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Short Communication

DNA Barcoding *Culicoides* Biting Midges (Diptera: Ceratopogonidae) in Northeast Brazil

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Abstract

Biting midges of the genus *Culicoides* are small insects associated with the transmission of several pathogens, which requires the correct identification of the species, for implementation of effective strategies against these insects. However, many species are difficult to identify only by morphological characters. Therefore, the use of molecular methods can help in the taxonomy and systematics of this group. Here, the DNA barcode approach was evaluated for nine species of *Culicoides* from the State of Maranhão, Brazil. We generated 39 sequences from a 476 bp (base pairs) fragment of the cytochrome *c* oxidase subunit I (*COI*) mitochondrial gene. To assess the usefulness of *COI* barcodes for the identification of these species, paired genetic distances from intra and interspecific comparisons and phylogenetic trees were generated in MEGA and RAxML/BEAST softwares, respectively. In addition, species delimitation was performed using the PTP, GMYC, and ABGD algorithms. The intra and interspecific genetic distances showed a clear distinction between them, demonstrating that, for the taxa studied, there can hardly be ambiguous identifications with barcodes. In the same sense, the phylogenetic reconstruction resulted in well-supported clades for all morphospecies analyzed.

Key words: molecular taxonomy, integrative taxonomy, molecular systematics, biting midges, species delimitation

Background

The genus *Culicoides* Latreille (Diptera: Ceratopogonidae) comprises about 1,347 species distributed worldwide (Borkent and Dominiak 2020). Females of these dipterans are hematophagous, i.e., they feed on the blood of animals and can attack birds and mammals, including humans; the bite is painful and can cause discomfort and damage to the skin of sensitive people (Mellor et al. 2000).

In addition, they can host and transmit pathogens (including viruses, protozoans, and filarial nematodes) to vertebrates, thus being able to cause various diseases (Mansonellosis, Oroupoche fever, Bluetongue virus, and Equine onchocerciasis). About 30 species of *Culicoides* have identified as competent BTV vectors worldwide (Slama et al. 2017, Federici et al. 2019.).

Some studies also detected the presence of *Leishmania* spp. in biting midge species, as in the species *Culicoides foxi* and *Culicoides ignacioi* that were found with Leishmania *braziliensis* DNA (Rebêlo et al. 2016). Therefore, is an important group in medical, veterinary, economic, and sanitary terms (Costa et al. 2013).

The inaccurate species-level identification may have significant impacts on control attempts (Bakhoum et al. 2018). Morphological

identification of these insects is mainly based on the pattern of light/dark spots on their wings (Felippe-Bauer 2003), and may involve dissection and microscopic mounting of specimens, becoming time-consuming and limited to the expertise of the identifier. Further, the conventional morphological characters are limited in the identification of cryptic species groups, which can be delimited with the help of molecular markers (e.g., Pagès et al. 2009).

One of these molecular approaches – DNA barcoding – is based on the sequencing and analysis of a fragment of the *cytochrome c oxidase subunit I* (*COI*) gene (mitochondrial DNA), and has been proposed as a rapid and authentic tool for species identification in the most varied animal taxa (Hebert et al. 2003). For *Culicoides*, this method is efficient to distinguish almost all analyzed species in the Old World (Linton et al. 2002, Lassen et al. 2012, Ander et al. 2013, Harrup et al. 2016). Thus, these data may provide a common platform for researchers from a wide range of studies regarding taxonomy, ecology, behavior, vector control, and vector–parasite relationship of this group. In addition to routine identification, DNA barcoding data provide information on additional taxonomic research through the elucidation of cryptic species and the resolution of species complexes.

Despite their great utility, the DNA barcodes of *Culicoides* are poorly studied in Brazil, and so far, only two studies has been carried out to use this approach for the identification of *C. insignis* Lutz, 1913 from northeast region (Rios et al. 2021), and 18 other species from the western Amazon (Carvalho et al. 2022). The state of Maranhão shows a great diversity of biting midges that are associated with peridomicile environments, including some pathogen vectors (Carvalho and Silva 2014; Bandeira et al. 2016, 2017), and the evaluation of the usefulness of this method in the identification and species delimitation for this state is of great importance from a public health point of view. Thus, this study aimed to employ and establish *COI*-barcodes as a reliable marker for species-level identification of *Culicoides* from the State of Maranhão.

Methods

The specimens used for molecular analyses were obtained in the following municipalities of the State of Maranhão, Northeast of Brazil: Barreirinhas (02° 48′46.43″ S; 43° 13′28.15″ W), Raposa (6° 31′0″ S; 44° 10′60″ W), São José de Ribamar (2° 39′56″ S; 44° 09′34″ W), and Vitória do Mearim (03° 27′43″ S; 44° 52′15″ W) (Fig. 1). For the capture of insects, CDC-type HP light traps were operated from 1800 hours to 600 hours of the next day. The captured specimens were taken to the Laboratório de Entomologia e Vetores (LEV) of the Universidade Federal do Maranhão, and then morphologically-identified following the proposal of Wirth and Blanton (1973), Wirth et al. (1988), and Spinelli et al. (2005). Subsequently, they were stored in 70% alcohol and incorporated into the entomological collection of LEV.

The DNA extraction was performed using the phenol/chloroform/isoamyl alcohol method adapted from Sambrook and Russell (2006). The polymerase chain reaction (PCR) was performed to amplify the mitochondrial gene *cytochrome c oxidase subunit I* (*COI*). Reactions were performed as described by Folmer et al. (1994), using the pair of primers LCO1490/HCO2198. The reactions were carried out with the following parameters: 95°C for 5 min; 35 cycles at 94°C for 1 min, 47°C for 1 min, and 72°C for 1 min; 72°C for 10 min. Amplified products were checked in 1% agarose gel electrophoresis, and positive samples were purified using the ExoSap-It kit (Amplied Biosystems) according to the manufacturer's instructions. Amplicons were sequenced on the ABI-Prism 3500 Genetic Analyzer platform (Amplied Biosystems) using the BigDye Terminator v3.1 cycle sequencing kit (Amplied Biosystems), and the forward primer. After manual inspection these sequences were submitted to GenBank database, and have been assigned Accession Numbers ON002312-ON002350.

Sequences were visualized and edited in the program BioEdit 5.0.9.0 (Hall 1999), and the alignment was performed using ClustalW (Thompson et al. 1994) in the Mega 7 software (Kumar et al. 2016). The intra and interspecific pairwise genetic distances (*p* distances) were estimated in MEGA, which were used to construct a sequence distribution graph to check the presence of the 'barcode gap' (Wiemers and Fiedler 2007). Phylogenetic gene trees were reconstructed using the maximum likelihood (ML) and bayesian inference (BI) methods in the RAxML 8.1.2 (Stamatakis 2014), and BEAST 2.6.7 (Bouckaert et al. 2014) software, respectively. In the first, heuristic searches were performed with 1,000 bootstrap pseudo replicates, using the nucleotide substitution model GTR+G+I, as indicated by JModelTest software (Darriba et al. 2012). For BI, we used the same model in addition to strict molecular clock and Yule tree as priors. Two independent runs were performed, each with 10,000,000 generations (sampling every 1,000), and log files were visualized in Tracer 1.7.1 (Rambaut et al. 2018) to check the convergence of the runs. Maximum clade credibility (MCC) tree was made with the trees (10% burn-in) using TreeAnnotator 2.5.2, and FigTree 1.4.4 was used to visualize and edit the trees. Clades were considered well supported when bootstrap and posterior probability values were greater than 70 and 0.95, respectively.

The species delimitation of *COI* sequences was carried out using three algorithms; Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012), Generalized Mixed Yule-Coalescent (GMYC) (Fujisawa and Barraclough 2013), and Poisson Tree Processes (PTP) (Zhang et al. 2013). These methods seek to group barcode sequences into genetic clusters, called Molecular Operational Taxonomic Unit (MOTU), using the similarity between sequences as criteria. The analysis of the ABGD (available at <https://bioinfo.mnhn.fr/abi/public/abgd/>) was performed with the parameters Pmin = 0.001, Pmax = 0.1, and X = 1.0, while the use of PTP and GMYC was made in the web-servers (available at <https://species.h-its.org/ptp/>, and <https://species.h-its.org/gmyc/>) with default values, ML and BI trees as input, respectively.

Results

In total, 39 individuals of nine species of the genus *Culicoides* were sequenced, (476 bp fragments) of the *COI* gene. The wing morphologies are shown in Fig. 2. The genetic distances of individuals of the same species had a variation from 0.0% to 4.6%, with most species showing a maximum divergence of 1.5%, except for the three sequences of *C. limai*, which presented maximum values of 4.6% (Table 1). When comparing specimens of different species, the values varied between 11.6% and 22.0% (Table 2). The pairwise genetic distance distribution of all sequences showed a bimodal pattern (Fig. 3), demonstrating a clear gap (barcode gap) between these classes of distances.

Phylogenetic analysis allowed the reconstruction of well-supported clades for all morphospecies analyzed (Fig. 4). In the same sense, the species delimitation generated by the PTP, GMYC, and ABGD algorithms indicated the presence of at least one MOTU for each morphospecies. These methods agreed in the vast majority of partitions, with a notable exception for the taxon *C. limai*, in which three MOTUs were assigned, one for each sequence, using the PTP algorithm, while GMYC splitted them into two groups (Fig. 4).

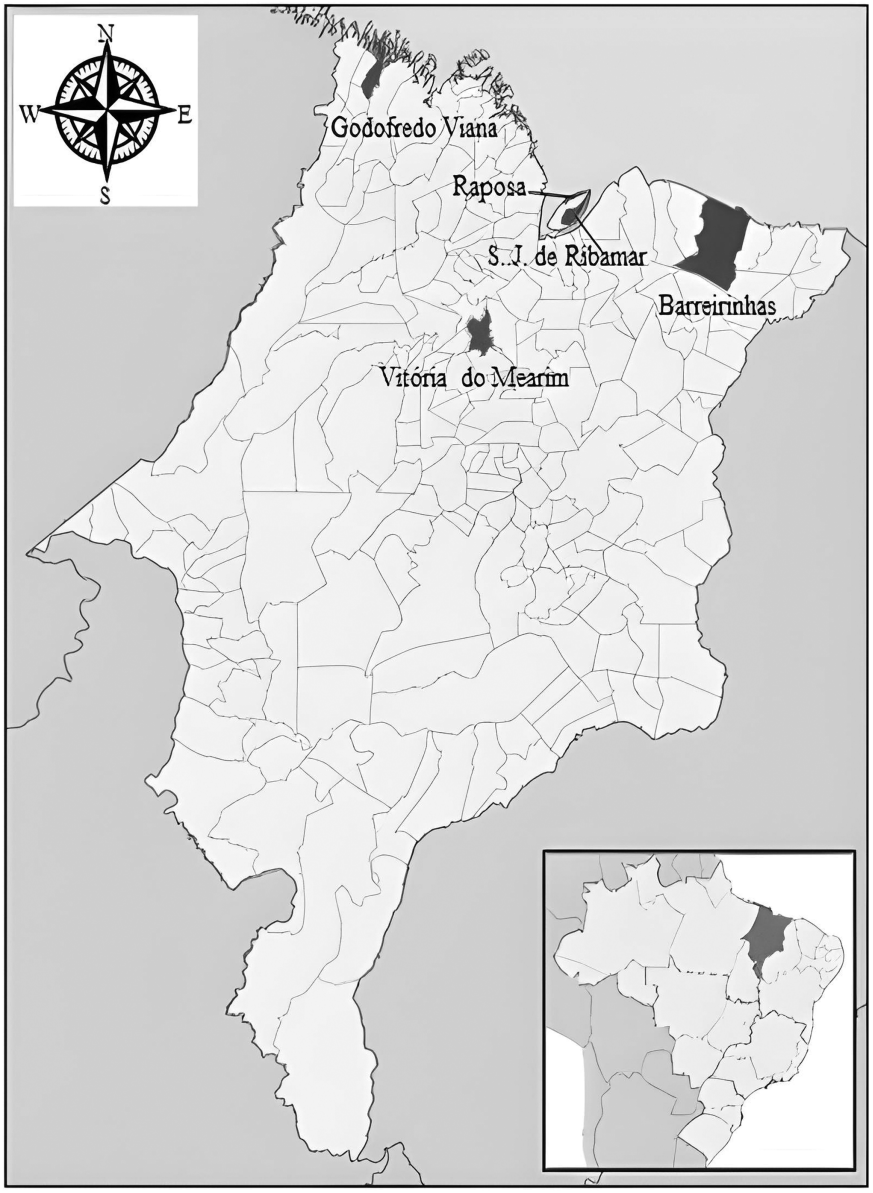

Fig. 1. Map of the state of Maranhão, Brazil, highlighting of the municipalities where *Culicoides* were captured.

Discussion

Our study presents one of the first DNA barcode efforts for different species in the genus *Culicoides* from Brazil, and the first for the state of Maranhão, which provides the basis for producing an updated inventory of valid species known in this region, developed through a data-supported integrative taxonomy approach. We generated for the first time *COI*-barcodes of the species *C. foxi* Ortiz, 1950, *C. guyanensis* Floch & Abonnenc, 1942, *C. ignacioi* Forattini, 1957, *C. iriartei* Fox, 1952, *C. limai* Barreto, 1944, and *C. ruizi* Forattini, 1954. We also generated new *COI* sequences for *C. furens* Poey, 1853, which had sequences generated for Old World specimens (Hadj-Henni et al. 2015), in addition of *C. leopoldoi* Ortiz, 1951, and *C. paucienfuscatus* Barbosa, 1947, which were previously processed in the western Amazon region of Brazil (Carvalho et al. 2022). Despite being restricted to specimens from the State of Maranhão, these sequences may help to better understand the taxonomy, systematics, and evolution of this genus on a continental scale, since this widely used fragment can be compared with other sequences in future studies.

The intra and interspecific genetic distances showed a clear distinction between them, demonstrating that, for these taxa, there can hardly be ambiguous identification with barcodes. This is a very promising result, despite the disparity detected in the intraspecific distances of *C. limai*. This gap between the classes of distances, despite being an interesting result, may indicate how homogeneous the analyzed populations are, which may reflect the proximity of the geographic areas studied. However, it may also be a consequence of the low sampling rate of this study, both in terms of the number of species and geographic locations, which directly influence the high interspecific distances and low intraspecific distances, respectively (Zhang et al. 2010, Bergsten et al. 2012, Pentinsaari et al. 2014, Phillips et al. 2019). Thus, the analysis of *COI*-barcodes with a more comprehensive sampling effort in relation to species diversity must reveal a lower identification success rate for close-related taxa, as the case of

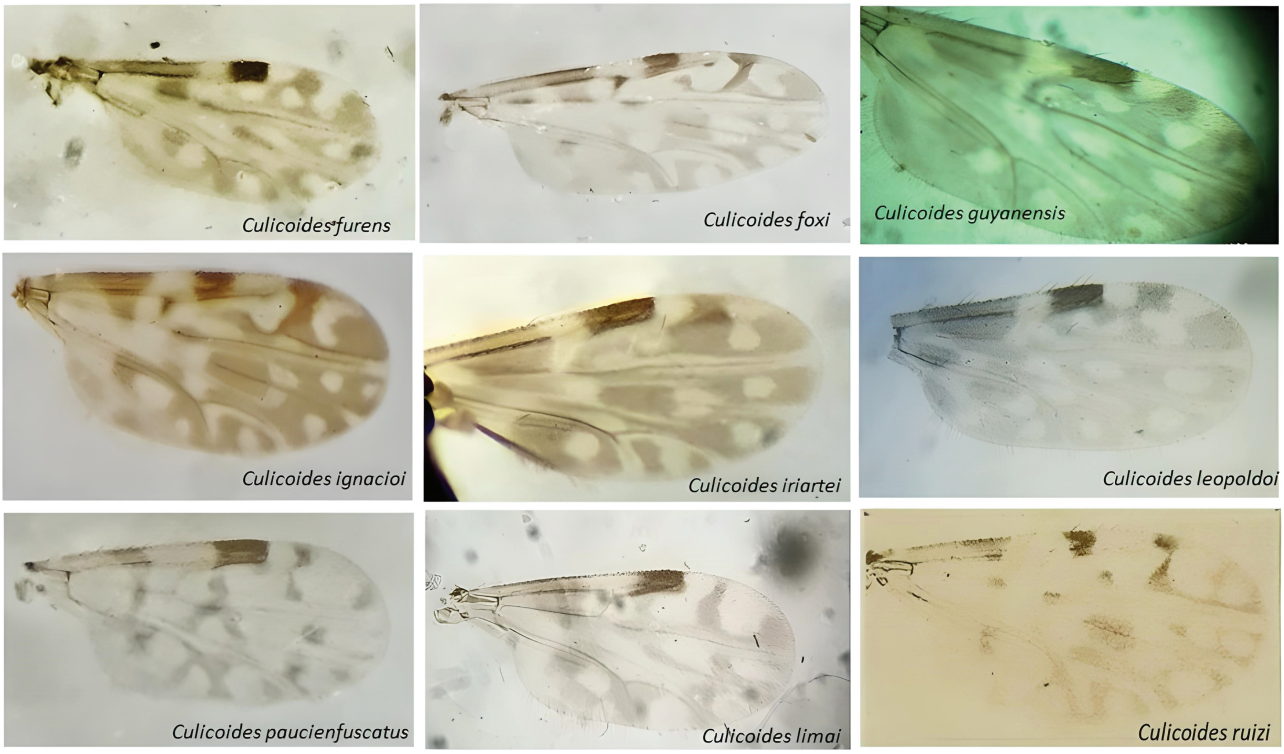

Fig. 2. *Culicoides* wing photos. The wings were photographed using a ×10 lens.

Table 1. List of *Culicoides* morphospecies of Maranhão, Brazil used in molecular analyses, number of sequences generated, and intraspecific pairwise distances of a fragment of the *COI* gene

Species	Collection site	Number of sequences	Maximum intraspecific distances (mean)
Culicoides foxi	Vitória do Mearim/Barreirinhas	4	0.006 (0.004)
C. furens	São José de Ribamar	7	0.011 (0.007)
C. guyanensis	Vitória do Mearim/Raposa	5	0.015 (0.009)
C. ignacioi	Vitória do Mearim/São José de Ribamar	4	0.006 (0.004)
C. iriartei	São José de Ribamar	6	0.015 (0.009)
C. leopoldoi	São José de Ribamar	6	0 (0)
C. limai	São José de Ribamar/Barreirinhas	3	0.043 (0.027)
C. paucienfuscatus	São José de Ribamar	2	0 (0)
C. ruizi	Barreirinhas	2	0 (0)

Table 2. Interspecific genetic distances of *Culicoides* morphospecies of Maranhão, Brazil

Species	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
(1) *Culicoides foxi*								
(2) *C. furens*	0.170							
(3) *C. guyanensis*	0.148	0.124						
(4) *C. ignacioi*	0.151	0.187	0.176					
(5) *C. iriartei*	0.172	0.154	0.131	0.220				
(6) *C. leopoldoi*	0.182	0.152	0.138	0.179	0.181			
(7) *C. limai*	0.180	0.153	0.137	0.193	0.172	0.172		
(8) *C. paucienfuscatus*	0.179	0.163	0.144	0.207	0.175	0.190	0.160	
(9) *C. ruizi*	0.120	0.155	0.131	0.116	0.172	0.175	0.164	0.182

Values generated from a fragment of the *COI* gene.

the nominal species *C. festivipennis*/*C. clastrieri*, and *C. salinarius*/*C. manchuriensis*, which are indistinguishable using *COI* sequences despite of some morphological differences (Ander et al. 2013). In the future, it is possible to expand the study areas and increase the number of species to check whether *COI*-barcodes enable correct molecular identifications in the state of Maranhão and Brazil.

Beyond the correct identification and delimitation of 100% of the analyzed taxa, the sequences of the *COI* gene indicate a possible

presence of cryptic species in the taxon *C. limai*. One of the main uses of molecular taxonomy is to identify different species that do not have phenotypic disparity (Hebert et al. 2004). The speciation process does not imply the morphological differentiation of taxa, especially in incipient stages of diversification, thus generating the so-called cryptic species (Bickford et al. 2007), which are of high relevance from an epidemiological point of view due to vector competence differences that these lineages may harbor (e.g., Besansky 1999). In the case of *Culicoides*, species morphologically identified as *C. huffi* and *C. jacobsoni* in Thailand also have high values of intraspecific nucleotide divergence values (10–12%) (Jomkumsing et al. 2021), which are similar to interspecific comparisons, demonstrating that these morphospecies may represent distinct biological entities. In our study, despite not being significantly discrepant, *C. limai* barcodes have high genetic variability, in addition to being splitted into three and two distinct MOTUs by PTP/GMYC delimitations, demonstrating that this taxon deserves attention for future integrative taxonomy studies, aiming to describe a possible morphological variation that confirms the presence of different species. In the territory of the State of Maranhão, there are two species of this group in which the morphological identification is trick – *C. limai* and *C. boliviensis*. The identification of both species requires expertise of the identifier, as the wing spots,

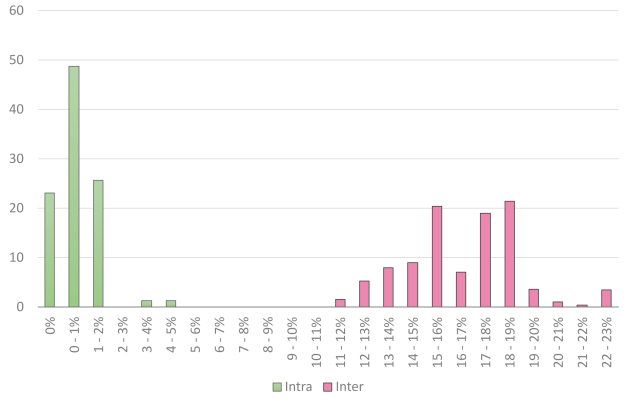

Fig. 3. Frequency distribution of intra and interspecific pairwise genetic distances of *Culicoides* spp. of Maranhão, Brazil, generated from a fragment of the *COI* gene.

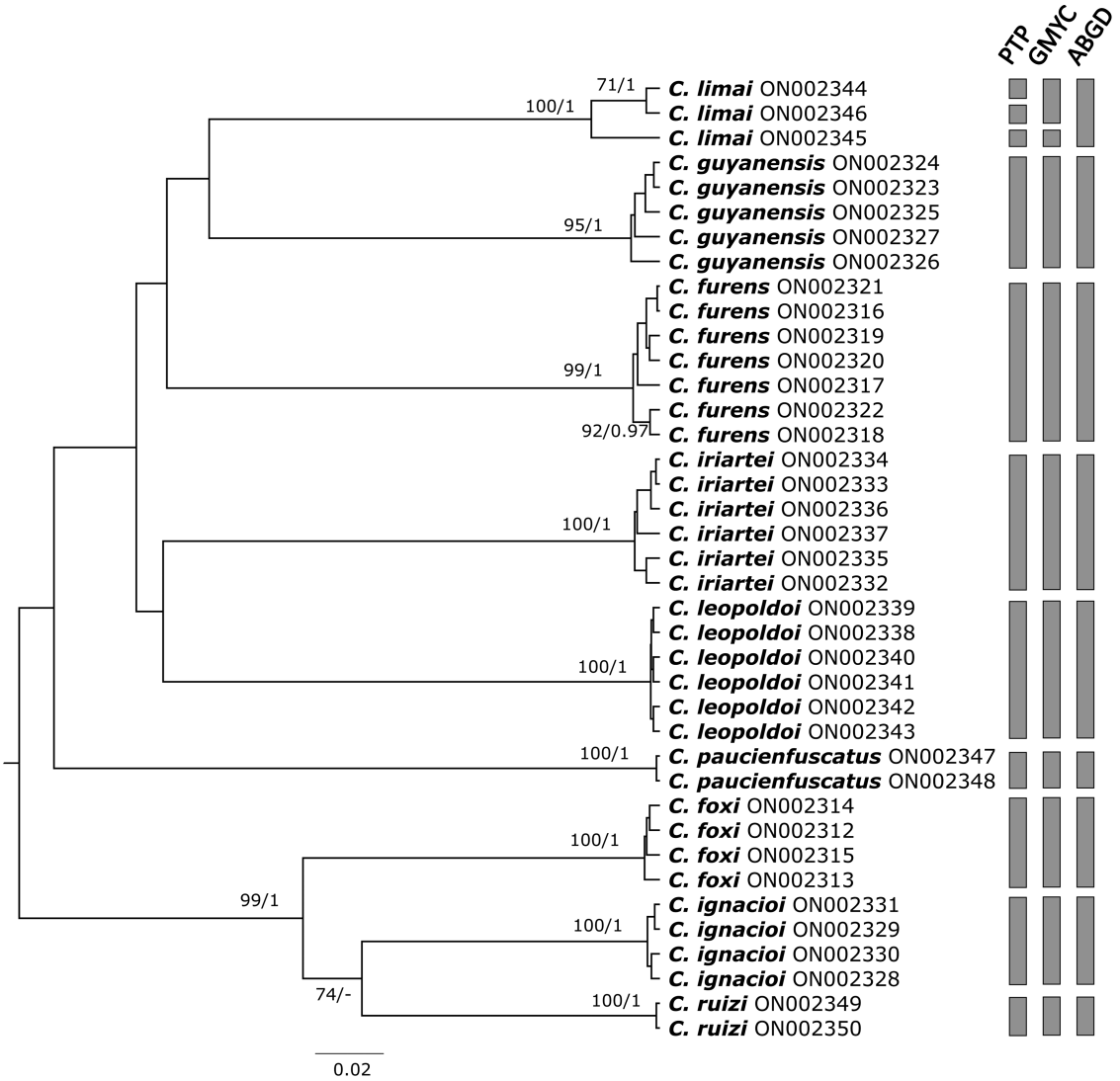

Fig. 4. Phylogenetic gene tree reconstructed using a fragment of the *COI* gene of *Culicoides* spp. from Maranhão, Brazil. Values near nodes indicate bootstrap support and posterior probabilities greater than 70 and 0.95, respectively. The tip labels indicate the analyzed nominal species and its GenBank accession number. Gray bars indicate species delimitation made by PTP, GMYC, and ABGD algorithms.

the main diagnostic feature used in the taxonomy of these insects, does not work for this complex, being delimited only by the color pattern on the legs. Therefore, it is necessary to look for new morphological markers.

Regarding what was discussed, the DNA barcoding showed high efficiency in the identification of *Culicoides* spp. from the state of Maranhão. The sequencing of the first *COI* sequences for some species of this genus in Brazil opens a wide range of research aimed at the integrative taxonomy of these insects. The absence of specialists and the great difficulty of morphological taxonomy due to the small size of the specimens creates a large gap in knowledge of *Culicoides*, especially with taxonomy and systematics, disciplines that will have a positive impact on the production and analysis of new DNA sequences, that encourage academic production and elucidation of hypotheses that were previously very difficult to be answered by traditional methods.

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