Resurrection of Anopheles goeldii from synonymy with Anopheles nuneztovari (Diptera, Culicidae) and a new record for Anopheles dunhami in the Brazilian Amazon

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Nucleotide sequences of the internal transcribed spacer 2 (ITS2) rDNA and partial sequences of the cytochrome c oxidase subunit I (COI) mtDNA and white gene nDNA were obtained from specimens of Anopheles nuneztovari s.l. A. nuneztovari s.l. was obtained from specimens in Macapá (state of Amapá), Óbidos, Prainha and Almeirim (state of Pará), Itacoatiara and Parintins (state of Amazonas), Brazil, and compared with previously published sequences of A. nuneztovari A. Results of the Bayesian phylogenetic analyses performed using either COI or combined ITS2, COI and white gene sequences suggest that An. nuneztovari B/C is distinct from specimens collected in the Amazonas/Solimões River basin. Anopheles goeldii, currently in synonymy with An. nuneztovari, was described from individuals collected in Belterra (= Fordlândia) in the Tapajós River, state of Pará, Southern Amazonas River. Morphological comparisons of the characteristics of the male genitalia indicated that An. nuneztovari A and An. goeldii are similar but distinct from An. nuneztovari B/C by the apex of the aedeagus. In considering the results of the phylogenetic analyses and morphological comparisons, An. goeldii is resurrected from synonymy with An. nuneztovari. Additionally, Anopheles dunhami is reported for the first time in Parintins. This species can be distinguished from An. goeldii by characters of the male genitalia and molecular data.

Key words: Anopheles - Nyssorhynchus - ITS2 - COI - white gene - Bayesian phylogeny

Anopheles nuneztovari s.l. was recorded from western Panama to northern South America (Faran 1980). This taxon is a primary vector of human Plasmodium spp. in Venezuela and Colombia (Gabaldón et al. 1975), and in the Brazilian Amazon An. nuneztovari s.l. was reported infected with Plasmodium spp. in states of Pará (Arruda et al. 1986, Póvoa et al. 2001) and Amapá (Galardo et al. 2007). Additionally, specimens collected in Pará were found infected with Tucuruí, Caraípé and Arumateua arboviruses of the Anopheles A serogroup, Bunyavirus, Bunyaviridae (Travassos da Rosa et al. 1992).

Chromosomal, molecular, morphological and behavioral polymorphisms have been observed in An. nuneztovari, supporting the hypothesis of a species complex (Elliott 1972, Kitzmiller et al. 1973, Panday 1977, Steiner et al. 1980, Fritz et al. 1994, Hribar 1994, Linley et al. 1996, Conn et al. 1998, Tadei et al. 1998, Scarpassa et al. 1999, 2000). Conn (1990) and Conn et al. (1993) described three chromosomal cytotypes in An. nuneztovari and suggested that cytotype A occurs in Brazil, cytotype B in Western Venezuela, southeast of the Andes Mountains and cytotype C in North Colombia and Western Venezuela, northeast of the Andes Mountains. When using the white gene sequence data, Mirabello and Conn (2008) found three sympatric lineages in the Brazilian Amazon.

Hribar (1994), while comparing the male genitalia of specimens of An. nuneztovari A, B and C, suggested that the aedeagus of An. nuneztovari A was similar to that of Anopheles goeldii illustrated by Rozeboom and Gabaldón (1941) (plate III, 3). An. goeldii was described from males, females, larvae and eggs collected in Belterra (= Fordlândia), state of Pará, Brazil (Rozeboom & Gabaldón 1941). However, based on specimens obtained in Guiana, Floch and Abonnenc (1946) synonymized An. goeldii with An. nuneztovari. Later, Lane (1953) corroborated the synonymy of An. goeldii with An. nuneztovari and also included Anopheles dunhami as a synonym. Faran (1980) accepted the synonymy of An. goeldii with An. nuneztovari but also considered that the former could be a valid species. Subsequently, Gabaldón (1981) observed that An. goeldii was a valid species and reported characteristics of the fourth-instar larva and male genitalia that could distinguish both species. Specimens of An. nuneztovari could be distinguished from those of An. goeldii by having strongly sclerotized aedegal leaflets, whereas in the latter species the leaflets are weakly sclerotized (Gabaldón 1981). By examining specimens collected along the Amazonas/Solimões River basin, Bergo et al. (2007) hypothesized that An. goeldii was a valid species. These authors noted that the

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male genitalia of *An. nuneztovari* from Amapá did not agree with the illustrations reported in the published literature from Venezuela (Cojedes, type-locality) and that they are similar to those represented in the description of *An. goeldii* (plate III, 3).

In this study, we analyzed specimens collected in several localities in states of Amapá, Pará and Amazonas (Brazil) using sequence data from the rDNA second internal transcribed spacer 2 (ITS2), a fragment of the single copy nuclear *white* gene and a fragment of the cytochrome c oxidase subunit I (COI) of the mitochondrial genome. These markers were employed (i) to examine the taxonomic status of *An. nuneztovari* A that occurs along the Solimões/Amazonas River basin and (ii) to test the hypothesis that *An. goeldii* is a valid species.

**MATERIALS AND METHODS**

**Mosquito collection** - Larvae were collected in ground-water habitats and reared to the adult stage to obtain adults linked with larval and pupal exuviae and male genitalia. Vouchers consisting of larval and/or pupal exuviae and dissected male genitalia mounted in Canada balsam on microscope slides are deposited in the Entomological Collection of Faculdade de Saúde Pública, Universidade de São Paulo, Brazil (FSP-USP). Details about field collections are in Table I.

**DNA extraction** - DNA was isolated using the protocol described by Wilkerson et al. (1993), with minor modifications. Phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol extractions were conducted using Phase Lock Gel (Eppendorf®) following the manufacturer’s instructions. DNA extraction from this study is retained dry at -70°C in the FSP-USP for future reference.

**Amplification and sequencing** - ITS2 PCR products were amplified in a 10 µL reaction mix containing: 1 X PCR buffer (20 mM Tris-HCl, 10 mM [NH₄]₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X -100), 0.1 mM of each dNTP (Eppendorf®), 5 picomoles of each primer and 0.5 U of *Taq* DNA Polymerase with Thermo Pol Buffer (New England BioLabs® Inc). One µL of genomic DNA (1/800th) was used in each PCR reaction. The PCR amplification of the ITS2 region was carried out using the 5.8SF (5' - ATC ACT CGG CTC GTG GAT CG -3') and 28SR (5' - ATG CTT AAA TTT AGG GGG TAG TC-3') primers (Djadid et al. 2007). PCR amplification consisted of 2 min denaturation at 94°C, 40 cycles at 94°C, 60°C and 72°C for 30 sec, followed by a 10 min extension at 72°C. PCR products were electrophoresed in 1% TBE agarose gels stained with ethidium bromide. PCR products were purified using PEG precipitation (20% polyethylene glycol 8000/2.5 M NaCl). Two specimens were successfully directly sequenced; 18 were cloned into pGem-T Easy Vector (Promega, Madison, WI, USA). Two to four positive clones per sample, yielding a total of 62 clones, were used in sequencing reactions performed with the ITS2 primer set. For analysis, the partial sequences of the 5.8S and 28S rRNA gene regions were excluded.

COI fragments of ~ 500 base pairs were amplified using the primers C1-J-1718 (5' - GGA GGA TTT GGA AAT TGA TTA GTT CC-3') and C1-N-2191 (5' - CCC GGT AAA ATT AAA ATA TAA ACT TC-3') (Simon et al. 1994). PCR reactions were carried out in a 50-µL reaction mix containing: 1 X PCR buffer, 0.2 mM dNTP (Eppendorf®), 10 picomoles of each primer, 1 U of *Taq* DNA Polymerase with Thermo Pol Buffer (New England BioLabs® Inc) and 1 µL of DNA template (1/100th). PCR amplification protocol consisted of 2 min denaturation at 95°C, 5 cycles at 94°C for 40 sec, 37°C for 40 sec, and 72°C for 40 sec, 35 cycles at 94°C for 40 sec, 48°C for 40 sec, and 72°C for 1 min, followed by a final extension at 72°C for 7 min.

**White** gene fragments of ~ 600 bp were amplified using WZ2E (5' - AAY TAY AAY CCI GCC GAY TTY TA - 3') and WZ11X (5' - TTI ARR AAR AAI CCI CCR AA - 3') (Besansky & Fahey 1997). PCR amplicons were used as template in a second PCR using the internal primers W1F (5' - GAT CAA RAA GAT CTG YGA CTC GTT- 3') and W2R (5' - GCC ATC GAG ATG GAG GAG CTG - 3') (designed for the study by MAM Sallum). The first PCR reactions were conducted in a 10 µL reaction mix containing: 1 X PCR buffer, 0.2 mM dNTP (Eppendorf®), 10 picomoles of each primer and 0.4 U *Taq* DNA Polymerase with Thermo Pol Buffer (New England BioLabs® Inc.). One µL of genomic DNA (1/1000th) was used per PCR reaction. For the second PCR, 1 µL of the

**TABLE I**

| Specimens   | Locality   | Coordinates | Species             |
|-------------|------------|-------------|---------------------|
| AP20-104, AP15-115 | Amapá, Macapá | 0'16'17.5"N 50°53'53.3"W | *Anopheles goeldii* |
| BRAM03-01, BRAM06-04 | Amazonas, Itacoiara | 3°08'48.3"S 58°23'39.7"W |               |
| BRAM07-101, BRAM7-103 | Amazonas, Itacoiara | 2°54'56.1"S 90°02'91.7"W |               |
| BRAM12-106 | Amazonas, Parintins | 2°38'21.7"S 56°39'0.96"W |               |
| BRAM13-08 | Amazonas, Parintins | 2°38'66.2"S 56°38'29.6"W | *Anopheles dunhami* |
| BRAM14-03, BRAM14-05 | Pará, Obidos | 2°32'31.9"S 57°45'36.0"W |               |
| BRAM15-05, BRAM16-01 | Pará, Prainha | 2°04'49.3"S 53°35'27.1"W |               |
| BRAM22-01, BRAM22-101 | Pará, Almeirim | 1°28'34.1"S 52°44'35.4"W |               |
| BRAM25-01, BRAM25-07 | Amazonas, Parintins | 2°38'66.2"S 56°38'29.6"W | *Anopheles goeldii* |
| BRAM10-06, BRAM13-07, BRAM13-106, BRAM13-103 | Amazonas, Parintins | 2°38'66.2"S 56°38'29.6"W |               |
products of the first PCR was employed, using the same protocol in 25 μL. PCR amplification protocol consisted of a cycle at 94ºC for 5 min, 40 cycles at 94ºC for 30 sec, 52ºC for 30 sec, and 72ºC for 60 sec, followed by a final extension at 72ºC for 10 min. For the second PCR, the profile was similar except for the annealing temperature for which we adopted 55ºC for 30 sec.

Sequencing reactions were carried out in both directions using the PCR primers. Big Dye Terminator Kit v.3.1 (PE Applied Biosystems, Warrington, England) was employed for those specimens that were electrophoresed in an ABI Prism 3100, Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), or ABI 3130x1 Genetic Analyzer, or ABI 377 Sequencer. For those specimens processed in the MegaBACE™ DNA Analysis Systems 1000 (GE Healthcare), we used DY Enamic™ ET Terminator Kit (with Thermo Sequenase™ II DNA Polimerase). The sequences have been deposited in GenBank (COI accession numbers EU848313 - EU848336; ITS2 accession numbers EU848337 - EU848400; white gene accession numbers EU848401 - EU848416).

**Sequence alignment** - Nucleotide sequences were edited using either Chromas Lite version 2.01 (Tecneleydisum Pty Ltd. 2007) or BioEdit version 7.0.5.3 (Hall 1999). Sequences alignments were performed in BioEdit and adjusted by visual inspection using MacClade version 4.0 (Maddison & Maddison 2000). The ITS2 length was inferred using sequences from Fritz et al. (1994), but the nucleotide positions differ due to intragenomic and interspecific polymorphisms. Accuracy of nucleotide sequence alignments (COI and white gene) was examined using amino acid sequence alignment. White gene intron position was determined based on the Anopheles albimanus white gene sequence (U73839). Sequence similarities were assessed using FASTA search (http://www.ncbi.nlm.nih.gov/BLAST/).

**Sequence analysis** - Intraspecific sequence differentiation was assessed using mean uncorrected P distance in Phylogenetic Analysis Using Parsimony (PAUP) (Swofford 2003). Genetic distance under the Kimura-2-parameter model implemented in PAUP was employed to examine COI sequences. ITS2 consensus sequences from clones of the same individual were generated in BioEdit program and used for Bayesian phylogenetic analysis.

**Phylogenetic analysis** - Modeltest version 3.7 (Posada & Crandall 1998) was employed to choose a model using the Akaike Information Criterion. MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003) was employed for Bayesian phylogeny. The model was chosen for each gene separately. Constant sites were removed because they have no effect on the topology. Duplicate sequences were also removed. Combined analysis (ITS2, partial COI, and partial white gene) used a model partitioned by gene, with free partition rates. To compare An. nuneztovari A, B/C and our specimens, sequences of ITS2 and COI of An. nuneztovari from Colombia were downloaded from GenBank and designated as follows: Guaramito 1 (AY028083, AF368094), Guaramito 2 (AY028082, AF368089), Choco 1 (AY028121, AF368078), Sitronella 1 (AY028095, AF368104), Sitronella 2 (AY028094, AF368102), Tibu 1 (AY028097, AF368106) and Tibu 2 (AY028108, AF368115). As outgroups, we used sequences of An. dunhami.

**RESULTS**

**ITS2 rDNA** - From 20 specimens analyzed, only BRAM 13-106 and BRAM13-06 (both An. dunhami) were directly sequenced and showed 100% similarity. Specimens of An. dunhami had similar sequences, differing in tandem repeats regions and in a few sites that were individual-specific. The remaining samples, including two specimens of An. dunhami, showed superimposed chromatogram peaks due to intragenomic variation, especially in two GA repeats at positions 274-291 and 352-361.

In An. nuneztovari A, ITS2 length ranged from 358 to 373 bp, and in some individuals the difference among clones was 10 bp long. A large number of variants were observed for each specimen. Differences in fragment size across clones were mainly due to insertions/deletions in dinucleotide tandem repeat regions. The number of these GA repeats at positions 274-291 was neither specific to any morphological form nor associated with geographical location. Rare single base polymorphisms in otherwise highly conserved regions may have been cloning artifacts. Pairwise distance between clones, the number of clones sequenced and the variants found from each individual are shown in Table II. Due to intragenomic polymorphisms, consensus sequences from ITS2 were generated for our specimens and these were compared to sequences of An. nuneztovari s.l. from Brazil, Colombia, Venezuela, Bolivia and Suriname, published by Fritz et al. (1994) and Sierra et al. (2004), and sequences available in GenBank [U92350, U92351, U925343 (Danoff-Burg & Conn 1997, unpublished)]. No fixed ITS2 differences were observed among cytotypes A, B and C. All polymorphisms observed represent intraspecific variation because they were detected among the clones generated from a single individuals (Fig. 1). Sequence differences among An. nuneztovari A and An. dunhami are shown in Fig. 2.

**COI mtDNA** - We compared sequences of 493 bp generated for 16 individuals of An. nuneztovari A, from which 11 haplotypes were found. Under the Kimura two-parameter model, the genetic distance among the specimens of An. nuneztovari A varied from 0-0.02490 (the highest distances were detected among BRAM7-103 and BRAM13-08; BRAM16-1 and BRAM7-103/BRAM15-5; BRAM15-5 and BRAM13-08), whereas among An. nuneztovari B/C (Colombian specimens) distances ranged from 0-0.00407. By comparing the haplotypes of An. nuneztovari A with those of An. nuneztovari B/C, we observed that the genetic distance ranged from 0.00818-0.02071, the lowest distance was between BRAM22-101 and AF368102, whereas the highest was among BRAM13-08/BRAM16-01 and AF368115, AF368094 and AF368106. Genetic distances among An. nuneztovari A and An. dunhami ranged from 0.01436-0.03343, whereas among An. dunhami ranged from 0-0.01232.
Nuclear white gene - A 606-630 bp fragment of the white gene flanking the fourth intron was analyzed. Intron IV was present in all specimens and variation in this region was responsible for the length differences among sequences. Among An. nuneztovari A, four haplotypes were detected, three haplotypes were unique and the fourth haplotype was observed in the remaining 13 individuals. Sequences generated from specimens of An. dunhami showed poly C and T. In An. nuneztovari A, homopolymers were observed, but the number of repeats was lower. In these individuals, the intron region was 85-88 bp in length, whereas in An. dunhami it was 92-106 bp. Few polymorphisms were observed in exons III and IV; most were detected between An. nuneztovari A and An. dunhami. In An. nuneztovari A, substitution occurred in four positions in the intron and three in the exon regions, in which all substitution represented synonymous mutations.

Phylogenetic analyses - Because there were few informative sites, the topologies generated from a single marker showed little resolution either under the parsimony criterion (not shown) or Bayesian approach. Consequently, Bayesian analysis was conducted using the COI data partition, as it was the most informative of the data, and combined data sets (ITS2, COI and white gene). Alignment of the COI was 493 bp; of these only 27 (5.47%) sites were variable and 22 (4.46%) were parsimony informative. For Bayesian analysis, a GTR model with no among-site rate variation was used. Results of the phylogenetic analyses using COI data set showed that all specimens from Colombia are separated from specimens from the Amazonas/Solimões River basin; however, the split leading to Colombian (An. nuneztovari B/C) and Brazilian (An. nuneztovari A) clusters was only moderately supported (Fig. 3).

Bayesian phylogenetic analysis for the combined data (73 variable sites) was carried out using a partitioned model (COI: GTR, white: GTR, ITS2: JC) with no among-site heterogeneity but with free partition rates. Except for AF461749 (from Acre, Brazil), all Brazilian and Surinamese (Borokopondo) specimens clustered together in a distinct clade from Colombian (Tibu, Guaramito, Sitronella and Choco), Venezuelan (Zulia and Barinas) and Bolivian (Beni) specimens. Bayesian probabilities are shown in Fig. 4. In all analyses, An. dunhami sequences clustered together in a strongly supported group.

DISCUSSION

The ITS2 has been employed for identification of Anopheles species. However, comparisons between An. nuneztovari A from the Amazonas/Solimões River basin and published data from the literature of An. nuneztovari A, B and C revealed no fixed difference among the Brazilian and Colombian/Venezuelan populations.

Sierra et al. (2004) showed that specimens of An. nuneztovari A from Suriname and Brazil differ from those of cytotypes B/C by divergences in number of GA repeats in the second microsatellite region, and a G > T transversion at positions 356-361. We found that the number of GA repeats is variable in the populations of An. nuneztovari A from the Solimões/Amazon River basin but always fewer than five. In Colombian/Venezuelan sequences generated by Fritz et al. (1994) and Sierra et al. (2004), the number of GA repeats was always five.

| Specimens | N of clones sequenced (variants) | ITS2 length | Mean uncorrected p distance |
|-----------|---------------------------------|-------------|-----------------------------|
| AP20-104  | 4 (3)                           | 369-373     | 0                           |
| AP15-105  | 5 (3)                           | 361-369     | 0.0027-0.0081               |
| BRAM03-01 | 3 (3)                           | 361-369     | 0.0026-0.0080               |
| BRAM06-04 | 4 (2)                           | 361-369     | 0.00054                     |
| BRAM07-101| 3 (3)                           | 359-361     | 0.0027-0.0082               |
| BRAM07-103| 2 (2)                           | 369-371     | 0                           |
| BRAM12-106| 3 (3)                           | 361-371     | 0.0027-0.0080               |
| BRAM14-03 | 2 (2)                           | 369-371     | 0.00054                     |
| BRAM15-05 | 3 (3)                           | 361-369     | 0.0054-0.0081               |
| BRAM16-01 | 3 (2)                           | 361-369     | 0.00083                     |
| BRAM22-01 | 5 (5)                           | 361-371     | 0.0027-0.0138               |
| BRAM22-101| 4 (4)                           | 361-369     | 0.00054                     |
| BRAM25-01 | 5 (2)                           | 361-371     | 0.00054                     |
| BRAM25-07 | 3 (3)                           | 358-360     | 0.00055                     |
| BRAM13-113| 4 (3)                           | 358-360     | 0.0028-0.0055               |
| BRAM13-07 | 3 (3)                           | 358-360     | 0.00054                     |
| BRAM13-08 | 3 (2)                           | 358-360     | 0.00054                     |
| BRAM13-06 | Directly sequenced              | 360         | -                           |
| BRAM13-106|                                  | 360         | -                           |
Fig. 1: partial internal transcribed spacer 2 sequence alignment of specimens of *Anopheles goeldii* from Brazil and Surinam, and *Anopheles nuneztovari* from Bolivia, Colombia and Venezuela. FBeni, FBrokopondo, FPuraquequara 1, FPuraquequara 2 and FCapanema sequences were retrieved from Fritz et al. (1994); AY028121, AY028125, AF461749, U92343, U92350 and U92351 were downloaded from GenBank.

Fig. 2: alignment of the complete internal transcribed spacer 2 (ITS2) sequences of *Anopheles dunhami*, *Anopheles goeldii* from Brazil, and *Anopheles nuneztovari* from Colombia and Venezuela. Lounibos et al. (1998), whereas GenBank is the sequence available in GenBank under accession U92326. BRAM1306, BRAM13-07, BRAM13106 and BRAM13113 are ITS2 sequences of *An. dunhami* generated in this study; L22462 and AY028095 are ITS2 sequences of *An. nuneztovari B/C* and AP20-104 of *A. goeldii*. 
In our analyses, the G > T transversion was both variable among clones obtained from a single individual and found in 12 out of 16 individuals of *An. nuneztovari*. Additionally, several clones showed a polymorphic A insertion after the G > T transversion in specimens with 3 GAs. The A insertion was absent in specimens from Suriname and Brazil published by Fritz et al. (1994), but was observed by Onyabe and Conn (1999) in other Brazilian populations. These authors also showed that T (transversion) + A (insertion) is more frequent in specimens from states of Pará and Amazonas than in those from state of Rondônia (Brazil). In fact, T + A was frequent in our clones, and it is possible that the Rondônia population studied by Onyabe and Conn (2008) represents a distinct lineage as suggested by Mirabello and Conn (2008). Although our results suggest that the ITS2 is not a good marker for separating *An. nuneztovari* cytotypes A, B and C, mainly due to intragenomic polymorphisms, differences in heterogeneity can be indicative of distinct cryptic species (Onyabe & Conn 1999). Intragenomic polymorphisms were reported for *An. nuneztovari* by Fritz et al. (1994) in individuals from Boa Vista, state of Roraima, and Puraquequara, state of Amazonas (Brazil), and Suriname. Later, Onyabe and Conn (1999) observed less intragenomic heterogeneity in Colombian/Venezuelan specimens than in Brazilian populations.

Fritz et al. (1994) observed that specimens from Colombia and both sides of the Andes in Venezuela had identical ITS2 sequences. Similarly, Sierra et al. (2004) observed that two of 46 specimens from Colombia varied in one bp and suggested that *An. nuneztovari* cytotypes B and C were conspecific. Genetic similarities between cytotypes B and C were corroborated by random amplified DNA analysis (PCR-RAPD) of populations from Western and Northeastern Colombia (Posso et al. 2003). Moreover, Onyabe and Conn (1999) observed differences among Colombian and Brazilian populations that could be indicative of two or more species. Our results corroborate Onyabe and Conn (1999) hypothesis that the Brazilian populations are more heterogeneous than those...
from Venezuela and Colombia and thus may represent a different species.

Results of the phylogenetic analyses using the COI data set indicated that *An. nuneztovari* A is a different species than *An. nuneztovari* B/C. Similar results were obtained in the analyses using the combined data, where all Brazilian specimens, except for AF46174, clustered separately from Colombian and Venezuela specimens, indicating that *An. nuneztovari* A is not conspecific with B/C.

Morphological polymorphisms in male genitalia have been reported for *An. nuneztovari* s.l. and have raised questions about the taxonomic status of some morphological forms, especially those from Brazil and Venezuela (Fig. 5A-E). In considering the description of the *An. nuneztovari* holotype by Savage (1986) and the illustration of this species by Gabaldón (1940), we observed that the male genitalia of specimens of *An. nuneztovari* A collected in the Amazonas/Solimões River basin (Fig. 5A) differ from those of *An. nuneztovari* s.s. (Fig. 5D) in the characteristics of the apex of the aedeagus, but are similar to those of *An. goeldii* (Fig. 5C). By comparing genitalia from our specimens with the holotype and one paratype of *An. goeldii*, we observed that *An. nuneztovari* A is morphologically more similar to the paratype (Fig. 5C) than to the holotype (Fig. 5B). These differences in the aedeagal apex of the holotype and paratype may represent morphological polymorphism within *An. goeldii*, not representative of distinct species as suggested by Bergo et al. (2007). Considering the differences observed among DNA sequences and based on characters observed in the male genitalia, we hereby propose the resurrection of *An. goeldii* from synonymy with *An. nuneztovari*.

Specimen BRAM13-113 was identified as *An. nuneztovari* by Bergo et al. (2007) based on male genitalia and pupal characteristics; however, ITS2, COI and white gene sequences indicate that this is a specimen of *An. dunhami*. Only two ITS2 sequences of *An. dunhami* are available in GenBank, AF462378 and U92326. By comparing AF462378 with the consensus sequences generated in this study, we observed that they share 83-85% similarity, suggesting that they were generated from an individual of a distinct species (Marrelli et al. 2006). When comparing the U92326 ITS2 sequence with that published by Lounibos et al. (1998), we found that they are distinct but share the same GenBank accession number. The major disagreement between the sequences is represented by a 14 bp insertion (TACCGAAGCAACG) at

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**Fig. 5:** details of male genitalia. A: *Anopheles nuneztovari* (Venezuela, image preparation #1353 WRBU); B: *Anopheles goeldii* (holotype); C: *An. goeldii* (paratype, image preparation #1356 WRBU); D: *An. goeldii* (Amapá, Brazil); E: *Anopheles dunhami* (lectotype number #58031WRBU).
positions 305-318 in U92326. We assume it is most likely a sequencing error. Other polymorphisms among our sequences and U92326 (Lounibos et al. 1998) are shown in Fig. 2 and may represent either individual variation or a cloning artifact. In considering male genitalia characters, the aedeagal leaflets are absent and the apex is somewhat rounded in specimens BRAM13-06, BRAM13-106 and BRAM13-07, whereas in BRAM13-113 (Bergo et al. 2007) (Fig. 1C, D) the leaflets are short, poorly sclerotized, and the apex of the aedeagus is somewhat conical. The results of this study indicate that the male genitalia of An. dunhami exhibit morphological polymorphisms that should be taken into consideration for species identification. An. dunhami was reported in Tefé and Tabatinga (5º47'22S 65º24'32W, Amazonas, Brazil) (Lounibos et al. 1998), both localities in the Solimões River basin. This is the first record of An. dunhami from Parintins municipality along the Amazonas River. The influence of vector behaviour on malaria transmission and consequently mixture of information about genetics, vector competence, biting behavioral and distribution of these taxa.

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REFERENCES

Arruda M, Carvalho MB, Nussenzeig RS, Ferreira AW, Cochrane AH 1986. Potential vectors of malaria and their different susceptibility to Plasmodium falciparum and Plasmodium vivax in Northern Brazil identified by immunoassay. Am J trop Med Hyg 35: 873-881.

Bergo ES, Souto RNP, Galardo AKR, Nagaki SS, Calado DC, Salum MAM 2007. Systematic notes on Anopheles Meigen (Diptera: Culicidae) species in the state of Amapá, Brazil. Mem Inst Oswaldo Cruz 102: 373-376.

Besansky NJ, Fahey GT 1997. Utility of the white gene in estimating phylogenetic relationships among mosquitoes. Mol Biol Evol 14: 442-454.

Conn JE 1990. A genetic study of the malaria vector Anopheles nuneztovari from Western Venezuela and Colombia. J Am Mosq Control Assoc 6: 400-405.

Conn JE, Mitchell SE, Cockburn AF 1998. Mitochondrial DNA analysis of the neotropical malaria vector Anopheles nuneztovari. Genome 41: 313-327.

Conn JE, Puertas YR, Seawright J 1993. A new cytotype of Anopheles nuneztovari from Western Venezuela and Colombia. J Am Mosq Control Assoc 9: 294-301.

Djadid ND, Ghelizadeh S, Tafsiri E, Romi R, Gorodeev M, Zakeri S 2007. Molecular identification of palearctic members of Anopheles maculipennis in Northern Iran. Malar J 17: 6.

Elliott R 1972. The influence of vector behaviour on malaria transmission. Am J Trop Med Hyg 21: 755-763.

Faran ME 1980. Mosquito studies (Diptera, Culicidae) XXXIV. A revision of the Albimanus section of the subgenus Nyssorhynchus of Anopheles. Contrib Amer Entomol Inst 15: 1-215.

Floch H, Abonnenc E 1946. Sur A. nunez-tovari et A. pessoi aus Guyane Francaise. Table d’identification des Nyssorhynchus guyanais. Inst Pasteur Guyane Territ Inini Publ 126: 1-5.

Fritz GN, Conn J, Cockburn AF, Seawright JA 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of Anopheles nuneztovari (Diptera: Culicidae). Mol Biol Evol 11: 406-416.

Gabaldón A 1940. Estudios sobre anofelinos. Serie I. 1. Descripción de Anopheles (Nyssorhynchus) nunez-tovari n. sp. y consideraciones sobre una subdivisión del grupo Nyssorhynchus (Diptera: Culicidae). Pabl Div Malar 5: 3-7.

Gabaldón A 1981. Anopheles nuñez-tovari: importante vector y agente de malaria refractaria en Venezuela. Bol Dir Malaria San Amb 21: 28-38.

Gabaldón A, Martin GG, Sifontes R 1975. Necesidades en el campo de la investigacion del Programa Nacional de Eradicacion y Control de la Malaria de Venezuela. Bol Dir Malarial San Amb 15: 263-285.

Galardo AKR, Arruda M, Couto AARA, Wirtz R, Lounibos P, Zimmermann R 2007. Malaria vector incrimination in three rural riverine villages in the Brazilian Amazon. Am J Trop Med Hyg 76: 461-469.

Hall TA 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95-98.

Hribar L 1994. Geographical variation of male genitalia of Anopheles nuneztovari Gabaldón. Mosq Syst 26: 132-144.

Kitzmiller JB, Kreutzer RD, Tallarfero E 1973. Chromosomal differences in populations of Anopheles nuneztovari. Bull WHO 48: 435-445.

Lane J 1953. Neotropical Culicidae, Vol. 1, Editora da Universidade de São Paulo, São Paulo, 548 pp.

Linley JR, Lounibos LP, Conn JE, Duzak D, Nishimura N 1996. A description and morphometric comparison of eggs from eight geographic populations of the South American malaria vector Anopheles (Nyssorhynchus) nuneztovari. J Am Mosq Control Assoc 12: 275-292.

Lounibos LP, Wilkerson RC, Conn JE, Hribar LJ, Fritz GN, Danoff-Burg JA 1998. Molecular, morphological and chromosomal discrimination of cryptic Anopheles (Nyssorhynchus) (Diptera: Culicidae) from South America. J Med Entomol 35: 830-838.

Maddison WP, Maddison DR 2000. MacClade: analysis of phylogeny and character evolution, Version 4.0., Sinauer, Sunderland, MA.

Marrelli MT, Sallum MA, Mariniot O 2006. The second internal transcribed spacer of nuclear ribosomal DNA as a tool for Latin American anopheline taxonomy: a critical review. Mem Inst Oswaldo Cruz 101: 817-832.

Mirabello L, Conn JE 2008. Population analysis using the nuclear white gene detects Pliocene/Pleistocene lineage divergence within in Anopheles nuneztovari in South America. Med Vet Entomol 22: 109-119.

Onyabe DY, Conn JE 1999. Intragenomic heterogeneity of a ribosomal DNA spacer (ITS2) varies regionally in the neotropical malaria vector Anopheles nuneztovari (Diptera: Culicidae). Insect Mol Biol 8: 435-442.

Panday RS 1977. A. nuneztovari and malaria transmission in Surname. Mosq News 37: 728-737.
Peyton EL 1993. *Anopheles (Nyssorhynchus) dunhami*, resurrected from synonymy with *Anopheles nuneztovari* and validated as a senior synonym of *Anopheles trinkae* (Diptera: Culicidae). *Mosq Syst* 25: 151-156.

Posada D, Crandall KA 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14: 817-818.

Posso CE, González R, Cárdenas H, Gallego G, Duque MC, Suarez MF 2003. Random Amplified Polymorphic DNA Analysis of *Anopheles nuneztovari* (Diptera: Culicidae) from Western and Northeastern Colombia. *Mem Inst Oswaldo Cruz* 98: 469-476.

Póvoa MM, Wirtz RA, Lacerda RNL, Miles MA, Warhurst D 2001. Malaria Vectors in the municipality of Serra do Navio, state of Amapá, Amazon Region, Brazil. *Mem Inst Oswaldo Cruz* 96: 179-184.

Ronquist F, Huelsenbeck JP 2003. *Mr Bayes 3*: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-1574.

Rozeboom LE, Gabaldón A 1941. A summary of the “tarsimaculatus” complex of *Anopheles* (Diptera: Culicidae). *Am J Hyg* 33: 88-100.

Savage HM 1986. Identification and location of the holotype and paratypes of *Anopheles (Nyssorhynchus) nuneztovari* Gabaldón (Diptera: Culicidae). *Mosq Syst* 18: 279-283.

Scarpassa VM, Tadei WP, Surez MF 1999. Population structure and genetic divergence in *Anopheles nuneztovari* (Diptera: Culicidae) from Brazil and Colombia. *Am J Trop Med Hyg* 60: 1010-1018.

Scarpassa VM, Geurgas S, Azeredo-Espin AML, Tadei WP 2000. Genetic divergence in mitochondrial DNA of *Anopheles nuneztovari* (Diptera: Culicidae) from Brazil and Colombia. *Genet Mol Biol* 23: 71-78.

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

Simon FF, Beckenbach A, Crespi B, Liu H, Flook P 1994. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann Entomol Soc Am* 87: 651-701.

Steiner WWM, Kitzmiller JB, Osterbur DL 1980. Gene differentiation in chromosome races of *Anopheles nuneztovari* Gabaldón. *Mosquito Syst* 12: 306-319.

Swoford DL 2003. PAUP*. *Phylogenetic Analysis Using Parsimony (and other methods), Version 4.01b*, Sinauer Associates, Sunderland, MA.

Tadei WP, Thatcher BD, Santos JMM, Scarpassa VM, Rodrigues IB, Rafael MS 1998. Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. *Am J Trop Med Hyg* 59: 325-335.

Technelysium Pty Ltd. 2007. *Chromas lite version 2.01*. Available from: http://www.technelysium.com.au/chromas_lite.html.

Travassos da Rosa JFS, Travassos da Rosa APA, Dégallier N, Vasconcelos PF da C 1992. Caracterização e relacionamento antigênico de três novos Bunyavirus no grupo *Anopheles A* (Bunyaviridae) dos arbovírus. *Rev Saude Publ* 26: 173-178.

Willerson RC, Parsons TJ, Albright DG, Klein TA, Braun MJ 1993. Random amplified polymorphic DNA (RAPD) markers readily distinguish cryptic mosquito species (Diptera: Culicidae: *Anopheles*). *Insect Mol Biol* 1: 205-211.