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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Morphology, Systematics, Evolution

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

T. F. Silva-do-Nascimento,1 R. C. Wilkerson,2 R. Lourenço-de-Oliveira,1 and F. A. Monteiro3


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 (Marreli 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 km² subjected to annual flooding in the rainy season, from November to June (Galdino and Resende 2000). Blood-fed females were collected...
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 species with the Biosys-1 software (Swofford and Seander 1981).

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean sample size/locus</th>
<th>Mean no. of alleles/locus</th>
<th>Polymorphic loci (%)</th>
<th>Mean heterozygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ho</td>
</tr>
<tr>
<td>An. triannulatus</td>
<td>28.4 (2.4)</td>
<td>1.8 (0.3)</td>
<td>0.45</td>
<td>0.142 (0.071)</td>
</tr>
<tr>
<td>An. halophylus</td>
<td>30.5 (3.1)</td>
<td>1.5 (0.2)</td>
<td>0.27</td>
<td>0.054 (0.024)</td>
</tr>
<tr>
<td>New species</td>
<td>19.0 (1.5)</td>
<td>1.6 (0.2)</td>
<td>0.36</td>
<td>0.061 (0.035)</td>
</tr>
</tbody>
</table>

*Standard error values are in parentheses

* A locus was considered polymorphic if the frequency of the most common allele was ≤0.95.
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 (Table 1). The analysis of the variant individuals with both species; Mpi was diagnostic (sensu Ayala 1983) 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).

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

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

<table>
<thead>
<tr>
<th>Locus</th>
<th>An. triannulatus</th>
<th>An. halophylus</th>
<th>New species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase-1</td>
<td>−</td>
<td>−</td>
<td>−0.050 ns</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase-1</td>
<td>−0.033 ns</td>
<td>−0.058 ns</td>
<td>−0.017 ns</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase-2</td>
<td>0.036 ns</td>
<td>−0.043 ns</td>
<td>−0.161 ns</td>
</tr>
<tr>
<td>Manose-6-phosphate-isomerase</td>
<td>0.044 ns</td>
<td>0.340 ns</td>
<td>−</td>
</tr>
<tr>
<td>Malate dehydrogenase-1</td>
<td>−0.026 ns</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>−0.085 ns</td>
<td>−0.084 ns</td>
<td>−</td>
</tr>
</tbody>
</table>

−, a single allele was detected; ns, nonsignificant ($P > 0.05$).
sympathy 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 claspsate, 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 lead 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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