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DNA PROBES FOR THE *ANOPHELES PUNCTULATUS* COMPLEX

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Abstract. Genomic DNA probes were made for two recently identified members of the *Anopheles punctulatus* complex; *Anopheles* sp. near *punctulatus* from Papua New Guinea and *Anopheles farauti* No. 7 from the Solomon Islands. The probes are species-specific and with the use of ^{32}P labeling sensitive enough so that a squash blot of only a small segment of the mosquito is required for identification. The 119-basepair (bp) probe for *An.* sp. near *punctulatus* and the 1,106-bp probe for *An. farauti* No. 7 have been sequenced in full and the probes have been tested on field collected specimens. These probes now make it possible to distinguish *An.* sp. near *punctulatus* and *An. farauti* No. 7 from the other eight members of the *An. punctulatus* complex. A pan-species probe was also made from the 18S ribosomal DNA that binds to DNA from all members of the complex. These three probes complete the set required for distinguishing all known members of the *An. punctulatus* complex by DNA hybridization.

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. Three species, *An. punctulatus* Doenitz, *An. koliensis* Owen, and *An. farauti* Laveran were recognized on morphologic grounds.[1] Cross-mating and polytene chromosomal studies showed *An. farauti* could be separated into at least three species, *An. farauti* No. 1, 2, and 3.[2,3] Subsequent allozyme analysis using 35 loci confirmed these classifications and revealed the presence of three additional species in Papua New Guinea now designated *An. farauti* No. 4, 5, and 6.[4] To facilitate rapid identification of large mosquito collections, DNA hybridization probes were developed for these eight species within the *An. punctulatus* complex.[5–7]

Recently, allozyme analysis has identified two new species within the punctulatus complex.[8,9] One from the Solomon Islands was a sibling species of *An. farauti* and was called *An. farauti* No. 7; the second from the Western Province of Papua New Guinea was morphologically similar to *An. punctulatus* and was designated *An.* species near *punctulatus.*

In this paper, we report the development of DNA probes for these two species. We also report the development of a pan-species probe that can be used as a control to evaluate the amount of mosquito DNA bound to the hybridization filter. These three probes thus complete the reagents required for a system for large scale identification of all currently known members of the *An. punctulatus complex.*

MATERIALS AND METHODS

Mosquito DNA. *Anopheles* sp. near *punctulatus* adults and larvae were collected from the Rentoul River area of the Western Province, Papua New Guinea,[8] while *An. farauti* No. 7 adults were from Mamara on the island of Guadalcanal in the Solomon Islands.[9]

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 100 μl of phenol/chloroform/isoamyl alcohol (25:24:1), then precipitated by the addition of 50 μl of 7.5M ammonium acetate and 300 μl of 100% ethanol at −70°C for 15 min and pelleted in a microfuge for 20 min. The pellet was then washed with 75% ethanol, dried, then reconstituted in 20–50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 10 μg/ml of RNase.

Genomic library construction. Genomic DNA from *An. farauti* No. 7 was digested to completion with the restriction endonuclease *Hind* III and *An.* species near *punctulatus* was digested with *Sau* 3A I. The fragments were ligated into dephosphorylated pUC19 (Stratagene, La Jolla, CA) at the *Hind* III and *Bam* HI sites, respectively, then used to transform *Escherichia coli* (DH5α).[10] Colonies were replica-plated prior to screening by colony hybridization.[9,11] Genomic DNA extracted from the other species in the complex were used as the heterologous DNA while the single species constituted the homologous DNA. Radiolabeling was performed using ^{32}P random hexamer priming kit (Pharmacia, Uppsala, Sweden). Colonies that gave a strong signal with homologous DNA but not heterologous DNA were selected as potential species-specific probes.

Probe optimization. The specificity of the probe for *An. farauti* No. 7 was improved by subcloning a fragment. The probe was digested with *Pst* I, electrophoresed on a 1.5% agarose gel, Southern blotted onto a nylon matrix (Hybond-N; Amersham, Buckinghamshire, United Kingdom) and differentially probed with labeled homologous and heterologous genomic DNA. Fragments specific for homologous DNA were subcloned into pUC19 at the *Pst* I site.

Sensitivity and specificity of probes. Dot blots of approximately 30 ng of DNA from each member of the complex were spotted onto a nylon membrane (Hybond-N; Amersham). The DNA was denatured in 0.5 M NaOH, 1.5 M NaCl for 2 min, neutralized in 1 M Tris-HCl pH 7, 1.5 M NaCl for 5 min, washed briefly in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and fixed to the membrane by baking in a vacuum oven at 80°C for 2 hr. The membranes were hybridized using random-primed ^{32}P-labeled plasmids (specific activity = 0.05 μCi/ng) containing the probe insert at a final 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), at 65°C overnight. Membranes were washed twice for 30 min in 2× SSC, 0.1% SDS; twice for 30 min in 0.1× SSC, 0.1% SDS at 65°C; and exposed to autoradiograph film for 4–16 hr.

Pan-species probe. An 850-basepair (bp) segment from the 5′ end of the 18S ribosomal DNA (rDNA) was amplified from *An. farauti* No. 1 genomic DNA using primers derived from *Drosophila melanogaster* (5′GAGGGAGCCTGAGAAACGGCTAC3′, 5′CCTTCCGTCAATTCCTTTAAGTTTC 3′) with 20 ng of template cycling 35 times between 94°C, 54°C, and 72°C for 1 min each. The product was excised from a 1.0% agarose gel and purified using Gene Clean (Bio 101, Inc., La Jolla, CA). The purified DNA was labeled with ^{32}P-dCTP using random hexamer primers. Hybridization was as described above except the filters were washed twice in 2× SSC, 0.1% SDS for 30 min each at 65°C, then placed on film for 16 hr.

Sequencing of cloned inserts. The *An.* species near *punctulatus* probe was sequenced using M13-20 and reverse dye-primers as recommended in the Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The *An. farauti* No.7 probe was digested with *Sau* 3AI, and a 600-bp fragment of the 3′ end was subcloned into pUC19 at the *Bam* HI site and sequenced in both directions using dye primer chemistry and M13 universal primers. From this sequence, a reverse primer (5′CGACATTCGGGCGCTCG3′) was designed to enable the 5′ end of the original 1,100-bp clone to be sequenced. This was obtained using dye terminator chemistry. The double-stranded sequence analysis was performed in both directions according to the manufacturer's recommendations on a 370A DNA Sequencer (Applied Biosystems).

RESULTS

Genomic DNA probes were isolated by differential screening genomic DNA libraries using homologous and heterologous DNA. Clones that preferentially hybridized with homologous DNA were selected and tested for specificity and sensitivity. A single clone of 8 kb from the *An. farauti* No. 7 library was isolated after the screening of 20,000 bacterial colonies (ampicillin-selected plasmid clones). This clone, however, cross-reacted with *An. farauti* No. 5 genomic DNA, a species only found to date in the highlands of Papua New Guinea.[4] A *Pst* I digest of this 8-kb insert gave a 1,106-bp subfragment that was specific for *An. farauti* No. 7. The *An.* species near *punctulatus* probe had a 119-bp insert and required 2,000 bacterial colonies to be screened. It displayed no-cross reactivity with other members of the complex.

Amplification of the pan-species probe using rDNA primers required 35 cycles and generated an 850-bp product. This product was sized in a 0.8% agarose gel, purified from the gel, and used as template for radiolabeling. This probe hybridized to genomic DNA from all members of the complex and signal strength was proportional the amount of DNA on the filter.

In Figure 1, the specificity of each of the probes is compared with the pan-species rDNA probe on dot blots of genomic DNA. In Figure 2, previously identified mosquitoes from the Western Province of Papua New Guinea wer squashed onto a nylon filter that was subsequently probed stripped, and reprobed with ^{32}P-labeled *An. punctulatus* spe cies-specific probe (A), *An.* sp. near *punctulatus* probe (B) and pan-species 18S probe (C).

Species-specific probe sequences have been submitted t Genbank with accession numbers of U21088 for *An. farau* No. 7 and U21089 for *An.* sp. near *punctulatus*. No signifi cant homology was found with any of the probes to sequenc es contained in Genbank (Release 87.0 and weekly update to 8/95).

DISCUSSION

Genomic probes for two members of the *An. punctulatu* complex and a pan-species DNA probe are described in thi paper. Unlike some genomic probes developed for identify ing anophelines, the two new species-specific probes do nc consist of tandem arrays of relatively short repeating se quences,[5,6,12–14] but reflect a longer multicopied sequenc format similar to other *An. punctulatus* genomic DN probes.[7] Selection of short repeats would not have occurre if there had been significant homology between repeat se quences in other species. However, regardless of the natu of the sequences isolated, the probes adequately identifie field material of the two new species.

The most specific clone for *An. farauti* No. 7 was foun after screening 20,000 colonies. The main difficulty wa finding probes that distinguished between *An. farauti* No. and *An. farauti* No. 1. The only clone not displaying cros reactivity to *An. farauti* No. 1 reacted with DNA from A *farauti* No. 5, an allopatric species, so far only found in th highland of Papua New Guinea. However, the cross-reactiv sequence could be deleted resulting in a probe with absolu specificity for *An. farauti* No. 7. The DNA probe for A *farauti* No. 1 was initially selected to distinguish *An. farau* No. 1 from *An. punctulatus, An. koliensis,* and *An. farau* No. 2 and No. 3, and was later found to hybridize with A *farauti* No. 7 DNA.[5] A close relationship between *An. fa auti* No. 1 and No. 7 has also been noted by Foley a others.[8] These points suggest that *An. farauti* No. 7 may ha only recently evolved as a new species and thus does n contain many autonomous regions.

These results highlight a need for caution when usi DNA probes for identifying species: these probes can on be relied upon to distinguish species identified at the tir probes are selected. In the case of *An. farauti* No. 1 and N 7, although it is now known that the *An. farauti* No. 1 pro hybridizes to the DNA of *An. farauti* No. 7, these two sp cies can be readily distinguished since the *An. farauti* No. probe does not hybridize with genomic DNA from *An. fa auti* No. 1.

The species-specific probe for *An.* sp. near *punctulat* required a 10 times less screening to isolate a species-sp cific probe (2,000 colonies). Allozyme analysis suggests th unlike *An. farauti* No. 7, *An.* sp. near *punctulatus* has lit affinity with other members of the complex,[8] and that probably evolved as a new species far earlier than *An. fara* No. 7.

The pan-species DNA probe was amplified from the end of the 18S rDNA and seems to be conserved and i

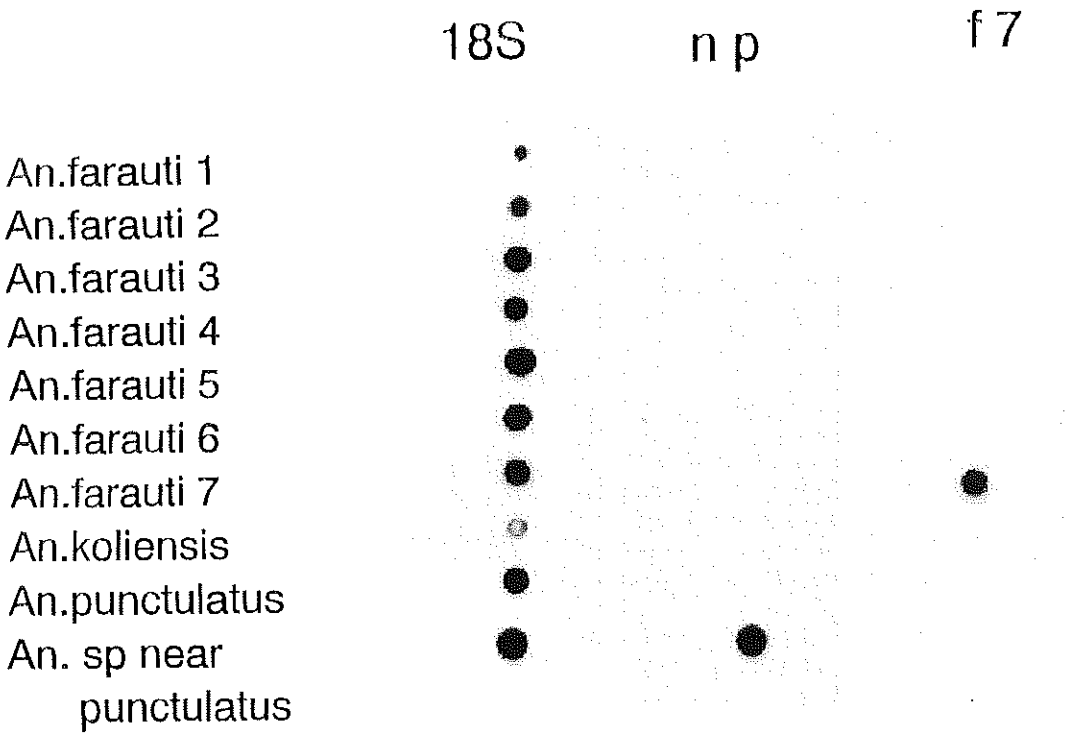

FIGURE 1. Dot blot of genomic DNA from each member of the *Anopheles punctulatus* complex probed by the pan-species (18S) probe, *An.* sp. near *punctulatus* DNA probe (np), and the *An. farauti* No. 7 DNA probe (f7).

high copy number. The rDNA probe has been a useful tool for determining the amount of DNA on the membrane and has been required in cases of inadequate squashing, poor specimen condition, and after multiple stripping of the filter. Some difficulties have been experienced when no species-specific probe has given an unequivocal positive signal. The condition of the sample may lead to low levels of DNA transferring to the membrane and this may be interpreted incorrectly, resulting in failure to identify a sample as belonging to a species already characterized. However, such technical difficulties can now be reassessed using the rDNA pan-species probe. This allows a classification of negative

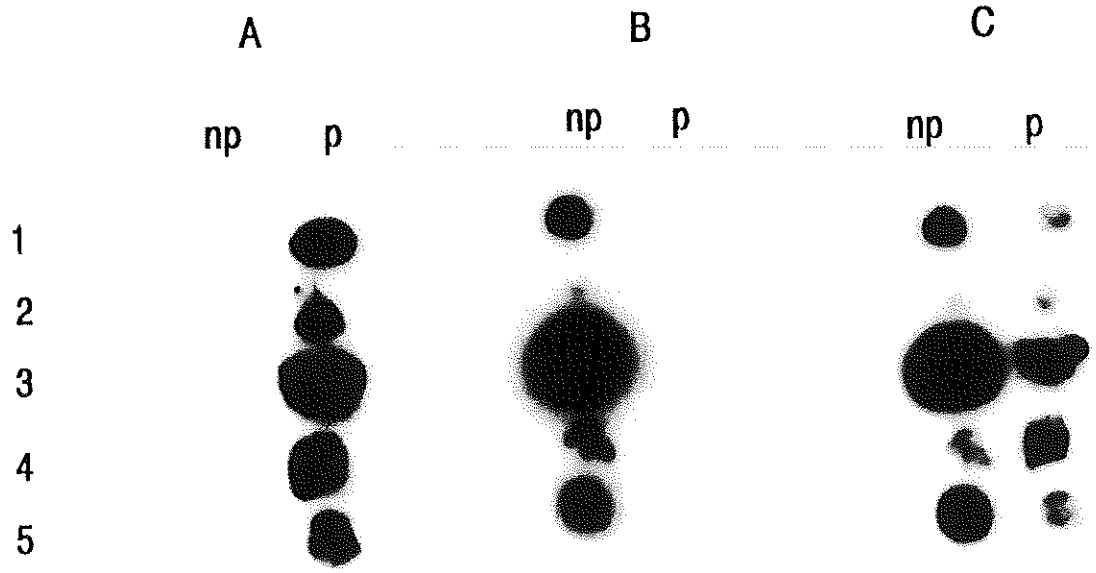

FIGURE 2. Squash blot of *Anopheles* (*An.*) sp. near *punctulatus* (np) and *An. punctulatus* (p) from the Western Province of Papua New Guinea. In panel A, the blot was probed with the *An. punctulatus* DNA probe.[7] In panel B, the blot was reprobed with *An.* sp. near *punctulatus* DNA probe. In panel C, the blot was reprobed with the 18S pan-species DNA probe. The blot was stripped between each probing. Rows 1–5 are sections of five individual mosquitoes. 1 = head; 2 = 2 × legs; 3 = abdomen; 4 = head; 5 = 1/4 abdomen.

and weak signals into three categories: 1) when the rDNA signal is strong and all species-specific probe signals are weak or negative, the species does not correspond to a previously identified species; 2) when the rDNA signal is weak, the ratio of species-specific probe signal to rDNA signal can be used for typing the sample thus extending the proportion of samples that can be typed; and 3) when the rDNA is very weak, and the sample cannot be typed.

Recently, an alternative method of typing individual specimens has been described,[15] based on the amplification of the internal transcribed spacer 2 (ITS2) region of the ribosomal RNA gene, followed by restriction length polymorphism analysis. This method independently supports the validity of the species status originally determined by polytene chromosome banding patterns and allozyme analysis.[3,4,8,9] There has been complete concordance between DNA hybridization and polymerase chain reaction (PCR) techniques to the original classification for all species so far,[15] indicating that these are fixed differences in at least two independent loci, making it even more likely that the 10 species described for this complex are valid.

The DNA hybridization and PCR techniques form complementary typing procedures. The DNA hybridization is best suited for screening large collections. For example, in the laboratory of the investigators, thousands of mosquitoes have been typed using this method. Such numbers are not feasible using PCR. In theory, the nylon membranes containing squash-blotted mosquito DNA may need to be probed up to 11 times (10 species probes and one pan-species probe) to identify all members of the complex. In practice, it would be unusual to need more than five separate probings to identify most of the mosquitoes in a collection since the maximum number of species found in one area so far is four. Around Hudini village near Madang, *An. farauti* No. 1, *An. farauti* No. 4, *An. koliensis,* and *An. punctulatus* were identified. Some *An. koliensis* and *An. punctulatus* species identified by morphology were in fact *An. farauti* No. 4 when subjected to allozyme analysis, demonstrating the problems with morphologic identification.[4]

Some mosquitoes will remain unidentified after several cycles of hybridization due to poor sample preparation, incorrect identification of the mosquito as a member of the *An. punctulatus* complex, rare species making further probing unproductive, or a new species. In these cases, the PCR technique will provide definitive identification if a portion of the mosquito is retained. Thus, the combination of DNA hybridization using the 11 probes developed for this complex along with PCR identification should provide a means of identifying both the highly abundant as well as the rare or unusual specimens in large collections, facilitating epidemiologic studies on these species.

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