8 % of the spectral variation, the adaxial (ii) dataset showed 97.6 % of the spectral variation, and the abaxial (iii) dataset was the most representative with a spectral variation of 98.1 % (Fig. 2). For the abaxial (iii) dataset, individuals belonging to the same species tended to group more cohesively and consequently less mixed compared to the remaining two datasets (adaxial+abaxial [i] and adaxial [ii]) (Fig. 2).

All datasets had high predictive results in the identification of species (correct predictions higher than 90 %) for both models (PLS and LDA) and validation techniques (K-fold and LOO) (Table 1). Among the best percentages for plant discrimination (over 96 %) were the LDA model with the (iii) abaxial dataset for both the K-fold and leave-one-out validation techniques, and the PLS-DA model with the (i) adaxial+abaxial dataset and leave-one-out validation.

The adaxial+abaxial (i) dataset alone had the best percentage only for the PLS model and leave-one-out validation (96.7 %), and both validations in a similar way

resulted in elevated correct identifications for the three datasets tested (adaxial+abaxial, adaxial, abaxial).

All individuals of *M. crispata* and *M. megalophylla* were 100 % correctly predicted in both models and validations tests, with no confusion of readings with any sample related to any other species (Figs. 3, 4, Table 2). For six species, *M. dictyophylla*, *M. geminata*, *M. lindbergii*, *M. lycopodioides*, *M. percussa*, and *M. reptans*, the correct prediction of the identities of individuals in all models and validations ranged from 90 to 100 % (Figs. 3, 4, Table 2).

Two species (15.3 %), *M. persicariifolia* and *M. squamulosa*, had correct predictions between 80 and 100 % among the models and validations tests (Figs. 3, 4). For *M. baldwinii*, the abaxial (iii) dataset underperformed in both the LDA and PLS-DA models and validations (Figs. 3, 4, Table 2). Additionally, in the PLS-DA model for this species, the adaxial+abaxial (i) dataset had 88 % and 81 % correct predictions for the K-fold and LOO validations, respectively. The remaining models and validations recovered 100 % correct predictions. For *M. persicariifolia*, the lowest prediction value (83 %) was found in the PLS model for the abaxial dataset, for both validations; the remaining models and validations recovered 100 % correct predictions. Regarding *M. squamulosa*, only the abaxial (iii) dataset had 100 % correct predictions in both models and validations (Figs. 3, 4).

The two species with the lowest percentages of correct predictions were *M. thurnii* and *M. vacciniifolia*. For *M. thurnii*, of the 12 different combinations of the datasets and tests, only two recovered one of the lowest percentages of correct predictions (75 %): the PLS model, with the adaxial (ii) dataset, for both validations. Four tests recovered more than 90 % correct predictions, and six had 100 % correct

identifications (Figs. 3, 4, Table 2). For this species, the PLS-DA models underperformed compared to the LDA models.

Microgramma vacciniifolia was the species with the lowest percentage of correct predictions (73 %), which was found by the PLS model with the adaxial+abaxial and adaxial datasets; although, for the abaxial dataset there was 100 % accuracy in the identifications (data not shown). Prediction errors for *M. vacciniifolia* individuals occurred mainly with spectra associated with *M. geminata* and *M. squamulosa* in both models. Even so, the lowest percentage observed was 73 % in the PLS model (Fig. 4).

DISCUSSION

This is the first time that Fourier-transform near-infrared spectroscopy (FT-NIR) was tested for discriminating ferns species. Our results show that FT-NIR is a powerful tool that can be easily applied to species identification using spectral data of leaves. For all different scenarios tested (species, datasets, models, and validations), more than 85 % had an accuracy equal or greater than 90 % (Figs. 3, 4, Table 2), with an average above 93 % (Table 1).

Regarding the accuracy of FT-NIR at species identification, we recognize the importance of using well-defined species circumscriptions as premises and well-identified samples for constructing spectral models. In this work, when incorrectly identified specimens were used (an individual of *M. reptans* incorrectly determined as *M. baldwinii*), the accuracy decreased to 85.5 %, and after redoing the analysis with the correct identification, the correct prediction of the individuals of *M. baldwinii* reached 93.4 %.

Ferns are characterized by the presence of sori, which are usually on the abaxial surface of the fronds (Christenhusz and Chase 2014). Some lineages exhibit leaf

dimorphism, with leaves morphologically and physiologically specialized for photosynthesis or reproduction, and in some cases, there are extreme differences between both types (Wagner and Wagner 1977). In our study, we used species that are both monomorphic (*M. baldwinii*, *M. dictyophylla*, *M. geminata*, *M. lindbergii*, *M. lycopodioides*, *M. megalophylla*, *M. percussa*, *M. persicariifolia*, and *M. thurnii*) and dimorphic (*M. crispata*, *M. reptans*, *M. squamulosa*, and *M. vacciniifolia*). The dataset for the abaxial leaf surface, which can be more affected by the presence of sori, had (on average) higher percentages of discriminating samples than the other tested datasets (Table 1). Given our results, we believe the presence of sori has minimal influence on the spectral readings and subsequent discrimination power among species. However, this can vary among different lineages, and further tests controlling for fertile and sterile frond spectral readings are recommended.

One of the species that was more difficult to discriminate was *M. vacciniifolia*, where ca. 40 % of the samples were incorrectly predicted as *M. squamulosa* (Figs. 3, 4, Table 2). These species are sympatric in eastern and central Brazil and exhibit wide morphological variation (Almeida 2014). Our results using near-infrared spectroscopy (NIR) could be revealing inconsistencies in their current taxonomic circumscriptions. Also, the existence of hybrids between these species (Sota 1973) might explain the related spectral readings and the lower percentage of correct predictions. The technique has been shown to detect differences in the physical and biochemical compositions expressed in plant samples, even between closely related species, populations, and hybrids (Atkinson et al. 1997; Cui et al. 2012; Espinoza et al. 2012; Humphreys et al. 2008). Spectral properties have been shown to directly reflect phylogenetic patterns (Asner et al. 2014; Meireles et al. 2020), so it is expected that existing intra-specific variation between individuals and populations can be captured in

the spectral model with a broad sampling of phenotypic and geographic variation within species.

Our results show that the best models and validations can, on average, correctly predict the identification of species 96.7 % of the time when using all wavelengths to construct the models, which is comparable to previously published taxonomic works. Fan et al. (2010) tested and indicated the reliability of the technique at discriminating Ephedra plants from different habitats and collection seasons, while Lang et al. (2017) showed the effectiveness of the technique at discriminating species, genera, and families of tree species from eighteen different angiosperm families. Prata et al. (2018) demonstrated for the first time that near-infrared spectroscopy on leaves of subspecies of the *Pagamea guianensis* complex can discriminate taxa with high precision. For groups of closely related plants, the technique has shown excellent results for species of *Protium* (Burseraceae), confirming the differences in spectral signatures among species (Damasco et al. 2019).

There are many sources of potential variation that could impact the spectral variation in leaves (Asner et al. 2014). The chemical composition and other structural characteristics of leaves vary within and between species, as a result of the developmental stage and a combination of environmental factors, ontogeny, and composition of the plant epidermis (Mediavilla et al. 2009, 2014). Young and mature leaves generally exhibit differences in morphology, structure, and biochemical composition. The development process in plants causes changes in leaf structure, and these changes can be expressed in the spectra of the leaf through changes of certain compounds such as pigments, water and an increase in the mass of the secondary cell wall components (Ustin and Jacquemoud, 2020). We controlled phenological sources of variation using only fully developed leaves in individuals that already presented fertile

leaves. Moreover, to avoid variation coming from the presence of epiphylls, such as bryophytes, lichens, or fungi, or response to herbivory and infections that can also affect chemical composition, we made a rigorous selection of specimens with clean, undamaged leaves. Chemical composition in leaves can vary with soil properties, as already shown for canopy trees (Asner et al. 2014). Despite this variation, FT-NIR was still able to discriminate between species.

The high predictive power of FT-NIR at discriminating fern species, presented here, is superior to that observed for that group using a single region of two combined DNA barcodes, for which the best performance was 75 % correct predictions (Li et al. 2011; Wang et al. 2016). Identifying ferns and other plant groups using DNA barcoding is an expensive and time demanding technique and different lineages require specific combinations of molecular markers, which can make this technique complicated (Li et al. 2011; Lima et al. 2018). However, barcoding gametophytes has shown promising results for identifying species of ferns, which represents a great contribution to what is known about the evolution of this group (Schneider and Schuettpelz 2006). Our work does not intend to minimize the importance of other techniques used in plant systematics, but rather tested the reliability and effectiveness of FT-NIR at discriminating species in a group known to be problematic. Further, it highlights the potential of using this method in studies about plant systematics.

CONCLUSION

Our results show that near-infrared spectroscopy (NIR) is a highly effective, cost-effective, and non-destructive technique that can be used to discriminate closely related species. In addition to the possibility of obtaining spectral data quickly with minimal damage to samples, the technique provides greater reliability at discriminating

morphologically similar fern species, as previously found for some angiosperms. The accuracy of the identifications is comparable to and surpasses that of DNA barcoding, even for species from highly diverse and heterogeneous areas, such as tropical forests. We believe that NIR has great potential to be used in integrative taxonomic studies that aim to better understand species circumscriptions in the fern lineage.

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FIGURES AND CAPTIONS

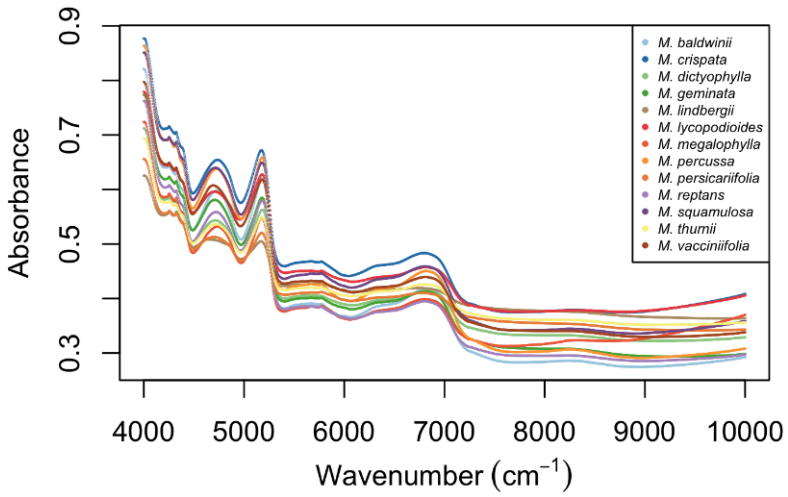

Figure 1-Average near-infrared spectral data for the thirteen sampled *Microgramma* species.

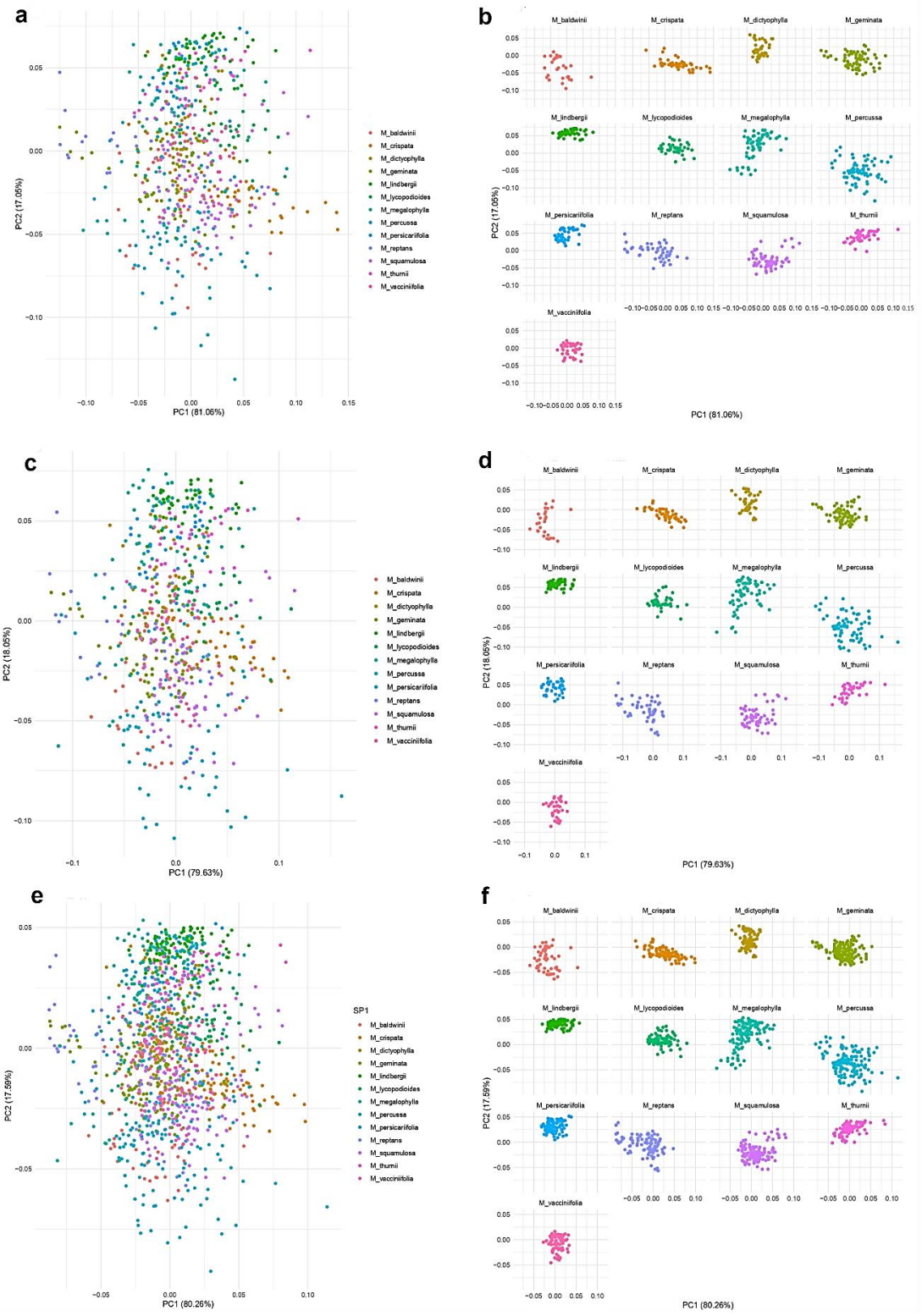

Figure 2-Principal Component Analysis (PCA) plot of the first two principal component axes for spectral data. **a**. Abaxial surface dataset (iii), all species. **b**. Abaxial surface dataset (iii), individually represented species. **c**. Adaxial surface dataset (ii), all species. **d**. Adaxial surface dataset (ii), individually represented species. **e**. Adaxial+abaxial surface dataset (i), all species. **f**. Adaxial+abaxial surface dataset (i), individually represented species.

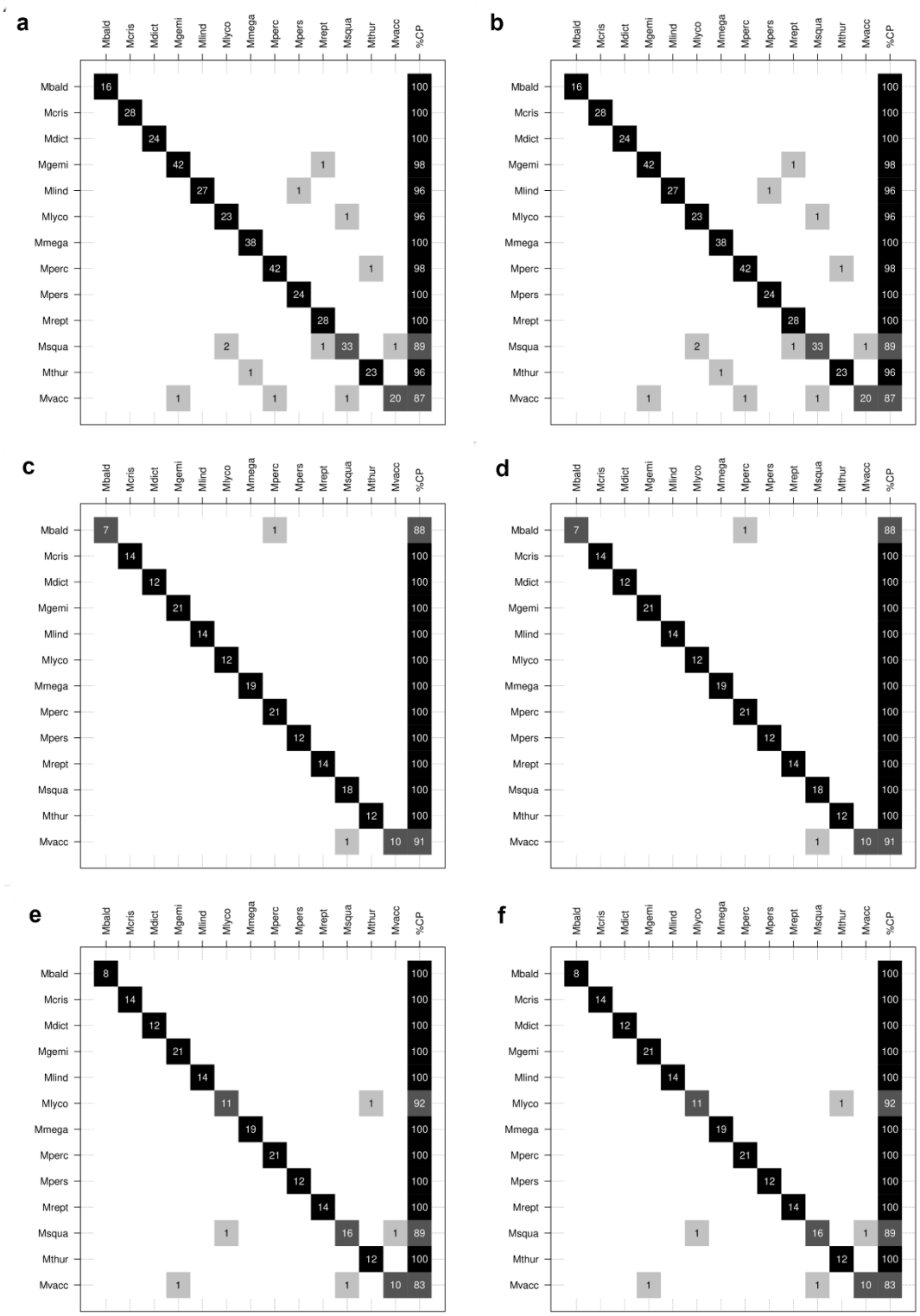

Figure 3-Confusion matrices resulting from the linear discriminant analysis (LDA) for the LOO and K-fold validations. **a**. LOO validation, adaxial+abaxial surface data. **b**. K-fold validation, adaxial+abaxial surface data. **c**. LOO validation, abaxial surface data only. **d**. K-fold validation, abaxial surface data only. **e**. LOO validation, adaxial surface data only. **f**. K-fold validation, adaxial surface data only. The names of the species observed are in rows and columns. The values on the diagonal correspond to correct predictions and those outside the diagonal correspond to incorrect predictions. Abbreviations: Mbald = *M. baldwinii*; Mcris = *M. crispata*; Mdict = *M. dictyophylla*; Mgemi = *M. geminata*; Mlind = *M. lindbergii*; Mlyco = *M. lycopodioides*; Mmega = *M. megalophylla*; Mperc = *M. percussa*; Mpers = *M. persicariifolia*; Mrept = *M. reptans*; Msqua = *M. squamulosa*; Mthur = *M. thurnii*; Mvacc = *M. vacciniifolia*.

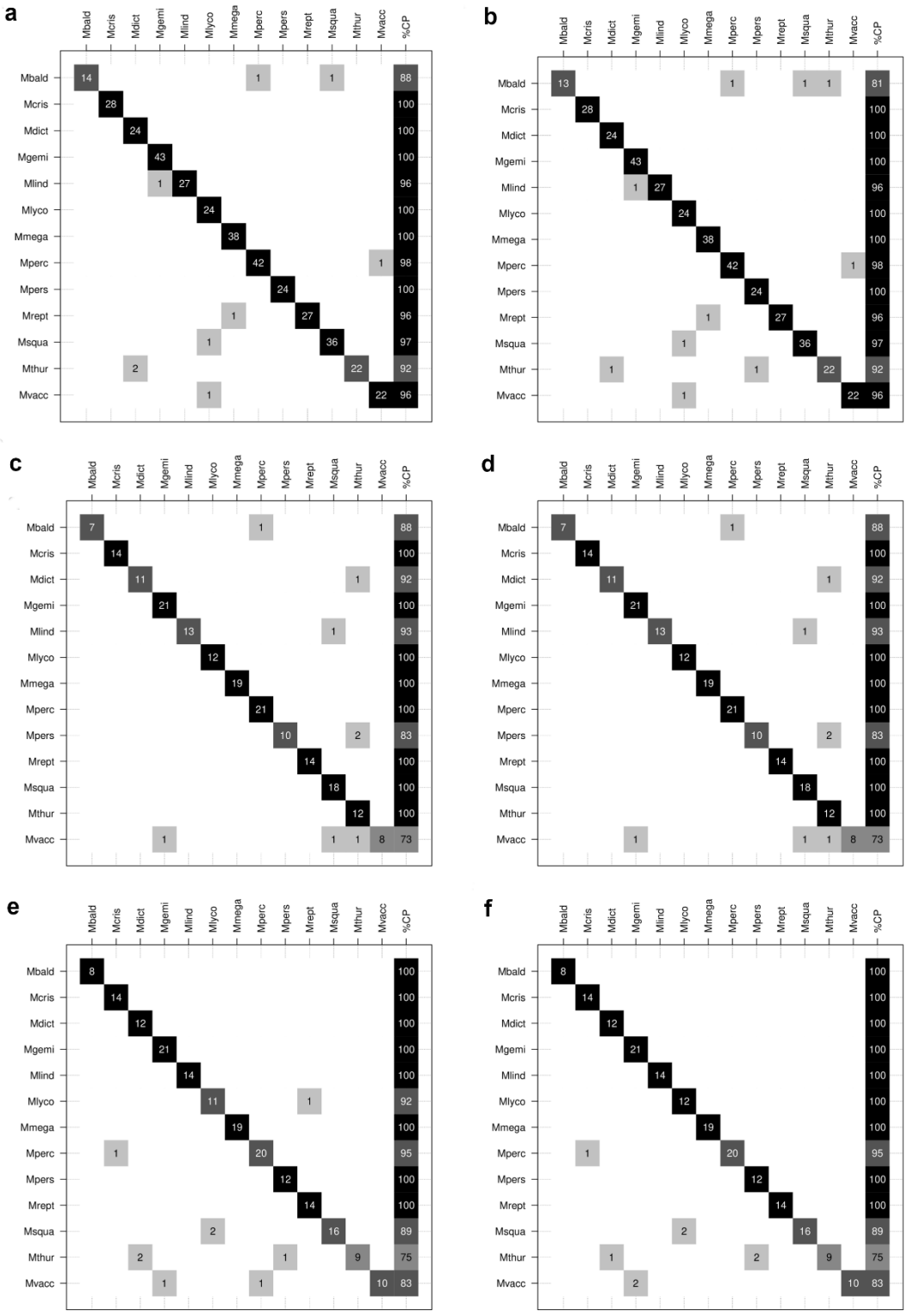

Figure 4- Confusion matrices resulting from the Partial Least Squares Discriminant Analysis (PLS) in the LOO and K-fold validations. **a**. K-fold validation, adaxial+abaxial surface data. **b**. LOO validation, adaxial+abaxial surface data. **c**. LOO validation, abaxial surface data only. **d**. K-fold validation, abaxial surface data only. **e**. K-fold validation, adaxial surface data only. **f**. LOO validation, adaxial surface data only. The names of the species observed are in rows and columns. The values on the diagonal correspond to correct predictions and those outside the diagonal correspond to incorrect predictions. Abbreviations: Mbald = *M. baldwinii*; Mcris = *M. crispata*; Mdict = *M. dictyophylla*; Mgemi = *M. geminata*; Mlind = *M. lindbergii*; Mlyco = *M. lycopodioides*; Mmega = *M. megalophylla*; Mperc = *M. percussa*; Mpers = *M. persicariifolia*; Mrept = *M. reptans*; Msqua = *M. squamulosa*; Mthur = *M. thurnii*; Mvacc = *M. vacciniifolia*.

TABLES

Table 1-Average percentage of correct identifications using a discriminant analysis and all of the FT-NIR spectrum wavelength data (1000–2500 nm) for the three datasets, (i) adaxial+abaxial leaf surfaces, (ii) adaxial surface only and (iii) abaxial surface only.

	LDA		PLS-DA	
Dataset	K-fold	LOO	K-fold	LOO
Adaxial+abaxial	95.3	93.0	96.2	96.7
Adaxial	95.4	95.8	93.9	94.3
Abaxial	96.2	96.2	94.6	95.1

Table 2-Summary of the percentage of incorrect predictions per species analyzed among the different datasets, analyses, and validations tested. LDA = Linear Discriminant Analysis. PLS= Partial Least Squares Discriminant Analysis. Abbreviations: Mbald = *M. baldwinii*; Mcris = *M. crispata*; Mdict = *M. dictyophylla*; Mgemi = *M. geminata*; Mlind = *M. lindbergii*; Mlyco = *M. lycopodioides*; Mmega = *M. megalophylla*; Mperc = *M. percussa*; Mpers = *M. persicariifolia*; Mrept = *M. reptans*; Msqua = *M. squamulosa*; Mthur = *M. thurnii*; Mvacc = *M. vacciniifolia*.

Dataset	Analysis	Validation	Mbald	Mcris	Mdict	Mgemi	Mlind	Mlyco	Mmega	Mperc	Mpers	Mrept	Msqua	Mthurn	Mvacc
Abaxial	LDA	LOO	12%	0	0	0	0	0	0	0	0	0	0	0	9%
		K-fold	12%	0	0	0	0	0	0	0	0	0	0	0	9%
	PLS	LOO	12%	0	8%	0	7%	0	0	0	17%	0	0	0	27%
		K-fold	12%	0	8%	0	7%	0	0	0	17%	0	0	0	27%
Adaxial	LDA	LOO	0	0	0	0	0	8%	0	0	0	0	11%	0	17%
		K-fold	0	0	0	0	0	8%	0	0	0	0	11%	0	17%
	PLS	LOO	0	0	0	0	0	0	0	5%	0	0	11%	25%	17%
		K-fold	0	0	0	0	0	8%	0	5%	0	0	11%	25%	17%
Both	LDA	LOO	0	0	0	2%	4%	4%	0	2%	0	0	11%	4%	13%
		K-fold	0	0	0	2%	4%	4%	0	2%	0	0	11%	4%	13%
	PLS	LOO	19%	0	0	0	4%	0	0	2%	0	4%	3%	8%	4%
		K-fold	12%	0	0	0	4%	0	0	2%	0	4%	3%	8%	4%

APPENDIX

Tables S1–Specimens used for spectral data capture. Herbaria acronym (in parentheses) follow Thiers (2020 onwards: http://sweetgum.nybg.org/science/ih/).

Taxon	Country	Voucher
Microgramma crispata	Brazil	Almeida 4430 (HSTM)
Microgramma crispata	Brazil	Souza 1565 (BHCB)
Microgramma crispata	Brazil	Krieger 10715 (BHCB)
Microgramma crispata	Brazil	Lima 126 (HSTM)
Microgramma crispata	Brazil	Salino 13919 (HSTM)
Microgramma crispata	Brazil	Dittrich 2187 (HSTM)
Microgramma lycopodioides	Brazil	Suemitsu 522 (HSTM)
Microgramma lycopodioides	Brazil	Almeida 4222 (HSTM)
Microgramma lycopodioides	Brazil	Giacomin 2577 (HSTM)
Microgramma lycopodioides	Brazil	Giacomin 1791 (HSTM)
Microgramma lycopodioides	Panama	Salino 15437 (BHCB)
Microgramma lycopodioides	Brazil	Viana 3386 (BHCB)
Microgramma lycopodioides	Bolivia	Almeida 3130 (BHCB)
Microgramma lycopodioides	Brazil	Salino 10067 (BHCB)
Microgramma lycopodioides	Brazil	Giacomin 2002 (HSTM)
Microgramma lycopodioides	Brazil	Giacomin 1933 (BHCB)
Microgramma dictyophylla	Brazil	Almeida 2605 (BHCB)
Microgramma dictyophylla	Brazil	Vidal 739 (BHCB)
Microgramma dictyophylla	Brazil	Almeida 2230 (HSTM)
Microgramma dictyophylla	Brazil	Almeida 2588 (HSTM)
Microgramma dictyophylla	Brazil	Freitas 33 (INPA)

Microgramma persicariifolia	Brazil	Almeida 2738 (HSTM)
Microgramma persicariifolia	Brazil	Mota 2411 (BHCB)
Microgramma persicariifolia	Brazil	Almeida 2203 (BHCB)
Microgramma persicariifolia	Brazil	Salino 304 (BHCB)
Microgramma persicariifolia	Brazil	Suemitsu 500 (HSTM)
Microgramma thurnii	Brazil	Almeida 2563 (HSTM)
Microgramma thurnii	Brazil	Almeida 2587 (BHCB)
Microgramma thurnii	Brazil	Araújo 115 (INPA)
Microgramma thurnii	Brazil	Acevedo 8152 (INPA)
Microgramma thurnii	Brazil	Almeida 3757 (INPA)
Microgramma vacciniifolia	Brazil	Souza 1565 (BHCB)
Microgramma vacciniifolia	Brazil	Almeida 2314 (HSTM)
Microgramma vacciniifolia	Brazil	Salino 6573 (HSTM)
Microgramma vacciniifolia	Brazil	Giacomin 1707 (HSTM)
Microgramma vacciniifolia	Brazil	Salino 8166 (HSTM)
Microgramma vacciniifolia	Brazil	Salino 14386 (HSTM)
Microgramma vacciniifolia	Brazil	Salino 5380 (HSTM)
Microgramma vacciniifolia	Brazil	Salino 1800 (HSTM)
Microgramma vacciniifolia	Brazil	Almeida 4802 (HSTM)
Microgramma vacciniifolia	Brazil	Salino 6116 (HSTM)
Microgramma baldwinii	Brazil	Almeida 4596 (HSTM)
Microgramma baldwinii	Brazil	Ribeiro 2728 (INPA)
Microgramma baldwinii	Brazil	Vieira 937 (INPA)
Microgramma baldwinii	Brazil	Poole 1645 (INPA)
Microgramma baldwinii	Brazil	Nelson 304 (INPA)

Microgramma baldwinii	Brazil	Freitas 602 (INPA)
Microgramma baldwinii	Brazil	Nee 46235 (INPA)
Microgramma squamulosa	Brazil	Giacomin 1655 (HSTM)
Microgramma squamulosa	Bolivia	Almeida 3105 (BHCB)
Microgramma squamulosa	Brazil	Almeida 3175 (HSTM)
Microgramma squamulosa	Brazil	Almeida 3363 (HSTM)
Microgramma squamulosa	Brazil	Almeida 2310 (HSTM)
Microgramma squamulosa	Brazil	Lima 65 (HSTM)
Microgramma squamulosa	Brazil	Dittrich 1655 (HSTM)
Microgramma squamulosa	Brazil	Almeida 4878 (HSTM)
Microgramma geminata	Brazil	Salino 10900 (HSTM)
Microgramma geminata	Brazil	Salino 14326 (HSTM)
Microgramma geminata	Brazil	Salino 2313 (HSTM)
Microgramma geminata	Brazil	Almeida 3071 (HSTM)
Microgramma geminata	Brazil	Salino 8208 (HSTM)
Microgramma geminata	Brazil	Salino 6679 (HSTM)
Microgramma geminata	Brazil	Salino 6574 (HSTM)
Microgramma geminata	Brazil	Salino 2560 (HSTM)
Microgramma geminata	Brazil	Salino 1655 (HSTM)
Microgramma lindbergii	Brazil	Almeida 3068 (HSTM)
Microgramma lindbergii	Brazil	Almeida 228 (HSTM)
Microgramma lindbergii	Brazil	Salino 4449 (HSTM)
Microgramma lindbergii	Brazil	Salino 3760 (HSTM)
Microgramma lindbergii	Brazil	Salino 929 (HSTM)
Microgramma lindbergii	Brazil	Echternacht 238 (HSTM)

Microgramma percussa	Brazil	Albuquerque 198 (INPA)
Microgramma percussa	Brazil	Albuquerque 281 (INPA)
Microgramma percussa	Brazil	Coelho 88 (INPA)
Microgramma percussa	Brazil	Albuquerque 717 (INPA)
Microgramma percussa	Brazil	Madison 618 (INPA)
Microgramma percussa	Brazil	Maia 58 (INPA)
Microgramma percussa	Brazil	Amaral 317 (INPA)
Microgramma percussa	Brazil	Ferreira 3498 (INPA)
Microgramma percussa	Brazil	Quaresma 9 (INPA)
Microgramma megalophylla	Brazil	Rodrigues 2554 (INPA)
Microgramma megalophylla	Brazil	Rodrigues 8840 (INPA)
Microgramma megalophylla	Brazil	Silva 105 (INPA)
Microgramma megalophylla	Brazil	Silva 1637 (INPA)
Microgramma megalophylla	Brazil	Albuquerque 1041 (INPA)
Microgramma megalophylla	Brazil	Coelho 41 (INPA)
Microgramma megalophylla	Brazil	Madison 190 (INPA)
Microgramma megalophylla	Brazil	Amaral 500 (INPA)
Microgramma reptans	Brazil	Almeida 2630 (HSTM)
Microgramma reptans	Brazil	Costa 4 (HSTM)
Microgramma reptans	Brazil	Almeida 2185 (HSTM)
Microgramma reptans	Brazil	Silva 35 (HSTM)
Microgramma reptans	Brazil	Giacomin 2576 (HSTM)
Microgramma reptans	Brazil	Fraga 3990 (INPA)
Microgramma reptans	Brazil	Madison 44 (INPA)
Microgramma reptans	Brazil	Madison 412 (INPA)

Microgramma reptans	Brazil	Freitas 590 (INPA)
Microgramma reptans	Brazil	Freitas 650 (INPA)
Microgramma reptans	Brazil	Mota 2413 (INPA)
Microgramma reptans	Brazil	Almeida 3596 (INPA)

3. CAPÍTULO 2

Paiva, D. N. A.; Almeida, T. E. Species delimitation in the *Microgramma vacciniifolia* complex (Polypodiaceae) base on morphometric analyses. Manuscrito formatado a ser submetido para o periódico *Plant Systematics and Evolution*.

Species delimitation in the *Microgramma vacciniifolia* complex (Polypodiaceae) based on morphometric analyses

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ABSTRACT

The genus *Microgramma* (Polypodiaceae) comprises 30 species widely distributed in the neotropical and paleotropical regions. Historically, the genus has been marked by controversial classification and the wide distribution combined with variable morphology, substantially aggravates the delimitation of species. In order to investigate the morphological variation between species of that genus and their circumscriptions, we evaluated 296 specimens referring to the currently accepted species *Microgramma vacciniifolia*, *M. crispata*, using *M. geminata* and *M. mauritiana* as outgroups. We used morphometric analyses with a total of nineteen morphological features. The data were treated through multivariate analyzes such as Principal Component Analysis (PCA), Canonical Discriminant Analysis (CDA), and Cluster Analysis (CA). Our results provide support for the four previously recognized taxa, with features such as: leaves length and leaves width at half its length as main characters to differentiate species, and length of leaves and petiole as features that most discriminate between *M. vacciniifolia* and *M. crispata*. Despite the great contribution of morphometry in the delimitation between groups, we emphasize the need for integrative analyzes to fully elucidate the taxonomic inconsistencies between species with marked levels of overlapping.

Keywords: ferns; morphometrics; multivariate analyzes; plant species delimitation; neotropical ferns.

INTRODUCTION

The Neotropical region is responsible for the highest levels of biodiversity on the planet (Pütz et al. 2014). In these forests, epiphytic plants stand out for the high number richness, contributing to approximately 30% of the diversity of plants in the world (Krömer and Gradstein, 2016). Epiphytic ferns occupy the second position (29%) of the richest groups of epiphytes, playing a crucial role in maintaining biodiversity (Dubuisson et al. 2009). An example of an expressive epiphytic group is the genus *Microgramma* C.Presl (Polypodiaceae), widely distributed in the tropics with 29 Neotropical and one Paleotropical species occurring in Africa and the Indian Ocean islands (Almeida 2014).

Morphologically, the genus is characterized by presenting small leaves, that can be monomorphic to dimorphic; anastomosed venation; rhizome with peltated, opaque scales; rounded, oblong to linear sori, in one or rarely several rows between the costa and the blade margin (Salino et al. 2008; Almeida 2014). They can be defined as predominantly epiphytic plants, occasionally rupicolous or terrestrial and preferentially inhabit humid forests, but they can also be found in savanna formations and seasonal forests (Rocha et al. 2014).

The genus is monophyletic, closely related to Niphidium J.Sm. and *Campyloneurum* C.Presl as a sister group (Salino et al. 2008; Almeida et al., submitted). Over time numerous studies have discussed generic and species circumscription, with *Microgramma dictyophylla* (Kunze ex Mett.) de la Sota and with *Microgramma percussa* (Cav.) de la Sota often recognized in Pleopeltis, and also *M. chrysolepis* (Hook). Crabbe is treated as Polypodium (Kreier et al. 2007; Salino et al. 2008; Sanín et al. 2019; Schneider et al. 2004). According to the most recent studies (Salino et al. 2008; Almeida 2014; Almeida et al., submitted), the inclusion of *M. percussa* and *M.*

dictyophylla in *Microgramma* was confirmed, with the exclusion of *Microgramma chrysolepis* of that genus (Almeida et al. 2017). The morphological variation and the wide distribution of some species in the genus demand for studies that clarify controversial circumscriptions and taxonomic relations (Salino et al. 2008; Almeida et al., submitted).

Microgramma vacciniifolia (Langsd. & Fisch.) Copel., for example, is distributed throughout Tropical America (Almeida 2014). Its occurrs in Argentina, Bolivia, Brazil, Colombia, Paraguay, Peru, Uruguay, and Venezuela. Part of its morphological variation shows similarity to *Microgramma crispata* (Fée) R.M.Tryon & A.F.Tryon, a species endemic of the Brazilian Atlantic Forest (Almeida 2020). Both occur in sympatry in eastern Brazil (Almeida 2020; Fig. 1) and are sister species, part of the *Vacciniifolia* clade, along with *M. geminata* (Schrad.) R.M.Tryon & A.F.Tryon and *M. mauritiana* (Willd.) Tardieu (Almeida et al., submitted). *Microgramma crispata* presents patent rhizome scales, while *M. vacciniifolia* presents adherent rhizome scales, characteristics used to differentiate them. On the other hand, a form of *M. crispata* occurring in Chapada Diamantina Brazil, in the ecotone of Cerrado and Caatinga with morphological differences in the leaves and related characters compared to the most coastal populations of the species suggests that *M. crispata* might also be more variable than previously thought (Almeida, pers. comm.). *Microgramma mauritiana* is described with marked morphological variation and is distributed throughout sub-Saharan Africa, Madagascar, off the coast of Africa, and the Indian Ocean Islands (Fig. 1). Recent data indicates an event of long-distance dispersion of the originally. Neotropical ancestral to Africa, followed by speciation (Janssen et al. 2007; Almeida et al. submitted).

Microgramma geminata can present different habits, such as epiphytic or terrestrial, being and endemic to eastern Brazil (Almeida 2020; Fig. 1A). The

morphological variation observed for many plant species, such as those described here, suggests a possible relationship between morphological divergence, geographic isolation, and hybridization between taxa (Friis and Balslev 2005).

In this study, we used morphometric analyzes to investigate the morphological variation between species of the genus *Microgramma*. (1) to test whether *Microgramma vacciniifolia* and *Microgramma crispata* can be distinguished only by morphology and (2) determine which morphological characters are most informative for the distinction between the studied *Microgramma* species.

MATERIALS AND METHODS

Specimens analyzed–Images of 296 specimens of *Microgramma vacciniifolia* (131) and *M. crispata* (69) as the ingroup, and 96 specimens of *M. mauritiana* (47) and *M. geminata* (49) as the outgroup, were measured. The specimens are deposited in the following herbaria: BHCB, NY, RB, ESA, HB, UEC, ALCB, EAC, VIC, P, BR, B, C, FURB, BM, R, VIES, US, ASE, GH, IAC, FMNH, G, UFP, HURFS, RFA, FUEL, UFR, and HUSC (acronyms according to Thiers (2020 onwards: https://sweetgum.nybg.org/science/ih/) (Appendix). After data selection through descriptive analysis, 134 specimens were excluded because they did not present all morphological characters. One-hundred and sixty-two samples were used in subsequent analyzes: sixty-six specimens of *M. vacciniifolia*, twenty of *M. crispata*, thirty-six of *M. mauritiana*, and forty of *M. geminata*.

Morphological characters–Informative morphological characters were chosen by consulting taxonomic references in the group (e.g. Almeida 2014). Initially, 25 characters (10 measurements in the sterile leaves and 15 measurements in the fertile leaves) were selected for the morphometric analysis, such as length and width of the leaves, angle measurements for the apex and base, length and diameter of the petiole,

quantifications of the sori and their association with the costa, and rhizome length and diameter (Table 1; fig. 2).

Linear measurements–Linear measurements were obtained from images of the selected species and specimens using ImageJ® (downloaded from https://imagej.nih.gov/ij/, ImageJ 1.52h, Java 1.8.0_112) (Schneider et al. 2012). Each image was calibrated only once based on the scale present in the image. Landmarks were added before the linear measurements and standardized whenever necessary (Fig. 2).

Statistical analyses–The analyzes were performed using the statistical program R, version 4.0.2 (R Core Team 2020), and the Vegan package (Oksanen et al. 2011). The morphological data matrix was standardized with the mean of each variable and divided by the standard deviation. Pearson's correlation coefficients were applied for all characters in the data set (Michener and Sokal 1957), and highly correlated morphological characters were excluded from the analyzes (DMSL, DSC, MWSL, MWFL, WHSL, WHFL; Table 1). After this analysis, 19 of the initial 25 morphological characters were retained: seven related to the sterile leaves (LSL, DBSL, BASL, BBSL, SASL, PLSL, PDSL; Table 1), eleven related to the fertile leaves (LFL, DMFL; DBFL; BAFL, BBFL, SAFL, SN; SA; SR; PLFL, PDFL; Table 1) and a character measured in samples with both leaves (RD; Table 1) (Fig. 2).

Boxplots were generated to visualize data distribution for the 19 morphological characters in the four studied species (Fig. 3). The obtaining of axes that best explained the variation of the data was conducted using the Principal Component Analysis (PCA), an ordering technique that reduces data dimensionality, extracting new axes with maximum variance (Fu 2015). Canonical Discriminant Analysis (CDA) (Mardia et al. 1994) was used to identify the most significant differences in the separation between the

four groups, and separately, only for *Microgramma crispata* and *Microgramma vacciniifolia*, considering the residual correlations between the averages of the observations.The success of the canonical discriminating functions was assessed using the cross-classification matrix, and the percentage of errors and successes between the tested groups was highlighted. Finally, a cluster analysis was performed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) based on Euclidean distance.

RESULTS

The result of the descriptive analyzes shows maximum, minimum, and average values for all morphological characters in the four studied species, in addition to the overlap of these traits in most species (Fig. 3).

The characters measured for *M. vacciniifolia*, such as the angle of symmetry at the sterile leaves (SASL) and diameter of the petiole of sterile leaves (PDSL), for example, have high variance, as opposed to the base angle of sterile leaves (BBSL) and apex angle of fertile leaves (BAFL) for the same species, which present low variation.

Principal component analysis and canonical discriminant analysis–The ordering of the characters for the four species represented in two dimensions was responsible for explaining 82.7 % of the observed variation (Fig. 4). Axes 1 (PC1) and 2 (PC2) showed a gradient of separation among species with *M. mauritiana* and *M. geminata* arranged to the left side of the first axis, and *M. vacciniifolia* and *M. crispata* on the right-side axis. The latter species also are distinctively distant to each other.

The diameter of the petiole of sterile leaves PDSL (1), the distance between the base and the maximum width of fertile leaves DBFL (1), the total length of fertile leaves LFL (0,97), and the total length of sterile leaves LSL (0,93) are the characters most strongly correlated with the first component. The most strongly correlated characters in component two are the length of the petiole at the sterile leaves PLSL (-

o,77), the length of the petiole at the fertile leaves PLFL (0,76), and the number of sori SN (0,81).

In the first Canonical Discriminant Analysis (all four species) the first two axes of the canonical discriminant analysis (CDA) contributed with 90.8 % of the observed variation (Fig. 5), and showed the almost complete separation of samples of *M. geminata* and *M. mauritiana*, as also observed in the PCA (Fig. 4), and despite the slight overlap, the canonical variables explain more than 90 % of the accumulated proportion (Table 2). Specimens of *M. crispata* overlap almost completely with specimens of *M. vacciniifolia*.

The morphological characters that most contribute to the differentiation between the four species on the first canonical axis are the total length of fertile leaves (LFL), the number of sori (SN), rhizome diameter (RD), and distance between the base and the maximum width of fertile leaves (DBFL), while on the second canonical axis are diameter of the petiole of sterile leaves (PDSL), the diameter of the petiole of fertile leaves (PDFL), sori area (SA), and distance between sori (SR).

Based on the morphological traits retained by canonical discrimination as more informative to discriminate species, the cross-classification matrix (Table 3) shows that the percentage of correct predictions for *M. crispata* reached 89 %; *M. geminata* 94 %; *M. mauritiana* 87 %, and *M. vacciniifolia* 85 %.

The total classification error reached 12.4 % and was mainly represented by *M. vacciniifolia*, *M. mauritiana* and *M. crispata*. *Microgramma crispata* had one incorrect prediction as *M. vacciniifolia*; *M. geminata* had one incorrect prediction as *M. crispata*; *M. mauritiana* had two incorrect predictions as *M. geminata* and *M. vacciniifolia*; and *M. vacciniifolia* had three incorrect predictions as *M. crispata* and one as *M. geminata* (Table 3).

Microgramma crispata* x *Microgramma vacciniifolia– The second canonical discriminant analysis including only *M. vacciniifolia* and *M. crispata* (Fig. 6) showed that the first canonical axis greater than 1 (1.6), and responsible for explaining 100 % of the total variance.

Some morphological traits do little to discriminate samples from these taxa, facilitating the occurrence of overlaps (Fig. 6A) such as the number of sori (SN), the distance between sori (SR), and symmetry angle of sterile leaves (SASL). On the other hand, characters that most contribute to the differentiation between *M. vacciniifolia* and *M. crispata* are the length of the petiole of fertile leaves (PLFL), the angle of the apex of sterile leaves (BASL), the distance between the half and the maximum width of fertile leaves (DMFL), the diameter of the petiole of sterile leaves (PDSL), and the total length of sterile leaves (LFL) (Fig. 6B).

The percentage of correct classifications for *M. crispata* reached 89 %, while for *M. vacciniifolia* it was 97 %, and the total correct classification observed was 94 %.

Cluster analysis–Based on the morphological characters, the cluster analysis is equivalent to what was observed in discriminant analyzes (Figure 7). The dendrogram illustrates three clusters formed according to morphological similarities. The blue cluster is formed by *M. geminata* and *M. mauritiana*, reflecting the homogeneity within the group due to a higher morphological association. This grouping differs from the clusters *M. crispata* (red) and *M. vacciniifolia* (green), taking into account the phenotypic heterogeneity among the last-mentioned groups. The cophenetic correlation resulting from the analysis (0.80) confirmed the reliability in the formation of the groups.

DISCUSSION

According to the morphometric analysis in association with the multivariate analyzes, the *Microgramma* part of taxa belonging to the *Vacciniifolia* clade studied here are quantitatively distinct, showing, however, some overlap.

Multivariate analyzes for all species– We confirm the morphological complexity between the species studied in the genus *Microgramma*. *Microgramma geminata* is the most distinct species morphologically, in addition to being the one with the best index of correct answers in classification, and, consequently, it is the species best supported in this group (Table 3). *Microgramma geminata*, which has a close morphological relationship with *M. mauritiana* (Fig. 5), is characterized by monomorphic leaves ranging from 10 to 18 cm in length in the case of fertile ones (Pena and Labiack at al. 2019), while *M. mauritiana* with fertile leaves ranging from 3 to 18 cm, confirming its marked dimorphism. Although the species mentioned are significantly differentiable, the phylogenetic relationships of *M. mauritiana*, the only paleotropical species in the genus (Almeida 2014) inside the *Vacciniifolia* clade are still not well resolved (Almeida et al., submitted).

The discriminant analyzes showed that *Microgramma vacciniifolia* is associated morphologically with *M. crispata*, 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 others angle measurements are not informative enough to discriminate between them. This may be related to the intraspecific variation (Menini Neto et al. 2013; Labiak et al. 2015; Menini Neto et al. 2019) of *M. vacciniifolia* (Almeida 2014), which can be compared to the *M. mauritiana*, which is highly variable and has been treated as a species complex (Almeida et al., submitted).

The distinction between *Microgramma vacciniifolia* and *Microgramma crispata*–Multivariate analyzes showed that despite the overlap in phenotypic characters, both species can be taxonomically differentiated (Table 4; Fig. 7). However, the observed overlap (Fig. 6A) indicates that the delimitation of polymorphic species, such as those sampled in this work, should not be based only on morphological characters, despite the technique being highly informative (Chang et al. 2003; Pelser and Houchin 2004; Whang et al. 2002).

Microgramma crispata has leaves ranging from 4.5–10 cm in length (sterile and fertile), here we find a higher standard, with sterile and fertile leaves ranging from 2–12 cm in length. According to Almeida (2014) the measure of the length of the leaves is a determining feature for discriminating *M. crispata* and *M. vacciniifolia*. Based on this research, in addition to measures of length, we included measures associated with the width of the leaves.

The occurrence in sympatry and consequently ecological aspects related to the distribution of species can greatly explain the observed morphological overlap (Baleeiro et al. 2016). *Microgramma vacciniifolia* has a wide geographical distribution (Fig. 1), while *M. crispata* shows a more restricted distribution overlapping with extent of occurrence of *M. vacciniifolia* (Fig. 1A). The overlap observed in the analysis can indicate the clinal nature of the morphological traits in question. Furthermore, as sister species occurring sympatrically, the occurrence of phenomena like hybridization and introgression cannot be discarded (Pinheiro and Barros 2007). Other morphometric studies have shown that widespread plants can exhibit more easily a broader morphological variation (Pinheiro and Barros 2007; Ribeiro et al., 2007), favoring phenomena such as those previously mentioned.

The existence of hybrids has already been documented in the genus. *Microgramma mortoniana* de la Sota, for example, has been described as a hybrid between the *M. squamulosa* and *M. vacciniifolia*. Its recognition is mainly based mainly on distribution overlap and intermediate states of morphological traits (Sota 1973). Like *M. crispata* and *M. vacciniifolia*, other *Microgramma* species also present different levels of overlap, like *M. reptans* and *M. tobagensis* (Smith et al. 2018).

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. 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.

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. The use of morphometric analysis provides reliable evidence (Pinheiro and Barros 2007; Melo and Borba 2011; Kaplan and Marhold 2012; Baleeiro et al. 2016; Leal et al. 2016; Ramírez-Valencia and Sanín 2017; Sanín et al. 2019); the use of molecular markers can provide faster identifications (Chen et al. 2010; De Mattia et al. 2011; De Vere et al. 2012; Fazekas et al. 2012; Lima et al. 2018; Sigel et al. 2018); and near-infrared spectroscopy (NIR), a reliable technique, can generate fast and low-cost identifications (Workman et al. 2008; Lang et al. 2017; Prata et al. 2018; Damasco et al. 2019; Paiva et al.2021).

We believe that for a comprehensive and quality taxonomic approach, especially when referring to species identification through morphometry, wide access to the

sample and morphological characters is essential. We suggest special attention regarding the digital samples available in virtual herbariums, so that the visualization of morphological traces, such as leaves, for example, is better explored in this type of study.

CONCLUSIONS

Based on our results, we found that *M. geminata*, *M. mauritiana*, *M. vacciniifolia*, and *M. crispata* can be recognized as morphologically distinct species. Although *M. crispata* and *M. vacciniifolia* can be recognized as distinct taxa with the support of some morphological traits, however, there is a high overlapping morphological variation between those species. We suggest the use of integrative systematic techniques as an aid in delimiting this and other taxonomically complex groups.

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FIGURES AND CAPTIONS

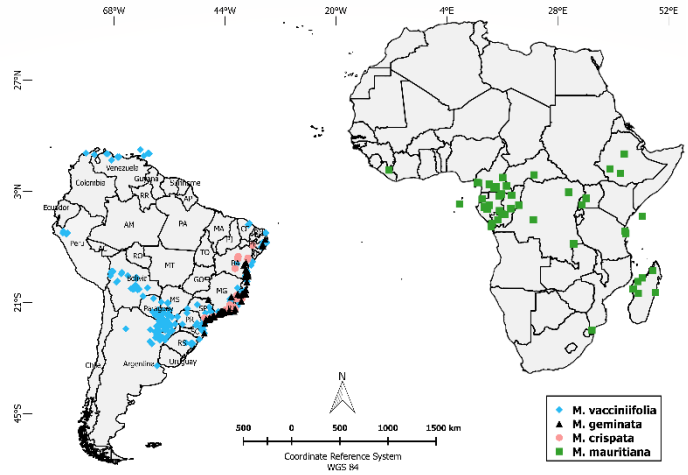


Figure 1- Map showing the distribution of the species used in the study: *Microgramma crispata*, pink circles; *Microgramma geminata*, black triangles; *Microgramma mauritiana*, green squares; *Microgramma vacciniifolia*, blue diamonds.

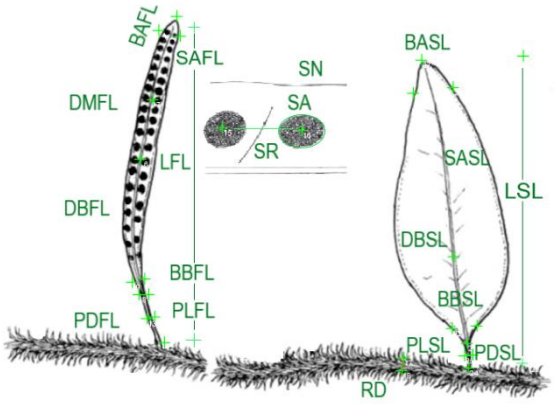
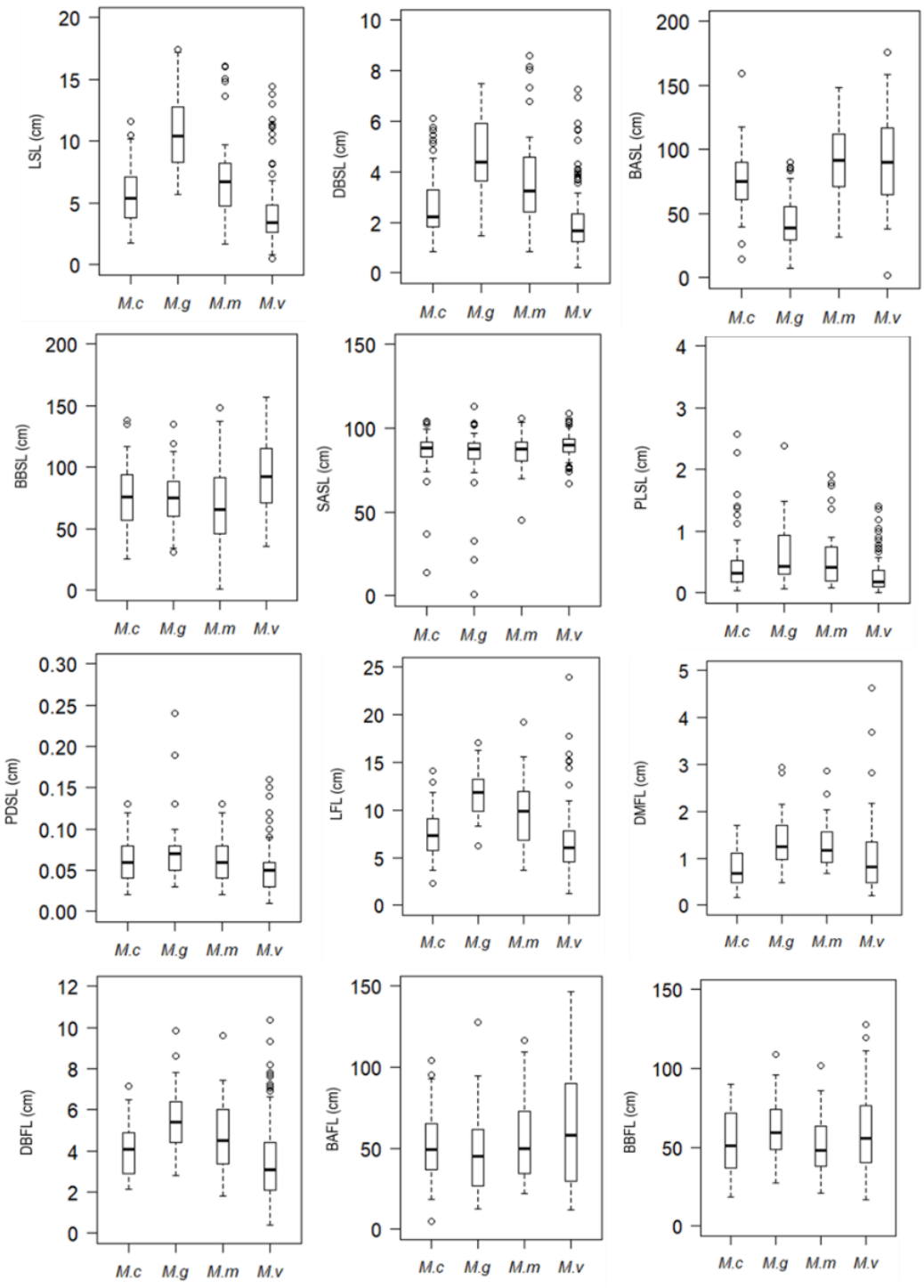


Figure 2-Illustration of a fertile (left) and sterile (right) leaves showing the nineteen morphological characters retained for multivariate analyzes. The sori are highlighted in the upper middle part of the figure. BAFL = Blade apex angle of fertile leaves; BASL = Blade apex angle of sterile leave; BBFL = Blade base angle of fertile leaves; BBSL = Blade base angle of sterile leave; DBFL = Distance between blade base and maximum width of fertile leaves; DBSL = Distance between sterile leave blade base and the maximum width; DMFL = Distance between half and the maximum width of fertile leaves; LFL = Fertile leaves length; LSL = Sterile leave length; PDFL = Petiole diameter of fertile leaves; PDSL = Petiole diameter of sterile leave; PLFL = Petiole length of fertile leaves; PLSL = Petiole length of sterile leave; RD = Rhizome diameter; SA = Sori area; SAFL = Symmetry angle of fertile leaves; SASL = Symmetry angle of sterile leave; SN = Sori number; SR = Sori distance.


LSL (cm)
DBSL (cm)
BASL (cm)
BBSL (cm)
SASL (cm)
PLSL (cm)
PDSL (cm)
LFL (cm)
DMFL (cm)
DBFL (cm)
BAFL (cm)
BBFL (cm)
M.c
M.g
M.m
M.v

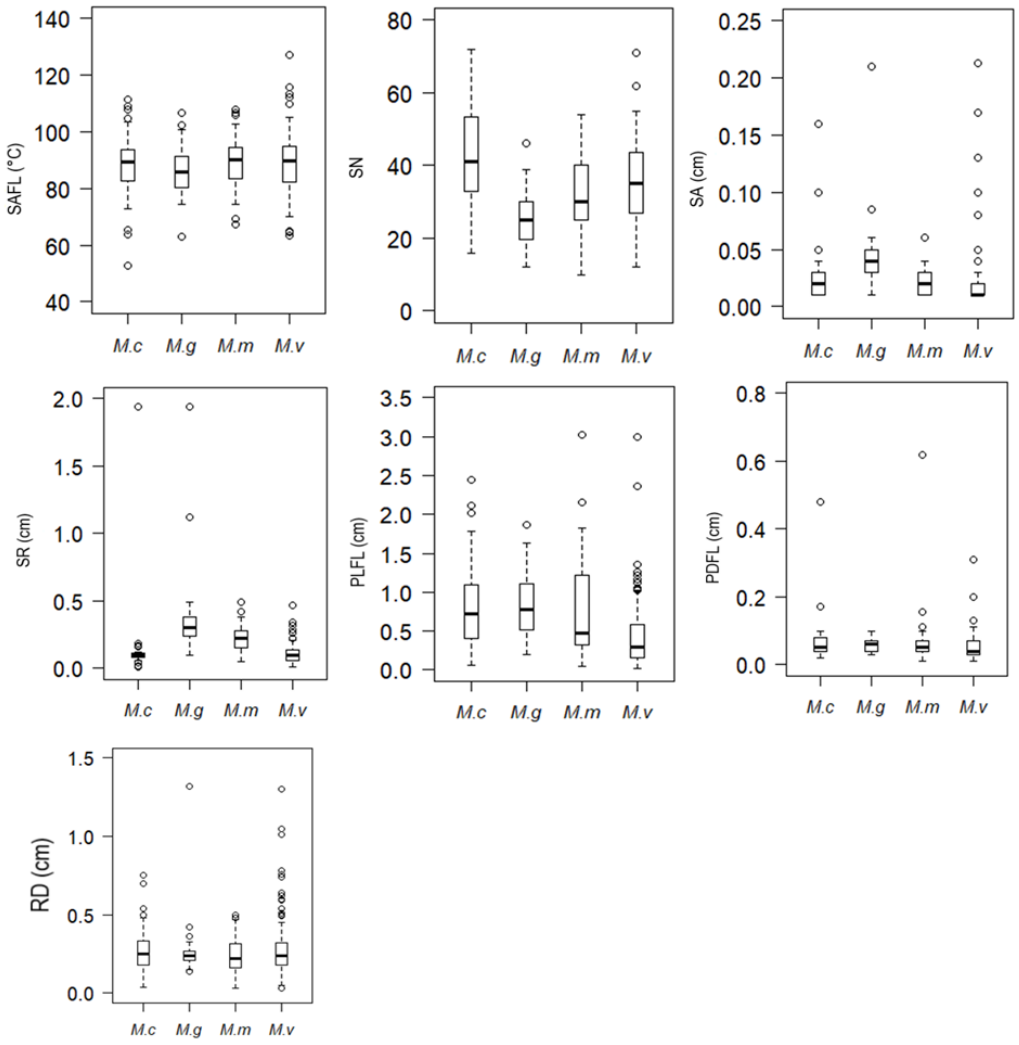


Figure 3-Box plots showing descriptive analysis of the 19 morphological characters for four species of *Microgramma*. Abbreviations: M.c = *Microgramma crispata*; M.g = *Microgramma geminata*; M.m = *Microgramma mauritiana*; M.v = *Microgramma vacciniifolia*. BAFL = Blade apex angle of fertile leaves; BASL = Blade apex angle of sterile leave; BBFL = Blade base angle of fertile leaves; BBSL = Blade base angle of sterile leave; DBFL = Distance between blade base and maximum width of fertile leaves; DBSL = Distance between sterile leave blade base and the maximum width; DMFL = Distance between half and the maximum width of fertile leaves; LFL = Fertile leaves length; LSL = Sterile leave length; PDFL = Petiole diameter of fertile leaves; PDSL = Petiole diameter of sterile leave; PLFL = Petiole length of fertile leaves; PLSL = Petiole length of sterile leave; RD = Rhizome diameter; SA = Sori area; SAFL = Symmetry angle of fertile leaves; SASL = Symmetry angle of sterile leave; SN = Sori number; SR = Sori distance.

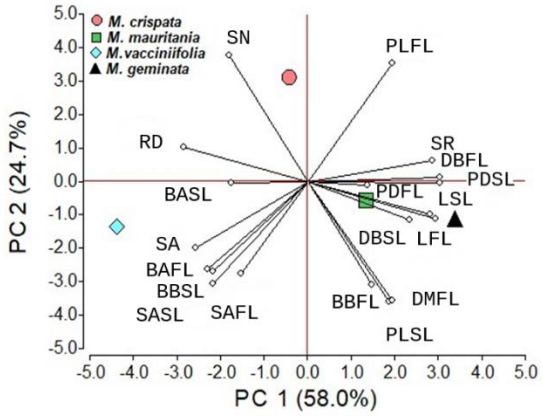


Figure 4- Biplot graph with the projection of the 19 morphological characters sampled for *M. crispata*, *M. geminata*, *M. mauritiana*, and *M. vacciniifolia*. BAFL = Blade apex angle of fertile leaves; BASL = Blade apex angle of sterile leave; BBFL = Blade base angle of fertile leaves; BBSL = Blade base angle of sterile leave; DBFL = Distance between blade base and maximum width of fertile leaves; DBSL = Distance between sterile leave blade base and the maximum width; DMFL = Distance between half and the maximum width of fertile leaves; LFL = Fertile leaves length; LSL = Sterile leave length; PDFL = Petiole diameter of fertile leaves; PDSL = Petiole diameter of sterile leave; PLFL = Petiole length of fertile leaves; PLSL = Petiole length of sterile leave; RD = Rhizome diameter; SA = Sori area; SAFL = Symmetry angle of fertile leaves; SASL = Symmetry angle of sterile leave; SN = Sori number; SR = Sori distance.

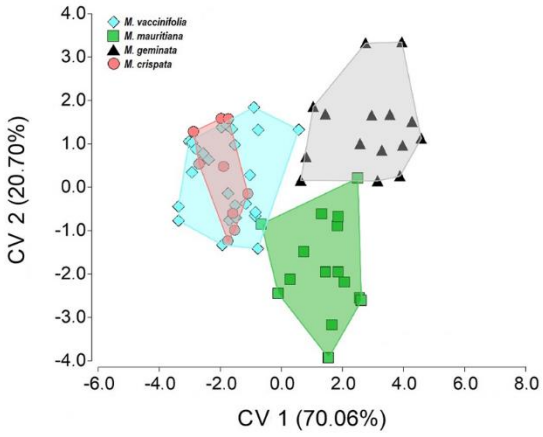


Figure 5- Canonical Discriminant Analysis (CDA) based on 19 morphological characters for *M. crispata* (pink circles), *M. geminata* (black triangles), *M. mauritiana* (green squares), and *M. vacciniifolia* (blue diamonds).

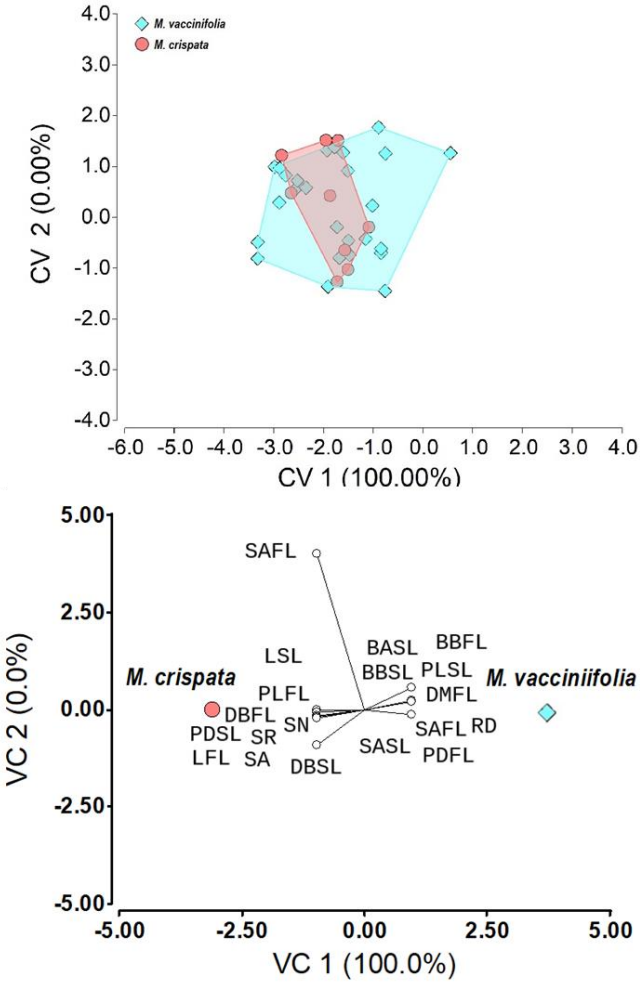


Figure 6- Canonical Discriminant Analysis (CDA) based on 19 morphological characters for *M. crispata* (pink circles) and *M. vacciniifolia* (blue diamonds). B. Projection of the sampled morphological characters. BAFL = Blade apex angle of fertile leaves; BASL = Blade apex angle of sterile leave; BBFL = Blade base angle of fertile leaves; BBSL = Blade base angle of sterile leave; DBFL = Distance between blade base and maximum width of fertile leaves; DBSL = Distance between sterile leave blade base and the maximum width; DMFL = Distance between half and the maximum width of fertile leaves; LFL = Fertile leaves length; LSL = Sterile leave length; PDFL = Petiole diameter of fertile leaves; PDSL = Petiole diameter of sterile leave; PLFL = Petiole length of fertile leaves; PLSL = Petiole length of sterile leave; RD = Rhizome diameter; SA = Sori area; SAFL = Symmetry angle of fertile leaves; SASL = Symmetry angle of sterile leave; SN = Sori number; SR = Sori distance.

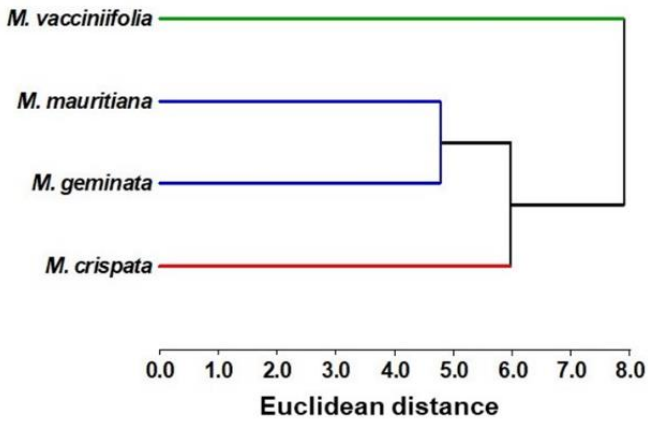


Figure 7-UPGMA dendrogram of 162 samples, based on the morphological characters for *M. crispata*, *M. geminata*, *M. mauritiana*, and. *M. vacciniifolia*.

TABLES

Table 1- Characters used in the morphometric analysis

Character	**Code**	**Measurement unit**
Sterile leaves		
1. Length	LSL	cm
2. Leaves width at half its length	WHSL	cm
3. Maximum leaves width	MWSL	cm
4. Distance between half and the maximum width of the leaves	DMSL	cm
5. Distance between the blade base and the maximum width	DBSL	cm
6. Blade apex angle	BASL	degrees
7. Blade base angle	BBSL	degrees
8. Symmetry angle	SASL	degrees
9. Petiole length	PLSL	cm
10. Petiole diameter	PDSL	cm
Fertile leaves		
11. Length	LFL	cm
12. Leaves width at half its length	WHFL	cm
13. Maximum leaves width	MWFL	cm
14. Distance between half and the maximum width of the leaves	DMFL	cm
15. Distance between blade base and maximum width	DBFL	cm
16. Blade apex angle	BAFL	degrees
17. Blade base angle	BBFL	degrees
18. Symmetry angle	SAFL	degrees
19. Sori number	SN	units
20. Sori area	SA	cm
21. Sori distance	SR	cm
22. Distance between sori and costa	DSC	cm
23. Petiole length	PLFL	cm
24. Petiole diameter	PDFL	cm
Rhizome		
25. Diameter	RD	cm

Table 2- Canonical Discriminant Analysis. Variation explained by the first two axes of canonical variables.

VC	Eigenvalue	Variance	Cumulative variance
1	4,31	70,06	70,06
2	1,28	20,70	90,76

Table 3-Classification matrix for the four studied *Microgramma* species.

Taxon	***M. crispata***	***M. geminata***	***M. mauritiana***	***M. vacciniifolia***	Total	% correct predictions
M. crispata	8	0	0	1	9	89
M. geminata	1	14	0	0	15	94
M. mauritiana	0	1	13	1	15	87
M. vacciniifolia	3	1	0	22	26	85
Total	12	16	13	24	65	88

APPENDIX

Specimens used for morphometrics analyses. Herbaria acronym (in parentheses) follow Thiers (2020 onwards: https://sweetgum.nybg.org/science/ih/). Supplemental file – Raw morphometric data. https://drive.google.com/file/d/12KLWDo1o-4PGWbpjwWPgYkYIqfNarerc/view?usp=sharing

Taxa	**Voucher**	**Country**
M. crispata	Almeida 2314 (BHCB)	Brazil
M. crispata	Almeida 2390 (BHCB)	Brazil
M. crispata	Almeida 2400 (BHCB)	Brazil
M. crispata	Batista 92 (ESA)	Brazil
M. crispata	Boldrin 177 (NY)	Brazil
M. crispata	Boldrin s.n. (HUSC)	Brazil
M. crispata	Bovini 3673 (BHCB)	Brazil
M. crispata	Brade 6598 (NY)	Brazil
M. crispata	Brade 9393 (NY)	Brazil

M. crispata	Braga 618 (BHCB)	Brazil
M. crispata	Breier 427 (UEC)	Brazil
M. crispata	Breier 834 (UEC)	Brazil
M. crispata	Crestani 197 (ALCB)	Brazil
M. crispata	Cribari 2386 (MBML)	Brazil
M. crispata	Cruz 7 (RBR)	Brazil
M. crispata	Damasceno 11(RBR)	Brazil
M. crispata	Davidse 11929 (MO)	Brazil
M. crispata	Dittrich 2187 (HSTM)	Brazil
M. crispata	Dittrich s.n. (BHCB)	Brazil
M. crispata	Glazian 2072 (P)	Brazil
M. crispata	Glazian 423 (P)	Brazil
M. crispata	Glazian 5282 (B)	Brazil
M. crispata	Glazian 5283 (B)	Brazil
M. crispata	Glaziou s.n. (R)	Brazil
M. crispata	Graçano 221 (VIC)	Brazil
M. crispata	Guedes 11931 (ALCB)	Brazil
M. crispata	Guedes 19057 (EAC)	Brazil
M. crispata	Hatschbach 75840 (MBM)	Brazil
M. crispata	Irwin 30783 (NY)	Brazil
M. crispata	Irwin 32402 (NY)	Brazil
M. crispata	Jardim 5582 (UFRN)	Brazil
M. crispata	Kollmann 179 (MBML)	Brazil
M. crispata	Krieger 10715 (FURB)	Brazil
M. crispata	Krieger 2690 (CESJ)	Brazil
M. crispata	Lima 126 (HSTM)	Brazil
M. crispata	Lino 38 (RFA)	Brazil

M. crispata	Lino 5 (RFA)	Brazil
M. crispata	Lino 83 (RFA)	Brazil
M. crispata	Lopes s.n. (EAC)	Brazil
M. crispata	Luiz s.n. (BHCB)	Brazil
M. crispata	Lute 2127 (BHCB)	Brazil
M. crispata	Mexia 1870 (BM)	Brazil
M. crispata	Mexia 5172 (NY)	Brazil
M. crispata	Mizoguchi 629 (NY)	Brazil
M. crispata	Moura 60 (FUEL)	Brazil
M. crispata	Paula 6179 (ESA)	Brazil
M. crispata	Pena 603 (VIES)	Brazil
M. crispata	Queiroz 4271 (NY)	Brazil
M. crispata	Reis 26 (RFA)	Brazil
M. crispata	Rodal 107 (PEUFR)	Brazil
M. crispata	Rodal 478 (NY)	Brazil
M. crispata	Rose s.n. (NY)	Brazil
M. crispata	Saka 220 (ASE)	Brazil
M. crispata	Sales 605 (NY)	Brazil
M. crispata	Salino 13919 (HSTM)	Brazil
M. crispata	Santiago 744 (UFP)	Brazil
M. crispata	Schwarstburd 2562 (VIC)	Brazil
M. crispata	Schwartsburd 3612 (VIC)	Brazil
M. crispata	Schwartsburd 3613 (VIC)	Brazil
M. crispata	Schwartsburd 3725 (VIC)	Brazil
M. crispata	Souza 1565 (BHCB)	Brazil
M. crispata	Souza 4806 (ESA)	Brazil
M. crispata	Souza 7420 (ESA)	Brazil

M. crispata	Valadão 582 (ALCB)	Brazil
M. crispata	Valente s.n (BHCB)	Brazil
M. crispata	Viana 1762 (ASE)	Brazil
M. crispata	Xavier 59 (UFP)	Brazil
M. crispata	Zárate 154 (EAC)	Brazil
M. crispata	Zink s.n. (P)	Brazil
M. geminata	Cárdenas 7325 (CO)	Bolivia
M. geminata	Almeida 2313 (NY)	Brazil
M. geminata	Almeida 3071 (NY)	Brazil
M. geminata	Alston 352 (BHCB)	Brazil
M. geminata	Araújo 4151(RB)	Brazil
M. geminata	Araújo 4349 (RB)	Brazil
M. geminata	Araújo 8399 (RB)	Brazil
M. geminata	B. 211 s.n. (B)	Brazil
M. geminata	Barreto 1611(ESA)	Brazil
M. geminata	Blanchet s.n. (NY)	Brazil
M. geminata	Brade 6277 (FMNH)	Brazil
M. geminata	Carvalho 4231(BHCB)	Brazil
M. geminata	Dittrich 464 (MBM)	Brazil
M. geminata	Dusén 173 (NY)	Brazil
M. geminata	Dusen s.n. (G)	Brazil
M. geminata	Folli 2737 (NY)	Brazil
M. geminata	França 5176 (HUEFS)	Brazil
M. geminata	Gadichaud s.n. (CO)	Brazil
M. geminata	Glaziou 4663 (B)	Brazil
M. geminata	Gueiros 199 (UFP)	Brazil
M. geminata	Gueiros 222 (UFP)	Brazil

M. geminata	Hance 18103 (BM)	Brazil
M. geminata	Kuhn s.n. (B)	Brazil
M. geminata	Lopes 302 (MBM)	Brazil
M. geminata	Matos 315 (NY)	Brazil
M. geminata	Matos 315 (UPCB)	Brazil
M. geminata	Melo 4080 (HUEFS)	Brazil
M. geminata	Melo 4979 (HUEFS)	Brazil
M. geminata	Melo s.n. (EAC)	Brazil
M. geminata	Morais 8 (HUEFS)	Brazil
M. geminata	Pastore 1032 (BHCB)	Brazil
M. geminata	Prado 1489 (NY)	Brazil
M. geminata	Prado 1580 (NY)	Brazil
M. geminata	Prado 375 (NY)	Brazil
M. geminata	Salino 10239 (BHCB)	Brazil
M. geminata	Salino 1655 (BHCB)	Brazil
M. geminata	Salino 5362 (ESA)	Brazil
M. geminata	Salino 6574 (BHCB)	Brazil
M. geminata	Santos 7 (CEPEC)	Brazil
M. geminata	Silva 506 (R)	Brazil
M. geminata	Silva 58322 (NY)	Brazil
M. geminata	Thomas s.n. (NY)	Brazil
M. geminata	Zink s.n. (BM)	Brazil
M. geminata	Zink s.n. (BM)	Brazil
M. geminata	Knapp 7119 (NY)	Peru
M. geminata	Knapp 7159 (MO)	Peru
M. geminata	Knapp 7960 (MO)	Peru
M. geminata	Knapp 8462 (MO)	Peru

M. geminata	Knapp 8467 (MO)	Peru
M. mauritiana	Carrisso 520 (BM)	Angola
M. mauritiana	Zink s.n. (BM)	Angola
M. mauritiana	Thompson 1392 (NY)	Cameroon
M. mauritiana	Zenker 644 (NY)	Cameroon
M. mauritiana	Cameron 833 (NY)	Gabon
M. mauritiana	Reitsma 1428 (NY)	Gabon
M. mauritiana	Benl 206 (BM)	Guinea
M. mauritiana	Bruun 44 (BM)	Guinea
M. mauritiana	Carvalho 4755 (NY)	Guinea
M. mauritiana	Jacques 7017 (NY)	Guinea
M. mauritiana	Jfey 187 (GH)	Guinea
M. mauritiana	Zink s.n. (BM)	Guinea
M. mauritiana	Baldwin 5938 (GH)	Liberia
M. mauritiana	Baldwin 9400 (GH)	Liberia
M. mauritiana	Cook 27 (GH)	Liberia
M. mauritiana	Cook 99 (GH)	Liberia
M. mauritiana	Fay 1207 (NY)	Liberia
M. mauritiana	Harley 21 (GH)	Liberia
M. mauritiana	Linder 1926 (GH)	Liberia
M. mauritiana	Quansah 88135 (BM)	Madagascar
M. mauritiana	R. D 1363 (BM)	Madagascar
M. mauritiana	Chase 4483 (BM)	Mozambique
M. mauritiana	Pedro 8919 (BM)	Mozambique
M. mauritiana	Olorunfami 94246 (NY)	Nigeria
M. mauritiana	Ross 267 (BM)	Nigeria
M. mauritiana	Z.O. 94226 (NY)	Nigeria

M. mauritiana	B.322 (NY)	Sierra Leone
M. mauritiana	F. 1302 (NY)	Sierra Leone
M. mauritiana	Fay 1031 (NY)	Sierra Leone
M. mauritiana	Fay 1103 (NY)	Sierra Leone
M. mauritiana	Fay 1104 (NY)	Sierra Leone
M. mauritiana	Fay 1199 (NY)	Sierra Leone
M. mauritiana	Fay 1305 (NY)	Sierra Leone
M. mauritiana	Pine 79 (GH)	Sierra Leone
M. mauritiana	Reitsma 746 (NY)	Sierra Leone
M. mauritiana	H. 979 (BM)	South Africa
M. mauritiana	L.A. s.n. (GH)	South Africa
M. mauritiana	Rudatis 1068 (BM)	South Africa
M. mauritiana	Schlechter 296 (GH)	South Africa
M. mauritiana	Shelpe 5204 (BM)	South Africa
M. mauritiana	Shelpe 5204 (GH)	South Africa
M. mauritiana	Strey 3683 (NY)	South Africa
M. mauritiana	Zink s.n. (BM)	South Africa
M. mauritiana	Eggeling 2134 (BM)	Uganda
M. mauritiana	Milburn 25 (BM)	Uganda
M. mauritiana	Taylor 1683 (B)	Uganda
M. mauritiana	Taylor 3233 (BM)	Uganda
M. vacciniifolia	Arbo 1454 (NY)	Argentina
M. vacciniifolia	Biganzoli 401 (MO)	Argentina
M. vacciniifolia	C.H 724 (G)	Argentina
M. vacciniifolia	Chocarro 1632 (MO)	Argentina
M. vacciniifolia	Ekman 69 (MO)	Argentina
M. vacciniifolia	Krapovickas s.n. (IAC)	Argentina

M. vacciniifolia	Krapovickas16512 (MO)	Argentina
M. vacciniifolia	Morel 4263 (BHCB)	Argentina
M. vacciniifolia	Morel 5823 (BHCB)	Argentina
M. vacciniifolia	Orbigny 79 (P)	Argentina
M. vacciniifolia	Pedersen 1129 (MO)	Argentina
M. vacciniifolia	Pedersen 3924 (NY)	Argentina
M. vacciniifolia	Pellegrini 11(NY)	Argentina
M. vacciniifolia	Pickett 12 (ASU)	Argentina
M. vacciniifolia	Schinini 24501 (C)	Argentina
M. vacciniifolia	Schinini 27437 (C)	Argentina
M. vacciniifolia	Torres 132 (HUEFS)	Argentina
M. vacciniifolia	Tressens 3576 (MO)	Argentina
M. vacciniifolia	Arroyo 39 (C)	Bolivia
M. vacciniifolia	Beck 2951(C)	Bolivia
M. vacciniifolia	Cutler 7075 (C)	Bolivia
M. vacciniifolia	Lewis 37212 (C)	Bolivia
M. vacciniifolia	Mandon 136 (P)	Bolivia
M. vacciniifolia	Murakami 1756 (MO)	Bolivia
M. vacciniifolia	Nee 34233 (US)	Bolivia
M. vacciniifolia	Nee 38979 (MO)	Bolivia
M. vacciniifolia	Paz 1378 (MO)	Bolivia
M. vacciniifolia	Pérez 1648 (MO)	Bolivia
M. vacciniifolia	Steinbach 7915 (C)	Bolivia
M. vacciniifolia	Williams 1040 (NY)	Bolivia
M. vacciniifolia	Almeida 3226 (UEC)	Brazil
M. vacciniifolia	Amorim 6444 (NY)	Brazil
M. vacciniifolia	Batista 92 (UEC)	Brazil

M. vacciniifolia	Beetle 1692 (US)	Brazil
M. vacciniifolia	Brade 41281 (MO)	Brazil
M. vacciniifolia	Braga 7454 (NY)	Brazil
M. vacciniifolia	Breier 975 (UEC)	Brazil
M. vacciniifolia	Dusén (P)	Brazil
M. vacciniifolia	Dusén 15208 (C)	Brazil
M. vacciniifolia	Edwards 2302 (NY)	Brazil
M. vacciniifolia	Eugenio s.n. 1941 (C)	Brazil
M. vacciniifolia	Evans (C)	Brazil
M. vacciniifolia	Fernandes 132 (C)	Brazil
M. vacciniifolia	Ferreira 29 (NY)	Brazil
M. vacciniifolia	Fiashi s.n (NY)	Brazil
M. vacciniifolia	Fontana 224 (BHCB)	Brazil
M. vacciniifolia	Gadelha 3860 (NY)	Brazil
M. vacciniifolia	Hatschbach 10769 (NY)	Brazil
M. vacciniifolia	Jaramillo 334 (SESJ)	Brazil
M. vacciniifolia	Labiak 4136 (BHCB)	Brazil
M. vacciniifolia	Labroy 4 (P)	Brazil
M. vacciniifolia	Leoni 9 (UEC)	Brazil
M. vacciniifolia	Lopes 308 (UFP)	Brazil
M. vacciniifolia	Matos 279 (NY)	Brazil
M. vacciniifolia	Mexia 4154 (NY)	Brazil
M. vacciniifolia	N.B s.n. (G)	Brazil
M. vacciniifolia	Oliveira 741(BHCB)	Brazil
M. vacciniifolia	Prado 1515 (NY)	Brazil
M. vacciniifolia	Prance 6948 (C)	Brazil
M. vacciniifolia	Rambo 41733 (NY)	Brazil

M. vacciniifolia	Rose 19631 (NY)	Brazil
M. vacciniifolia	Salino 10909 (BHCB)	Brazil
M. vacciniifolia	Salino 4443 (BHCB)	Brazil
M. vacciniifolia	Salino 5142 (BHCB)	Brazil
M. vacciniifolia	Santana 17994 (BHCB)	Brazil
M. vacciniifolia	Santana 323 (BHCB)	Brazil
M. vacciniifolia	Schmalz 10 (MO)	Brazil
M. vacciniifolia	Shepherd 8600	Brazil
M. vacciniifolia	Silva 19 (NY)	Brazil
M. vacciniifolia	Silva 5189 (NY)	Brazil
M. vacciniifolia	Silva s.n. (C)	Brazil
M. vacciniifolia	Silva-Almeida 412 (NY)	Brazil
M. vacciniifolia	Simões 26 (NY)	Brazil
M. vacciniifolia	Ulbricht 142 (NY)	Brazil
M. vacciniifolia	Wagner s.n. (P)	Brazil
M. vacciniifolia	Haught 4248 (GH)	Colombia
M. vacciniifolia	C. s.n (P)	França
M. vacciniifolia	Elliot s.n (NY)	Grenada
M. vacciniifolia	J. s.n (P)	Nicaragua
M. vacciniifolia	Aguayo 226 (MO)	Paraguay
M. vacciniifolia	Aguayo 281	Paraguay
M. vacciniifolia	Aguayo 319 (MO)	Paraguay
M. vacciniifolia	Aguayo 459 (MO)	Paraguay
M. vacciniifolia	Billiet 3080 (MO)	Paraguay
M. vacciniifolia	Casas 6062 (MO)	Paraguay
M. vacciniifolia	Casas 6062 (NY)	Paraguay
M. vacciniifolia	Degen 746 (MO)	Paraguay

M. vacciniifolia	Desconhecido (NY)	Paraguay
M. vacciniifolia	Fiebrig 5127 (MO)	Paraguay
M. vacciniifolia	Hahn 701 (MO)	Paraguay
M. vacciniifolia	Kuntze s.n (NY)	Paraguay
M. vacciniifolia	Mereles 3168 (P)	Paraguay
M. vacciniifolia	Mereles 5292 (MO)	Paraguay
M. vacciniifolia	Mereles 7587 (MO)	Paraguay
M. vacciniifolia	Morong 574 (C)	Paraguay
M. vacciniifolia	Morrone 210 (M0)	Paraguay
M. vacciniifolia	Ramella 2777 (G)	Paraguay
M. vacciniifolia	Ramirez 41435 (MO)	Paraguay
M. vacciniifolia	Rojas 12470 (NY)	Paraguay
M. vacciniifolia	Rojas 3193 (MO)	Paraguay
M. vacciniifolia	Soria 2724 (MO)	Paraguay
M. vacciniifolia	Vavrek 484 (MO)	Paraguay
M. vacciniifolia	Velasquez 13113 (MO)	Paraguay
M. vacciniifolia	Zardini 10066 (MO)	Paraguay
M. vacciniifolia	Zardini 10769 (MO)	Paraguay
M. vacciniifolia	Zardini 13717 (MO)	Paraguay
M. vacciniifolia	Zardini 15555 (MO)	Paraguay
M. vacciniifolia	Zardini 15809 (NY)	Paraguay
M. vacciniifolia	Zardini 16781(MO)	Paraguay
M. vacciniifolia	Zardini 22053 (NY)	Paraguay
M. vacciniifolia	Zardini 33954 (NY)	Paraguay
M. vacciniifolia	Zardini 3928 (MO)	Paraguay
M. vacciniifolia	Zardini 558 (MO)	Paraguay
M. vacciniifolia	Zardini 5881(MO)	Paraguay

M. vacciniifolia	Zardini 6316 (NY)	Paraguay
M. vacciniifolia	Camilo 3502 (MO)	Peru
M. vacciniifolia	Sembrera 3726 (MO)	Peru
M. vacciniifolia	Vargas 6029 (C)	Peru
M. vacciniifolia	Woytkowski 6938 (MO)	Peru
M. vacciniifolia	Andrews 579 (NY)	Trinidad and Tobago
M. vacciniifolia	Britton 886	Trinidad and Tobago
M. vacciniifolia	Broadway 3445 (NY)	Trinidad and Tobago
M. vacciniifolia	Broadway s.n. (P)	Trinidad and Tobago
M. vacciniifolia	Cowan (NY)	Trinidad and Tobago
M. vacciniifolia	Jenmam s.n. (NY)	Trinidad and Tobago
M. vacciniifolia	Roques 15692 (MO)	Trinidad and Tobago
M. vacciniifolia	Gilbert 24 (BHCB)	Uruguay
M. vacciniifolia	Buting 12480 (MO)	Venezuela
M. vacciniifolia	F.L 718 (P)	Venezuela
M. vacciniifolia	Falcon 345 (MO)	Venezuela
M. vacciniifolia	Miller 159 (C)	Venezuela

ANEXOS

Comentários à coordenação do PPGBEES:

O trabalho trata de um tema atual e relevante, além de apresentar uma metodologia inovadora para a delimitação de espécies em samambaias.

A introdução está de acordo com as normas apresentadas.

O capítulo 1 está bem escrito. A introdução contextualiza o tema de forma adequada e os objetivos estão bem definidos. Os resultados foram promissores para o grupo de estudo escolhido. Entretanto, seria interessante que espécies morfologicamente mais semelhantes fossem tratadas em análises separadas, para testar mais especificamente a robustez do método na diferenciação de complexos quanto ao método.

O capítulo 2 também está bem estruturado. Os objetivos estão claramente expostos e a metodologia está bem elaborada. Neste caso, um complexo é analisado com uma abordagem morfométrica. Os resultados apontam para o reconhecimento das espécies analisadas, exceto pela alta sobreposição entre M. vaccinifolia e M. crispata, o que já era esperado. O artigo conclui que a forma mais adequada de diferenciação entre estes táxons seria uma análise integrada, utilizando vários métodos.

Os dois artigos mostram a aplicação de métodos biossistematicos que podem ser usados integrados aos usuais na diferenciação de espécies. Mostra que resultados promissores podem ser obtidos com técnicas de custo menos elevadas, de forma a melhor representar nossa biodiversidade. Considero que os resultados promissores e a adequada análise dos resultados obtidos foram possibilitadas pela escolha do grupo de plantas. O conhecimento prévio e detalhado da taxonomia do gênero foi estratégico para uma discussão mais aprofundada sobre as análises realizadas e os resultados alcançados.

Portanto, considero a dissertação aprovada. Não tenho correções a fazer no manuscrito apresentado.

Avaliação final do projeto de dissertação de mestrado

I - Aprovado (X)

Indica que o revisor aprova a dissertações sem correções ou com correções mínimas.

II - Aprovado com Correções ( )

indica que o avaliador aprova o projeto com correçõe extensas,mas que a dissertação não precisa retornar ao avaliador para reavaliação

III- Necessita Revisão ( )

indica que há necessidade de reformulação do trabalho e que avaliadorquer reavaliar a nova versão da dissertação antes de emitir uma decisão final

IV- Reprovado ( )

indica que a dissertação não é adequada, nem com modificações substanciais

Identificação do membro da banca: Lana da Silva Sylvestre

Data: 23/11/2020

Assinatura: 

Comentários à coordenação do PPGBEES:

Gostaria de parabenizar a autora da dissertação e a sua orientadora pelo excelente trabalho, ele apresenta uma abordagem pertinente e inovadora para o grupo das samambaias. Encaminho o texto em PDF com pequenos comentários que visam contribuir para o aprimoramento da dissertação.

Avaliação final do projeto de dissertação de mestrado

I - Aprovado (X)

Indica que o revisor aprova a dissertações sem correções ou com correções mínimas.

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indica que há necessidade de reformulação do trabalho e que avaliadorquer reavaliar a nova versão da dissertação antes de emitir uma decisão final

IV- Reprovado ( )

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Identificação do membro da banca: Marcelo Guerra Santos

Data: 22 de novembro de 2020.

Assinatura: 

Comentários à coordenação do PPGBEES:

A dissertação está muito bem escrita. Demonstra que o aluno desenvolveu capacidade intelectual esperada para um mestrando e até além. Há um problema metodológico no capítulo 2 que não invalida o trabalho. O mesmo capítulo necessita de um pouco mais de atenção, mas ainda assim o trabalho está acima da média das dissertações por mim avaliadas. As recomendações neste caso estão no PDF.

A introdução, na minha opinião, ainda é muito científica. Há termos muito técnicos e para um leigo (que seria o público de uma divulgação científica) me parece inadequada. Fiz uma série de sugestões para o autor pensando sempre no público alvo. Reconheço o esforço despendido, uma vez que fazer escrita não acadêmica pode ser extremamente desafiador para quem não está acostumado.

Avaliação final do projeto de dissertação de mestrado

I - Aprovado (X)

Indica que o revisor aprova a dissertações sem correções ou com correções mínimas.

II - Aprovado com Correções ( )

indica que o avaliador aprova o projeto com correçõe extensas,mas que a dissertação não precisa retornar ao avaliador para reavaliação

III- Necessita Revisão ( )

indica que há necessidade de reformulação do trabalho e que avaliadorquer reavaliar a nova versão da dissertação antes de emitir uma decisão final

IV- Reprovado ( )

indica que a dissertação não é adequada, nem com modificações substanciais

Identificação do membro da banca: André Luís de Gasper

Data: 05/11/2020

Assinatura: 

Comentários à coordenação do PPGBEES:

A aluna cumpriu todos os requisitos para obtenção do título de mestre e mostra evolução considerável em relação ao texto apresentado na qualificação. A dissertação se divide em três capítulos, sendo o primeiro capitulo uma introdução curta, no formato indicado pelo programa, e os outros dois capítulos no formato de artigo com os resultados obtidos ao longo do mestrado. O conjunto de dados obtidos é extenso e as análises empregadas são adequadas para os objetivos propostos. Destaco também que os manuscritos apresentados estão bem encaminhados, mas o segundo manuscrito (Capítulo 3), em especial, ainda exija uma atenção maior na descrição e interpretação dos resultados. Em termos gerais, penso que ainda é importante uma revisão da fluidez do texto (em especial, da transição entre os parágrafos) e de algumas figuras/tabelas. Para o capítulo 3 também julgo muito importante que sejam especificados os objetivos no ultimo parágrafo da Introdução. Disponibilizo o arquivo pdf da dissertação com meus comentários e sugestões acerca do que pode ser melhorado visando a entrega da versão final da dissertação e também uma futura submissão do capítulo 3 a algum periódico.

Avaliação final do projeto de dissertação de mestrado

I - Aprovado (X)

Indica que o revisor aprova a dissertações sem correções ou com correções mínimas.

II - Aprovado com Correções ( )

indica que o avaliador aprova o projeto com correçõe extensas,mas que a dissertação não precisa retornar ao avaliador para reavaliação

III- Necessita Revisão ( )

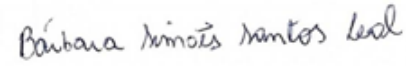
indica que há necessidade de reformulação do trabalho e que avaliadorquer reavaliar a nova versão da dissertação antes de emitir uma decisão final

IV- Reprovado ( )

indica que a dissertação não é adequada, nem com modificações substanciais

Identificação do membro da banca: Bárbara Simões Santos Leal

Data: 19/11/2020

Assinatura: 

Comentários à coordenação do PPGBEES:

A dissertação é uma contribuição cientifica original e adequada à área sistemática biológica. Indiquei pontos sensíveis no próprio texto (envio em anexo). O segundo capítulo precisa de uma reformulação mais radical mais particularmente quanto á introdução, a apresentação dos resultados e a revisão da língua, que eu recomendo fortemente que sejam para a versão final.

Avaliação final do projeto de dissertação de mestrado

I - Aprovado ( )

Indica que o revisor aprova a dissertações sem correções ou com correções mínimas.

II - Aprovado com Correções (X)

indica que o avaliador aprova o projeto com correçõe extensas,mas que a dissertação não precisa retornar ao avaliador para reavaliação

III- Necessita Revisão ( )

indica que há necessidade de reformulação do trabalho e que avaliadorquer reavaliar a nova versão da dissertação antes de emitir uma decisão final

IV- Reprovado ( )

indica que a dissertação não é adequada, nem com modificações substanciais

Identificação do membro da banca: Thiago Jose de Carvalho André

Data: 20 de Novembro de 2020

Assinatura: