Am. J. Trop. Med. Hyg., 70(6), 2004, pp. 583–590
Copyright © 2004 by The American Society of Tropical Medicine and Hygiene

A SINGLE MULTIPLEX ASSAY TO IDENTIFY MAJOR MALARIA VECTORS WITHIN THE AFRICAN *ANOPHELES FUNESTUS* AND THE ORIENTAL *AN. MINIMUS* GROUPS

CLAIRE GARROS, LIZETTE L. KOEKEMOER, MAUREEN COETZEE, MARC COOSEMANS, AND SYLVIE MANGUIN

Institute of Research for Development, Centre of Biology and Management of Populations, Montpellier, France; Vector Control Reference Unit, National Institute for Communicable Diseases, Johannesburg, South Africa; Department of Clinical Microbiology and Infectious Diseases, School of Pathology of the National Health Laboratory Service and University of Witwatersand, Johannesburg, South Africa; Department of Parasitology, Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium

Abstract. The African *Anopheles funestus* and the Oriental *An. minimus* groups are closely related and composed of major malaria vectors in Africa and Southeast Asia, respectively. None of the species of either the *An. funestus* or the *An. minimus* group can be identified with absolute certainty using the adult morphology. Polymorphisms present on the internal transcribed spacer 2 (ITS2) of ribosomal DNA allowed the development of 10 primers that combined with an universal forward primer lead to a simple and sensitive multiplex allele-specific polymerase chain reaction (AS-PCR). Moreover, the possible additional amplification of the entire ITS2 allows one to detect other anopheline species in sympatry with members of both groups not included in this assay and serves as a control band. This universal PCR method permits the discrimination of 10 species within the subgenus *Cellia*, among which figure three major malaria vectors, and constitutes a very efficient and powerful tool to improve our knowledge on these species distribution and biology. Not only restricted to anophelines, this AS-PCR could also be developed and applied to other insect groups.

INTRODUCTION

Molecular methods for species identification have received great attention in recent years. The methods have been applied to important groups of mosquito species complexes. Particularly important are complexes containing vectors of malaria and other vector-borne diseases. Correct and precise identification of the target species has direct medical and practical implications, such as in vector control. In the past, mosquito taxonomy has been achieved mostly by using morphologic characteristics, cytogenetic and isoenzyme markers. However, there are a variety of circumstances in which the molecular approach has greatly improved the accuracy of species identification. This not only applies to sibling species, but also to members of closely related groups with overlapping morphologic characters. Vectorial and behavioral variations found among these species groups or complexes constitute the major reason for the need of accurate and precise identification.

The *Anopheles funestus* and *An. minimus* groups belong to the *Myzomyia* series, subgenus *Cellia*,[1] and are closely related based on various cytogenetic and molecular studies (Garros C and others, unpublished data).[2,3] They are probably considered distinct only because of their geographic separation.[4] In spite of this genetic proximity, both groups had seldom been studied jointly (Garros C and others, unpublished data).[2,3]

On the Afrotropical continent, the *An. funestus* group consists of nine species, namely *An. brucei*, *An. confusus*, *An. fuscivenosus*, *An. leesoni*, and *An. rivulorum*, as well as the *An. funestus* subgroup, which is composed of *An. aruni*, *An. funestus*, *An. parensis*, and *An. vaneedeni*.[5–7] Although *An. leesoni* does not belong to the *An. funestus* group, following the recommendation of Harbach,[1] who included it in the *An. minimus* group, it was included here within the members of the *An. funestus* group because of its morphologic similarities and sub-Saharan distribution. Beside the anthropophilic and endophilic *An. funestus*, which is a highly efficient malaria vector, the other species of the group are mainly zoophilic and play little or no role in malaria transmission.[5,6,10] Realizing the difficulties of morphologic identification and the need to elucidate the role of individual species in malaria transmission, molecular methods have been introduced for distinguishing members of the *An. funestus* group.[8,9,11,12] A polymerase chain reaction (PCR)–single strand conformation polymorphism (SSCP) assay can identified four species: *An. funestus*, *An. leesoni*, *An. rivulorum*, and *An. vaneedeni*.[8] The inclusion of *An. parensis* revealed overlapping banding patterns with *An. vaneedeni*. A sequence length variation in the internal transcribed spacer 2 (ITS2) of ribosomal DNA was identified by its amplification and permitted the identification of *An. parensis*.[11] Very recently, a species-specific PCR assay was developed for differentiating these five members of the group.[12]

Similarly, in the Oriental region, the *An. minimus* group is composed of nine species: *An. aconitus*, *An. filipinae*, *An. flavirostris*, *An. fluviatilis*, *An. mangyanus*, *An. minimus* species A and C, belonging to the *An. minimus* complex,[1] *An. pampanai*, and *An. varuna*.[4] *Anopheles minimus s.l.* is most commonly associated with rivers and streams throughout Southeast Asia, where it is one of the primary vectors of human malaria. These species are difficult to distinguish at the adult stage due to overlapping characteristics.[4] However, associated immature skins can be useful for differentiating the species, except those of the *An. minimus* complex. These species play different roles in malaria transmission, requiring vector and non-vector species to be differentiated with reliable markers. Molecular methods were recently developed for distinguishing different species of the group. Two PCR-based techniques, an allele-specific amplification (ASA) and an SSCP were elaborated for distinguishing, respectively *An. minimus* A from C; and both species A and C along with *An. aconitus* and *An. varuna*.[13] A PCR-restriction fragment length polymorphism (RFLP) method was also designed for the identification of these four species, as well as for *An. pampanai*, *An. culicifacies* B, *An. jeyporiensis,* and the hybrids between *An. minimus* species A and C.[14] Very recently, a single multiplex PCR assay, using sequence characterized amplified region (SCAR) markers derived from individual random amplified polymorphic DNA markers, was developed for an easy and reliable identification of *An. minimus* A and

C, and their hybrids, as well as the three closely related species of the *An. minimus* group (*An. aconitus*, *An. varuna*, and *An. pampanai*).[15,16]

Since the *An. funestus* and *An. minimus* groups include major malaria vectors on both the African and Asian continents, there is a tremendous need for accurate and precise identification methods for members of these two groups. Moreover, the standardization of molecular identification methods is important for their transfer to field laboratories for faunistic studies, as well as for scientists working on phylogenetic relationships. Therefore, based on a previous study by Koekemoer and others,[12] the aim of the present work was to develop a multiplex PCR assay, based on one-step amplification, targeting *An. funestus*- and *An. minimus*-specific parts of the gene encoding the ITS2 spacer, for rapid, easy, and reliable identification of the most common species of both groups.

MATERIALS AND METHODS

Mosquito collection. The samples used in the study originated from various localities in Africa and Southeast Asia (Table 1). After field capture, all mosquitoes were first identified in field conditions on the basis of their morphology. The morphologic identification of the members of the *An. funestus* group were performed according to Gillies and De Meillon[6] and Gillies and Coetzee;[5] the species of the *An. minimus* group were identified by use of a standard key for medically important anophelines of this region.[17] Morphologic identification was then generally checked with specific molecular methods (Table 1): members of the *An. funestus* group were differentiated using the PCR-SSCP and/or the PCR-multiplex,[8,12] whereas the allele-specific method[15] (SCAR-multiplex) and the RFLP (ITS2 restriction)[14] were used for identifying the members of the *An. minimus* group, except for 30 specimens from Thailand (*An. minimus* A from Rayong Province and *An. minimus* C from Kanchanaburi Province) that were only identified based on their morphologic characteristics.

Extraction of DNA. Genomic DNA was extracted from single individual adult mosquitoes, whole specimens or mosquito parts, such as legs and wings (material from Kenya and Madagascar), according to the procedure of Linton and others.[18]

Amplification by PCR and sequencing of the ITS2 region. The ITS2 primers from the 5.8S and 28S coding regions flanking the variable ITS2 region were used to amplify the genomic DNA.[14] 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 2.5 μL of 10× reaction buffer (Qiagen, Valencia, CA), 200 μM of each dNTP, 64 nmol of primer, 0.5 units of *Taq* DNA polymerase (Qiagen), and 3 μL of DNA template. Amplification conditions were composed of an initial hot start at 94°C for two minutes, 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 a final extension at 72°C for 10 minutes. One negative control was included. To confirm amplifications, 7 μL of the PCR-product was subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The remaining PCR product (18 μL) was loaded onto a 1.5% agarose gel and subjected to electrophoresis. The PCR product was excised from the gel and purified using QIAquick spin columns (Qiagen) and products were electrophoresed on a 1% agarose gel to quantify the purifi-

TABLE 1

Mosquito collection sites, number of specimens (n = 63 for the *Anopheles funestus* group, n = 150 for the *An. minimus* group), methods used for specimen identification, and their references

Species	Country, locality	No. of specimens	Identification method*
An. funestus (n = 26)	Burkina Faso, Boromo village	5	
	Cameroon, Simbock village	5	
	Senegal, Kedougou village	5	
	Madagascar, Ambohimena village	2	Multiplex PCR (12)
	South Africa, KwaZulu Natal Province	5	
	Angola, Calueque village	2	
	Uganda, Apac District	2	
An. leesoni	South Africa, Northern Province	7	
An. parensis	Kenya, Mwea and Baringo villages	10	Multiplex PCR, SSCP (12, 8)
An. rivulorum	Kenya, Jaribuni village	10	
An. vaneedeni	South Africa, Mpumalanga and Tzaneen Provinces	10	
An. aconitus (n = 14)	Cambodia, Rattanakiry Province	1	
	Laos, Vientiane Province	6	SCAR multiplex, ITS2 restriction (15, 14)
	Vietnam, Khanh Hoa Province	7	
An. minimus A (n = 66)	Cambodia, Rattanakiry Province	11	
	Laos, Vientiane Province	9	SCAR multiplex, ITS2 restriction (15, 14)
	Thailand, Kanchanaburi Province	4	
	Thailand, Rayong Province	18	Only morphology
	Vietnam, Khanh Hoa and Hoa Binh Provinces	24	SCAR multiplex, ITS2 restriction (15, 14)
An. minimus C (n = 27)	Thailand, Kanchanaburi Province	12	Only morphology
	Vietnam, Hoa Binh Province	15	
An. pampanai (n = 34)	Cambodia, Rattanakiry Province	15	
	Thailand, Kanchanaburi Province	1	SCAR multiplex, ITS2 restriction (15, 14)
	Vietnam, Khanh Hoa Province	18	
An. varuna	Vietnam, Khanh Hoa Province	9	

* PCR = polymerase chain reaction; SSCP = single-strand conformation polymorphism; SCAR = sequence characterized amplified region; ITS2 = internal transcribed spacer 2.

cation. Double-strand PCR products were directly sequenced by MWG-Biotech (Ebersberg, Germany) with either primer ITS2A or ITS2B. At least two individuals per species were sequenced in both strands. GenBank accession numbers are as follows: *An. funestus* AY259143, *An. parensis* AY 259148, *An. rivulorum* AY255105, *An. vaneedeni* AY259147, *An. leesoni* AY255107, *An. aconitus* AY255106, *An. minimus* species A AY255108, *An. minimus* species C AY255109, *An. pampanai* AY255110, and *An. varuna* AY255111.

Primer design and allele-specific PCR. From the sequence of individual specimens from each species, specific primers were designed and their specificity were tested. The ITS2 sequences of all 10 species were aligned using BioEdit.[19] Moreover, sequences from previous studies obtained through GenBank were also used in the alignment for improving primer design.[12,14] The location for primer sequences was chosen on the criteria of at least three nucleotide differences between species and PCR products easily distinguishable by agarose gel electrophoresis. The oligonucleotide primers were synthesized by MWG-Biotech. Primer names, sequences, and sizes of the PCR products are shown in Table 2. To check the length of the amplified fragment and assess primer specificity, each primer was tested with samples of different locations of the considered species, as well as the other nine species. Amplification was done in a final volume of 25 μL containing 2.5 μL of 10× buffer (Qiagen), 200 μM of each dNTP, 64 nmol of each primer, 0.5 units of *Taq* polymerase, and 3 μL of DNA template. After an initial denaturation step at 94°C for two minutes, 30 cycles were programmed as follows: 94°C for 30 seconds, 45°C for 30 seconds, 72°C for 40 seconds, and a final extension at 72°C for five minutes. Amplimers were resolved by electrophoresis on a 3% agarose gel with Tris-borate-EDTA buffer and stained with ethidium bromide.

Multiplex PCR. To detect simultaneously each of the 10 anopheline species, all primers were used to develop a one-step reaction. In a final volume of 25 μL, PCR conditions were as follows: 2.5 μL of 10× reaction buffer (Qiagen), 200 μM of each dNTP, 0.16 nmol of each primer, 0.5 units of *Taq* polymerase (Qiagen), and 3μL of DNA template. The PCR cycles were as follows: one cycle at 94°C for two minutes, followed by 40 cycles at 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 40 seconds. An additional autoextension at 72°C for five minutes was included at the end for one cycle. The PCR products were subjected to electrophoresis on a 3% agarose gel stained with ethidium bromide.

Validation of the assay. To determine the reliability of the new PCR assay, a large sample of specimens from both groups and from different localities over both continents, Africa and Southeast Asia, were tested (Table 1). Standards used for the design of the primers were run as positive controls on the electrophoresis gel.

RESULTS

Sequencing of ITS2. The length of ITS2 varies from 850 basepairs (bp) for *An. funestus* and *An. vaneedeni*, to 600 bp for *An. parensis*, 540 bp for *An. rivulorum*, and 520 bp for members of the *An. minimus* group and *An. leesoni*. Nucleotide alignment of the amplified ITS2 region for the 10 species of the *An. funestus* and *An. minimus* groups is shown in Figure 1. The ITS2 nucleotide length obtained by sequencing each species was 730 bp for *An. funestus*, 550 bp for *An. parensis*, 460 bp for *An. rivulorum*, 502 bp for *An. vaneedeni*, and 404–470 bp for *An. leesoni* and the five members of the *An. minimus* group. Due to difficulties in direct sequencing, it was possible to sequence 60% of the complete ITS2 region. The loss of 40% of the sequence does not interfere with a good alignment and the primer design.

Primer design. Our strategy for the ITS2 allele-specific amplification followed the approach of Scott and others[20] to distinguish members of the *An. gambiae* complex. Primers were designed using the sequences provided in Figure 1. We designed six primers for the five species of the *An. minimus* group as well for *An. leesoni*. We named the primers specific for the *An. minimus* group ACO, MIA, MIC, PAM, and VAR for *An. aconitus*, *An. minimus* species A, *An. minimus* species C, *An. pampanai*, and *An. varuna*., respectively. For *An. funestus*, *An. parensis*, *An. rivulorum*, and *An. vaneedeni*, we used the four primers FUN, PAR, RIV, and VAN, respectively, as designed by Koekemoer and others.[12] Preliminary assays of amplification with the primers for the *An. funestus* group on *An. minimus* species showed that the primer specific for *An. leesoni* (LEE), designed by Koekemoer and others,[12] hybridizes with the ITS2 of both species of the *An. minimus* complex (Garros C, unpublished data). Therefore, we designed a new primer specific for *An. leesoni*, which was named LEE2.

Criteria for primer design was that the PCR products after

TABLE 2
Primers designed for *Anopheles* species diagnostic assay with respective Tm*

Species	Primer name	Sequence (5′ to 3′)	Size of the product (bp)	Tm (°C)
Universal forward primer	ITS2A	TGT GAA CTG CAG GAC ACA T		54.5
An. minimus A	MIA	CCC GTG CGA CTT GAC GA	310	57.6
An. minimus C	MIC	GTT CAT TCA GCA ACA TCA GT	180	53.2
An. aconitus	ACO	ACA GCG TGT ACG TCC AGT	200	56.0
An. varuna	VAR	TTG ACC ACT TTC GAC GCA	260	53.7
An. pampanai	PAM	TGT ACA TCG GCC GGG GTA	90	58.2
An. leesoni	LEE2	GCT AAG TAC AGT GCC ACT GT	280	57.3
An. funestus	FUN	GCA TCG ATG GGT TAA TCA TG	460	52.4
An. vaneedeni	VAN	TGT CGA CTT GGT AGC CGA AC	555	58.0
An. rivulorum	RIV	CAA GCC GTT CGA CCC TGA TT	400	58.8
An. parensis	PAR	TGC GGT CCC AAG CTA GGT TC	235	60.5

* The internal transcribed spacer 2 (ITS2A) is the universal primer that binds to the same position on the ITS2 DNA for all 10 species, while the specific primers (MIA to RIV) bind at different places on the ITS2 DNA of the corresponding species. bp = basepairs; Tm = melting temperature.

```
             1                                                  50                                                 100
  funestus   TGTGAACTGC AGGACACATG AACACCGACA CGTTGAACGC ATATTGCGCG TCGGACGATT AAACCCGGCC GATGCACACA TTCTTGAGTG CCTATCAATT
 vaneedeni   TGTGAACTGC AGGACACATG AACACCGACA CGTTTGACGC ATATTGCGCG TCGGACGATT AAACCCGGCC GATGCACACA TTCTTGAGTG CCTATCAATT
  parensis   TGTG ITS2A  ACACATG AACACCGACA CGTTGAACGC ATATTGCGCG TCGGACGATT AAACCCGGCC GATGCACACA TTCTTGAGTG CCTATCAATT
 rivulorum   TGTG        ACACATG AACACCGACA CGTTGAACGC ATATGGCGCA TCGGACGTCT ACAACCGGCC GATGCACACA TTCTTGAGTG CCTACCAAAT
   leesoni   TGTGAACTGC AGGACACATG AACACCGACA CGTTGAACGC ATATGGCGCA TCGGACGTTT AAACCCGACC GATGTACACA TTCTTGAGTG CCTACCAATT
  minimusA   TGTGAACTGC AGGACACATG AACACCGACA CGTTGAACGC ATATGGCGCA TCGGACGTTT AAACC  PAM  TACACA TTCTTGAGTG CCTACCAATT
  minimusC   TGTGAACTGC AGGACACATG AACACCGACA CGTTGAACGC ATATGGCGCA TCGGACGTTT AAACC       TACACA TTCTTGAGTG CCTACCAATT
  aconitus   TGTGAACTGC AGGACACATG AACACCGACA CGTTGAACGC ATATTGCGCA TCGGACGTTT AAACCCGACC GATGTACACA TTCTTGAGTG CCTACCAAAT
  pampanai   TGTGAACTGC AGGACACATG AACACCGACA CGTTGAACGC ATATGGCGCA TCGGACGTTT TACCCCGGCC GATGTACACA TTCTTGAGTG CCTACCAATT
    varuna   TGTGAACTGC AGGACACATG AACACCGACA CGTTGAACGC ATATGGCGCA TCGGACGTTT CAACCCGACC GATGTACACA TTCTTGAGTG CCTACCAATT

           101                                                 150                                                 200
  funestus   CCTTGATATA CA..ACAAAC CAAACTTCAG GG..TGGAGC GTGC.....C ACAATAGAAC ACTAT..... .GGCGAGCAG CCCGT..... ...CTAGTGT
 vaneedeni   CCTTGATATA CA..ACAAAC CAAACTTCAG GG..TGGAGC GTGC.....C ACAATAGAAC ACTAT..... .GGCGAGCAG CCCGT..... ...CTAGTGT
  parensis   CCTTAATATA CA..ACAAAC CAAACTTCAG GG..TGGAGC GTGC.....C ACAATAGAAC ACTAT..... .GGCGAGCAG CCCGT..... ...CTAGTGT
 rivulorum   CCTTGTTACA CA...ATTGC CTGACTACAG TGGTGCACGC GTGC....AG CGACGAGAGT GCTAT..... .GGCGAGA.. CCCACGT..      GTGC
   leesoni   CCTTGTTACA CA..AT.AAT CTAACTACA. TG..GCGCCC GTGT....AC AGACG...GC ATCAT..... .GGCGAGCAG CCCGCCT..  MIC ATGT
  minimusA   CCTTGTTACA CA..CA.ACT CTAACTACA. TG..GCGCCC GTGT....AC GGACG...GC ATCAT..... .GGCGAGCAG CCCGCCTT.      ATGT
  minimusC   CCTTGTTACA CA..AT.ATT CTAACTACA. TG..GCGCCC GTGT....AC GGACG...GC ATCAT..... .GGCGAGCAG CCCGCCTA.. ...CTGATGT
  aconitus   CCTTGTTACA CA..AA.TAC ATAACTACA. GG..ACGGGC GTGCTAC.AC AGTCAAGTGA AACATTCC.. GGGCGAGCAG CCCGCCA... ...CGGAAGT
  pampanai   CCTTTATACA CATACA.TAC ATAACTACA. GG.CGCGCAC GTAAAGTGAC CGATTAAAAA AAAAGCCCTC AGGCGAGCAG CCCGCTTCGA AGGCTGGGA
    varuna   CCTTGATACA CAAACATAAC CGAACTACAG TG.TGCGCAC GCAT...... AGCGAGAAC .ATTCGG.... GGGCGAGCAG CCCGCAC... ...CCTGAAT

           201                                                 250                                                 300
  funestus   CG.TGGGGGA AACACGCTTC CACACTGTGC ATAATGGCGT GCTCGGG... ...AC  PAR  TGGG...AC CGCAGGGCGC TGAAAGTAAA GGGG.TGAAC
 vaneedeni   CG.TGGGGGA AACACGCTTC CACACTGTGC ATAATGGCGT GCTCGGG... ...AC       TGGG...AC CGCAGGGCGC TGAAAGTAAA GGGGATGAAC
  parensis   CG.TGGGGGA AACACGCTTC CACACTGTGC ATAATGGCGT GCTCGGA... ...ACCTAGC TTGGG...AC CGCAGGGCGC TGAAAGTAAA GGGGATGAAC
 rivulorum   TGCTGACCAA CACGCGCCTG CCCACTGTGC ATAATGACGT GCTCGGGAAG CGATTCTCGG GGGCGTTGAA TTACGAGCGA .GATGGGGG. ....CGG..T
   leesoni   TGCTGAATGA .ACACGTGAG CGCACTGTGC ATAATAGCGT GCAAGGC.TC GTCTCCTAC. .CGGG...A. CCTTGGGCGC TGAAA..GGT ...AAGG..C
  minimusA   TGCTGAATGA .ACACGTGAG CGCACTGTGC ATCATTGCGT GCAGGGC.CC GTCTCCTAC. .CGGG...A. CCTTGGGCGC TGAAAA.GGT ...AAGG..C
  minimusC   TGCTGAATGA .ACACGTGAG CGCACTGTGC ATCATTGCGT GCAGGGC.CC GTCTCCTAC. .CGGG...AA CCTTGGGCGC         GT ...AAGG..C
  aconitus   CACTGGACGT .ACACGCTGT CGGACTGTGC ATCTTGGCGT GCTTGGT.TC CACACCCAGA GCGGGGTGAA CCTCGGGCGC  VAR   GT ...AAGG..C
  pampanai   CACTG       TGTGCG CGCACTGTGC ATCATGGCAT GCTTGGTACC GTCTCCTAC. .CGG....AA CCATGGGTGC         GT ...GAGG..C
    varuna   TGCATA ACO  CGGGGC CACACTGTGC AGCATGACGT ACAAGCTTGA GCTGGCTTGA TTGAG..... .CTCGTGCGT CGAAAGTGGT CAAAAGG..C

           301                                                 350                                                 400
  funestus   CGCATAAATC GCACGCACG. ...TAAACGC GCACACACAC AAATAGAGTG AGACGTATCG TAGGATACCG CTA.AGAGTA CGTTGTGAAA CATGGGGAAA
 vaneedeni   CGCATAAAT LEE2 CG. ...TAAGAG. .......... ..TATGAGTG AGAGGTATCG TAAGATACAG TGAGAGAGTA CGTTGTGAAA AATGGAGA..
  parensis   CGCATAAAT      CGC AAATACACAC ACTCACACCC  MIA  GTG TGAGGTATCG TAAGATACAG TGAGAGAGTA TGTTGTGGAA AATTGTGAA.
 rivulorum   ATTCTGTTG       CAC CCCACGGGTG ATGATGGCGC       GCT GCGTGTTGCG CAA..GACGT C.CTGACAGC CCCGGCAG..
   leesoni   AGTACAGTGG CACTGTACTT AGCGGGGGTG CAGCGT...C AAGTCGCAC. ..GGGA..CG AA..CTTCGG CTATGGACGA C.CTGAGATA CCCGGCAGCC
  minimusA   AGTACAGTGT CACTGTACAA TTTGGGGGTG CATCGT...C AAGTCGCAC. ..GGGT..CG AA..CTTCGG CTATGGACGA C.CTGAGATA CCCGGCAGCC
  minimusC   AGTACAGTGT CACTGTACAA TTTGGGGGTG CAGCGT...C AAGTCGCAC. ..GGGT..CG AA..CTTCGG CTATGGACGA C.CTGAGATA CCCGGCAGCC
  aconitus   AGAGTACAAT CT.TGTACA. ..CCAGGGTA CAGCGT...C AAGTCGCAC. ..GGGT..CG AA..CTTCGG CTATGGACGA C.CTGAGAAC CCCGGCAGCC
  pampanai   AATAC..... ....GAACTG TATCAGGGCG C.GTGT...C AAGTCGCACA .CGGGT..CG AA..CTTCGG CTATGGATGA C.CTGAACAC CCCGGCAGCC
    varuna   AGTACG.... .AACGAAC.G TAGCAGGGAG T.GCGC...C GAGTCACACA .CGGGT..CG AA..CTTCGA CAATGGACGA C.TTGCGAAC CCCGGCAGCC

           401                                                 450                                                 500
  funestus   TTCAATCGAA AACCTCTTTG ATGTCCAAGA TTTCGTTGAC CGTATCCGTC GTAATACTGG ATCAACGTGC TTGGGGGAAA ACGTCAAAGG GTTTTATAAT
 vaneedeni   TTCATTTGAA AACCTCTTTG ATGTCCAAGA TTTCGTTGAC CGTA       AATACTGG ATCAACGTGC TTGGGGGAGC ACATCTTGGG GTTT...AAT
  parensis   TTCAGTTGAA AACCTCTTTG ATGTCCAAGA TTTCGTTGAC CGTA  RIV  AATACTGG ATCAACGTGC TTGGGAGGAA ACATCTTAGT GGTCAACCCG
 rivulorum   .AC...CTAC ACCAGGATTG TCGGGCTCTG AATCAGGGT. .....C.... ........GA A.CGGCTTGC CGGGTCGCGT AATGCGCG.. ..........
   leesoni   TAC...TAAC CCCAGGCTAG TCGATCCGGT TCCAGGGGTT ....AC.... ........GA ATCATCCGGC CGAGTCGTGT AACGCGTGAC GACCCATACG
  minimusA   TAC...TAAC ACCAGGCTTG TCGACCAGGT TCCAGGGGTT ....AC.... ........GA ATCATCCGGC CGAGTCGTGT AACGCGTGAC GACCCATACG
  minimusC   TAC...TAAC ACCAGGCTTG TCGACCAGGT TCCAGGGGTT ....AC.... ........GC ATCATCCGGC CGAGTCGTGT AACGCGTGAC GACCCATACG
  aconitus   TAC...TAAC ACCAGGCTAG CCGATCCGGG TCCAGGGGTT ....AC.... ........GC ATCATCCGGC CGAGTCGTGT AACACGTG.C AACCCTTACG
  pampanai   TAC...TAAC ACCAGGCTTG TCGCGCAGGT TTCAGGTGCT TACTAC.... ........GC TTCATCCAGC CGAGTCG... ....CGTGTT GACC...ACG
    varuna   TAC...TAAC ACCAGGCTTG TCGATC.... .CACGGGTTC CTACAT.... ........GT TTCATCCTGC GAAGTCAAGT AAAACGTGTC GTGATGGGCT

           501                                                 550                                                 600
  funestus   AGTGGTGCAT GATTAACCCA TCGATGCCCG AGGGGAACAT GTTGTCCAAT ACAATAGTGG TGCAGTTGGC TCGACATGCT CGGGGGGAGA CATCGTGGGT
 vaneedeni   CGTGGTGTAT ..          CCGATGCCCG AGGGGAACAT GTTGTCCAAT T.TTTAGTGG TGCA.TTGGC TCGACATGCT CGGGGAGAAA CATCGAGGGT
  parensis   TGTGGAGTAT ..   FUN    CGATGTGTG CAG......T GTTGT..... .......TGC GGATATCGGT TCGCTATGCC C......... ..........
 rivulorum   .......... ..          CTGTGC.... .......... TCG....... .......TGT CTTCGTAGCC C......... .GATAGGCAA CCACCTGTAC
   leesoni   .GTGGG.... .......CCC CTGGTGCATG ....AAA..T TCC....... .......TAC CTAAGTAGGC CTCAAGTGAT .GTGTGACAA CCCCCTGAAT
  minimusA   .GTGGG.... .......CCC CTGTGGCATG AGAAAAC..T TAC....... .......TTC ATAAGTAGGC CTCAAGTGAT .GTGTGACAA CCCCCTGAAT
  minimusC   .GTGCA.... .......CCC GTATTGAGTG GGCAACC..T TCC....... .......TTT CTACGTAGGC CTCAAGTGAT .GTGTGACAA CCC.......
  aconitus   CATGGA.... .......CCC TACTTGCTT. ....TCC..T TTC....... .......GC ATATGTAGGC CTCAAGTGAT .GTGTGACAA CCCCCTGAAT
  pampanai   ...ATA.... .......CCG CCAATGCCCG ......C..T TTC....... .......ATT GCATGTAGGC CTCAAGTGAT .GTGTGACAA CCC.......
    varuna   CTCTGG.... .......CCA CGGGGACCGT ACTTGCTGGT TTT....... .......TGT CCAAGTAGGC CTCAAGTGAT .GTGTGACAA CCCCC.....
```

FIGURE 1. (Continued on next page).

amplification would be specific for each species and easily visualized on an agarose gel. The oligonucleotide sequence for each primer and the melting temperature (Tm) are shown in Table 2. Since both groups are closely related and some species exhibited very similar ITS2 sequences, such as the *An. minimus* complex and *An. leesoni*, the MIA (*An. minimus* A) and MIC (*An. minimus* C) primers had only one base difference from the ITS2 sequence of *An. leesoni*. In spite of this, the species specificity of all the primers designed was conserved.

The universal forward primer (ITS2A) is located in the conserved 5.8S gene, whereas the species-specific reverse

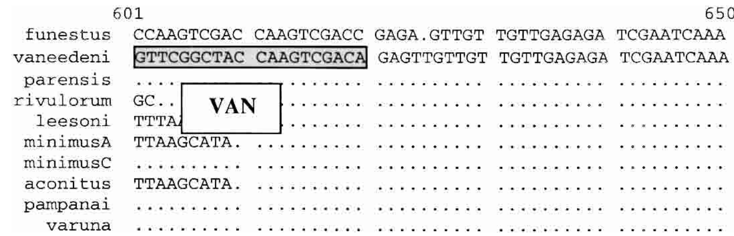

FIGURE 1. Alignment of the nucleotide sequence of the internal transcribed spacer 2 (ITS2) from 10 species of the *Anopheles funestus* and *An. minimus* groups. Shaded boxes indicate primer selection sites, white boxes indicate primer names for the polymerase chain reaction assay, and dots indicate an absence of the specific nucleotide at the indicated position. PAM = pampanai; MIC = minimus C; PAR = parensis; VAR = varuna; ACO = aconitus; LEE2 = leesoni; MIA = minimus A; RIV = rivulorum; FUN = funestus; VAN = vaneedeni.

primers are within the ITS2 spacer region. Each of the 10 species-specific primers works in combination with the universal forward primer ITS2A to give a good and reliable amplification signal. Ten species of both the *An. funestus* and *An. minimus* groups, five for each group, can be identified by the combination of 11 primers in our multiplex PCR. One of the 11 primers is the forward primer (ITS2A).

Lengths of amplified species-specific products were 90 bp for *An. pampanai*, 180 bp for *An. minimus* C, 200 bp for *An. aconitus*, 235 bp for *An. parensis*, 260 bp for *An. varuna*, 280 bp for *An. leesoni*, 310 bp for *An. minimus* A, 400 bp for *An. rivulorum*, 460 bp for *An. funestus*, and 555 bp for *An. vaneedeni* (Figure 2 and Table 2).

Multiplex PCR. The 11 primers can all be combined in a multiplex PCR mixture for the simultaneous amplifications of all 10 species (Figure 2). Therefore, each unknown specimen can be identified without performing 10 separate PCRs. We also tested the possibility of amplifications of the entire ITS2 region jointly with the species-specific fragment in the multiplex mixture. We combined the reverse universal primer ITS2B, which anneals to the 28S subunit, with the 10 specific primers and the universal ITS2A, for a total of up to 12 primers. This double amplification was tested on all 10 species and gave good amplification signals for all species and for both fragments. The amplification of both fragments, the ITS2 and the species-specific one, for five species of both groups, three from the *An. funestus* group (*An. vaneedeni*, *An. parensis*, and *An. rivulorum*) and two from the *An. minimus* group (*An. minimus* A and *An. aconitus*) is shown in Figure 3.

The PCR conditions were optimized with respect to a number of parameters (DNA polymerase and primers). The aim was to maximize the yield of the desired product while retaining specificity. To reduce the cost of the method and achieve adequate specificity, the lowest concentration of *Taq* polymerase that still provides a good resolution of bands on an agarose gel was selected (0.05 units). Since some diagnostic fragments only differ by 20 bp, it is important to use a 3% agarose gel and provide ample migration.

Validation of the multiplex PCR assay. The multiplex PCR assay was validated on specimens previously identified by molecular methods and/or morphology (Table 1). The DNA from the standard specimens was subjected to electrophoresis on an agarose gel as positive controls. A total of 213 specimens were tested for the validation of the PCR, including 63 specimens for the *An. funestus* group and 150 specimens for the *An. minimus* group. Two specimens of the *An. minimus* group from Cambodia and all 12 *An. minimus* C from Thailand (Kanchanaburi Province) were misidentified by morphology due to difficult field conditions for a precise identification. One specimen from Cambodia that was identified as *An. minimus* A was identified by the multiplex assay as *An. pampanai*, and second specimen that was identified as *An. minimus* A was identified by the multiplex assay as *An. minimus* C. The 12 samples from Kanchanaburi Province, which were all morphologically identified as *An. aconitus*, were identified by the multiplex assay as *An. minimus* C. This was

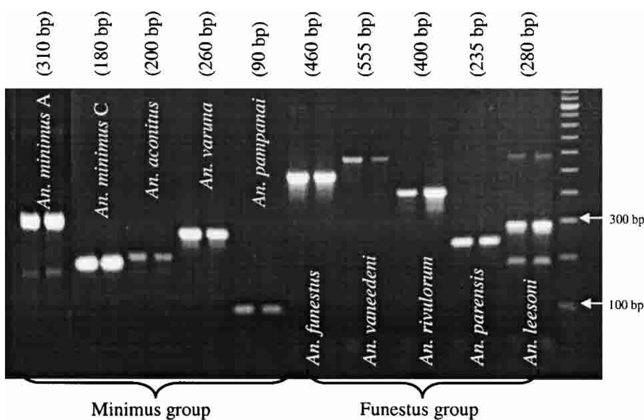

FIGURE 2. Amplified fragments using the species-specific polymerase chain reaction assay for identifying members of the *Anopheles funestus* and *An. minimus* groups. The fragment sizes of the DNA ladder is indicated in basepairs (bp) on the right side.

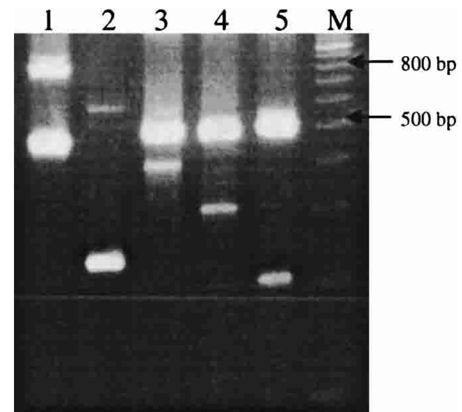

FIGURE 3. Amplification of the entire internal transcribed spacer 2 and species-specific fragments for five *Anopheles* species. Lane 1, *An. vaneedeni*; lane 2, *An. parensis*; lane 3, *An. rivulorum*; lane 4, *An. minimus* A; lane 5, *An. aconitus*. The fragment sizes of the DNA ladder (lane M) are indicated in basepairs (bp).

confirmed by testing the two specimens from Cambodia and five specimens from Kanchanaburi Province with the RFLP assay of Van Bortel and others.[14]

DISCUSSION

Precise knowledge of the biology and distribution of species has been limited by the absence of reliable diagnostic characteristics. Morphologic criteria are often difficult to apply because of a number of biologic and/or technical issues (shared overlapping characteristics, inadequate sampling, laboratory-rearing difficulties, and preservation of specimens). Therefore, molecular markers are increasingly being used to resolve identification issues. An allele specific-PCR (AS-PCR) has already provided a powerful diagnostic tool for the study of anopheline species complexes, such as *An. maculipennis*,[21] *An. gambiae*,[22] *An. quadrimaculatus*,[23] *An. fluviatilis*,[24] and *An. dirus*.[25]

Allele-specific strategies for discriminating closely related species involve 1) the use of universal primers able to amplify target sequences in virtually all species, and 2) species-specific primers for the identification of the target species. Our method used a universal forward primer that binds to the 5′ end of ITS2 in species in the *An. funestus* and *An. minimus* groups, whereas the reverse primers are species-specific and hybridize at different positions along the ITS2. This method has the advantage that any unknown *Anopheles* species can be amplified by universal primers, and the amplification allows unique identification of the expected species from the *An. funestus* and/or *An. minimus* groups with the specific banding pattern. The forward and reverse primers might be so universal that they will potentially amplify the ITS2 sequence of any Diptera. Moreover, the possibility of easily amplifying the entire ITS2 plays the role of a positive control in checking the quality of the amplification. Therefore, in a one-step PCR assay, the anopheline fauna of a specific region can be screened.

The validation of the multiplex PCR showed that two specimens of *An. pampanai* and *An. minimus* C from Cambodia were misreferenced in the collection instead of a misidentification by molecular and morphologic methods. The specimens from Thailand originally identified as *An. aconitus*, but later identified as *An. minimus* C by molecular methods, illustrates the difficulty in making precise identifications within the *An. minimus* group using only adult morphology. The presence of *An. minimus* C in the province of Kanchanaburi Province has already been demonstrated.[13,15]

No species hybrids between members of the *An. funestus* group have been reported either from the field or the laboratory. This is not the case for the *An. minimus* group, in which rare hybrids (less than 1%) between *An. minimus* species A and C have been reported in some regions of Vietnam.[14,15] We tested two hybrids of *An. minimus* A and C identified by ITS2 restriction.[14] The primer MIA, which is specific for *An. minimus* A, binds to the ITS2 sequence of the hybrids. This was verified after sequencing the ITS2 of the hybrids (Garros C and others, unpublished data). The multiplex PCR assay reached its limit by not identifying hybrids between *An. minimus* A and C. However, the low number of base differences existing in the ITS2 hybrid sequence, compared with those of both species of the *An. minimus* complex, makes it difficult to design hybrid primers that will not bind with the two former species. The development of a multiplex PCR that could identify the 10 most common species of both groups, including the hybrids between *An. minimus* A and *An. minimus* C, is possible if a more variable locus is used. The intergenic spacer might be a better region. However, male hybrids might be impossible to identify if all ribosomal DNA repeats were located on the X chromosome, as in the case of *An. gambiae*.[26] No information is available on the precise position of ribosomal DNA in *An. minimus*.

Primer design strategy was targeted at the ribosomal subunit DNA, which characterized by alternate well-conserved regions and variable ones. Molecular identification methods for anopheline species complexes have used mainly the ribosomal DNA locus.[12,23,24,27–33] This locus has many advantages: it is represented in multiple copies throughout the genome in mosquitoes and leads to high amplification signal. It contains highly variable regions that facilitates the selection of primer binding sites for each species to generate specific amplification products of different lengths. More precisely, both ITS1 and ITS2 regions show relatively high levels of interspecies variations, which allows us to design species-specific diagnostic assays.[19,24,32]

Many malaria control laboratories in Africa and Asia are already equipped to perform PCR assays; therefore, no additional equipment will be needed to identify members of both *An. funestus* and *An. minimus* groups. The multiplex PCR assay is rapid, cheap, sensitive, and easy to use, and can be easily adapted to countries in these regions. Both male and female mosquitoes and any life stage can be identified. Preservation is simple since specimens can be stored desiccated with silica gel or in ethanol. Only small quantities of material (1–2 legs) are needed for identification, leaving the rest of the body parts for additional analysis, such as sporozoite detection, blood meal analysis, and population genetics or insecticide resistance status.

In conclusion, the multiplex AS-PCR reported in this study represents a rapid and efficient method that is applicable on a routine basis for the identification of members of the *An. funestus* and *An. minimus* groups. This one-step PCR method constitutes a very powerful tool in large surveys of anopheline populations and widespread collections from Africa and Southeast Asia, and will assist in the improvement of the current knowledge on species distribution of both groups. Moreover, this method does not rely on skillful interpretation; therefore, no subjective bias is introduced in the identification. This is the first AS-PCR developed for identifying *Anopheles* species from two different continents. Malaria control programs on both continents could select the primers specific to either the *An. funestus* or *An. minimus* fauna present in their study sites. These assays will help improving our knowledge in the Arabic corridor or Indian region where some species distributions are unresolved.[4,5] The improvement of the existing assays and their standardization for both continents will aid vector control research. This kind of universal AS-PCR could be applied to other groups of insects or organisms living in sympatry and involved in agricultural or medical problems.

Received July 28, 2003. Accepted for publication September 12, 2003.

Acknowledgments: This study was made possible because of the assistance of a number of people who helped with mosquito sampling

on both continents. We are especially grateful to D. Fontenille (Institute of Research for Development, Montpellier, France), S. Laventure (Institut Pasteur de Madagascar, Antananarivo, Madagascar), G. Le Goff (Institute of Research for Development, Antananarivo, Madagascar), J. Mwangi and G. Otsyula (Kenya Medical Research Institute, Nairobi, Kenya), J. La Grange (Department of Health, Nelspruit, Mpumalanga, South Africa), T. Furemele (Department of Health, Tzaneen, Northern Province, South Africa), K. Hargreaves (Department of Health, Jozini, KwaZulu/Natal, South Africa), G. Kloke (Foray Consultants, Maputo, Mozambique), B. Ntomwa (Malaria Control Program, Oshakati, Namibia), T. Byembabazi (Malaria Control Program, Kampala, Uganda), and the entomology team of the National Institute of Malarialogy, Parasitology and Entomology (Hanoi, Vietnam) for assistance with field collections or supply of specimens. We also thank V. Baimai and M. Savanasoojarit (Mahidol University, Bangkok, Thailand) for specimens from Thailand.

Financial support: This work was partially supported by an EC grant, INCO-DC research project ERBIC 18CT970211, and a VIHPAL 2000 grant from the French Ministry of Research to Sylvie Manguin.

Authors' addresses: Claire Garros and Sylvie Manguin, Center of Biology and Management of Populations, Campus International de Baillarguet, CS 30016, 34988 Montferrier sur Lez, France, Lizette L. Koekemoer and Maureen Coetzee, Vector Control Reference Unit, National Institute for Communicable Diseases, PO Box 1038, Johannesburg, South Africa, Marc Coosemans, Department of Parasitology, Prince Leopold Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium.

Reprint requests: Claire Garros or Sylvie Manguin, Center of Biology and Management of Populations, Campus International de Baillarguet, CS 30016, 34988 Montferrier sur Lez, France, Telephone: 33-4-99-62-33-28/27, Fax: 33-4-99-62-33-45, E-mails: garros@mpl.ird.fr and manguin@mpl.ird.fr.

REFERENCES

1. Harbach RE, 1994. Review of the internal classification of the genus *Anopheles* (Diptera: Culicidae): the foundation for comparative systematics and phylogenetic research. *Bull Entomol Res 84:* 331–342.
2. Green CA, 1982. Cladistic analysis of mosquito chromosome data (*Anopheles* (*Cellia*) Myzomyia). *J Hered 73:* 2–11.
3. Pape T, 1992. Cladistic analysis of mosquito chromosome data in *Anopheles* subgenus *Cellia Myzomyia. Mosq Syst 24:* 1–11.
4. Harrison BA, 1980. The *Myzomyia* Series of *Anopheles* (*Cellia*) in Thailand, with emphasis on intra-interspecific variations (Diptera: Culicidae). Medical entomology studies -XIII. *Contr Am Entomol Inst 17:* 1–195.
5. Gillies MT, Coetzee M, 1987. *A Supplement to the Anophelinae of Africa South of the Sahara (Afrotropical Region).* Publication no. 55. Johannesburg, South Africa: Publications of the South African Institute for Medical Research.
6. Gillies MT, De Meillon B, 1968. *The Anophelinae of Africa South of the Sahara*. Publication no. 54. Johannesburg, South Africa: Publications of the South African Institute for Medical Research.
7. Hackett BJ, Gimnig J, Guelbeogo W, Costantini C, Koekemoer LL, Coetzee M, Collins FH, Besansky NJ, 2000. Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* and *An. rivulorum*, and uncover a cryptic taxon. *Insect Mol Biol 9:* 369–374.
8. Koekemoer LL, Lochouarn L, Hunt RH, Coetzee M, 1999. Single-strand conformation polymorphism analysis for identification of four members of the *Anopheles funestus* (Diptera: Culicidae) group. *J Med Entomol 36:* 125–130.
9. Koekemoer LL, Coetzee M, Hunt RH, 1998. *Hpa* II endonuclease distinguishes between two species in the *Anopheles funestus* group. *Insect Mol Biol 7:* 273–277.
10. Wilkes TJ, Matola YG, Charlwood JD, 1996. *Anopheles rivulorum*, a vector of human malaria in Africa. *Med Vet Entomol 10:* 108–110.
11. Koekemoer LL, Hargreaves K, Hunt RH, Coetzee M, 2002. Identification of *Anopheles parensis* (Diptera: Culicidae) using ribosomal DNA internal transcribed spacer (ITS2) sequence variation. *Afr Entomol 10:* 235–239.
12. Koekemoer LL, Kamau L, Hunt RH, Coetzee M, 2002. A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. *Am J Trop Med Hyg 66:* 804–811.
13. Sharpe RG, Hims MM, Harbach RE, Butlin RK, 1999. PCR-based methods for identification of species of the *Anopheles minimus* group: allele-specific amplification and single-strand conformation polymorphism. *Med Vet Entomol 13:* 265–273.
14. Van Bortel W, Trung HD, Roelants P, Harbach RE, Backeljau T, Coosemans M, 2000. Molecular identification of *Anopheles minimus s.l.* beyond distinguishing the members of the species complex. *Insect Mol Biol 9:* 335–340.
15. Kengne P, Trung HD, Baimai V, Coosemans M, Manguin S, 2001. A multiplex PCR-based method derived from random amplified polymorphic DNA (RAPD) markers for the identification of species of the *Anopheles minimus* group in Southeast Asia. *Insect Mol Biol 10:* 427–435.
16. Manguin S, Mouchet J, Coosemans M, 2001. Molecular identification of sibling *Anopheles* species: example of the *Anopheles minimus* and *Anopheles dirus* complexes, major malarial vectors in Southeast Asia. *Med Trop (Mars) 61:* 463–469.
17. IMPE, 1987. *Keys to the Anopheles in Vietnam (Adults-Pupae-Larvae).* Hanoi, Vietnam: Department of Entomology, Institute of Malarialogy, Parasitology and Entomology.
18. Linton YM, Harbach RE, Chang Moh S, Anthony TG, Matusop A, 2001. Morphological and molecular identity of *Anopheles* (*Cellia*) *sundaicus* (Diptera: Culicidae), the nominotypical member of a malaria vector species complex in Southeast Asia. *Syst Entomol 26:* 357–366.
19. Hall TA, 1999. BioEdit: a user-friendly biological sequencer alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser 41:* 95–98.
20. Scott JA, Brogdon WG, Collins FH, 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *Am J Trop Med Hyg 49:* 520–529.
21. Porter CH, Collins FH, 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). *Am J Trop Med Hyg 45:* 271–279.
22. Paskewitz SM, Wesson DM, Collins FH, 1993. The internal transcribed spacers of ribosomal DNA in five members of the *Anopheles gambiae* species complex. *Insect Mol Biol 2:* 247–257.
23. Cornel AJ, Porter CH, Collins FH, 1996. PCR diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. *J Med Entomol 33:* 109–116.
24. Manonmani A, Townson H, Adeniran T, Jambulingam P, Sahu SS, Vijayakumar T, 2001. rDNA-ITS2 polymerase chain reaction assay for the sibling species of *Anopheles fluviatilis. Acta Trop 78:* 3–9.
25. Walton C, Handley JM, Kuvangkadilok C, Collins FH, Harbach RE, Baimai V, Butlin RK, 1999. Identification of five species of the *Anopheles dirus* complex from Thailand, using allele-specific polymerase chain reaction. *Med Vet Entomol 13:* 24–32.
26. McLain DK, Collins FH, 1989. Structure of rDNA in the mosquito *Anopheles gambiae* and rDNA sequence variation within and between species of the *A. gambiae* complex. *Heredity 62:* 233–242.
27. Beebe NW, Ellis JT, Cooper RD, Saul A, 1999. DNA sequence analysis of the ribosomal DNA ITS2 region for the *Anopheles punctulatus* group of mosquitoes. *Insect Mol Biol 8:* 381–390.
28. Beebe NW, Maung J, van den Hurk AF, Ellis JT, Cooper RD, 2001. Ribosomal DNA spacer genotypes of the *Anopheles bancroftii* group (Diptera: Culicidae) from Australia and Papua New Guinea. *Insect Mol Biol 10:* 407–413.
29. Collins FH, Paskewitz SM, 1996. A review of the use of ribosomal DNA to differentiate among cryptic *Anopheles* species. *Insect Mol Biol 5:* 1–9.

30. Fritz GN, 1998. Sequence analysis of the rDNA internal transcribed spacer 2 of five species of South American human malaria mosquitoes. *DNA Seq 8:* 215–221.
31. Harbach RE, Townson H, Mukwaya LG, Adeniran T, 1997. Use of rDNA-PCR to investigate the ecological distribution of *Anopheles bwambae* in relation to other members of the *An. gambiae* complex of mosquitoes in Bwamba County, Uganda. *Med Vet Entomol 11:* 329–334.
32. Linton YM, Samanidou-Voyadjoglou A, Harbach RE, 2002. Ribosomal ITS2 sequence data for *Anopheles maculipennis* and *An. messeae* in northern Greece, with a critical assessment of previously published sequences. *Insect Mol Biol 11:* 379–383.
33. Fettene M, Temu EA, 2003. Species-specific primer for identification of *Anopheles quadriannulatus* sp. B (Diptera: Culicidae) from Ethiopia using a multiplex polymerase chain reaction assay. *J Med Entomol 40:* 112–115.