POLYMERASE CHAIN REACTION DIAGNOSIS AND THE CHANGING PATTERN OF VECTOR ECOLOGY AND MALARIA TRANSMISSION DYNAMICS IN PAPUA NEW GUINEA

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Abstract. The ecology and behavior of most of the 11 known members of the Anopheles punctulatus group remain unresolved and only the morphologic species An. farauti, An. koliensis, and An. punctulatus are known as vectors of malaria in Papua New Guinea. Of 1,582 mosquitoes examined morphologically, 737 were identified as An. farauti s.l., 719 as An. koliensis, and 126 as An. punctulatus. All specimens identified morphologically as An. punctulatus were shown to be An. punctulatus by polymerase chain reaction–restriction fragment length polymorphism analysis, but the An. farauti and An. koliensis morphotypes consisted of three or more species including An. farauti s.s., An. farauti No. 2, and An. farauti No. 4. The biting cycles and role in malaria transmission of some of these species are described here for the first time. We also show evidence that An. koliensis could be a sub-complex of two or more species. The epidemiologic implications of our findings are discussed.

INTRODUCTION

The taxonomic status of malaria vectors in disease-endemic countries in the western Pacific region is complex because of the involvement of cryptic species. The main vectors of malaria in Papua New Guinea, West Papua (Indonesia), the Solomon Islands, and Vanuatu are members of the Anopheles punctulatus group of mosquitoes, but other species such as An. bancroftii, An. kawari, and An. longirostris may have a minor role in the transmission of the disease. Over the years, as the battery of taxonomic tools has evolved, the number of species identified within the An. punctulatus group has increased. Cross-mating experiments and allozyme analysis have now shown at least 11 sibling species within the group: An. punctulatus, An. sp. near punctulatus, An. clowi, An. koliensis, and An. farauti Nos. 1 to 7. Some of these species are very similar morphologically and the standard method for identification based on proboscis morphology and sector spot on the costa is proving to be quite unreliable in differentiating them. Analysis by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) of the ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) is currently the most reliable and sensitive method for distinguishing between members of the group. It is based on a specific banding pattern for each of the sibling species, but in some cases slightly different patterns may be associated with variants of the same species.

Anopheles farauti s.l. is a species sub-complex consisting of at least seven sibling species that are morphologically indistinguishable. Six species within this sub-complex are known to exist in Papua New Guinea: An. farauti s.s. (formally An. farauti No. 1) and five others designated An. farauti Nos. 2 to 6. The most widely distributed of these is An. farauti s.s., which is found throughout the coastal areas. Anopheles farauti Nos. 2, 3, and 4 are found in the coastal and inland areas, while An. farauti no. 5 and An. farauti No. 6 have only been found in the highlands. Anopheles farauti No. 7 has only been observed in the Solomon Islands. Schmidt and others recently described morphologic markers and presented keys for adult females, fourth instar larvae, and pupae that identify most specimens of An. farauti Nos. 1 to 3 and renamed them An. farauti, An. hinesorum, and An. torresiensis, respectively. Other members of the An. punctulatus group include An. sp. near punctulatus and An. clowi.

The ecologic status and vectorial capacity of the different members of the An. punctulatus group in Papua New Guinea remain largely unresolved, thus impeding the design and implementation of proper plans for the control of malaria transmission. Currently, only the morphologic species of An. farauti s.l., An. koliensis, and An. punctulatus are known as vectors of malaria. In this paper, we describe the adult ecology and role in malaria transmission of other members of the group including An. farauti No. 2 and An. farauti No. 4. Ecologic differences between different genotypes associated with An. koliensis are described for the first time.

MATERIALS AND METHODS

Study area. Entomologic surveys were conducted in 14 coastal and inland villages in the neighboring Provinces of Madang and East Sepik, Papua New Guinea (Figure 1). Both areas are characterized by a tropical wet climate. Rainfall is the main climatic feature that shows marked seasonality, with a wet season normally occurring from October to May and a dry season from June to September. Annual rainfall averages 3,400 mm. Dwellings are often made of bush materials with elevated floors and sago palm leaf roofs. Walls are either of woven split bamboo or sago palm stalk with large unscreened windows. Malaria is highly endemic in both areas and people are exposed to Plasmodium falciparum, P. vivax, and P. malariae throughout the year.

In Madang Province, mosquitoes were collected from 10 villages located less than 50 km from Madang town. Five villages were located on the coastal plains less than 15 meters above sea level with shallow soils on coral limestone. The other villages were located in the foothills of mountain ranges where heavy clay soils support lowland hill forest at altitudes varying from 15 to 500 meters.

In the East Sepik Province, mosquitoes were collected in four villages in the Wosera-Gawi District. The major ecologic feature in the Wosera area is the Amugu River, a permanent stream of greatly variably discharge. The altitude of this re-
region is approximately 180 meters above sea level. The Papua New Guinea Institute of Medical Research field site for malaria vaccine studies is located in the Wosera area. Ethical approval for this research was granted by the Papua New Guinea Medical Research and Advisory Committee.

Mosquito sampling. Mosquitoes were collected using the all-night landing catch and Centers for Disease Control light trap methods as described previously. Anthropophagic mosquitoes were captured outdoors between 6:00 PM and 6:00 AM as they attempted to feed on humans seated on benches with feet and legs bared to the knee. The Centers for Disease control light traps were operated inside houses between 6:00 PM and 6:00 AM. Collected mosquitoes were kept in polystyrene cups in cool boxes and brought to the laboratory for further processing.

Species identification using morphologic techniques. Mosquitoes were sorted and examined microscopically using keys described by Belkin. Morphologic characteristics based on the color of the proboscis and the presence of a sector spot were used to separate members of the *An. punctulatus* group.

![Figure 1](image)

**Figure 1.** Map showing the locations of the mosquito collection sites in the Madang (MP) and East Sepik (ESP) provinces in Papua New Guinea.

![Figure 2](image)

**Figure 2.** Species composition of mosquitoes identified morphologically as **A.** *Anopheles farauti* s.l. and **B.** *Anopheles koliensis.*
into *Anopheles punctulatus*, *An. farauti*, and *An. koliensis*. Following morphologic identification, the specimens were placed individually in coded vials and kept dry in tins containing silica gel for future PCR-RFLP identification.

**Species identification using molecular techniques.** Sibling species of the *An. punctulatus* complex were distinguished by a PCR-RFLP method based on the amplification of the ITS2 region of the rDNA. However, sample processing did not involve isolation of DNA and only a portion of a dry mosquito leg served as template DNA for the PCR. The leg segment was added directly to the PCR mixture in a final reaction volume of 20 μL that contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM MgCl₂, 0.2 mM dNTP, 6% dimethylsulfoxide, 2 units of Taq polymerase (Gibco-BRL, Gaithersburg, MD), and 0.6 μM of each primer: ITS2A, 5’-TGTGAACTGCAGGACACAT-3’ and ITS2B, 5’-TATGCTTAAATTCAAGGGGT-3’. Cycling involved an initial denaturation at 94°C for five minutes, then 40 cycles of 94°C for one minute, 51°C for one minute, and 72°C for two minutes, followed by a final at 72°C for seven minutes. The ITS2 PCR products were subjected to electrophoresis on a 1% agarose gel containing 0.5 g/mL of ethidium bromide and visualized on an ultraviolet UV transilluminator. The PCR products were then digested with five units of Msp I (final volume of 20 μL) and the resulting products resolved by electrophoresis on a 10% polyacrylamide gel, stained in a solution of 0.5 μg/mL of ethidium bromide, and visualized on an ultraviolet transilluminator.

**Detection of malaria parasites by an enzyme-linked immunosorbent assay (ELISA).** Performing the PCR assay on a segment of the mosquito leg allowed the rest of the specimen to be processed by an ELISA to determine malaria infection status. Individual head-thoraces were tested for *P. falciparum*, *P. vivax* (PV210), and *P. vivax* variant (PV247) circumsporozoite antigens according to the methods described by Wirtz and others. Plates were read by an ELISA reader (Molecular Devices Corp., Sunnyvale, CA) and samples were considered positive if absorbance values (range = 0–2.00) at 414 nm exceeded twice the mean of the negative control values.

**RESULTS**

**Species composition and distribution.** Of a total of 1,582 mosquitoes examined morphologically, 737 were identified as *An. farauti* s.l., 719 as *An. koliensis*, and 126 as *An. punctulatus*. The PCR-RFLP was used to verify the morphologic classifications and also to distinguish between sibling species. All specimens identified morphologically as *An. punctulatus* were shown to be *An. punctulatus* by PCR-RFLP analysis. However, similar analysis showed the *An. farauti* and *An. koliensis* morphotypes to consist of several species (Figure 2): five for the *An. farauti* morphotype (*An. farauti* s.s., *An. farauti* No. 4, *An. farauti* No. 2, *An. koliensis*, and *An. punctulatus*) and three for the *An. koliensis* morphotype (*An. farauti* No. 4, *An. koliensis*, and *An. punctulatus*). *Anopheles punctulatus*, *An. koliensis*, and *An. farauti* No. 4 were found in both Wosera and Madang, but *Anopheles farauti* No. 2 was found only in the Wosera area where *An. farauti* s.s. was not observed.

![Figure 3](image-url)
The species composition of the typical coastal morphotype, An. farauti, was analyzed for specimens collected from different distances from the ocean (Figure 3). Anopheles farauti s.s. accounted for 75.8% of specimens collected less than 2 km from the ocean, but An. farauti No. 4 (18.3%) was also common this close to the coast. Only 3.6% of the specimens caught more than 2 km from the ocean were identified as An. farauti s.s., and this species was not observed beyond 5 km inland. Anopheles farauti s.s. was therefore not found in the Wosera area. Conversely, An. farauti No. 2 was only observed among mosquitoes collected from the Wosera study sites, which were located more than 30 km from the coast. Anopheles farauti No. 4 was commonly found in coastal (< 5 km) and inland villages located less than 30 km from the ocean, although 2 of the 195 specimens analyzed from the Wosera area (> 30 km) were An. farauti No. 4. Most (82.1%) of the An.

farauti morphologic specimens collected 5–15 km from the ocean were identified as An. koliensis.

**Anthropophily and biting cycles.** Human-seeking mosquitoes included all species identified in this study. The majority (44.6%) of biting by An. punctulatus occurred during the early hours of the morning between 2:00 AM and 6:00 AM. However, three species within the An. farauti sub-complex (Figure 4) showed a tendency towards early evening biting with activity peaking before midnight. However, the cycles for An. farauti s.s., An. farauti No. 2, and An. farauti No. 4 were slightly different. Anopheles farauti s.s. had a more uniform biting activity between 6:00 PM and 6:00 AM with a slight peak for the period 9:00–10:00 PM. Anopheles farauti No. 2 and An. farauti No. 4 were predominantly early biters, with the proportion of mosquitoes caught between 6:00 PM and 10:00 PM being 66.1% and 40.6%, respectively. When the two-sample Kolmogorov-Smirnov test for equality of distribution functions was applied, we showed that the biting cycles of An. farauti s.s. and An. farauti No. 4 were similar ($P = 0.542$). However, the biting cycle for An. farauti No. 2 was significantly different from An. farauti s.s. and An. farauti No. 4 ($P < 0.001$).

A closer examination of the PCR-RFLP results for the ITS2 region for An. koliensis showed three different genotypes (Figure 5). All three banding patterns were similar to the pattern already described for An. koliensis, but completely different from the patterns described for other species within the An. punctulatus group. The three variants of An. koliensis were designated as M (Madang), W (Wosera), and MW (Madang-Wosera) based on collection site. The MW corresponds to the classic type (with bands of 86, 113, 197, and 207 basepairs), whereas M and W are described here for the first time. The MW variant was the most common and was predominant in the Wosera area, but occasionally found in Madang. The M variant, with major bands of 87, 92, 113 (double), and 197 basepairs, was found only along the Madang coast, no more than 5 km from the ocean. The W variant, with bands of 87, 92, 111, 113, 140, and 202 basepairs, was found only in the Wosera area, more than 30 km from the coast. The specific bands of the Wosera genotype were never found in combination with the specific bands of the M and MW variants. Conversely, we found a hybrid PCR-RFLP pattern for M and MW, which contained the combination of both banding patterns, indicating interbreeding between these two populations. Intra-individual polymorphism was detected as an unresolved banding pattern (very faint bands) in mosquito samples from all three variants (Figure 5).

The biting cycles of the W and MW variants were similar ($P = 0.99$), but the M variant exhibited a biting cycle that was significant different from both of them ($P < 0.001$). The M variant had a late biting cycle, with majority of the biting occurring after midnight as opposed to the W and MW variants, which had a peak biting activity between 6:00 PM and midnight (Figure 6).

**Sporozoite rates.** A total of 583 Madang mosquitoes identified morphologically as An. farauti were processed by the ELISA and 13 were found to be positive for P. falciparum or P. vivax (Table 1). The PCR analysis of the positive mosquitoes showed five An. farauti s.s., four An. farauti No. 4, three An. koliensis, and one An. punctulatus. All mosquito species, except An. punctulatus, were positive for more than one Plasmodium species.

All 12,516 mosquitoes collected in the Wosera area in 1999 and 2000 were processed by the ELISA and the sporozoite rates for the three morphotypes are given in Table 2. Anopheles punctulatus and An. koliensis were positive for P. falciparum and both variants of P. vivax, but assays on 467 individual head-thoraces of An. farauti s.s showed no positive mosquitoes.

**DISCUSSION**

Analysis of morphologic characteristics is still the most widely used technique for taxonomic and ecologic studies in malaria entomology because it is inexpensive and easily applicable in the field. Until recently, malaria vector species identification in Papua New Guinea was based on morphologic characteristics; it was believed that the color of the proboscis and the presence of a sector spot on the wings could reliably distinguish between species and only three members of the An. punctulatus group of mosquitoes were known to exist in the country: An. farauti, An. koliensis, and An. punctulatus. A wide diversity in epidemiologically important characteristics soon suggested a more complex group of morphologically indistinguishable sibling species as demonstrated using other techniques. Once again, as reported by Cooper and others when identifying members of the An. punctulatus in Papua New Guinea, we found morphologic identification to be unreliable for delineating adult females to species within the An. punctulatus group as shown when using PCR-RFLP analysis. Anopheles farauti No. 4 and An. koliensis, which are phylogenetically very close, were morphologically indistinguishable and thus confused on several occasions. Our simplified PCR assay, which did not involve DNA extraction but only the addition of a small portion of the mosquito leg into the PCR mixture, was found to be...
cheaper, quicker, safer, and very effective. Although Beebe and Saul had previously indicated that this is possible, to our knowledge, this is the first time that it has actually been put to practice and with good results.

Specimens with An. farauti morphologic characteristics occurred in all five species found in the two provinces. In the Wosera area of the East Sepik Province, where An. farauti s.s. appears to be absent, An. farauti No. 4 was very rare and An. farauti No. 2 was predominant. Falsely identified specimens were very uncommon in those identified morphologically as An. farauti or An. koliensis. This observation is in good agreement with the phylogenetic analysis based on the ITS regions of the rDNA. Overall, our findings suggest that morphologic criteria could be relied upon for An. farauti No. 2, An. koliensis, and An. punctulatus in the Wosera area, but caution should be exercised in the interpretation of the findings of the

Figure 6. Biting cycles of the Madang (M), Wosera (W), and Madang-Wosera (MW) variants of Anopheles koliensis caught in the Madang and East Sepik provinces of Papua New Guinea.
Table 1
Species composition and infection status of sporozoite-positive Anopheles farauti morphotype specimens collected in Madang Province, Papua New Guinea*

<table>
<thead>
<tr>
<th>Species</th>
<th>PF</th>
<th>PV 210</th>
<th>PV 247</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. farauti</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>An. farauti No. 4</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>An. koliensis</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>An. punctulatus</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>7</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

* Mosquitoes were tested for Plasmodium falciparum (PF), P. vivax (PV 210), and a P. vivax variant (PV247).

Table 2
Sporozoite rates for Anopheles punctulatus, An. farauti s.l., and An. koliensis found in the Wosera area of East Sepik Province, Papua New Guinea*

<table>
<thead>
<tr>
<th>Morphotype</th>
<th>No. tested</th>
<th>PF</th>
<th>PV 210</th>
<th>PV 247</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. punctulatus</td>
<td>5,612</td>
<td>1.1</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>An. koliensis</td>
<td>6,437</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>An. farauti</td>
<td>467</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>12,516</td>
<td>0.6</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* Mosquitoes were tested for Plasmodium falciparum (PF), P. vivax (PV210), and a P. vivax variant (PV240).
vector status of mosquitoes, particularly those that are members of species complexes, and the mapping of their distribution is an important prerequisite. Mosquito control strategies must be designed using knowledge of mosquito biology susceptible to specific control measures.

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