Isoenzymatic Variability among Five *Anopheles* Species Belonging to the *Nyssorhynchus* and *Anopheles* Subgenera of the Amazon Region, Brazil

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An isoenzymatic comparative analysis of the variability and genetics differentiation among Anopheles *species was done in populations of* An. (Nys.) intermedius *and* An. (Ano.) mattogrossensis *of the* Anopheles *subgenus, and of* An. darlingi, An. albitarsis *and* An. triannulatus *of the* Nyssorhynchus *subgenus, with the aim of detecting differences between both subgenera and of estimating the degree of genetic interespecific divergence. Samples from Macapá, State of Amapá and Janauari Lake, near Manaus, State of Amazonas, were analyzed for eight isoenzymatic loci. Analysis revealed differences in the average number of alleles per locus (1.6-2.3) and heterozygosity (0.060-0.284). However, the proportion of polymorphic loci was the same for* An. (Nys.) darlingi, An. (Nys.) triannulatus *and* An. (Ano.) mattogrossensis *(50%), but differed for* An. (Nys.) albitarsis *(62.5%) and* An. (Ano.) intermedius *(25%). Only the IDH1 ($P > 0.5$) locus in all species studied was in Hardy-Weinberg equilibrium. The fixation index demonstrated elevated genetic structuring among species, based on values of $F_{st} = 0.644$ and genetic distance (0.344-0.989). Genetic difference was higher between* An. (Nys.) triannulatus *and* An. (Ano.) intermedius *(0.989) and smaller between* An. (Nys.) albitarsis sensu lato *and* An. (Nys.) darlingi *(0.344). The data show interspecific genetic divergence which differs from the phylogenetic hypothesis based on morphological characters.*

Key words: *Nyssorhynchus* - *Anopheles* - genetic variability - isozymes - Amazon - Brazil

The main vectors of human malaria in the Amazon are *Anopheles* mosquitoes of the *Nyssorhynchus* subgenus. Among them, *An. darlingi* is the main vector in Brazil, specially in the Amazon, also contributing to malaria endemicity throughout its distribution (WHO 1984, 1988). *An. albitarsis sensu lato* also transmits malaria in some regions of the country (Deane 1986, Tadei et al. 1993, Tadei & Dutary-Thatcher 2000). This species has recently been found positive by ELISA for *Plasmodium falciparum*, *P. vivax* and *P. malariae*, in the Brazilian states of Amapá and Roraima, and probably plays an important role in malaria transmission in these areas (Póvoa et al. 2001, Silva-Vasconcelos et al. 2002). *An. triannulatus sensu lato* has been found infected by *P. vivax* and *P. falciparum* in immunosorbent (ELISA) and immunoradiometric assays (IRMA) being considered a potential vector (Arruda et al. 1986, Tadei et al. 1988, 1991, Branquinho et al. 1993, Tadei & Dutary-Thatcher 2000). *An. darlingi* and *An. albitarsis* belong to the Argyritarsis Section whereas *An. triannulatus* belongs to the Albimanus Section, both in the *Nyssorhynchus* subgenus. The *Anopheles* subgenus has relatively few studies on the biology and genetics of the species comprised in this subgenus, in South America. This, despite the fact that some of the species in the *Anopheles* subgenus occur sympatrically with malaria vectors of the *Nyssorhynchus* subgenus, and at times biting man, although they have not been found to be infected with *Plasmodium* (Tadei et al. 1993). *An. (Ano.) intermedius* and *An. (Ano.) mattogrossensis*, of the Laticorn Section, are examples (Harbach 1994).

The species studied present a wide geographical distribution. *An. darlingi* has been recorded from Mexico to North Argentina, from east of the Andes to the Atlantic Coast of South America. In Brazil, it occurs in all states except for Santa Catarina and Rio Grande do Sul (Forattini 1962). *An. albitarsis* is the species of largest geographical distribution in Brazil, also extending to all countries of South America east of the Andes, from North of Guatemala to the North of Argentina (Rosa-Freitas 1988). *An. triannulatus* has been recorded throughout South America and in some regions of Central America (Lane 1953, Forattini 1962, Faran & Linthicum 1981). *An. intermedius* is distributed in all South America up to Mexico in Central America (Forattini 1962, Morales-Ayala 1971). *An. mattogrossensis* has been registered in all Amazonian countries (Forattini 1962, Garcia & Ronderos 1962, Ferreira 1964). In some regions of their wide distribution, all five species occur sympatrically. *An. intermedius* and *An. mattogrossensis* present exophilic habits, whereas *An. darlingi*, *An. albitarsis* and *An. triannulatus* present endophilic and exophilic habits, according to the geographic region (Deane et al. 1988, Tadei et al. 1993).

An additional complication for the correct taxonomy and geographic distribution is the fact that adult females of many *Anopheles* species are difficult to distinguish morphologically and most of them are treated as species

Research supported by the Program for the Protection of Brazilian Rainforest/MCT, Subprogram C&T/PPD G-7, and CTPETRO.

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Received 7 May 2002
Accepted 12 December 2002

complexes. That is the case for the complex *An. albitarsis* (Kreutzer et al. 1976, Steiner et al. 1982, Rosa-Freitas et al. 1990, Narang et al. 1993). Recent studies with enzymes electrophoresis and RAPD-PCR (random amplified polymorphic DNA-polymerase chain reaction), demonstrated the existence of four species in the *albitarsis* complex (Rosa-Freitas et al. 1990, Narang et al. 1993, Wilkerson et al. 1995a). Although members of the *albitarsis* complex have been incriminated as important vectors in the transmission of malaria, their actual role as vectors in a given area might be difficult to determine as they are morphologically indistinguishable (Wilkerson et al. 1995b). Morphological, behavioral and epidemiological differences have been observed in *An. triannulatus*, rendering more complicated the taxonomic status of this species, which in some cases is considered a highly polymorphic species, but in others two varieties (Consoli & Lourenço-de-Oliveira 1994, Silva do Nascimento 1995).

Although in the past the taxonomic status of *An. darlingi* was controversial (WHO 1984, 1988) due to small morphological variation (e.g. in the scales of hind 3-5 tarsomeres in specimens of the Belize populations, Harbach et al. 1993), little interpopulational variation has been found based on morphological, isoenzymatic and DNA analyses pointing for the existence of a single species (Linthicum 1988, Freitas-Sibajev et al. 1995, Manguin et al. 1999, Santos et al. 1999).

Genetic structure studies in *Anopheles* species have been intensified, mostly in the *Nyssorhynchus* subgenus, due to its epidemiological importance in malaria transmission. Nonetheless, little is known on the genetics of other species in the *Anopheles* subgenus in South America, such as *An. intermedius* and *An. mattogrossensis*.

The present study was conducted to determine the interpopulational genetics divergence and variability among three species of the *Nyssorhynchus* subgenus, *An. darlingi*, *An. triannulatus* and *An. albitarsis*, which includes the most important species in the transmission of malaria in the New World. It also includes two species in the *Anopheles* subgenus, *An. intermedius and An. mattogrossensis*, which have not been incriminated as vectors.

MATERIALS AND METHODS

Specimens and collection sites - Specimens of *An. darlingi* and *An. triannulatus* were from Mazagão (00º05'15.1''S, 51º17''21.4''W), *An. albitarsis* and *An. intermedius* from Rodovia Pacoval (00º02'19.8''N, 51º03'40.7''W), km 4, all near Macapá, State of Amapá. Specimens of *An. mattogrossensis* were from Janauari Lake (03º08'00.5''S, 60º00'34.7''W), near Manaus. Adults of the subgenus *Nyssorhynchus* were collected in areas of peri-domiciliary (human and cattle baits) and intradomiciliary (human bait), and those of the subgenus *Anopheles* were collected only in peri-domiciliary areas (cattle bait) between 18:00 and 22:00 h. Individual females were allowed to lay their eggs separately. Eggs were reared to larvae following the methodology described by Santos et al. (1981).

Electrophoretic analyses - Eight loci were analyzed: Esterase (EST5 - E.C.3.1.1.1.), Leucine aminopeptidase (LAP2, LAP5 - E.C.3.4.11), Isocitrate dehydrogenase (IDH1 - E.C.1.1.1.42), Phosphoglucomutase (PGM1 - E.C.2.7.5.1), Phosphoglucose isomerase (PGI1 - E.C.5.3.1.9), Xanthine dehydrogenase (XDH1 - E.C.1.2.1.37) and 6-Phosphogluconate dehydrogenase (6-PGDH - EC 1.1.1.44). Larvae were homogenized individually in 15 µl 0.5% β-mercaptoethanol. Filter paper Whatman no.3 (0.5 cm x 0.6 cm) was used to absorb the homogenate supernatant for horizontal electrophoresis. Isozymes were separeted in two types of electrophoretic support: starch (Sigma) gel, at a concentration of 12% and, starch-agarose gel at concentrations of 2% and 1%, respectively. The three buffer systems used were CA-7: gel buffer-0.009 M Tris, 0.003 M citric acid, pH 7.10; electrode buffer-0.135 M Tris, 0.040 M citric acid, pH 6.90 (Steiner & Joslyn 1979), modificated Poulik: gel buffer- 0.017 M Tris, 0.0023 M citric acid, pH 8.00; electrode buffer- 0.3 M boric acid, pH 8.00 (Contel 1980) and TEMM: electrode buffer-0.1 M Tris, 0.1 M maleic anhydride, 0.01 M EDTA, 0.01 M $MgCl_2$, pH 7.40, and a 1:15 dilution was used in the gel (Harris & Hopkinson 1976) and staining systems were those described in Contel (1980).

Statistical analyses - Five larvae from each progeny were used for each single enzymatic system and about 100 individuals, representative of 20 progeny were analyzed to infer the Mendelian inheritance standards of electromorphs for each isoenzyme locus. Isoenzymatic variation among species was analyzed using the Biosys-1 software (Swofford & Selander 1981). Genetic distance was calculated according to Nei (1978). Cluster grouping was done employing the UPGMA method (unweighted pair-group method, arithmetic average) using Biosys-1.

RESULTS

Of the eight loci analyzed, only PGI1 was monomorphic in the five species. The XDH1 locus was polymorphic for *An. mattogrossensis* and *An. triannulatus*. The 6PGDH locus was polymorphic for *An. albitarsis* and *An. mattogrossensis*. The LAP5 and PGM1 loci were polymorphic for all species. The EST5 locus revealed four alleles in the three species of the *Nyssorhynchus* subgenus, two alleles for *An. mattogrossensis* and one allele for *An. intermedius*, both in the *Anopheles* subgenus. LAP2 was monomorphic only for *An. darlingi* and IDH1 only for *An. mattogrossensis* (Table I). Only IDH1 displayed frequencies agreement to the distribution predicted by random mating for all the species studied. All other loci showed deviation of the genetic equilibrium by Hardy-Weinberg tests.

The average number of alleles per loci varied between 1.6 ± 0.30 for *An. intermedius* to 2.3 ± 0.4 for *An. triannulatus*. Out of the five species analyzed, *An. triannulatus*, *An. albitarsis* and *An. mattogrossensis* showed larger polymorphism (P = 0.75). *An. albitarsis* revealed high heterozygozity values (Ho = 0.196 ± 0.075; He = 0.284 ± 0.084). EST5, LAP2 and LAP5 loci (with Ho = 0.605, 0.451, 0.455, respectively) contributing to this result (Tables I, II).

Genetic distance (D) values (0.344-0.989) were consistent with interspecific genetic divergence. *An. (Nys.) triannulatus* and *An. (Ano.) intermedius* showed higher

TABLE I

Frequencies of alleles at eight loci in five *Anopheles* species belonging to the *Nyssorhynchus* and *Anopheles* subgenera, Amazon Region, Brazil

		Anopheles				
		Subgenus *Nyssorhynchus*			Subgenus *Anopheles*	
Locus	Allele	*An. triannulatus*	*An. darlingi*	*An. albitarsis*	*An. intermedius*	*An. mattogrossensis*
EST5						
(N)		104	143	105	101	93
	A	0.135	0.150	0.143	1.000	0.871
	B	0.572	0.605	0.367	0.000	0.129
	C	0.260	0.231	0.490	0.000	0.000
	D	0.034	0.014	0.000	0.000	0.000
χ^2 *H-W*		6.912	38.215 *a*	20.955 *a*	-	0.226
H		0.586	0.558	0.605	0.000	0.225
LAP2						
(N)		95	112	86	87	133
	A	0.842	1.000	0.657	0.983	0.970
	B	0.158	0.000	0.343	0.017	0.030
χ^2 *H-W*		27.228 *a*	-	18.570 *a*	56.998 *a*	0.111
H		0.266	0.000	0.451	0.034	0.058
LAP5						
(N)		84	114	87	77	114
	A	0.940	0.627	0.351	0.870	0.952
	B	0.060	0.373	0.649	0.130	0.048
χ^2 *H-W*		0.301	26.020 *a*	1.477	0.061	0.265
H		0.112	0.468	0.455	0.226	0.092
IDH1						
(N)		98	154	112	123	100
	A	0.000	0.000	0.000	0.016	0.000
	B	0.005	0.006	0.018	0.955	0.000
	C	0.964	0.932	0.982	0.028	1.000
	D	0.031	0.062	0.000	0.000	0.000
χ^2 *H-W*		0.115	0.783	0.028	0.244	-
H		0.069	0.128	0.035	0.086	0.000
PGM1						
(N)		115	178	120	106	103
	A	0.000	0.011	0.117	0.000	0.000
	B	0.104	0.885	0.804	0.071	0.000
	C	0.865	0.104	0.079	0.929	0.000
	D	0.030	0.000	0.000	0.000	0.728
	E	0.000	0.000	0.000	0.000	0.272
χ^2 *H-W*		23.982 *a*	97.121 *a*	7.488	0.571	5.071 *b*
H		0.240	0.206	0.333	0.131	0.396
PGI1						
(N)		112	112	112	110	110
	A	0.000	0.000	1.000	1.000	1.000
	B	0.000	1.000	0.000	0.000	0.000
	C	1.000	0.000	0.000	0.000	0.000
χ^2 *H-W*		-	-	-	-	-
H		0.000	0.000	0.000	0.000	0.000
XDH1						
(N)		120	120	120	37	37
	A	0.000	0.000	0.000	1.000	0.284
	B	0.000	0.000	0.000	0.000	0.716
	C	0.975	1.000	1.000	0.000	0.000
	D	0.025	0.000	0.000	0.000	0.000
χ^2 *H-W*		0.065	-	-	-	17.401 *a*
H		0.049	0.000	0.000	0.000	0.407
6-PGD						
(N)		56	56	112	117	101
	A	1.000	0.000	0.741	0.000	0.119
	B	0.000	1.000	0.259	1.000	0.881
χ^2 *H-W*		-	-	113.632 *a*	-	0.365
H		0.000	0.000	0.384	0.000	0.209

N: number of 4th instar larvae analyzed; *a*: P < 0.001; *b*: P < 0.05; H: heterozygosity; -: monomorphic locus

D (0.989) and *An. (Nys.) albitarsis* and *An. (Nys.) darlingi* displayed lower D (0.344), thus separating the subgenera (Table III). The presence of diagnostic alleles in PGI1 (B and C alleles), XDH1 (C and D alleles only in the *Nyssorhynchus* subgenus, and A and B alleles in the *Anopheles* subgenus) and PGM1 loci (A allele in the *Nyssorhynchus* subgenus and to allele E in *A. mattogrossensis* of *Anopheles* subgenus) also contributed to these results (Table I).

Species were separated in two groups, corresponding to both subgenera according to genetic distance values among species. Group I clustered the three species in the *Nyssorhynchus* subgenus. *An. darlingi* and *An. albitarsis* (Argyritarsis Section) had the highest identity value (I = 0.709) indicating low genetic differentiation and a close phylogenetic relationship, separated from *An. triannulatus* (Albimanus Section). Group II consisted of the two species of the *Anopheles* subgenus (Figure).

DISCUSSION

Genetic, biochemical and morphological studies in anopheline populations have contributed to the elucidation and identification of species complexes, which are of importance to a better knowledge of the malaria transmission and control. Cryptic species are very common in *Anopheles.* The *Nyssorhynchus* subgenus, which includes

TABLE II

Genetic variability at eight loci in five *Anopheles* species belonging to the *Nyssorhynchus* and *Anopheles* subgenera, Amazon Region, Brazil

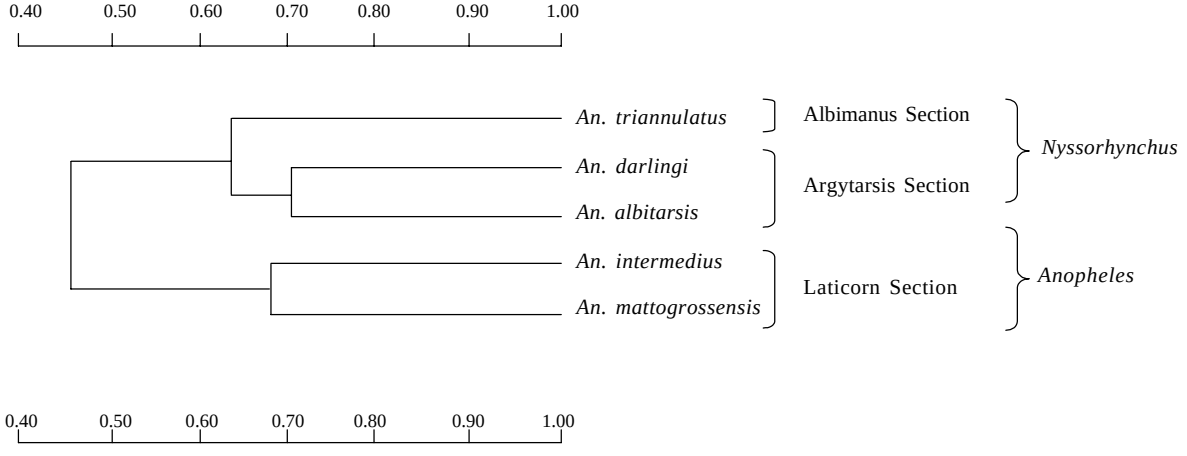
Population	Mean sample size/locus	Mean no. of alleles/locus	% polymorphic loci [a]	Mean Heterozygosity	
				Observed	Expected [b]
An. (*Nys.*) *triannulatus*	98.0 ± 7.3	2.3 ± 0.4	75	0.146 ± 0.073	0.166 ± 0.070
An. (*Nys*.) *darlingi*	123.6 ± 18	2.0 ± 0.4	50	0.171 ± 0.093	0.171 ± 0.080
An. (Nys.) albitarsis	106.8 ± 4.7	2.0 ± 0.3	75	0.196 ± 0.075	0.284 ± 0.084
An. (*Ano.*) *intermedius*	94.8 ± 9.8	1.6 ± 0.3	50	0.060 ± 0.031	0.060 ± 0.030
An. (*Ano.*) *mattogrossensis*	98.9 ± 9.8	1.8 ± 0.2	75	0.149 ± 0.056	0.175 ± 0.058

a: a locus was considered polymorphic if more than one allele was detected; *b*: expected heterozygosity of Hardy-Weinberg; estimate Nei's unbiased (Nei 1978)

TABLE III

Genetic identity (above diagonal) and distance coefficients (below diagonal) among five *Anopheles* populations (*Nyssorhynchus* and *Anopheles* subgenera) from Amazon Region, Brazil

Specie	1	2	3	4	5
1 *An.* (*Nys.*) *triannulatus*	*****	0.592	0.671	0.372	0.454
2 *An.* (*Nys.*) *darlingi*	0.524	*****	0.709	0.413	0.545
3 *An.* (*Nys.*) *albitarsis*	0.399	0.344	*****	0.399	0.566
4 *An.* (*Ano.*) *intermedius*	0.989	0.884	0.919	*****	0.689
5 *An.* (*Ano.*) *mattogrossensis*	0.789	0.607	0.568	0.373	*****



Unweighted pair-group method, arithmetic avarege dendrogram for five *Anopheles* species based on their genetic identity values (cophenetic correlation = 0.868).

species of great epidemiological importance in Brazil, had studies on genetic differentiation focused mostly on the level of the genetic variability of the populations and for cryptic species recognition (Rosa-Freitas et al. 1990, 1998, Santos et al. 1992, 1999, Narang et al. 1993, Wilkerson et al. 1995a, b, Manguin et al. 1999, Scarpassa et al. 1999). Even so, genetic studies with other sugbenera are still incipient, which might be related to their lack of importance in the malaria transmission.

Geographical differentiation in *An. darlingi* populations has been observed in chromosomes (Kreutzer et al. 1972, Tadei et al. 1982), morphology (Harbach et al. 1993), behavior (Charlwood & Hayes 1978, Charlwood & Wilkes 1979, Roberts et al. 1987, Rosa-Freitas et al. 1992) and hydrocarbon profile (Rosa-Freitas et al. 1992). Isozymes, RAPD and sequencing of the gene ITS2 showed however, no significant differences in Brazilian and other South America populations (Freitas-Sibajev et al. 1995, Manguin et al. 1999, Santos et al. 1999). For isoenzymes, most diagnostic loci that distinguish *An. darlingi* from other members of the *Nyssorhynchus* subgenus are monomorphic throughout their distribution area. This was also observed by us for LAP2 and PGI1 loci. PGI1 was also monomorphic for *An. albitarsis, An. triannulatus, An. intermedius* and *An. mattogrossensis* for different alleles. The EST5, LAP5, PGM1, IDH1, XDH1 and 6-PGD loci showed variation for two or more species.

Our data showed that most loci analyzed were polymorphic for each of the five species, with a relative elevated specific variability ($0.50 < P < 0.75$) when compared with other studies. Fritz et al. (1995) found a low percentage of polymorphic loci ($20.8 < P < 58.3$) among populations of *An. nuneztovari, An. trinkae* and *An. rangeli* from Brazil, Ecuador, Bolivia and Venezuela. These results might have been influenced by the small sample size, although other variability measures (mean heterozygosity, mean number of alleles) were not affected. In a study based on 33 enzymatic loci (Manguin et al. 1995), low polymorphism was found in populations of *An. pseudopunctipennis* (from Grenada and Chile, P = 12.1 and 39.4, respectively), *An. franciscanus* (P = 33.3 from California, USA) and, *An. crucians* (P = 39.4 from Belize) belonging to the subgenus *Anopheles*, and *An. albimanus* (P = 30.3) belonging to the subgenus *Nyssorhynchus*. According to the authors, the low polymorphism of *An. pseudopunctipennis* from Grenada is caused by the lack of heterozygotes due to the geographical isolation. However, in the mainland populations of *An. pseudopuctipennis* the polymorphism values ranges from 39.4% to 78.8%. Similar results were found by Scarpassa et al. (1999) in a study based on 19 enzymatic loci of the *An. nuneztovari* populations from Brazil and Colombia, in which the polymorphism varied from 31.3 to 56.6%. Rodriguez (1998) also observed low polymorphism in populations from Manaus of *An. (Ano.) intermedius* (P = 35.3, Ho = 0.051) and from Amapá of *An. (Ano.) mattogrossensis* (P = 47.1, Ho = 0.085), based in 18 isoenzymatic loci.

In our study, genetic variability (including locus polymorphism, heterozygosity, mean number of alleles/locus) was similar to other species of the *Nyssorhynchus* subgenus (Narang 1980, Steiner et al. 1982, Santos et al. 1985, 1992, 1999, Santos 1992, Fritz et al. 1995) and of the *Anopheles* subgenus (Lanzaro et al. 1990, Narang & Seawright 1994). Out of the five species, *An. (Ano.) intermedius* and *An. (Nys.) darlingi* displayed the lowest variability and degree of polymorphism for all the eight loci analyzed (P = 0.50). However, regarding heterozygosity, the lowest value found was for *An. (Ano.) intermedius* (0.060 ± 0.31), whereas *An. (Nys.) triannulatus* and *An. (Nys.) albitarsis* showed the highest polymorphism (P = 0.75) and more alleles per locus (2.3 ± 0.4 and 2.0 ± 0.3).

Genetic distances were the lowest between *An.* (*Nys.*) *albitarsis* and *An.* (*Nys.*) *darlingi* (0.344), and the highest between *An. (Nys.) triannulatus* and *An. (Ano.) intermedius* (0.989). These data are consistent with the interspecific limits for genetic variation, according to the values found for *An. rangeli, An. trinkae* and *An. nuneztovari* (0.319-0.440) (Fritz et al. 1995) and for *An. pseudopunctipennis, An. franciscanus, An. crucians* and *An. albimanus* (0.335-2.355) (Manguin et al. 1995).

The results from polymorphism presented in this paper showed interspecific genetic divergence separating the species in two groups, in accordance with their subgenera. Considering the genetic distance, the closest genetic relationship belonged to *An. darlingi* and *An. albitarsis* while the highest genetic divergence was between *An. darlingi* and *An. triannulatus*. Genetic distance values grouped the first two species in the same cluster and separated *An. triannulatus* from all other species. These data are in part discordant with the phylogenetic relation based on morphologic characters for the subgenus *Nyssorhynchus*, conducted by Sallum et al. (2000), who grouped *An. albitarsis* (Argyritarsis Section) and *An. triannulatus* (Albimanus Section) in the same clade, separated from *An. darlingi* (Argyritarsis Section).

ACKNOWLEDGEMENTS

To Dr Raul Guerra de Queiroz for help with the English version of the manuscript and the technicians of the Malaria and Dengue Vectors Laboratory for their technical support in the collections and identification of the mosquitoes. To the anonymous reviewers for their comments which greatly improved this manuscript.

REFERENCES

Arruda M, Carvalho MB, Nussenzweig RS, Maracic M, Ferreira AW, Cochrane AH 1986. Potential vectores of malaria and their different susceptibility to *Plasmodium falciparum* and *Plasmodium vivax* in northern Brazil identified by immunoassay. *Am J Trop Med Hyg 35*: 873-881.

Branquinho MS, Lagos CB, Rocha RM, Natal D, Barata JM, Cochrane AH, Nardin E, Nussenzweig RS, Kloetzel JK 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.

Charlwood JD, Hayes J 1978. Variações geográficas no ciclo de picadas do *Anopheles darlingi*, Root no Brasil. *Acta Amazonica 8*: 601-603.

Charlwood JD, Wilkes TJ 1979. Studies on the age composition of samples of *Anopheles darlingi*, Root in Brazil. *Bull Entomol Res 69*: 337-342.

Consoli RAGB, Lourenço-de-Oliveira R 1994. *Principais Mosquitos de Importância Sanitária no Brasil*, Fiocruz, Rio de Janeiro, 228 pp.

Contel EPB 1980. *Variabilidade Proteica em Populações Naturais de Abelhas da Amazônia*, PhD Thesis, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 158 pp.

Deane LM 1986. Malaria vectores in Brazil. *Mem Inst Oswaldo Cruz 81*(Suppl. II): 5-14.

Deane LM, Ribeiro CD, Lourenço-de-Oliveira R, Oliveira-Ferreira JA, Guimarães E 1988. Study on the natural history of malaria in areas of the Rondonia State, Brazil and problems related to its control. *Rev Inst Med Trop São Paulo 30*: 153-156.

Faran ME, Linthicum KJ 1981. A handbook of the Amazonian species of *Anopheles* (*Nyssorhynchus*) (Diptera, Culicidae). *Mosq Syst 13*: 1-81.

Ferreira E 1964. Distribuição geográfica dos anofelinos no Brasil e sua relação com o estudo atual da erradicação da malária. *Rev Bras Malar D Trop 16*: 329-348.

Forattini OP 1962. *Entomologia Médica*, Vol. 1, Faculdade de Higiene e Saúde Pública, São Paulo, 662 pp.

Freitas-Sibajev MGR, Conn J, Mitchell SE, Cockburn AF, Seawright JA, Momen H 1995. Mitochondrial DNA and morphological analyses of *Anopheles darlingi* populations from Brazil (Diptera: Culicidae). *Mosq Syst 27*: 78-99.

Fritz GN, Bermudez H, Seawright JA 1995. Genetic differentiation and diagnostic loci of *Anopheles nuneztovari*, *An. trinkae*, and *An. rangeli* (Diptera: Culicidae). *J Med Entomol 32*: 663-672.

Garcia M, Ronderos RA 1962. Mosquitos de la República Argentina. 1. Tribu Anophelini (Diptera-Culicidae-Culicinae). *An Com Inv Cient Prov Bs As 3*: 103-112.

Harbach RE 1994. Review of the internal classification of the genus *Anopheles* (Diptera: Culicidae): the foundation for comparative systematics and phylogenetic research. *Bull Entomol Res 84*: 331-342.

Harbach RE, Roberts DR, Manguin S 1993. Variation in the hindtarsal markings of *Anopheles darlingi* (Diptera: Culicidae) in Belize. *Mosq Syst 25*: 192-197.

Harris H, Hopkinson DA 1976. *Handbook of Enzyme Electrophoresis in Human Genetics*, North-Holland Publishing Co., Amsterdam, 323 pp.

Kreutzer RD, Kitzmilller JB, Ferreira E 1972. Inversion polymorphism in the salivary gland chromosomes of *Anopheles darlingi*. *Mosq News 32*: 555-556.

Kreutzer RD, Kitzmiller JB, Rabbani MG 1976. Cytogenetically distinguishable sympatric and allopatric populations of the mosquito *Anopheles albitarsis*. *Acta Amazonica 6*: 473-481.

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

Lanzaro GC, Narang SK, Seawright JA 1990. Speciation in an Anopheline (Diptera: Culicidae) mosquito: enzyme polymorphism and the genetic structure of populations. *Ann Entomol Soc Am 83*: 578-585.

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

Manguin S, Roberts DR, Peyton EL, Fernandez-Salas I, Barreto M, Fernandez-Loayza R, Spinola RE, Granaou RM Rodriguez MH 1995. Biochemical systematics and population genetic structure of *Anopheles pseudopunctipennis*, vector of malaria in Central and South America. *Am J Trop Med Hyg 53*: 362-377.

Manguin S, Wilkerson RC, Conn JE, Rubio-Palis Y, Danoff-Burg JA, Roberts DR 1999. Population structure of the primary malaria vector in South America *Anopheles darlingi*, using isozyme, random amplified polymorphic DNA, internal transcribed spacer 2, and morphologic markers. *Am J Trop Med Hyg 60*: 364-376.

Morales-Ayala F 1971. A list of the mosquitoes of Peru (Diptera: Culicidae). *Mosq Syst 3*: 138-145.

Narang SK 1980. Genetic variability in natural populations, evidence in support of the selectionist view. *Experientia 36*: 50-51.

Narang SK, Seawright JA 1994. Genetic differentiation among members of species complexes in anopheline mosquitoes (Diptera: Culicidae). In RC Sobti, *The Eukaryotic Chromosome-structural and Functional Aspects*, Narosa, New Delhi, p. 59-96.

Narang SK, Klein TA, Perera OP, Lima JB, Tang AT 1993. Genetic evidence for the existence of cryptic species in the *Anopheles albitarsis* complex in Brazil: allozymes and mitochondrial DNA restriction fragment length polymorphisms. *Biochem Gen 31*: 97-112.

Nei M 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics 89*: 583-590.

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

Roberts DR, Alecrim WD, Tavares AM, Radke MG 1987. The house-frequenting, host-seeking and resting behavior of *Anopheles darlingi* in Southeastern Amazonas, Brazil. *J Am Mosq Control Assoc 3*: 433-441.

Rodriguez GAD 1998. *Padrões Isoenzimáticos e Variabilidade Genética em Anopheles (Anopheles) intermedius Chagas, 1908 e Anopheles (Anopheles) mattogrossensis Lutz & Neiva, 1911 (Diptera: Culicidae) da Amazônia Brasileira*, MSc Thesis, Instituto Nacional de Pesquisas da Amazônia, Manaus, 107 pp.

Rosa-Freitas MG 1988. *Anopheles albitarsis Lynch-Arribálzaga, 1878 (Diptera; Culicidae): Um Estudo Comparativo de Caracteres Morfológicos, Bioquímicos e Comportamentais de Populações de Dez Localidades*, MSc Thesis, Instituto Oswaldo Cruz, Rio de Janeiro, 105 pp.

Rosa-Freitas MG, Broomfield G, Priestman A, Milligan PJM, Momen H, Molyneux DH 1992. Cuticular hydrocarbons, isoenzymes and behavior of three populations of *Anopheles darlingi* from Brazil. *J Am Mosq Control Assoc 8*: 357-366.

Rosa-Freitas MG, Deane LM, Momen H 1990. A morphological, isoenzymatic and behavioural study of ten populations of *Anopheles* (*Nyssorhynchus*) *albitarsis* Lynch-Arribalzaga, 1878 (Diptera: Culicidae) including from the type-locality - Baradero, Argentina. *Mem Inst Oswaldo Cruz 85*: 275-289.

Rosa-Freitas MG, Lourenço-de-Oliveira R, Carvalho-Pinto CJ, Flores-Mendoza C, Silva-do-Nascimento TF 1998. Anopheline species complexes in Brazil. Current knowledge of those related to malaria transmission. *Mem Inst Oswaldo Cruz 93*: 651-655.

Sallum MAM, Schultz TR, Wilkerson RC 2000. Phylogeny of Anophelinae (Diptera Culicidae) based on morphological characters. *Ann Entomol Soc Am 93*: 745-775.

Santos JMM 1992. *Variabilidade Genética em Populações Naturais de Anopheles (Nyssorhynchus) darlingi Root, 1926 (Diptera: Culicidae)*, PhD Thesis, Instituto Nacional de Pesquisas da Amazônia, Manaus, 150 pp.

Santos JMM, Contel EPB, Kerr WE 1981. Biologia de anofelinos amazônicos. Ciclo biológico, postura e estádios larvais de *Anopheles darlingi* Root, 1926 (Diptera: Culicidae) da Rodovia Manaus/Boa Vista. *Acta Amazonica 11*: 789-797.

Santos JMM, Contel EPB, Kerr WE 1985. Biology of Amazo-

nian mosquitoes. III. Esterases isozymes in *Anopheles darlingi*. *Acta Amazonica 15*: 167-177.

Santos JMM, Lobo JA, Tadei WP, Contel EPB 1999. Intrapopulational genetic differentiation in *Anopheles* (*N.*) *darlingi* Rot, 1926 (Diptera: Culicidae) in the Amazon region. *Gen Mol Biol 22*: 325-331.

Santos JMM, Tadei WP, Contel EPB 1992. Biologia de anofelinos amazônicos. XIV. Isoenzimas de esterase em *Anopheles triannulatus* (Neiva & Pinto, 1922). *Acta Amazonica 22*: 219-228.

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

Silva do Nascimento TF 1995. *Estudo Taxonômico e Notas sobre a Biologia de Anopheles triannulatus (Neiva & Pinto, 1922) de Oito Localidades*, MSc Thesis, Rio de Janeiro, Instituto Oswaldo Cruz, 87 pp.

Silva-Vasconcelos A, Kató MYN, Mourão EN, Souza RTL, Lacerda RNL, Sibajev A, Tsouris P, Póvoa MM, Momen H, Rosa-Freitas MG 2002. Biting indices, host-seeking activity and natural infection rates of anopheline species in Boa Vista, Roraima, Brazil from 1996 to 1998. *Mem Inst Oswaldo Cruz 97*: 151-161.

Steiner WWM, Joslyn DJ 1979. Electrophoretic techniques for the genetic studies of mosquitoes. *Mosq News 39*: 35-54.

Steiner WWM, Narang S, Kitzmiller JB, Swofford DL 1982. Genetic diversity and evolution in neotropical *Anopheles* (subgenus *Nyssorhynchus*). In WWM Steiner, WJ Tabachnick, KS Rai, SK Narang (eds), *Recent Developments in the Genetics of Insect Disease Vectors*, Stipes, Champaign, IL, p. 523-550.

Swofford DL, Selander RK 1981. BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *J Hered 72*: 281-283.

Tadei WP, Dutary-Thatcher B 2000. Malaria vectors in the Brazilian Amazon: *Anopheles* of the subgenus *Nyssorhynchus*. *Rev Inst Med Trop São Paulo 42*: 87-94.

Tadei WP, Ferreira AW, Ávila SLM, Nussenzweig RS, Xavier PA, Lima IENS 1991. Prevalence of *Plasmodium* spp. in *Anopheles* spp. in goldmining areas of Amapá State, Brazil detected by immuno-enzimatic assay. IV International Congress on Malaria and Babesiosis (Abstracts), Fiocruz/Foundation Internationale Laveran, Rio de Janeiro, ab. 10.34.

Tadei WP, Santos JMM, Costa WLS, Scarpassa VM 1988. Biologia de anofelinos amazônicos XII. Ocorrência de espécies de *Anopheles*, dinâmica de transmissão e controle da malária na zona urbana de Ariquemes (Rondônia). *Rev Inst Med Trop São Paulo 30*: 221-251.

Tadei WP, Santos JMM, Rabbani MG 1982. Biologia de anofelinos amazônicos. V. Polimorfismo cromossômico de *Anopheles darlingi* Root (Diptera: Culicidae). *Acta Amazonica 12*: 353-369.

Tadei WP, Santos JMM, Scarpassa VM, Rodrigues IB 1993. Incidência, distribuição e aspectos ecológicos de espécies de *Anopheles* (Diptera: Culicidae), em regiões naturais e sob impacto ambiental da Amazônia brasileira. In EJG Ferreira, GM Santos, ELM Leão, LA Oliveira (eds), *Bases Científicas para Estratégias de Preservação e Desenvolvimento da Amazônia*, Instituto Nacional de Pesquisas da Amazônia, Manaus, Vol. 2, p. 167-196.

WHO-World Health Organization 1984. Malaria vector species complexes and intra-specific variations: relevance for malaria control and orientation for further research. Report of an informal consultation of the Scientific Working Group on Applied Field Research in Malaria. TDR/FIELDMALSWG 930/84.3.

WHO-World Health Organization 1988. Report on a technical consultation on research in support of malaria control in the Amazon Basin. TDR/FIELDMAL/SC/AMAZ/88.3.

Wilkerson RC, Gaffigan TV, Lima JB 1995a. Identification of species related to *Anopheles* (*Nysssorhynchus*) *albitarsis* by random amplified polymorphic DNA-polymerase chain reaction (Diptera: Culicidae). *Mem Inst Oswaldo Cruz 90*: 721-732.

Wilkerson RC, Parsons TJ, Klein TA, Gaffigan TV, Bergo E, Consolim J 1995b. Diagnosis by random amplified polymorphic DNA polymerase chain reaction of four cryptic species related to *Anopheles* (*Nyssorhynchus*) *albitarsis* (Diptera: Culicidae) from Paraguay, Argentina, and Brazil. *J Med Entomol 32*: 697-704.