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Amazonian malaria vector anopheline relationships interpreted from ITS2 rDNA sequences

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Abstract. Species identification of anopheline mosquitoes (Diptera: Culicidae) can be problematic because many of them belong to complexes of morphologically similar species, often with contrasted ecology, behaviour and vectorial importance. The application of DNA-based diagnostics has proved to be useful for distinguishing between such species. We determined ribosomal DNA sequences of the second internal transcribed spacer (ITS2) from samples of 16 species of *Anopheles* captured in the Amazon Basin, Brazil. Length of the ITS2 varied from 323 to 410 base pairs, with GC content ranging from 50.7% to 66.5% and sequence identity from 25% to 99% between species. Maximum-likelihood PAUP analysis separated two distinct groups of species conforming with the recognized subgenera *Anopheles* (represented by *eiseni*, *mattogrossensis*, *mediopunctatus* and *peryassui*) and *Nyssorhynchus* (represented by 12 spp.). For the latter group, the neighbour-joining tree generated from rDNA sequence ITS2 relationships is compatible with the morphological taxonomic key established for these Amazonian species: *albitarsis*, *aquasalis*, *benarrochi*, *braziliensis*, *darlingi*, *deaneorum*, *dunhami*, *evansae*, *nuneztovari*, *oswaldoi*, *rangeli* and *triannulatus*. These ITS2 sequence data proved to be a useful tool for species identification and, potentially, to solve taxonomic problems.

Key words. *Anopheles*, *Nyssorhynchus*, ITS2, malaria vectors, molecular diagnostics, ribosomal DNA, second internal transcribed spacer, species identification, Amazon, Brazil.

Introduction

Malaria remains endemic throughout tropical Africa and many parts of Asia, central and South America, transmitted by anopheline mosquitoes. In Brazil, nearly all the malaria transmission (> 99%) occurs in the Amazon Basin inhabited by almost 20 million people, representing approximately 12% of the Brazilian population. The incidence of malaria in Brazil has increased during recent years, with > 630 000 reported cases in 1999. Among the problems making it difficult to control malaria transmission in the Amazonian region are massive human migration and agricultural development, parasite resistance to antimalaria drugs, changes in behaviour of malaria vector anopheline mosquitoes and insecticide resistance (Singer & de Castro, 2001).

About 60 species of *Anopheles* are known to occur in Brazil; they are classified in five subgenera: *Stethomyia*,

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Nyssorhynchus, *Lophopodomyia*, *Kerteszia* and *Anopheles sensu stricto* (Causey *et al*., 1946; Faran & Linthicum, 1981; Sallum *et al*., 1999; Wilkerson & Sallum, 1999). Among more than 30 species of anophelines in Amazonia, *An. darlingi* is generally regarded as the main malaria vector (Deane, 1986), while an increasing number of other *Anopheles* species have been implicated as Amazonian vectors of *Plasmodium falciparum*, *P. vivax* and *P. malariae* causing human malaria (Lounibos & Conn, 2000; Tadei & Dutary-Thatcher, 2000; Conn *et al*., 2002; Fritz *et al*., 2004). Therefore, it is necessary to develop accurate and reliable methods for the identification of neotropical malaria vectors.

Whereas morphological characters are often satisfactory for mosquito species identification, the genus *Anopheles* includes many complexes of isomorphic or similar sibling species (Hackett, 1939; Davidson, 1964; White, 1978, 1979; Peyton, 1990; Reinert *et al*., 1997; Rosa-Freitas *et al*., 1998; Schmidt *et al*., 2001; Coluzzi *et al*., 2002) and this may result in imprecise morphological identification of captured mosquitoes. For most of these cases, the problem of vector identification has been solved by the application of DNA-based methods. Frequently mitochondrial and ribosomal DNA sequences provide useful taxonomic and phylogenetic information (Walton *et al*., 1999; Sallum *et al*., 2002). The internal transcribed spacers, ITS1 and ITS2, (Fedoroff, 1979) of rDNA genes have proved to be useful for differentiating between closely related species of mosquitoes (Paskewitz & Collins, 1990; Porter & Collins, 1991; Paskewitz *et al*., 1993a; Crabtree *et al*., 1995; Collins & Paskewitz, 1996; Miller *et al*., 1996; Severini *et al*., 1996; Lounibos *et al*., 1998; Marinucci *et al*., 1999; Hackett *et al*., 2000; Manonmari *et al*., 2001; Garros *et al*., 2004; Wilkerson *et al*., 2004). In addition, the ITS2 sequences can help to resolve phylogenetic relationships at different taxonomic levels, including recently diverged taxa, such as sibling species of mosquitoes (Collins & Paskewitz, 1996; Xu & Qu, 1997; Walton *et al*., 1999).

In this study of ribosomal DNA we determined and compared nucleotide sequences of the ITS2 region of 16 neotropical anopheline species, including several known malaria vectors from the Amazon Region. The sequence data obtained are useful for species identification and, potentially, to solve taxonomic problems among such mosquitoes.

Materials and methods

Mosquitoes

Table 1 lists the anopheline species and origins of samples studied. Adult mosquitoes were collected during July – August 1998 in localities of the States of Acre (Plácido de Castro, 10°20′ S, 67°11′ W) and Rondônia (São Miguel, 8°49′ S, 63°54′ W), Western Amazon Region of Brazil. Most of the mosquito species were captured in Shannon traps and some of them were aspirated from human baits (*An. darlingi* and *An. albitarsis* s.l). Material of *An. aquasalis* came from the FIOCRUZ colony maintained in the State of Rio de Janeiro. Only adult female specimens were used for analysis, primarily identified morphologically by C.F.M. according to keys and descriptions by Faran (1980), Faran & Linthicum (1981), Rosa-Freitas (1989) and/or Consoli & Lourenço-de-Oliveria (1994), and then immediately immersed in isopropanol. Two species (*An. evansae* and *An. aquasalis*) were provided and identified by researchers indicated in the acknowledgements. Field-collected specimens were taken to the Laboratory of Insect Vectors, Department of Parasitology, University of São Paulo and stored at room temperature until DNA extraction. As far as possible, two samples (different field collections) of each

Table 1. Anopheline species analysed, abbreviations used and their population origin.

Genus (subgenus) species	Abbreviations	Samples	Origin	Material
Anopheles (*Anopheles*) *eiseni* Coquillett	eise	1	Rondônia	field
Anopheles (*Anopheles*) *mattogrossensis* Lutz & Neiva	matto	2	Rondônia	field
Anopheles (*Anopheles*) *mediopunctatus* Theobald s.l.	medio	1	Rondônia	field
Anopheles (*Anopheles*) *peryassui* Dyar & Knab	pery	2	Rondônia	field
Anopheles (*Nyssorynchus*) *albitarsis* Lynch-Arribalzaga s.l.	albi-1	1	Acre	field
	albi-2 & 3	2	Rondônia	field
Anopheles (*Nyssorynchus*) *aquasalis* Curry	aqua	2	Rio de Janeiro	colony, adult
Anopheles (*Nyssorynchus*) *benarrochi* Gabaldon, Cova Garcia & Lopes	bena-1 & 2	2	Rondônia	field
Anopheles (*Nyssorynchus*) *braziliensis* Chaga	braz	1	Rondônia	field
Anopheles (*Nyssorynchus*) *darlingi* Root	darl-1	1	Acre	reared, F1
	darl-2	1	Rondônia	reared, F1
Anopheles (*Nyssorynchus*) *deaneorum* Rosa-Freitas	dean-1 & 2	2	Acre	field
Anopheles (*Nyssorynchus*) *dunhami* Causey	dunh	1	Acre	field
Anopheles (*Nyssorynchus*) *evansae* Brethes	evan	2	Rondônia	field
Anopheles (*Nyssorynchus*) *nuneztovari* Gabaldon	nunez	1	Acre	field
Anopheles (*Nyssorynchus*) *oswaldoi* Peryassu	osw-1	1	Acre	reared, F1
	osw-2	1	Rondônia	field
Anopheles (*Nyssorynchus*) *rangeli* Gabaldon, Cova Garcia & Lopes	rang	2	Acre	field
Anopheles (*Nyssorynchus*) *triannulatus* Neiva & Pinto	trian	2	Rondônia	field

species were processed, but for several scarce species we obtained only one sample. The selection of *Anopheles* species was based on their geographical distribution, predominance and their importance as vectors of human malaria.

DNA extraction

Genomic DNA was extracted from individual mosquitoes according to the procedure of Malafronte *et al.* (1999). They were homogenized in 50 µL of lysis buffer (100mMTris-HCl, pH 7.5; 100 mM NaCl; 100 mM EDTA; 1% SDS; 1 mg/mL proteinase K) and incubated for 1 h at 60°C. Genomic DNA was extracted with phenol (2×) phenol/chloroform (1×) chloroform/isoamylic (1×) and precipitated by cold ethanol overnight. After centrifugation (5 min, 12 000 g), the pellet was dissolved in 1 mL of TE containing 10 mg/mL RNAse. The DNA was precipitated a second time by addition of 20% (v/v) of sodium acetate 3 M and 1 mL of isopropanol. The suspension was kept for 15 min at room temperature and centrifuged (30 min, 12 000 g). The pellet was washed with 1 mL of 70% ethanol, dried, dissolved in 20 µL of distilled water and stored at −20°C.

ITS2 amplification, cloning and sequencing

The rDNA ITS2 regions were amplified from genomic DNA samples following the protocol described by Porter & Collins (1991), with some modifications, and using the following primers:

CP16 (5′–GCGGGTACCATGCTTAAATTTAGGG-GGTA–3′)
and
CP17 (5′–GCGCCGCGGTGTGAACTGCAGGACA-CATG–3′)

The PCR reactions were performed for 25 cycles (94°C for 1 min, 50°C for 2 min and 72°C for 2 min) and the products were visualized by ethidium bromide staining after electrophoresis in a 1.2% agarose gel.

PCR products containing the ITS2 and part of the flanking 5.8 S and 28 S genes were cloned into pGEM-T easy vector plasmid (Promega, Madison, WI) vector and used to transform *E. coli* strain Dh5α. The cloned fragments were sequenced in both forward and reverse direction using ABI Prism dGTP BigDye Terminator Ready Reaction Kit (Perkin Elmer, Foster City, CA). Four or five clones from each mosquito were sequenced.

DNA sequence analysis

The sequences were aligned initially using the CLUSTAL W (1.60) (Thompson *et al.*, 1994). Obvious misaligned nucleotides were manually adjusted and the boundaries of the ITS2 were deduced by comparisons with rDNA sequences of other anophelines species (Porter & Collins, 1991; Fritz *et al.*, 1994; Beebe *et al.*, 1999). Only the ITS2 sequences were used for determination of guanine-cytosine (CG) content and phylogenetic analyses. Aligned sequences were examined with the program MEGA (Molecular Evolutionary Genetics Analysis, version 1.01) (Kumar *et al.*, 1993) and a similarity matrix was constructed. Relationships between individuals were assessed by neighbour-joining (NJ) method with nucleotides distances (p-distance) with 500 replications in the bootstrap test. The tree was rooted using the ITS2 sequence of the culicine mosquito *Aedes aegypti* (Linnaeus) from Wesson *et al.* (1992) as the outgroup (GenBank/EMBL accession number: M95126). Phylogenetic analyses based on the obtained sequences were conducted using maximum-likelihood (PAUP 4.0) (Swofford, 1998). Two analyses were performed, one taking into consideration the complete sequences and another in which only the nucleotides involved in the secondary structure of the ITS2 molecules were considered. The nucleotides involved in secondary structure were determined by comparison with the *An. nuneztovari* ITS2 sequence and structure (Fritz *et al.*, 1994). Nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL Data Bank and their respective accession numbers are indicated in Table 2.

Results and discussion

Ribosomal DNA sequences were generated for the ITS2 of 16 neotropical anopheline species (Fig. 1) including the

Table 2. ITS2 length (base pairs) and percentage GC for 23 samples of 16 species of Amazonian *Anopheles* (abbreviations as in Table 1) and their Accession Numbers in the GenBank/EMBL.

Species abbrev.	Length (bp)	GC (%)	Accession number
albi-1	355	54.9	AF462385
albi-2	354	54.5	AF462386
albi-3	353	54.7	AF462387
aqua	348	54.3	AF462376
bena-1	370	55.4	AF462383
bena-2	367	55.3	AF462384
braz	351	50.7	AF461753
darl-1	410	59.3	AF462388
darl-2	409	59.2	AF462389
dean-1	355	54.9	AF461751
dean-2	355	55.8	AF461752
dunh	341	54.2	AF462378
eise	331	53.5	AF462380
evan	364	66.5	AF461750
matto	331	52.8	AF461754
medio	323	59.1	AF462379
nunez	364	55.4	AF461749
osw-1	353	53.5	AF055068
osw-2	351	54.1	AF055069
pery	332	52.1	AF461755
rang-1	342	53.8	AF462381
rang-2	343	53.6	AF462382
trian	400	58.2	AF462377

most important Amazonian malaria vectors. *Anopheles darlingi* is the most anthropophilic and endophilic species among the Amazonian anophelines. Its geographical distribution and density are related to malaria and these mosquitoes are frequently found infected with malaria parasites; it is not unusual for more than one *Plasmodium* species to be found in the same *An. darlingi* specimen (Tadei *et al.*, 1998). All these characteristics place *An. darlingi* as the major malaria vector in Brazil.

Among the local or secondary malaria vectors included, *An. aquasalis* is an important vector of human malaria in coastal Brazil, despite its generally exophilic and zoophilic behaviour. Investigations on variability of *An. aquasalis* (Conn *et al.*, 1993; Linley *et al.*, 1993; Maldonado *et al.*, 1997) have not demonstrated complex speciation (Fairley *et al.*, 2002). *Anopheles triannulatus* is not considered to be a vector in Brazil, but it was found naturally infected with oocysts in some areas out of Brazilian Amazon (Deane, 1988; Oliveira-Ferreira *et al.*, 1990).

Anopheles nuneztovari is suspected to comprise a species complex, being a primary malaria vector in Venezuela and Colombia (where its ITS2 sequences are quite uniform despite chromosomal polymorphism: Sierra *et al.*, 2004), but *An. nuneztovari* has less vectorial capacity in the Brazilian Amazon, where it seems to be incapable of causing malaria outbreaks in the absence of a more efficient vector such as *An. darlingi* (Fritz *et al.*, 1994; Linley *et al.*, 1996; Tadei & Dutary-Thatcher, 2000). *Anopheles dunhami*, which has never been implicated as a human malaria vector, is confused with *An. nuneztovari* due to their morphological

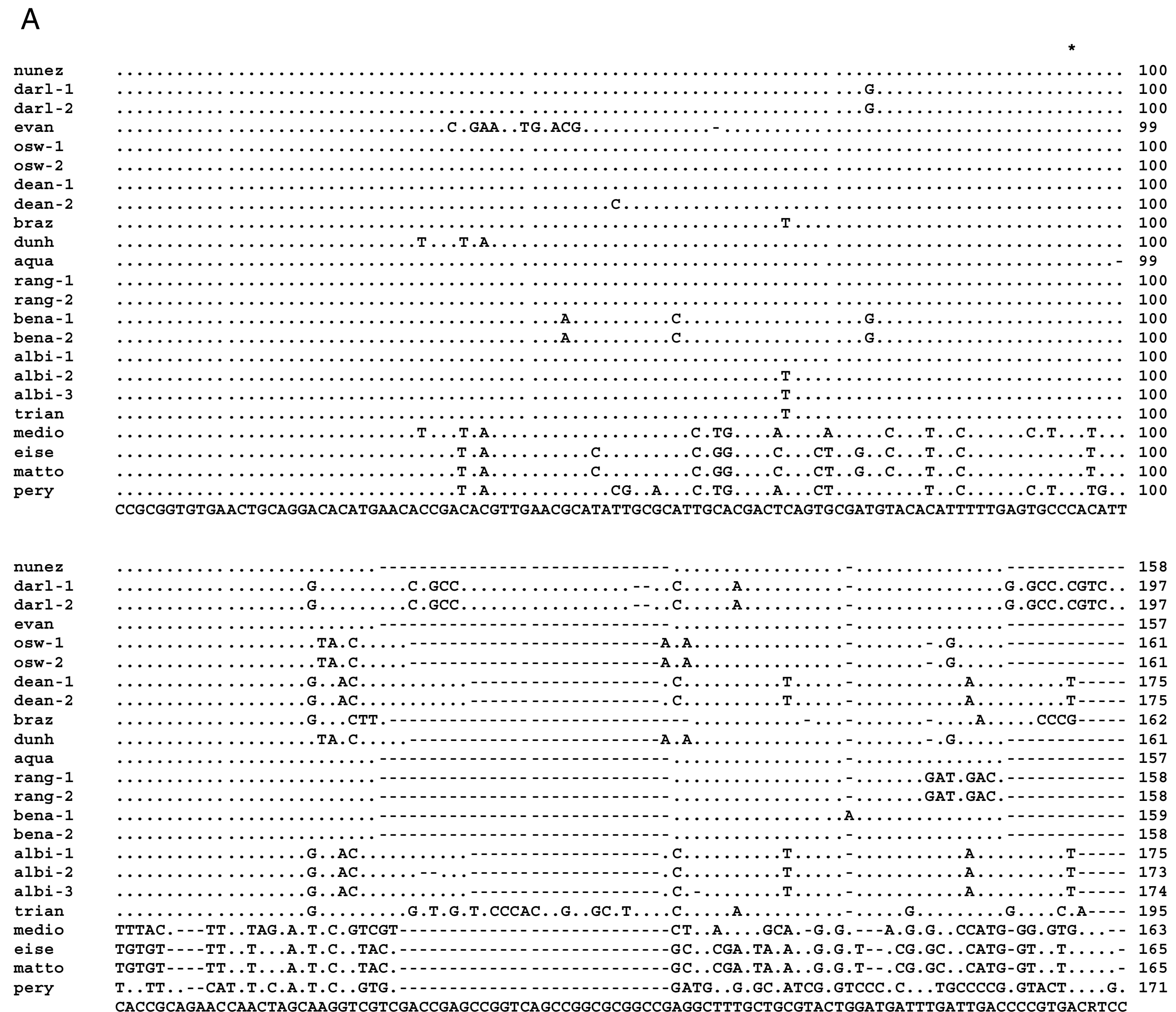

Fig. 1. Alignment of nucleotide sequences (5′ to 3′) of the ITS2 and flanking 5.8 S (nucleotides 1–100) and 28 S (the 26 end nucleotides) coding regions of ribosomal DNA of the 16 anopheline species from the Amazon Region. Taxa are abbreviated as in Table 1 (* indicate where the ITS2 sequence begins and ends).

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B

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nunez     ------------......-..GC.............................-.......A.....C..--...............-.....---C.. 237
darl-1    ..................GTGG..............................-.......------...--...............C...C.T-.... 287
darl-2    ..................GTGG..............................-.......------...--...............C...C.T-.... 287
evan      ------------......-..GC.............................-.......A.....C..--...............-.....---C.. 237
osw-1     ------------.AT...C-...C............................-.......--......C.--..............-.....-..... 240
osw-2     ------------.AT...C-...C............................-.......--......C.--..............-.....-..... 240
dean-1    -------------------.................................-.................-...............-.....-..... 249
dean-2    -------------------.................................-.................-...............-.....-..... 249
braz      -------------GT....-T.G........................TG.T.........-----...A..CGT............ATCT.----... 241
dunh      ------------.AT...C-...C............................-.......--......C.--..............-.....-..... 240
aqua      -------------.....C-................................-.......--.......C--G.............-.....---C.. 234
rang-1    -------------.....C-................................-.......---.......--..............-.....-..... 233
rang-2    -------------.....C-................................-.......---.......--..............-.....-..... 233
bena-1    ------------......-..GC.............................-.......A.....C..--...............-.....---C.. 238
bena-2    ------------......-..GC.............................-.......A.....C..--...............-.....---C.. 237
albi-1    -------------------.................................-.................-...............-.....-..... 249
albi-2    -------------------.G...............................-.................-...............-.....-..... 245
albi-3    -------------------.................................-.................-...............-.....-..... 248
trian     -------------A..C..GTG............................A.-.A...TCC.....G...................-.....C..... 279
medio     -------------G..ACC.AC-GTTCC.T..A..ACCG..AA.....T.CACTC..CTTCC.C..CC..CA-CAAG..ACC.GCGGT----------- 236
eisi      -------------G...TG.ACTGA..CACG..T..CGTG.AAC..A.T..CA.AA.CGT.C...C.CAA..-CT..C...CTG.GGT.----------- 240
matto     -------------G...TG.ACTGA..CACG..T..CGTG.AAC..A.T..CA.AA.CGT.C...C.CAA..-CT..C...CTG.GGT.----------- 240
pery      -.-----------TAA..G.GT.A..GCTCCGC........T...CTTAAAACC.TGAA......CTACC-CT.CAA..CGG.CGC----------- 246
          CAACCGGACGCGCCCGTGTCCAATCAAGCATTGAAGGACTGTGGCGTGGTGGGRTGCACCGTGTGTGTGTGTCGTTGCTTAATACGTGACTCATYTCT

nunez     ............................---..........................T...-----.-.................C..G.CG........ 328
darl-1    .C...T......................---.........................C.A...---.-....................AT.C.A..G.--- 377
darl-2    .C...T......................---.........................C.A...---.-....................AT.C.A..G.--- 377
evan      ............................---..........................T...-----.-.........-.......C..G.-G........ 326
osw-1     ............................---..................CC......------------.A....................C..CAT.-- 323
osw-2     ............................---..................CC......------------.A....................C..CAT.-- 323
dean-1    ...........G................---..................CC.......A..T-------...............A.......-------- 332
dean-2    ...........G................---..................CC.......A..T-------...............A.......-------- 332
braz      ....C......AACA......TGGGCTGT.A..................CC......A------------A...............A.T.C.A..G.--- 327
dunh      .........................A..---..................C.......-------------....................C..CA--.-- 321
aqua      ............................---..............................A-----.-.-......-............C.T.---C-- 318
rang-1    ............................---...................C.....CAAC.A-----.-..................A.G..TA------ 317
rang-2    ............................---...................C.....CAAC.A-----.-..................A.G..TA------ 317
bena-1    ....G.......................---..........................T...-----.-.................CC.GG-......... 327
bena-2    ............................---..........................T...-----.-.................C..G.CG........ 328
albi-1    ...........G................---..................CC.......A..T-------...............A.......-------- 332
albi-2    ...........G................---..................CC.......A..T-------...............A........A------ 329
albi-3    ...........G.........-......---..................CC.......A..T-------...............A.......-------- 330
trian     ...........A....T........C....AG.................C.....AC.AC......T.....................------------ 367
medio     --..ACCAG..C..CAC--CTCTC.CTG.AT...AGGGGCGTG.T.T.TGC.CG.ACC.C---------AT.C.TTGG.TACG.CG.C..G.G.------ 317
eise      TC...CCA...C..CACAGCTT..GCTA...-.CAGGCGAGTGTT.-AT..ACA.TGC.C---------.ACCAT.G....ACTCGAATG..AA------ 323
matto     TC...CCA...C..CACAGCTT..GCTA...-.CAGGCGAGTGTT.-AT..ACA.TGC.C---------.ACCAT.G....ACTCGAATG..AA------ 323
pery      ----.TCGTT.G.AATC.T.CTTC.A.A.T.CAC..TCGC..T...T.CTT..C..TAG.---------.T.GATT.GTT..CA..CACG...A------ 327
          CTGGTATCACATCTGGAGCGGGCTATCCCCCAGTCACAATCCCCAGCGAAATGTGCAGCTACAGCTCGCGGTAGCCCCGATGTGGAGGACAMCGSAATCG
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Fig. 1. Continued.

similarity. This species was first described by Causey (1945), then synonymized with *An. nuneztovari* by Lane (1953), but subsequently reinstated by Peyton (1993). *Anopheles dunhami* has been found only in a few localities in the State of Amazonas, whereas *An. nuneztovari* is widespread around the Amazon Region and both differ from *An. trinkae* (Lounibos *et al.,* 1998).

Anopheles oswaldoi has been considered an important vector of malaria parasites in certain parts of Brazil, whereas in other localities it has no role in transmission (Deane *et al.*, 1948; Deane, 1986; Hayes *et al.*, 1987; Lourenço-de-Oliveira *et al.*, 1989; Klein *et al.*, 1991a, b; Rubio-Palis *et al.*, 1992; Branquinho *et al.*, 1993, 1996). These findings are consistent with data indicating that *An. oswaldoi sensu lato* constitutes a complex of sibling species (Branquinho *et al.*, 1993, 1996; Marrelli *et al.*, 1999a). Artificial feeding of *An. oswaldoi sensu stricto* and *An. konderi*, a sibling species, with *Plasmodium vivax*-infected blood showed the production of sporozoites reaching the salivary glands only in *An. oswaldoi s. s.* (Marrelli *et al.*, 1999b).

The *Anopheles albitarsis* complex has at least four sibling species, including *An. deaneorum*, *An. marajoara* and *An. albitarsis sensu stricto* (Wilkerson *et al.*, 1995). *Anopheles deaneorum* is the only morphologically distinguishable member of this complex, and it was implicated as an important malaria vector in areas from Rondônia, Brazil (Klein *et al.*, 1991a, 1991b). Due to socio-economic changes, *An. marajoara* is becoming more important as a vector in Amapa State (Conn *et al.*, 2002).

As discussed above, most of the species belonging to subgenus *Nyssorhynchus* examined in this study (Table 1) are malaria vectors, whereas *An. dunhami* and *An. evansae* have

C

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nunez    .....................-..............T..............--.----..................-...GA--TG.--........A.. 416
darl-1   G.A--...........A...T.G.G...AA..C....CA..C........A-.----GAG.GA.CGA..AA.....-.....--------T.G....C.C 461
darl-2   G.A--...........A...T.G.G...AA..C....CA..C........A-.----GAG.GA.CGA..AA.....-.....--------T.G....C.C 461
evan     .....................-..............T.................-------.C.............-...G---TG.--........A.. 415
osw-1    -----................-.C.......T.-.........C..........A--...................-....-----.T--..G...AC.C 407
osw-2    -----................-.C.......T.-.........C.........----...................-....-----.T--..G...AC.C 405
dean-1   -------.T..............---.T..A.C....ACTAGG..............--.TG...............-...........--.G..GAC.-A 416
dean-2   -------.T..............---.T..A.C....ACTAGG..............--.TG...............-...........--.G..GAC.-A 416
braz     -----.T.......CG.T...-TCTT.....T...GT..ACC.....AGA.A-----T......................AA.-------TT....AT.G 409
dunh     -----................-.C.......T.-..................A---....................-....-----.T--..G...AC.. 403
aqua     T---...........C....-TCT.......T..ACAC.A...G...C......TG--AA................-......TG.G---.G....... 408
rang-1   T----T...............-.....................G........------..................-...GT--.----TG...A.. 400
rang-2   T----T...............-.....................G........------..................-...GT--..A--.TG...A.. 401
bena-1   .....................G..............T....................C..................-...AA--TG.--........A.. 422
bena-2   .....................-..............T.................----..................-...GAA-TG.--........A.. 419
albi-1   -------.T..............---.T..A.C....ACTAGG..............--.TG...............-...........--.G..GAC.-A 416
albi-2   -----...T.......A......---.T..A.CA...ACTTGG..............--.TG...............-...........--.G...AC.-A 415
albi-3   -------.T..............---.T..A.C....ACTTGG..............--.TG...............-...........--.G...AC.-A 414
trian    ---------T..A.GC.A.CT---.CCTCA.AG...GC.T.G.T....T.......-.................C.....A...G.....G.GGC..A 453
medio    -------G.GTGTGC.AA......GC.-.C.....G.TT.GG.TTGTCACC.C-----.TTCCGT...TTGCT...GG.A..---------.G.GT.A..C 396
eise     -------CTG.GT.CC.GGCA..AAT...AGTCT.G.T..TG.GACACACTC.-----ATGTTACCT.TT.CT.C...TG..--------AT.G.GC.AG 403
matto    -------CTG.GT.CC.TGCA..AAT...AGTCT.G.T..TG.GACACACTC.-----ATGTTACCT.TT.CT.C...TG..--------AT.G.GA.AG 403
pery     -------CTA.GA..C..AG..AAA.CACCTTCA.......C.TAT.T.GA..-----AG.TGG.C-.TTG.C.A.....C.--------ATCTT.TT-C 405
         CGGACCCTCCCTCAAAGCCAGTCTCATGTGACACCACACCAAVAGAGAGAGAGAGAGACCAAACGTACCCTGAAGCAAACGTGCACACTTGCACACGGGT

                                                 *
nunez    .............C.-AT.A...A..........GT.........T..............-................ 491
darl-1   TGT...............G.AC...GATCTAG....TAG....T................-................ 537
darl-2   TGT............-..G.AC...GATCTAG....TAG....T................-................ 536
evan     .............C.-AT.A...A..........GT.........T..............-................ 491
osw-1    .....A.....T..A-..G..C...TC.GA...GA.A......--...............-................ 480
osw-2    ...........T..A-..G..C...TC..A...GA........--...............-................ 478
dean-1   ......TCA.GA.C..A.A..AT..---------..C--.....................-.......C........ 482
dean-2   ......TCA.GA.C...A..AT..---------..C--......................-.......C........ 482
braz     TGTG.T.A..C.ACA-A.A..A..A.A-------..CGAC.CATG...............-................ 478
dunh     ....---------CA-..G..C...TC.GA...GA.A......--..C............-................ 468
aqua     ..AACT..GC.T...-CT.A.CT..T..AC...T.----------...............-................ 474
rang-1   .CTACT.------.T-C.A...T..CAT.CCTTCT..G...G..................-................ 469
rang-2   .CTACT.------.T-C.A...T..CAT.CCTTCT..G...G..................-................ 470
bena-1   .............C.-AT.A...A..........GT.........T..............-................ 497
bena-2   .............C.-AT.A...A..........GT.........T..............-................ 494
albi-1   ......TCA.GA.C..A.A..AT..---------..C--.....................-................ 482
albi-2   ......TCA.GA.C...A..AT..---------..C--......................-................ 481
albi-3   ......TCA.GA.C...A..AT..---------..C--......................-................ 480
trian    ...TGAG...........A..AT.............C--.....................-................ 527
medio    CGC..CTA.-..C.T....T.G.GAC---------------------A...A.......-.G................ 450
eise     .AATG..A..C.C.T...TG..GTAG---------------------A...........-.G................ 458
matto    .AATG..A..C.C.T...TG..GTAG---------------------A...........-.G................ 458
pery     AGTG.A.G.T...GA.TG.G.AG.A----------------------A...A.......-.G................ 459
         GTGCAGCTCATCGAGCGCCCGTACGAGAGAGAGAACACCGATCACAAGTGGGCCTCAAAATAATGTGTGACTACCCC
```

Fig. 1. Continued.

not been reported as involved in human malaria transmission. Likewise *An. rangeli* and *An. benarrochi* are not regarded as vectors in Brazil, although they have been implicated in Peru (Hayes *et al.*, 1987; Flores-Mendoza *et al.*, 2004).

In addition to those 12 species of subgenus *Nyssorhynchus*, we analysed the ITS2 of four species of subgenus *Anopheles*. Among these, *An. mattogrossensis*, *An. peryassui* and *An. mediopunctatus sensu lato* occasionally transmit human malaria (Tadei & Dutary-Thatcher, 2000). Also *An. mediopunctatus* is often associated with simian malaria (Lourenço-de-Oliveira, personal communication) and susceptible to experimental infection with human malaria (Klein *et al.*, 1991a, b). Sallum *et al.* (1999) reported that the *An. mediopunctatus* complex includes three sibling species with indistinguishable adult females.

For the 16 spp. of neotropical anophelines investigated, we compared the ITS2 rDNA sequences. PCR products containing the complete ITS2 and flanking sequences that include 100 base pairs (bp) at the 3′-end of 5.8 S gene and 26 bp at the 5′-end of 28 S were cloned, sequenced and aligned (Fig. 1). To assess possible intra-individual ITS2 variability, four to five clones from each specimen were sequenced, but no inconsistencies were detected. For some of these species of *Anopheles* (*aquasalis*, *evansae*, *mattogrossensis*, *peryassui*, *trianulatus*) the ITS2 sequences obtained from separate individuals were identical, so that only one of them is shown in the alignment. Comparisons of our data with ITS2 sequences previously published for *An. darlingi*, *An. nuneztovari* and *An. oswaldoi* (Fritz *et al.*, 1994; Malafronte *et al.*, 1999; Manguin *et al.*, 1999; Marrelli *et al.*, 1999a) showed much consistency, so the few observed differences may represent intraspecific polymorphism.

The GC contents of the ITS2s varied from 50.7% in *An. braziliensis* to 66.5% in *An. evansae* (Table 2). These

percentages are similar to the values determined for other mosquito species, which fall in the range of 50–70% (Porter & Collins, 1991; Wesson *et al.*, 1992; Paskewitz *et al.*, 1993b; Fritz *et al.*, 1994; Cornel *et al.*, 1996; Xu & Qu, 1997; Beebe *et al.*, 1999).

We found considerable heterogeneity of ITS2 size among species (Table 2). The lengths ranged in size from 323 bp in *An. mediopunctatus* to 410 bp in *An. darlingi*. The species of subgenus *Anopheles* (*eiseni*, *mattogrossensis*, *mediopunctatus*, *peryassui*) had smaller ITS2 sequences, ranging from 323 to 332 bp, while the species of subgenus *Nyssorhynchus* had ITS2 sequences ranging in size from 341 to 410 bp. Afrotropical members of the *An. gambiae* complex (subgenus *Cellia*) have ITS2 of ~426 bp (Paskewitz *et al.*, 1993b), while in the Australasian *An. punctulatus* group the determined lengths ranged from 549 to 563 bp (Beebe *et al.*, 1999). Among anopheline mosquitoes, the longest ITS2 sequences have been found in members of the Oriental *An. dirus* complex, in the range 710–716 bp (Xu & Qu, 1997).

In addition to differences in ITS2 lengths and CG contents, rDNA sequences showing substantial interspecific divergence were identified (Fig. 1), and these could be used to distinguish between most of the species studied. The sequences CCGAATTTGACG at position 34 of *An. evansae*, TGGGCTGTC at position 263 of *An. braziliensis*, GAGAGAGCGA and GCGCCGCGTC at positions 426 and 184 of *An. darlingi* are examples of such divergences. Indels of distinct lengths, preceded and followed by variable nucleotides, are also useful for species identification purposes. Such indels are found at positions 126 and 146 of *An. nuneztovari* and equivalent positions of the sequences obtained for other anophelines. It is important to note that no intra-individual and intraspecific variations were observed for these sequences.

A matrix of ITS2 similarity was calculated with all the obtained sequences (Table 3) and the results showed values ranging from 25% to 99%. The lowest observed value of identity was 25% between *An. mediopunctatus* and *An. darlingi*. The highest identity between two species was 99% between *An. mattogrossensis and An. eiseni*, differing in only two sites.

Comparing the findings of ITS2 sequences and morphological characters used for species identification, revealed three circumstances: (1) species had different ITS2 sequences and distinctive morphological characters; (2) different ITS2 sequences were identified that allow the identification of species which lack distinctive morphological characters; and (3) no distinctive ITS2 sequences were found to differentiate species belonging to complexes of sibling species.

Anopheles darlingi and *An. braziliensis* exemplify the first circumstance, having no particular difficulties with their identification based on morphological characters (Rosa-Freitas *et al.*, 1992; Manguin *et al.*, 1999). ITS2 differences between *An. darlingi* and other anophelines are prominent, but there are no pressing taxonomic questions to be solved thereby. Table 3 shows that *An. darlingi* ITS2 differs by 35–75% from sequences of the other 15 spp. included in this study.

Anopheles braziliensis has been found naturally infected with *P. vivax and P. falciparum*, but its involvement in human malaria transmission is not clear (Consoli & Lourenço-de-Oliveira, 1994). Our ITS2 analysis of *An. braziliensis* showed its sequence to be very different from the other anophelines. Although morphological characters are

Table 3. Similarity matrix for the ITS2 sequences of individual anophelines from Amazon Region. Taxa are abbreviated as in Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1 nunez	–																						
2 darl-1	65	–																					
3 darl-2	65	99	–																				
4 evan	98	65	65	–																			
5 osw-1	80	63	63	79	–																		
6 osw-2	80	63	63	79	98	–																	
7 dean-1	69	61	61	69	69	69	–																
8 dean-2	69	62	61	69	70	70	99	–															
9 braz	65	57	57	64	68	69	61	61	–														
10 dunh	78	61	61	77	94	94	69	69	68	–													
11 aqua	79	62	62	79	78	78	68	68	63	76	–												
12 rang-1	78	63	63	77	76	76	70	69	64	77	77	–											
13 rang-2	78	63	63	77	76	76	70	69	64	77	77	99	–										
14 bena-1	95	64	64	95	78	79	68	68	64	76	78	76	76	–									
15 bena-2	98	65	65	98	80	80	69	69	65	78	79	77	77	96	–								
16 albi-1	69	61	61	69	69	69	100	99	61	69	68	70	70	68	69	–							
17 albi-2	69	62	62	69	70	70	96	96	61	69	68	70	70	68	70	96	–						
18 albi-3	68	61	61	68	69	69	98	98	61	68	67	69	69	67	69	98	96	–					
19 trian	62	61	61	62	61	62	70	70	56	62	60	60	60	62	63	70	69	69	–				
20 medio	28	25	25	28	28	28	28	28	30	28	29	29	29	28	29	28	29	28	26	–			
21 eise	30	28	28	30	29	30	29	29	30	29	31	31	31	30	30	29	29	29	30	48	–		
22 matto	30	28	28	30	29	30	29	29	31	29	31	31	31	30	30	29	30	29	30	48	99	–	
23 pery	31	30	30	31	32	33	31	31	32	32	32	33	33	31	31	31	31	31	30	34	39	39	–

available to identify these species, DNA-based methods can be applied to specimens and situations unsuitable for morphological taxonomy. For example, DNA may be analysed from specimens in all developmental stages, of both sexes, preserved in alcohol, dried, fresh or frozen (Crampton & Hill, 1997). Furthermore, molecular techniques may be applied with very small samples, such as a single mosquito leg or dissected tissues such as midguts and salivary glands used for oocysts and sporozoites detection.

Readily discernible morphological characters may not exist for the identification of some species of anophelines (White, 1977, 1979). Taxonomic reviews of anophelines belonging to subgenus *Nyssorhynchus* (Faran, 1980; Linthicum, 1988) have highlighted many problems in the morphological distinction of specimens within the Albimanus Section of the Oswaldoi Group. Characters, such as the basal dark band in the hindtarsomere II of *An. oswaldoi*, have a large range of variation, overlapping the range of the same characters for non-vectors *An. rangeli and An. evansae*, confusing the identification of these species (Consoli & Lourenço-de-Oliveira, 1994). By contrast, *An. oswaldoi* has 21% difference of the ITS2 sequence compared to *An. evansae*, and 24% compared with A*n. rangeli*, whereas *An. evansae* has 23% difference from *An. rangeli* as previously identified by Fritz (1998). These interspecific ITS2 differences make it easy to separate these three species at the molecular level.

The local vector *An. nuneztovari* and *An. dunhami*, which has never been found infected with human malaria, are almost indistinguishable morphologically. However, the ITS2 sequences of these two species differ by 22% and therefore may help in the correct identification of these mosquitoes. These are examples of the second circumstance mentioned above in which ITS2 may be useful for the identification of morphologically similar species. Based on the identified sequence differences, specific primers could be designed to anneal to different regions of the ITS2, so that a unique-sized PCR product would be amplified for each of these species.

The third mentioned circumstance, in which ITS2 sequences did not help to solve taxonomic problems, was found in two complexes of closely related species. In the Argyritarsis Section (Albitarsis Group), the *An. albitarsis* complex has at least four sibling species (Wilkerson *et al.*, 1995) but, unfortunately, we found only 2–4% differences between their ITS2 sequences, insufficient for reliable molecular identification (Table 3). Secondly, specimens morphologically identified as *An. oswaldoi* differed by 2% of their ITS2 sequence. Previously described differences in biting behaviour and ITS2 sequences of contrasted *An. oswaldoi* populations have been used to support the hypothesis that *An. oswaldoi s. l.* includes several morphologically indistinguishable species (Marrelli *et al.*, 1999a, b). Whether the differential sequences for mosquitoes of the Albitarsis and Oswaldoi Complexes represent different sibling species or intraspecific variation cannot be clarified without further sampling and analysis. Such low levels of variation would support a hypothesis of very recent or incipient speciation in these two complexes. The limited values of ITS2 difference are comparable to those obtained with members of the *An. gambiae* complex, having seven members that cannot all be distinguished morphologically, and for which the ITS2 sequences do not show enough differences to be used for species identification (Paskewitz *et al.*, 1993b).

The aligned complete sequences (Fig. 1) from the 16 species were compared by maximum-likelihood analysis (PAUP 4.0) (Swofford, 1998), but their phylogenetic relationships remain unresolved. In addition to considering the complete sequence, we performed another analysis considering only those nucleotides involved in the possible secondary structure of the ITS2 molecule. This analysis was performed by comparing the sequences that we determined with the sequence and structure described by Fritz *et al.* (1994) for the ITS2 of *An. nuneztovari*. Again, the obtained

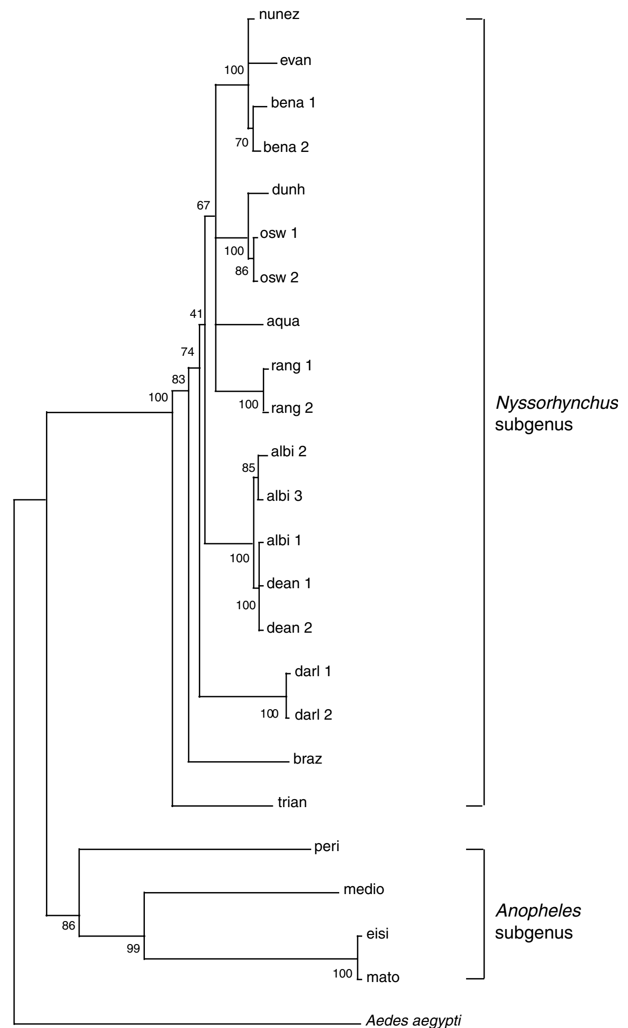

Fig. 2. A neighbour-joining bootstrap tree based on rDNA ITS2 sequence data for 23 samples of 16 species of *Anopheles* from Amazonia. The tree was rooted with *Aedes aegypti* ITS2 sequence (GenBank/EMBL: M95126). Numbers show bootstrap confidence levels (from 500 replications) above the branches tested. Species names abbreviated as in Table 1.

tree showed unresolved branches (data not shown), so more species and samples are needed to provide a robust phylogenetic analysis of *Nyssorhynchus*.

Using the distance values found in the similarity matrix, a neighbour-joining (NJ) tree was produced (Fig. 2) which separated the two subgenera as distinct groups. Within *Nyssorhynchus*, seven species belonging to the Oswaldoi Group of Albimanus Section (Harbach, 2004) clustered together, as shown at the top of Fig. 2, comprising *An. nuneztovari*, *An. evansae*, *An. benarrochi*, *An. dunhami*, *An. oswaldoi*, *An. aquasalis* and *An. rangeli*. However, a substantial distance separated *An. triannulatus*, which also belongs to the Albimanus Section. Species belonging to the Argyritarsis Section were all separated in distinct clades, except for *An. albitarsis* and *An. deaneorum*, which grouped together. This result was expected, as these two species are classified in the Albitarsis Complex on morphological grounds. Although the ITS2 sequences were of little value for phylogenetic interpretation, considerable nucleotide divergences were found, which can be useful to help in species identification and solving taxonomic questions.

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