Distribution and proportion of anopheline mosquitoes identified by the PCR-RFLP analysis method in Wewak and Maprik Districts of East Sepik Province, Papua New Guinea

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Abstract: Entomological surveys were done as a part of a malaria control project at nine sites in East Sepik Province. Adult mosquitoes were collected by the human-bait method and the number of anopheline mosquitoes by 177 night × people collections was 3,631. Species identification was done by the PCR-RFLP method at the ITS 2 region of the rDNA. Five kinds of anopheline mosquitoes, i.e., Anopheles farauti 1, An. farauti 2, An. farauti 4, An. koliensis and An. punctulatus were identified. Anopheles koliensis was the most common, then An. punctulatus, An. farauti 1, and followed by An. farauti 4 and An. farauti 2. Anopheles farauti 1 was distributed mainly in coastal areas and An. farauti 2 in inland areas. Anopheles farauti 4 was found only in Kairiru Island and this is the first report of the distribution of this species in an island. Anopheles koliensis and An. punctulatus were widely distributed throughout the districts; however, the distribution of An. koliensis was sporadic. Negative correlations were found between the collected numbers of An. farauti 1 and An. farauti 2 and those of An. koliensis and An. punctulatus.

Key words: Papua New Guinea (PNG), anopheline mosquitoes, human-bait method, PCR-RFLP method, ITS2 region

INTRODUCTION

Anopheline mosquitoes have been exhaustively studied throughout the world because of their role as vectors of malaria. Several anopheline species have been incriminated as vectors of malaria in Papua New Guinea (Hii et al., 2000). The members of the Anopheles punctulatus group

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are the main vectors (Burkot et al., 1988). This group originally consisted of *An. farauti*, *An. koliensis* and *An. punctulatus*. They were classified by the morphological characteristics of their proboscis (Rozeboom and Knight, 1946). *Anopheles farauti* has an all-black scaled labium, *An. koliensis* has a ventral patch of white-scales on the apical half of the labium and *An. punctulatus* has an almost entirely white-scaled apical half of the labium. In succeeding years, cross-mating experiments and allozyme analysis have shown that there are at least 11 sibling species within this group: *An. farauti* 1 to 7, *An. koliensis*, *An. punctulatus*, *An. sp. near punctulatus* and *An. clowi* (Bryan, 1973; Mahon and Miethke, 1982; Foley et al., 1993, 1994, 1995). Some of these species are very similar morphologically and the standard method for identification based on proboscis morphology is proving to be unreliable in differentiating them. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) region is currently the most reliable and sensitive method for differentiating each member of the *An. punctulatus* group (Beebe and Saul, 1995). It is based on a specific banding pattern for each of the sibling species. ITS regions undergo an elevated mutation rate compared to transcribed genes and have been used to distinguish closely related mosquito species (Collins and Paskewitz, 1996; Cornel et al., 1996). No less than ten species within the *An. punctulatus* group are known to exist in Papua New Guinea: *An. farauti* 1 to 6, *An. koliensis*, *An. punctulatus*, *An. sp. near punctulatus* and *An. clowi* (Beebe and Cooper, 2002, Cooper et al., 2002). The most widely distributed of these is *An. farauti* 1 which is found throughout the coastal areas, *An. koliensis* and *An. punctulatus* which are common and widespread throughout PNG. *Anopheles farauti* 2, 3, and 4 are found in the coastal and inland areas, while *An. farauti* 5 and *An. farauti* 6 have been found only in the highlands. *Anopheles clowi* is a rare species with sparse and limited distribution (Rozeboom and Knight, 1946; Cooper et al., 2000). *Anopheles farauti* 7 has only been observed in the Solomon Islands (Foley et al., 1994). Schmidt and others recently described morphologic markers and presented keys for adult females, fourth instar larvae, and pupae and renamed *An. farauti* 1 to 3 and 7 to *An. farauti*, *An. hinesorum*, *An. torresiensis* and *An. irenicus*, respectively (Schmidt et al., 2001, 2003). An integrated cooperative research for the malaria control project between the University of Papua New Guinea and Tokyo Women's Medical University was performed from 2001 to 2004 under Japan International Cooperation Agency (JICA) partnership project program. Entomological surveys were done in the East Sepik Province. In this paper, the results of the surveys are presented.

**Materials and Methods**

Surveys were conducted in the East Sepik Province of Papua New Guinea in February-March 2002, February-March and July-August 2003, February 2004. Mosquitoes were collected from two islands and seven villages in the Province. The islands were Walis and Kairiru, and two villages of Dagua and Boiken are located in the coastal area. The other villages are situated in the inland area. Wingei and Warabung are near a highway, Kaboibus and Jawia are located at the foothills of mountain ranges and Witupe in the inland plains. These sites are indicated in Fig. 1.

Adult mosquitoes were collected outdoor using the human-bait method between 6 pm and 6 am of the following morning at hourly intervals. Identification of morphological species of the collected mosquitoes was done first. Then the specimens were preserved in 100% ethanol and carried back to Japan. More samples were analyzed for the identifica-
Fig. 1. Map of the study region (○; study site). Island areas: Walis, Kairiru; Coastal areas: Dagua, Boiken; Foothill areas: Kaboibus, Jawia; Highway areas: Wingwi, Warabung; Plain area: Witupe.

The sequences of primers were the same as those used by Beebe and Saul (1995). PCR conditions were: initial denaturation at 94°C for 1 min, then 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1.5 min and extension at 72°C for 0.5 min and subsequent final extension at 72°C for 10 min. The PCR products were digested with Msp I restriction enzyme at 37°C for 1 hour. The digested products were electrophoresed on 5% polyacrylamide gel, stained in a solution of 0.5 μg/ml of ethidium bromide, and visualized using an ultraviolet transilluminator. The patterns of RFLP were compared with those of Beebe and Saul (1995) for identifying sibling species.
RESULTS

1. The numbers of collected and analyzed mosquitoes

All collected anopheline mosquitoes as identified by the morphological method at nine sites by 177 night×people collections are shown in Table 1. Three species of mosquitoes, i.e. An. farauti, An. koliensis and An. punctulatus were identified. Most of them are An. farauti and An. koliensis (1,923; 53.0% and 1,454; 40.0% respectively). The rest (254; 7.0%) are An. punctulatus. Finally, a total number of 663 mosquitoes was analyzed by the PCR-RFLP method, i.e. 241 An. farauti, 205 An. koliensis and 217 An. punctulatus.

2. The RFLP banding patterns and nucleotide sequences

The RFLP banding patterns of 663 mosquitoes analyzed using PAGE (Fig. 2) showed the same patterns as those of agarose gel reported by Beebe and Saul (1995) except for 29 mosquitoes. The latter were excluded from further analysis. The nucleotide sequences of each sibling species were compared with those reported by Beebe et al. (1999, 2000). Five sibling species identified this way include An. farauti 1, An. farauti 2, An. farauti 4, An. koliensis and An. punctulatus. Though the RFLP pattern was similar with that by Beebe and Saul (1995) but other than An. farauti 1 all other (An. farauti 2, An. farauti 4, An. punctulatus and An. koliensis) species showed some sort of dissimilarities. As mentioned, the sequence of An. farauti 1 (570 bp) was completely identical to the pattern sequence in Beebe's report. But in the case of An. farauti 2, the sample from Warabung (567 bp) has shown five deletions and one insertion more than that of Beebe (571 bp) and the similarity was 98.05%. Besides these, An. farauti 4 from Kairiru Island (638 bp) has shown maximum size variation including 20 deletions and 2 insertions more than that of Beebe (656 bp) and the similarity was 98.11%. Again, An. koliensis from Witupe (602 bp) had seven deletions and two insertions more than that of Beebe (607 bp) and the similarity was 99.00%. Finally, An. punctulatus from Warabung (568 bp) had one deletion and two insertions more than that of Beebe (567 bp) and the similarity was 98.94%. These highly similar findings show that the amplified ITS2 regions in the present experiment are identical to those of Beebe's experiment.

3. Identification by proboscis morphology and PCR-RFLP analysis

Table 2 shows the relations of the results by morphological identification and RFLP analysis. Out of 241 mosquitoes identified as An. farauti, 156 were An. farauti 1, 31 were farauti 2, 32 were farauti 4, 5 were koliensis and 3 were punctulatus including 14 unknown. Of the 205 morphologically identified as An. koliensis, 195 were koliensis, 5 punctulatus, 3 farauti 2, 1 farauti 4 and 1 unknown. In the case of An. punctulatus, 180 were punctulatus, 17
Table 1. The number of mosquitoes collected and analyzed.

<table>
<thead>
<tr>
<th>Island</th>
<th>Coastal</th>
<th>Foothill</th>
<th>Highway</th>
<th>Plain</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Walis</td>
<td>Kairiru</td>
<td>Dagua</td>
<td>Boiken</td>
<td>Kaboibus</td>
</tr>
<tr>
<td>An. farauti</td>
<td>292 (45)</td>
<td>1,069 (49)</td>
<td>344 (45)</td>
<td>166 (50)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>An. koliensis</td>
<td>0 (0)</td>
<td>207 (50)</td>
<td>1,133 (41)</td>
<td>27 (27)</td>
<td>5 (5)</td>
</tr>
<tr>
<td>An. punctulatus</td>
<td>4 (4)</td>
<td>23 (23)</td>
<td>85 (48)</td>
<td>5 (5)</td>
<td>15 (15)</td>
</tr>
<tr>
<td>Total</td>
<td>296 (49)</td>
<td>1,299 (122)</td>
<td>1,562 (134)</td>
<td>198 (82)</td>
<td>28 (28)</td>
</tr>
<tr>
<td>Night (N)×People (P)</td>
<td>16</td>
<td>26</td>
<td>64</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>No./N×P</td>
<td>18.50</td>
<td>49.96</td>
<td>24.42</td>
<td>33.00</td>
<td>1.66</td>
</tr>
</tbody>
</table>

Numbers in parentheses show those mosquitoes analyzed using PCR-RFLP.

Table 2. Number of mosquitoes collected per night×people.

<table>
<thead>
<tr>
<th>Island</th>
<th>Coastal</th>
<th>Foothill</th>
<th>Highway</th>
<th>Plain</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Walis</td>
<td>Kairiru</td>
<td>Dagua</td>
<td>Boiken</td>
<td>Kaboibus</td>
</tr>
<tr>
<td></td>
<td>No./N×P (%)</td>
<td>No./N×P (%)</td>
<td>No./N×P (%)</td>
<td>No./N×P (%)</td>
<td>No./N×P (%)</td>
</tr>
<tr>
<td>An. farauti</td>
<td>18.25 (98.6)</td>
<td>13.46 (26.9)</td>
<td>5.14 (21.0)</td>
<td>27.67 (83.8)</td>
<td>0.06 (3.6)</td>
</tr>
<tr>
<td>An. koliensis</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.24 (14.3)</td>
</tr>
<tr>
<td>An. punctulatus</td>
<td>0 (0)</td>
<td>0.31 (0.6)</td>
<td>1.28 (5.2)</td>
<td>1.00 (3.0)</td>
<td>1.12 (67.9)</td>
</tr>
<tr>
<td>Unidentified</td>
<td>0.25 (1.4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.06 (3.6)</td>
</tr>
<tr>
<td>Total</td>
<td>18.50 (100)</td>
<td>49.96 (100)</td>
<td>24.42 (100)</td>
<td>33.00 (100)</td>
<td>1.66 (100)</td>
</tr>
</tbody>
</table>
were *koliensis*, 5 were *farauti* 4, 1 was *farauti* 1 and 14 were unknown. The banding patterns of these 29 unknown mosquitoes showed three varieties and these patterns were very stable. A single band variety at about the 500 bp level was shown by 16 mosquitoes. Again, a double-banding pattern at about the 130 and 200 bp level was shown by 4 mosquitoes. Finally, a four-banding pattern at about the 70, 120, 130 and 150 bp level was shown by 9 mosquitoes.

4. The distribution of species

The total number of mosquitoes analyzed by the PCR-RFLP method was 663. As mentioned earlier, five sibling species, i.e. *An. farauti* 1, *An. farauti* 2, *An. farauti* 4, *An. koliensis* and *An. punctulatus* were identified from these specimens. The proportionate distribution of these sibling species is summarized in Table 3. *Anopheles farauti* 1 was found mainly in islands and coastal areas: Walis, Kairiru, Dagua and Boiken. On the contrary, *An. farauti* 2 was found mainly in inland areas: Kaboibus, Warabung, Witupe and Wingei. *Anopheles farauti* 4 was isolated only from Kairiru Island. *Anopheles koliensis* was found to be a major species spread over coastal and inland areas of Kairiru, Dagua and Boiken and also foothills, highways and inland plains of Kaboibus, Warabung and Witupe. *Anopheles punctulatus* were also comparatively widespread in these areas except in Walis Island. Furthermore, there were negative correlations found between the numbers of *An. farauti* 1 and *An. farauti* 2 (correlation coefficient; $-0.617$) and *An. koliensis* and *An. punctulatus* (correlation coefficient; $-0.635$).

5. The species proportion at each site

Table 3 shows the number of mosquitoes collected per night. *Anopheles farauti* 1 was found as the sole species in Walis Island whereas four kinds of mosquitoes were caught at Kairiru Island and listed according to abundance: *An. farauti* 4 (54.4% of the collection), *An. farauti* 1 (26.9%), followed by *An. koliensis* and *An. punctulatus* (18.0% and 0.6%, respectively). In coastal areas, three kinds of mosquitoes (Anopheles farauti 1, An. koliensis and An. punctulatus) were caught at both sites of Dagua and Boiken. *Anopheles koliensis* was the main species in Dagua while *An. farauti* 1 was most abundant in Boiken. Among foothill areas, *An. punctulatus* was the only species caught at Jawia (97.7%). On the other hand, four kinds were caught at Kaboibus and the main species was also *An. punctulatus* (67.9%). Similarly, in the highway areas of Wingei *An. punctulatus* was the major species (83.7%) caught while two species of *An. farauti* 1 and *An. farauti* 2 were very few. At Warabung three kinds of species, namely: *An. koliensis*, *An. punctulatus* and *An. farauti* 2, were caught but these species did not vary much in number (38.6%, 34.3% and 22.9%, respectively). Moreover, in inland plain areas, three kinds of mosquitoes were caught, namely: *An. koliensis*, *An. farauti* 2 and *An. punctulatus*. The main species was *An.
koliensis (58.7%).

6. Biting activity

The number of mosquitoes collected per night × people was 18.50 at Walis, 49.96 at Kairiru, 24.42 at Dagua, 33.00 at Boiken, 1.66 at Kaboibus, 1.79 at Jawia, 10.75 at Wingei, 4.38 at Warabong, 23.00 at Witupe, respectively (Table 1). These results show that mosquitoes actively attack humans in the islands and coastal areas; on the contrary, they are not so active in foothills and highway areas. In the plain areas the activity was moderate.

Discussion

The density of the different species depends on the places surveyed. In this study, the collected number of An. farauti was about 1.32 times that of An. koliensis and about 7.57 times that of An. punctulatus. This result was relatively similar to that of Benet’s survey in Madang and Wosera-Gawi District (Benet et al., 2004). They also found three kinds of anopheline mosquitoes and the number of An. farauti was 1.03 times that of An. koliensis and 5.85 times of An. punctulatus. There is resemblance in the physiological conditions of these areas surveyed. On the contrary, Hii’s survey (Hii et al., 2000) in the Wosera district of East Sepik Province showed that An. koliensis was the most common species collected, followed by An. punctulatus and An. farauti. The five species in this study were similar in showing very high (more than 98%) nucleotide sequences at the ITS2 region as compared with those of Gene Bank. This fact shows that the PCR could amplify ITS2 regions correctly. Beebe and Saul (1995) reported RFLP banding patterns of the ITS2 region by the restriction enzyme of Msp I in 10 cryptic species. The banding patterns of 634 samples out of 663 analyzed correspond to one of Beebe’s patterns; thus, the sibling species of these samples could be decided. But the remaining 29 samples showed three kinds of patterns other than Beebe’s. These three kinds of patterns were constant and all samples had the same patterns. These three kinds of nucleotide sequences should be confirmed and further studies should be done for identifying these species. Benet et al. (2004) described three variant patterns in An. koliensis and suggested the possibility of a subspecies-complex of An. koliensis.

The surveyed region is divided into five areas, i.e. island, coastal, foothill, highway and plain areas. The disposition of An. farauti 1 in island and coastal areas was the same as those of the reports in Papua New Guinea by Charlwood et al. (1986) and in Solomon Islands and Vanuatu by Foley et al. (1994). Cooper et al. (2002) stated that 50% of the collections was made less than 1 km from the coast and 75% of the collections was made within 10 km of the coast. The number of An. farauti 2 was supposed to be very few in this region. So this species was only collected in the foothill, highway and plain areas and not collected in island and coastal areas. Cooper and Frances (2002) also stated that this species is not collected by the human-bait method and is unlikely to be involved in malaria transmission. But in Solomon Islands, this species is very common in inland areas (reported by Foley et al. (1994)) and popular in the area between 10 km and 100 km from the coast in Papua New Guinea according to the report by Cooper et al. (2002). An. farauti 4 was only collected in Kairiru Island in the present study. This species is said to be distributed throughout the islands and occasionally found near the coast (Cooper et al. 2002). This might be the first report of An. farauti 4 from an island in Papua New Guinea. The unique distribution of this species in this region might be attributed to its being a volcanic island. The distribution of An. koliensis has been reported as a very common species throughout PNG by Charlwood et al. (1986), Foley et al. (1994) and Cooper et al. (2002). This species was the second most common species in our survey but its
habitat varied widely and there was no collection at some sites i.e., Walis Island, Jawia and Wingei. The distribution of *An. punctulatus* has been also reported as a very common species throughout PNG by the same authors. In our study, this species was the third most common species from eight sites except in Walis Island.

The negative correlation found between the numbers of *An. farauti* 1 and *An. farauti* 2 is supposed to be reasonable because *An. farauti* 1 is said to be distributed in coastal areas while *An. farauti* 2 prefers inland areas. On the other hand, the negative correlation between *An. koliensis* and *An. punctulatus* seems unpopular because these two species have been reported as very common throughout PNG and have the same preference for inland areas. It is therefore possible that there might be competition between these species as to habitat.

At Walis Island, only *An. farauti* 1 was caught except for a small number of unknown species. This might be caused by the presence of corals. In coral islands, the penetration of water into the land is relatively high, so the condition is not suitable for keeping water; thus, mosquito larvae are not able to survive to adult stage to maintain a population. In Kairiru Island, four species of anopheline mosquitoes were found among human and livestock habitation unlike in Walis Island. Cooper et al. (2002) noted the presence of positive correlation with human habitation in *An. farauti* 1, *farauti* 2, *farauti* 4 and *koliensis*. In the coastal areas, there was a difference in the density of the three species: *An. farauti* 1, *An. koliensis* and *An. punctulatus* between Dagua and Boiken. This difference was thought to be caused by the many permanent pools in Dagua and many streams in Boiken. The foothill area is situated at an elevation of about 300 m above sea level and about 30 km from the coast. The mosquitoes collected were very few because people live on the ridge of the mountain and streams run at the bottom of deep ravines. So the habitats of mosqui-

to larvae were very few around houses because of the steepness of the land. The highway area is about 150 m above sea level and about 35 km from the coast. In this area, the distribution of the three kinds of species of *An. farauti* 2, *An. koliensis* and *An. punctulatus* in Warabung is similar to the report by Cooper et al. (2002) on the height and distance. But *An. koliensis* was not collected at Wingei. Witupu is situated in a plain at about 80 m above sea level and at a distance of 50 km from the coast. The distribution of the three species: *An. farauti* 2, *An. koliensis* and *An. punctulatus*, was also similar to the report of Cooper et al. (2002). But the number of mosquitoes collected was higher than those in the highway areas because of many breeding sites in the plain.

The mean biting activity per night × people was 18.62. This number was almost the same when compared with the report of 2.9 per man × hour by Samarakwickrema et al. (1992) in Solomon Islands. Blood smear examinations were done at the same time of mosquito collections by Tsukahara et al. (2003). The positive cases of human malaria were 15.2% (76/499) at Walis, 18.8% (242/1,288) at Kairiru, 21.4% (367/1,711) at Dagua, 21.5% (216/ 1,004) at Boiken, 34.3% (250/717) at Koboibus, 27.5% (471/1,711) at Jawia, 25.8% (288/ 1,115) at Wingei, 26.1% (253/967) at Warabung and 33.5% (380/1,134) at Witupu. The positive cases at Koboibus, Jawia and Warabung were relatively high despite the low biting activity. There was no direct correlation between malaria positive cases of people and mosquito biting activity.

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