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DNA PROBES FOR IDENTIFYING THE MEMBERS OF THE *ANOPHELES PUNCTULATUS* COMPLEX IN PAPUA NEW GUINEA

NIGEL W. BEEBE, DESMOND H. FOLEY, ALLAN SAUL, LEANNE COOPER, JOAN H. BRYAN, AND THOMAS R. BURKOT

Tropical Health Program, Queensland Institute of Medical Research, Brisbane, Queensland, Australia; Tropical Health Program and Entomology Department, University of Queensland, Saint Lucia, Australia

Abstract. Genomic DNA probes were made for five members of the *Anopheles punctulatus* complex of mosquitoes found in Papua New Guinea. Specific DNA probes were developed for *An. punctulatus, An. koliensis,* and three sibling species, *An. farauti* No. 4, *An. farauti* No. 5, and *An. farauti* No. 6, by differentially screening total genomic DNA libraries of individual species and sibling species with homologous DNA against heterologous DNA labeled with ^{32}P. Probes ranged from 273 to 630 bp. Identification of species can be made from squash or dot blots using only a segment of the mosquito (i.e., head, thorax, abdomen, or even legs), allowing for concurrent analysis of the remainder of the mosquito for other epidemiologic characteristics.

The mosquitoes known collectively as the *Anopheles punctulatus* complex are the most important vectors of human malaria in Papua New Guinea, Irian Jaya (Indonesia), the Solomon Islands, and Vanuatu. Members of the complex are also important vectors of human filariasis caused by *Wuchereria bancrofti* in parts of the southwest Pacific.

In Papua New Guinea, three morphologic species, *An. farauti, An. koliensis,* and *An. punctulatus,* have been described.[1] Cross-mating studies revealed three isomorphic species designated *An. farauti* Nos. 1, 2, and 3.[2,3] Banding patterns of the fourth instar larval salivary gland polytene chromosomes also differentiate these three species.[4] In addition to *An. koliensis, An. punctulatus,* and *An. farauti* Nos. 1, 2, and 3, the presence of three additional isomorphic species in Papua New Guinea designated *An. farauti* Nos. 4, 5, and 6 were revealed by the analysis of 35 allozyme patterns.[5,6]

Unfortunately, these species cannot be distinguished by morphology, and further investigations have shown that morphologic characteristics are unreliable even for distinguishing *An. punctulatus, An. koliensis,* and *A. farauti* s.l.[5] To evaluate the relative importance of these species as vectors and to monitor control programs directed against the important vectors, rapid, reliable, and inexpensive ways of identifying very large numbers of adult mosquitoes are needed.

The use of species-specific genomic DNA probes that hybridize with DNA from mosquito squash blots on nylon membranes would facilitate such studies since individual sample handling is minimized. In addition all reagents are stable at room temperature, and the procedure is compatible with isotopic, chromogenic, and chemiluminescence detection systems,[7] enabling large numbers of mosquitoes to be analyzed inexpensively. The key requirement is to develop probes with the required specificity and sensitivity. Probes have been described for members of the *An. gambiae*[8,9] and *An. dirus* complexes,[10] and the Australian members of the *An. punctulatus* complex.[7,11] In this paper, species-specific genomic DNA probes for members of the *An. punctulatus* complex found in Papua New Guinea, *An. punctulatus, An. koliensis,* and *An. farauti* Nos. 4, 5, and 6, are described.

MATERIALS AND METHODS

Mosquito DNA

Isofemale lines of mosquitoes identified by electrophoretic keys[4] were used to construct genomic DNA libraries. *Anopheles farauti* No. 4 and *An. koliensis* were collected from Gonoa village in Papua New Guinea near Madang, *An. punctulatus* from Buksak village near Madang, *An. farauti* No. 5 from Ketarabo village near Goroka, and *An. farauti* No. 6 from Hiwanda village near Tari.[5]

Mosquitoes were homogenized in 100 μl of lysis buffer (10 mM Tris-HCl, 2 mM EDTA, 400 mM NaCl, 1% sodium dodecyl sulfate

[SDS], pH 8) containing proteinase K (0.1 mg/ml). Following a 2-hr incubation at 65°C, mosquito DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and then precipitated by the addition of 50 μl of 7.5 M ammonium acetate and 300 μl of ethanol at −70°C for at least 15 min. The DNA was pelleted in a microfuge for 20 min prior to washing with 75% ethanol, dried, and reconstituted in 20–50 μl of TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) containing 10 μg/ml of RNase.

Genomic library construction

Anopheles punctulatus and *An. farauti* No. 5 DNA were digested to completion with *Sau 3A* I and ligated into dephosphorylated pBluescript II KS+ (Stratagene, La Jolla, CA) at the *Bam* HI site. *Anopheles koliensis* and *An. farauti* Nos. 4 and 6 DNA were digested with *Hind* III and ligated into dephosphorylated pUC19 (New England Biolabs, Beverly, MA) at the *Hind* III site. *Escherichia coli* was transformed[12] and replica plated[13] prior to screening by colony hybridization.[7] The DNA to be used as probes was labeled by the incorporation of ^{32}P dCTP using random hexamer priming (Pharmacia, Uppsala, Sweden).

Probe insert size determination and optimization

Recombinant plasmids were cleaved at the *Eco* RI restriction site and separated on a 0.8% agarose gel, and the sizes were estimated by comparison with molecular weight markers. Probes with inserts larger than 1,200 bp or those that cross-reacted with other members of the complex were further digested with *Rsa* I and *Bam* HI, electrophoresed on a 1.5% agarose gel, Southern blotted onto a nylon matrix (Hybond-N; Amersham, Arlington Heights, IL), and differentially probed by comparing hybridization with ^{32}P-dCTP–labeled homologous and heterologous genomic DNA. The DNA fragments specific for homologous DNA were subcloned into pUC19 at the *Hinc* II or *Bam* HI sites.

Sensitivity and specificity of probes

The genomic DNA was quantified in a 0.8% agarose gel containing 0.5 μg/ml of ethidium bromide. Eight two-fold serial dilutions of DNA from each species in the complex were spotted onto a nylon membrane. Quantities of DNA ranged from 50 ng to 0.375 ng per spot. The DNA on the membrane was denatured in 0.5 M NaOH, 1.5 M NaCl for 2 min, neutralized in 1 M Tris, 1.5 M NaCl for 5 min, washed briefly in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), and fixed to the membrane by baking in a vacuum oven at 80°C for 2 hr. The membranes were hybridized with the respective plasmid DNA containing the probe labeled by random hexamer priming with α^{32}P-dCTP to give a specific activity of 0.05 μCi/ng of DNA. Probes were used at a concentration of 10 ng/ml in a minimal volume of phosphate buffer (0.263 M Na_2HPO_4, 7% SDS, 1.0 mM EDTA, pH 8.0, 1% bovine serum albumin) overnight at 65°C. Membranes were washed twice for 30 min in 2× SSC, 0.1% SDS at 65°C; twice for 30 min in 0.1× SSC, 0.1% SDS at 65°C; and exposed to autoradiographic film for 4–16 hr. Following exposure of the autoradiograph, bound label was stripped by washing the membrane in boiling 0.1% SDS, rinsing once in 2× SSC, and the membrane was reprobed. The selectivity of the probes was determined from the quantity of DNA that gave a signal equivalent to the 50-ng DNA sample of the most intense spot of a heterologous DNA sample. For example, the 1.5-ng DNA spot of *An. farauti* No. 6 gave a spot similar to that of the 50-ng spot of *An. farauti* Nos. 4 and 5 and thus has a selectivity of 32. The sensitivity was determined as the minimum quantity of DNA that gave a clearly visible spot after an overnight exposure.

Squash blots of stored material

Probes were tested on mosquitoes collected in the field to verify specificity and determine sensitivity. Abdomens of individual mosquitoes typed as *An. punctulatus, An. koliensis*, and *An. farauti* No. 6 by allozyme were squashed onto nylon membranes and probed with the probes described in this paper. A collection of *An. farauti* s.l. collected in the Wosera district of Papua New Guinea was kindly provided By Dr. J. Hii (Institute of Medical Research, Papua New Guinea). Other mosquitoes in this collection were typed as *An. farauti* No. 4. by allozyme analysis. No further field collections of *An. farauti* No. 5 have been available for testing.

The sensitivity of the probe for *An. farauti*

TABLE 1
Summary of the species-specific DNA probes

Species	Size of insert (base pairs)	No. of colonies screened	Vector (restriction enzyme site)	GenBank accession number
An. punctulatus	532	1,000	pBluescript (*Bam* HI)	L13085
An. koliensis	273	10,000	pUC19 (*Hind* III)	L13084
An. farauti no. 4	630*	15,000	pUC19 (*Bam* HI)	L13086
An. farauti no. 5	392	2,000	pBluescript (*Bam* HI)	L13087
An. farauti no. 6	526*	15,000	pUC19 (*Hinc* II)	L13088

* Subcloned from probe isolated in a primary screen.

No. 6 was tested on field-collected material. The head and three legs of mosquitoes stored either in 70% ethanol, dried, or frozen were squashed directly onto a nylon membrane wet with 10% SDS. The membrane was then denatured, neutralized, rinsed, and fixed as mentioned above. The hybridization method and washing conditions used are as mentioned above. Exposure time on the film was 16 hr.

Sequencing of cloned inserts

Recombinant probes were amplified with *Taq* polymerase (Promega, Madison, WI) using forward and reverse universal primers for 25 cycles at 94°C, 55°C, and 72°C for 1 min each. The products were electrophoresed on a 0.8% agarose gel and the amplified insert was excised and extracted with Gene Clean (Bio 101, La Jolla, CA). Sequencing was performed with universal forward and reverse dye-primers as recommended in the *Taq* Dye Primer Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The double-stranded sequence analysis was performed in both directions according to the manufacturer's recommendations on a 370A DNA Sequencer (Applied Biosystems).

RESULTS

Isolation of genomic DNA probes for species in the *An. punctulatus* complex was done by differential screening of genomic DNA libraries with homologous DNA compared with heterologous genomic DNA. The original probe isolated for *An. farauti* No. 4 was 4 kb and cross-reacted with *An. farauti* No. 6 DNA. A 700-bp subfragment was species specific and this was cloned into pUC19. Similarly, the original probe for *An. farauti* No. 6. was also large (3.6 kb) and a smaller 600-bp *Rsa* I subfragment was chosen as the final probe. Details of the probes isolated are given in Table 1. Probes hybridizing with homologous and heterologous DNA are shown in Figure 1. All probes had a high specificity. The *An. punctulatus* probe gave a strong signal on 0.39 ng of DNA after a 4-hr exposure and failed to react with 50 ng of DNA from any of the other species under these conditions. Probes for *An. koliensis* and *An. farauti* Nos. 4 and 5 gave similar intensities with 0.79 ng of homologous DNA to 50 ng of heterologous DNA and thus had a specificity of approximately 63, where the specificity equals the heterologous cross-reactivity divided by the homologous sensitivity (50 ng/0.97 ng = 63.3), and the 0.39-ng DNA sample was visible for each of these following an overnight exposure. The probe for *An. farauti* No. 6 was both the least sensitive and least specific, with a specificity of 32 and a sensitivity of 0.78 ng following an overnight exposure. As shown in Figure 1, the membranes could be repeatedly stripped and reprobed without an unacceptable loss of signal.

The specificity of probes for *An. punctulatus*, *An. koliensis*, and *An. farauti* Nos. 4 and 6 were verified by probing squash blots of mosquitoes typed by allozyme analysis. Each mosquito was tested against a panel of probes: in all cases probes agreed with the allozyme typing (data not shown). The sensitivity of the *An. farauti* No. 6 probe was investigated further. As shown in Figure 2, DNA extracted by squashing heads or legs from mosquitoes stored frozen, dried, or in alcohol was readily visualized following an overnight exposure.

Probe sequences have been submitted to Genbank (Los Alamos, NM) and the accession numbers are detailed in Table 1. The *An. koliensis* probe had a 102-bp repeat bounded by palindromic sequences. None of the other probes contained internally repetitive sequences. All of the probes had stretches of open reading frames, but

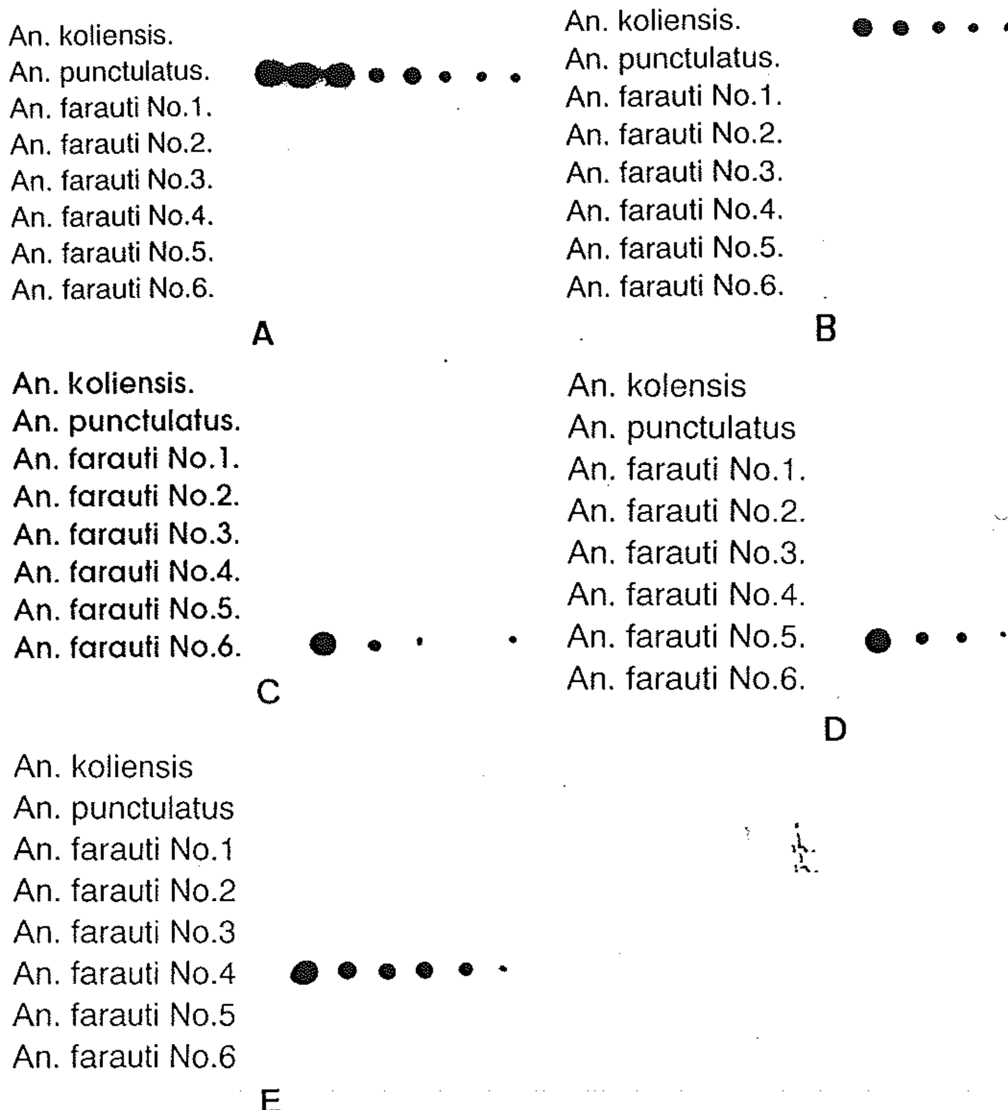

FIGURE 1. Sensitivity and specificity of dot blots of genomic DNA representing the *Anopheles punctulatus* complex in eight, two-fold serial dilutions (from left to right), (50–0.78 ng) hybridized with ^{32}P-labeled probes, stripped, and reprobed in the following order: **A**, *An. punctulatus;* **B**, *An. koliensis;* **C**, *An. farauti* No. 6; **D**, *An. farauti* No. 5; and **E**, *An. farauti* No. 4.

there is no information to show if these are translated. No significant homology was found with any of the probes to sequences contained in Genebank (Version 7.2, release 75.0 [2/93], last weekly update 3/15/93).

DISCUSSION

Genomic probes that are able to distinguish members of the *An. punctulatus* complex are described in this paper. Unlike previous reports of genomic probes for identifying mosquitoes, these probes do not consist of tandem arrays of relatively short repeating sequences.[7,9,11] Only one probe, *An. koliensis,* contained an internal repeat; two copies of a 102-bp sequence. Although this study failed to isolate probes based on short repeat structures, this does not indicate that they are absent from this species. Because of differential screening procedures, they would

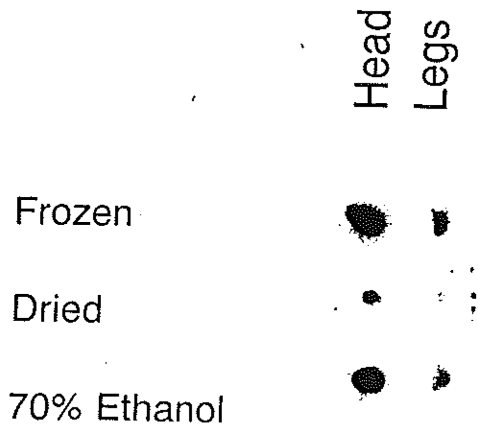

FIGURE 2. Squash blot of the head and legs of *Anopheles farauti* No. 6 mosquitoes that had been stored frozen, dried, or in 70% ethanol and probed with ^{32}P-labeled *An. farauti* No. 6 species-specific probe.

not have been isolated if there was significant homology between repeat sequences in more than one species.

Regardless of the nature of the sequences isolated, they do fulfill criteria needed as probes for identifying these members of the *An. punctulatus* complex. The probes are species specific. The probes were generated using DNA from isofemale lines but with the exception of *An. farauti* No. 5 for which no additional field material has been available, they have all displayed the expected concordance when retested on field-caught mosquitoes typed by allozymes.

The sensitivity of the probes allows species identification with only a fraction of the mosquito (i.e., head, thorax, abdomen, or legs) squash-blotted onto a membrane. The remainder of the mosquito may be concurrently analyzed for epidemiologically important characteristics such as host blood meal source and/or human parasite detection. The simplicity of squash blots minimizes field preparation of specimens. Squashes of legs result in less binding of DNA to the filter due to difficulty in breaking up the chitin and should be noted when comparing the results. Furthermore, identification may be made using mosquitoes collected and stored frozen, air-dried, or fixed in 70% ethanol or isopropanol. A useful point when using squash blots is to determine the amount of DNA bound to the membrane. This could be done using a heterologous probe such as one for the rDNA, where any selectivity problems can be evaluated.

The probes described in this report have a comparable sensitivity to the probes developed earlier for *An. farauti* Nos. 1, 2, and 3.[7] Since these probes have been used successfully with nonradioactive detection systems (e.g., chemiluminescence and chromogenic detection[7,14]), we would expect that the new probes will also be suitable for nonradioactive detection systems, thus allowing laboratories without access to radiochemicals the opportunity to use them.

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Authors' addresses: Nigel W. Beebe, Allan Saul, and Leanne Cooper, Tropical Health Program, Queensland Institute of Medical Research, The Bancroft Centre, 300 Herston Road, Brisbane, Queensland, Australia 4029. Desmond H. Foley and Joan H. Bryan, Tropical Health Program and Entomology Department, University of Queensland, Saint Lucia, Australia 4067. Thomas R. Burkot, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, PO Box 2087, Fort Collins, CO 80522.

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