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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)

RESUMO - *Anopheles* (*Nyssorhynchus*) *oswaldoi* (Peryassú) compreende complexo de espécies crípticas na América do Sul. Espécimes de duas localidades situadas no leste da Mata Atlântica foram empregados para caracterizar morfologica e molecularmente *An. oswaldoi s.s.* Foram observadas e avaliadas variações na forma do ápice do edeago da genitália 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, genitália masculina, identificação

ABSTRACT - *Anopheles* (*Nyssorhynchus*) *oswaldoi* (Peryassú) comprises a species complex in South America. Specimens from two localities in east Mata Atlântica were characterized both at the morphological and molecular level as *An. oswaldoi s.s.* Intraspecific variation of the shape of the apex of the aedeagus of the male genitalia of specimens of *Anopheles* (*Nyssorhynchus*) *oswaldoi s.s.* from Vale do Ribeira, Mata Atlântica, São Paulo State, Brazil, was observed. Distinctive aedeagus of the specimens from Vale do Ribeira, Mata Atlântica, were evaluated, illustrated and compared to that of *An. oswaldoi s.s.*

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 immature 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-Açú 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 (24º42'54"S, 44º52'52"W), Pariquera-Açú 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 larvae. 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, female), 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 94ºC, 34 cycles at 94ºC, 57ºC and 72ºC for 30 sec each, followed by a 10 min extension at 72ºC. 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 -70ºC 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-Açú, SP, were compared with the sequences of *An. oswaldoi s.s.* deposited in GenBank (Espírito Santo, EF457228-37; São Paulo EF457238-9). 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-Açú 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

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                                                                                                              100
EF457237 CACCGCAGAACCAACTAGCATAGCCGTCGAAAGCTTTGCTGCGTACTGATGATTGGTTGACCATGTGCCAACCAAGCATTGAAGGACTGTGGCGTGGTGG
EF457239 ....................................................................................................
SP22-9   ....................................................................................................
SP22-21  ....................................................................................................
SP22-33  ....................................................................................................
SP22-70  ....................................................................................................
SP22-103 ....................................................................................................
SP22-104 ....................................................................................................

                                                                                                              200
EF457237 GTGCACCGTGTGTGCGTCGTTGCTTAATACGACTCATTCTCTGGTATCACATCTGGAGCGGGCTAACCAGTCACAATCCCCAGCGACATGTGCAGATAGC
EF457239 ....................................................................................................
SP22-9   ....................................................................................................
SP22-21  ....................................................................................................
SP22-33  ....................................................................................................
SP22-70  ....................................................................................................
SP22-103 ....................................................................................................
SP22-104 ....................................................................................................

                                                                                                              300
EF457237 CCCGATGTGGAGGACCAACATCCTCCCTCAAAGCCAGCCCATGTGATACACACCAACAGAGAGAGACCAAACGTACCCTGAAGCAACGTATGCGCACACG
EF457239 ....................................................................................................
SP22-9   ....................................................................................................
SP22-21  ....................................................................................................
SP22-33  ....................................................................................................
SP22-70  ....................................................................................................
SP22-103 ....................................................................................................
SP22-104 ....................................................................................................

                                                      345
EF457237 CGTGCAACTCATTGAAGCGCACGATCGAAAGAGAACCGATCAAGT
EF457239 .............................................
SP22-9   .............................................
SP22-21  .............................................
SP22-33  .............................................
SP22-70  .............................................
SP22-103 .............................................
SP22-104 .............................................
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Fig 1 Alignment of nucleotide sequences of the ITS2 region of *Anopheles oswaldoi s. s.* (EF457239, SP22-9, SP22-21, SP22-33, SP22-70, SP22-103, SP22-104 are from Pariquera Mirim, Pariquera-Açú São Paulo State, Brazil; EF457237 is from Fazenda Marianelli, Lagoa do Macuco, Espírito Santo State, Brazil). Dots indicate similar residues.

characteristics to ensure accurate species identification. This is the case in most species of *Anopheles* (*Nyssorhynchus*). In the revision of the Albimanus Section, Faran (1980) distinguished *An. evansae* (Brethes) (as *An. noroestensis* Galvão and Lane) from *An. oswaldoi s.l., An. galvaoi* and *An. aquasalis* Curry by the shape of the apex of the aedeagus, which is truncate in *An. evansae*, whereas it is rounded in the other species. Additionally, Peyton (1993) validated and resurrected *An. dunhami* Causey from the synonymy of *An. nuneztovari* based on characteristics of the male genitalia, including aspects of the aedeagus, and Florez-Mendoza *et al*. (2004) considered *An. konderi* to be a valid name based on the shape of the apex of the aedeagus.

In considering characteristics of the aedeagus, Hribar (1994) analyzed morphological variation of male genitalia structures of several specimens of *An. nuneztovari* 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 cytotypes A and C. Interestingly, Sierra *et al* (2004) examined the variability of four populations of *An. nuneztovari* from Colombia using the second internal transcribed spacer (ITS2) of nuclear ribosomal DNA sequences and demonstrated the cytotypes B and C to be conspecific. Consequently, the male genitalia characteristics observed by Hribar (1994) which distinguished the cytotype B from both A and C may represent morphological polymorphisms.

According to Faran (1980), male genitalia characteristics of *An. oswaldoi s.s.* are somewhat similar to those of *An. galvaoi*. These taxa can be distinguished by aspects of the ventral claspette, i.e., the length of the setae along basal margin of basal lobules and shape and size of the preapical plate. Additionally, Faran (1980) reported that in *An. galvaoi*, the apex of the aedeagus was rounded, about as wide as it is long, distinguishing it from that of *An. oswaldoi* which is longer. Consequently, in considering only the apex of the aedeagus to distinguish between *An. oswaldoi s.s.* and *An. galvaoi*, it is plausible to consider that a specimen with an anomalous aedeagus could be misidentified as *An. galvaoi*. Although the variation on the shape of the apex of the aedeagus reported for the specimens obtained in Pariquera Mirim, Pariquera-Açú, the fourth-instar larvae and the adult female were identified as *An. oswaldoi s.l.* Because *An. oswaldoi s.l.* 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* (Flores-Mendoza *et al* 2004) and other unnamed species of the complex that occur in Brazil (Sallum *et al* 2008).

Fig 2 A - Ventral claspette of *Anopheles oswaldoi s.s.* from São Paulo State, Brazil; B, C, D - Aedeagus of *An. oswaldoi s.s.* from São Paulo State, Brazil; E, F - Ventral claspette and aedeagus of *Anopheles oswaldoi s.s.* from Espírito Santo State, Brazil.

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References

Bergo E S, Souto R N P, Galardo A K R, Nagaki S S, Calado D C, Sallum M A M (2007) Systematic notes on *Anopheles* Meigen (Diptera: Culicidae) species in the State of Amapá, Brazil. Mem Inst Oswaldo Cruz 102: 373-376.

Branquinho M S, Araújo M S, Natal D, Marrelli M T, Rocha M R, Taveira F A L, Kloetzel J K (1996) *Anopheles oswaldoi* a potential malaria vector in Acre, Brazil. Trans R Soc Trop Med Hyg 90: 233.

Faran M E (1980) Mosquito studies (Diptera: Culicidae) XXXIV. A revision of the *Albimanus* section of the subgenus *Nyssorhynchus* of *Anopheles*. Cont Amer Entomol Inst 15: 1-215.

Flores-Mendoza C, Peyton E L, Wilkerson R C, Lourenço-de-Oliveira R (2004) *Anopheles* (*Nyssorhynchus*) *konderi* Galvão and Damasceno: neotype designation and resurrection from synonymy with *Anopheles* (*Nyssorhynchus*) *oswaldoi* (Peryassú) (Diptera: Culicidae). Proc Entomol Soc Washington 106: 118-132.

Gwinne D T (1998) Genitally does it. Nature 393: 734-735.

Hribar L J (1994) Geographic variation of male genitalia of *Anopheles nuneztovari* (Diptera: Culicidae). Mosq Syst 26: 132-144.

Lane J (1953) Neotropical Culicidae, v I, Universidade de São Paulo, São Paulo, 1112p.

Linthicum K J (1988) A revision of *Argyritarsis* section of the subgenus *Nyssorhynchus* of *Anopheles* (Diptera: Culicidae). Mosq Syst 20: 98-271.

Kumar S, Tamura K, Nei M (2004) *MEGA*3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings Bioinform 5:150-163.

Maddison D R, Maddison W P (2000) MacClade 4: analysis of phylogeny and characters evolution, version 4.0, Sunderland. MA, Sinauer Associates, Inc.

Marrelli M T, Malafronte R S, Flores-Mendoza C, Lourenço-de-Oliveira R, Kloetzel J K, Marinotti O (1999) Sequence analysis of the second internal transcribed spacer of ribosomal DNA in *Anopheles oswaldoi* (Diptera: Culicidae). J Med Entomol 36: 679-684.

Motoki M T, Linton Y M, Ruiz F, Flores-Mendoza C, Sallum M A M (2007) Redescription of *Anopheles oswaldoi* (Peryassú, 1922), with formal lectotype designation. Zootaxa 1588: 31-51.

Peryassú A (1922) Duas novas espécies de mosquitos do Brasil. A Folha Médica 3: 179.

Peyton E L (1993) *Anopheles* (*Nyssorhynchus*) *dunhami*, ressurected from synonymy with *Anopheles nuneztovari* and validated as a senior synonym of *Anopheles trinkae* (Diptera: Culicidae). Mosq Syst 25: 151-156.

Pires H H R, Barbosa S E, Margonari C, Jurberg J, Diotaiuti L (1998) Variations of the external male genitalia in three population of *Triatoma infestans* Klug 1834. Mem Inst Oswaldo Cruz 93: 479-483.

Ruiz F, Quiñones M L, Erazo H F, Calle D A, Alzate J F, Linton Y M (2005) Molecular differentiation of *Anopheles* (*Nyssorhynchus*) *benarrochi* and *Anopheles* (*Nys*.) *oswaldoi* from southern Colombia. Mem Inst Oswaldo Cruz 100: 155-160.

Sallum M A M, Marrelli M T, Nagaki S S, Laporta G Z, Santos C L S (2008) Insights into *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae) species from Brazil. J Med Entomol 45: 970-981.

Scarpassa V M, Conn J E (2006) Molecular differentiation in natural populations of *Anopheles oswaldoi sensu lato* (Diptera: Culicidae) from the Brazilian Amazon, using sequences of the COI gene from mitochondrial DNA. Gen Mol Res 5: 493-502.

Schulmeister S (2003a) Genitalia and terminal abdominal segments of male basal Hymenoptera (Insecta): morphology and evolution. Org Divers Evol 3: 253-279.

Schulmeister S (2003b) Simultaneous analysis of basal Hymenoptera (Insecta): introducing robust-choice sensitivity analysis. Biol J Linn Soc 79: 245-275.

Sierra D M, Velez I D, Linton Y M (2004) Malaria vector *Anopheles* (*Nyssorhynchus*) *nuneztovari* comprises one genetic species based on homogeneity of nuclear ITS2 rDNA. J Med Entomol 41: 302-307.

Thompson J D, Gibson T J, Plewniak F, Jeanmougin F, Higgins D G (1997) The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876-4882.

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