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ORIGINAL ARTICLE

Characterization of *Mesocriconema* species associated with grapevine decline disease in southern Brazil

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Abstract

The grapevine decline disease (GDD) is a complex disease that causes substantial losses in grape production. Ring nematode (*Mesocriconema xenoplax*) is frequently detected in symptomatic vineyards. Unfortunately, this nematode is frequently mis-identified due to its similarities with other species of this genus. In this study, the hypothesis was tested that there is a species complex of *Mesocriconema* associated with GDD in southern Brazil. This hypothesis was based on a previous result that identified different Mesocriconema species in vineyards in southern Brazil including *M. xenoplax*, *M. curvatum*, *M. rusticum*, *M. sphaerocephalum*, *M. ornatum*, and another seven undefined species, using only morphometric data. This current study provides the first characterization of *Mesocriconema* species associated with GDD, their distribution, and variability, through the use of morphological and molecular analysis in an integrative approach.

Keywords Ring nematode · *Vitis* sp. · Integrative taxonomy · DNA barcode

Introduction

Among the several plant health problems that affect the vineyards, those caused by plant parasitic nematodes (PPNs) require attention due to the damage to the crop levels and distribution (Téliz et al. 2007; Gomes et al. 2009; Divers et al. 2019). Several species have already been reported parasitizing *Vitis* spp., such as species of the root-knot nematode (*Meloidogyne* spp.), the ring nematode (*Mesocriconema* spp.), the dagger nematode (*Xiphinema* spp.), the citrus nematode (*Tylenchulus semipenetrans*), and the root-lesion nematode (*Pratylenchus* spp.) (Walker and Stirling 2008; Askary et al. 2018). Nowadays, *Mesocriconema* species (morphotypes) have been found associated with grapevine decline disease (GDD) on several regions of the world, as the southern Brazil (Divers et al. 2019).

Historically, the taxonomic status of the genus *Mesocriconema* is controversial, and taxonomists have not yet reached a consensus on the validity and composition of the species, (Cordero et al. 2012; Powers et al. 2014). For the

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identification of these nematodes, in addition to morphological observations, conventional morphometric measurements have been often used as the number of annuli in the body and the position of certain organs in relation to these annuli. Even using these observations, it is still difficult to identify *Mesocriconema* species, due to the existence of closely related taxa (sister species), in which the differences are so slight that doubts arisen whether some specimens are really another dissimilar species or cryptic species (Cordero et al. 2012; Kaur et al. 2012).

In view of the undeniable difficulty in identifying *Mesocriconema* species and the importance that this group of nematodes represents for viticulture, our study aimed (*i*) to identify and characterize (morphological and molecular approaches) the *Mesocriconema* species complex associated with GDD in southern Brazil, (*ii*) to study the diversity of the *Mesocriconema* species, and (*iii*) to elucidate the phylogenetic relationship between *Mesocriconema* species based on COI (cytochrome oxidase subunit I) sequences. To our knowledge, this is the first comprehensive study to identify *Mesocriconema* species associated with GDD in Brazil.

Material and methods

Nematode collection

Fifteen populations of *Mesocriconema* spp. collected by Divers (2018) in vineyards with symptoms of GDD in Rio Grande do Sul and Santa Catarina were used in our study. Here, we used the unified species concept, reformulated by Queiroz (2005), to recognize the species, and information for each specimen examined is provided in Tables 1, 2, 3.

Morphologic and morphometric analysis

Temporary slides were prepared from 15 adult females of each population of *Mesocriconema*, where each nematode was photographed with a video camera (Leica DFC 295) attached to an optical microscope (Leica DM 1000) at 20×, 40×, and 100× magnification, using the LAS Core software, version 3.7 (Leica Microsystems 2021). A set of standard measurements was obtained from each specimen that included the following measurements, percentages, and ratios: L (body length), St (stylet length), Ø (longest body width), Oes (length of esophagus), t (tail length), L' (distance from the anterior end to the anus), V (distance from the anterior end to the vulva), VL (distance from the posterior end to the vulva), VA (distance from the vulva to the anus), VB (diameter of the body at the vulva), RB (width of annules at midbody), R (number of annules), RA (number of anastomoses on the body cuticle), RV (number of annules from posterior end to vulva), Ran (number of annules from anus to posterior end), Rvan (number of annules from vulva to anus), Roes (number of annules in the esophageal region), Rex (number of annules from anterior end to 1st annuli after the excretory pore), a (L/Ø), b (L/Oes), c (L/t), d (t/VL), V% (V × 100/ L), V' (V × 100/ L'), St%L, St × 100/L, St%Oes, and St × 100/Oes.

The morphometric data were submitted to principal component analysis (PCA), using a correlation matrix. Firstly, a PCA was performed to select the number of principal

Table 1 Sample identification number collected in vineyard with symptoms of grapevine decline disease in southern Brazil, rootstocks, geographic coordinates, and sampler

SIN	Rootstock	Geographic coordinates	Municipality/state	Sampler
Mayer[/1]	Capdeboscq	31°28′35.02″ S 52°34′17.17″ W	Pelotas/RS[/2]	Divers (2018)
1	Paulsen 1103	27°2′0.23″ S 51°8′6.008″ W	Videira/SC[/3]	Divers (2018)
2	Paulsen 1103	27°2′0.23″ S 51°8′5.045″ W	Videira/SC	Divers (2018)
7	VR043-43	27°2′023″ S 51°8′6.008″ W	Pinheiro Preto/SC	Divers (2018)
8	Own-rooted	27°3′20.29″ S 51°14′2.593″ W	Pinheiro Preto/SC	Divers (2018)
9	Own-rooted	27°2′22.81″ S 51°15′4.064″ W	Pinheiro Preto/SC	Divers (2018)
10	Paulsen 1103	Not identified	Tangará/SC	Divers (2018)
11	Paulsen 1103	Not identified	Tangará/SC	Divers (2018)
12	Paulsen 1103	Not identified	Tangará/SC	Divers (2018)
367	Paulsen 1103	29°14′923″ S 51°14′376″ W	Caxias do Sul/RS	Divers (2018)
369	Own-rooted	29°14′477″ S 51°14′363″ W	Caxias do Sul/RS	Divers (2018)
376	Paulsen 1103	29°12′102″ S 51°33′16″ W	Garibaldi/RS	Divers (2018)
378	Paulsen 1103	29°12′64″ S 51°33′64″ W	Garibaldi/RS	Divers (2018)
380	Not identified	29°14′811″ S 51°38′334″ W	Garibaldi/RS	Divers (2018)
422	Paulsen 1103	29°04′505″ S 51°14′246″ W	Flores da Cunha/RS	Divers (2018)

[/1] Soil samples from peach tree plants

[/2] Rio Grande do Sul

[/3] Santa Catarina

Table 2 Sample identification number, frequency, and species of *Mesocriconema* detected by morphology and morphometry in the vineyard samples

SIN	Species	Frequency (%)
Mayer[/1]	*M. xenoplax*	100.0
1	*M. xenoplax*	76.3
	M. curvatum	26.7
2	*M. xenoplax*	80.0
	M. rusticum	20.0
7	*M. xenoplax*	40.0
	M. curvatum	46.7
	M. rusticum	13.3
8	*M. xenoplax*	100.0
9	*M. xenoplax*	73.3
	M. curvatum	26.7
10	*M. xenoplax*	73.3
	M. curvatum	26.7
11	*M. xenoplax*	60.0
	M. curvatum	40.0
12	*M. xenoplax*	66.7
	M. rusticum	33.3
367	*M. xenoplax*	100.0
369	*M. xenoplax*	80.0
	M. rusticum	20.0
376	*M. xenoplax*	73.3
	M. curvatum	26.7
378	*M. xenoplax*	66.7
	M. curvatum	33.3
380	*M. xenoplax*	60.0
	M. rusticum	40.0
422	*M. xenoplax*	73.3
	M. curvatum	26.7

[/1] Sample from peach tree plants

components (PC) capable of retaining the largest variance. Then, a second PCA was performed to obtain the clusters. The analyses were performed using the packages FactoMineR (Husson et al. 2018) and factoextra (Kassambara and Mundt 2020), in the R software (version 4.0) (R Development Core Team 2022).

DNA extraction and polymerase chain reaction

Following photo-documentation and measurement, each nematode specimen was submitted to the protocol by Powers et al. (2014). For this, one female from each *Mesocriconema* population was removed from the temporary glass slide, placed on a coverslip in a 18 µL of sterile water, and ruptured with a micropipette tip. Then, each specimen was ruptured and transferred to a reaction tube (0.2 mL) and stored at 4 °C.

Amplification of fragments of the COI mitochondrial gene was performed using the primers COI-F5 (5′-AAT WTWGGTGTTGGAACTTCTTGAAC-3′) and COI-R9 (5′-CTTAAACATAATGRAAATGGCAACATATAGTC-3′), described by Powers et al. (2014). The polymerase chain reaction (PCR) was conducted in a BioRad T100 Thermocycler (30 µl), consisted of 9 µl of total DNA, 2.4 µl of each primer (20 µM), 15 µl of GoTaq® Green Master Mix (Promega), and 1.2 µl of sterile nuclease-free deionized water. PCR conditions included an initial hot start and 5′ at 94 °C followed by 50 cycles of 30″ at 94 °C denaturation, 30″ at 48 °C annealing, and 1.5′ at 72 °C with a ramping rate of 0.5 °C by second for elongation step. A final 5′ extension at 72 °C completed this process. PCR products were sent for sequencing.

Phylogenetic analysis

The nucleotide sequences were compared by the Blastn (http://www.ncbi.nlm.nih.gov/blast/), submitted to GenBank database (Table 4), and aligned with ClustalW available in software MEGA 11 (Kumar et al. 2021). The phylogenetic analyses were based on Jukes-Cantor method and using maximum likelihood model and complete deletion (500 replicates). Other *Mesocriconema* sequences were selected and included in the analysis for comparison. In addition, *Discocriconemella limitanea* (KU552168) was used as an outgroup. The measures of distinction of haplotype groups were evaluated using Arlequin v. 3.5.2.2 (Excoffier and Lischer 2010).

Results

Three species of *Mesocriconema* were identified: *M. xenoplax*, *M. curvatum*, and *M. rusticum* (Table 2). *Mesocriconema xenoplax* was detected at all samples besides to be the predominant species as well as the most frequent, except only for sample 7, with 40.0% frequency, whose *M. curvatum* was predominant (46.7%). *M. curvatum* was detected in eight samples, followed by *M. rusticum* in four samples. Among the samples from Santa Catarina and Rio Grande do Sul state vineyards, only in the samples 8 and 367 M. *xenoplax* was identified unlike the others that there was mixture of species. In the Mayer sample, from a peach orchard with the syndrome Peach Tree Short Life, only the species *M. xenoplax* was also detected, according to the previous identification by Divers (2018).

Data obtained from morphological, morphometric, and allometric characterizations are presented in Table 3. Regarding the morphology, the *M. xenoplax* female specimens (Fig. 1A1) differed from *M. curvatum* (Fig. 1B1) and *M. rusticum* (Fig. 1C1) mainly by the shape of the vulva,

Table 3 Morphometric data from females of *Mesocriconema* species collected in vineyards with symptoms of GDD in southern Brazil

	Mean µm (range µm)		
	M. xenoplax	*M. curvatum*	*M. rusticum*
Measurements			
L	655.9 (754.6–565.3)	576.4 (589.2–502.0)	534.0 (539.5–507.6)
St	80.7 (86.5–71.3)	68.9 (72.7–64.8)	57.1 (60.5–49.0)
Ø	59.3 (68.6–52.3)	43.6 (48.5–44.7)	44.0 (44.6–40.5)
Oes	154.9 (173.8–135.8)	124.6 (125.0–120.6)	108.6 (107.2–99.4)
t	29.8 (37.3–22.1)	27.4 (28.6–23.2)	22.8 (23.4–22.1)
L'	627.0 (718.4–550.6)	509.0 (522.0–477.5)	521.3 (514.6–482.2)
V	613.4 (706.3–535.2)	499.2 (512.3–468.6)	493.1 (505.1–475.0)
VL	40.1 (48.0–30.2)	37.2 (38.2–31.5)	31.2 (33.7–32.0)
VA	12.4 (14.3–10.9)	7.6 (7.8–5.4)	6.1 (6.1–5.7)
VB	42.1 (47.8–36.6)	40.7 (42.2–34.9)	36.7 (38.7–30.1)
RB	6.2 (7.1–5.5)	6.1 (6.8–5.4)	6.5 (6.5–5.6)
Nº annuli	-	-	-
R	101.4 (110.0–96.0)	97.5 (98.0–84.0)	99.4 (109.0–94.0)
RSt	14.3 (16.0–11.0)	14.1 (15.0–12.0)	13.2 (15.0–10.0)
RV	7.7 (9.0–7.0)	6.5 (7.0–5.0)	6.3 (8.0–6.0)
Ran	5.5 (6.0–5.0)	4.0 (7.0–4.0)	5.1 (6.0–5.0)
Rvan	2.0 (2.0–2.0)	1.3 (2.0–1.0)	1.2 (2.0–1.0)
Roes	24.3 (25.0–23.0)	25.5 (26.0–22.0)	23.8 (26.0–18.0)
Rex	28.6 (30.0–27.0)	29.6 (31.0–28.0)	29.1 (30.0–26.0)
RA	0.0 (0.0–0.0)	5.0 (7.0–3.0)	5.0 (7–2)
Ratios	-	-	-
a	11.1 (12.1–9.4)	10.8 (12.0–9.2)	11.7 (13.3–9.9)
b	4.2 (4.8–3.6)	3.8 (4.0–3.6)	4.0 (4.6–3.5)
c	22.3 (25.6–18.9)	21.2 (32.0–17.0)	23.8 (30.8–18.9)
d	0.7 (1.0–0.6)	0.8 (0.9–0.5)	0.7 (0.8–0.6)
Percentages	-	-	-
V%	93.5 (96.3–90.6)	93.5 (95.0–92.4)	93.9 (95.6–92.3)
V'	97.8 (98.6–96.6)	98.2 (98.8–97.4)	98.1 (98.8–96.9)
St%L	12.4 (14.2–10.5)	13.7 (11.4–10.7)	12.8 (16.0–10.1)
St%Oes	52.1 (55.2–47.3)	51.7 (53.0–48.4)	51.1 (56.7–46.4)

being sigmoid (slight curvature). The presence of lobes on the vulva was one of the most difficult characteristics to observe in the analyses. On the other hand, despite the ease of observing the annulation of the specimens, its classification is of great difficulty, given the subtle differences between irregular and smooth annuli margins, as well as the classification of the tail shape. A striking feature that was very useful to separate *M. xenoplax* from the other species was the absence of anastomoses.

The morphometric and allometric variables under analysis had their mean values within the reported range, for all identified species, according to the original descriptions and additional studies (Raski 1952; Brzeski et al. 2002; Cordero et al. 2012; Powers et al. 2014). It was possible to observe significant and essential differences for species identification; for example, the variables L, St, Oes, t, and R were of great importance for the separation between *M. xenoplax* and the other species. This analysis was compatible with the molecular identification, although the efficacy of the Powers' protocol was extremely low (20%). Only 32 specimens had their fragments amplified (Table 4), with six populations obtained from Santa Catarina and two from Rio Grande do Sul. The amplified fragments were of 721 base pairs, except for individuals from population 1, where there was no amplification of any individual.

Despite of the reduced number of sequenced specimens, the analysis of the COI sequences corroborated the identification, a priori, obtained from morphological and morphometric approaches. The sequences of *M. xenoplax* showed 99.7% of similarity with the strain N9103 (MN711236), as well as the sequences of *M. curvatum* and *M. rusticum* showed 99.8 and 99.7% of similarity with the individuals TN11 (MN734383) and N8878 (MN711111), respectively.

Table 4 *Mesocriconema* species genetically identified with their identification number of the specimen (INS), sample identification number (SIN), and GenBank accession number (GBAN)

INS	Species	SIN	GBAN	INS	Species	SIN	GBAN
MxB5	*M. xenoplax*	Mayer[1/]	OP418003	MxB233	*M. xenoplax*	11	OP431937
MxB10	*M. xenoplax*	Mayer	OP428648	MxB235	*M. xenoplax*	11	OP431938
MxB15	*M. xenoplax*	8	OP431930	MxB236	*M. xenoplax*	11	OP431939
MxB20	*M. xenoplax*	8	OP431931	MxB239	*M. xenoplax*	11	OP431940
MxB40	*M. xenoplax*	369	OP431941	McB53	*M. curvatum*	378	OP431950
MxB44	*M. xenoplax*	369	OP431942	McB200	*M. curvatum*	9	OP431947
MxB45	*M. xenoplax*	369	OP431943	McB203	*M. curvatum*	9	OP431949
MxB46	*M. xenoplax*	378	OP431934	McB208	*M. curvatum*	9	OP431948
MxB50	*M. xenoplax*	378	OP431935	McB221	*M. curvatum*	7	OP431944
MxB209	*M. xenoplax*	9	OP431932	McB223	*M. curvatum*	7	OP431945
MxB210	*M. xenoplax*	9	OP431933	McB224	*M. curvatum*	7	OP431946
MxB213	*M. xenoplax*	7	OP428649	MrB34	*M. rusticum*	369	OP431951
MxB216	*M. xenoplax*	7	OP428652	MrB35	*M. rusticum*	369	OP431952
MxB222	*M. xenoplax*	7	OP428650	MrB167	*M. rusticum*	2	OP431953
MxB225	*M. xenoplax*	7	OP428651	MrB178	*M. rusticum*	2	OP431954
MxB232	*M. xenoplax*	11	OP431936	MrB219	*M. rusticum*	7	OP431955

[1/]Sample from peach tree plants

Furthermore, through PCA analysis (Fig. 2), the species were grouped into three distinct groups, although there were overlaps among the groups. The first two PCs explained 87% of the variance. The first PC was positive for four characteristics (St, Oes, L, and R), while the second PC was positive only for R and L. We observed low intraspecific variability in *M. xenoplax*, while greater variability was observed between *M. curvatum* and *M. rusticum*.

In the maximum likelihood tree (Fig. 3), there was no grouping trend by region of origin. However, the separation of the specimens according to the haplotype group was observed, suggesting a high variability within of each species. In the haplotype analyses, ten haplotypes shared within the populations were identified, as can be seen by the haplotype network (Fig. 4). Consensus tree from the *Mesocriconema* dataset produced strong node support values for haplotype groups, except for group 4 (0.56). Group 10 had a bootstrap value of 1.0. The MxB45 isolate, the only representative of group 5, showed a bootstrap value of 1.0 with an isolate from the USA (N2893). Isolates from groups 7, 6, and 8 had a bootstrap value of 0.97, 0.92, and 0.90, respectively.

Based on specific diagnostic traits or measurements, the haplotype groups generated by COI analysis each appear to be associated with morphospecies: groups 1 and 2 associated with *M. rusticum*, groups 3 and 4 associated with *M. curvatum*, and groups 5 to 10 associated with *M. xenoplax*. Isolates from haplotype groups 5 to 10 corresponded morphologically to *M. xenoplax* and evidently belong to a monophyletic group. Similarly, isolates from haplotype group from groups 1 and 2 generally conform to *M. rusticum* and belonged to a monophyletic group. On the other hand, isolates from haplotypes 3 and 4, despite corresponding morphologically to *M. curvatum*, apparently belong to a paraphyletic group.

Haplotype 1 was composed by specimens from samples 7 and 369. Haplotype 2 was represented by specimens from samples 2 and 369. Haplotypes 3 and 4, which included only specimens of *M. curvatum*, were represented by specimens from sample 7, 9,and 378. The other haplotypes (5 to 10) were composed by specimens from all samples, except population 2. Several median vectors among the haplotypes of the different species were observed, indicating that the sampling needs to be larger for the variability to be better represented. From the haplotype network, it can be observed that some of the populations studied have more than one haplotype.

The distribution of haplotypes according to the collection site is provided in Fig. 5. Due to the greater number of individuals sequenced, the population from Pinheiro Preto (sample 7; Table 4) showed the greatest variability, being represented by eight of the ten identified haplotypes. On the other hand, only one haplotype was identified in the population from Videira (sample 2), in which only two specimens were sequenced. In the populations from Tangará (sample 11) and Caxias do Sul (sample 369), five haplotypes were identified for each and the two populations share haplotype 10. Similarly, a population from Pinheiro Preto shares the same haplotypes with the populations from other municipalities, except the population from Videira. Haplotypic diversity was also observed for the population from peach tree plants, where two haplotypes were identified (H9 and H10).

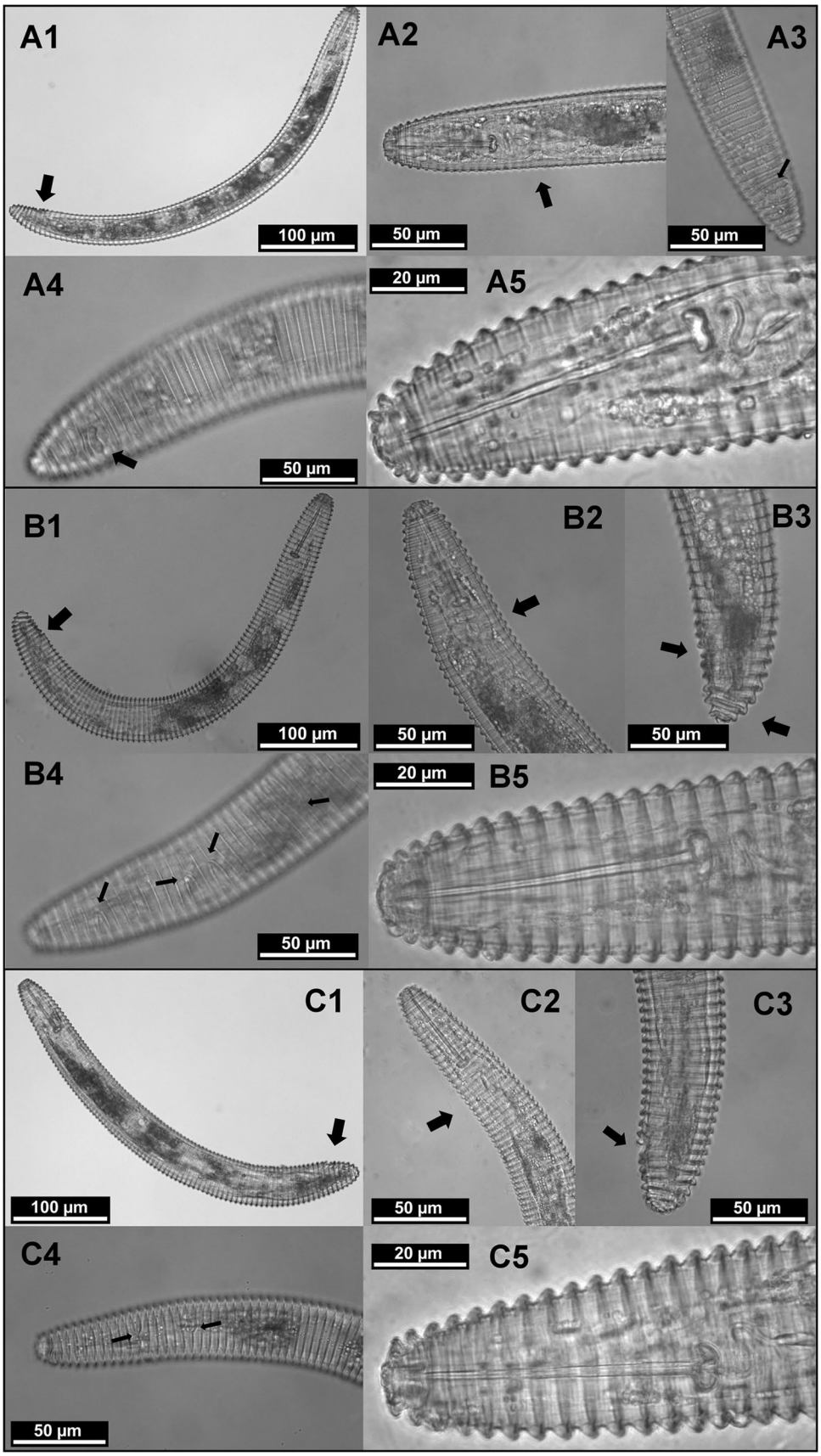

Fig. 1 **A1** *Mesocriconema xenoplax*. **A2** Esophageal region. **A3** and **A4** Conical-rounded tail and anterior vulval lip with lobes. **A5** Lip region and annuli margin. **B1** *Mesocriconema curvatum*. **B2** Esophageal region. **B3** Rounded tail and vulval lip. **B4** Anastomoses. **B5** Lip region and annuli margin. **C1** *Mesocriconema rusticum*. **C2** Esophageal region. **C3** Rounded tail and vulval lip. **C4** Anastomoses. **C5** Lip region and annuli margin. Scale bars: 20 µm, 50 µm, and 100 µm

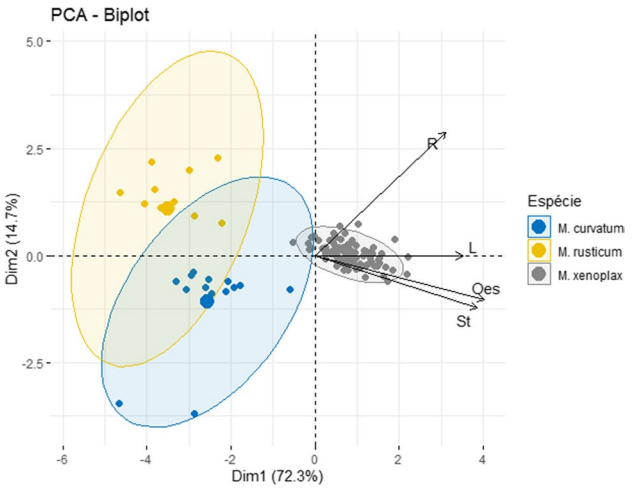

Fig. 2 Biplot obtained from morphometric data of *Mesocriconema* populations subjected to principal component analysis (PCA). L: body length, St: stylet length, Oes: esophagus length, R: number of body annuli. Other data were obtained from Cordero et al. (2012)

Discussion

Our results are partially according with the results reported by Divers (2018), where six species were identified morphologically and morphometrically. In that study, the species *M. sphaerocephalum*, *M. ornatum*, and seven undefined species were not identified, possibly morphospecies, as reported by Powers et al. (2014), which can be explained, in part, by the number of sequenced copies.

The genus *Mesocriconema* is still the point of discussions among taxonomists due to the great similarity between the species (Olson et al. 2017). Reproduction in this genus occurs by mitotic parthenogenesis and males are extremely rare (Brzeski et al. 2002). *Mesocriconema* males are believed to play little or no role in reproduction and that mitochondrial and nuclear genomes are clonally inherited, with parthenogenesis assumed to be the primary mode of reproduction for this genus (Powers et al. 2014).

In genera such as *Mesocriconema*, asexual reproduction is generally regarded as an evolutionary dead end, and the difficulties for lineages to adapt to the environment are significantly greater due to the possible lack of genetic plasticity (Castagnone-Sereno and Danchin 2014). However, unlike the sexual species, the parthenogenic mitotic ring nematode species are remarkably widespread and polyphagous, capable of parasitizing a wide range of hosts (Schreiner et al. 2012; Powers et al. 2014). Although this may reflect, in part, the stability of agricultural environments, the extreme parasitic success of these clonal species points to them as a remarkable evolutionary paradox in relation to current theories about the benefits of sexual reproduction and may be a way to understand the events of speciation in a group where new species are increasingly being discovered (Castagnone-Sereno and Danchin 2014).

Although the genus *Mesocriconema* still require studies, it is known that for other plant parasitic nematodes with the same type of reproduction, most of the genome is composed by pairs of homologous segments that presumably evolved independently of the absence of sexual recombination (Bird et al. 2015). In recent studies on other biological systems, this observation suggests that functional innovation could emerge from such a peculiar genome architecture, which may, in turn, explain the adaptive capacity of these asexual parasites (Nyguyen et al. 2019).

Studies carried out by De Ley et al. (2005) indicated that populations of *Mesocriconema* in Southern California were genetically heterogeneous, with differentiation between coastal and inland populations, which could correlate with differences in reproductive capacity in grape rootstock varieties planted in the populations' collection areas. Similarly, Cordero et al. (2012) reported high morphometric variation among *M. xenoplax* populations from different regions and host species. Among the nine populations studied by Cordero et al. (2012), all were morphometrically distinct, even within the delimitation range of *M. xenoplax*. Similar results were observed in the present study, where marked morphometric variations were observed between populations of this species.

It is important to point out how difficult it is to identify *Mesocriconema* species, due to the closely related taxa; that is, the differences between the species are so small that they come to question whether some individuals are really another species or are morphotypes, individuals with morphological variations belonging to the same species (Cordero et al. 2012; Kaur et al. 2012). There are an estimated 487 valid species in the Criconematidae family, according to the latest review, which makes it even more difficult to establish boundaries between them (Geraert 2010). Assuming that morphological distinction is the criterion used to establish these species, the perspective that each morphospecies would compose new species would result in a significant increase in the number of species in a taxon that is already presumed to be "hyperdiverse," as observed in *Caenorhabditis* (Puillandre et al. 2012; Dey et al. 2013).

Powers et al. (2014) reported that some of the species of the genus *Mesocriconema*, considered cosmopolitan in their distribution, are multispecific polyphyletic groupings and an accurate assessment of the distributions of the species of this genus should be performed to better understand the genetic variability of this group of nematodes. In that study, the authors were successful in amplifying 242 specimens, but did not provide information about the efficiency of the protocol for amplification of mitochondrial DNA (mtDNA). In our study, the protocol proposed by

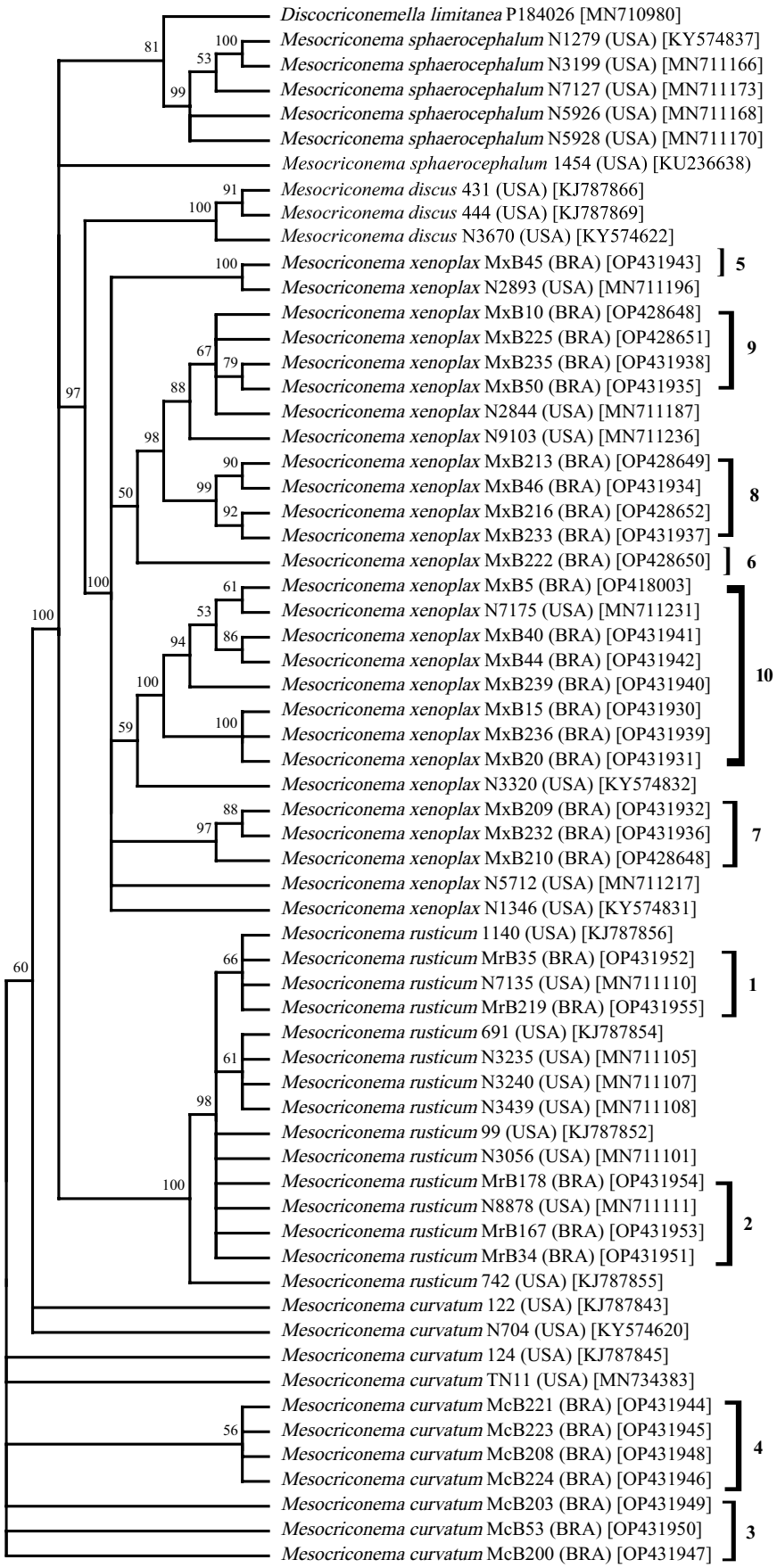

Fig. 3 Maximum-Likelihood tree of COI nucleotide from 42 sequences of *Mesocriconema* species of this study and sequences from Powers et al. (2014). Bootstrap support values > 50% (500 replications) are provided at the nodes. *Discocriconemella limitanea* P184026 was used as an outgroup. Haplotype groups have been bracketed and given a group number

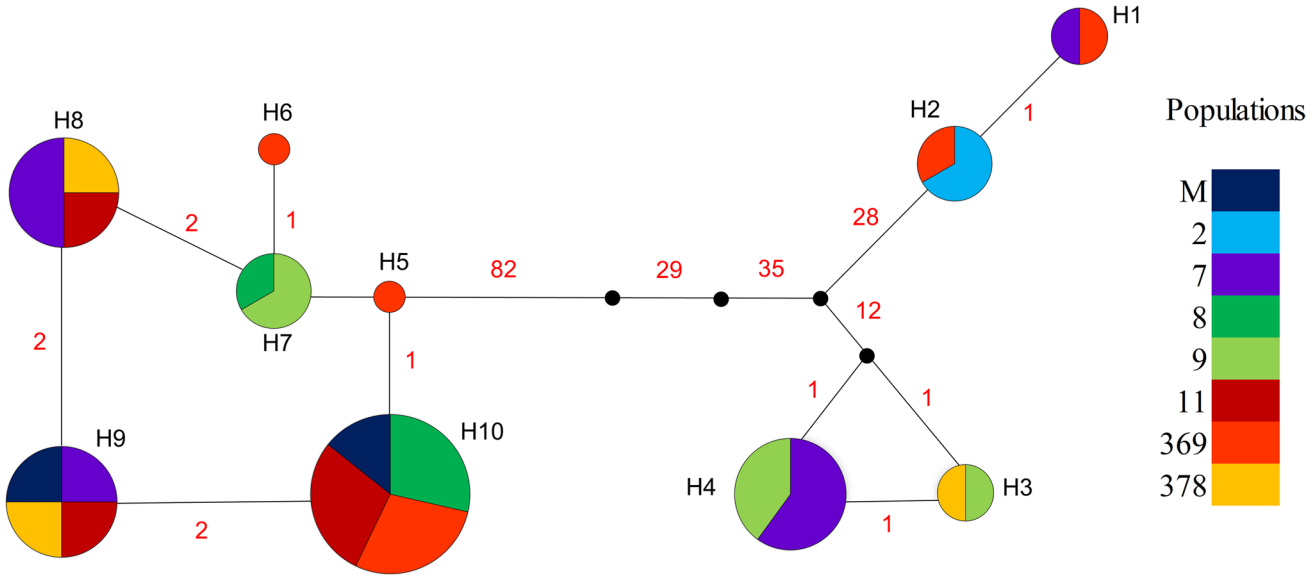

Fig. 4 Haplotype network of *Mesocriconema* species. Haplotype groups (H) are represented as circles connected by hash marks indicating base pair changes between haplotypes (numbers in red). The size of the circles is proportional to the number of individuals conforming to the haplotype. Colored circles indicate haplotypes from different *Mesocriconema* populations. Nodes in black are medium vectors and indicate haplotypes not detected and separated by mutations. The M in the legend means Mayer population

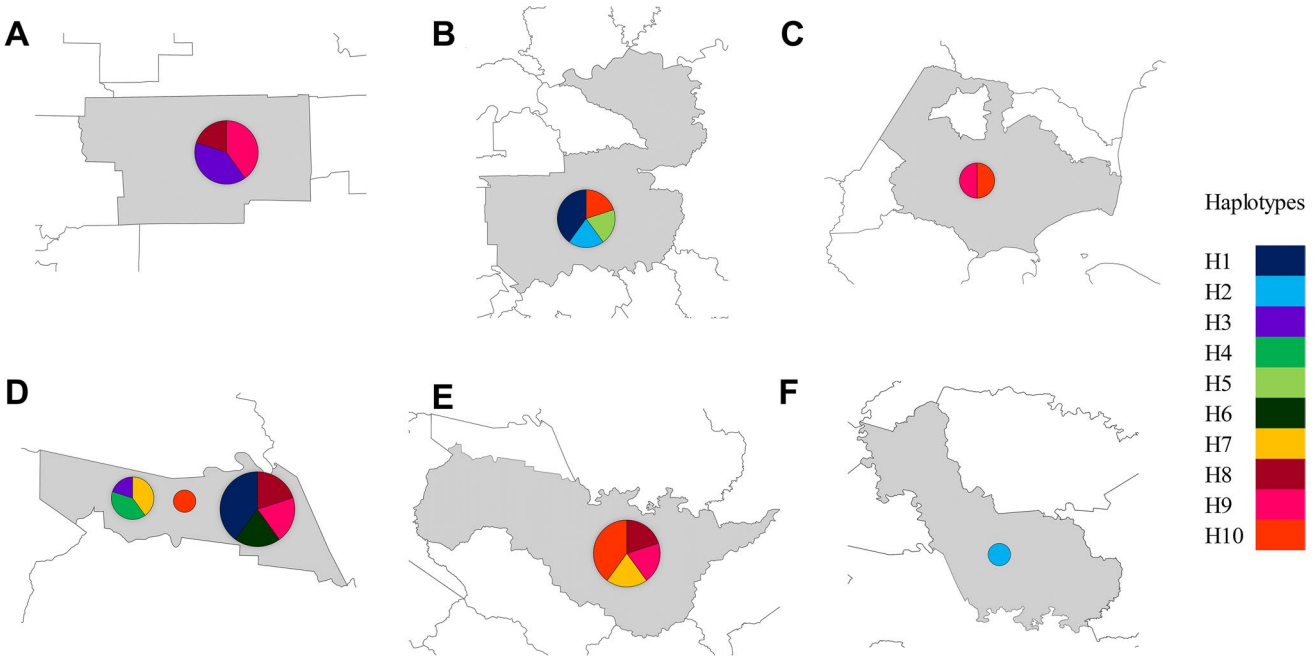

Fig. 5 Haplotype distribution map of *Mesocriconema* species in the sampled areas**.** Each circle represents a population, and the size of the circle is proportional to the number of individuals conforming to the haplotype

the authors showed low efficiency, and among the factors that may have contributed to this result is the access to the target DNA, since after agarose gel electrophoresis, it was possible to observe a large amount of primers, suggesting that they did not anneal to the target fragment and there was no amplification. Considering the location of mtDNA, the difficulty of accessing the target fragments is implied, as they are extranuclear molecules, located inside the mitochondria and protected by the inner membrane of the mitochondrial matrix.

As for the morphological characters, the greatest difficulties to delimit the species were observed for *M. curvatum*, because great variation between the characters was observed. Following the study by Powers et al. (2014) as a model, the authors also reported this difficulty. Furthermore, it is not clear in the taxonomic literature which diagnostic characteristics delimit *M. curvatum* from the others, often observing the overlapping of characters. According to the original description of *M. curvatum*, one of the most important features is the observation of the submedian lobes, which have a conical shape and the first labial ring divided into irregular labial plates. However, these characteristics are only visualized by means of scanning electron microscopy, a technique not used in the present study.

According to Brzeski et al. (2002), separation of *M. xenoplax* is based on the shorter stylet of *M. curvatum* and the shape of the vagina of *M. curvatum* (straight) versus the shape of the vagina associated with *M. xenoplax* (sigmoid). The tail shape is described as variable, rounded with a conical tip. Geraert (2010) also reported that juveniles had irregular annuli margins, but adults may have smooth annuli margins.

Mesocriconema rusticum is a cosmopolitan species and is generally associated with cover vegetation (Wouts 2006). According to Powers et al. (2014), the species can be confused with *M. discus* due to the similarities in the lip region of the two species, mainly the presence of large truncates submedian lobes. Some authors consider that the tail shape can be used to delimit these two species, where *M. rusticum* has a truncated end and a slight dorsal curvature. On the other hand, the shape of the anterior lip of the vulva differs from that of *M. xenoplax*, with no rounded lobes as in the latter.

Although the separation of species was confirmed by phylogenetic analysis, the grouping generated was not clear enough to understand the relationships between populations, probably due to the reduced number of specimens representing each population, reflecting on the formation of groups. Additionally, a greater number of specimens need to be included in this analysis, as well as in the analysis of haplotype diversity so that the results become more representative for the regions sampled.

It is quite likely that additional samplings of *Mesocriconema* will continue to reveal high inter- and intraspecific variability among criconematids associated with GDD in southern Brazil, given the diverse environments in which these parasites occur. This would not be unexpected. In addition, the characters used to delimit the species need to be revised. Molecular analyses on some well-studied nematode taxa, such as *Caenorhabditis* (Kiontke et al. 2011) and *Globodera* (Handoo et al. 2012), revealed variations that forced reconsideration of diagnostic characters. In the case of *Mesocriconema*, the morphological variation in key diagnostic characters within the species and the overlapping of morphological characters among them creates great difficulties in the identification and recognition of boundaries. Obvious examples include varying the degree of crenation at the annuli margins, the anterior vulval lip, and the shape of the vagina. These diagnostic characters need to be systematically reassessed within the context of phylogenetic groupings to fully access the information content of taxonomic units. This insight, in turn, will allow the nematode taxonomy to better integrate and contribute to biodiversity issues.

Our study provides the first information on the diversity of *Mesocriconema* species associated with grapevine decline in Brazil, through an integrative approach, exposing the existence of a species complex in areas with a history of the disease. In addition, we found high inter- and intraspecific variability among the specimens studied, despite the need for greater representation of populations.

Author contribution All authors contributed to the study conception and design. The collection of samples, morphology, and morphometry were performed by WRS and MD. The genetic characterization was performed by WRS, GSC, SOM, JTS, and DRB. Data analysis and interpretation, as well as the original draft of the manuscript, were carried out by WRS, JVAF, and CBG. All authors revised all previous versions and approved the manuscript.

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Data availability Original data and data sets analyzed in this study are available by request from the corresponding author.

Declarations

Conflict of interest The authors declare no competing interests.

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