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Mitochondrial COI gene as a tool in the taxonomy of mosquitoes *Culex* subgenus *Melanoconion*

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ABSTRACT

The subgenus *Melanoconion* is the second largest subgenus within the genus *Culex*, with 160 described species. Several of the species are proven vectors of arboviruses, including West Nile virus, Venezuelan equine encephalitis virus complex and Eastern equine encephalomyelitis virus. Species of *Melanoconion* are well distributed from southern North America to most countries of South America and display the highest species diversity in tropical regions. Taxonomical identification within this group has been primarily based on morphological characters, with the male genitalia as the source of the most solid diagnostic features. The difficulty in reaching accurate species determinations when studying specimens of *Culex* (*Melanoconion*) has been extensively documented as a real limitation to expand knowledge of these insects. We tested the utility of the mitochondrial gene *COI* as a complementary tool in the taxonomy of *Melanoconion*. Using a data set of 120 *COI* sequences from *Culex* specimen captured in several localities in Brazil, the utility of *COI* barcodes for species delimitation is discussed through the evaluation of genetic divergences among specimens and the clustering patterns of species in three topologies obtained with Neighbor Joining, Maximum Likelihood and Bayesian phylogenetic inference. For all specimens included in this study a previous morphological examination was performed, and most of the taxonomical determinations were corroborated using the *COI* barcode. We generated *COI* sequences that belong to 48 species of *Melanoconion*, with a mean intraspecific K2P genetic divergence of 3%; and all interspecific divergence values higher than the intraspecific divergence values. This is the first comprehensive study of subgenus *Melanoconion*, with evidence of *COI* as a useful and accessible DNA barcode.

1. Introduction

Mosquitoes of the genus *Culex*, subgenus *Melanoconion*, are recognized as a highly diverse group of great taxonomic difficulty. Moreover, several species currently classified within this subgenus, have been proven to be vectors of several arboviruses, including West Nile virus, Venezuelan equine encephalitis virus complex and Eastern equine encephalomyelitis virus (Mitchell et al., 1985, 1987; Powers et al., 1997; Turell et al., 2000, 2005, 2006; Ferro et al., 2003; Cupp et al., 2003, 2007; Weaver et al., 2004). Currently, the 160 described species are subdivided into two major sections designated as Spissipes Section and Melanoconion Section, for which there is scarce knowledge of their taxonomy and phylogeny exists (Torres-Gutierrez and Sallum, 2015). The major taxonomical revision of the subgenus focused on the Spissipes Section (Sallum and Forattini, 1996), providing descriptions and keys to identify the 22 species of this section known at the time. On the other hand, the Melanoconion Section, composed of 137 species, remains as a complex and poorly described taxonomic group.

Traditionally, accurate identification of species of the subgenus *Melanoconion* depends upon the study of the male genitalia. Morphological characters of the fourth-instar larvae and pupae are greatly informative as well (Forattini and Sallum, 1992, 1993, 1995).

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Although Sallum and Forattini (1996) fully described the morphological features of female adults of species, some key characters (i.e. presence, shape and color of groups of scales on certain parts of the body) depend on how specimens are sampled and handled in the field. In most cases, female specimens suffer considerable damage during their transition from the field to the laboratory, which in turn, prevents taxonomists from a confident identification. Because of this, selective sampling practices, such as, male-oriented sampling techniques (rare and highly species-specific), or a more common immature-oriented sampling effort, which involves the keeping of live larvae and pupae in insectaries are necessary (Gaffigan and Pecor, 1997). Rearing mosquitoes under laboratory conditions provides higher chances of obtaining both female and male individuals; as well as skins of larvae and pupae from a given population. However, this practice poses great challenges in terms of time, resources and expertise. In this context, new, genetic approaches to improve our taxonomical knowledge and skills are highly expected.

Modern techniques have introduced the use of a DNA-based species identification system (Hebert et al., 2003). This involves a complementary identification tool using a standardized sequence of a 658-base-pair cytochrome *c* oxidase subunit I (cox1 or COI) gene region, as the DNA barcode, for members of the animal kingdom. The use of *COI* for delimiting species has been considered an effective tool to identify taxa for which morphology-based identifications are difficult. Additional advantages of using DNA barcodes are the ability to identify various life stages of species, and facilitate the discovery and description of new species. With this knowledge the creation of a major database has been possible, where information regarding voucher specimens, origins and other records of sequenced specimens is rigorously kept as a public source (Hebert and Gregory, 2005). With a public database of *COI* sequences established (CBOL, www.barcodeoflife.org), this information can be used as reference libraries of *COI* sequences from reliably identified voucher specimens. The recent literature contains a wealth of information regarding *COI* sequences for several groups of arthropods, mainly the insect orders Lepidoptera, Coleoptera, Hemiptera and Diptera (Hajibabaei et al., 2006; Hemmerter et al., 2007; Kim et al., 2012; Woodcock et al., 2013; Dinca et al., 2015; Gwiazdowski et al., 2015; Blagoev et al., 2016).

The resolution delivered by *COI* barcodes in previous research efforts with mosquito species (Diptera: Culicidae) is highly variable. Motoki et al. (2009) found that *COI* was unable to separate individuals of *Anopheles deaneorum* from *Anopheles marajoara* when studying South American samples. Also, Sallum et al. (2010) argued that analyses using *COI* sequences alone were unable to separate specimens belonging to *An. albertoi* from *An. strodei*, and Bourke et al. (2013) documented a low frequency of identification when comparing *COI* sequences of *An. albertoi* and *An. strodei* to reference data. In other studies, *COI* gene was found as an effective complementary identification tool for Culicidae species by Cywinska et al. (2006); Ashfaq et al. (2014) and Rozo-Lopez and Mengual (2015). A study from Brazil by Demari-Silva et al. (2011) tested the potential of a 478 base-pair fragment of *COI* to study phylogenetic relationships of 17 *Culex* species (including several subgenera), and reported the mitochondrial gene as a useful tool. Comparatively, Laurito et al. (2013), when studying species of *Culex* (*Culex*) from Argentina and Brazil, recorded that only a 42% of their samples clustered together with their conspecifics when using Neighbor Joining analysis. These authors concluded that the *COI* gene does not have enough information for separating species within subgenus *Culex*. The available information concerning the utility of *COI* as a tool to delineate *Culex* species is still scarce, in this study we showed the utility and effectiveness of *COI* gene as a tool in the taxonomical identification of species of *Culex*, subgenus *Melanoconion*.

2. Material and methods

2.1. Sample data

Our data included 120 specimens (ingroup), from 39 species defined by morphological characters including male genitalia, female, and larval and pupal exuviae (Rozeboom and Komp (1950), Sirivanakarn (1983), Pecor et al. (1992) and Sallum and Forattini (1996)). An additional 17 specimens required confirmation, due to ambiguous species assignment based on morphological characters, and therefore were employed as test-species (all undetermined species are marked with "near", i.e. *Cx.* near *vaxus*). Additional specimens corresponded to the outgroup; two sequences of *Culex* (*Culex*) *mollis* and one sequence retrieved from GenBank belonging to *Cx.* (*Cx.*) *quinquefasciatus* (Table 1). The mosquitoes used in this study were collected during the development of several research projects at the School of Public Health of the University of São Paulo, throughout the last decade in various regions of Brazil. Specimens were preserved in 95% ethanol at −80 °C or dried inside plastic tubes with silica gel. All male individuals have the corresponding dissected genitalia mounted on microscope slides and deposited in the Coleção Entomológica de Referência, Universidade de São Paulo (FSP-USP) as vouchers (Supplementary data 1). The female individuals have Supporting information on a database containing the exact geographical information (for most cases geographical coordinates) of collection sites, as well as records of other individuals of the same species collected at the same localities (such individuals are pinned as part of the entomological collection).

Additionally, any *COI* sequences available in public databases (GenBank) of representatives of the subgenus *Melanoconion* were incorporated in the study. The list of *COI* sequences (nine species; 23 specimens) of subgenus *Melanoconion* currently available on GenBank – in January 2016 – are presented in Table 1.

2.2. DNA extraction, amplification and sequencing

Total genomic DNA extractions were performed using the complete mosquito specimens following a salting out protocol (modified from Miller et al., 1988). All extraction products were stored at −20 °C. The amplification of *COI* was performed using the primers recommended by Folmer et al. (1994), LCO1490 (5′GGT CAA ATC ATA AAG ATA TTG G 3′) and HCO2198 (5′TAA ACT TCA GGG TGA CCA AAA AAT CA 3′), to obtain a sequence of 658 base pairs. PCR reactions of 25 μL contained: 1 μL DNA; 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$ (Invitrogen); 0.1 μM of each primer; 200 μM each dNTPs (Amresco, Solon, OH, USA); 0.625 U of Platinum® *Taq* DNA Polymerase, High Fidelity (Life Technologies); the remaining volume of ultrapure H_2O. Amplification consisted of a 3-min denaturation step at 94 °C and 35 cycles of 94 °C, 55 °C and 72 °C for 1 min each, followed by a 7-min extension at 72 °C. *COI* products were purified using PEG precipitation (20% polyethylene glycol 8000/2.5 M NaCl). PCR products of all specimens were electrophoresed in 1% TAE agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., Hayward, USA), to verify the size of expected products as well as their quality.

Sequencing reactions were carried out in both directions using 0.5 μL of the ABI Big Dye Terminator Kit version 3.1 (PE Applied Biosystems, Warrington, England), 3.6 pmol/μL each primer; 2 μL buffer (5 mM MgCl2, 200 mM Tris-hCl, pH 9.0), 5–20 ng of DNA purified product and ultra pure water to reach a final volume of 10 μL. Thermocycler conditions followed 96 °C and 50 °C for 15 s each, and 60 °C for 4mins (25 cycles). Sequencing reactions were purified in Sephadex G50® columns (GE Healthcare). Sequences were analyzed on an ABI Prism 3100 – Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), following the Sanger method. All DNA extracts from the specimens are kept as voucher DNA at −80 °C