A COCKTAIL POLYMERASE CHAIN REACTION ASSAY TO IDENTIFY MEMBERS OF THE ANOPHELES FUNESTUS (DIPTERA: CULICIDAE) GROUP

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Abstract. Anopheles funestus Giles is a major malaria vector in Africa belonging to a group of species with morphologically similar characteristics. Morphological identification of members of the A. funestus group is difficult because of overlap of distinguishing characteristics in adult or immature stages as well as the necessity to rear isofemale lines to examine larval and egg characters. A rapid rDNA polymerase chain reaction (PCR) method has been developed to accurately identify five members of the A. funestus group. This PCR is based on species-specific primers in the ITS2 region on the rDNA to identify A. funestus (=505bp), Anopheles vaneedeni Gillies and Coetzee (=587bp), Anopheles rivulorum Leeson (=411bp), Anopheles leesonii Evans (=146bp), and Anopheles parensis Gillies (=252bp).

INTRODUCTION

Mosquitoes of the genus Anopheles transmit the human malaria parasites Plasmodium falciparum, P. vivax, P. malariae, and P. ovale. In Africa, two species groups within this genus contain the major malaria vector species. The Anopheles gambiae Giles complex and the Anopheles funestus Giles group are well known for their important role in malaria transmission.1 Anopheles arabiensis Patton and A. gambiae s.s are both members of the A. gambiae complex, whereas A. funestus is the only major vector in the A. funestus group.1 The vectorial and behavioral variation found within these species groups or complexes is the major reason why accurate identification is needed.1,2

A polymerase chain reaction (PCR) assay was published by Scott and others,3 which made identification of members of the A. gambiae complex easy, fast, and reliable. Today many laboratories in Africa are equipped to perform this assay, which aids in their malaria control programs. Identification of the A. funestus group is more complicated because of the lack of an easy and fast identification method.

The A. funestus group comprises nine members: A. funestus, Anopheles vaneedeni Gillies and Coetzee, Anopheles leesonii Evans, Anopheles rivulorum Leeson, Anopheles parensis Gillies, Anopheles fuscivenosus Leeson, Anopheles aruni Sobti, Anopheles brucei Service, and Anopheles confusus Evans and Leeson. Anopheles funestus is the most anthropophilic and endophilic member of the group and is a highly efficient vector of malaria.3 The other members of the group are mainly zoophilic and only A. rivulorum has been implicated as a minor vector in Tanzania.4 A. vaneedeni has been shown to be a vector under laboratory conditions5 but has not been shown to be associated with malaria transmission in nature. The distribution of A. funestus is widespread throughout subtropical Africa, and it can be found in sympathy with other members of the group.1,2

A. funestus is widespread over most of the malarious areas of Africa, extending from northern Sudan to South Africa and across West Africa to northern Mali and Senegal.1 It is also common in Madagascar.6 A. parensis and A. confusus are found in eastern Africa from Kenya and Tanzania in the North to KwaZulu/Natal Province in South Africa.3 A. rivulorum and A. leesonii are widespread, occurring from Ethiopia through to the northern parts of South Africa and across West Africa.1 The other members of the group are localized: A. vaneedeni occurs only in Mpumalanga and the Northern Province in South Africa, A. aruni from Zanzibar, A. fuscivenosus from Zimbabwe, and A. brucei from Nigeria.1,2

With insecticide resistance on the increase throughout Africa,7–11 it is essential to have proper species identification to investigate the way in which insecticide resistance develops in each species or population as well as to study the migration of resistance genes between populations. Furthermore, studies in South Africa have shown that four members of the A. funestus group can be collected resting indoors: A. funestus, A. rivulorum, A. parensis, and A. leesonii.10 This underlines the importance of identifying members of the A. funestus group.

Species identification in the past has mainly been performed using either morphological or cytogenetic methods. Morphological identification is dependent on characters found in various life stages and, therefore, means that each wild-caught female has to produce an egg batch so that eggs, larvae, and adult progeny from that particular female can be studied. This is time consuming and can lead to a percentage of specimens not properly identified because of overlap between species (e.g., A. funestus, A. parensis, and A. vaneedeni).5 Mortality is high during the rearing process, which is also time consuming, taking between 4 and 6 weeks before identification is made.

Polytene chromosome identification is much faster than using morphology. Green and Hunt12 and Green13 have published chromosome maps showing fixed inversion differences of the banding arrangements in A. funestus, A. rivulorum, A. leesonii, A. fuscivenosus, A. parensis, and A. confusus. A. vaneedeni can be distinguished from A. funestus using a polymorphic inversion on Arm 2; however, if the inversion is homozygous for the standard arrangement, then these two species cannot be separated.12 Other disadvantages are that only half-gravid females can be identified. If wild-caught females are unfed or fully gravid, they cannot be identified. A. funestus males are frequently collected inside houses (unpublished data) and also cannot be identified using this method.

Molecular methods have been reported, but these too have limitations.14–16 The PCR-SSCP assay15 is able to distinguish between A. rivulorum, A. funestus, A. vaneedeni, and A. leesonii. However, this method cannot be used to distinguish
between *A. vaneedeni* and *A. parensis* (unpublished data). The latter two species are frequently found in South Africa and are morphologically similar to *A. funestus.* Hackett and others' ITS2 PCR was used to distinguish between *A. funestus* and *A. rivulorum* only. When applied to other members of the group, overlap in fragment size occurs (unpublished data).

Molecular identification methods for species complexes have mainly used the rDNA locus because it is represented in multiple copies throughout the genome in mosquitoes, and it contains highly variable regions. One transcription unit consists of three coding regions, 18S, 28S, and a small 5.8S gene, which are separated by noncoding regions called the internal transcribed Spacer Regions 1 and 2 (ITS1 and ITS2). The transcription units are separated by intergenic spacer regions (IGSs). The ITS regions show relatively high levels of intraspecies variation but not as high as the IGS region. Variation found in these regions makes it possible to design species-specific diagnostic assays.

In this article, we report on the use of a PCR assay that is able to rapidly identify five of the most commonly found members of the *A. funestus* group: *A. funestus, A. vaneedeni, A. parensis, A. leesoni,* and *A. rivulorum.*

### MATERIALS AND METHODS

**Mosquito collection.** Field collections and a priori identifications. The samples used in this study came from various localities (Table 1). All samples were transported to the laboratory where females were held in individual tubes for egg laying. Egg morphology was examined before eggs were placed in bowls with aerated water. Larvae were fed on a high-protein diet consisting of ground brewers’ yeast tablets and dog biscuits. Fourth instar larvae and pupal pellets were stored initially in ethanol and then permanently mounted on microscope slides to be used with pinned adults for morphological identification. The remainder of the adults emerging from individual egg batches were frozen in liquid nitrogen. Morphological identification was performed according to Gillies and De Meillon1 and Gillies and Coetsee.2

**DNA extraction.** Genomic DNA from single mosquitoes was extracted using the method described by Collins and oth-
ers. DNA pellets were resuspended in 200 μL 1XTE, and 2 μL was used in a 100-μL PCR mixture.

*Extraction using STE buffer.* A mosquito leg was homogenized in a sodium-Tris-EDTA (EDTA) buffer (0.1 M NaCl; 10 mM Tris, pH 8.6; 1 mM EDTA) and incubated at 94°C for 10 to 12 minutes. Cell debris was precipitated by centrifuging for 1 minute. We used 0.4 μL DNA for PCR. The remaining homogenate was stored at −20°C for future use.

**PCR amplification of the ITS2 region.** ITS2 primers from the 5.8S and 28S coding region flanking the variable ITS2 region were used to amplify the genomic DNA. The primers were as follows: ITS2A: 5’-TGT GAA CTG CAG GAC ACA T 3’; ITS2B: 5’-TAT GCT TAA ATT CAG GGG GT 3’.

The PCR mixture contained the following: 10 μL of the 10× reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3), 1.5 mM MgCl₂, 8.25 pmol per primer of each primer, 200 μM of each dNTP, and two units thermostable taq DNA polymerase, overlaid by a drop of mineral oil. Amplification conditions were as follows: initial hot start at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 40 seconds, and final extension at 72°C for 10 minutes. Two negative controls were included. The first was a DNA extraction negative control in which extractions were performed without any DNA. The second control was a negative control containing all the substances in the PCR mixture except DNA template. To confirm successful amplification, 5 μL of the product was electrophoresed on a 1% agarose gel stained with ethidium bromide. The remaining PCR product was diluted 1:100 in deionized distilled water; 0.4 μL was used in PCR reactions alongside unknown specimens.

**Sequencing of ITS2 PCR products.** QIAquick spin columns (Qiagen) and products electrophoresed on 1% agarose gel stained with ethidium bromide. Theremaining PCR product (95 μl) was loaded on NuSieve GTG agarose (Biowhittaker Molecular Applications, East Rutherford, NJ) low-melting-point agarose. PCR product was excised from the gel and purified using QIAquick spin columns (Qiagen) and products electrophoresed on 1% agarose gel stained with ethidium bromide to determine the success of purification. At least three individuals per species were sequenced to achieve the sequencing data reported.

**Sequencing of ITS2 PCR products.** QIAquick purified products were quantified using the 1-Kb-plus DNA ladder (GIBCO-BRL) included with purified samples before electrophoresis. The 650-bp fragment was calculated at 8 ng/μL. Sequencing was done according to manufacturer’s specifications for use of the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Direct cycle sequencing was done using the ABI Prism Dye Deoxy Terminator Cycle Sequencing kit (PE Applied Biosystems). Sense strand was sequenced using the ITS2A primer; the antisense strand was sequenced using the ITS2B primer. An internal reverse primer (Van) (5’-TGT CGA CTT GTG AGC CGA AC 3’) was used to help in sequencing A. vaneedeni ITS2. Reaction products were purified using Centrisep spin columns (Princeton Separations).

Genebank accession numbers are as follow: A. leesoni, AY035719; A. vaneedeni, AY035718; A. parensis, AY035720; A. funestus, AF062512; A. funestus (Madagascar), AY035721; A. rivulorum, AF 210724.

**Sequence analysis and primer design.** DNA sequence data were analyzed using SeqMan II and MegAlign (DNASTAR). Location for primer sequences were chosen using the criteria that the primer sequence must have at least five nucleotide differences between the different species and that the

<table>
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<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>Tm (°C)</th>
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<tbody>
<tr>
<td>UV</td>
<td>TGT GAA CTG CAG GAC ACA T</td>
<td>55.34</td>
</tr>
<tr>
<td>FUN</td>
<td>GCA TCG ATG GGT TAA TCA TG</td>
<td>52.4</td>
</tr>
<tr>
<td>VAN</td>
<td>TGT CGA CTT GTG AGC CGA AC</td>
<td>58</td>
</tr>
<tr>
<td>RIV</td>
<td>CAA GCC GTT CCC TGA TT</td>
<td>58.8</td>
</tr>
<tr>
<td>PAR</td>
<td>TGG GTT CCC AAG CTA GT T C</td>
<td>60.5</td>
</tr>
<tr>
<td>LEES</td>
<td>TAC ACG GCC GGC ATG TAG TT</td>
<td>60.2</td>
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PCR products would be easily distinguishable using agarose electrophoresis (differing by at least 50 bp).

**Cocktail PCR.** Primer names, sequences as well as Tm temperatures are provided in Table 2. PCR conditions were as follows: 12.5-μL reactions contained the following: 1.25 μL 10× reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3), 1.5 mM MgCl₂, 3.3 pmol/primer of each primer, 200 μM of each dNTP, and 0.5 units thermostable taq DNA polymerase overlaid by a drop of mineral oil. PCR cycles were as follow: One cycle at 94°C for 2 minutes followed by 30 cycles by 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 40 seconds. An additional autoextension of 72°C for 5 minutes was included for one cycle at the end. PCR products were electrophoresed on 2.5% to 3% agarose gel stained with ethidium bromide.

**Standards used as positive controls.** Standard DNA was constructed by amplifying DNA from known specimens in 25 μL reaction mix; 8 μL were electrophoresed, and the size of the amplified product was confirmed using 1-Kb-plus ladder. The remaining PCR product was diluted 1:100 in deionized distilled water; 0.4 μL was used in PCR reactions alongside unknown specimens.

**Testing of primers.** Primers were tested on morphologically,1,2 cytogetically,12,13 or PCR-SSCP15 identified specimens to determine the reliability of the new PCR assay. A. parensis was identified from A. vaneedeni after SSCP analysis using the 650-bp fragment obtained after the ITS2 PCR (L. L. Koekemoer and others, unpublished data).

**RESULTS**

**Sequencing of the ITS2 fragment.** Nucleotide alignment of the amplified ITS2 region for the five species of the A. funestus group is shown in Figure 1. The ITS2 sequence is flanked at the 5′ end with the 5.8S ribosomal RNA gene and at the 3′ end with the 28S ribosomal RNA gene. The nucleotide length for each species varies. The spacer for A. funestus is approximately 704 bp,20 ≈681 bp for A. vaneedeni, ≈398 bp for A. leesoni, ≈380 bp for A. rivulorum,16 and ≈463 bp for A. parensis. The percentage homology between these five species is shown in Table 3. The sequences used for the development of primers for A. rivulorum (from South African specimens16) and A. funestus20 were accessed from Genbank.

**Primer design.** Primers were designed using the sequence provided in Figure 1. Criteria for primer design was that the PCR products after amplification would be unique for each species and had to be easily visualized on agarose gel. Table 2 provides the oligonucleotide sequences for each primer as well as the Tm temperature of the primers. Primer lengths varied between 19 bp for the universal forward primer (UV), which is the same as the original ITS2A primer used to am-

**TABLE 2**

<table>
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<th>Sequence (5’ to 3’)</th>
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<tr>
<td>UV</td>
<td>TGT GAA CTG CAG GAC ACA T</td>
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<td>TGT CGA CTT GTG AGC CGA AC</td>
<td>58</td>
</tr>
<tr>
<td>RIV</td>
<td>CAA GCC GTT CCC TGA TT</td>
<td>58.8</td>
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<tr>
<td>PAR</td>
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<td>60.2</td>
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plify the ITS 2 fragment, and 20 bp for the species-specific reverse primers. Tm of primers varies between 52.4°C and 60.5°C (see Table 2). The universal forward primer is in the conserved 5.8S gene, whereas the species-specific reverse primers are within the ITS2 spacer region.

Cocktail PCR. Lengths of amplified species-specific product were for *A. leesoni* ≈146 bp, *A. funestus* ≈505 bp, *A. vaneedeni* ≈587 bp, *A. parensis* ≈252 bp, and *A. rivulorum* ≈411 bp (Figure 2). All six primers (see Table 2) can be included in a cocktail PCR reaction mix simultaneously without any affect on amplification. Each unknown specimen can be identified without performing five separate PCR reactions.

Testing on morphological/cytogenetically identified specimens. Table 1 gives the origin of samples used to develop the PCR assay and in testing the species-specific PCR on specimens identified earlier by morphology, PCR-SSCP, PCR-ITS2, or cytogenetics. *A. parensis* cannot be identified by SSCP electrophoresis alone because *A. vaneedeni* and *A. parensis* gave overlapping banding patterns. An additional PCR (ITS2) was needed to identify *A. parensis* on a 650-bp fragment (L. L. Koekemoer and others, unpublished data).

Five individual mosquitoes, one from each species, were designated as standards, and DNA from these five specimens were run on every gel as a positive control. The positive controls were included for various reasons. First, lack of amplification of either the wild-caught specimens or the positive controls would indicate a problem in the amplification step, which might be the set-up in the cocktail master mix or the amplification itself. Second, wild-caught specimens for identification, collected by field teams, are not always preserved properly, and degradation of DNA is possible. If the positive controls amplify and the wild-caught specimens do not, it can be an indication that the DNA for those specimens might be too degraded to give any amplification. If there is no reason to believe that this might be the case, one needs to investigate the possibility that the unamplified specimens are not members of the *A. funestus* group or that they are other members of the *A. funestus* group not included in this study. The positive controls were also included to ensure that each primer is functional.

Amplicons were identified using the 1-Kb-plus markers as well as the positive controls. Hybrid specimens were mimicked by mixing DNA from various species together before amplification. Real hybrids could not be used because of the lack of colonized members of the *A. funestus* group. No hybrids have been recorded from nature using cytogenetic identification methods. Figure 3 shows the results of hybrid DNA after amplification.
DISCUSSION

Sequencing of the ITS spacer region. Alignment between \textit{A. funestus}, \textit{A. vaneedeni}, and \textit{A. parensis} showed a large degree of sequence similarity (72–74\%; Table 3). The 5' end of the ITS2 spacer region showed a high degree of similarity between all five species investigated. \textit{A. rivulorum} and \textit{A. leesoni} showed less similarity compared with \textit{A. funestus} (see Figure 1). Performing a BLASTn search (www.ncbi.nlm.nih.gov/blast/Blast.cgi) using the \textit{A. leesoni} nucleotide sequence revealed a very high-sequence homology (94\%) between \textit{A. leesoni} and the oriental species \textit{Anopheles minimus} Theobald sp. A and \textit{Anopheles fluviatilis} James. \textit{A. leesoni} only showed a 36\% to 38\% sequence similarity between other members of the \textit{A. funestus} group (see Table 3).

The \textit{A. funestus} group belongs to the series Myzomyia and is classified in this series by characters of the pharyngeal armature.\textsuperscript{1} The \textit{A. funestus} group itself is defined on characters of the larva while exhibiting great uniformity in adult characteristics. Green\textsuperscript{13} used polytene chromosome banding homologies to show that, whereas most of the members of the \textit{A. funestus} group are phylogenetically closely related, \textit{A. leesoni} could be grouped with \textit{A. fluviatilis}/culicifacies (\textit{A. minimus} was not included in his study). The molecular data presented here support this hypothesis.

PCR assay to identify members of the \textit{An. funestus} group.

We used the rDNA PCR assay to identify specimens from various localities (see Table 1). Correct identification was recorded for each specimen. Identification based on size difference can easily be done using a size marker or standard on a 2.5\% to 3\% agarose gel. In all specimens analyzed, both standard and size marker were included to confirm species identification. Electrophoresis was done for at least 2 hours or until size marker had separated sufficiently to exclude misidentifications.

Only small amounts of DNA are needed for amplification, and DNA extractions can be done on a single leg. DNA extraction\textsuperscript{17} is rapid and reliable but takes up to half a day before PCR can be done. A sodium-Tris-EDTA buffer was used for a fast extraction method, which only took 20 minutes to extract DNA when dealing with large sample sizes. This method worked equally well for amplification. No amplification was obtained using legs alone without performing an extraction method.

No species hybrids between members of the \textit{A. funestus} group have been reported either from nature or in the laboratory, the latter probably because of the lack of colonization of members of the \textit{A. funestus} group. The mixing of DNA of the various species gave some idea of what one might expect in the case of hybrid specimens. However, one would expect to only find hybrids using the species-specific PCR when using female specimens because the rDNA is X-linked.

\textit{A. aruni}, \textit{A. confusus}, \textit{A. brucei}, and \textit{A. fuscivenosus} were not included in this study because these species are mostly
PCR IDENTIFICATION OF THE *A. FUNESTUS* GROUP

Figure 1. Continued.
highly localized in their distribution and all are very rare.\textsuperscript{1}
None were identified from the various field collections performed during this study.

The use of this assay has shown that \textit{A. parensis} can be collected in fair numbers resting indoors (see Table 1). Whereas this has been reported before in South African populations,\textsuperscript{1} this was not the case in Kenya. Gillies and Furlong\textsuperscript{22} collected large numbers of \textit{A. parensis} on human bait outdoors. However, very few were collected resting indoors. Although this species is not considered to be important in malaria transmission, it may be necessary to reinvestigate its role in those areas in Kenya listed in Table 1, especially in \textit{Mwea}, where it was the only member of the \textit{A. funestus} group collected indoors.

Because many laboratories in Africa are already equipped to perform PCR identification of the \textit{A. gambiae} complex, no additional equipment will be needed to identify members of the \textit{A. funestus} group. The assay is rapid, sensitive, and easy to use. Both male and female mosquitoes of any life stage can be identified. Preservation is easy, and specimens can be stored desiccated on silica or in 75\% to 80\% ethanol. Only small quantities of material (one to two legs) are needed for identification, leaving the rest of the body parts for sporozoite detection, blood meal analysis, and other studies such as isoenzyme or population genetic studies.

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