

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 A collected 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 s.l. 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 obtained 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, Caraipé 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, north-east 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 aedeagal 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

Financial support: FAPESP (Grant 05/53973-0, to MAMS), CNPq (Grant 472485/2006-7, to MAMS), NIH AI RO154139 (to Jan E Conn).

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Received 5 July 2008

Accepted 19 November 2008

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. Template DNA 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 25 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 ThermoPol 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 GCI 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
Specimen codes, localities, geographical coordinates and species studied

Specimens	Locality	Coordinates	Species
AP20-104, AP15-115	Amapá, Macapá	0°16'17.5''N 50°53'53.3''W	<i>Anopheles goeldii</i>
BRAM03-01, BRAM06-04	Amazonas, Itacotiara	3°08'48.3''S 58°23'39.7''W	
BRAM7-101, BRAM7-103	Amazonas, Itacotiara	2°54'56.1''S 59°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	
BRAM14-03, BRAM14-05,			
BRAM15-05, BRAM16-01	Pará, Óbidos	2°32'31.9''S 57°45'36.0''W	
BRAM22-01, BRAM22-101	Pará, Prainha	2°04'49.3''S 53°35'27.1''W	
BRAM25-01, BRAM25-07	Pará, Almeirim	1°28'34.1''S 52°44'35.4''W	
BRAM13-06, BRAM13-07,			
BRAM13-106, BRAM13-103	Amazonas, Parintins	2°38'66.2''S 56°38'29.6''W	<i>Anopheles dunhami</i>

products of the first PCR was employed, using the same protocol in 25 μ L. PCR amplification protocol consisted of a cycle at 94°C for 5 min, 40 cycles at 94°C for 30 sec, 52°C for 30 sec, and 72°C for 60 sec, followed by a final extension at 72°C for 10 min. For the second PCR, the profile was similar except for the annealing temperature for which we adopted 55°C 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 3130xl Genetic Analyzer, or ABI 377 Sequencer. For those specimens processed in the MegaBACE™ DNA Analysis Systems 1000 (GE Healthcare), we used DYEnamic™ ET Terminator Kit (with Thermo Sequenase™ II DNA Polymerase). 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 (Tecnelysium 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), Sitronel-

la 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.

TABLE II
Sequence variability of ITS2 among clones from the same individual

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)	361-371	0.0026-0.0080
BRAM06-04	4 (2)	361-369	0-0.0054
BRAM07-101	3 (3)	361	0.0027-0.0083
BRAM07-103	3 (3)	359-361	0.0027-0.0082
BRAM12-106	2 (2)	369-371	0
BRAM14-03	3 (3)	361-371	0.0027-0.0080
BRAM14-05	2 (2)	369	0.0081
BRAM15-05	3 (3)	361-369	0.0054-0.0081
BRAM16-01	3 (2)	361-369	0-0.0083
BRAM22-01	5 (5)	361-371	0.0027-0.0138
BRAM22-101	4 (4)	361-369	0-0.0054
BRAM25-01	5 (2)	369	0-0.0027
BRAM25-07	3 (3)	369-371	0-0.0054
BRAM13-113	4 (3)	358-360	0-0.0055
BRAM13-07	3 (3)	358-360	0.0028-0.0055
BRAM13-08	3 (2)	361-369	0-0.0054
BRAM13-06	Directly sequenced	360	-
BRAM13-106		360	-

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 (Borokoponde) 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.

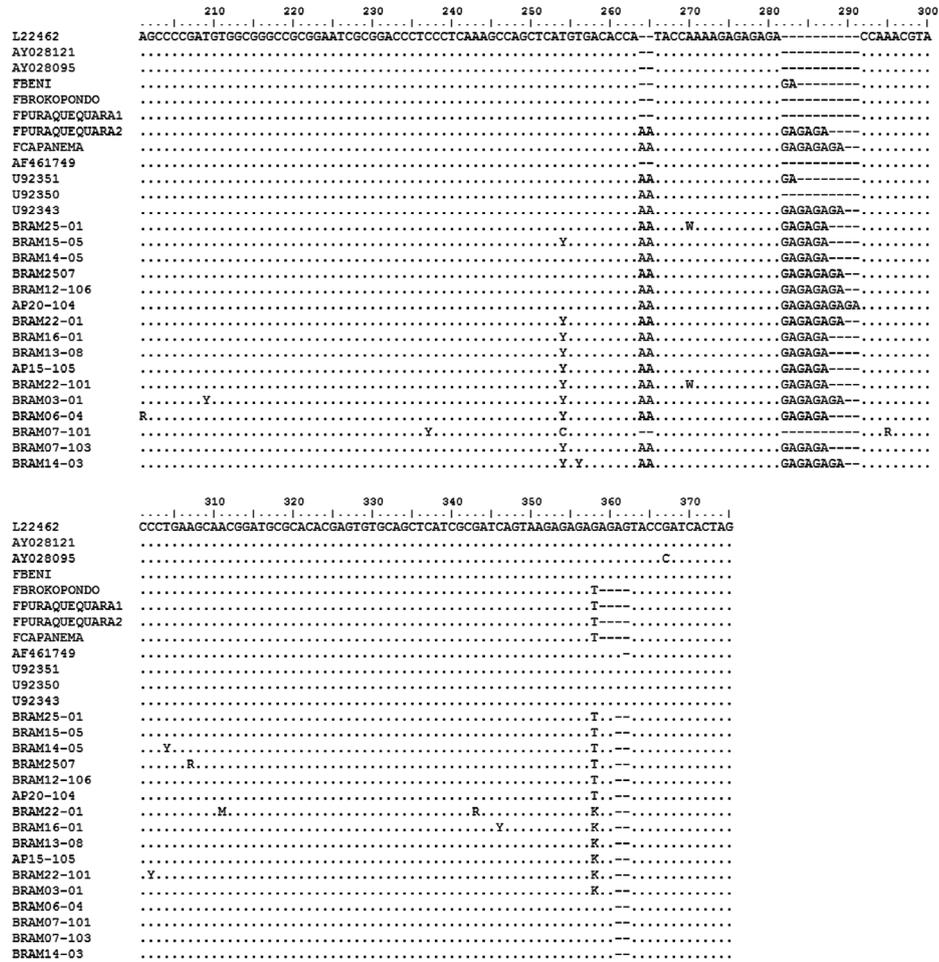


Fig. 1: partial internal transcribed spacer 2 sequence alignment of specimens of *Anopheles goeldii* from Brazil and Surinam, and *Anopheles nuneztovari* from Bolivia, Colombia and Venezuela. FBeni, FBrokoPondo, FPuraquequara 1, FPuraquequara 2 and FCapanema sequences were retrieved from Fritz et al. (1994); AY028121, AY028125, AF461749, U92343, U92350 and U92351 were downloaded from GenBank.

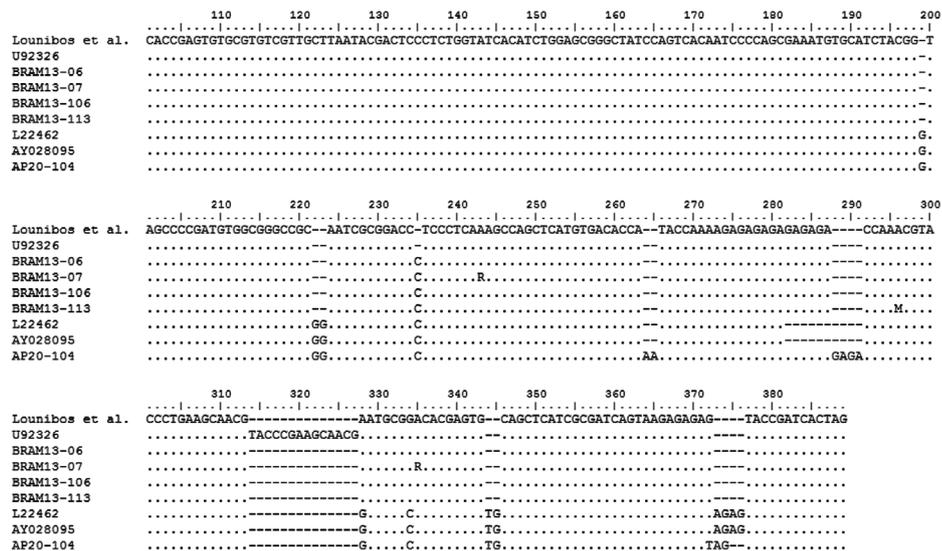


Fig. 2: alignment of the complete internal transcribed spacer 2 (ITS2) sequences of *Anopheles dunhami*, *Anopheles goeldii* from Brazil, and *Anopheles nuneztovari* from Colombia and Venezuela. Lounibos et al. corresponds to ITS2 sequence of *An. dunhami* presented by Lounibos et al. (1998), whereas 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 *An. goeldii*.

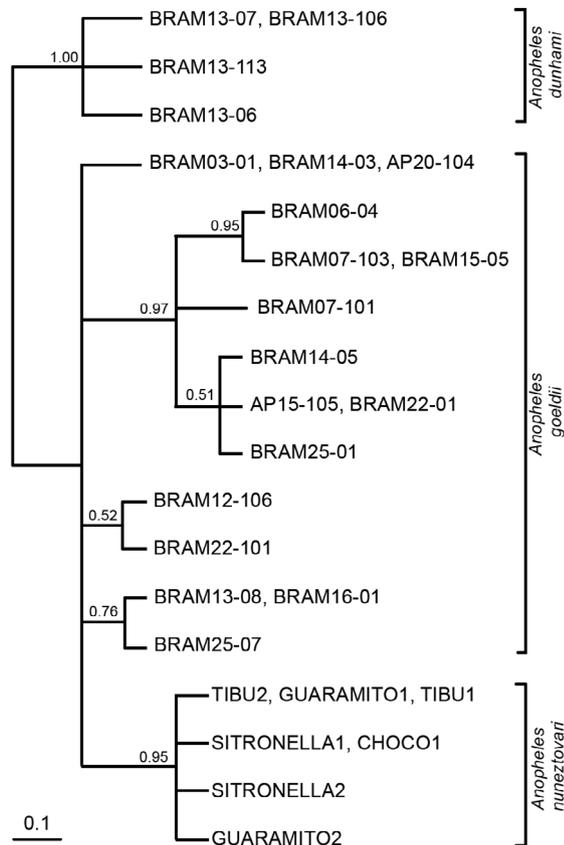


Fig. 3: bayesian topology generated under the GTR model employing the partial cytochrome c oxidase subunit I sequences of *An. dunhami*, *An. goeldii* from Brazil and *An. nuneztovari* from Colombia and Venezuela. Numbers on each branch represent posterior probabilities.

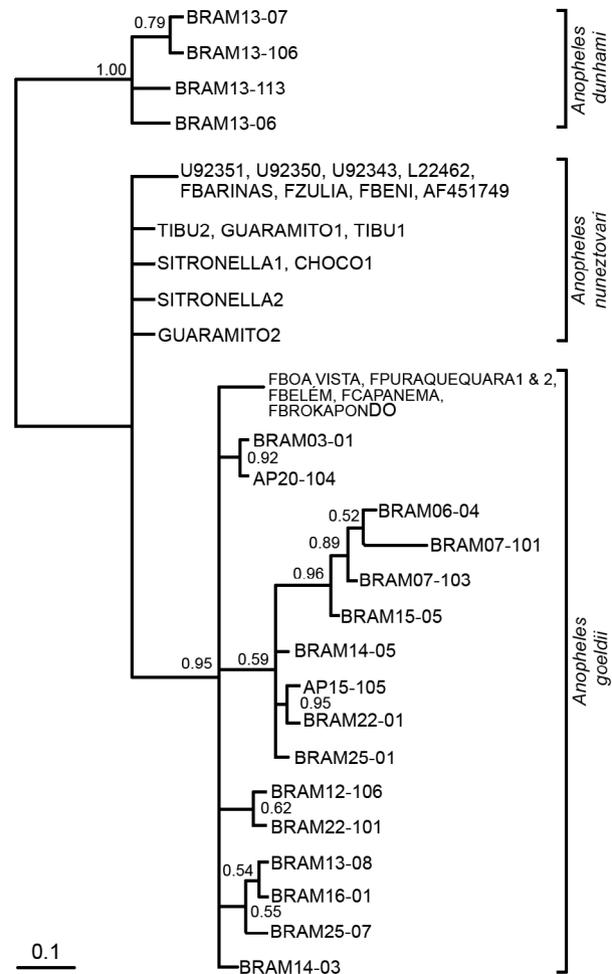


Fig. 4: bayesian topology generated under the GTR model employing the combined internal transcribed spacer 2, partial cytochrome c oxidase subunit I and *white* gene sequence data of *An. dunhami*, *An. goeldii* from Brazil and Surinam, and *An. nuneztovari* from Bolivia, Colombia and Venezuela. Numbers on each branch represent posterior probabilities.

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* A. 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 (1999) represents a distinct lineage as suggested by Mirabello and Conn (2008). Although our results show 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 (TACCCGAAGCAACG) at

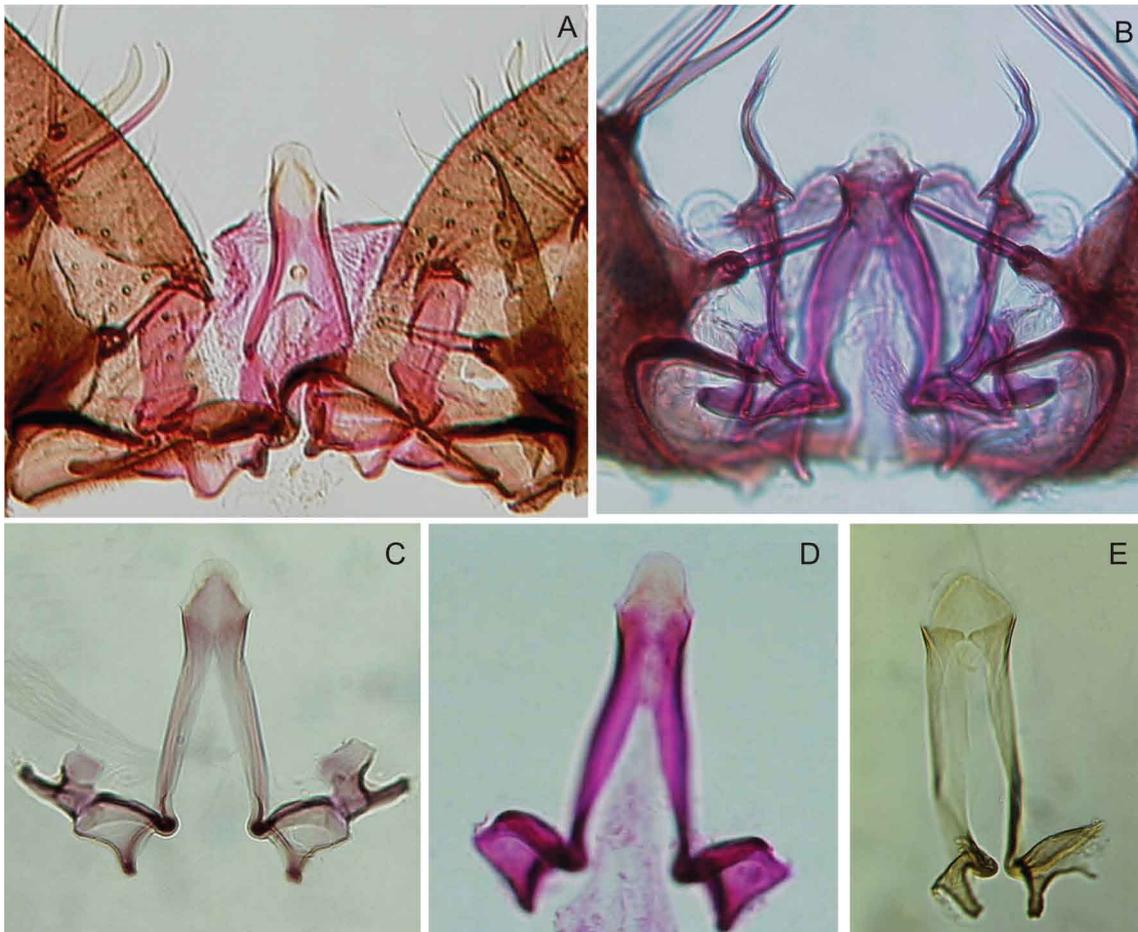


Fig. 5: details of male genitalia. A: *Anopheles nuneztovari* (Venezuela, image preparation #1353 WRBU); B: *Anopheles goeldii* (holotype); C: *An. goeldii* (paratype, image preparation #1356 WRBU); D: *An. goeldii* (Amapá, Brazil); E: *Anopheles dunhami* (lectotype number #58031WRBU).

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 (5°47'22S 65°24'32W, 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. In the adult stage, *An. nuneztovari* and *An. dunhami* can be distinguished by the shape of the apical part of the aedeagus (Peyton 1993), but it remains difficult to identify adult females. Morphological similarities between these species can be responsible for misidentifications and consequently mixture of information about genetics, vector competence, biting behavioral and distribution of these taxa.

ACKNOWLEDGEMENTS

To Richard C Wilkerson and J Stoffer (WRBU, USA), for the microphotos of the male genitalia of the holotype of *An. goeldii*, paratype of *An. goeldii*, lectotype of *An. dunhami* and non-type of *An. nuneztovari*, and to Henry Rupp kindly, reviewed the English.

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