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Identification of some Amazonian species of *Culex* (*Culex*) and *Culex* (*Melanoconion*) by morphotyping and barcoding

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Abstract

Culex spp. mosquitoes have idiosyncratic characteristics and its low variability makes difficult their identification. The aim of our study was to analyze the 5' region of the cytochrome oxidase subunit I gene (*co*I) for the taxonomic identification of *Culex* species which were previously morphotyped and diagnosed in *Culex* and *Melanoconion* subgenera at the field conditions. Ten specimen sequences were analyzed by the Automatic Barcode Gap Discovery (ABGD). All sequences showed 94-99% identity when compared to other *Culex* species sequences available from GenBank. Five initial partitions supported 80-88 species groups. Among them, eight sets contained the specimens of the present study. Of the 10 mosquito sequences, five did not form any consistent cluster, and the remaining showed some consistency in the taxonomic diagnosis at the field conditions. Our results suggest that some *co*I gene sequences of specimens may belong to species of the subgenus *Melanoconion*, whose 5' coI sequence is unknown or unpublished in GenBank.

Palavras-chave: ABGD, *co*I gene, HP trap, Neighbor-Joining method, Taxonomic Diagnosis.

Identificação de algumas espécies amazônicas de *Culex* (*Culex*) e *Culex* (*Melanoconion*) por meio de morfotipagem e barcoding

Resumo

Os mosquitos *Culex* spp. apresentam características idiossincráticas e sua baixa variabilidade dificulta sua identificação. O objetivo do nosso estudo foi analisar a região 5 'do gene da subunidade I do citocromo oxidase (*co*I) para a identificação taxonômica de espécies de *Culex* que foram previamente diagnosticadas em subgêneros *Culex* e *Melanoconion* em condições de campo. Dez sequências de espécimes foram analisadas pelo Automatic Barcode Gap Discovery (ABGD). Todas as sequências apresentaram 94-99% de identidade quando comparadas com outras sequências de espécies de *Culex* disponíveis no GenBank. Cinco partições iniciais suportaram 80-88 grupos de espécies. Entre eles, oito conjuntos continham os espécimes do presente estudo. Das 10 sequências de mosquitos, cinco não formaram nenhum cluster consistente, e as demais apresentaram alguma consistência no diagnóstico taxonômico nas condições de campo. Nossos resultados sugerem que algumas sequências do gene *co*I de espécimes podem pertencer a espécies do subgênero *Melanoconion*, cuja sequência 5' *co*I é desconhecida ou inédita no GenBank.

Palavras-chave: ABGD, Armadilha CDC-HP, gene *co*I, método Neighbour-Joining, Taxonomia.

Introduction

Culicidae represents an important taxon due to the medical and veterinary relevance. This family encompasses 3,556 valid species, distributed into 113 genera, belonging to Anophelinae and Culicinae subfamilies. The species of *Culex* genus are widely distributed around the world and harbor nearly of one fifth of mosquito species (Harbach, 2013). Members of *Culex* spp. are recognized as main vectors of important pathogens such as filarial species, encephalitis and

fever viruses, and avian malaria *Plasmodium* spp. (Eldridge, 2005), Oropouche (Cardoso et al., 2015) and Zika virus (Ferreira-de-Brito et al., 2016; Song et al., 2017).

Culex species were categorized into twenty six subgenera, while sections, series, groups, subgroups and complexes have been informally employed to organize similar species based on their morphological characters (Harbach, 2013). Few females of *Culex* spp. have idiosyncratic characteristics and its low variability makes difficult their identification. In contrast, male genitalia have remarkable structures being the main identification resource (Consoli & Lourenço-de-Oliveira, 1994; Harbach, 2011). Furthermore, several species complexes of *Culex* and *Melanoconion* subgenera can only be correctly identified with complementary information about adult females, their cibarial armature (Williams & Savage, 2009), and larval and pupal characters from exuviae (Demari-Silva, B., Vesgueiro, F. T., Sallum, M. A., Marelli, M. T., 2011; Torres-Gutierrez et al., 2016).

The obstacles in morphological taxonomy can be magnified if the specimens are captured and manipulated in the field - in most cases causing damages to the specimens structure and organ loss - which hinder reliable identification by taxonomists (Torres-Gutierrez et al., 2016). In this context, molecular identification approaches allow to determine and discover new species through the analysis of a small segment of the genome, representing an efficient tool that would facilitate the diagnosis of biological diversity (Hebert, P. D. N., Cywinska, A., Ball, S. L., deWaard, J. R., 2003). Besides, microsatellite loci and wing geometry have also been employed as biological markers to assess genetic microevolution in the populations of *Aedes aegypti* (Louise et al., 2015).

DNA barcoding using the subunit I of the mitochondrial *cytochrome C oxidase* gene (*co*I) have been widely used as DNA barcode for animal identification (Hebert et al., 2003, Hebert, P. D. N., Penton, E. H., Burns, J., Jansen, D. H., Hallwachs, W., 2004). The Barcode of Life Data System (BOLD), an online platform that collects, analyzes and publishes DNA barcode data from around the world (Ratnasingham & Hebert, 2007). Herein we analyze the 5' region of the *co*I for the taxonomic identification from specimens previously morphotyped and diagnosed in *Culex* and *Melanoconion* subgenera. This work was part of efforts to develop a draft field guide of *Culex* spp. based on specimens captured in the Brazilian Amazon, from forest remnants in the state of Rondônia.

Materials and Methods

The collections and field identification took place at three forest areas in the state of Rondônia, Brazil (Point 1: 8 º 53 ' 7.10 " S, 64 º 0 ' 55.90 " W; Point 2: 9 º 34 ' 58.50 " S, 64 º 51 ' 57.50 " W; Point 3: 9 º 15 ' 35.20 ", S 62 º 54 ' 13.3 " W). Mosquito captures were done with HP light traps (Pugedo et al., 2005) and BG-sentinel traps, baited with carbon dioxide (CO_2, dry ice), between 6:00 pm and 6:00 am, in 2015 and 2016. The specimens were taxonomically identified by stereoscopic microscopy according to the dichotomous keys proposed by Consoli & Lourenço-de-Oliveira (1994), Forattini (2002), and Lane (1953).

The great majority of *Culex* mosquitos was identified only in genus/subgenus level. Hence, mosquitoes were categorized into morphotypes, based on general structures and features in its head, pleura, thorax, abdomen, and legs, and after that grouped in pools. For the barcode analysis, we target specimens from the same mosquito pool and the most frequent morphotypes.

DNA was individually extracted from mosquito legs using the DNeasy Blood & Tissue kit (Qiagen). The PCR was performed with 0.4 μM of each primer LCO1490 and HCO2198 (Hebert et al., 2003), 10 ng – 62 ng of DNA, 3 U HotMaster™ Polymerase on 25 μL mix reaction. PCR products were purified using PureLink™ Quick Gel extraction kit (Invitrogen™).

Sequencing was performed at Oswaldo Cruz Foundation Platform Facilities (RPT01H and RPT01E). Sequences edition and multiple alignment were performed using the MEGA7 (Kumar et al., 2016), and the consensus sequences were built using BioEdit 7.2.6. (Hall, 1999). Consensus sequences were used to query GenBank most similar hits with BLASTn tool. The BLASTx was used to check for stop codons and nucleotide substitutions.

The descriptive statistics and Phylogenetic analyses were performed on MEGA7 (Kumar, S., Stecher, G., Tamura, K., 2016). Phylogenetic tree was generated using new and harvested sequences, by the Neighbor-Joining (NJ) method and K2P distance (Kimura, 1980), to evaluate the cluster patterns among species (1000 bootstrap replicates) (Kumar et al., 2016). Species delimitation was estimated using ABGD software (Puillandre, N., Lambert, A., Brouillet, S., Achaz, G., 2012).

Results and Discussion

The *co*I amplification was obtained from 37 individuals whose were morphologically identified in one species and nine morphotypes. Due to the low quality of sequences, only 10 specimen sequences (630 bp, 455 conserved and 149 variable sites) were analyzed, belonging to six *Culex* morphotypes (Table 1). The average nucleotide composition was A = 28.7 %, T = 39 %, C = 16.3 % and G = 16 %, in agreement with previously described for other insect groups (Torres-Gutierrez et al., 2016).

Sequences showed 94-99 % identity when compared to other *Culex* species sequences gathered from GenBank. Torres-Gutierrez and colleagues (2016) employed barcode identification of *Melanoconion* mosquitoes and considered the sequence similarity percentages between 98 and 100 % as acceptable threshold of agreement in the intraspecific pairwise comparisons.

For the mosquito sequence analysis, 127 *co*I gene sequences of *Culex* species from GenBank were included in our database, to guarantee a more consistent result in the species identification. ABGD was employed for initial species delineation, with a previous intraspecific divergence range of 0.001-0.1, which resulted in five initial partitions supporting 80-88 species groups. Among them, eight sets contained the specimens of the present study. In the original tree, eleven subtree branches were compressed (CS) to improve graphical representation (Figure 1, Supplementary Data).

Our study considered that the GenBank sequences of the *Culex* species previously defined were taxonomically confirmed by an expert entomologist. Thus, we supposed that the ABGD grouping of our sequences with the deposited sequences were due to both specimen sequences represent the same species, nearly species or complex integrant (Table 1). Hence, species confirmation was performed using the dataset phylogenetic analysis.

Table 1. Initial delimitation of *Culex* species using ABGD of the studied morphotype sequences and sequences dataset deposited in GenBank (access code). The consistency between taxonomic identification in the field and ABGD analysis was also described.

Initial Group	Morphotype	GenBank Sequence	Identification Concordance
1	*Culex (Melanoconion)* sp. 1	HE600697 *Cx.* (*Culex*) *brethesi*; HE605120 *Cx.* (*Culex*) *eduardoi*; KF919200 *Cx.* (*Culex*) *camposi*; KF919226 (*Cx. maxi*); KF919232 *Cx.* (*Culex*) *saltanensis*; KF919233 *Cx.* (*Culex*) *surinamensis*; KM592996 *Culex* sp.; KX671403 *Cx.* (*Culex*) *coronator*; KX671406 *Cx.* (*Culex*) *usquatus*.	Error: misleading subgenus
2	*Culex* (*Melanoconion*) sp. 2a	Atratus Group	-
3	*Culex* (*Melanoconion*) sp. 2b	Conspirator Group	-
4	*Culex* (*Melanoconion*) sp. 3	KX7798889 *Cx.* (*Melanoconion*) nr. *portesi*;	Concordance in subgenus
5	*Culex* (*Melanoconion*) sp. 4a *Culex* (*Melanoconion*) sp. 4b	Spissipes Section	-
6	*Culex* (*Culex*) sp. 5a	X779788 (*Culex bastagarius*), KX779789 (*Culex bastagarius*)	Error: misleading subgenus
7	*Culex* (*Culex*) sp. 5b	Atratus Group	-
8	*Culex* (*Melanoconion*) sp. 6a *Culex* (*Melanoconion*) sp. 6b	KX779819 (*Culex idottus*)	Concordance in subgenus

The specimen *Cx.* (*Melanoconion*) sp. 1 was erroneously identified as belonging to the *Melanoconion* subgenus and did not consistently form a cluster with any specific Genbank sequence (Figure 1 and Table 1). However, this sequence matched with eight species from the Pipiens Group, *Culex* subgenus (Figure 1). The *co*I barcoding of *Cx.* (*Culex*) spp. may not contain enough information for the species distinction (Laurito et al. 2013), and Pipiens group have been previously depicted as a complex assemblage (Harbach 2011, 2012). Thus, some groups may need more markers to consistently evaluated the relationships among species, or the species within these groups are, in fact, very similar genetically.

The *Cx.* (*Mel.*) sp. 2a sequence assembled with the *Cx.* (*Mel.*) *zeteki* and a specimen of *Culex* sp. (bootstrap 76, MID = 0.034, Figure 1), belonging to the Atratus Group. By the other hand, the sequence of *Cx.* (*Mel.*) sp. 2b was pooled in between *Cx.* (*Mel.*) *lucifugus* (bootstrap 95) and *Cx.* (*Mel.*) *aliciae* (bootstrap 97) groups. The *Cx.* (*Mel.*) *lucifugus* cluster contains a sequence deposited as *Cx.* (*Mel.*) near *aliciae*, which needs morphological review for further taxonomic conclusions (Torres-Guiterrez et al 2016), and corroborates that, based on available sequences, the limits between *Cx.* (*Mel.*) *lucifugus* and *Cx.* (*Mel.*) *aliciae* is not well determined Those facts raise some hypotheses to the position of the specimen *Cx.* (*Mel.*) sp. 2b: it belongs to one of the two species of the Conspirator groups; it composes a genetically similar species, but not yet morphologically described.

Another point is that the two specimens from the same morphotype (Initial Group 2) belong to different genetic clusters [*Cx.* (*Mel.*) sp. 2a to the Atratus Group and *Cx.* (*Mel.*) sp. 2b to Conspirator Group], and were wrongly pooled, indicating an inconsistency during the field identification through the quick guide.

Culex (*Melanoconion*) sp. 3 sequence clustered with a GenBank specimen identified as *Culex* (*Mel.*) nr. *portesi* (Figure 1). This cluster also included *Cx.* (*Mel.*) *panocossa*, with high bootstrap (100). These two species belong to morphologically different groups, Vomerifer and Ocossa respectively (Sallum, 1994), hence their relationships deserve further analysis.

Cx. (*Melanoconion*) sp. 4 - "a" and "b" specimens - were grouped into an in a strongly supported clade (bootstrap 100, MID = 0.008) and differing from all *Culex* sequences included. The *Cx.* (*Melanoconion*) sp. 4 sequences compose a sister group of *Cx. crybda* and *Cx. ribeirensis* (Figure 1). Both species were described in the Spissipes Section, Crybda Group and Pedroi Subgroup and share morphological similarities (Sallum et al., 1996). This indicate that this morphotype may represent a newly sequenced species from the same group/subgroup. This Subgroup also includes *Cx. adamesi* and *Cx. pedroi* (Sallum & Forattini, 1996), with no *co*I sequence record for the former.

The *Culex* (*Culex*) sp. 5a specimen had 100% of similarity with two *Cx.* (*Mel.*) *bastagarius* sequences from GenBank (bootstrap 92), with a discordance in the subgenus identification. Sirivanakarn (1982) presents a review of the subgenus *Melanoconion*, in which the Bastagarius Group can be distinguish from the Atratus Group based on morphological characteristics. Since these mosquitoes were caught in air suction traps, which usually damage mosquitoes, the barcoding tool was useful for correcting any misleading diagnosis. Another specimen from the same pool, *Cx.* (*Culex*) sp. 5b clustered with *Cx.* (*Mel.*) *ensiformis*, however there was a low support for this relationship (bootstrap 20, MID = 0.031, Figure 1).

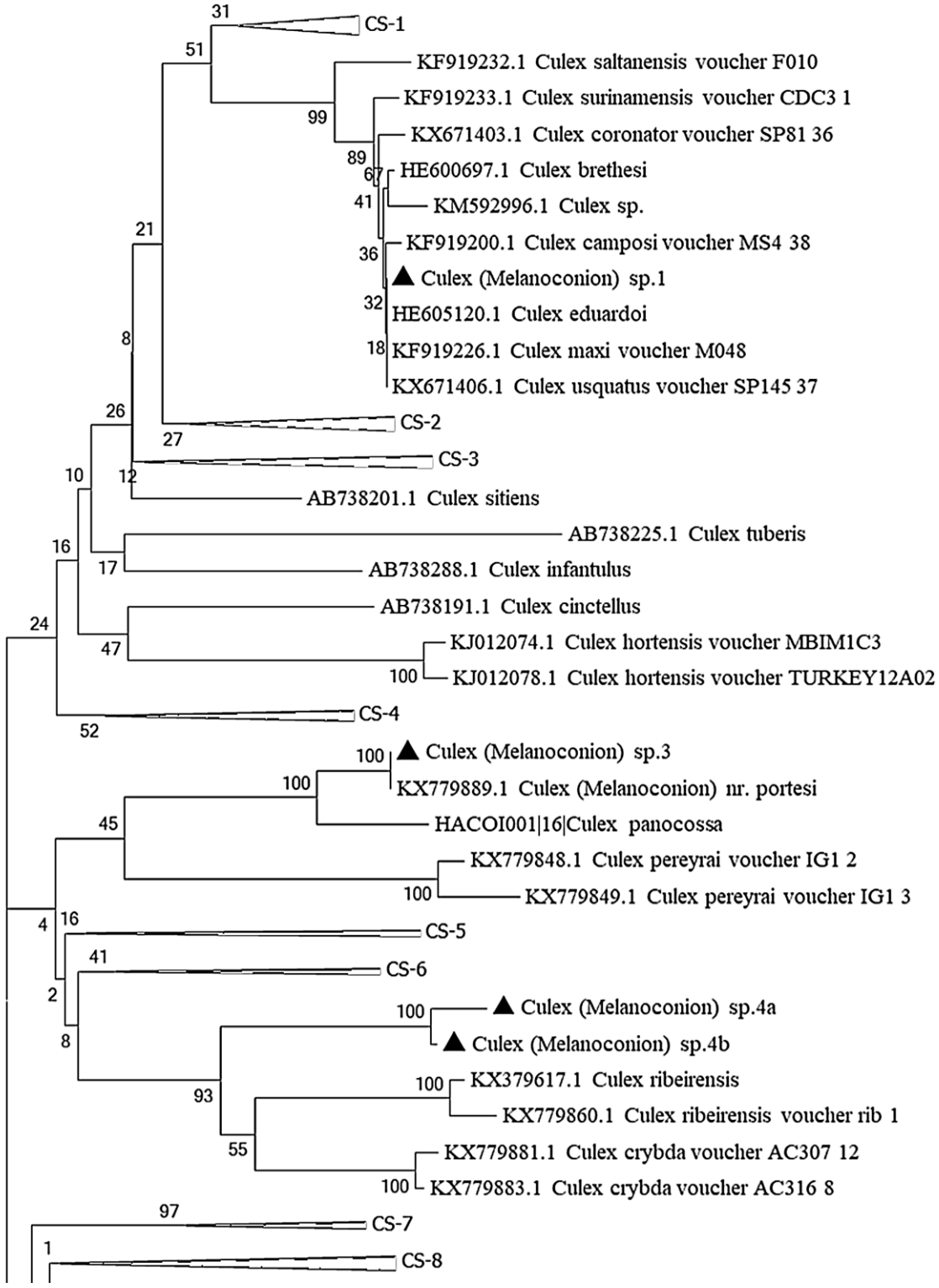

Figure 1. Phylogenetic tree from the *co*I gene sequence data set of *Culex* morphotypes of this study and from *Culex* deposited on GenBank.

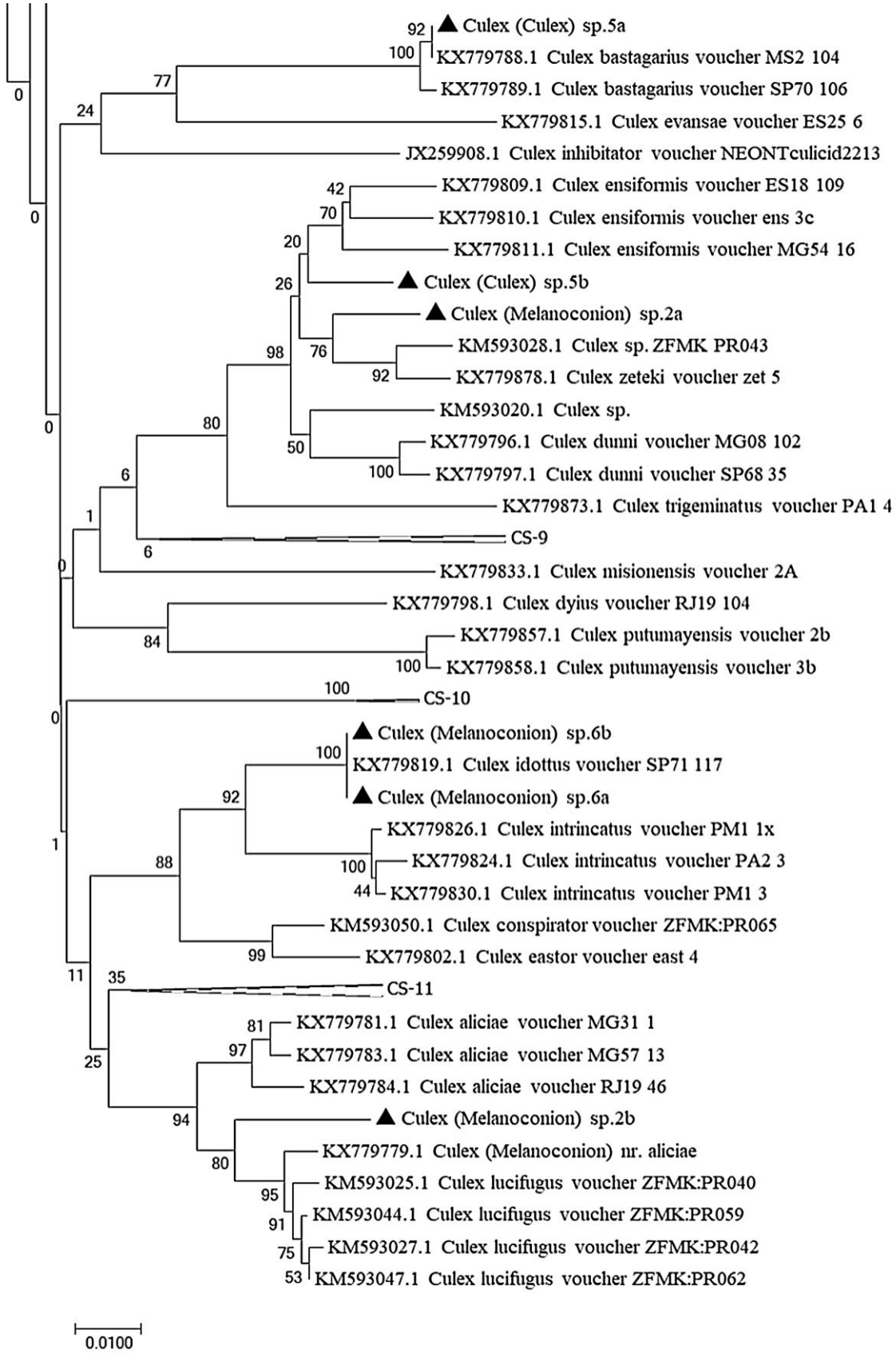

Figure 1. (Continuation) Phylogenetic tree from the *co*I gene sequence data set of *Culex* morphotypes of this study and from *Culex* deposited on GenBank.

When analyzing primary clade, a higher bootstrap (98) includes *Cx.* (*Mel.*) *zeteki* and *Cx.* (*Mel.*) *dunni*, which together with *Cx.* (*Mel.*) *ensiformis* constitute a representative part of the Atratus Group (Gutierrez, 2015). This result suggest that our specimen belongs to the Atratus Group and to a *Melanoconion* species which sequence was not available in GenBank. Likewise, *Cx. atratus*, *Cx. caribeanus*, *Cx. commevynensis* and *Cx. trigeminatus* compose part of this group (Kobayashi, 1999), and *co*I sequences have been found only for the last two species.

The *co*I sequences of two individuals morphologically identified as *Cx.* (*Melanoconion*) sp. 6 formed a strongly supported clade (bootstrap 100) with the *Cx.* (*Mel.*) *idottus* and mean intraspecific distance (MID) of 0.000. Similarly, Torres-Gutierrez and colleagues (2016) analyzed two females of this species which presented 100% pairwise identity. *Cx. idottus* has been captured in several Brazilian states (Gomes et al., 2007; Dibo et al., 2011; Hutchings, R. S. G.; Sallum, M. A. M.; Hutchings, W.R., 2011), and we assumed that our morphotype 6 correspond to this species.

Studies involving virology inquiries in mosquitoes and capture of potential wild vectors are usually done in remote locations and may face challenges in determining mosquito species before storage. 200 and 160 species were recorded to the *Culex* and *Melanoconion* subgenera, respectively (Harbach, 2013) and clusters are not uncommon in the taxonomic keys due to the existence of complexes or species absence in these keys (Forattini, 2002). Besides this, the paired combination of morphological and molecular taxonomic information may aid in the status resolution of a species (Collins et al., 2014). To some species, our results demonstrated that the ABGD was efficient in its initial delimitation. As highlighted by Puillandre et al. (2012), the software partition should not be interpreted as a final discrimination of the species, but rather a first hypothesis of species partitioning on which further analysis is required.

Hoyos-López et al. (2016) investigated the presence of arboviruses in mosquitoes from Colombia and, similarly, grouped mosquitoes into pools, based on similarities in the morphological characteristics, due to the difficulty of species differentiation of *Melanoconion* and *Culex* subgenera. In the present study, some *Culex* species were grouped into morphotypes, for which pictorial diagnosis were established, with textual description of the main morphological traits, with subsequent analysis of the *co*I gene for confirmation of the species grouped in morphotypes.

DNA barcode technique using the *co*I gene have been an efficient tool to distinguish morphologically similar and sympatric species. *Aedes aegypti*, *Aedes albopictus* and *Aedes scutellaris* were clearly separated, assessing their phylogenetic position in relation to other species of Culicidae. Besides, wing geometric morphometry could also help the morphological identification of these three species (Sumruayphol et al., 2016).

Hernández-Triana and colleagues (2019) have shown that the combination of morphological feature analysis and DNA Barcode is an effective approach for identifying British mosquitoes, for monitoring invasive species, and for detecting hidden diversity within species groups. However, although most of the specimens were differentiated by the *co*I gene, certain species could not be distinguished using this genetic marker, mainly within the genera *Aedes*, *Anopheles* and *Culex*. The use of *co*I also generated problems of identification of *Culiseta* species (*Cs. fumipennis*, *Cs. litorea* and *Cs. morsitans*) within the BOLD and NCBI databases. These outcomes demonstrate that further researches must conciliate the use of molecular techniques and morphological characteristics for the delineation of Culicidae species.

Conclusions

Our findings sustain that molecular barcoding is a valuable tool in the morphological taxonomy and diagnosis of *Culex* species. Furthermore, the occurrence of indistinguishable species in some complexes suggested that other markers than *co*I should be evaluated as barcoding targets for *Culex* mosquitoes.

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