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Molecular differentiation in natural populations of *Anopheles oswaldoi* sensu lato (Diptera: Culicidae) from the Brazilian Amazon, using sequences of the COI gene from mitochondrial DNA

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ABSTRACT. *Anopheles* (*Nyssorhynchus*) *oswaldoi* (Peryassú, 1922) s. l., which has been incriminated as a potential human malaria vector in Western Brazilian Amazon, may constitute a cryptic species complex. However, the most recent study with isozymes indicated high similarity among samples from the States of Acre, Amazonas and Rondônia in the Brazilian Amazon. In the present study, 45 individuals were sequenced from Sena Madureira (State of Acre), Coari (State of Amazonas), São Miguel (State of Rondônia), and Moju (State of Pará), using the cytochrome oxidase I gene from mitochondrial DNA. Twenty-five haplotypes were identified in the four localities, and no haplotype was shared among them. The lowest haplotype number was detected in the Coari sample. The dendrogram based on maximum parsimony analysis yielded four groups: I) haplotypes 1, 2, 3, 4, and 5 from Sena Madureira and

haplotypes 17 and 18 from São Miguel; II) haplotypes 13 to 16 and 19 to 22 from São Miguel; III) haplotypes 23 to 25 from Moju, and IV) haplotypes 6 to 9 from Sena Madureira and haplotypes 10 to 12 from Coari. The genetic distance (uncorrected *p*) obtained among the four groups ranged from 0.08 to 5.3%, whereas the highest values (4.97 to 5.3%) were found between groups I (Sena Madureira) and III (Moju). Based on male genitalia identification, it was suggested that group I may be *A. oswaldoi* s. s. whereas group IV may be *A. konderi*. Groups II and III could constitute other lineages or species within *A. oswaldoi* s. l., whose taxonomic status remains to be clarified. These results suggest that additional studies are necessary using samples of *A. oswaldoi* s. l. from a larger geographic area.

Key words: *Anopheles oswaldoi* s. l., Malaria, Population genetics, mtDNA, Molecular taxonomy, Brazilian Amazon

INTRODUCTION

Anopheles (*Nyssorhynchus*) *oswaldoi* sensu lato (s. l.) has a large geographic distribution, from Costa Rica to northern Argentina, including Trinidad (Faran, 1980).

A. oswaldoi (Peryassú, 1922) was described based on the morphology of adults collected in Vale do Rio Doce (Espírito Santo State, Brazil), whereas *Anopheles konderi* Galvão and Damasceno, 1942, was described using male genitalia from specimens collected in Coari (Amazonas State, Brazil). Both species have high morphological similarity and can be separated only by the apical portion of the mesosoma of male genitalia. *A. oswaldoi* has an elongate mesosoma, much longer than wide and without spines. *A. konderi* has a rounded mesosoma with tiny pointed spines (small protuberances) on both lateral sides (Causey et al., 1946). Because of this morphological similarity between the two species, *A. konderi* was considered as a junior synonym of *A. oswaldoi* for almost five decades (Lane, 1953; see revision in Scarpassa, 2005). Later, revised morphological studies using all stages (larval, pupal and adult) indicated that *A. konderi* may be a valid species (Flores-Mendoza et al., 1998), and currently, *A. konderi* was resurrected from synonymy with *A. oswaldoi* (Flores-Mendoza et al., 2004). Based on the second internal transcribed spacer sequences of ribosomal DNA, Marrelli et al. (1999) found four groups that may correspond to at least four cryptic species: one may correspond to *A. oswaldoi* s. s., another to *A. konderi*, and two other groups may correspond to species for which morphological identification remains to be clarified.

The morphological data from Flores-Mendoza et al. (1998) suggested that *A. oswaldoi* and *A. konderi* coexist sympatrically in Acre State. In this state, the populations of *A. oswaldoi* s. l. were incriminated as a potential human malaria vector based on enzyme-linked immunosorbent assays and analysis of dissected salivary gland (Branquinho et al., 1993, 1996). Therefore, because the taxonomic status of *A. oswaldoi* s. l. is still controversial, molecular analysis and

population genetic studies are necessary to better understand the evolutionary relationships of this "species group". However, it is interesting that the most recent study using 20 isozyme loci showed high genetic similarity and the absence of diagnostic loci among samples from the States of Acre, Amazonas and Rondônia in the Brazilian Amazon (Scarpassa, 2005), indicating that additional studies are required.

The aim of the current study was a preliminary estimate of the intra- and inter-populational genetic variability of four samples of *A. oswaldoi* s. l. from the Brazilian Amazon, using sequences of the cytochrome oxidase, subunit I (COI) gene of mitochondrial DNA (mtDNA).

MATERIAL AND METHODS

Mosquito collection

Specimens of *A. oswaldoi* s. l. were collected from four localities from the Brazilian Amazon: Sena Madureira (SMA), State of Acre (09° 03' S; 68° 39' W), Coari (COA), State of Amazonas (04° 05' S; 63° 08' W), Municipality of São Miguel (SMI), State of Rondônia (08° 36' S; 63° 49' W), and Moju (MOJ), State of Pará (1° 52' S; 48° 45' W). The samples from Moju were obtained in July 1998 to 1999, and the samples from the remaining localities were collected in June 2001 to August 2002. In the SMA site, fifteen specimens were examined, while in COA, SMI and MOJ localities, 12, 15 and three specimens were examined, respectively (Table 1). The three mosquitoes examined from MOJ were wild-caught females. The mosquitoes from the remaining localities were offspring (F_1) of laboratory-reared progeny, where from one to two individuals of each progeny were used in the analyses. Adult (females) and offspring (eggs) were identified using the keys of Gorham et al. (1967), Faran (1980) and the report of Lounibos et al. (1997). The male genitalia were identified when possible according to the key of Causey et al. (1946). Nine, eight and nine progeny were assayed from SMA, COA, and SMI, respectively, whereas the identifications of male genitalia were performed on three, four, and one progeny, respectively. In the sample from SMA, two progeny were identified as *A. oswaldoi* and one as *A. konderi*; all four progeny were *A. konderi* in COA, and the single progeny identified in SMI was *A. konderi*. No male genitalia identification was conducted in specimens from MOJ, because the sequences analyzed were from wild-caught females. Thus, because we were unable to identify the male genitalia from all progenies used in the present study, the samples analyzed are reported as *A. oswaldoi* s. l.. Following the identifications, the mosquitoes were kept in a -80°C freezer until the analyses. Two individuals of *A. rangeli* Gabaldón, Cova-Garcia and Lopes, 1940, and two of *A. nuneztovari* Gabaldón, 1940, were used as outgroups, because they are morphologically and phylogenetically closely related to *A. oswaldoi*, and are included in the same section (Albimanus) of the *Nyssorhynchus* subgenus (Faran, 1980).

DNA extraction, PCR amplification, purification, and DNA sequencing

Total genomic DNA was extracted from whole mosquitoes using a phenol and chloroform method, as described in Sambrook and Russel (2001). The fragment size of 1226 bp of the COI gene of mtDNA was amplified using the primers and PCR conditions described by Zhang and Hewitt (1997). The PCR products were then checked on 1% agarose minigels, stained with

ethidium bromide and analyzed under UV light. The PCR purified product was then sequenced using a BigDye Terminator Cycle sequencing kit. All fragments were sequenced in both directions. An automated DNA sequencer from Applied Biosystems (ABI PRISM 377 XL) was used.

Statistical analysis

All sequences were aligned by eye using Sequencher Program, version 3.0 (Gene Codes Corporation, Ann Arbor, MI, 1995) and were imported into PAUP*, version 4.0 (Swofford, 2003), where the maximum parsimony and pairwise distance (uncorrected *p*) analyses were performed. Identical haplotypes were identified using MacClade, version 3.07 (Maddison and Maddison, 1997). The identical haplotypes were considered as a single unique sequence. In the maximum parsimony analysis, the uninformative characters were excluded, and all characters were unordered, with equal weight, and parsimony-informative. Bootstrapping (Felsenstein, 1985) was conducted using 1000 pseudoreplicates. The uncorrected *p* sequence divergence was used, due to the low level of divergence among the haplotypes. Haplotypes are deposited in GenBank under the accession numbers DQ784827 to DQ784851.

RESULTS

Forty-five specimens of *A. oswaldoi* s. l. were sequenced and the fragment size obtained was 1226 bp of the COI gene (mtDNA), from which 25 haplotypes were identified (Table 1). In the SMA and SMI, the highest haplotype diversity was detected with 9 and 10 haplotypes, respectively. The lowest haplotype diversity was in COA with three haplotypes. In MOJ, the three specimens sequenced yielded three different haplotypes. None of the four localities shared haplotypes. Table 2 shows the 25 haplotypes with their respective variable positions for the four samples of *A. oswaldoi* s. l. as well as four haplotypes (26 to 29) of the two outgroups.

The average nucleotide composition, including the two outgroups, was 29.94% A, 39.68% T, 15.65% C, and 14.74% G, with an A + T bias of 69.62%. In the ingroup *A. oswaldoi* s. l., the 45 sequences had 123 (10.03%) polymorphic sites and 108 (8.81%) were phylogenetically informative. Including the two outgroups, *A. rangeli* and *A. nuneztovari*, the data had 176 (14.36%) polymorphic sites, of which 163 (13.30%) were phylogenetically informative.

Of the 123 variable sites within *A. oswaldoi* s. l., nine (7.32%) substitutions were detected at the first codon position, two (1.63%) at the second position, and 112 (91.02%) were observed at the third codon position. Of the 123 variable sites, 96 (78.05%) were transitions, and most of them occurred at the third codon and were from T $\leftrightarrow$ C. Of the 27 (21.95%) transversions, all occurred at the third codon position.

The maximum parsimony analysis (Figure 1) yielded a dendrogram that suggests that the samples of *A. oswaldoi* s. l. analyzed are monophyletic with respect to two outgroups, *A. rangeli* and *A. nuneztovari* (84% bootstrap support). The most parsimonious tree generated a tree-length of 270, a consistency index of 0.670 and a retention index of 0.882. The figure shows that most haplotypes were grouped geographically, except the haplotypes 6, 7, 8, 9, 17, and 18 (see also Table 1). This dendrogram shows four groups: I) haplotypes 1, 2, 3, 4, and 5 from SMA, and the haplotypes 17 and 18 from SMI; II) haplotypes 13 to 16 and 19 to 22 from

Table 1. Haplotype frequency in four samples of *Anopheles oswaldoi* sensu lato from the Brazilian Amazon.

Haplotype	Sample				
	Sena Madureira	Coari	São Miguel	Moju	Total
1	2 (0.13)	-	-	-	2 (0.04)
2	1 (0.07)	-	-	-	1 (0.02)
3	1 (0.07)	-	-	-	1 (0.02)
4	3 (0.20)	-	-	-	3 (0.08)
5	2 (0.13)	-	-	-	2 (0.04)
6	2 (0.13)	-	-	-	2 (0.04)
7	1 (0.07)	-	-	-	1 (0.02)
8	1 (0.07)	-	-	-	1 (0.02)
9	2 (0.13)	-	-	-	2 (0.04)
10	-	8 (0.66)	-	-	8 (0.20)
11	-	2 (0.17)	-	-	2 (0.04)
12	-	2 (0.17)	-	-	2 (0.04)
13	-	-	1 (0.07)	-	1 (0.02)
14	-	-	1 (0.07)	-	1 (0.02)
15	-	-	3 (0.20)	-	3 (0.08)
16	-	-	1 (0.07)	-	1 (0.02)
17	-	-	1 (0.07)	-	1 (0.02)
18	-	-	1 (0.07)	-	1 (0.02)
19	-	-	4 (0.24)	-	4 (0.10)
20	-	-	1 (0.07)	-	1 (0.02)
21	-	-	1 (0.07)	-	1 (0.02)
22	-	-	1 (0.07)	-	1 (0.02)
23	-	-	-	1 (0.33)	1 (0.02)
24	-	-	-	1 (0.33)	1 (0.02)
25	-	-	-	1 (0.33)	1 (0.02)
Total	15	12	15	03	45

The haplotype frequency is shown in parentheses.

SMI; III) haplotypes 23 to 25 from MOJ, and IV) haplotypes 6 to 9 from SMA and haplotypes 10 to 12 from COA. All clades were well supported (Figure 1). The maximum likelihood analysis yielded a tree with identical topology (data not shown).

The genetic distance based on the uncorrected *p* ranged from 0.08 to 5.3% among the four groups from Figure 1. The highest values (4.97 to 5.3%) were obtained among the haplotypes 1, 2, 3, 4, and 5 from SMA (group I) and the haplotypes 23, 24, and 25 from MOJ (group III). Between groups I and IV, it was 4.5 to 5.1%, and between groups I and II, 3.7 to 4.2%. Otherwise, the genetic distance within the groups was lower than among groups: group I ranged from 0.08 to 2.8%; group II from 0.08 to 0.6%; group III from 0.3 to 0.7%, and group IV from 0.08 to 1.3%. Between *A. oswaldoi* s. l. and the outgroup *A. rangeli* the genetic distance was 3.99 to 6.61%, and between *A. oswaldoi* s. l. and *A. nuneztovari* the distance was 5.63 to 7.59%, suggesting that *A. oswaldoi* s. l. and *A. rangeli* are more closely related than *A. oswaldoi* s. l. and *A. nuneztovari*, at least for the mitochondrial genome.

Table 2. Variable positions in the 1226 bp of the COI gene for 25 haplotypes of *Anopheles oswaldoi* sensu lato, two haplotypes (26 and 27) of *Anopheles rangeli* and two haplotypes (28 and 29) of A*nopheles nuneztovari*.

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                  1111111111111111222222222223333333333333333333344444444444444455555555555566666666666666777777777
      113366788990111224455677899011344457890011223455556677899011223444566790013666789901124556788990001234 4
H     471325469284069286928149217948625845295814045703692847028469281369547640951036513684701065803351470873 9

 1    GCTCGTATCTAATTTTTTTTAACATTCCGTTAAGTCTTATGTATTTAATCTTAGCCCTTCTTTAAAAATAGTCACTACATTATTTTGAATTACTGTTATATTA
 2    ....A.....................................A.....................................................................
 3    ........T.....................................................................................A.............
 4    ...............................................................................................................
 5    ...............................................................................................................
 6    AT.TA..CT.....A...C...TG..TT....TA.T..G.A.T.C...CTC..A.TT.......T....T..T..A.T..C...A.T...CG.CA.C.A.CC.
 7    AT.TA..CT.....A...C...T...TT...GTA.T..G.A.T.C...CTC..A..T.......T.G..T..TG.AGT..C...A.T...C..CA...A..C.
 8    AT.TA..CT.....A...C...T...TT....TA.T..G.A.T.C...CT...ATTT.......TG...T..T..A.T..C..CA.T...C..CA...A....
 9    AT.TA..CT.....A...C...T...TT....TA.T..G.A.T.C...CTC..A.TT.......TG...T..T..A.T..C...A.T..AC..CA...A..CT
10    AT.TA..CT.....G...C...T...TT....TA.T..G.A.T.C...CTC..AT.T.......T....T..T..AGT..C...A.T......CA...A..C.
11    AT.TA..CT.....G...C...T...TTA...TA.T..G.A.T.C...CTC..AT.T.......T....T..T..AGT..C...A.T......CA...A..C.
12    AT.TA..CT.....A...C..GT...TT...GTA.T....A.T.C...CTC..A..T.......T....T..T.TAGT..C...A.T...C..CA...A..C.
13    AT..A.GCT..G..A.......T...TT....TA......A.TC.C...TC..A.T...T.............TT..T........A.......A.C..G...
14    ATC.A.GC......A.......T...TT....TA......A.TC.....TC..A.T...T.............TT..T........A.......A.C..G...
15    AT..A.GC......A.......T...TT....TA......A.TC.....TC..A.T...T.............TT..T........A.......A.C..G...
16    AT..A.GC......A.......T...TT....TA......A.TC.....TC..A.T...T.............TT..T........A.......A.C..G...
17    ........T.........C.........TT.............A.............AT...CT..................TG.......A.....T.A........
18    ..C.....T.........C.........TT.............A.............AT...CT..................TG.......A.....T.A........
19    AT..A.GC......A.......T...TT....TA......A.TC.....TC..A.T...T.............TT..T........A.......A.C..G...
20    AT..A.GC......A.......T...TT....TA......A.TC.....TC..A.T...T.............TT..T........A.......A.C..G...
21    AT..A.GC.A....A.......T...TT....TA......A.TC.....TC..A.T...T.............TT..T........A.......A.C..G...
22    AT..A.GC......A.......T...TT....TA......A.TC.....TC..A.T...T.............TT..T........A.......A.C..G...
23    A..TAC..TC....A.CC...GT.C.TT....TA.T......T......TCATATT...T.C.GTG....A.T.T..T...G....T......CA...A..C.
24    A..TAC..TC....G.CC....T.C.TT....TA.T......T......T.ATATT...T.C.GTG....A.T.T..T...G....T..A...CA...A..C.
25    A..TAC..TC....A.CC...GT.C.TT....TA.T......T......T.ATATT...T.C.GTG....A.T.T..T...G....T......CA...A..C.
26    AT.TAA.CT....CA....C.GTG..TT....TA...A..A.T...T.CTC..ATTT...C.C.T....T.AT.T..A..CT..AAT.G...T.AC.CAGC..
27    AT.T.A.CT....CA....C.GT...TT....TA...A..A.T...T.CTC..ATTT...C.C.T....TAAT.T..A..CT..AAT.....T.AC.CAGC..
28    AT..A..CT.T.A.GA.C..TGT..CTT.AC.TAGTCA.AAATC...T.T..GA.TTC.T..C.T..CCT..T.T..A.ACTC..ATT....T.A........
29    AT..AAGC..T.A.GA.C..TGTG.CTT.AC.TAATCA.AAATC...T.....A..TC.T..C...G.CT..T.T..A.ACTC..ATT....T.A.C......
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Continued on next page

Table 2. Continued.

H	1111111111111111111111111111 7777777788888888888888888888888899999999999900000000000001111111111111122 5667899900011222333455667778899990112577788990011123445670124556668899902 8470547803928147039814392591403462173914709564703957042199212171391703656
1	AAATACATACTTATTTTTTAAATAAAGTTCACTATCTACTTACATAAAACTTTGCTCTTAACGCTAATATAGA
2	..........T....
3	.G..........T.....G.....
4	..........T....
5	..........TC....
6	...A.T...T...AC..C.G.............T....CCT......T....T.T.....ATA.....TA.
7	.GTA.T.C.....AC..C..............T....CCT......T....T.T.....ATA.....TA.
8	...A.T...T...A...C.......G.........T....CCT......T....T.T.....ATA.....TA.
9	...A.T...T...A...C..............T....CCT......T....T.T.....ATA..C..TA.
10	.G.A.T.C.T...AC.................T....CCT......T....T.T.....ATA.....TA.
11	.G.A.T.C.T...AC.................T....CCT......T....T.T.....ATA.....TA.
12	.G.A.T.C.T...AC..C.............G.T....CCT......T....T.T.....ATA.....TA.
13	...AG....T..........GG........T....T....CTT....TTTC..AT.T.....A.A..C..GA.
14	...AG....T..........G........T....T....CCT....TTTC..AT.T.....A.A..C..GA.
15	...AG....T..........GG........T....T....CCT....TTTC..AT.T.....A.A..C..GA.
16	...AG....T..........G........T....T....CCT..G.TTTC..AT.T.....A.A.GC..GA.
17	.....TG.GT...A.............T...........CT..TG..TC....AT.C..TA.A.....TA.
18	.....TG.GT...A.............T...........CT..TG..TC....AT.C..TA.A.....TA.
19	...AG....T..........G........T....T....CCT....TTTC..AT.T.....A.A..C..GA.
20	...AG....T..........G........T....T.....CT....TTTC..AT.T.....A.A..C..GA.
21	...AG....T..........G........T....T....CCT..G.TTTC..AT.T.....A.A..C..GA.
22	...AG....T..........G........T....T....CCT..G.TTTC..AT.T.....A.A..C..GA.
23	.G.C.TG.....G.A.....................TC.TTTC.....C..AT.T.....ATA.....TA.
24	.G.C.T......G.A..C....C.............TC.TTTC....TC..AT.T.....ATA..C..TA.
25	.G.C.TG.....G.A..C....C.............TC.TTTC....TC..AT.T.....ATA.....TA.
26	G....T...T...A..C.AG...GT..C.....T..A.T.CTT......T..CAT.T...T.ATA..C.CTAC
27	G....T...T...A..C.AG...GT..C.....T..A.T.CTT......T..CAT.T...T.ATA....CTAC
28	...A.T...TAC.A.CC.G..C....A.C..TA.A..G..CTT.C..T..CC.AT.TC..T.ATA...T.TA.
29	...A.T...TAC.A..C.G..C....A.C..TA.A..GA.CTT.CG.T..CC.AT.TC.GT.ATAG..T.GA.

H = haplotype. 1 to 25 = haplotypes detected within *A. oswaldoi* s. l.; 26 and 27 = haplotypes detected in *A. rangeli*; 28 and 29 = haplotypes observed in *A. nuneztovari*. Haplotype locations are cited in Table 1. Note: dots refer to identity with reference to haplotype 1.

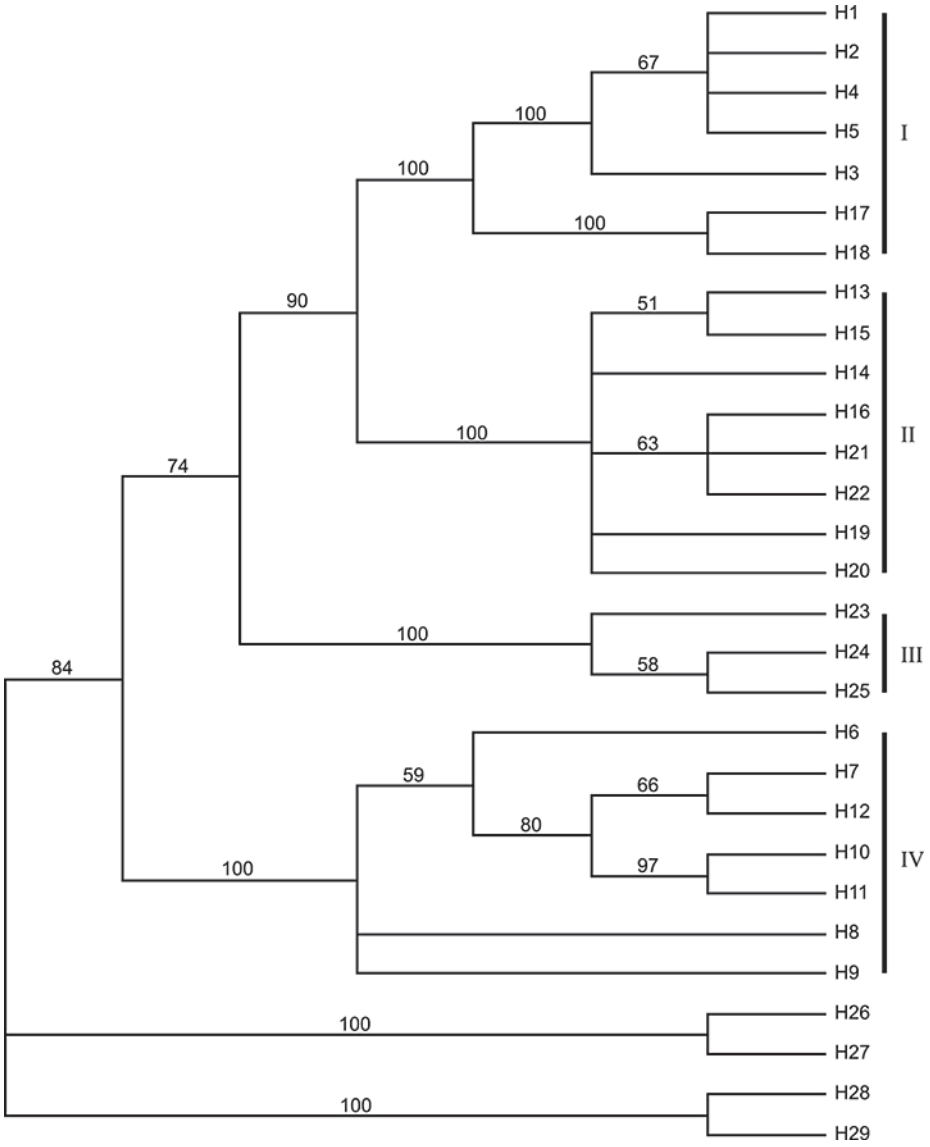

Figure 1. Dendrogram based on maximum parsimony analysis for 25 haplotypes (H1 to H25) obtained within *Anopheles oswaldoi* s. l.. The bootstrap proportions are above the branches. Haplotype designations within *A. oswaldoi* s. l. are in Table 1. *A. rangeli* and *A. nuneztovari* were used as outgroups. H26 and H27 = *A. rangeli*; H28 and H29 = *A. nuneztovari*.

DISCUSSION

The A + T composition (69.62%) is similar to other insect mtDNA (Clary and Wolstenholme, 1985; Bernasconi et al., 2000; Mirabello and Conn, 2006). The high frequency of transitions in the third codon position among the haplotypes of *A. oswaldoi* s. l. is typical of populations of the same species/lineages or of recently diverged species (Moritz et al., 1987;

Simon et al., 1994). When species have been separated for a long time, the accumulation of third position substitutions is expected to have reached saturation (Pedersen, 1996), by the increase of transversions and substitutions in the first and second positions. These results were not observed in the present study, supporting a close relationship among the samples of *A. oswaldoi* s. l., despite the four distinct groups seen in Figure 1.

Curiously, none of the four samples of *A. oswaldoi* s. l. shared haplotypes indicating an absence of gene flow among them, with evidence of populations genetically structured. However, the apparent absence of gene flow may be due to the failure of detecting intermediate samples among localities, but this can only be determined with additional sample collections and analyses.

The low haplotype number detected in COA is similar to results obtained with isozymes (Scarpassa, 2005), suggesting that in the past a strong bottleneck effect or founder effect may have occurred in this population.

Based on the male genitalia identification, we suggest that group I may be *A. oswaldoi* s. s. while group IV may be *A. konderi*. Groups II and III could comprise other lineages or species within *A. oswaldoi* s. l., which remains to be clarified. Conversely, the isozyme studies did not indicate differences among samples from these same localities (Scarpassa, 2005). These distinct results may be explained by differences in the evolutionary rate between the mitochondrial genome and the nuclear genome (isozymes). Also, because of the maternal inheritance of mtDNA, its effective population size is smaller than that of the nuclear genome and, therefore, should be more sensitive to population subdivision (Roderick, 1996).

These data suggest that it is possible to separate *A. konderi* from *A. oswaldoi* using sequences from COI gene. However, further investigations are necessary in *A. oswald*oi s. l. from a larger geographic area.

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REFERENCES

Bernasconi MV, Valsangiacomo C, Piffaretti JC and Ward PI (2000). Phylogenetic relationships among Muscoidea (Diptera: Calyptratae) based on mitochondrial DNA sequences. *Insect Mol. Biol*. 9: 67-74.

Branquinho MS, Taipe-Lagos CB, Rocha RM, Natal D, et al. (1993). Anophelines in the State of Acre, Brazil, infected with *Plasmodium falciparum*, *P. vivax*, the variant *P. vivax* VK247 and *P. malariae*. *Trans. R. Soc. Trop. Med. Hyg*. 87: 391-394.

Branquinho MS, Araújo MS, Natal D, Marrelli MT, et al. (1996). *Anopheles oswaldoi* an important potential malaria vector in Acre, Brazil. *Trans. R. Soc. Trop. Med. Hyg*. 90: 233.

Causey OR, Deane LM and Deane MP (1946). Studies on Brazilian Anophelines from the northeast and Amazon regions. II. An illustrated key by male genitalic characteristics for the identification of thirty-four species of Anophelini, with a note on dissection technique. *Am. J. Hyg. Monogr. Ser*. 18: 21-34.

Clary DO and Wolstenholme DR (1985). The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol*. 22: 252-271.

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

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.

Flores-Mendoza C, Marrelli MT, Lourenço-de-Oliveira R, Neves JF, et al. (1998). Variações fenotípicas em populações de *Anopheles oswaldoi*. In: 6ª Reunião Nacional de Pesquisas em Malária. Livro de Resumos, Goiânia, GO, Brazil, 59.

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

Gorham JR, Stojanovich CJ and Scott HG (1967). Clave ilustrada para los anofelinos de Sudamerica Oriental. Communicable Disease Center, Public and Human Health Services, Atlanta, GA, USA.

Lane J (1953). Neotropical Culicidae. Vol. I. Universidade de São Paulo, São Paulo, SP, Brazil.

Lounibos LP, Duzak D and Linley JR (1997). Comparative egg morphology of six species of the Albimanus section of *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae). *J. Med. Entomol.* 34: 136-155.

Maddison WP and Maddison DR (1997). MacClade: analysis of phylogeny and character evolution. Version 3.07. Sinauer Associates, Massachusetts.

Marrelli MT, Malafronte RS, Flores-Mendoza C, Lourenço-de-Oliveira R, et al. (1999). Sequence analysis of the second internal transcribed spacer of ribosomal DNA in *Anopheles oswaldoi* (Diptera: Culicidae). *J. Med. Entomol.* 36: 679-684.

Mirabello L and Conn JE (2006). Molecular population genetics of the malaria vector *Anopheles darlingi* in Central and South America. *Heredity* 96: 311-321.

Moritz C, Dowling TE and Brown WM (1987). Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Ann. Rev. Ecol. Syst.* 18: 269-292.

Pedersen BV (1996). A phylogenetic analysis of Cuckoo Bumblebees (*Psithyrus*, Lepeletier) and Bumblebees (*Bombus*, Latreille) inferred from sequences of the mitochondrial gene cytochrome oxidase I. *Mol. Phylog. Evol.* 5: 289-297.

Roderick GK (1996). Geographic structure of insect populations: gene flow, phylogeography, and their uses. *Annu. Rev. Entomol.* 41: 325-352.

Sambrook J and Russel D (2001). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Scarpassa VM (2005). Isozyme similarity in *Anopheles oswaldoi* sensu lato (Diptera: Culicidae) from the Amazon Region, Brazil. *J. Med. Entomol.* 42: 319-326.

Simon C, Frati F, Beckenbach A, Crespi B, et al. (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.

Swofford DL (2003). PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sinauer Associates Inc., Sunderland, MA, USA.

Zhang DX and Hewitt GM (1997). Assessment of the universality and utility of a set of conserved mitochondrial COI primers in insects. *Insect Mol. Biol.* 6: 143-150.