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Intraespecific Variation on the Aedeagus of *Anopheles oswaldoi* (Peryassú) (Diptera: Culicidae)

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Variação Intraespecífica no Edeago de *Anopheles oswaldoi* (Peryassú) (Diptera: Culicidae)

**ABSTRACT** - *Anopheles (Nyssorhynchus) oswaldoi* (Peryassú) compreende complexo de espécies cripticas na América do Sul. Espécimes de duas localidades situadas no leste da Mata Atlântica foram empregados para caracterizar morfologicamente e molecularmente *An. oswaldoi s.s.*. Foram observadas e avaliadas variações na forma do ápice do edeago da genitalia masculina de espécimes de *Anopheles (Nyssorhynchus) oswaldoi s.s.* do Vale do Ribeira, Mata Atlântica, estado de São Paulo, e nas sequências do segundo espaçador interno transcrito (ITS2). Os espécimes com edeagos distintos apresentaram seqüências idênticas de ITS2. Os tipos distintos de edeago encontrados nos exemplares do Vale do Ribeira, Mata Atlântica, foram ilustrados.

PALAVRAS-CHAVE: ITS2, *Nyssorhynchus*, ITS2, genitalia masculina, identificação

**KEY WORDS:** ITS2, *Nyssorhynchus*, ITS2, male genitalia, identification

*Anopheles (Nyssorhynchus) oswaldoi* (Peryassú) comprises a species complex in South America (Marrelli et al 1999, Ruiz et al 2005, Scarpassa & Conn 2006). It is considered an efficient malaria vector in some regions of Brazil (Branquinho et al 1996), while a secondary or unimportant vector in others.

*Anopheles oswaldoi s.s.* is morphologically similar and thus was considered conspecific with *Anopheles konderi* Galvão and Damasceno by Lane (1953). Recently, Flores-Mendoza et al (2004) validated and resurrected *An. konderi* from synonymy with *An. oswaldoi*. Motoki et al. (2007), using morphological characters of all life stages except the eggs, redescribed *An. oswaldoi s.s.* and designated the lectotype. Results of these studies showed that males of *An. oswaldoi s.s.* and *An. konderi* can be distinguished by the shape of the apical part of the aedeagus, which is usually longer than broad and somewhat ovate in *An. oswaldoi s.s.*, whereas it is broader than long and somewhat conical in *An. konderi* (Flores-Mendoza et al 2004, Motoki et al 2007).

Male genitalia characteristics are important and largely used to distinguish among species of *Anopheles (Nyssorhynchus)* (Faran 1980, Linthicum 1988, Bergo et al 2007, Motoki et al 2007). However, Hribar (1994) observed geographic variation in the male genitalia characteristics of *Anopheles nuneztovari* Gabaldón from several regions. Similarly, morphological variation on the male genitalia was reported in other groups of insects. Schulmeister (2003a, b) observed morphological variations in the terminal abdominal segments of Hymenoptera species, whereas Pires et al (1998) studied distinct male genitalia characteristics in three *Triatoma infestans* Klug populations.

While examining the male genitalia of specimens of *An. oswaldoi s.l.* collected in Pariquera Mirim district, Pariquera-Açú, São Paulo State, Brazil, we observed differences in the shape of apex of the aedeagus. In considering only the shape of the apex of the aedeagus, those specimens could be misidentified as *An. galvaoi* Causey, Deane and Deane, when using Faran’s (1980) identification key, because the apex of the aedeagus either was broader than long or as broad as long. However, based on the characteristics of the
immediate stages and adult male and female, those specimens were identified as *An. oswaldoi* s.l. To confirm the identity of these morphologically variant specimens, the second internal transcribed spacer (ITS2) of nuclear ribosomal DNA was sequenced for those specimens obtained in Pariquera Mirim district, Pariquera-Açu and compared with those generated by Motoki et al. (2007) for *An. oswaldoi* s.s.

### Material and Methods

Immature collections were conducted in Pariquera Mirim (24°42′54″S, 44°52′52″W), Pariquera-Açu municipality, São Paulo State, Brazil. The ITS2 nucleotide sequences were derived from six individually reared adult male specimens, with associated fourth-instar larval and/or pupal exuviae, and the adult male genitalia were slide mounted and kept as vouchers. Species identification was based on both characters of both the male genitalia and fourth-instar larva. Specimens examined are as follow: SP22-9 (Le, Pe, male), SP22-13 (Le, Pe, female), SP22-17 (Le, Pe, female), SP22-21 (Le, Pe, male, DNA), SP22-33 (Le, Pe, male, DNA), SP22-48 (Le, Pe, female), SP22-54 (Le, Pe, male, DNA), SP22-55 (Le, Pe, female), SP22-63 (Le, Pe, female), SP22-70 (Le, Pe, male, DNA), SP22-80 (Le, Pe, female), SP22-103 (Pe, male, DNA) and SP22-104 (Pe, male, DNA).

DNA was extracted from the field collected specimens, following the DNA extraction protocol provided by the QiAgen DNeasy® Blood and Tissue Kit (QiAgen Ltd., Crawley, UK). All buffers were supplied in the kit. The elution step was repeated and stored in a separate tube, as DNA often remains bound to the membrane following the first elution. One μl of the first elution was used as DNA template in the PCR reactions. Amplification of the ITS2 region was carried out using the 5.8SF (- ATC ACT CGG CTC GTG GAT CG -) and 28SR (- ATG CTT AAA TTT AGG GGG TAG TC -) primers recommended by Ruiz et al. (2005). PCR products were amplified in 25 μl reaction mix containing: 1 μl DNA; 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl2; 2.5 μl DMSO; 5 pM of each primer; 200 μM each dNTPs; and 2.5 U New England Biolabs® Taq polymerase. PCR amplification protocol consisted of a 2-min denaturation at 94°C, 34 cycles at 94°C, 57°C and 72°C for 30 sec each, followed by a 10 min extension at 72°C. PCR products were electrophoresed in 1% TAE agarose gels stained with ethidium bromide. ITS2 PCR amplicons were purified directly from bands excised from agarose gel using the QIAQuick Gel Extraction kit, and directly sequenced.

Sequencing reactions were carried out in both directions using the PCR primers and the Big Dye Terminator Kit v.3.1 (PE Applied Biosystems, Warrington, England). Sequences were analyzed on a 377 - ABI Sequencer (Applied Biosystems, Foster City, CA, USA).

Template DNA from this study is retained at -70°C in the Faculdade de Saúde Pública (FSP-USP) for future reference (DNA reference numbers SP22-9, SP22-21, SP22-33, SP22-70, SP22-103 and SP22-104). Linked immature exuviae and male genitalia slides of specimens used for DNA extraction are deposited in the FSP-USP collection. Sequences were edited using Sequencer Navigator (version 1.0.1, PE Applied Biosystems®, ) aligned in CLUSTAL X (Thompson et al. 1997) and optimized manually in MacClade version 4.3 (Maddison and Maddison 2000). Sequence similarity of the ITS2 sequences generated in this study (GenBank accession number FJ 425910-FJ425915), with those previously available in GenBank (EF457237 & EF457239, Motoki et al. 2007) were assessed using FASTA search (http://www.ncbi.nlm.nih.gov/BLAST), and aligned using MEGA3 software (Kumar et al. 2004).

### Results

The ITS2 sequences obtained from adults of *An. oswaldoi* s.s. collected in Pariquera Mirim, Pariquera-Açu, SP, were compared with the sequences of *An. oswaldoi* s.s. deposited in GenBank (Espírito Santo, EF457228-37; São Paulo EF457228-9), ITS2 sequences of all specimens were identical (Fig 1), and thus the identity of all specimens used in this study were determined as *An. oswaldoi* s.s.

The male genitalia characteristics of specimens of *An. oswaldoi* s.s. from Espírito Santo state and São Paulo state are very similar (Fig 2A, E), except for six specimens collected in Pariquera Mirim, Pariquera-Açu municipality, that showed a dissimilar aedeagus apex when compared with those of specimens of *An. oswaldoi* s.s. described by Motoki et al (2007). By examining these specimens, we observed three distinct aedeagus apex shapes, i.e., the width and length of the apex of aedeagus is the same (Fig 2B,C), the apex is broader than long (not shown), and the apex is slightly longer than broad (Fig 2D), but less than in the specimens of *An. oswaldoi* s.s. (Fig 2F).

### Discussion

Molecular and morphological data are convenient to test relative rates of evolution (Huber 1993). In insects, the male genitalia characters are complex and the differences in genitals may have evolved as barriers to insemination. Additionally, the genital structures may have diverged through the sexual selection that occurs after insemination for any device that increases fertilization success (Gwinne 1998). Schulmeister (2003a, b) simultaneously analyzed morphological and molecular characters of the terminal abdominal segments to characterize Hymenoptera. The characters of male genitalia provided support for some nodes in the phylogenetic tree. Pires et al (1998) employed both the presence and absence of denticles on endosoma process of the external male genitalia of three populations of *T. infestans*, two from Brazil (Minas Gerais and Bahia) and one from Bolivia (Cochabamba Valley) to evaluate intraspecific variation. These differences on the external male genitalia were used to characterize three populations of *T. infestans*.

Having said that, it is noteworthy that in the Culicidae, it is usually necessary to examine the male genitalia...
In considering characteristics of the aedeagus, Hribar (1994) analyzed morphological variation of male genitalia structures of several specimens of *An. nuneztoi* cytotype A (Amazonian), B (western Venezuela, southeast of the Andes) and C (from Colombia and western Venezuela northwest of the Andes), and concluded that, despite of geographical variations, there are four characters that distinguish cytotype B from both A and C may represent morphological polymorphisms.

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Anopheles nuneztoi is a species complex, it is important to evaluate any morphological difference observed specially in the male genitalia to ascertain the species identification. Furthermore, other species of the complex can be distinguished by characteristics of the apex of the aedeagus, for example *An. konderi* (Florez-Mendoza et al. 2004) and other unnamed species of the complex that occur in Brazil (Sallum et al. 2004).
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