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

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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*.

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

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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

Table 1
Taxon ID, location and geographic coordinates of *Nyssorhynchus* specimens used in this study. Outgroups *An. intermedius* and *An. minor* are from the subgenus *Anoppheles*.

Species	Specimen ID	State, municipality	Geographic coordinates
An. albitarsis B	SP09(-)-3	São Paulo, Dourado	22°4′S, 48°27′W
An. albitarsis s.s.	VP06(1)-1	São Paulo, Pindamonhangaba	22°58′S, 45°27′W
An. antunesi	VP11-a	São Paulo, Pindamonhangaba, Pico do Itapeva	22°46′S, 45°31′W
An. antunesi s.s.	VP11-b	São Paulo, Pindamonhangaba, Pico do Iatapeva	22°46′S, 45°31′W
An. argyritarsis	MG04 3	Minas Gerais, Frutal	19°59′S, 40°0′W
*An. braziliensis*1	AP21(39)-3	Amapá, Macapá, São José do Mata Fome	0°13′N, 50°58′W
*An. braziliensis*2	SP16(-)-3	São Paulo, Dourado	22°6′S, 48°26′W
*An. darlingi*1	AP13(3)-6	Amapá, Macapá, São José do Mata Fome	0°13′N, 50°58′W
*An. darlingi*2	AP17(1)-10	Amapá, Macapá	0°16′N, 50°54′W
*An. darlingi*3	AC20(21)-100	Acre, Acrelândia	10°7′S, 66°55′W
*An. deaneorum*1	AC01-7	Acre, Acrelândia	9°42′S, 67°5′W
*An. deaneorum*2	AC02-2	Acre, Acrelândia	9°43′S, 67°6′W
*An. dunhami*1	BRAM13-7	Amazonas, Parintins	2°37′S, 56°40′W
*An. dunhami*2	BRAM13-6	Amazonas, Parintins	2°37′S, 56°40′W
*An. dunhami*3	BRAM13-113	Amazonas, Parintins	2°37′S, 56°40′W
*An. evansae*1	SP12(-)-28	São Paulo, Dourado	22°4′S, 48°27′W
*An. evansae*2	SP18(-)-27	São Paulo, Dourado	22°8′S, 48°23′W
An. galvaoi	SP18(-)-111	São Paulo, Dourado	22°8′S, 48°23′W
*An. goeldii*1	BRAM03-01	Amazonas, Itacoatiara	3°9′S, 58°24′W
*An. goeldii*2	BRAM22-101	Pará, Prainha	2°5′S, 53°35′W
*An. konderi*1	AP15-11	Amapá, Macapá	0°16′N, 50°54′W
*An. konderi*2	AP21(43)	Amapá, Macapá, São José do Mata Fome	0°13′N, 50°58′W
*An. lanei*1	CJ02-02	São Paulo, Pindamonhangaba, Pico do Itapeva	22°46′S, 45°31′W
*An. lanei*2	CJ02-03	São Paulo, Pindamonhangaba, Pico do Itapeva	22°46′S, 45°31′W
*An. lutzii*1	SP02(9)-2	São Paulo, Pariquera Açu	24°45′S, 47°57′W
*An. lutzii*2	SP02(10)-5	São Paulo, Pariquera Açu	24°45′S, 47°57′W
An. marajoara	AP05(1)-4	Amapá, Macapá, Reserva de Curiaú	0°8′N, 51°3′W
*An. oswaldoi*1	ES08(11)-7	Espírito Santo, Jaguaré	19°2′S, 39°57′W
*An. oswaldoi*2	SP03-6	São Paulo, Pariquera Açu	24°44′S, 47°50′W
*An. parvus*1	MG07(9)-1	Minas Gerais, Frutal	20°2′S, 49°5′W
*An. parvus*2	PR28(18)-01	Paraná, Guaira	24°16′S, 54°17′W
*An. rangeli*1	AC18-120	Acre, Acrelândia	9°41′S, 67°8′W
*An. rangeli*2	AC15-4	Acre, Acrelândia	9°41′S, 67°8′W
*An. strodei*1	MG07(3)-3	Minas Gerais, Frutal	20°2′S, 49°5′W
*An. strodei*2	VP06(5)-1	São Paulo, Pindamonhangaba	22°58′S, 45°27′W
*An. strodei*3	SPR04-7	Paraná, São José dos Pinhais	25°44′S, 49°16′W
*An. triannulatus*1	ES03(3)-1	Espírito Santo, Sooretama	19°3′S, 40°1′W
*An. triannulatus*2	AP17(4)-1	Amapá, Macapá	0°16′N, 50°54′W
*An. triannulatus*3	AC01-108	Acre, Acrelândia	9°42′S, 67°5′W
*An. triannulatus*4	SP09(-)-2	São Paulo, Dourado	22°4′S, 48°27′W
*An. rondoni*1	PR28(34)	Paraná, Guaira	24°16′S, 54°17′W
*An. rondoni*2	PR28(55)	Paraná, Guaira	24°16′S, 54°17′W
*An. rondoni*3	PR28(36)- 2	Paraná, Guaira	24°16′S, 54°17′W
An. intermedius	SP22-106	São Paulo, Pariquera Açu, Pariquera Mirim	24°43′S, 47°53′W
An. minor	ES10-100	Espírito Santo, Santa Teresa	19°8′S, 40°0′W

and codon position. Optimal evolutionary models were determined for isolated partitions using the Akaike Information Criterion in Modeltest, and for partition combinations using maximum likelihoods in P4 (Foster, 2004). These optimal models for *white*, ND6 and combined sequence data were then used in MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Each analysis consisted of two runs to provide additional confirmation of convergence of posterior probability distribution. Each run was 4 million generations long, and the first 3 million were discarded as burnin. The MCMCMC strategy was used, with 4 heated chains; adequate mixing was achieved by setting the chain "temperature" to 0.15, 0.10 and 0.05 for *white*, ND6 and combined genes, respectively. Convergence of topology between the two runs was monitored using the average standard deviation of split frequencies – this index consistently fell to below 0.013 in the post-burnin samples. Convergence was also monitored by noting that the PSRF values were all approx-

Table 2
Data characteristics described by gene (combined, *white* and ND6 genes) and codon position.

Gene	Codon position	Unique sequences	Sites	Constant	Variable	Parsimony informative
white + ND6		41	1088	650	438	347
	1		363	256	107	83
	2		363	310	53	44
	3		362	84	278	220
white		38	563	319	244	179
	1		188	135	53	38
	2		188	155	33	26
	3		187	29	158	115
ND6		38	525	331	194	153
	1		175	121	54	42
	2		175	155	20	16
	3		175	55	120	95

Table 3
Model choice selection for gene (combined, *white* and ND6 genes) and codon position.

Gene	Codon position	Modeltest choice	Model used[a]
White + ND6		GTR + I + G	GTR + I + G
	1	GTR + I + G	GTR + I + G
	2	TVM + I + G	GTR + I + G
	3	GTR + G	GTR + G
White		TVM + I + G	GTR + I + G
	1	K81 + I + G	GTR + I + G
	2	TVM + I + G	GTR + I + G
	3	HKY + G	HKY + G
ND6		TVM + I + G	GTR + I + G
	1	TIM + I + G	GTR + I + G
	2	TVM + G	GTR + G
	3	K81uf + G	HKY + G

[a] The rate matrices used were the closest available in MrBayes that were more parameter-rich; the only exception was for ND6 position 3 where the HKY + G model had almost as high an AIC as the K81uf + G model but with 1 less parameter.

imately 1 in the post-burnin samples. The consensus of post-burnin samples is shown, where numbers refer to percent posterior probability, with nodes with less than 70% PP collapsed. *An. intermedius* (Peryassú) and *An. minor* Costa Lima were used as outgroups in all phylogenetic analyses.

3. Results

Mosquitoes collected from various localities described in Table 1 were firstly identified by morphological analysis of the larval exuviae and male genitalia and DNA was then extracted from each individual as described in Section 2. Sections of the single copy nuclear *white* gene and the mitochondrial ND6 gene were amplified and sequenced. The alignment of sequences from 45 individuals yielded 38 unique sequences for both the *white* and ND6 genes, and 41 unique sequences for the combined genes (Table 2). A total of 1088 sites were included in the analyses, consisting of 252 sites from Exon 3 and 311 sites from Exon 4 of the *white* gene, and 525 sites from the ND6 gene (Table 2). Difficulties with aligning the intron in the *white* gene meant that this segment (site position 253–362) was excluded from further analyses.

The optimal evolutionary models for the sequence partitions are shown in Table 3. Where the most appropriate rate matrix was unavailable in MrBayes (i.e. TVM and K81), the most similar rate matrix available was selected (i.e. GTR). The best evolutionary model for *white* and ND6 genes was TVM + I + G (substituted with the GTR + I + G model in Bayesian analyses), partitioned by codon position with APRV (free among-partition rate variation). The best model for combined genes was the GTR + I + G model without APRV (Including APRV did improve the log likelihood estimate significantly), and which partitioned the data by gene and codon position (six partitions in total, see Table 4). Parsimony and maximum likelihood analyses (not shown) produced trees with near complete basal polytomies across all species and so were excluded from further analyses.

The Argyritarsis Section is one of three Sections described in the subgenus *Nyssorhynchus*, based on larval, pupal and adult character morphology. It consists of 12 species. Of these, eight are included in the current study (Figs. 1 and 2). Our results show no evidence to support the Argyritarsis Section as a natural grouping. Within this section, the Albitarsis Group (consisting of *An. albitarsis*, *An. albitarsis B*, *An. marajoara* and *An. deaneorum*) is monophyletic in the ND6 (Fig. 2) and combined (Fig. 3) data sets, with strong branch support (Bayesian posterior probability, BPP, of 100%). In the *white* gene analysis, this group is less well resolved (Fig. 1; 89%). *An. albitarsis sensu stricto* is recovered as a sister species to *Anopheles deaneorum* in the *white* gene (Fig. 1; with BPP of 99%) while in ND6 it is sister to the remaining Albitarsis species (Fig. 2; with 98% BPP). Across combined genes, *An. albitarsis s.s.* is again recovered as sister to *An. deaneorum*, and the basal node of the Albitarsis complex connects *An. marajoara* with all remaining Albitarsis species (Fig. 3). *An. braziliensis*, *An. darlingi* and *An. lanei* are recovered as monophyletic species in all analyses.

The Myzorhynchella Section currently consists of four species (*An. antunesi*, *An. lutzii*, *An. nigritarsis*, and *An. parvus*), and all but one (*An. nigritarsis*) is included in the current study. We find the Myzorhynchella Section monophyletic in the *white* gene with BPP of 86% (Fig. 1), but no support for such monophyly at the ND6 gene (Fig. 2). Across combined genes, the Myzorhynchella Section again gains support for monophyly, with BPP of 92% (Fig. 3). *An. lutzii* and the two *An. antunesi* sequences from a distinct clade (with greater than 99% BPP) in all analyses, with the first *An. antunesi* sister to a clade consisting of *An. lutzii* and the second *An. antunesi* (hereafter referred to as "near_Antunesi"). There is therefore strong evidence for a species complex in *An. antunesi*.

The Albimanus Section consists of 20 species subdivided into Albimanus and Oswaldoi Groups. The former group includes only *An. albimanus*, whereas the latter has 19 species, subdivided into two subgroup and two species complex. Our study includes 10 of these species (see Figs. 1–3). Monophyly in the Oswaldoi Group (all

Table 4
Partition schemes for the combined genes, with and without among-partition rate variation (APRV), detailing ln likelihoods, AIC values (from Modeltest) and ln marginal likelihoods (from P4). The same data and tree were used in all ML optimizations.

	Partition	APRV	ln likelihood	Free parameters	AIC	ln marginal likelihood
A	None	n/a	−7133.2	10	14286.4	
B	By gene	–	−6888.72	20	13817.44	
		+	−6888.69	21	13819.38	−6969.58
C	By codon	–	−6963.6	29	13985.2	
	Position	+	−6907.17	31	13876.34	−7063.1
D	By gene and codon	–	−6641.32	49	13380.64	
	Position	+	−6561.85	54	13231.7	−6661.61

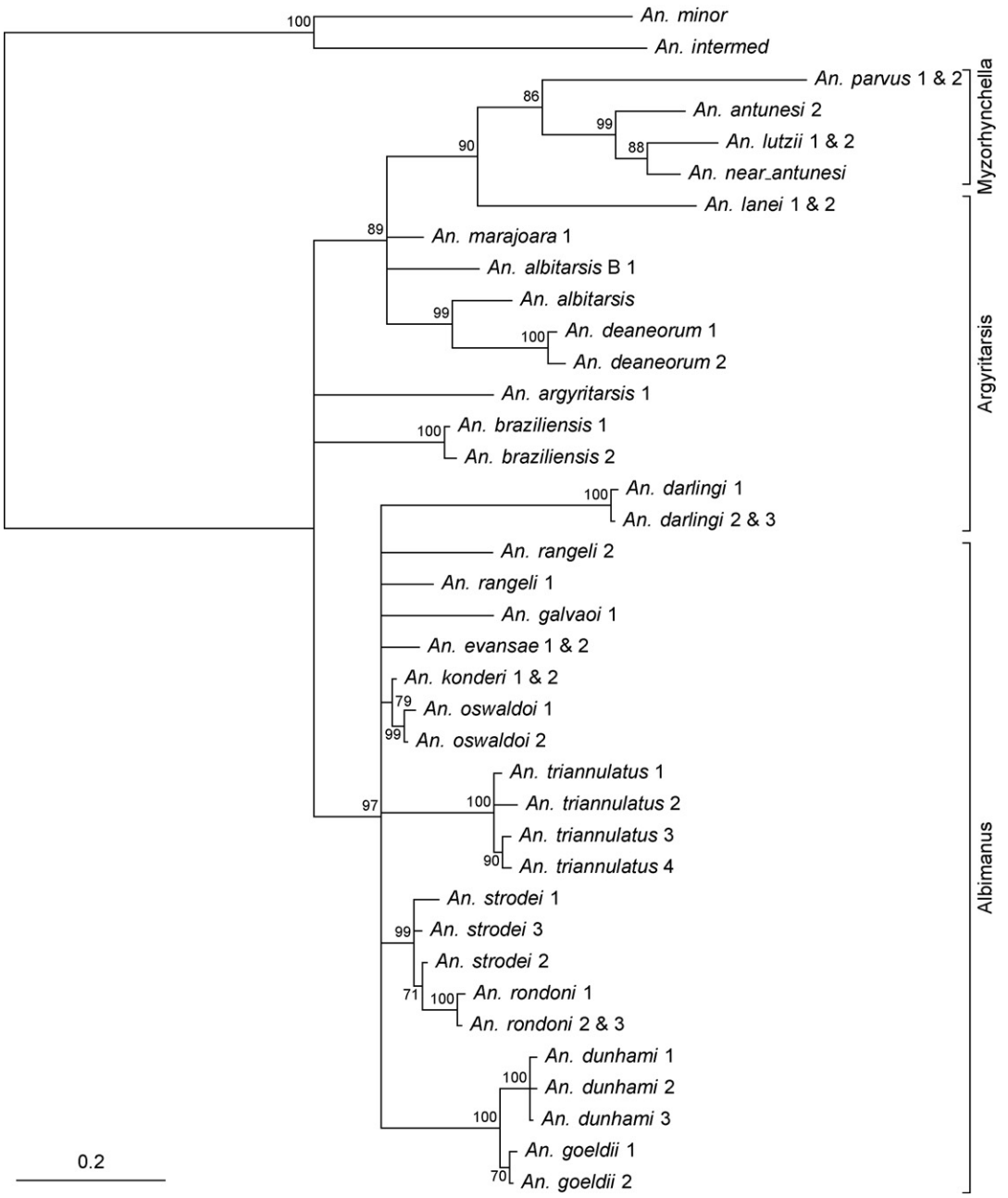

Fig. 1. Bayesian phylogenetic analysis of the *white* gene. The data was partitioned by codon position, and with models assigned as described in Table 3. Numbers at branches indicate Bayesian posterior probability (>70%).

of the Albimanus Group except for *An. triannulatus* from the Triannulatus Subgroup; 85% BPP) and Subgroup (all of the Albimanus Group except for *An. triannulatus*, *An. rondoni* and *An. strodei*; 84% BPP) is weakly supported at combined genes (Fig. 3), but not supported when analyzed individually. The *white* gene data recovers a clade consisting of all 10 species from the Albimanus Section plus *An. darlingi*, with 97% BPP (Fig. 1), but this clade is lost when combined with ND6 gene data (Fig. 3). *An. oswaldoi* is weakly clustered with *An. konderi* at the *white* gene (with 79% BPP; Fig. 1), but is strongly clustered with *An. evansae* at the ND6 gene (with 95% BPP; Fig. 2). Across combined genes the *An. oswaldoi*/*An. evansae* group is recovered again, with enhanced support (100% BPP; Fig. 3). *An. dunhami* and *An. goeldii* form a strongly supported clade in all three data sets (>91% BPP). The monophyly of the Strodei subgroup (consisting of *An. strodei* and *An. rondoni*) is strongly supported in all three data sets (>99% BPP). While *An. rondoni* is monophyletic, *An. strodei* is paraphyletic with respect to *An. rondoni* across all data sets. Data from the Albimanus Section therefore provide strong evidence for a species complex in *An. strodei*.

4. Discussion

The monophyly of the subgenus *Nyssorhynchus* is strongly supported in studies of morphological (Sallum et al., 2000; Harbach and Kitching, 2005) and molecular (Krzywinski et al., 2001a,b) data. However, the relationships within *Nyssorhynchus* are poorly understood, and to date there is a dearth of genetic data with which to analyse relationships within the subgenus. While the findings from our study provide phylogenetic support for some of the groupings within the subgenus, disparity between ND6 and *white* gene trees is also evident. However, much of this disparity is not strongly supported, particularly in the ND6 gene, and is perhaps indica-

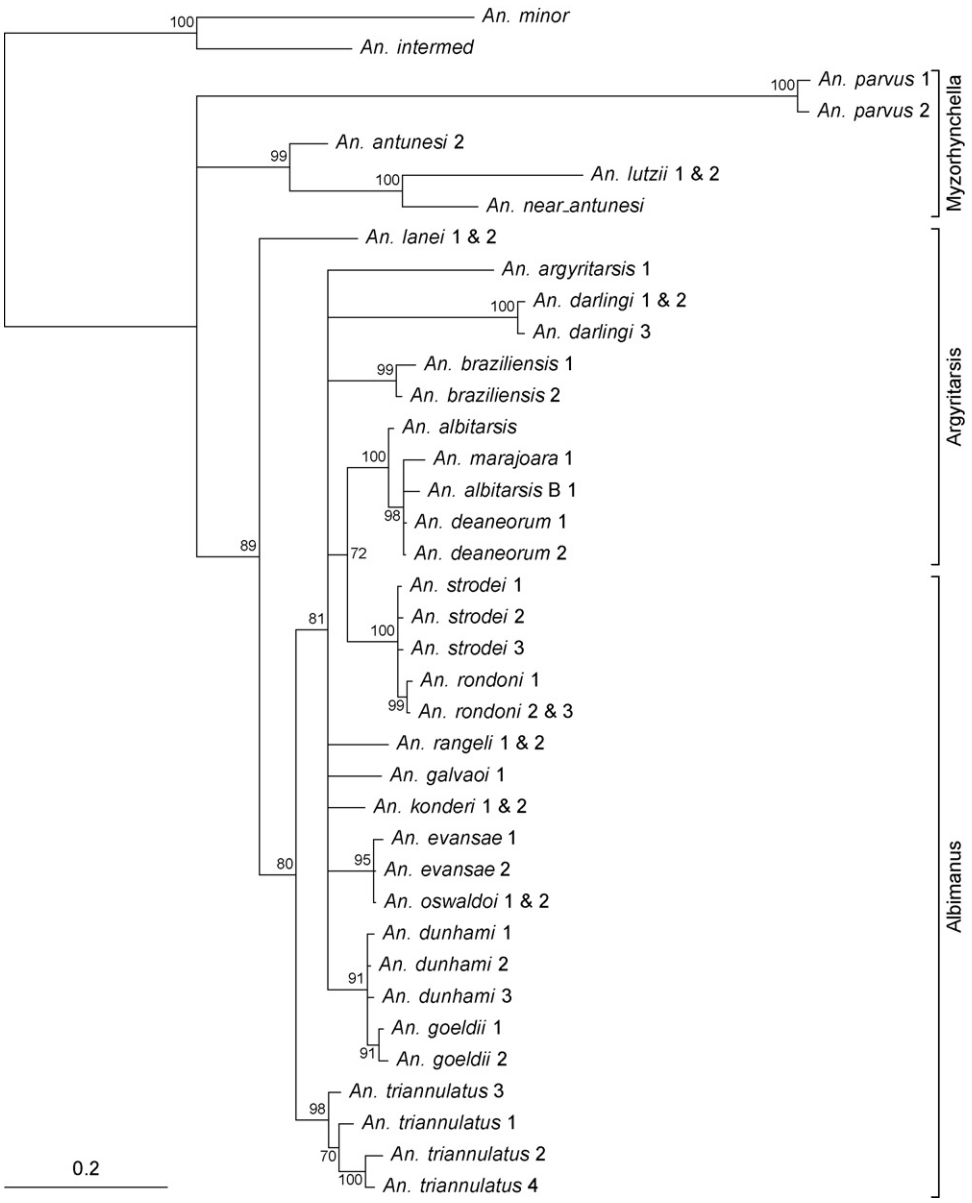

Fig. 2. Bayesian phylogenetic analysis of the ND6 gene. The data was partitioned by codon position, and with models assigned as described in Table 3. Numbers at branches indicate Bayesian posterior probability (>70%).

tive of ND6 being less phylogenetically informative than the *white* gene.

The Myzorhynchella Section is monophyletic and a sister group to the remaining species in *Nyssorhynchus* when analysed across combined genes, although conflicting relationships are recovered at the two different genes (monophyly versus paraphyly). Despite little published molecular data available for the Myzorhynchella Section, Sallum et al. (2000) and Harbach and Kitching (2005) morphological analyses of Anophelinae also recovers Myzorhynchella species (*An. lutzii* and *An parvus*) as a sister group to all other *Nyssorhynchus* species. However, in neither study was Myzorhynchella Section found to be monophyletic. Our analysis strongly supports *An. lutzii*-"near_Antunesi"-*An. antunesi* as a natural grouping and the relative position of "near_Antunesi" is suggestive of a new, undescribed species that is closely related to *An. lutzii*. This finding is supported by recent ITS2 and morphological analysis of *An. antunesi* from Serra da Mantiqueira, southeastern Brazil (Nagaki et al., unpublished).

The *An. albitarsis* species complex is particularly important from a human health perspective. *An. deaneorum* and *An. albitarsis sensu lato* (Klein et al., 1991c), *An albitarsis* E (Póvoa et al., 2006) and *An. marajoara* (Conn et al., 2002) are important vectors of human malaria in Western Amazonia, the State of Roraima, and Northeastern Amazonia, respectively. The complex has previously been recovered as a monophyletic clade using the second internal transcribed spacer (ITS2) of ribosomal DNA (Marrelli et al., 2006). Our analyses also show some support for the *An. albitarsis* complex as a natural group. However, paraphyly with *An. lanei*/Myzorhynchella Section at the *white* gene makes the phylogenetic status of the complex equivocal. This disparity between gene trees is also observed in the *An. albitarsis* complex.

Discriminating between species of *An. albitarsis* complex has proved problematic, with some species, such as *An. albitarsis* E, particularly difficult to resolve (Li and Wilkerson, 2005, 2007; Brochero et al., 2007). The geographic distribution of the *An. albitarsis* species complex is poorly described, particularly along the

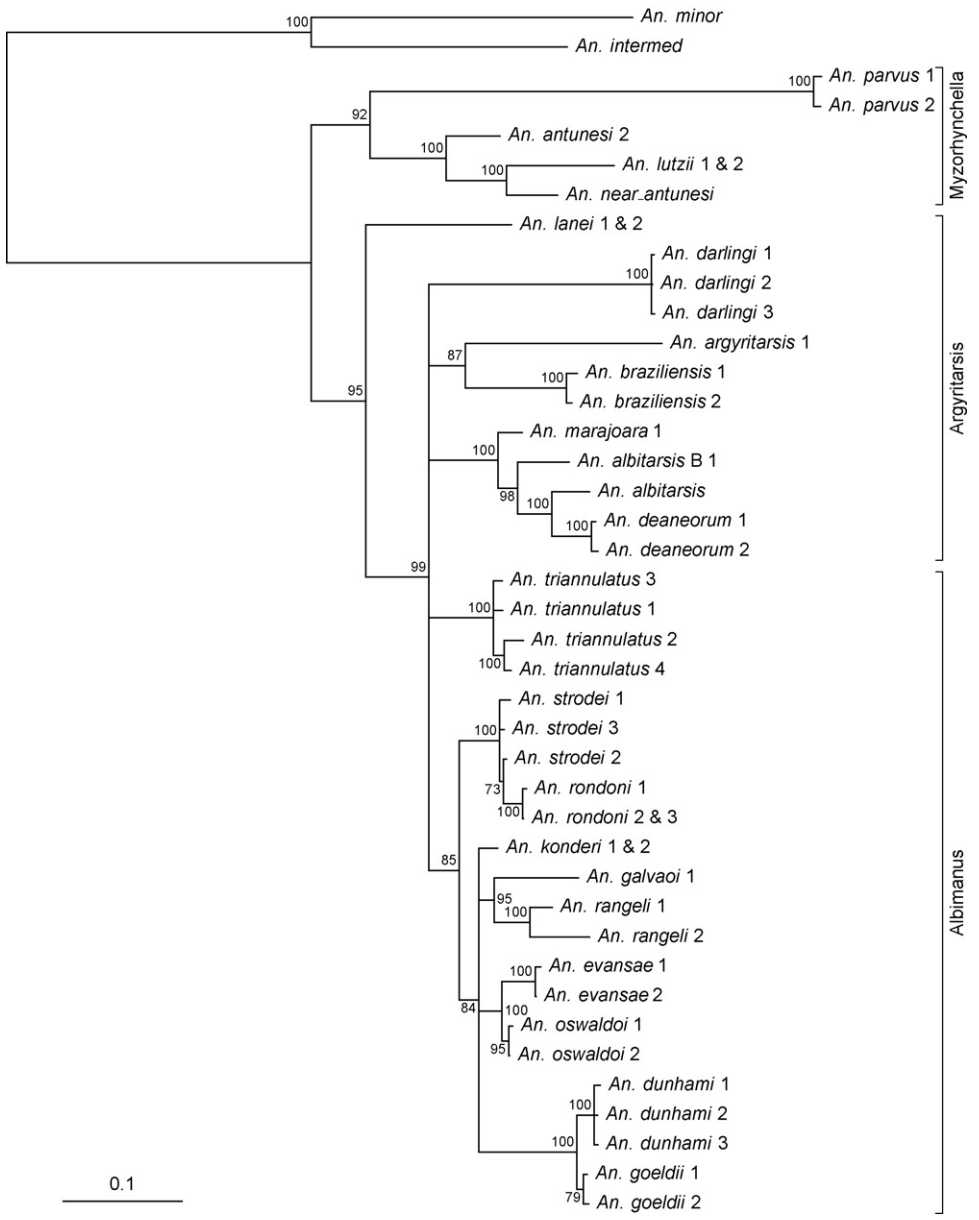

Fig. 3. Bayesian phylogenetic analysis of combined *white* and ND6 data. The data was partitioned as described in Table 4(D), that is by gene and by codon, and with models assigned as described in Table 3. Numbers at branches indicate Bayesian posterior probability (>70%).

Brazilian coast and savannah, and there is a need to address this paucity of information if species diversity and delineation is to be properly understood. The specimens in our study come from within known ranges. However, our analyses provide conflicting patterns to species relationships in this complex. Although *An. albitarsis* is recovered as sister to *An. deaneorum*, and *An. marajoara* is sister to the remaining species in the Albitarsis complex when analyzed across combined genes, these relationships are not recovered at the ND6 gene. At this gene *An. deaneorum*, *An. marajoara*, and *An. albitarsis* are unclustered within a clade, which provides support for a species complex. These clear differences in tree topology among genes are also apparent in the literature, with Wilkerson et al. (2005) identifying (*An. albitarsis*, *An. albitarsis* B) and (*An. marajoara*, *An. deaneorum*) groupings at mitochondrial COI and ND4 and rDNA ITS2 and D2 genes, and Lehr et al. (2005) recovering similar relationships at the complete COI gene, albeit with *An. marajoara* paraphyletic with respect to *An. deaneorum*. Brochero et al. (2007) study of the Albitarsis complex (with the inclusion of *An. albitarsis* F) at the *white* gene recognizes the different phylogenetic relations observed between genes, but considers the *white* gene topology potentially the most parsimonious solution due to a single intron loss event resolving *An. marajoara* and *An. albitarsis* E as sisters to all remaining species of *An. albitarsis* complex (Merritt et al., 2005).

Conflicting patterns among gene trees are widely reported in the literature (Maddison, 1997; Nichols, 2001; Degnan and Rosenberg, 2009). Discordance among gene trees of closely related species may be attributed to various biological processes such as natural selection or genetic drift (Rieseberg et al., 1996; Maddison, 1997; Satta et al., 2000; Martin and Burg, 2002). Concatenating data from multiple loci has been seen as a solution to such discordance by resolving the dominant phylogenetic signal across all loci (Rokas et al., 2003; Steppan et al., 2005). However, in their study of the *An. gambiae* complex, Besansky et al. (2003) found that discordant tree topologies could be attributed to introgression and reproductive isolation, and stated the "total evidence" approach, i.e. concatenating multiple loci for analyses, may well obscure important genetic differences between closely related species, leading to increasing confidence in the "wrong answer". Therefore, where conflicting

tree topologies exist between closely related species, such as in the Myzorhynchella Section and *An. albitarsis* complex, a more cautious loci-by-loci assessment of tree topologies across additional loci is recommended to understand such species phylogenies.

Morphological analysis has previously recovered *An. strodei* and *An. rondoni* as nearest relatives within the Albimanus Section with the principal morphological differences occurring at the larval clypeal index (Sallum and Wilkerson, 1997). In a review of ITS2 data from Latin American anophelines, Marrelli et al. (2006) placed *An. strodei* and *An. rondoni* within a well supported monophyletic clade but found that *An. strodei* was paraphyletic with respect to *An. rondoni*. Our results recover the same highly supported clade (*An. strodei–An. rondoni*) with *An. strodei* paraphyly. However, our results also provide strong support for monophyly in *An. rondoni*. Marrelli et al. (2006) suggest that *An. strodei* paraphyly may be evidence of a species complex, and this is supported by Sallum et al. (unpublished) morphological and COI, ITS2 and *white* gene sequence data analyses of *An. strodei* that suggest the presence of at least four different species in Brazil. Our results are consistent with these findings. Although *An. strodei* is a vector of malaria (Oliveira-Ferreira et al., 1990) and one of the most widely distributed *Anopheles* species in South America (Faran, 1980), study specimens to date have been sourced from a limited area, mainly South East Brazil (Minas Gerais, Paraná, and São Paulo). It is therefore appropriate to propose a more comprehensive assessment of genetic diversity in order to describe the full extent of *An. strodei* speciosity.

The monophyletic clade of *An. dunhami* and *An. goeldii* is supported across all analyses. Support for their species status is recovered across all analyses. This close association is generally consistent with morphological data for the species. Until recently *An. dunhami* and *An. goeldii* were synonomized with *An. nuneztovari*, but have since been elevated to species within the Oswaldoi Subgroup (Peyton, 1993; Calado et al., 2008, respectively). *An. dunhami* and *An. goeldii* can be distinguished from each other by characters of the male genitalia and molecular data, and *An. goeldii* appears to be more closely associated with *An. nuneztovari* than *An. dunhami* across multiple loci (Calado et al., 2008). Recognizing the relationships between *An. nuneztovari*, *An. dunhami* and *An. goeldii* is significant as *An. nuneztovari* is an important vector of malaria in South America, particularly in Colombia and Venezuela (Rey and Renijifo, 1950; Gabaldón and Guerrero, 1959; Tadei and Dutary-Thatcher, 2000) while *An. dunhami* has never been implicated as a malaria vector. The recent reclassification of *An. goeldii* to the status of species and its very close association with both *An. nuneztovari* and *An. dunhami* warrants an assessment of its capacity to vector malaria and underlines the potential utility of this group in the study of disease-refractory genes.

Our results find an association between *An. konderi*, *An. oswaldoi* and *An. evansae*, with strong support for (*An. oswaldoi–An. konderi*) and (*An. oswaldoi–An. evansae*) at alternate genes, and with the third species of the triplet unclustered in the previous clade. This relationship is broadly consistent with previous studies. *An. konderi* has previously been synonomized with *An. oswaldoi* (Lane, 1953; Faran, 1980). The resolution of these species is extremely difficult and is normally achieved through assessment of morphological variation in the aedeagus of the male genitalia (Causey et al., 1946; Marrelli et al., 2006).

The results of the current study did not unequivocally resolve any of the three recognized Sections (Albimanus, Argyritarsis, Myzorhynchella) within *Nyssorhynchus*. The status of Myzorhynchella was ambiguous by displaying monophyly and paraphyly at alternate genes. There is evidence of complexes in several species, such as *An. antunesi*, *An. deaneorum* and *An. strodei*. Our study shows a clear need for further analyses at multiple loci to resolve discordance among trees and test whether ambiguous groups, such as Myzorhynchella, are indeed natural. A more detailed assessment of genetic diversity is also recommended for those species identified as potential complexes and this is particularly important if species are to be effectively incriminated in vector based malaria control measures.

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