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ORIGINAL ARTICLE

COI barcodes for the identification of anthropophilic Biting Midges (Diptera: Ceratopogonidae: *Culicoides*) from the Brazilian Amazon

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Abstract. The genus *Culicoides* is the best known of the family Ceratopogonidae. Hematophagous females of the genus typically feed on the blood of vertebrate animals and in the Brazilian Amazon often on the blood of human beings. Amazon region anthropophilic *Culicoides* bites can provoke allergic reactions and transmit *Mansonella ozzardi* as well as the Oropouche virus. Past integrated taxonomy studies, combining morphometric and molecular analyses, have revealed hidden disease vector biodiversity and cryptic species with epidemiological and disease control relevance and have provided new tools to assist with vector identification. For this study we used light traps set in 12 distinct sites from three different Amazon states: Rondonia (1 site), Amazonas (3 sites) and Para (8 sites). We captured 12 different species of *Culicoides* representing seven different subgenera: *C. foxi, C. fusipalpis, C. hylas, C. insignis, C. plaumanni, C. pseudodiabolicus, C. ruizi, C. debilipalpis, C. glabrior, C. jurutiensis, C. paraensis, C. paucienfuscatus.* Between two and nine specimens were barcoded of each species. Neighbor joining and Maximum likelihood phylogenetic analysis with these COI barcodes showed the utility of these barcode sequences for species identification by clustering the barcode sequences into bootstrap-supported, species-specific monophyletic groups. Although this barcoding analysis did not resolve relationships between the species studied, it did reveal cryptic diversity within *C. paucienfuscatus, C. glabrior*, *C. plaumanni, C. insignis* and *C. pseudodiabolicus.* Two-dimensional geometric morphometrics, using eight wing-vein landmarks, robustly separated the analyzed species and raised questions about the validity of the subgenus *Haematomyidium*. Importantly, our GM wing landmark analysis separated *C. paraensis* from all the other analyzed species suggesting this type of analysis could be harnessed for epidemiological monitoring of this key Amazon-region vector species.

INTRODUCTION

The genus *Culicoides* is nested within the Ceratopogonidae family and contains some of the physically smallest Dipterans of medical importance (Lane & Crosskey, 1993). Females are hematophagous, blood-feeding on vertebrate animals, including humans (Lane & Crosskey, 1993). The bites of *Culicoides* can cause allergic reactions and can transmit a range of pathogens, which cause disease in animals and humans (Lane & Crosskey, 1993). For example, epizootic hemorrhagic disease, Schmallenberg disease, Bluetongue, Oropouche fever and mansonellosis are all transmitted by *Culicoides* (Ta-Tang et al., 2018; Sick et al., 2019). With regional concern growing about: emergent zoonotic diseases, the under-reporting of Oropouche virus infections and the public health importance of mansonellosis, there is an attendant growing need for new tools to assist with the rapid and reliable identification of *Culicoides* species for effective regional epidemiological surveillance

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(Lowe et al., 2020; Mohapatra et al., 2024; Portela et al., 2024).

Borkent and Dominiak recognize 52 extinct and 1,399 extant species of *Culicoides*, of which 301 occur in the Neotropical Region (Borkent & Dominiak, 2020). Of the 151 of these that have been recorded in Brazil, 123 species have been reported in the Brazilian Amazon Basin (Borkent & Dominiak, 2020; Santarém & Felippe-Bauer, 2022). The Borkent taxonomic framework for the genus *Culicoides* organizes species into 33 subgenera and 38 informal groups of species (Borkent, 2016b). This framework, like most of the existing taxonomic frameworks for *Culicoides*, is based mostly on morphological data and thus requires specialist morpho-taxonomists to make use of it.

Like in many areas of taxonomy, access to reliable identifications of *Culicoides* species has been broadened by the generation and depositing of reference mitochondrial "barcode" COI DNA sequences in public databases (Linton et al., 2002; Yildirim et al., 2019; Carvalho et al., 2022). As well as providing useful reference sequences that non-specialist can use to make reliable species levels identifications of *Culicoides*, the past COI barcoding studies reporting these reference sequences have also revealed potentially new species and "species complexes", unseen at the morphological level (Pagès & Sarto i Monteys, 2005; Pagès et al., 2009; Ander et al., 2013; Henni et al., 2014; Slama et al., 2014). Subspecies of greatly varying epidemiological importance have been reported in many medically important dipteran species that have been studied including blackflies, sandflies and mosquitoes (Adler et al., 2010; Hernandez-Triana et al., 2012; Fontaine et al., 2015; Georgiadou et al., 2015). The current paucity of such reports from *Culicoides* could thus be explained by the limited number of studies that have investigated *Culicoides* molecular genetics and thus highlights the need for studies in this area.

Historically, the McAlpine Ground plan of Dipteran wing venation has been extremely valuable for medical entomology (Lane & Crosskey, 1993). Because of their two-dimensional shape and because they contain veins that encompass natural anatomical points, Dipteran wings are ideal for marking landmarks and thus have emerged as a popular target for Geometric Morphometric (GM) studies of medically important Diptera including: sandflies (Godoy et al., 2014; Freitas et al., 2015; Giordani et al., 2017; Godoy et al., 2018); mosquitoes from the genera *Anopheles* (Calle et al., 2008; Lorenz et al., 2012), *Aedes* (Jirakanjanakit & Dujardin, 2005; Vidal et al., 2012) and *Culex* (Demari-Silva et al., 2014). While, thus, wing venation is not the only morphological trait that can be used in GM analysis, it is the trait that is currently most commonly used in GM analysis, not just for Dipterans, but for most insects (Jirakanjanakit & Dujardin, 2005; Godoy et al., 2014, 2018; Freitas et al., 2015; Giordani et al., 2017) .

Geometric Morphometric (GM) has also been used for a limited number of taxonomic studies on species of *Culicoides* and thus while it is not yet used routinely for *Culicoides* identifications its potential for this has been demonstrated (Augot et al., 2010; Muñoz-Muñoz et al., 2011; Henni et al., 2014). Interestingly, insect wing GM has recently been used in conjunction with deep learning as a highly effective way of separating medically important closely related species, without the need of expert taxonomists, suggesting that epidemiological surveillance of these species could be made radically more accessible and indeed accurate (Pataki et al., 2021; de Lima et al., 2023). Such technologies are the future for rapid and cheap epidemiological entomological surveillance and can be used in conjunction with citizen science for rapid and cost-effective vector surveillance. AI training, however, depends on reliable reference data which is not presently available for all the *Culicoides* of the Amazon region (Cannet et al., 2023a, b, c).

In this study we have thus collected and identified *Culicoides* from wide range of mansonellosis and Oropouche endemic and non-endemic sites spanning three states in the Brazilian Amazon region. Our study provides useful reference data from field-caught specimens from 12 different species collected from 12 widely dispersed localities and shows the potential of both COI barcoding and GM for facilitating *Culicoides* identification in the Brazilian Amazon region and beyond.

MATERIAL AND METHODS

Field collection and identifications of adult female *Culicoides*

Adult female *Culicoides* used in this study were collected between 2016 and 2018 using Center of Disease Control (CDC) style light traps at eight distinct locations within the Brazilian Amazon (Farias et al., 2020). Briefly, standard battery-operated CDC light traps were suspended 1.5 m from the soil surface using twine knotted to convenient outdoor solid structure such as a tree. Traps were left overnight, being set at approximately 6 pm and emptied at approximately 6 am. Fig. 1 shows the distribution of the sites used in this study, which were within the Brazilian states of Amazonas, Pará and Rondônia. Fig. 1 also shows the location of these states within the national borders of Brazil. Exact coordinates for the two sites within Amazonas state are as follows: Rio Pardo, in the municipality of Presidente Figueiredo, –1°49′01.0″S, 60°19′03.7″W; Tefé, in the municipality of Tefé, 3°19′34.2″S, 64°42′39.0″W. Exact coordinates for the five sites within the state of Pará are as follows: Pedra Branca, within the municipality of Itaituba, 4°07′28.1″S, 55°47′30.6″W; Serra da Escama, within the municipality of Óbidos, 1°54′57.4″S, 55°30′22.7″W; Boca do Figueiredo, on the Nhamundá River, in the municipality of Oriximiná, 1°53′21.7″S, 56°05′33.9″W; Ícatu community, close to Lago Sapucuá, in the municipality of Oriximiná 1°43′59.5″S, 56°06′14.9″W; an urban area in the municipality of Oriximiná 1°46′21.8″S, 55°51′34.7″W. The Rondônia collection site used for this study is within the municipality of Porto Velho, 8°37′44.5″S, 63°42′34.7″W. After collection, the *Culicoides* were sorted into morphotypes and stored in tubes containing absolute alcohol. Species-level morphological identifications were made by mounting relevant dissected *Culicoides* tissues between a slide and a coverslip using the phenol-balsam method as described by Wirth & Marston (1968) and identifying species with keys from Wirth & Blanton (1959), Spinelli et al. (1993), and Santarém et al. (2015); subgeneric classifications were based on Borkent (2016a).

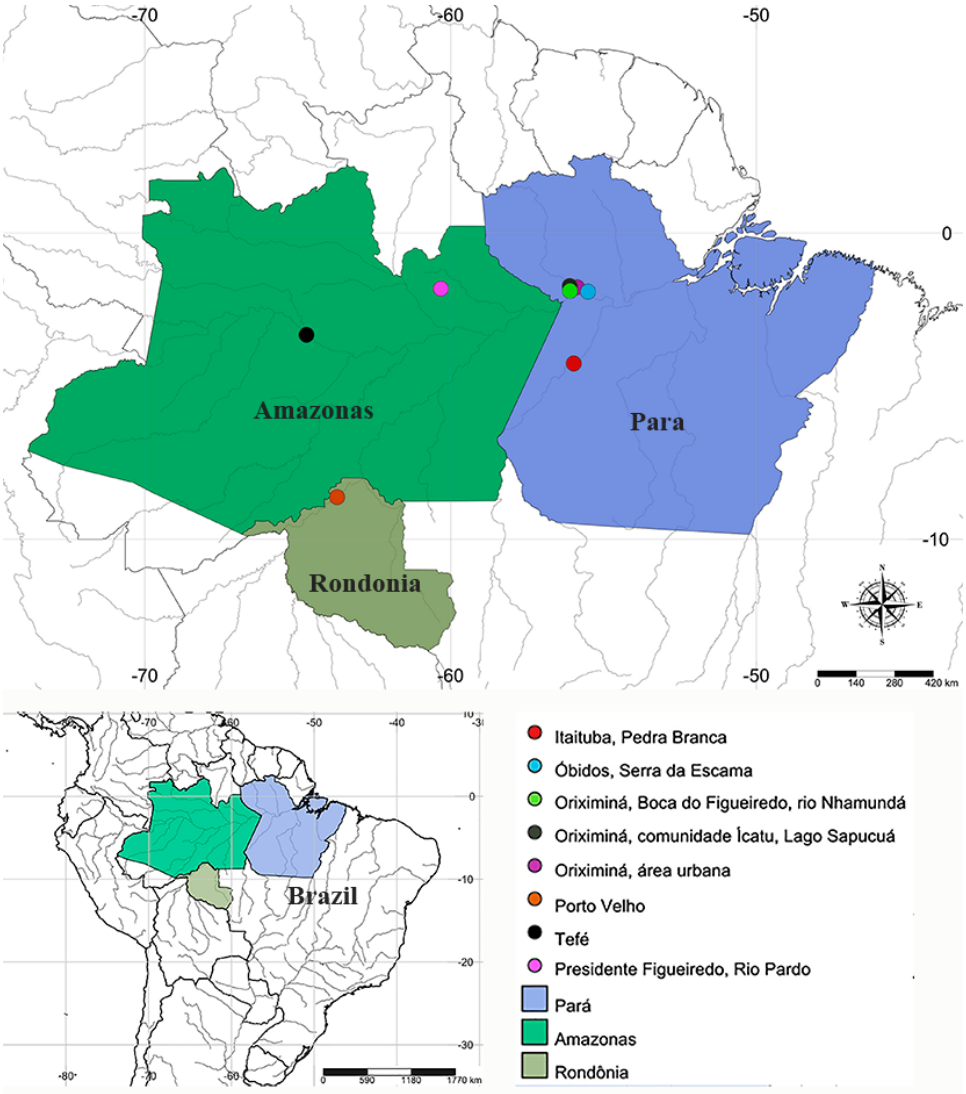

Fig. 1. These maps show the *Culicoides* collection sites used for this study.

PCR amplification of partial *Culicoides* COI sequences from 12 different *Culicoides* species from the Brazilian Amazon region

Individual morphologically identified adult female *Culicoides* had their DNA extracted using the QIAGEN DNeasy® Blood & Tissue kit and protocol. Up to 10 specimens from each morphologically identified species were used for this, with the exact number of specimens for each species being dependent on the number of insects captured in the sampling section of this work. Partial COI barcode gene sequences were amplified from *Culicoides* DNA using 1 µL of the DNA; LepF ATT CAA CCA ATC ATA AAG ATA TTG G; and LepR TAA ACT TCT GGA TGT CCA AAA AAT CA primers and a promega GoTaq® DNA Polymerase reaction mix (Cêtre-Sossah et al., 2004; Bellis et al., 2013; Henni et al., 2014; Slama et al., 2014). The PCR amplifications were performed using the following thermocycling conditions: initial denaturation at 94°C, 3 min; 5 cycles of denaturation at 94°C, 30 s; annealing at 45°C, 1 min and 30 s; extension at 68°C, 1 min; followed by 35 cycles of denaturation at 94°C, 30 s; annealing at 51°C, 1 min and 30 s; extension at 68°C, 1 min; final extension at 68°C, 10 min. PCR products were visualized in a 2.0% agarose gel containing Gel Red™. PCR amplicons were purified using Wizard® SV Gel kit and PCR Clean-Up System (Promega® Fitchburg, Wisconsin, USA) kit and protocol and quantified using a NanoDrop®. PCR products were sequenced in the forward and reverse directions using a BigDyeTM Terminator Cycle Sequencing kit (v. 3.1 – Applied Biosystems) and an ABI Prism 3100 Genetic Analyzer.

Culicoides partial COI barcoding gene sequence analysis

Matching forward and reverse nucleotide sequences, generated from Sanger sequencing PCR products, were orientated, edited and aligned to produce partial COI consensus sequences from a total of 50 *Culicoides*-origin DNA samples (Table 1). These sequences were aligned with two reference sequences: *Atrichopogon* sp. (HM431965.1) and *Forcipomyia* sp. (KX450776.1). The reference sequences were downloaded from GenBank (Tamura et al., 2011). A 381-nucleotide alignment was then used to produce a distance matrix and two phylogenetic trees. MEGA Software was also used to construct a neighbor-joining tree using, implementing Kimura's two-parameter correction method (Saittou & Nei, 1987). A maximum likelihood (ML) tree was constructed from the same alignment using PhyML v. 3.0 (Guindon et al., 2010). The robustness of phylogenetic groupings generated from both our neighbor joining and ML trees, was tested with 1000 bootstrap replicates.

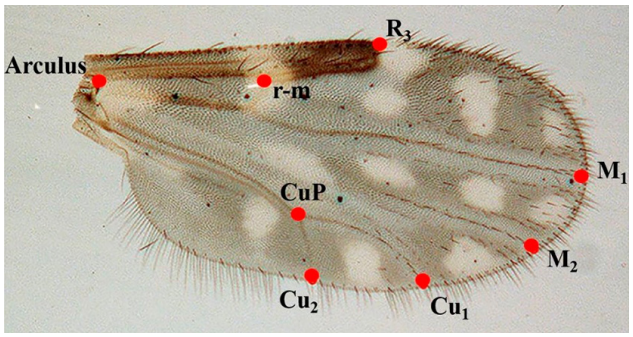

Fig. 2. Wings of a female *Culicoides paraensis*. Red circles show the eight anatomical landmarks – ARPs.

Geomorphic morphometrics wing analysis of 12 species of *Culicoides* from the Brazilian Amazon

For our Geometric Morphometric Dipteran Wing Analysis (GMDWA) *Culicoides* specimen right wing images were obtained from female specimens with a JVC KY-F55B digital camera mounted on an optical microscope using a 10× objective. Eight easily recognizable anatomical reference points (ARPs), which have been used in previous GMDWA, were selected for our analysis (Muñoz-Muñoz et al., 2011; Henni et al., 2014). All 8 ARPs are shown in Fig. 2 and have been given names based on how they related to the McAlpine (1981) wing venation ground plan, which has been used extensively for dipteran taxonomy for decades (McAlpine, 1981; Lane & Crosskey, 1993). ARP-1 "Arculus" is situated between the bifurcation of the basal arc and the radial vein; ARP-2 "r-m" is situated between the bifurcation of r-m with R_1; ARP-3 "R_3" is situated on the costal apex; ARP-4 "M_1" is situated at the terminal apex of the M_1 vein; ARP-5 "M_2" is situated at the terminal apex of the M_2 vein; ARP-6 "Cu_1" is situated at the terminal apex of the CuA_1 vein; ARP-7 "Cu_2" is situated at the terminal apex of the CuA_2 vein; ARP-8 "CuP" is situated between the bifurcation of CuP with the CuA vein (Fig. 2). For our Geometric morphometric analysis, the eight ARPs placed on each of our 248 wing photographs were transformed into shape coordinates, aligned and compared by procrustes superimposition using the TPS software suite from the integrated morphometrics software suite. Canonical Variable Analysis (CVA) were used to identify differences between the WVGPA of different species and subgenera.

RESULTS

COI barcoding of 12 Amazon region *Culicoides* species

Using classical morphological taxonomy we identified 12 species of *Culicoides* from our collections (Table 1), with six species from the *Hoffmania* Fox subgenus: *C. foxi* Ortíz, *C. fusipalpis* Wirth & Blanton, *C. hylas* Macfie, *C. insignis* Lutz, *C. plaumanni* Spinelli, *C. pseudodiabolicus* Fox and *C. ruizi* Forattini; four species from the *Haematomyidium* Goeldi subgenus: *C. debilipalpis* Lutz, *C. glabrior* Macfie, *C. jurutiensis* Felippe-Bauer, *C. paraensis* Goeldi; and one species from the *reticulatus* group: *C. paucienfuscatus* Barbosa. A total of 43 representative C01 sequences recovered for this study were deposited in GenBank, five of the species they were obtained from had no previous sequence deposits at the time of submission: *C. foxi*, *C. fusipalpis*, *C. plaumanni*, *C. ruizi*, and *C. jurutiensis*. The sequences deposited correspond a short DNA Barcode fragment (381bp) and have been assigned the following accession numbers: OP087650-OP087651 (*C. foxi*), OP103649-OP103651 (*C. fusipalpis*), OP088715-OP088717 (*C. hylas*), OP087401-OP087408 (*C. insignis*), OP093625-OP093627 (*C. plaumanni*), OP093746-OP093754 (*C. pseudodiabolicus*), OP093625-OP093627 (*C. ruizi*), OP088727-OP088729 (*C. debilipalpis*), OP103652-OP103656 (*C. glabrior*), OP103657-OP103658 (*C. jurutiensis*), OP103713-OP103714 (*C. paraensis*) and OP103659-OP103661 (*C. paucienfuscatus*). Aligning these sequences allowed us to identify 216 (56.7%) conserved nucleotides positions and 165 (43.3%) polymorphic nucleotide positions. Among the polymorphic sites, 163 (98.8%) were parsimony-informative sites and two (1.2%) were singletons.

The phylogenetic analyses carried out using the Neighbor-Joining and Maximum Likelihood reconstruction methods were equally effective in differentiating the 12 Amazonian Biting midge species. A phylogenetic tree built using the Neighbor-Joining grouped all the *Culicoides* barcode sequences into species-specific clusters with 100% bootstrap support (Fig. 3). The same bootstrap-supported species clusters were also obtained with Maximum analy-

Table 1. List of collection sites and species of *Culicoides* used in Genetic Analysis (GA) and geometric morphometric (GM) of this study.

Species	Nº sample to GM	Nº sample to GA	Location
C. foxi	16	2	Amazonas, Presidente Figueiredo, Rio Pardo
C. fusipalpis	20	3	Amazonas, Presidente Figueiredo, Rio Pardo
C. hylas	26	3	Amazonas, Presidente Figueiredo, Rio Pardo
C. insignis	0	1	Pará, Óbidos, Serra da Escama
C. insignis	0	4	Pará, Oriximiná, Nhamundá, Boca do Figueiredo
C. insignis	23	3	Pará, Oriximiná, Sapucuá, Comunidade do Icatú
C. plaumanni	14	3	Amazonas, Presidente Figueiredo, Rio Pardo
C. pseudodiabolicus	34	7	Amazonas, Presidente Figueiredo, Rio Pardo
C. pseudodiabolicus	0	1	Pará, Itaituba, Pedra Branca
C. pseudodiabolicus	0	1	Amazonas, Tefé
C. ruizi	24	7	Pará, Oriximiná, Sapucuá, Comunidade do Icatú
C. debilipalpis	16	3	Amazonas, Presidente Figueiredo, Rio Pardo
C. glabrior	29	5	Amazonas, Presidente Figueiredo, Rio Pardo
C. jurutiensis	5	2	Amazonas, Presidente Figueiredo, Rio Pardo
C. paraensis	19	2	Rondônia, Porto Velho
C. paucienfuscatus	23	3	Pará, Oriximiná, Sapucuá, Comunidade do Icatú

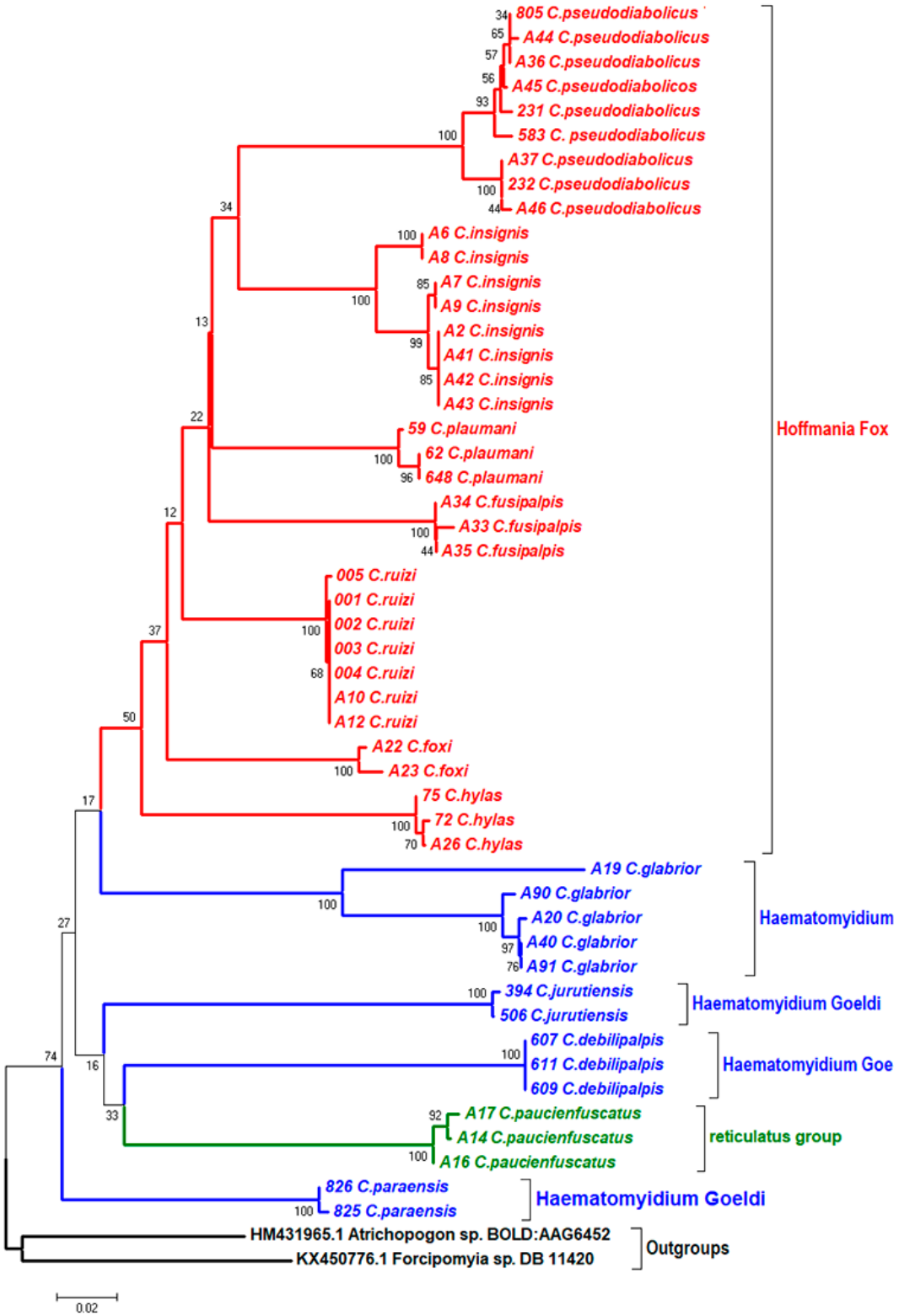

Fig. 3. Phylogenetic analysis of *Culicoides* sp. specimens using a short COI DNA barcode. A rooted neighbor-joining tree was generated with the Kimura 2 parameter model and 381 bp sequences.

sis. Although some of the ML-obtained clusters were not as strongly supported as they were in the Neighbor-Joining analysis, all had 83% or higher bootstrap support (Fig. 4). Unfortunately, however, neither our neighbor-joining nor or maximum likelihood analysis was not able to resolve relationships between any of the species we obtained COI sequences from. Our phylogenetic analysis thus showed that the 381bp COI sequences that we have deposited in GenBank are adequate for species-level identification, but not for resolving between species relationships. Neighbor-Joining analysis also identified Bootstrap-supported subclusters within seven of the species clusters: *C. pauc-*

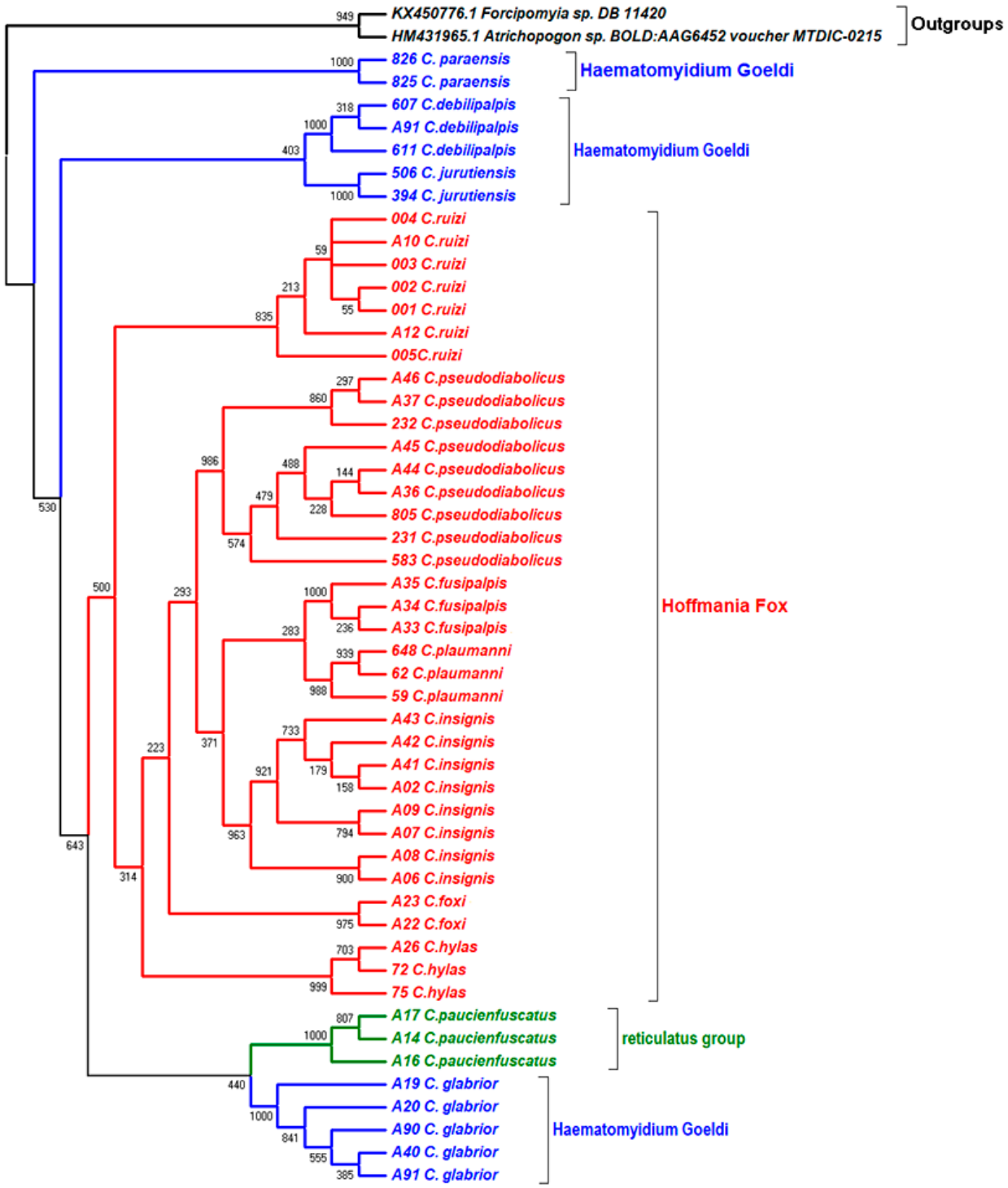

Fig. 4. Phylogenetic analysis of *Culicoides* sp. specimens using the DNA Barcode. A rooted maximum likelihood tree was generated with the GTR +I + G model and 381 bp DNA Barcode sequences.

ienfuscatus, *C. glabrior*, *C. hylas*, *C. ruizi*, *C. plaumanni*, *C. insignis* and *C. pseudodiabolicus* (Fig. 3). Five of these bootstrap-supported clusters were also recovered with maximum likelihood analysis: *C. paucienfuscatus*, *C. glabrior*, *C. hylas*, *C. plaumanni*, *C. insignis.* Tables 2 and 3 provide pair-wise genetic distance measurements made between reference COI sequences from each of the 12 *Culicoides* species used in our study. As can be seen, the greatest between species distance measurements were seen in comparisons between *C. glabrior* and *C. jurutiensis* and the smallest between species differences were observed between *C. foxi* and *C. ruizi* (Table 3). Table 2 provides measurements of within species genetic diversity for each of the 12 species that we have studied.

Geometric morphometry of female wings of Amazon region *Culicoides*

Fig. 5 shows a 2-dimensional canonical variate analysis based on Procrustes superimpositions comparisons of

Table 2. Measures of intrapopulation genetic diversity of the 12 species of *Culicoides* collection in the Brazilian Amazon.

Species	S	NS	H	π	Hd
C. debilipalpis	3	0	1	0.00000	0.00000
C. glabrior	5	50	4	0.05433	0.90000
C. jurutiensis	2	1	2	0.00263	1.00000
C. paraensis	2	1	2	0.00262	1.00000
C. hylas	3	2	3	0.00350	1.00000
C. foxi	2	4	2	0.01050	1.00000
C. fusipalpis	3	2	3	0.00350	0.66667
C. insignis	8	14	3	0.01612	0.71429
C. plaumanni	3	3	2	0.00525	0.66667
C. pseudodiabolicus	9	15	7	0.01772	0.94444
C. ruizi	7	1	2	0.00075	0.28571
C. paucienfuscatus	3	3	3	0.00612	1.00000

S – number of sequences; H – number of haplotypes; NS – number of segregating sites; Hd – haplotype diversity; π – nucleotide diversity.

shape coordinates calculated from eight anatomical reference points placed on 248 left-wing photographs taken of 12 distinct species of *Culicoides* captured in the Brazilian Amazon. As can be seen, the shape coordinates of both *C. paraensis* and *C. glabrior* are in clusters entirely distinct from the other shape co-ordinate clusters generated for all other analyzed species and that there is only very limited overlap in the clusters generated from *C. paucienfuscatus* and *C. ruizi* and between *C. debilipalpis* and *C. jurutiensis*. The shape coordinate clusters generated for all the other seven species, while all distinct, all share considerable overlap in the morphological space they occupy on in the canonical variable axes used in our analysis.

DISCUSSION

DNA barcoding is a robust way of identifying Amazon region *Culicoides* species

In line with previous studies on *Culicoides* and, indeed, many other studies on insect genera from the family Diptera, all 12 *Culicoides* species that we studied were robustly separated in our phylogenetic analysis, even though the COI sequences that we recovered were quite short. Also like many previous barcoding studies on *Culicoides* and related Dipterans, our analysis revealed cryptic diversity (Linton et al., 2002; Hernandez-Triana et al., 2012; Conceição et al., 2013; Carvalho et al., 2022). Genetic variation was detected in 11 of the 12 species analyzed, with the three COI barcode sequences obtained from *C. debilipalpis* proving invariant. Of the nine species that we obtained enough sequences (i.e $n \geq 2$) to detect substructuring within, we observed five of the species-specific sequence clades contained distinct bootstrap supported clusters nested within them in both the NJ and ML analysis and two additional species-specific sequence clades that had bootstrap supported clusters within them that were only bootstrap-supported in the NJ analysis. Even though our analysis of intra-specific sequence diversity was limited, it still detected substantial levels of genetic variation among most of the Amazon region species we analyzed, consistent with the common occurrence of species complexes. As such variation has proven to be epidemiologically significant for numerous disease vectors, our work highlights the need for detailed follow-up investigations on vector species such as *C. paraensis*, which is a known disease vector of both mansonellosis and the Oropouche virus (Sakkas et al., 2018; Ta-Tang et al., 2018).

Integrating GM and barcoding analysis for a better understanding of Amazon region systematics

Although our barcoding analysis was able to robustly identify species and indeed uncover genetic substructuring below the morpho-species level, it failed to resolve between species relationships. This has been a common problem for COI barcoding studies performed on Diptera family insects even when full length COI sequences have been used in the analysis (Linton et al., 2002; Hernandez-Triana et al., 2012; Conceição et al., 2013; Carvalho et al., 2022). Interestingly, while our GM morphometric analysis of *Culicoides* did not entirely resolve the relationships between the *Culicoides* species we studied, it did provide some insights into subgenera. Most notably our analysis suggested that *C. paraensis* and *C. glabrior* do not belong to the same genus and thus that the subgenus "*Haematomyidium*" is of questionable validity. Although single gene COI analysis has rarely provided insights into intra species relationships within the Dipteran family, using this gene in conjunction with other variable gene sequences in Multi Loci Sequence Typing (MLST) has often proved capable of resolving Dipteran family between-species relationships (Adler et al., 2010; Harbach, 2011). A MLST investigation into the "*Haematomyidium*" could thus provide a more definitive answer on the validity of this subgenus. Similarly, MLST analysis could help to mitigate the limitations of

Table 3. Genetic distance between 12 Amazon-region species of *Culicoides* based on COI DNA sequences.

Species	1	2	3	4	5	6	7	8	9	10	11	12
C. jurutiensis	0.000											
C. ruizi	0.199	0.000										
C. debilipalpis	0.232	0.229	0.000									
C. foxi	0.225	0.118	0.272	0.000								
C. fusipalpis	0.248	0.129	0.253	0.152	0.000							
C. glabrior	0.303	0.218	0.284	0.264	0.237	0.000						
C. hylas	0.217	0.142	0.241	0.162	0.145	0.280	0.000					
C. insignis	0.237	0.119	0.248	0.132	0.125	0.248	0.159	0.000				
C. paraensis	0.215	0.170	0.199	0.213	0.195	0.233	0.170	0.217	0.000			
C. paucienfuscatus	0.219	0.189	0.218	0.195	0.229	0.237	0.213	0.181	0.225	0.000		
C. plaumanni	0.257	0.119	0.258	0.156	0.136	0.253	0.180	0.132	0.214	0.195	0.000	
C. pseudodiabolicus	0.300	0.172	0.233	0.182	0.169	0.230	0.196	0.143	0.225	0.210	0.161	0.000

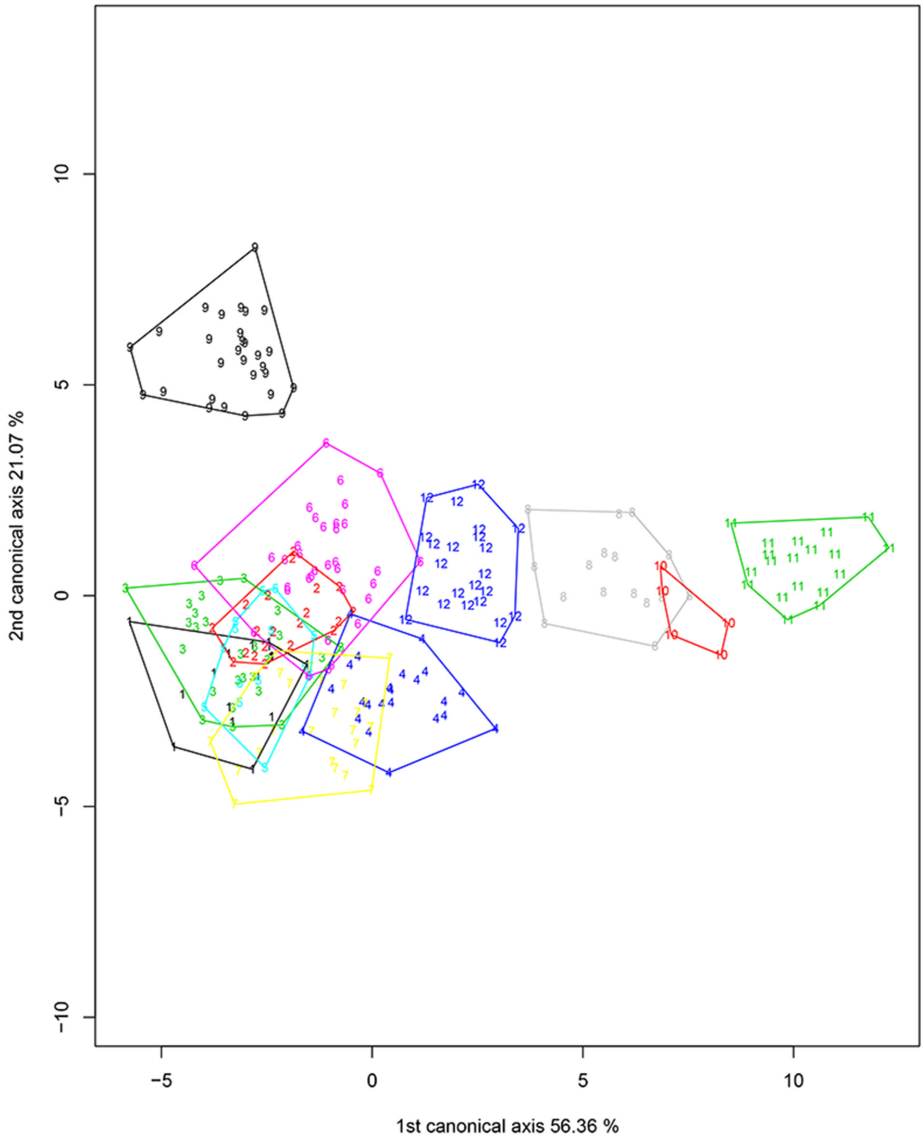

Fig. 5. Morphological space of canonical variables (CVs) 1 and 2 yielded by discriminant analysis of wing principal components (CV1 with 56.36% and CV2 with 21.07%), showing the clusters formed of 12 species of *Culicoides*. Subgenus *Hoffmania* (1 to 7): 1 – *C. foxi*, 2 – *C. fusipalpis*, 3 – *C. hylas*, 4 – *C. insignis*, 5 – *C. plaumanni*, 6 – *C. pseudodiabolicus*, 7 – *C. ruizi*. Subgenus *Haematomyidium* (8 to 11): 8 – *C. debilipalpis*, 9 – *C. glabrior*, 10 – *C. jurutiensis*, 11 – *C. paraensis*, 12 – *C. paucienfuscatus*.

using COI barcoding in isolation, which can sometimes give a false impression of a species' diversity. For example, *Wolbachia* infections can reduce COI barcode diversity in a given species by driving one specific mitochondrial haplotype through the population and NUMT pseudogenes mistook for *COI* gene sequences can make a species look more genetic diverse than it actually is (Jiggins, 2003; Song et al., 2008; Cariou et al., 2017; Crainey et al., 2018; Deng et al., 2021).

GM morphometric analysis for vector monitoring of Amazon region *Culicoides*

Both *M. ozzardi* and the Oropouche virus are transmitted by the highly anthropophilic *C. paraensis*, which is found widely throughout the amazon region (Feitoza et al., 2023). These attributes make *C. paraensis* probably the most medically important *Culicoides* species in the Amazon region (Feitoza et al., 2023). While our barcoding sequences for this species could be used to assist with laboratory-based surveillance of this species, arguably our GM analysis has potential more utility. Public health resources in the Brazilian Amazon are thinly stretched dealing with a wide range of infectious disease that have disease burdens far greater than those presently recognized for Oropouche and mansonellosis (Ta-Tang et al., 2021; Mohapatra et al., 2024; Portela et al., 2024). It is unlikely that wide-ranging molecular entomological monitoring of *C. paraensis* distribution and biting will be financed either in the Brazilian amazon or beyond. In situation such as this, researchers have increasingly been calling for the deployment of citizen science approaches as a practical way of monitoring neglected tropical diseases (Delgado-Noguera et al., 2022; Peng et al., 2023). It has been proposed, for example, that cellular phone applications could use wing venation landmark analysis and AI deep learning to identify different mosquito species as a way of assisting with the monitoring

of arbovirus vectors such as *Ae. aegypti* and *Ae. albopictus* (Cannet et al., 2024a, b, c). Our work here has shown that the wing venation patterns of *C. paraensis* are distinct from all 11 other anthropophilic Amazon region *Culicoides* species that we analyzed. Our 8 ARP-based GM analysis thus suggest that *Culicoides* wings could be targeted for the development of AI-tools for *C. paraensis* entomological monitoring both in the Brazilian Amazon and beyond.

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REFERENCES

Adler P.H., Cheke R.A. & Post R.J. 2010: Evolution, epidemiology, and population genetics of black flies (Diptera: Simuliidae). — *Infect. Genet. Evol.* **10**: 846–865.

Ander M., Troell K. & Chirico J. 2013: Barcoding of biting midges in the genus *Culicoides*: a tool for species determination. — *Med. Vet. Entomol.* **27**: 323–331.

Augot D., Sauvage F., Jouet D., Simphal E., Veuille M., Couloux A., Kaltenbach M.L. & Depaquit J. 2010: Discrimination of *Culicoides obsoletus* and *C. scoticus*, potential bluetongue vectors, by morphometrical and mitochondrial cytochrome oxidase subunit I analysis. — *Infect. Genet. Evol.* **10**: 629–637.

Bellis G.A., Dyce A.L., Gopurenko D. & Mitchell A. 2013: Revision of the *Immaculatus* group of *Culicoides* Latreille (Diptera: Ceratopogonidae) from the Australasian Region with description of two new species. — *Zootaxa* **3680**: 15–37.

Borkent A. 2016a: *The Subgeneric Classification of Species of* Culicoides *– Thoughts and a Warning.* URL: https://wwv.inhs.illinois.edu/files/5014/6532/8290/CulicoidesSubgenera.pdf

Borkent A. 2016b: *Numbers of Extant and Fossil Species of Ceratopogonidae.* URL: https://www.inhs.illinois.edu/files/4014/6785/5847/WorldCatalogtaxa.pdf

Borkent A. & Dominiak P. 2020: Catalog of the Biting Midges of the World (Diptera: Ceratopogonidae). — *Zootaxa* **4787**: 1–377.

Calle D.A., Quiñones M.L., Erazo H.J. & Jaramillo N. 2008: Discriminación por morfometría geométrica de once especies de *Anopheles* (*Nyssorhynchus*) presentes en Colombia. — *Biomedica* **28**: 371–385.

Cannet A., Simon-Chane C., Histace A., Akhoundi M., Romain O., Souchaud M., Jacob P., Sereno D., Gouagna L.C., Bousses P., Mathieu-Daude F. & Sereno D. 2023a: Wing Interferential Patterns (WIPs) and machine learning for the classification of some *Aedes* species of medical interest. — *Sci. Rep.* **13**: 17628, 12 pp.

Cannet A., Simon-Chane C., Histace A., Akhoundi M., Romain O., Souchaud M., Jacob P., Sereno D., Volf P., Dvorak V. & Sereno D. 2023b: Species identification of phlebotomine sandflies using deep learning and wing interferential pattern (WIP). — *Sci. Rep.* **13**: 21389, 9 pp.

Cannet A., Simon-Chane C., Akhoundi M., Histace A., Romain O., Souchaud M., Jacob P., Sereno D., Mouline K., Barnabe C., Lardeux F., Boussès P. & Sereno D. 2023c: Deep learning and wing interferential patterns identify *Anopheles* species and discriminate amongst Gambiae complex species. — *Sci. Rep.* **13**: 13895, 13 pp.

Cariou M., Duret L. & Charlat S. 2017: The global impact of *Wolbachia* on mitochondrial diversity and evolution. — *J. Evol. Biol.* **30**: 2204–2210.

Carvalho L.P.C., Costa Silva G., Pereira Júnior A.M., Paulo P.F.M., Silva G.S., Carioca A.P.M., Rodrigues B.L., Pessoa F.A.C. & Medeiros J.F. 2022: DNA barcoding of genus *Culicoides* biting midges (Diptera: Ceratopogonidae) in the Brazilian Amazon. — *Acta. Trop.* **235**: 106619, 8 pp.

Cêtre-Sossah C., Baldet T., Delécolle J.C., Mathieu B., Perrin A., Grillet C. & Albina E. 2004: Molecular detection of *Culicoides* spp. and *Culicoides imicola*, the principal vector of African horse sickness (AHS) in Africa and Europe. — *Vet. Res.* **35**: 325–337.

Conceição P.A., Crainey J.L., Almeida T.P., Shelley A.J. & Luz S.L. 2013: New molecular identifiers for *Simulium limbatum* and *Simulium incrustatum sl* and the detection of genetic substructure with potential implications for onchocerciasis epidemiology in the Amazonia focus of Brazil. — *Acta. Trop.* **127**: 118–125.

Crainey J.L., Marín M.A., Silva T.R.R.D., de Medeiros J.F., Pessoa F.A.C., Santos Y.V., Vicente A.C.P. & Luz S.L.B. 2018: *Mansonella ozzardi* mitogenome and pseudogene characterisation provides new perspectives on filarial parasite systematics and CO-1 barcoding. — *Sci. Rep.* **8**: 6158, 11 pp.

Delgado-Noguera L.A., Hernández-Pereira C.E., Ramírez J.D., Hernández C., Velasquez-Ortíz N., Clavijo J., Ayala J.M., Forero-Peña D., Marquez M., Suarez M.J. et al. 2022: Tele-entomology and tele-parasitology: A citizen science-based approach for surveillance and control of Chagas disease in Venezuela. — *Parasite Epidemiol. Control* **19**: e00273, 9 pp.

de Lima V.R., de Morais M.C.C. & Kirchgatter K. 2023: Integrating artificial intelligence and wing geometric morphometry to automate mosquito classification. — *Acta. Trop.* **249**: 107089, 7 pp.

Demari-Silva B., Suesdek L., Sallum M.A.M. & Marrelli M.T. 2014: Wing geometry of *Culex coronator* (Diptera: Culicidae) from South and Southeast Brazil. — *Parasit Vectors* **7**: 174, 9 pp.

Deng J., Assandri G., Chauhan P., Futahashi R., Galimberti A., Hansson B., Lancaster L.T., Takahashi Y., Svensson E.I. & Duplouy A. 2021: *Wolbachia*-driven selective sweep in a range expanding insect species. — *BMC Ecol. Evol.* **21**: 1–17.

Farias E.S., Almeida J.F., Pereira-Silva J.W., Coelho L.S., Ríos-Velásquez C.M., Luz S.L.B. & Pessoa F.A.C. 2020: Diversity of biting midges *Culicoides* (Diptera: Ceratopogonidae), potential vectors of disease, in different environments in an Amazonian rural settlement, Brazil. — *Rev. Soc. Bras. Med. Trop*. **53**: e20200067, 8 pp.

Feitoza L.H.M., de Carvalho L.P.C., da Silva L.R., Meireles A.C.A., Rios F.G.F., Silva G.S., Medeiros de Paulo P.F., Pessoa F.A.C. & Julião G.R. 2023: Influence of meteorological and seasonal parameters on the activity of *Culicoides paraensis* (Diptera: Ceratopogonidae), an annoying anthropophilic biting midge and putative vector of Oropouche virus in Rondônia, Brazilian Amazon. — *Acta. Trop.* **243**: 106928, 8 pp.

Fontaine M.C., Pease J.B., Steele A., Waterhouse R.M., Neafsey D.E., Sharakhov I.V., Hall A.B., Evdoxia Kakani F.C.E., Mitchell S.N., Wu Y.C., Smith H.A., Love R.R., Lawniczak M.K., Slotman M.A., Emrich S.J., Hahn M.W. & Besansky N.J. 2015: Extensive introgression in a malaria vector species complex revealed by phylogenomics. — *Science* **347**: 1258524, 20 pp.

Freitas M.T.S., Ríos-Velasquez C.M., Costa C.R. Jr., Figueiredo C.A., Aragão N.C., Balbino T.C.L., Pessoa F.A.C.

& Balbino V.Q. 2015: Phenotypic and genotypic variations among three allopatric populations of *Lutzomyia umbratilis*, main vector of *Leishmania guyanensis*. — *Parasit Vectors* **8**: 448, 10 pp.

Georgiadou S.P., Makaritsis K.P. & Dalekos G.N. 2015: Leishmaniasis revisited: current aspects on epidemiology, diagnosis and treatment. — *J. Transl. Int. Med*. **3**: 43–50.

Giordani B.F., Andrade A.J., Galati E.A.B. & Gurgel-Gonçalves R. 2017: The role of wing geometric morphometrics in the identification of sandflies within the subgenus *Lutzomyia*. — *Med. Vet. Entomol.* **31**: 373–380.

Godoy R.E., Galati E.A.B., Estrela P.C., Souza N.A., Santos T.V., Sousa L.G. & Rangel E. 2014: Comparative study of the phlebotomine sand fly species (Diptera: Psychodidae: Phlebotominae) of the genera *Nyssomyia* Barretto, 1962, *Bichromomyia* Artemiev, 1991 and *Migonemyia* Galati, 1995 vectors of American cutaneous leishmaniasis in Brazil. — *Zootaxa* **3838**: 501–517.

Godoy R.E., Shimabukuro P.H., Santos T.V., Pessoa F.A.C., Cunha A.E.F.L., Santos F.K.M., Vilela M.L., Rangel A.F. & Galati E.A.B. 2018: Geometric morphometry of the head in sand flies (Diptera: Psychodidae: Phlebotominae), an alternative approach to taxonomy studies. — *Zootaxa* **4504**: 566–576.

Guindon S., Dufayard J.F., Lefort V., Anisimova M., Hordijk W. & Gascuel O. 2010: New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. — *Syst. Biol.* **59**: 307–321.

Harbach R.E. 2011: Classification within the cosmopolitan genus *Culex* (Diptera: Culicidae): The foundation for molecular systematics and phylogenetic research. — *Acta. Trop.* **120**: 1–14.

Henni H.L., Sauvage F., Ninio C., Depaquit J. & Augot D. 2014: Wing geometry as a tool for discrimination of *Obsoletus* group (Diptera: Ceratopogonidae: *Culicoides*) in France. — *Infect. Genet. Evol.* **21**: 110–117.

Hernandez-Triana L.M., Crainey J.L., Hall A., Fatih F., Mackenzie-Dodds J., Shelley A.J., Zhou X., Post R.J., Gregory T.R. & Hebert P.D.N. 2012: DNA barcodes reveal cryptic genetic diversity within the blackfly subgenus *Trichodagmia* Enderlein (Diptera: Simuliidae: *Simulium*) and related taxa in the New World. — *Zootaxa* **3514**: 43–69.

Jiggins F.M. 2003: Male-killing *Wolbachia* and mitochondrial DNA: selective sweeps, hybrid introgression and parasite population dynamics. — *Genetics* **164**: 5–12.

Jirakanjanakit N. & Dujardin J.P. 2005: Discrimination of *Aedes aegypti* (Diptera: Culicidae) laboratory lines based on wing geometry. — *Southeast Asian J. Trop. Med. Public Health* **36**: 858–861.

Lane R.P. & Crosskey R.W. 1993: *Medical Insects and Arachnids*. Chapman & Hall, London, xv + 723 pp.

Linton Y.M., Mordue A.J., Cruickshank R.H., Meiswinkel R., Mellor P.S. & Dallas J.F. 2002: Phylogenetic analysis of the mitochondrial cytochrome oxidase subunit I gene of five species of the *Culicoides imicola* species complex. — *Med. Vet. Entomol.* **16**: 139–146.

Lorenz C., Marques T.C., Sallum M.A.M. & Suesdek L. 2012: Morphometrical diagnosis of the malaria vectors *Anopheles cruzii*, *An. homunculus* and *An. bellator*. — *Parasit Vectors* **5**: 257, 7 pp.

Lowe R., Lee S., Lana R.M., Codeço C.T., Castro M.C. & Pascual M. 2020: Emerging arboviruses in the urbanized Amazon rainforest. — *B.M.J.* **371**: m4385, 5 pp.

McAlpine J.F. 1981: Morphology and terminology – adults. In McAlpine J.F., Peterson B.V., Shewell G.E., Teskey H.J., Vockeroth J.R. & Wood D.M. (eds): *Manual of Nearctic Diptera, Vol. 1*. Research Branch, Agriculture Canada (Monograph No. 27), Ottawa, pp. 9–63.

Mohapatra R.K., Mishr S., Satapathy P., Kandi V. & Tuglo L.S. 2024: Surging Oropouche Virus (OROV) cases in the Americas: A public health challenge. — *New Microbes New Infect.* **59**: 101243, 2 pp.

Muñoz-Muñoz F., Talavera S. & Pagès N. 2011: Geometric morphometrics of the wing in the subgenus *Culicoides* (Diptera: Ceratopogonidae): from practical implications to evolutionary interpretations. — *J. Med. Entomol.* **48**: 129–139.

Pagès N. & Sarto i Monteys I. 2005: Differentiation of *Culicoides obsoletus* and *Culicoides scoticus* (Diptera: Ceratopogonidae) based on mitochondrial cytochrome oxidase subunit I. — *J. Med. Entomol.* **42**: 1026–1034.

Pagès N., Muñoz-Muñoz F., Talavera S., Sarton V., Lorca C. & Núñez J.I. 2009: Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the subgenus *Culicoides* and development of species-specific PCR assays based on Barcode regions. — *Vet. Parasitol.* **165**: 298–310.

Pataki B.A., Garriga J., Eritja R., Palmer J.R., Bartumeus F. & Csabai I. 2021: Deep learning identification for citizen science surveillance of tiger mosquitoes. — *Sci. Rep.* **11**: 4718, 12 pp.

Peng N.Y., Hall R.N., Huang N., West P., Cox T.E., Mahar J.E., Mason H., Campbell S., O'Connor T., Read A.J. et al. 2023: Utilizing molecular epidemiology and citizen science for the surveillance of lagoviruses in Australia. — *Viruses* **15**: 2348, 17 pp.

Portela C.S., de Araújo C.P.M., Sousa P.M., Simão C.L.G., de Oliveira J.C.S. & Crainey J.L. 2024: Filarial disease in the brazilian amazon and emerging opportunities for treatment and control. — *Curr. Res. Parasitol. Vector Borne Dis.* **5**: 100168, 10 pp.

Saitou N. & Nei M.1987: The neighbor-joining method: a new method for reconstructing phylogenetic trees. — *Mol. Biol. Evol.* **4**: 406–425.

Sakkas H., Bozidis P., Franks A. & Papadopoulou C. 2018: Oropouche fever: a review. — *Viruses* **10**: 175, 16 pp.

Santarém M.C. & Felippe-Bauer M.L. 2022: *Brazilian species of biting midges – espécies de maruins do Brasil (Diptera: Ceratopogonidae).* URL: https://portal.fiocruz.br/sites/portal.fiocruz.br/files/documentos/brazilian_species_of_biting_midges_2022_0.pdf

Santarém M.C.A., Farias E.S. & Felippe-Bauer M.L. 2015: *Culicoides castelloni* sp. nov. from the Brazilian Amazon Region with a revision of the *reticulatus* species group (Diptera, 63 Ceratopogonidae). — *An. Acad. Bras. Cienc.* **87**: 955–972.

Sick F., Beer M., Kampen H. & Wernike K. 2019: *Culicoides* biting midges – underestimated vectors for arboviruses of public health and veterinary importance. — *Viruses* **11**: 376, 19 pp.

Slama D., Haouas N., Remadi L., Mezhoud H., Babba H. & Chaker E. 2014: First detection of *Leishmania infantum* (Kinetoplastida: Trypanosomatidae) in *Culicoides* spp. (Diptera: Ceratopogonidae). — *Parasit. Vectors* **7**: 51, 3 pp.

Song H., Buhay J.E., Whiting M.F. & Crandall K.A. 2008: Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. — *Proc. Natl. Acad. Sci. U.S.A*. **105**: 13486–13491.

Spinelli G.R. & Wirth W.W. 1993: Los Ceratopogonidae de la Argentina (Insecta: Diptera). In Castellanos Z.A. (ed.): *Fauna de Agua Dulce de la Republica Argentina, Vol. 38(3)*. Profadu (Conicet), Buenos Aires, 124 pp.

Ta-Tang T.H., Crainey J.L., Post R.J., Luz S.L. & Rubio J.M. 2018: Mansonellosis: current perspectives. — *Res. Rep. Trop. Med.* **9**: 9–24.

Ta-Tang T.H., Luz S.L., Crainey J.L. & Rubio J.M. 2021: An overview of the management of mansonellosis — *Res. Rep. Trop. Med* **12**: 93–105.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M. & Kumar S. 2011: MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. — *Mol. Biol. Evol.* **28**: 2731–2739.

Vidal P.O., Carvalho E. & Suesdek L. 2012: Temporal variation of wing geometry in *Aedes albopictus*. — *Mem. Inst. Oswaldo Cruz* **107**: 1030–1034.

Wirth W.W. & Blanton F.S. 1959: Biting midges of the genus *Culicoides* from Panama (Diptera: Heleidae). — *Proc. U.S. Natl. Mus.* **109**: 237–482.

Wirth W.W. & Marston N. 1968: Method for mounting small insects on microscope slides in Canada balsam. — *Ann. Entomol. Soc. Am.* **61**: 783–784.

Yildirim A., Dik B., Duzlu O., Onder Z., Ciloglu A., Yetismis G. & Inci A. 2019: Genetic diversity of *Culicoides* species within the *Pulicaris* complex (Diptera: Ceratopogonidae) in Turkey inferred from mitochondrial COI gene sequences. — *Acta. Trop.* **190**: 380–388.

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