

Subset partitioning of the ribosomal DNA small subunit and its effects on the phylogeny of the *Anopheles punctulatus* group

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Abstract

A phylogenetic study, based on maximum parsimony, of ten species in the *Anopheles punctulatus* group of malaria vectors from the south-west Pacific was performed using structural and similarity-based DNA sequence alignments of the nuclear small ribosomal subunit (SSU = 18S). The structural alignment proved to be more informative than a computer generated similarity-based alignment. Analyses involving the full structural sequence alignment (2169 bp) and the helical regions (1547 bp) resolved a single tree of the same topology, while analyses using the similarity based alignment could not resolve the group. Studies on the three structural domains of the nuclear rDNA SSU identified domain 2 (769 bp) as the only region informative at the sibling-species level and resulted in the same tree as the full structural sequence and helical regions. The main conclusions of these studies were that the *An. punctulatus* group formed two clades: a Farauti clade containing members displaying an all black scaled proboscis (*An. farauti* 1–3 and 5–7) and a Punctulatus clade containing members that display some degree of white scaling on the proboscis (*An. farauti* 4, *An. punctulatus* and *An. species near punctulatus*). *Anopheles koliensis* can display either proboscis morphology and was positioned basal to the Farauti Clade. These results do not fully concord with those derived from the mitochondrial COII gene.

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Introduction

A number of the world's major malaria vectors are now known to consist of complexes or groups of closely related species that are either isomorphic or share overlapping morphological characters (Munstermann & Conn, 1997; Beebe & Cooper, 2000). The lack of diagnostic markers has hampered work on these mosquitoes with regard to disease transmission, control efforts and systematic studies. Identifying the levels of genetic division between species may shed light on the nature of evolutionary forces acting within and between species, and possibly assist in understanding the evolutionary dynamics of disease transmission and the phylogenetic relationships among these closely related mosquitoes.

The *Anopheles punctulatus* group of the subgenus *Cellia*, occurs throughout the south-west Pacific from the Moluccas east into Irian Jaya, Papua New Guinea (PNG), the Solomon Islands, Vanuatu and south to Northern Australia. This group contains the major malaria and filariasis vectors in this region (Lee *et al.*, 1987). The group originally consisted of *Anopheles farauti*, *Anopheles koliensis* and *Anopheles punctulatus* (Rozeboom & Knight, 1946). These species could be separated on proboscis morphology, with *An. farauti* having an all black scaled labium, *An. koliensis* with a ventral patch of white scales on the apical half of the labium and *An. punctulatus* with the apical half of the labium almost entirely white scaled (Rozeboom & Knight, 1946). Crossmating, cytogenetic and allozyme analysis have now identified twelve species within this group of which the following ten are the most common and widespread: *An. farauti* 1–7, *An. koliensis*, *An. punctulatus* and *An. species near punctulatus* (Bryan, 1973; Mahon & Miethke, 1982; Foley *et al.*, 1993, 1994, 1995). Morphological characters are no longer reliable for separating the members of this group; seven species are isomorphic while other members have been found to be polymorphic for characters previously believed to be diagnostic.

The rDNA gene family has been useful in identifying species-specific characters for several anopheline species, in particular the gene spacers, which evolve at higher rates than the rDNA genes, have provided useful markers for mosquito systematics (Beebe & Saul, 1995; Porter & Collins, 1991; Fritz *et al.*, 1994; Cornel *et al.*, 1996; Walton *et al.*, 1999). However, these regions may not be practical for phylogenetic analysis because the ITS sequences can prove difficult to align between species due to insertion/deletion indels (Beebe *et al.*, 1999; van Herwerden *et al.*, 1999). Moreover, heterogeneity within a species and heterogeneity between copies in the rDNA array have also been detected, while the selection of an outgroup can be complicated by the fact that ITS2 sequences of related species can contain little similarity (Rich *et al.*, 1997; Beebe *et al.*, 1999; Onyabe & Conn, 1999; Beebe *et al.*, 2000).

Ribosomes are responsible for protein assembly; they are abundant and have conserved structural regions (Hillis & Dixon, 1991). Ribosomal RNA gene transcripts produce a single strand of RNA that is subsequently folded to pair with itself forming a secondary structure composed of helical stems connected by unpaired loops. The RNA genes evolve in a unique manner dictated by their structure and function. The secondary structures, containing helices or stem loop regions, are folded into a three-dimensional tertiary structure held together by ribosomal proteins. These protein attachment sites are highly conserved and occur in the helices (Brimacombe *et al.*, 1990). The helices evolve through the formation of compensatory mutations (van de Peer *et al.*, 1993), while the single stranded regions connecting these helices do not and thus may display a different level of sequence variation. The folded SSU comprises three structural domains that also can be partitioned and viewed independently in an evolutionary analysis.

In this study the rDNA SSU gene was used to reconstruct a phylogeny for members of the *An. punctulatus* group: *An. farauti* 1–7, *An. koliensis*, *An. punctulatus* and *An. species near punctulatus*. The SSU's close physical linkage to the ITS2 suggests it evolves through similar

concerted evolutionary machinery, while its function restricts the rate of sequence change. Because an understanding of its secondary structure exists (van de Peer *et al.*, 1993), we compared SSU sequence alignments based on similarity to those alignments based on secondary structure. Secondary structure alignments were partitioned into subsets comprising the full structural sequence, helical regions (stems and loops) and nonhelical single stranded regions. The three structural domains that comprise the SSU were also examined.

Results and discussion

The SSU regions from single mosquitoes representing ten species in the *An. punctulatus* group were sequenced and deposited in GenBank (Table 1). Sequence length and GC content are summarized in Table 1. A transition : transversion ratio of 1 : 1 was identified by comparing closely related species sequences and counting transitions and transversions manually (*An. farauti* 2 and 6, *An. farauti* 1 and 7). Moreover, this ratio was confirmed using maximum likelihood analysis on the data set (data not shown). A summary of the phylogenetic analyses is given in Table 2. The SSU structural sequence alignment was deposited at the National Centre for Biotechnology Information (NCBI), as well as: <<http://www.science.edu.au/~davidm/Beebe18sAll.html>>; with the helical subset </Beebe18sHelix.html>; nonhelical subset </Beebe18sNonhelix.html>. The three domains were partitioned in accordance to the NCBI submission as follows: 1–642, domain 1; 643–1313, domain 2; 1314–2169, domain 3.

Maximum parsimony analysis was performed on these alignments because it will find the tree (or trees) that require fewest nucleotide changes. Maximum parsimony works best when sequence variation is small and the chance of homoplasy is rare (Felsenstein, 1977).

The nuclear SSU sequence alignments based on structure were found to be more informative than similarity-based alignments. The full structural sequence, helical

Table 1. Sequencing summary of *Anopheles* species SSU

Species	18S length bp	GC content %	Accession no.	Location
<i>An. annulipes</i>	2019	52.5	AF121053	Sydney NSW
<i>An. farauti</i> 1	2046	54.1	AF121054	152°10'E 4°12'S
<i>An. farauti</i> 2	2051	55.0	AF121055	145°53'E 17°25'S
<i>An. farauti</i> 3	1983	54.0	AF121056	146°6'S 17°36'S
<i>An. farauti</i> 4	2057	50.3	AF121057	142°10'E 3°30'S
<i>An. farauti</i> 5	2050	53.5	AF121058	145°20'E 6°1'S
<i>An. farauti</i> 6	2049	54.0	AF121059	138°55'E 4°5'S
<i>An. farauti</i> 7	2050	54.3	AF121060	138°55'E 4°5'S
<i>An. koliensis</i>	2095	55.0	AF121061	159°50'E 9°22'S
<i>An. punctulatus</i>	2102	54.0	AF121062	142°20'E 4°12'S
<i>An. sp. near punctulatus</i>	2073	53.5	AF121063	142°30'E 6°24'S

Table 2. Summary of parsimony analysis of the nuclear rDNA SSU data sets

Data sets	Sequence length (bp)	Trees found	Shortest tree length	DNA sequence variation ^a
Full (similarity)	2147	2	404	0.5–5.9
Full (structure)	2169	1	337	0.4–4.4
Helices	1547	1	261	0.5–5.1
Nonhelices	621	2	72	0.2–4.4
Domain 1	632	1	41	0–2.1
Domain 2	769	1	188	0.9–8.0
Domain 3	768	7	99	0.4–3.5

^aMean distances ($\times 100$) adjusted for missing data between species.

regions and domain 2 resolved the same single tree (Fig. 1). This tree separated the group into two main clades: one containing species with an all blacked scaled labium termed the Farauti clade and the other containing species with white scaling on the labium termed the Punctulatus clade. Basal to the Farauti clade was *An. koliensis* and basal to the Punctulatus clade was *An. farauti 4*.

The full structural sequence and helical regions showed similar levels of sequence variation between species, while domain 2 displayed an elevated level of variation. Sequence similarity over the entire region was greatest between *An. farauti 1* and 7, while sequence variation was greatest between *An. koliensis* and *An. punctulatus*. However, domain 2 showed greatest variation between *An. punctulatus* and *An. farauti 3*. A distance matrix of these three alignments is shown in Table 3.

Domain 1 displayed little phylogenetically useful information at the species level, although it resolved a single tree. The tree generated could not distinguish fully the Punctulatus and the Farauti clades because *An. farauti 1* and *An. farauti 7* contained identical sequences forming a polytomy at the base of the tree. *Anopheles koliensis* was basal to both the Punctulatus and the rest of the Farauti clade and exhibited the greatest sequence variation compared to *An. punctulatus* (2.1%). Domain 3 resulted in seven trees with a strict consensus identifying only the

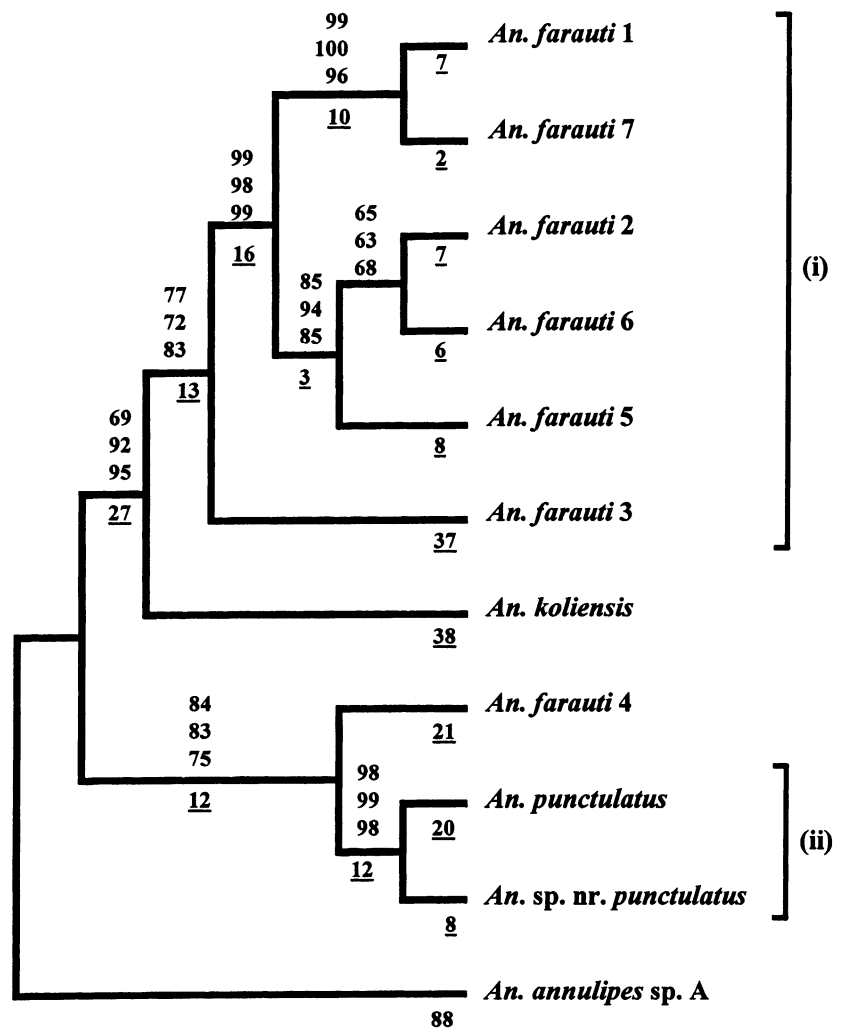


Figure 1. A phylogenetic tree of the members in the *An. punctulatus* group generated from a maximum parsimony analysis (PAUP 3.1.1.) of the helical regions, full structural sequence and domain 2. Bootstrap replicates (1000) are indicated as percentage values above the branches in descending order: helical regions, full structural sequence and domain 2. Below the line and underlined are the distance values which indicate amount of inferred evolutionary change. The Farauti clad (i) contains species of all black proboscis and the Punctulatus clad (ii) contains species of which the apical half of the proboscis is almost entirely white scaled.

Table 3. Structural alignment distance matrix of the nuclear rDNA SSU data sets (full sequence/helical regions/domain 2)

Species	Af2	Af3	Af4	Af5	Af6	Af7	Ak	Ap1	Asnp	Aann
<i>An. farauti</i> 1	1.4/1.4/2.4	3.1/3.0/5.8	3.6/4.1/6.5	1.4/1.2/1.9	1.3/1.3/1.8	0.4/0.5/0.9	3.2/3.8/5.6	4.1/4.2/7.9	3.3/3.5/6.7	5.5/5.7/8.8
<i>An. farauti</i> 2		3.1/3.3/6.6	3.1/3.8/6.3	0.8/0.8/1.6	0.6/0.8/1.2	1.1/1.1/1.9	3.4/4.2/5.8	3.7/4.1/7.7	3.0/3.5/6.6	5.7/6.1/9.1
<i>An. farauti</i> 3			3.7/3.9/7.2	3.1/3.1/5.8	3.2/3.4/6.1	2.8/2.7/5.5	3.2/3.7/4.8	3.9/4.0/8.0	3.5/3.5/7.5	5.2/5.3/8.5
<i>An. farauti</i> 4				3.3/3.7/6.3	3.0/3.5/6.0	3.3/3.7/6.0	3.5/4.1/6.2	2.3/2.5/3.6	1.8/2.1/2.4	4.8/5.2/6.8
<i>An. farauti</i> 5					0.8/0.8/1.2	1.1/0.8/1.5	3.3/4.0/5.6	3.8/3.9/7.6	3.1/3.3/6.6	5.8/6.0/9.0
<i>An. farauti</i> 6						1.1/1.0/1.5	3.3/4.0/5.2	3.8/4.0/7.5	3.1/3.4/6.3	5.8/6.1/8.9
<i>An. farauti</i> 7							3.0/3.5/5.4	3.8/3.8/7.4	3.0/3.2/6.2	5.2/5.4/8.5
<i>An. koliensis</i>								4.4/5.1/7.6	3.8/4.6/6.6	5.6/6.0/8.9
<i>An. punctulatus</i>									1.4/1.1/1.8	5.5/5.7/8.6
<i>An. sp. nr. punctulatus</i>										4.8/5.3/7.2

Mean distances ($\times 100$) adjusted for missing data.

closely related species of *An. farauti* 1 and 7, *An. punctulatus* and *An. species near punctulatus*, with the other branches collapsing. Sequence variation for this domain ranged from 0.4% (*An. farauti* 1 and 7) to 3.5% (*An. koliensis* and *An. punctulatus*).

Parsimony analysis of the alignment made using the GCG PILEUP program (Genetics Computer Group, Version 81994) to align the sequences (default settings) resulted in two trees of which a consensus could not resolve all branches. This tree was of similar topology to the tree in Fig. 1, except that *An. koliensis* and *An. farauti* 3 formed a separate clade basal to the Farauti clade and *An. farauti* 2, 5 and 6 collapsed into a polytomy.

Analysis of the nonhelical single stranded regions retained two trees that resolved only the Punctulatus clade, because the Farauti clade placed *An. farauti* 3 and *An. koliensis* as sister species on the same branch as *An. farauti* 1 and 7. *Anopheles farauti* 2, 5 and 6 again collapsed into a polytomy. Sequence variation ranged from 0.2% (*An. farauti* 1 and 7) and to 4.4% (*An. koliensis* 1 and *An. punctulatus*).

The full structural sequence, helical regions and domain 2 were further assessed for sampling error and branch confidence by performing 1000 bootstrap replicates; the results are shown in Fig. 1. These bootstrap values show slight variations between data sets with well supported branches identifying the Punctulatus clade species of *An. punctulatus* and *An. species near punctulatus* and the all black proboscis species of *An. farauti* 1–3 and 5–7. The lowest bootstrap values occur at the placements of *An. farauti* 2, 5 and 6 in the Farauti clade. A study by Foley *et al.* (1998) of Australasian anophelines (including the *An. punctulatus* group members), using the mitochondrial COII gene, supported this branch topology. However, their placement of *An. koliensis* as basal to both the Farauti and Punctulatus clades was not observed in our nuclear SSU analyses and nor was it well supported by their own data. Their placement of *An. species near punctulatus* basal to *An. farauti* 4 and *An. punctulatus* was also not well supported by COII gene analysis and did not concur with the SSU gene.

Both the full sequence and helical regions required sequencing of the whole SSU gene (~2000 bp), although the smaller region of domain 2 (769 bp) also generated the same tree using Maximum Parsimony. Furthermore, domain 2 displays sufficient levels of sequence variation between species that primers could be designed for allele specific amplification (ASA) if desired, although at present the ITS2 PCR-RFLP procedure appears sufficiently robust for species identification (Beebe & Saul, 1995). In comparison, domain 1 (632 bp) displayed sequence variation between species ranging from no difference between *An. farauti* 1 and 7–2.1% between *An. koliensis* and *An. punctulatus*, while domain 3 (768 bp) ranged from 0.4% to 3.5% for the same species comparisons.

There is some agreement between the morphological groupings as *Anopheles farauti* 1, 2, 3, 5, 6 and 7 have only ever been found to display an all black scaled proboscis (Foley *et al.*, 1993, 1994, 1995; Cooper *et al.*, 1997) and all group together in the Farauti clade. *Anopheles punctulatus* and *An. species near punctulatus* always display extensive white scaling on the apical half of the proboscis and both were grouped together into a separate clade (the Punctulatus clade) (Cooper *et al.*, 1997; Foley *et al.*, 1993, 1994, 1995). *Anopheles farauti* 4 and *An. koliensis* are both known to be polymorphic with regard to this character, because both species at times have displayed a farauti, koliensis and punctulatus like proboscis (Woodhill, 1946; Foley *et al.*, 1993). The inclusion of *An. farauti* 4 in the Punctulatus clade is not surprising and in agreement with the phenotype and genotype. Thus the positioning of *An. farauti* 4 basal to *An. koliensis* is also reasonable, although the non-Linnaean name assigned to this species can draw confusion.

For studies in mosquito systematics, domain 2 of the SSU, which can be amplified using the primers 18SA and 18SB, may provide a useful marker for species or intraspecific studies using either a PCR-RFLP procedure (Beebe & Saul, 1995), single stranded conformational polymorphism (Sharp *et al.*, 1999) or heteroduplex analyses (Beebe *et al.*, 2000).

Table 4. Primers used for the nuclear SSU amplification

Primer	Oligonucleotide sequence
18SAP1	5' GAGGGAGCCTGAGAAATGG 3'
18SH ^a	5' GATATACGCTGCTCAAAGG 3'
18SA	5' GAGGGAGCCTGAGAAATGG 3'
18SB	5' CCGTCAATTCCTTTAAGTTT 3'
18SE	5' AGACGCTACCCCTTCTTTCG 3'
18SAP2	5' CGGAAACCTTGTTACGACT 3'

^aUsed in the SSU 5' amplification of *An. farauti* 3.

Experimental procedures

Mosquitoes

Ten members of the *An. punctulatus* group were examined in this study. *Anopheles farauti* 2 and 3 were collected from Northern Queensland, Australia. *Anopheles koliensis* and *An. punctulatus*, *An. species near punctulatus* and *Anopheles farauti* 1, 4 and 5 were from field material collected in Papua New Guinea. *Anopheles farauti* 6 was from field material collected in Irian Jaya and *Anopheles farauti* 7 was from field material collected in Guadalcanal, Solomon Islands. Locality co-ordinates are summarized in Table 1. The outgroup for this study was *An. annulipes* obtained from material collected from Sydney, Australia. This species is considered closely related to the *An. punctulatus* group based on morphology and cytogenetics (Booth *et al.*, 1987).

For each mosquito species, identification was confirmed by PCR-RFLP analysis following DNA extraction using the techniques of Beebe & Saul (1995).

PCR primers, amplification and sequencing

Six primers producing overlapping fragments were designed for conserved regions of the nuclear SSU rDNA (Table 4). Two overlapping fragments of this gene were initially generated and subsequently sequenced in duplicate on an ABI 377 DNA sequencer. The 5' fragment was amplified by the complementary primers 18SA and 18SB and sequenced using these same primers and an internal primer 18SG. A separate primer was required to amplify the 5' region of *An. farauti* 3 (18SH replacing 18SA). The 3' product was amplified and sequenced using the primers 18SE and 18SAP2.

The polymerase chain reaction was carried out in 0.5 ml microfuge tubes in a 50- μ l volume on a Hybaid OmniGene thermal cycler. The final PCR mixture contained 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1 mM MgCl₂, 0.125 mM of each dNTP, 60 ng of each primer, 5% DMSO, 1.0 unit of *Taq* polymerase and 2–50 ng of purified genomic DNA. The DMSO was required to prevent the secondary structure of the SSU hindering amplification. Cycling involved an initial denaturation at 94 °C for 4 min, then thirty-five cycles of 94 °C for 1 min, 51 °C for 1 min, 72 °C for 1 min using minimum transition times. PCR products were purified using Bresa-Clean glassmilk chromatography according to manufactures' recommendations.

Data set construction

A similarity alignment was produced by the GCG PILEUP program (Genetics Computer Group, Version 81994) using default settings,

as altering these settings did not change the tree resolution or topology. The structural nuclear SSU rDNA sequence alignments were based on the procedure of van de Peer *et al.* (1994). Complete secondary structure was defined for the SSU molecule by aligning the principal secondary structure and subsequently refining the alignment utilizing higher order structural constraints based on compensative base substitutions (Gutell, 1996). Structural alignments of the SSU were produced using the DCSE sequence editor (de Rijk & de Wachter, 1993), and were modified for analysis using MacClade 3.07 (Maddison & Maddison, 1992). From this structural alignment, six data sets were constructed. The first data set contained the full SSU sequence, the second contained helices (stems and loops) and the third contained single stranded nonhelical regions. The next three data sets represented the secondary structure domains that make up the mature SSU RNA molecule, and were based on the domain structures of *Drosophila melanogaster* (van de Peer *et al.*, 1998).

Cladistic analysis

Maximum parsimony analysis was performed with the branch-and-bound option in the PAUP 3.1.1 program (Swofford, 1993). Once the tree was generated, missing data (i.e. gaps) were assigned a state that would be most parsimonious given the taxons placement in the tree and these states were then used to generate the distance matrix. To estimate sampling error, 1000 bootstrap replicates were performed on the full structural sequence, helical regions and domain 2 data sets.

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