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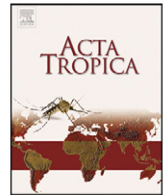

Phylogenetic relationships among species of *Anopheles* (*Nyssorhynchus*) (Diptera, Culicidae) based on nuclear and mitochondrial gene sequences

Brian P. Bourke[a,*], Peter G. Foster[b], Eduardo S. Bergo[c], Daniela C. Calado[a], Maria Anice M. Sallum[a]

[a] *Departamento de Epidemiologia, Faculdade de Saúde Pública, Universidade de São Paulo, Av. Dr. Arnaldo, 715 sala 200 Cerquiera Cesar, São Paulo, SP, Brazil*
[b] *The Natural History Museum, Department of Zoology, Cromwell Rd., London SW7 5BD, United Kingdom*
[c] *Superintendência de Controle de Endemias, Secretaria de Estado da Saúde de São Paulo, Araraquara, SP, Brazil*

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ABSTRACT

Phylogenetic relationships among 21 species of mosquitoes in subgenus *Nyssorhynchus* were inferred from the nuclear *white* and mitochondrial NADH dehydrogenase subunit 6 (ND6) genes. Bayesian phylogenetic methods found that none of the three Sections within *Nyssorhynchus* (Albimanus, Argyritarsis, Myzorhynchella) were supported in all analyses, although Myzorhynchella was found to be monophyletic at the combined genes. Within the Albimanus Section the monophyly of the Strodei Subgroup was strongly supported and within the Myzorhynchella Section *Anopheles antunesi* and *An. lutzii* formed a strongly supported monophyletic group. The epidemiologically significant Albitarsis Complex showed evidence of paraphyly (relative to *An. lanei*-Myzorhynchella) and discordance across gene trees, and the previously synonomized species of *An. dunhami* and *An. goeldii* were recovered as sister species. Finally, there was evidence of complexes in several species, including *An. antunesi*, *An. deaneorum*, and *An. strodei*.

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1. Introduction

Malaria is one of the most common infectious diseases worldwide with an estimated 250 million cases occurring annually (WHO, 2008). The primary tools for malaria control are insecticides for use against malaria vectors, and anti-malarial medicines in high transmission areas (RBM, 2008). However, poorly designed or implemented control measures and the spread of drug and insecticide resistant malaria parasites and vectors, respectively, make the threat posed by malaria serious. While the development of malaria vaccines remains among the highest priorities in the fight against malaria (Long and Hoffman, 2002), understanding the ecology, life history strategy and transmission efficiency of vector species are essential steps for the design and implementation of effective malaria control measures. In addition, genetic control strategies are now seen as a major course of action for the future and much of their development depends on the mapping of loci controlling malaria resistance traits in vector species (Marshall and Taylor, 2009). Thus, identifying complexes and clades of closely related species with varying degrees of malaria resistance could identify important candidate species for the study of variation and evolution in disease-refractory genes (Lehmann et al., 2009).

The vectors of human malaria are mosquitoes found solely within the genus *Anopheles* yet only about 15% of *Anopheles* species are known to transmit malaria and many of these vector species have very different vectoring capacities (Klein et al., 1991a,b). The ability to differentiate between vector and non-vector species is therefore essential if we are to target the correct species in malaria transmission, and effectively describe its geographical distribution and biology. However, the task of differentiating and delineating species is not a simple one when little or no apparent morphological variation exists among the many *Anopheles* species. For example, *An. maculipennis* was once considered a single species and the principal malaria vector in Europe, yet malaria was absent through large parts of its range: a phenomenon known as "Anophelism without malaria" (Jetten and Takken, 1994). The geographic distribution of malaria in Europe was only later understood with the recognition that *An. maculipennis* exists not as a single species but rather as a group or complex of several closely related species, each with different vector capacities. The existence of such complexes is common through much of *Anopheles*, including the epidemiologically important subgenus *Anopheles* (*Nyssorhynchus*).

The subgenus *Nyssorhynchus* contains the three most important malaria vectors in Latin America; *An. darlingi* Root (Tadei and Dutary-Thatcher, 2000), *An. nuneztovari* Gabaldón (Kitzmiller et al., 1973) and *An. albimanus* Wiedemann (Conn et al., 2002). This subgenus is divided into descending informal groupings based on morphological similarities that may or may not indicate natural relationships. It currently comprises of three Sections based

* Corresponding author. Tel.: +55 11 63627559.
E-mail address: bpbourke@usp.br (B.P. Bourke).

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on unique combinations of larval, pupal and adult characters (Peyton et al., 1992). The Albimanus Section is the largest with 20 species (including *An. albimanus* and *An. nuneztovari*; Harbach, 2004; Calado et al., 2008), the Argyritarsis Section contains approximately 12 species (including *An. darlingi*; Harbach, 2004; Lehr et al., 2005; Brochero et al., 2007), and the Myzorhynchella Section is the smallest with four valid species (Harbach, 2004).

Although *Nyssorhynchus* is a well supported monophyletic group (Sallum et al., 2000, 2002; Harbach, 2007), the phylogenetic relationships within the group are poorly understood. Many of the species are extremely difficult to differentiate morphologically, mainly due to their intraspecific variation and interspecific similarity (Faran, 1980), and are generally only reliably resolved by chromosome or DNA analyses. The literature suggests that the speciosity of *Nysshorhynchus* is underestimated and increasing numbers of previously recognized species contain species complexes e.g. *An. benarrochi* (Ruiz et al., 2005; Sallum et al., 2008), *An. marajoara* (Lehr et al., 2005), *An. nuneztovari* (Fritz et al., 1994; Conn et al., 1998), *An. oswaldoi* (Motoki et al., 2007), *An. strodei* (Sallum et al., unpublished).

Consequently, the main goals of this study are to determine the phylogenetic relationships within the subgenus *Nyssorhynchus*, ascertain candidate species complexes and identify potential disparities with the current predominantly morphologically based *Nyssorhynchus* classification (Harbach, 2004). Our phylogentic analysis is performed on 21 species from *Nyssorhynchus*, using the single copy nuclear *white* and mitochondrial NADH dehydrogenase subunit 6 (ND6) genes.

2. Material and methods

2.1. Mosquito collection

Specimens included male and female adults collected using a Shannon trap (Shannon, 1939) in the localities described in Table 1. Several specimens were offspring of field-caught females raised to adulthood in order to obtain morphological data from exuviae and male genitalia for correct species identification, while other individuals were taken as either larvae or pupae from water in immature habitats using a pan trap. Offspring of field-caught females were raised in distilled water which was replaced on a daily basis while field-caught larvae and pupae were raised in water collected from the field. Larvae and pupae were kept at room temperature and larvae were fed fish food (Tetramin) on a daily basis. Voucher material consisting of larval/pupal exuviae and male genitalia was produced for each individual, which was then deposited in Entomological Collection at Faculdade de Saúde Pública, Universidade de São Paulo, Brazil (FSP-USP).

2.2. DNA extraction

DNA was extracted from individual [adult] mosquitoes of each species according to the methods described by Wilkerson et al. (1993). DNA working solutions were 1:100 dilutions of initial extraction solution. All extractions were diluted to 100 μl with TE buffer and aliquots and DNA extraction solutions were retained for storage at −80 °C in the frozen collection of the Laboratório de Sistemática Molecular at the Faculdade de Saúde Pública, Universidade de São Paulo.

2.3. Amplification

The primers that were chosen for this study amplified fragments from the nuclear *white* gene and the mitochondrial NADH dehydrogenase subunit 6 (ND6) gene.

2.3.1. The white gene

The *white* gene was amplified using WZ2E and WZ11 primers (Besansky and Fahey, 1997). This amplification product then served as a template in a second PCR reaction using internal primers W1F (5′-GAT CAA RAA GAT CTG YGA CTC GTT-3′) and W2R (5′-GCC ATC GAG ATG GAG GAG CTG-3′). The initial PCR reaction contained 1 μl of DNA extraction solution in a total volume of 10 μl containing 1× PCR buffer (20 mM Tris–HCl, 10 mM $(NH_4)SO_4$, 10 mM KCl, 2 mM $MgSO_4$ and 0.1% Triton X-100), 0.2 mM of each dNTP (Eppendorf®), 10 pmol of each primer and 0.4 U *Taq* DNA Polimerase with Thermo Pol Buffer (New England BioLabs®Inc.). The reaction proceeded under the following temperature profile: 94 °C for 5 min, 40 cycles at 94 °C for 30 s, an annealing temperature of 52 °C for 30 s, and then 72 °C for 60 s followed by a final extension at 72 °C for 10 min. The second PCR reaction contained 1 μl of the first PCR product solution in a total volume of 25 μl containing the same reagent concentrations described above. This reaction proceeded under the same temperature profile as the first, except that the annealing temperature was changed to 55 °C. The final PCR product was then purified using PEG precipitation (20% polyethylene glycol 8000/2.5 M NaCl).

2.3.2. The ND6 gene

The ND6 gene was amplified by primers ND6F (5′-TCA CTA ACT CCC AAA CTT AAT AT-3′) and ND6R (5′-ATG GTG CAG GTA AAT CTA CTA ATG G-3′). The PCR reaction was conducted in a total volume of 50 μl containing 1 μl of DNA extraction solution, 1× PCR buffer, 0.2 mM of each dNTP, 10 pmol of each of the two primers, 1.5 U of *Taq* DNA Polimerase with Thermo Pol Buffer (New England BioLabs®Inc.). The reaction proceeded under the following temperature profile: 95 °C for 2 min, 5 cycles of 94 °C for 40 s, 37 °C for 40 s and 72 °C for 40 s, 45 cycles of 94 °C for 40 s, 48 °C for 40 s and a final extension at 72 °C for 7 min.

2.4. Sequencing and sequence alignment

Sequencing reactions were carried out in both directions using a Big Dye Terminator cycle sequencing kit v3.1 (Applied Biosystems) and Applied Biosystems 3130 DNA Analyzer (Applied Biosystems). The ND6 gene and Exon 3 and Exon 4 from the *white* gene were first aligned by nucleotide and then by amino acid using the *Drosophila* genetic code implemented in MacClade 4.08 OSX (Maddison and Maddison, 2003).

2.5. Phylogenetic analyses

Constant, variable and parsimony informative site counts were made using PAUP 4.0b10 (Swofford, 2004). Unweighted parsimony analyses were performed using PAUP 4.0b10 by using a heuristic search with tree bisection reconnection (TBR) branch-swapping and 1000 random taxon additions. Parsimony bootstrapping (Felsenstein, 1985) used 1000 pseudo-replicates, with 10 random taxon addition replicates per pseudo-replicate. Parsimony-uninformative characters were excluded from all the analyses.

Maximum likelihood analyses were performed using PHYML version 2.4.4 (Guindon and Gascuel, 2003). Optimal evolutionary models were determined for separate and combined genes using the Akaike Information Criterion (AIC) in Modeltest version 3.7 (Posada and Crandall, 1998). Support for each clade generated from data sets was assessed by 1000 bootstrap replicates.

For Bayesian analyses, a partitioning strategy was applied to the *white* and ND6 gene sequence data to allow different partitions to have their own model characteristics (composition, rate matrix, and among-site variation) and to allow for among-partition rate variation. The dataset could be left unpartitioned, partitioned by gene, partitioned by codon position, or partitioned by both gene

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