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A Phylogenetic Study of the *Anopheles punctulatus* Group of Malaria Vectors Comparing rDNA Sequence Alignments Derived from the Mitochondrial and Nuclear Small Ribosomal Subunits

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A phylogenetic study of the members of the *Anopheles punctulatus* group was performed using structural and similarity-based DNA sequence alignments of the small ribosomal subunit (SSU) from both the nuclear and the mitochondrial genomes. The mitochondrial SSU gene (12S, ~650 bp) proved to be highly restricted by its secondary structure and displayed little informative sequence variation. Consequently, it was considered unsuitable for a phylogenetic study of these closely related mosquito species. A structural alignment of the nuclear ribosomal DNA SSU (18S, ~2000 bp) proved to be more informative than similarity-based alignments. Analyses showed the *A. punctulatus* group to be monophyletic with two major clades; a Farauti clade containing members displaying an all-black-scaled proboscis (*A. farauti* 1–3 and 5–7) and the Punctulatus clade containing members displaying extensive white scaling on the apical half of the proboscis (*A. farauti* 4, *A. punctulatus*, and *An.* sp. near *punctulatus*). *Anopheles koliensis* was positioned basal to the Farauti clade.

Key Words: Anopheles; **mosquito; mtDNA; phylogeny; rDNA; SSU.**

INTRODUCTION

The internal classification of the genus *Anopheles* (Diptera: Culicidae) includes 458 species divided among six subgenera (Harbach, 1994). These subgenera are further divided into groups, complexes, and species. Groups and complexes are informal classifications that have become prominent with the proliferation of cryptic species within a number of anopheline taxa. Many of these cryptic species are important vectors of malaria throughout the world and the problem of accurate identification is a major concern in understanding the transmission of disease and in targeting the right species for control purposes. As morphological characters are widely used in the internal classification of the *Anopheles* genus (Harbach, 1994), the presence of cryptic species also presents problems for systematic and phylogenetic studies that are based on morphological characteristics.

The *Anopheles punctulatus* group, of the *Cellia* subgenus *Neomyzomyia* Series, occurs throughout the southwest Pacific from the Moluccas east through Irian Jaya, Papua New Guinea (PNG), the Solomon Islands and Vanuatu, and south into northern Australia. This group contains the major malaria and filariasis vectors in this region. Originally, the group consisted of three closely related species, *Anopheles farauti* Laveran, *Anopheles koliensis* Owen, and *Anopheles punctulatus* Donitz (Rozeboom and Knight, 1946). These species could be readily separated using proboscis morphology: *A. farauti* having an all-black-scaled proboscis; *A. koliensis* having a ventral patch of white scales, of varying size, on the apical half of the proboscis; and *A. punctulatus* having the apical half of the proboscis extensively or completely white-scaled (Rozeboom and Knight, 1946).

Crossmating, cytogenetic, and allozyme analyses have now identified 12 species within this group, the following 10 of which are fairly common and widespread: *A. farauti* 1–7, *A. koliensis*, *A. punctulatus*, and *A.* sp. near *punctulatus* (Bryan, 1973; Mahon and Miethke, 1982; Foley *et al.*, 1993, 1994, 1995). The two other members of this group, *Anopheles clowi* and *Anopheles rennellensis* (Rozeboom and Knight, 1946; Taylor and Maffi, 1971), have been rarely recorded and appear to have very limited distributions. As well as in the 7 isomorphic species of *A. farauti*, the presence or absence of white scaling on the proboscis can be extremely variable among a number of the species (Foley *et al.*, 1993; Woodhill, 1946). At present there is no reliable way of morphologically identifying any of the members of this group and identification using proboscis morphology should be approached with great caution.

For members of the *A. punctulatus* group, the ribosomal internal transcribed spacer 2 (rDNA ITS2) has

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been an informative region for identifying cryptic species within this group (Beebe and Saul, 1995). However, for molecular systematic studies this region was unsuitable due to its polymorphic nature within the rDNA array, difficulty in aligning sequence due to the high level of sequence variation appearing as insertion/deletion indels, and the fact that the ITS2 secondary structure is not well understood (Beebe *et al.*, 1999). The rDNA small subunit (SSU) may be more informative for evolutionary analysis as its structural function restricts the rate of sequence change and an understanding of its secondary structure exists (van de Peer *et al.*, 1993).

Ribosomes are responsible for protein assembly; they are abundant and have universally structurally conserved regions (Hillis and Dixon, 1991). 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 structure comprises helices or stem loop regions and evolves through the formation of compensatory mutations (van de Peer *et al.*, 1993). The RNA secondary structure molecule is subsequently folded into a three-dimensional tertiary structure held together by ribosomal proteins. Mitochondrial ribosomal genes perform the same function as nuclear rDNA genes despite being smaller and simpler. Moreover, the smaller mitochondrial RNA gene subunits exist only as one copy per genome and contain no spacer regions. Nonhelical single-stranded regions joining the domains are relatively conserved compared to the nuclear RNA, and substitutions in animal mitochondrial genomes has been shown to reflect deep levels of divergence (Simon *et al.*, 1990, 1994).

In this study we determined the SSU DNA sequence from both the mitochondrial and the nuclear genomes and used alignments based on secondary structure and similarity to investigate the evolutionary relationships among members of the *A. punctulatus* group.

MATERIAL AND METHODS

Mosquito DNA

Ten species of the *A. punctulatus* group were examined in this study; specimens of *A. clowi* and *A. rennellensis* were unavailable. *A. farauti* 1 specimens were from a colony that has been maintained at the Australian Army Malaria Institute (AMI) since 1965; this colony was established from specimens collected from Rabaul, PNG. *A. farauti* 2 and 3 were from field material collected in northern Queensland, Australia. *A. farauti* 4 and 5, *A. punctulatus*, *A. koliensis*, and *A.* sp. near *punctulatus* specimens were from field material collected in PNG. *A. farauti* 6 was from field material collected in Irian Jaya, Indonesia. *A. farauti* 7 was from field material collected from Guadacanal in the Solomon Islands. The *A. annulipes* specimens were collected from the Sydney area, New South Wales, Australia. Voucher specimens (DNA only) for all species except *A. farauti* 5 are held at the AMI, whereas *A. farauti* 5 is held by Dr. Desmond Foley, Department of Zoology and Entomology, University of Queensland, Brisbane, Australia (Email: D.Foley@mailbox.uq.edu.au).

Mosquitoes were identified to species status by PCR-RFLP analysis (Beebe and Saul, 1995). DNA was extracted from single mosquitoes using a modified Pat Roman method (Black and Munsterman, 1996) as follows. Mosquitoes (either partial/whole or larvae) were thoroughly ground in a 1.5-ml microfuge tube containing 50 μl of lysis buffer (1.0 M NaCl, 0.2 M sucrose, 0.1 M Tris–HCl (pH 9.0), 0.5 M EDTA, and 0.5% sodium dodecyl sulfate (SDS). The grinding implement consisted of a 0.5-ml microfuge tube (flange removed with scissors prior to autoclaving) inserted in the end of a 1-ml micropipette tip. Tubes were pulse-microfuged to concentrate the homogenate at the bottom of the tube prior to incubation at 65°C for 30 min. To each tube 7 μl of 8.0 M KAc was added and mixed; the tubes were placed on ice for 15–30 min and then microfuged for 15 min at 14,000 rpm. Supernatants were placed in a new tube, to which 100 μl of 100% EtOH was added, then incubated for 5 min at room temperature, and microfuged at 14,000 rpm for 15 min. Supernatants were removed, 100 μl of cold 70% EtOH was added, and tubes were centrifuged again at 14,000 rpm for 5 min. Supernatants were again removed; tubes were air dried and resuspended in 50 μl TE containing RNase (5 μg/ml).

PCR Primers, Amplification, and Sequencing

Primers were designed to conserved regions of both the nuclear and the mitochondrial SSU rDNA. The nuclear SSU region required five primers to generate overlapping fragments (Table 1). Two overlapping fragments of this gene were generated and subsequently sequenced on an ABI 377 DNA sequencer. The 5′ fragment was amplified by primers 18SA and 18SB and sequenced using these same primers and an internal primer 18SG. A separate primer (18SH) was required to amplify the 5′ region of *A. farauti* 3 (replacing 18A). The 3′ product was amplified and sequenced using the primers 18SE and 18AP2. The mitochondrial 12S gene was amplified and sequenced using the primers 12SA and 12SC.

The polymerase chain reaction was carried out in 0.5-ml microfuge tubes in a 50-μl volume using a Thermal cycler (Hybad Omnigene). Final PCR mixture contained 50 mM KCl, 10 mM Tris–HCl, pH 9.0, 1 mM MgCl, 0.125 mM each dNTP, 60 ng of each primer, 5% dimethyl sulfoxide (DMSO), 1.0 unit of *Taq* polymerase, and 2–50 ng of purified genomic DNA. Cycling

TABLE 1

Primers Used for Amplification of the Nuclear and Mitochondrial SSU

Primer	Oligonucleotide sequence
18SAP1	5′ GAGGGAGCCTGAGAAATGG 3′
18SH[a]	5′ GATATACGCTGCTCAAAGG 3′
18SA	5′ GAGGGAGCCTGAGAAATGG 3′
18SB	5′ CCGTCAATTCCTTTAAGTTT 3′
18SE	5′ AGACGCTACCCTTCTTTCG 3′
18SG	5′ TGAGGTTGAGCGATAACAGG 3′
18SAP2	5′ CGGAAACCTTGTTACGACT 3′
12SA	5′ CTCATTTTAAAAATGAGAGC 3′
12SD	5′ CATGTAAATTTTTGTGTG 3′

[a] Used in the 5′ amplification of *A. farauti* 3.

involved an initial denaturation at 94°C for 4 min and then 35 cycles of 94°C for 1 min, 51°C for 1 min, 72° for 1 min using minimum transition times. PCR products were purified using Bresa-Clean glassmilk chromatography according to manufacture's recommendations.

Data Set Construction

Data sets used for analysis contained subsets of the nuclear SSU rDNA (~2000 bp) and the mitochondrial 12S gene (~650 bp) for the 10 species of the *A. punctulatus* group. The outgroup used for this analysis was *A. annulipes;* this species belongs to the *Neomyzomyia* series of the subgenus *Cellia* and is regarded as the sister taxon to the *A. punctulatus* group (Foley *et al.*, 1998).

The nuclear SSU was aligned using two methods: sequence similarity and structural alignments. Similarity-based alignments utilized the PILEUP algorithm in GCG package (Genetics Computer Group, Version 8, 1994). Default values for gap-weight (5.0) and gap-length-weight (0.3) were used in the final analysis. The alternative SSU rDNA sequence alignment was 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 using compensatory base substitutions (Gutell, 1996). A structural alignment of the SSU was made using the DCSE sequence editor (de Rijk and de Wachter, 1993). File modifications for the different analyses were made using MacClade 3.07 (Maddison and Maddison, 1992).

The mitochondrial SSU (12S) region was shorter (~650 bp) than the nuclear SSU. Structural alignments were performed manually using *A. gambiae* as the structural template (SSU database, http://www-rrna.uia.ac.be/ssu/list/Mitochondria.html#AN).

Cladistic Analysis

Parsimony analysis was performed with the branch-and-bound option in the PAUP 3.1.1 program using default settings, and gaps were treated as missing data (Swofford, 1993). To analyze the robustness of the groups, 500 bootstrap replicates were performed on the data sets. To test whether the data sets were evolving in a clock-like mode, maximum-likelihood analysis was performed with default values with and without a molecular clock hypothesis, and likelihood values were then subjected to the likelihood ratio test (Phylip 3.57; Felsenstein, 1995). Transition:transversion (Ti:Tv) ratios were identified manually by comparing the closely related species sequences and counting transitions and transversions (*A. farauti* 2 with 6 and *A. farauti* 1 with 7). A Ti:Tv ratio of 1:1 was subsequently identified and used with empirical base frequencies, one rate class for nucleotide substitution across sites and global branch rearrangements.

RESULTS

Nuclear and mitochondrial SSU regions from single mosquitoes representing species in the *A. punctulatus* group were sequenced directly from PCR products. Nuclear SSU sequences ranged from 1983 to 2102 bp and the GC content from 52.5 to 55.0% (GenBank AF121053–AF121063). Mitochondrial SSU sequences ranged from 654 to 660 bp and GC content from 20.2 to 21% (GenBank AF121064–AF121074). The SSU structural alignments were deposited in the National Center for Biotechnology Information (NCBI) and can be accessed by a string query of the Popstudies division of Entrez (http://www.ncbi.nlm.nih.gov). Voucher specimens are held by the Australian Army Malaria Institute.

Nuclear 18S Gene

The SSU alignment generated by PILEUP contained 2151 characters and the alignment based on structure contained 2169 characters. A distance matrix for the sequences in the structural alignment is shown in Table 2. Sequence differences between species in the group ranged from 4.4% (*A. koliensis* and *A. punctulatus*) to 0.4% (*A. farauti* 1 and 7). Confidence levels were assessed in the parsimony analysis by performing 500 bootstrap replicates. The shortest trees found using the sequence alignment based on structure and on similarity were 337 steps and 414 steps, respectively.

The structural alignment of the nuclear SSU gene was the only data set that resolved a single tree. This tree contained two major clades. The first contained *A. farauti* 1, 2, 3, 5, 6, and 7 and *A. koliensis* and was termed the Farauti clade since the majority of its members had the *A. farauti*-like, all-black-scaled, proboscis. The other major clade contained *A. punctulatus, A.* sp. near *punctulatus,* and *A. farauti* 4 and was termed the Punctulatus clade since it contained members that can exhibit the *A. punctulatus*-like proboscis or at least some degree of white scaling on the proboscis (Fig. 1).

TABLE 2

Distance Matrix of the SSU Data Sets (18S Structure/18S Similarity/12S)

Species	Af2	Af3	Af4	Af5	Af6	Af7	Ak	Ap1	Asnp	Aann
A. farauti 1	1.4/1.7/0.5	3.1/2.9/0.6	3.6/4.4/2.1	1.4/1.2/0.5	1.3/1.3/0.8	0.4/0.5/0.9	3.2/2.8/2.1	4.1/4.8/2.6	3.3/4.2/2.9	5.5/5.4/4.0
A. farauti 2		3.1/3.2/0.5	3.1/4.5/2.0	0.8/1.0/0.3	0.6/1.0/0.6	1.1/1.4/0.9	3.4/2.8/2.0	3.7/4.8/2.3	3.0/4.2/2.3	5.7/5.9/3.8
A. farauti 3			3.7/4.5/2.0	3.1/2.7/0.5	3.2/3.0/0.6	2.8/2.8/1.2	3.2/3.6/2.1	3.9/4.1/2.3	3.5/4.4/2.8	5.2/5.8/3.7
A. farauti 4				3.3/4.1/1.8	3.0/4.0/2.0	3.3/4.4/2.8	3.5/5.1/2.8	2.3/2.3/3.2	1.8/1.7/3.1	4.8/6.0/3.5
A. farauti 5					0.8/0.7/0.5	1.1/1.0/1.1	3.3/3.3/2.0	3.8/4.6/2.3	3.1/4.1/2.3	5.8/5.7/3.7
A. farauti 6						1.1/1.1/1.5	3.3/3.3/2.4	3.8/4.7/2.6	3.1/4.3/2.4	5.8/5.2/3.5
A. farauti 7							3.0/2.8/2.3	3.8/4.9/2.9	3.0/4.3/3.7	5.2/5.2/4.6
A. koliensis								4.4/5.9/2.6	3.8/5.2/2.6	5.6/6.6/4.0
A. punctulatus									1.4/1.0/3.2	5.5/6.9/4.4
A. sp. nr. *punctulatus*										4.8/5.9/4.3

Note. Values are mean distances (×10) adjusted for missing data.

The similarity-based alignment resulted in two trees, one of which was resolved and differed from the structural alignment tree only in the placement of *A. farauti* 3 and *A. koliensis*, which grouped together basal to the Farauti-like clade. The second tree was unresolved though similar to the first tree except that *A. farauti* 5, 2, and 6 collapsed into a polytome. A strict consensus of the two trees resulted in the second unresolved tree.

The likelihood ratio test of Felsentein (1988), comparing the maximum-likelihood tree with the molecular clock to that without the molecular clock, rejected the molecular clock for all data sets. The transversion: transition ratios in these data sets were found to be 1:1,

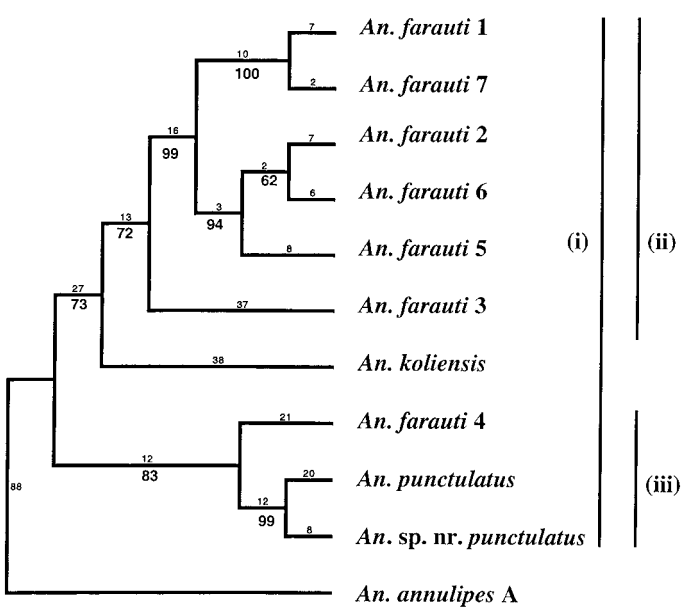

FIG. 1. Single most-parsimonious tree generated from the nuclear SSU structural alignment from members in the *A. punctulatus* group (i). The group separated into a Farauti-like clade (ii) and a Punctulatus-like clade (iii). Numbers above the line represent branch lengths and numbers below the line are percentage values of 500 bootstrap replicates.

complementing the earlier manual comparison of closely related species. The tree derived from these maximum-likelihood analyses was identical to the parsimony tree.

Mitochondrial 12S rDNA

The 664-bp sequence alignment of the mitochondrial 12S gene contained little structural flexibility, with most sequence differences manifesting as point mutations with insertion deletion sequence motifs being rare. Both the structural and the similarity alignments were found to be identical. Parsimony analysis of the full 12S sequence alignments resulted in 123 trees retained (shortest tree required 76 steps) and a strict consensus of these trees could distinguish only the Farauti and Punctulatus clades, with all species in each clade collapsing into a polytome. However, the Punctulatus clade now contained *A. koliensis*, and *A. farauti* 4 was positioned basal to both clades.

DISCUSSION

The genetic relationships among the *A. punctulatus* group of mosquitoes were assessed by comparison of both nuclear and mitochondrial SSU rDNA genes and tested alignments based on sequence similarity and secondary structure. The core of the SSU secondary structure is conserved across both nuclear and mitochondrial genomes, as these genes perform the same protein assembly task (De Rijk and de Wachter, 1993; van de Peer *et al.*, 1993).

The mitochondrial 12S gene was investigated because considerable sequence variation was present in the nuclear rDNA 18S gene and internal transcribed spacers (Beebe *et al.*, 1999; N. W. Beebe *et al.*, unpublished data), making them potentially inappropriate for phylogenetic studies. Unlike the nuclear SSU gene, the mitochondrial SSU is single copy in its genome, clonally inherited, and nonrecombining (Moritz, 1987). The main differences between these two genes were

size (nuclear ~2000 bp and mitochondrial ~650 bp) and GC content (nuclear ~54% and mitochondrial ~22%). The shorter mitochondrial SSU gene exhibited less sequence around which the core secondary structure could be maintained and was therefore restricted in its ability to mutate without affecting structure and thus function. Thus, the amount of informative sequence variation in the alignment of the *A. punctulatus* group members was limited, though it did reveal two separate clades resembling the Punctulatus and Farauti clades. However, it should be noted that, in regions of the gene more prone to sequence mutation, the elevated AT content of the mitochondrial genome may promote homoplasy.

Parsimony analysis of the nuclear SSU resolved a single tree for the *A. punctulatus* group only when the sequence alignment took into account the structure of the ribosomal RNA molecule. The similarity-based alignment resulted in two trees with a strict consensus giving an unresolved tree placing *A. farauti* 3 and *A. koliensis* as sister taxa basal to the Farauti clade.

The nuclear SSU data suggests that the *A. punctulatus* group is monophyletic, which is in agreement with Foley *et al.* (1998), who used the mitochondrial COII gene in a systematic study of many Australasian anophelines. These workers placed *A. koliensis* basal to both the Punctulatus and the Farauti clades, *A. farauti* 2 was positioned basal to *A. farauti* 5 and *A. farauti* 6, and *An.* sp. nr. *punctulatus* was basal to *A. punctulatus* and *A. farauti* 4. However, all these placements were not well supported. Nevertheless, the nuclear SSU gene and COII gene separately identified a Punctulatus and Farauti clade with *A. farauti* 4 paraphyletic to the Farauti clade.

Other observations supporting the monophyletic grouping of the *A. punctulatus* group include the size of the rDNA ITS2 (~700 bp), which appears to be unique to this group of anophelines (Beebe *et al.*, 1999). A weighted parsimony analysis of this region could not resolve a tree but did place *A. farauti* 4 with the Punctulatus-like species.

Phenotypically, there is some concordance between the morphological groupings based on proboscis morphology and the molecular classification presented here. *A. 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 were grouped into the Farauti clade on molecular characteristics. *A. punctulatus* and *A.* sp. near *punctulatus* always display extensive white scaling on the apical half of the proboscis and both were grouped together in the Punctulatus clade (Foley *et al.*, 1993, 1994, 1995; Cooper *et al.*, 1997). *A. farauti* 4 and *A. koliensis* are both known to be polymorphic with regard to this character, as both species have at times displayed farauti-, koliensis-, and punctulatus-like proboscises (Foley *et al.*, 1993; Woodhill, 1946). With *A. farauti* 4, white scaling on the proboscis may be more common than was originally thought and the inclusion of this species in the Punctulatus clade would be consistent with an agreement between phenotype and genotype. However, with *A. koliensis*, the typical proboscis type is with some white scaling on the apical half, and so its appearance basal to the Farauti clade and not in the Punctulatus clade presents an anomaly in these groupings with regard to morphology.

Absence of fossil records for any anophelines makes evolutionary studies on this group of mosquitoes difficult. In general, within the Australian region, New Guinea is considered to be a center for insect speciation and radiation, most often from ancestors of Oriental stock (Kikkawa *et al.*, 1981; Taylor, 1971). The ancestor of the *A. punctulatus* group probably originated in New Guinea, possibly from Oriental stock, as members of this group show genetic similarities with members of the *A. dirus* complex of Asia (Foley *et al.*, 1998; Beebe *et al.*, 1999). This migration of Asian mosquitoes into the Australasian region may have occurred as early as the late Miocene (11 mya) when the Indonesian islands were created, forming a link with Asia. However, throughout the Miocene and early Pliocene (5.3–3.4 mya) much of New Guinea was submerged, the presently recognized land mass appearing in the late Pliocene (3.4–1.6 mya). During the Pleistocene (1.6–0.01 mya) a series of ice ages lowered sea levels, creating links with southeast Asia, and it is more likely that it was during this time that the ancestors of the *A. punctulatus* group migrated into New Guinea from Asia. Other, possibly more recent, immigrants from Asia include *Anopheles subpictus* and *Anopheles karwari.*

As 10 of the known species of the *A. punctulatus* group occur in New Guinea, it is likely that much of the evolution of this group occurred there with southern, eastern, and elevational migration leading to their present distribution in northern Australia, Solomon Islands, and Vanuatu. Speciation within the group has probably occurred since the late Pliocene when the island of New Guinea emerged from the sea. Major fluctuations in sea levels during the Pleistocene would have created opportunities for speciation by both vicariance and dispersalist means.

Insect species dispersal south from the continuous wet of New Guinea to the more arid seasonal wet/dry of northern Australia is uncommon (Kikkawa *et al.*, 1981; Taylor, 1971). Only three members of the *A. punctulatus* group occur in northern Australia, *A. farauti* 1, 2, and 3. Of these, only *A. farauti* 3 appears to have adapted to the arid conditions of this region. It is abundant in areas of the Northern Territory of Australia with precipitation of <1500 mm and uncommon in the continuous wet conditions of Western Province, PNG with precipitation of >2500 mm (Cooper *et al.*, 1996, 1997). This species shows the most genetic divergence

of the Farauti-clade species. It, or its ancestors, may have migrated to northern Australia, a movement that would have been facilitated during the Pleistocene when glaciation lowered the sea levels, creating a land mass connecting northern Australia with the east–west length of New Guinea (Kikkawa *et al.*, 1981). These conditions remained up until the Holocene (0.01 mya), when subsequent warming and drying of the region created the Arafura Sea and the Torres Strait, isolating *A. farauti* 3 in a more arid climate.

Distribution studies on these species do not appear to offer any useful information with regard to interpreting their phylogeny. Dispersal has been affected by recent events, possibly human, and may have little to do with the ancestral age of the species. The evolution of *A. farauti* 1 and 2 and *A. punctulatus* appears to be quite recent; however, all three have wide distributions throughout the southwest Pacific. *A. farauti* 3 and *A. koliensis*, which are ancestral to these three species, have, by comparison, more restricted distributions (Cooper *et al.*, 1996, 1997, unpublished data).

The differences in biology and behavior among the members of the *A. punctulatus* group also appear not to be reliable characters for determining phylogenetic relationships. Small genetic distances separating species within a clade can reflect large behavioral and biological differences, possibly because these differences are too recent or subtle to be revealed in SSU sequence variation. *A. farauti* 1 and *A. farauti* 7 are very closely related species within the Farauti clades (0.4% sequence variation). *A. farauti* 7 was recently identified on Guadacanal in the Solomon Islands where it occurs both on the coast and inland (Foley *et al.*, 1995). It is conceivable that *A. farauti* 7 has evolved from *A. farauti* 1. However, certain aspects of the behavior and biology of these two species are quite dissimilar. *A. farauti* 7 occurs both coastally and inland and, unlike *A. farauti* 1, which will readily bite humans, appears to be exclusively zoophilic (Foley *et al.*, 1995). Both species will survive a saltwater tolerance test (Foley and Bryan, 2000); however, *A. farauti* 7 appears to be an inland freshwater species, whereas *A. farauti* 1 has a purely coastal distribution (Beebe *et al.*, 2000). Thus, within the nuclear SSU of the *A. punctulatus* group, behavioral and ecological differences may be concealed in small genetic distances.

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