Molecular Confirmation of the Specific Status of *Anopheles halophylus* (Diptera: Culicidae) and Evidence of a New Cryptic Species within *An. triannulatus* in Central Brazil

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ABSTRACT *Anopheles halophylus* Silva-do-Nascimento & Lourenço-de-Oliveira was recently described using morphological and biological variants in specimens previously identified as *Anopheles triannulatus* (Neiva & Pinto). Because these two species occur in sympatry in central Brazil, we used allozymes to determine the extent of gene flow to confirm that they are different species. Of 11 allozyme loci analyzed, one (*Mpi*) was found to be diagnostic for *An. halophylus* and *An. triannulatus*, confirming their specific status. This locus revealed a second sibling species within *An. triannulatus* sensu lato. *An. halophylus* and the new undescribed species were confirmed using random amplified polymorphic DNA markers that showed moderate genetic divergence among these three sympatric and closely related taxa (D = 0.145–0.428). Moreover, this marker indicates that *An. halophylus* and the new species are more closely related to each other than either is to *An. triannulatus*.

KEY WORDS *Anopheles*, taxonomy, Culicidae, allozymes, RAPD

Several important malaria vectors are members of morphologically similar (sometimes indistinguishable) species complexes. The *Anopheles* subgenus *Nyssorhynchus* is one of the most studied subgenera because of the importance of its members as malaria vectors in South and Central America.

Anopheles triannulatus (Neiva & Pinto) is a polymorphic species with a wide geographic distribution, ranging from Argentina to Nicaragua (Faran and Linthicum 1981), and recently reported from the island of Trinidad, in the West Indies (Chadee and Wilkerson 2005). Although the species is essentially zoophilic, it seems to be able to transmit malaria when in high densities (Charlwood and Wilkes 1981); thus, it has been considered to be a potential vector in some areas of Latin America (Rubio-Palis 1994). In Brazil, the morphological variability of this species has resulted in five names currently in synonymy (Faran 1980) and the designation of two subspecies (Galvão and Lane 1941). This issue was a matter of controversy because although they were recognized by some researchers (Deane et al. 1947), others still considered *An. triannulatus* to be a single polymorphic species (Faran 1980).

Recent studies have attempted to distinguish *An. triannulatus* from morphologically similar species based on multiplex polymerase chain reaction (PCR) assays (Fritz et al. 2004) as well as determine its phylogenetic position within the *Nyssorhynchus* subgenus (Marrelli et al. 2005).

Morphological studies on *An. triannulatus* from several localities in Brazil, Peru, and Argentina revealed the existence of two distinct sympatric forms in Salobra, central Brazil, *An. triannulatus* and the recently described *Anopheles halophylus* Silva-do-Nascimento and Lourenço-de-Oliveira, 2002. However, certain individuals seemed to share morphological characters with both *An. halophylus* and *An. triannulatus* (Silva-do-Nascimento and Lourenço-de-Oliveira, 2002) and could thus represent natural hybrids between them. We used allozymes and random amplification of polymorphic DNA (RAPD) to genetically compare sympatric *An. triannulatus* and *An. halophylus* from Salobra to corroborate their status as separate species and to shed light on the identity of the specimens presenting the variant morphology.

Materials and Methods

Mosquito Sampling and Collection Site. Two field trips were carried out in August 1996 and July 1997 to collect female *An. triannulatus* and *An. halophylus* (undescribed at the time) in Salobra (20°12′40″ S, 56°29′30″ W), Mato Grosso do Sul State, Brazil. Salobra is in the Pantanal region of Central Brazil, an area of nearly 140,000 km^2 subjected to annual flooding in the rainy season, from November to June (Galdino and Resende 2000). Blood-fed females were collected

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Table 1. Gene frequencies for 11 allozyme loci for sympatric *An. triannulatus*, *An. halophylus*, and the new species from Salobra, Brazil

Locus	Allele	Species		
		An. triannulatus	*An. halophylus*	New species
Fum	100 (n)	**1.000** (30)	**1.000** (23)	**1.000** (16)
α-Gpdh	108	0.019	0.000	0.000
	100 (n)	**0.981** (27)	**1.000** (41)	**1.000** (16)
Gpi	100 (n)	**1.000** (41)	**1.000** (51)	**1.000** (22)
Hk-1	100	**1.000**	**1.000**	**0.932**
	93 (n)	0.000 (24)	0.000 (27)	0.068 (22)
Hk-2	100 (n)	**1.000** (26)	**1.000** (27)	**1.000** (22)
Idh-1	104	0.047	0.020	0.031
	100	**0.953**	**0.922**	**0.938**
	102 (n)	0.000 (32)	0.059 (51)	0.031 (16)
Idh-2	102	0.000	0.000	0.156
	100	0.413	**0.946**	**0.812**
	103 (n)	**0.587** (23)	0.054 (37)	0.031 (16)
Mdh-1	104	0.048	0.000	0.000
	100 (n)	**0.952** (21)	**1.000** (36)	**1.000** (16)
Mdh-2	100 (n)	**1.000** (29)	**1.000** (45)	**1.000** (16)
Mpi	111	0.000	0.104	0.000
	109	0.000	**0.896**	0.000
	103	0.000	0.000	0.016
	100	0.000	0.000	**0.984**
	93	0.221	0.000	0.000
	86	**0.353**	0.000	0.000
	82	0.147	0.000	0.000
	78 (n)	0.229 (34)	0.000 (48)	0.000 (31)
Pgm	103	0.013	0.000	0.000
	100	**0.900**	**0.898**	**1.000**
	97	0.000	0.082	0.000
	95 (n)	0.087 (40)	0.020 (49)	0.000 (16)

n is number of individuals analyzed. Most frequent alleles per locus per species are in bold.

from horse bait between 1800 and 2100 hours for seven consecutive days on each occasion. Mosquitoes were identified (Consoli and Lourenço-de-Oliveira 1994), individually separated into labeled vials to lay eggs, and the progeny were separately reared. For *An. halophylus*, however, complete larval development was only achieved when brackish water from the local breeding sites was added to the rearing containers (Silva-do-Nascimento and Lourenço-de-Oliveira 2002). A single mosquito from each F1 was used for subsequent molecular analyses. Eggs, fourth instars, and adults of each progeny were stored in 4% glutaraldehyde, 70% ethanol, or pinned, respectively, for further morphological analyses.

Allozymes. Each mosquito was homogenized in 25 μl of lysis buffer (0.05 mM Tris-HCl, pH 8.0, 0.01 mM EDTA, 15 mM dithiothreitol, 0.01 mM ϵ-amino-*n*-caproic acid, and 1% Triton X-100). Two μl of each homogenate were loaded onto a 1% agarose gel and subjected to electrophoresis as described by Momen and Salles (1985) and stained according to Manchenko (1994). Specimens were analyzed for eight enzymes: fumarate hydratase (FUM, E.C. 4.2.1.2.), glucose phosphate isomerase (GPI, E.C. 5.3.1.9.), α-glycerophosphate dehydrogenase (α-GPDH, E.C. 1.1.1.8.), hexokinase (HK, E.C. 2.7.1.1.), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42.), malate dehydrogenase (MDH, E.C. 1.1.1.37.), mannose phosphate isomerase (MPI, E.C. 5.3.1.8.), and phosphoglucomutase (PGM, E.C. 2.7.5.1.). Genotype frequencies for each locus were obtained directly from the electrophoretic phenotypes. The most frequent electromorph for each enzyme was designated arbitrarily as the "100" reference allele, and motilities for the other electromorphs were determined relative to this common electromorph.

Genepop online (version 3.4) (Raymond and Rousset 1995) was used to calculate allele frequencies and agreement with Hardy–Weinberg (H-W) equilibrium. F_{IS} was computed as in Weir and Cockerham (1984). Goodness-of-fit to H-W expectations was assessed for each locus in each species by using the exact probability test available in Genepop. Levels of genetic variability (i.e., number of alleles per locus, percentage of polymorphic loci, and observed and expected heterozygosities) were calculated for the three species with the Biosys-1 software (Swofford and Selander 1981).

RAPD-Polymerase Chain Reaction (PCR). DNA isolation followed the protocol described in Wilkerson et al. (1993). Sixty-seven decamer primers (Operon Technologies, Alameda, CA) were screened and 14 (A8, A11, A19, B3, B7, B8, B10, B11, B15, C4, C10, C16, D15, and D16) were selected on the basis of their reproducibility, polymorphism, and efficiency in the PCR amplification. The reproducibility of the amplification was tested using the same DNA sample in three different PCR reactions. These primers were used to amplify five to six specimens of each of the three putative species from Salobra. The procedure was essentially as described in Wilkerson et al. (1995), except that 40 PCR cycles were used.

RAPD band patterns were interpreted following Wilkerson et al. (1993). It was assumed that the populations were in H-W equilibrium and that bands that comigrate are homologous. RAPD markers were analyzed using the software program TFPGA version 1.3 (Miller 1997) to calculate Nei's unbiased genetic distances and characterized using the unweighted pair group method with arithmetic means (UPGMA). Re-

Table 2. Genetic variability in *An. triannulatus*, *An. halophylus*, and the new species

Species	Mean sample size/locus	Mean no. of alleles/locus	Polymorphic loci (%)[a]	Mean heterozygosity	
				Ho	He
An. triannulatus	28.4 (2.4)	1.8 (0.3)	0.45	0.142 (0.071)	0.153 (0.074)
An. halophylus	39.5 (3.1)	1.5 (0.2)	0.27	0.054 (0.024)	0.057 (0.025)
New species	19.0 (1.5)	1.6 (0.2)	0.36	0.061 (0.035)	0.055 (0.031)

Standard error values are in parentheses

[a] A locus was considered polymorphic if the frequency of the most common allele was ≤0.95.

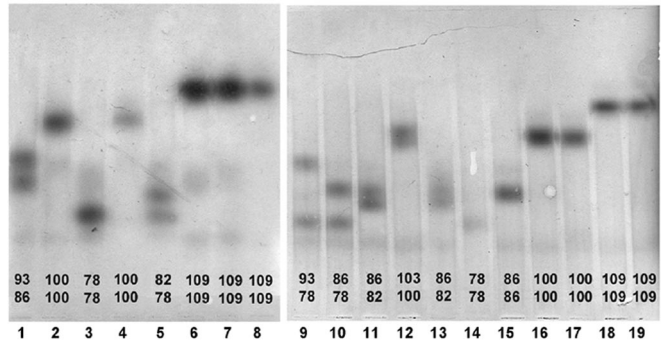

Fig. 1. *Mpi* agarose gel showing genotypes for *An. triannulatus* (samples 1, 3, 5, 9–11, and 13–15), *An. halophylus* (samples 6–8, 18, 19), and the new species (samples 2, 4, 12, 16, and 17).

liability of the groups in the phenogram was assessed through 1,000 bootstrap replicates of the alleles.

Results

Allozymes. In total, 123 specimens were analyzed. Allele frequencies for the 11 enzyme loci scored are given in Table 1. Six loci (*Fum*, *α-Gpdh*, *Gpi*, *Hk-2*, and *Mdh-1* and *-2*) were monomorphic for all samples. A locus was considered monomorphic if the frequency of the most common allele was >0.95 (Tables 1 and 2).

The *Mpi* locus was diagnostic (sensu Ayala 1983) between *An. triannulatus* and *An. halophylus*, corroborating their morphological separation. Furthermore, this locus revealed a third isolated genetic group composed of the variant individuals that share morphological characters with both *An. triannulatus* and *An. halophylus* (Fig. 1; Table 1). The analysis of the variant individuals together with either *An. triannulatus* or *An. halophylus*, would lead to strong departures from the H-W equilibrium ($F_{IS} = 0.521$, $P < 0.001$; $F_{IS} = 0.841$, $P < 0.001$, respectively) for this locus, as opposed to the lack of disequilibrium when the three species are analyzed separately ($F_{IS} = 0.044$, $P = 0.109$, for *An. triannulatus*, and $F_{IS} = 0.340$, $P = 0.059$, for *An. halophylus;* there is no F_{IS} value for the variant individuals as a single heterozygote was detected; Table 3). The presence of different alleles in the *Mpi* locus for the three putative species was observed not only when insects from the two consecutive field trips (1996 and 1997) were analyzed together but also when samples from each year were analyzed separately (data not shown). Significant (although not diagnostic, as with *Mpi*) differences in allele frequencies among the three species were detected for the *Idh-2* locus ($\chi^2 = 2.25$, df = 2, $P < 0.002$, between *An. halophylus* and the new species; $\chi^2 = 42.05$, df = 1, $P < 0.0001$, between *An. halophylus* and *An. triannulatus;* and $\chi^2 = 28.64$, df = 2, $P < 0.0001$, between the new species and *An. triannulatus*), whereas the *Pgm* loci presented differences only between *An. halophylus* and *An. triannulatus* ($\chi^2 = 11.68$; df = 3, $P < 0.001$).

No departures from H-W equilibrium were observed for the other four polymorphic loci (*Hk-1*, *Idh-1* and *-2*, and *Pgm*) for any of the three species ($-0.161 < F_{IS} < 0.340$, $P > 0.05$) (Table 3). The mean number of alleles per locus ranged from 1.5 to 1.8, the percentage of polymorphic loci from 27 to 45%, and the mean expected heterozygosity (*He*) from 0.055 to 0.153 (Table 2).

RAPD-PCR. Five *An. triannulatus*, six *An. halophylus*, and six individuals of variant morphology were amplified with 14 primers, producing 123 reproducible bands. Bands ranged in size from 310 to 2,300 bp. Average genetic distance (*D;* Nei 1978) between *An. triannulatus* and *An. halophylus* was 0.288; between *An. triannulatus* and the morphological variant was 0.428, and between *An. halophylus* and the morphological variant was 0.145, supporting the observations seen with allozymes.

A UPGMA dendrogram based on Nei's distances was constructed, revealing the existence of three well supported clusters (bootstrap values ≥78), each containing a single species consistent with the *Mpi* allozyme segregation of the three species (Fig. 2).

Table 3. Wright's fixation index (F_{IS}) for *An. triannulatus*, *An. halophylus*, and the new species

Locus	F_{IS}		
	An. triannulatus	*An. halophylus*	New species
Hexokinase-1	—	—	−0.050 ns
Isocitrate dehydrogenase-1	−0.033 ns	−0.058 ns	−0.017 ns
Isocitrate dehydrogenase-2	0.036 ns	−0.043 ns	−0.161 ns
Manose-6-phosphate-isomerase	0.044 ns	0.340 ns	—
Malate dehydrogenase-1	−0.026 ns	—	—
Phosphoglucomutase	−0.085 ns	−0.084 ns	—

—, a single allele was detected; ns, nonsignificant ($P > 0.05$).

Discussion

Allozyme and RAPD markers confirmed that there are genetic differences between *An. halophylus* and *An. triannulatus* and revealed the existence of a third sympatric species in the Triannulatus Complex. The lack of shared *Mpi* alleles and moderate RAPD-based genetic distances among the three forms collected in

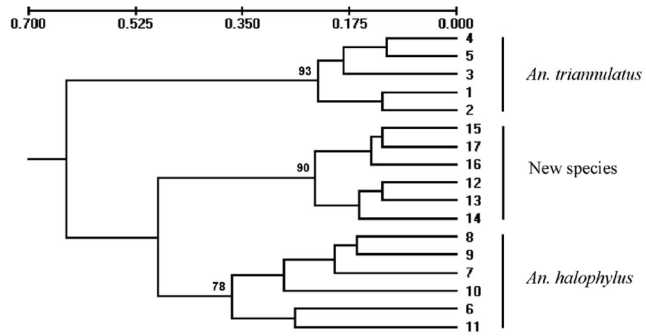

Fig. 2. UPGMA dendrogram constructed based on RAPD-derived distance matrix (Nei 1978) for *An. triannulatus*, *An. halophylus*, and the new undescribed species. Numbers represent individual mosquitoes tested using RAPD. Numbers to the left of each group are bootstrap support values.

sympatry in Salobra demonstrate the occurrence of three different biological species in that region. In addition to the *Mpi* diagnostic locus, there are significant gene frequency differences in the *Idh-2* locus among the three species (see Results).

The existence of fixed or diagnostic alleles between sympatric populations is evidence for a barrier to gene flow among the populations and that they are different species. The observation that the diagnostic difference at the *Mpi* locus was detected in independent collections in two consecutive years reinforces that conclusion and rules out the possibility of the specimens with intermediate morphology being natural hybrids between *An. halophylus* and *An. triannulatus.* Natural hybrids would present different combinations of the *Mpi* alleles present in both parental species (78, 82, 86, 93, 109, and 111), instead of the new allelic variants observed (alleles 100 and 103; Table 1; Fig. 1).

An. triannulatus, *An. halophylus*, and the new species presented heterozygosity values ranging from 0.055 ± 0.031 to 0.153 ± 0.074. These values are similar to what has been reported for other anopheline species (Manguin et al. 1999, Santos et al. 2004).

Salobra is located in the Pantanal region of central Brazil, which is an immense alluvial plain with a complex ecological system that includes seasonally flooded grasslands, gallery and dry forests, numerous river corridors, and lakes of fresh and brackish water. Salobra (Portuguese for brackish water) is at the Miranda River margins, one of the major tributaries of the Paraguay River. The Miranda River originates in the Serra da Bodoquena, where the calcareous soil contributes to the high calcium carbonate and chloride concentration in the water. During the rainy season, both fresh and brackish water habitats are present around Salobra, providing a variety of ecosystems for mosquito breeding. It was noted that the water of one breeding site of *An. halophylus* had a high concentration of chloride (from 2.0 to 4.0 mg/liter) that was critical for larval development. In contrast, *An. triannulatus* breeds solely in freshwater (Silva-do-Nascimento and Lourenço-de-Oliveira 2002). Therefore, the chemical composition of breeding site waters may be selective for species development in Salobra and elsewhere in the Paraguay basin.

Results of both allozyme and RAPD analyses of *An. triannulatus* and *An. halophylus* from Salobra are in accordance with previous morphological findings (Silva-do-Nascimento and Lourenço-de-Oliveira 2002) and reveal the existence of a third taxon in the Triannulatus Complex. Although females of the three species are morphologically indistinguishable, *An. triannulatus* and *An. halophylus* males, larvae, and eggs may be distinguished based on several characters (Silva-do-Nascimento and Lourenço-de-Oliveira 2002). The specimens that belong to the new species were initially classified as variants because they shared morphological characters with both *An. triannulatus* and *An. halophylus.* For example, the new species presents usually long lateral arms of the median plate of the spiracular apparatus similar to *An. triannulatus*, as opposed to the rudimentary or completely absent arms of *An. halophylus.* However, with respect to the male genitalia, the new species presents broad and usually directed proximally apicolateral lobes of the claspette, similarly to *An. halophylus*, but different from the narrow structures that characterize *An. triannulatus.* In addition, we cannot associate this taxon with any of the current *An. triannulatus* synonyms, which led us to conclude that it represents an undescribed species (see Silva-do-Nascimento and Lourenço-de-Oliveira 2002 for a discussion). Work toward the description and diagnosis of the new species is underway.

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