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Molecular differentiation of three closely related members of the mosquito species complex, *Anopheles moucheti*, by mitochondrial and ribosomal DNA polymorphism

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Abstract. Distinction between members of the equatorial Africa malaria vector *Anopheles moucheti* (Evans) *s.l.* (Diptera: Culicidae) has been based mainly on doubtful morphological features. To determine the level of genetic differentiation between the three morphological forms of this complex, we investigated molecular polymorphism in the gene encoding for mitochondrial cytochrome oxidase b (CytB) and in the ribosomal internal transcribed spacers (ITS1 and ITS2). The three genomic regions revealed sequence differences between the three morphological forms similar in degree to the differences shown previously for members of other anopheline species groups or complexes (genetic distance d = 0.047–0.05 for CytB, 0.084–0.166 for ITS1 and 0.03–0.05 for ITS2). Using sequence variation in the ITS1 region, we set up a diagnostic polymerase chain reaction (PCR) for rapid and reliable identification of each subspecies within the *An. moucheti* complex. Specimens of *An. moucheti s.l.* collected in Cameroon, the Democratic Republic of Congo (DRC), Uganda and Nigeria were successfully identified, demonstrating the general applicability of this technique.

Key words. *Anopheles moucheti*, allele-specific PCR, equatorial Africa, malaria vector, mitochondrial DNA, ribosomal DNA.

Introduction

Anopheles moucheti (Evans) *s.l.* is a major vector of human malaria in villages located close to rivers or slow-moving streams in equatorial Africa. This species has been reported to be responsible for high *Plasmodium* entomological inoculation rates that can reach 300 infective bites per person per year, with sporozoïte rates ranging up to 4% (Antonio-Nkondjio *et al.*, 2002a). Based on the distribution of clear spots on the wings of adults and subtle variations observable at the larval stage, three morphological forms have been described within this taxa (D'Haenens, 1961; Gillies & De Meillon, 1968; Brunhes *et al.*, 1998), comprising the type form *An. moucheti moucheti* Evans, *An. moucheti nigeriensis* Evans, which is most similar to the type form, and *An. moucheti bervoetsi* D'Haenens, which is considered to be a subspecies (Evans, 1931; Gillies & De Meillon, 1968; Gillies & Coetzee, 1987; Brunhes *et al.*, 1998).

In order to assess the taxonomic value of this morphological classification, direct observation of morphological variations within the progeny of field-collected females of all three forms in Cameroon was performed. The offspring from single females always belonged to at least two morphologically recognized types, and, usually, a mixture of all three forms was observed. These results raised doubts about the use of morphological

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character alone for the identification of adult members of the *An. moucheti* complex (Antonio-Nkondjio *et al.*, 2002b). Furthermore, allozyme and microsatellite analysis failed to detect genetic differences among morphological forms of *An. moucheti s.l.* collected in Cameroon (Antonio-Nkondjio *et al.*, 2002b, 2007; Annan *et al.*, 2003). Together these data demonstrate the confused status of these taxa and highlight the necessity for more robust means of identifying putative members of this important malaria vector.

DNA-based methods for the identification of closely related species have been used successfully with a number of anopheline complexes. Recent studies investigating polymorphism in mitochondrial and/or ribosomal DNA (rDNA) regions provided the foundations for developing diagnostic assays based on polymerase chain reaction (PCR) (Cohuet *et al.*, 2003; Kengne *et al.*, 2003) and for reconstructing phylogenetic relationships within several vector complexes (Danforth *et al.*, 2005). DNA from ribosomal genes was targeted for this study because of the high copy number and the presence in each cluster of a highly conserved coding region alternating with a less conserved spacer sequence that evolves at higher rates than the coding region. Molecular techniques are reliable and have the advantage of being applicable to any life stage and gender.

In the present study, we analysed molecular polymorphism in the internal transcribed spacers (ITS1 and ITS2) of rDNA and the cytochrome oxydase b (CytB) gene of mitochondrial DNA among the three morphological types of *An. moucheti s.l.* collected in Cameroon, the Democratic Republic of Congo (DRC), Uganda and Nigeria. Subsequently, a species-specific PCR assay was developed in order to differentiate the three taxa of the *An. moucheti* complex in a single reaction.

Materials and methods

Mosquito collection and morphological identification

Sample collection sites included three villages in Cameroon (Simbock 3°51′ N, 11°30′ E; Olama 3°24′ N, 11°18′ E; Nyabessan 2°80′ N, 10°25′ E), two villages in the DRC (Tsakalakuku 6°34′ S 17°35′ E and Kasombo 5°51′ S, 17°23′ E), two sites in Uganda (Bufumira 0°22′ S 32°20′ E and Buyovu Island 0°19′ S 32°30′ E) and a village (Akaka 06°27′ N, 03°24′ E) near Lagos, Nigeria. Tsakalakuku (DRC) and Akaka (Nigeria) are type localities of *An. m. bervoetsi* and *An. m. nigeriensis,* respectively. Mosquitoes were collected by pyrethrum spraying in bedrooms. Adult *An. moucheti s.l.* were separated from other anophelines, according to morphological identification keys (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987; Brunhes *et al.*, 1998), and further identified to subtype (e.g. *An. m. moucheti*, *An. m. nigeriensis* and *An. m. bervoetsi*) according to the size and distribution of the pale fringe spots and pale vein spots on the wings, following the identification keys of Gillies & De Meillon (1968) and Brunhes *et al.* (1998). In total, 102 specimens, including 68 *An. m. moucheti* (Cameroon, Uganda, DRC), 30 *An. m. bervoetsi* (DRC) and four *An. m. nigeriensis* (Nigeria) were used for sequence variation analysis.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from each mosquito according to the protocol defined by Cornel & Collins (1996), with the following modifications; incubation at 65° C for 10 min instead of 30 min in order to reduce processing time, and resuspension of the dried pellet in 20 μL instead of 100 μL nuclease-free water, for better storage of DNA. The ITS1 and ITS2 regions of the rDNA and the CytB on mtDNA were amplified in a 25-μL PCR reaction including: 20 ng template DNA, 2.5 μL 10× reaction buffer (Qiagen, Courtaboeuf, France), 1.5 mM MgCl2, 200 μM each dNTP (Eurogentec, Seraing, Belgium), 0.5 U Taq DNA polymerase (Qiagen) and 20 pmol of each primer (Table 1). Polymerase chain reaction conditions included an initial denaturation step at 94° C for 3 min, followed by 35 cycles for 30 s at 94° C, 45 s at 55° C and 45 s at 72° C, followed by a final extension step of 10 min at 72° C. The amplified products were electrophorized through 1.5% agarose gel.

The PCR products were sequenced directly in both directions using forward and reverse primers on an ABI Prism 3130 DNA sequencer (Applied Biosystems, Courtaboeuf, France). Sequences from different morphological forms were aligned using CLUSTAL X (Higgins & Sharp, 1988). Similarity and divergence estimates were derived using methods implemented in Mega3 (Kumar *et al.*, 2004) and DnaSP Version 3 (Rozas & Rozas, 1999) software programs. Consensus sequences of each subspecies of the *An. moucheti* complex have been deposited in the EMBL Nucleotide Sequence Database under accession numbers AM232661 for *An. m. moucheti*, AM232662 for *An. m. nigeriensis* and AM232663 for *An. m. bervoetsi*.

Table 1. Primers and annealing temperature used for amplification and sequencing of internal transcribed spacers 1 and 2 (ITS1 and ITS2) and cytochrome oxidase b (CytB).

Primer	Sequence (5′–3′)	Annealing temperature
19mer-Primer ITS1aF	CGAGCCGAGTGATCCACCG	52° C
18mer-Primer ITS1aR	TTGATTACGTCCCTGCCCTTT	50° C
20mer-Primer ITS2F	TGTGAACTGCAGGACACATG	50° C
20mer-Primer ITS2R	TATGCTTAAATTCAGGGGGT	49° C
28mer-Primer CytBF	GGACAAATATCATTTTGAGGAGCAACAG	54° C
27mer-Primer CytBR	ATTACTCCTCCTAGCTTATTAGGAATTG	53° C

Results

Analysis of ITS1, ITS2 and CytB sequences

Amplification by PCR using the primer pairs described in Table 1 revealed an apparent size polymorphism in the amplicons obtained for the ITS1 region, whereas unique amplicons were obtained for the ITS2 (~ 600 bp) and CytB (~ 500 bp) regions.

ITS1. Sequences were determined directly from the PCR products of a total of 102 *An. moucheti s.l.* specimens (four *An. m. nigeriensis,* 30 *An. m. bervoetsi* and 68 *An. m. moucheti*). Figure 1 presents the sequence alignment of consensus sequences for the ITS1 region for all three subspecies. Nucleotide changes (substitutions) as well as insertion/deletion polymorphisms leading to length variation were recorded. The smallest fragment was 629 bp long, in *An. m. bervoetsi*, and the longest was 667 bp, in *An. m. moucheti*. Mean genetic distances between consensus sequences of each subspecies were 0.084 for *An. m. moucheti* and *An. m. nigeriensis* (Amm vs. Amn), 0.166 between *An. m. nigeriensis* and *An. m. bervoetsi* (Amn vs. Amb) and 0.141 for *An. m. moucheti* and *An. m. bervoetsi* (Amm vs. Amb) (Table 2). This degree of differentiation between morphological forms is relatively high, indicating significant genetic divergence among the three taxa, which is comparable with that among species. It should be noted that nucleotide diversity was very low (0.00–0.002) among specimens from Cameroon, which might have been misidentified as *An. m. moucheti*, *An. m. nigeriensis* or *An. m. bervoetsi* based on morphological features. No fixed nucleotide differences were registered among forms in this country.

ITS2. A single band of ~ 500 bp was revealed after electrophoresis of PCR products. The length of sequenced fragments was 492 bp for *An. m. moucheti*, 504 bp for *An. m. nigeriensis* and 507 bp for *An. m. bervoetsi*. Mean distance within subspecies was very low, ranging from 0.000 (*An. m. nigeriensis*)

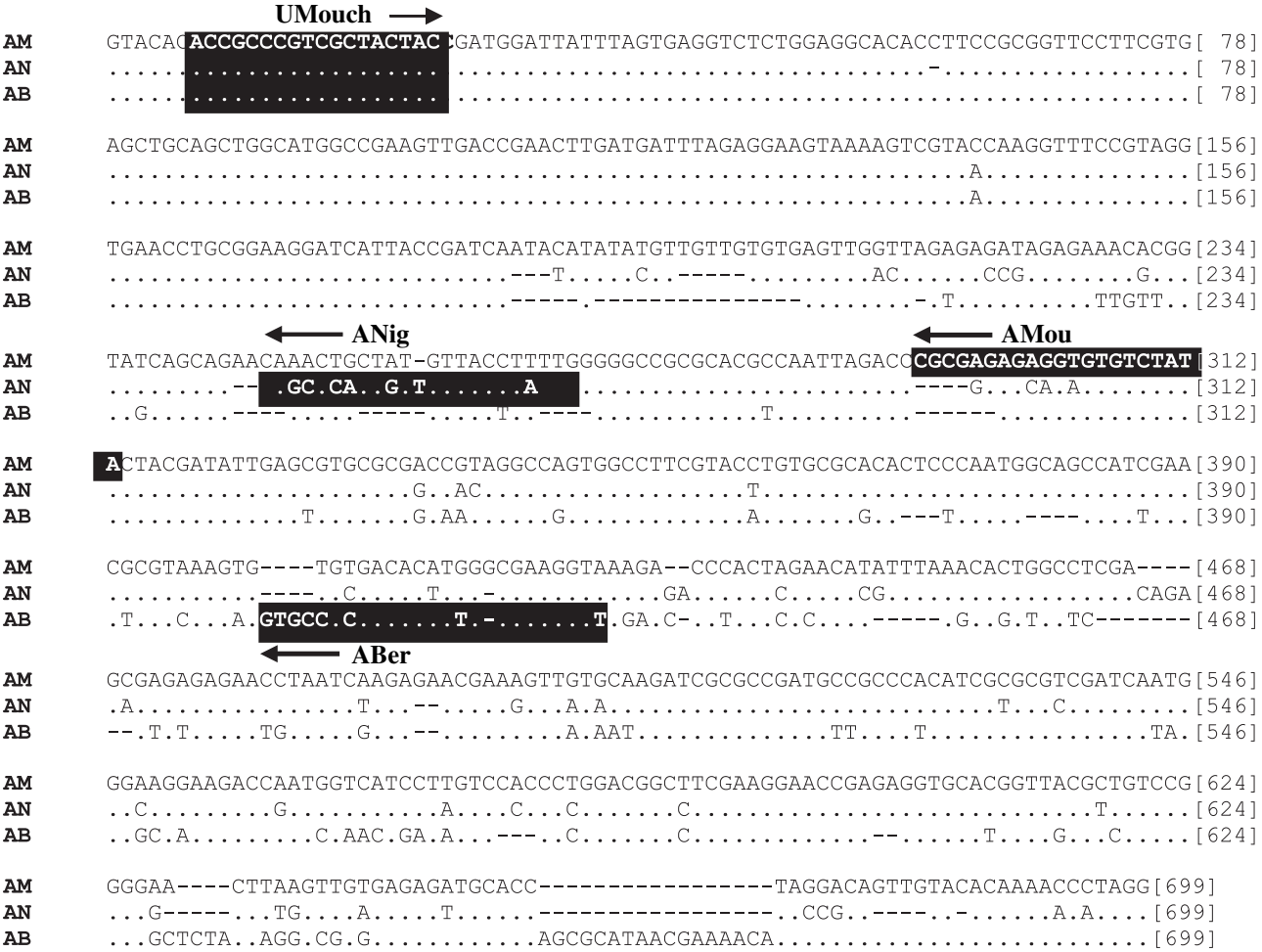

Fig. 1. Sequence alignment of the internal transcribed spacer (ITS)1 and flanking 18S and 5.8S consensus sequence from isolates representing each member of the *Anopheles moucheti* complex. The sequences in the alignment are as follows: *An. m. moucheti*, *An. m. nigeriensis* and *An. m. bervoetsi*. Consensus with *An. moucheti* is indicated by a dot (.); dashes (–) indicate an alignment space. Areas from which the primer was designed are underlined. The primers are: UMouch (universal primer); AMou (*An. m. moucheti*); ABer (*An. m. bervoetsi*), and ANig (*An. m. nigeriensis*).

Table 2. Pairwise genetic distance among three genes (ITS1, ITS2 and CytB) for each *Anopheles moucheti* subspecies vs. each other.

	ITS1			ITS2			CytB		
	Amb	Amm	Amn	Amb	Amm	Amn	Amb	Amm	Amn
Amb									
Amm	0.141			0.062			0.044		
Amn	0.166	0.084		0.055	0.03		0.047	0.035	

ITS, internal transcribed spacer; CytB, cytochrome oxidase b; Amb, *Anopheles moucheti bervoetsi*; Amm, *Anopheles moucheti moucheti*; Amn, *Anopheles moucheti nigeriensis*.

to 0.001 (*An. m. bervoetsi* and *An. m. moucheti*). The ITS2 sequence displayed a lower degree of divergence compared with the ITS1 sequence, ranging from 0.03 (Amm vs. Amn) to 0.063 (Amm vs. Amb) (Table 2). A total of 39 parsimony-informative sites out of 45 variable sites were observed. Overall, there were 30, 29 and 16 fixed indel or substitution differences between *An. m. moucheti*/*An. m. bervoetsi*, *An. m. nigeriensis*/*An. m. bervoetsi* and *An. m. moucheti*/*An. m. nigeriensis*, respectively. As for ITS1 sequences, we obtained a level of differentiation among subspecies indicating significant genetic divergence among the three taxa.

CytB. For the CytB gene, ~ 450 bp fragments were sequenced from 80 *An. moucheti s.l.* specimens (four *An. m. nigeriensis*, 19 *An. m. bervoetsi* and 57 *An. m. moucheti*). Of 30 variable sites, 28 were parsimony-informative. The sequences also showed a typical pattern of variability, with 26 first codon position differences fixed among morphological forms. As for ITS2, the CytB segment displayed low but consistent between-form divergence (0.035–0.047) (Table 2). The concordance of genetic variability of the three markers and the lack of intraspecific geographical variation suggests that the three members of the group are true species. ITS2 and CytB sequence data for *An. moucheti s.l.* are available on request.

Allele-specific PCR

Nucleotide sequence differences in the ITS1 region were used to design species-specific primers for the development of an allele-specific PCR assay for rapid identification of the three taxa. Primer selection was based on the principles described by Paskewitz & Collins (1990), Scott *et al.* (1993), Cornel & Collins (1996), Cohuet *et al.* (2003) and Kengne *et al.* (2003). A universal forward primer (UMouch) that anneals to the 5′-end of the ITS1 of all *An. moucheti s.l.* specimens was designed, together with three reverse primers AMou, ABer and ANig, respectively, specific to *An. m. moucheti*, *An. m. bervoetsi* and *An. m. nigeriensis*, respectively. Primer sequences were defined so that at least four nucleotides in their 3′-end would prevent hybridization to the alternative haplotypes and guarantee specificity of the assay. Primer sequences are indicated in Table 3.

The size of the diagnostic band was 378 bp for *An. m. bervoetsi*, 312 bp for *An. m. moucheti* and 249 bp for *An. m. nigeriensis* (Fig. 2). Various PCR conditions were tested in order to optimize amplification. The ideal mixture composition for diagnostic PCR was 1.5 mM MgCl2, 200 μM each dNTP (Eurogentec), 2.5 μL 10 × Taq buffer, 0.5 U Taq polymerase (Qiagen), and 10 ng of template DNA in 25 μL of final reaction volume. The amount of each primer used in the PCR assay was 20 pmol for UMouch and 10 pmol each for AMou, ABer and ANig. The PCR conditions included an initial denaturation step at 94° C for 5 min, followed by 30 cycles of 30 s at 94° C, 30 s at 62° C and 1 min at 72° C. After the final cycle, the products were extended for 7 min at 72° C and separated by electrophoresis on a 2% agarose gel.

Validation of the assay

To test the general application of the method, 202 field-collected *An. moucheti s.l.*, including 122 specimens from three sites in Cameroon (Simbock, Olama and Nyabessan), 22 specimens from two sites in Uganda (Bufumira and Buyovu Island), 54 specimens from two sites in the DRC (Tsakalakuku and Kasombo) and four specimens from one site in Nigeria (Akaka) were analysed. Samples ($n = 48$) from two locations in the DRC (Tsakalakuku and Kasombo) and from the unique location in Nigeria (Akaka) were identified as *An. m. bervoetsi* (DRC)

Table 3. Specific primers used for identification of three sibling species of the *Anopheles moucheti* group. Umouch, the universal primer, binds to the same position of internal transcribed spacer (ITS)-1 DNA from all members of the group. AMou, Aber and ANig bind only to ITS1 DNA from *An. m. moucheti*, *An. m. bervoetsi* and *An. m. nigeriensi*, respectively.

Primer	Sequence (5′–3′)	MT	Identified species	Size of PCR product
19mer-UMouch	ACCGCCCGTCGCTACTACC	54° C	–	–
21mer-AMou	TATAGACACACCTCTCTCGCG	52° C	*An. m. moucheti*	312 bp
22mer-ABer	CAGTTCTGCTGCTACCAACAAT	52° C	*An. m. bervoetsi*	378 bp
21mer-ANig	CTAGGTAACAACAGTGGGCTG	52° C	*An. m. nigeriensis*	249 bp

MT, melting temperature; PCR, polymerase chain reaction.

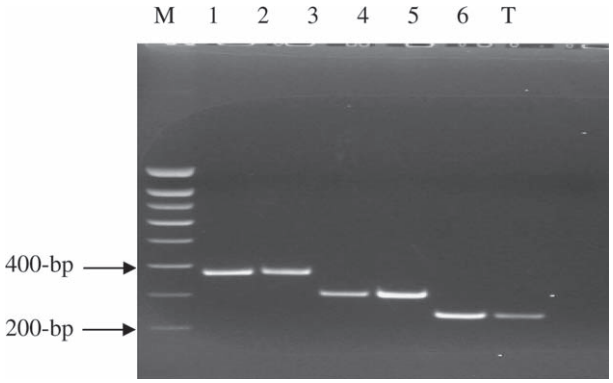

Fig. 2. Allele-specific polymerase chain reaction (PCR) assay of different members of the *Anopheles moucheti* complex. A photograph of 2% agarose gel showing amplified products from a single PCR that contains the universal primer and the three specific primers. Lane M, 100-bp DNA ladder; lanes 1–2, *An. m. bervoetsi*, (378 bp); lanes 3–4, *An. m. moucheti* (312 bp); lanes 5–6, *An. m. nigeriensis* (249 bp). Lane T is a negative control.

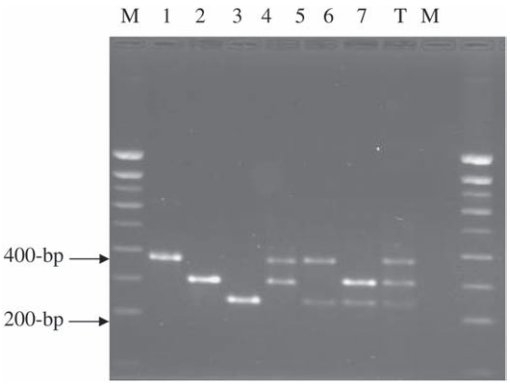

Fig. 3. Polymerase chain reaction results from different molecular forms in the *An. moucheti* complex and mixtures of DNA to simulate hybrids and mixed samples. Lane M, 100-bp DNA ladder; lane 1, *An. m. bervoetsi*; lane 2, *An. m. moucheti*; lane 3, *An. m. nigeriensis*; lane 4, *An. m. bervoetsi* + *An. m. moucheti* (1/1); lane 5, *An. m. bervoetsi* + *An. m. nigeriensis* (1/1); lane 6, *An. m. moucheti* + *An. m. nigeriensis* (1/1); lane 7, *An. m. bervoetsi* + *An. m. moucheti* + *An. m. nigeriensis* (1/1/1). Lane T is a negative control.

and *An. m. nigeriensis* (Nigeria) subspecies, respectively. Samples from the three sites in Cameroon (Simbock, Olama and Nyabessan) were identified as *An. m. moucheti*. Thirty-one of the 122 *An. moucheti s.l.* specimens from Cameroon, which showed doubtful morphological identification because of overlapping in diagnostic morphological features on the wing, were identified as *An. moucheti* subspecies. Under the allele-specific PCR assay conditions, no products were generated that would result in a false positive identification of *Anopheles gambiae* Giles *s.s.*, *Anopheles arabiensis* Patton, *Anopheles funestus* Giles, members of the *Anopheles nili* (Theobald) group, *Anopheles marshallii* (Theobald) and *Anopheles hancocki* Edwards from equatorial Africa (data not shown). Mixed DNA to simulate artificial/putative hybrids in nature was amplified in the same reaction conditions. Amplified fragments of expected sizes were obtained in all combinations (Fig. 3).

Discussion

Given the number of sibling species involved in malaria transmission in many areas of tropical Africa, fast and reliable identification is a basic prerequisite for appropriate malaria vector control programmes (Hougard *et al.*, 2002; Fontenille *et al.*, 2005). Here, we assessed the level of molecular polymorphism in the ITS1 and ITS2 rDNA regions and the mitochondrial gene CytB among *An. moucheti* subspecies from Central Africa.

In southern Cameroon, where Brunhes *et al.* (1998) suggested that *An. m. nigeriensis* was synonymous with *An. m. moucheti*, it was important to evaluate to what extent the country's three morphotypes were genetically distinct. The sequence of all individuals of each morphological form from Cameroon was relatively homogenous for the entire length of the three DNA markers (ITS1, ITS2 and CytB). Sequences of *An. m. moucheti* from Uganda were identical to those of *An. m. moucheti* from Cameroon, suggesting that the same unique subspecies *An. m. moucheti* is distributed across the different sites sampled in Cameroon and Uganda, although they are geographically distant. Our results are in agreement with isozyme data from Antonio-Nkondjio *et al.* (2002b), suggesting that morphological variation within the natural population of *An. moucheti* in Cameroon is not indicative of speciation. As a consequence, the morphological features used by Brunhes *et al.* (1998) to classify members of this group in Cameroon are inadequate.

By contrast, sequences of *An. moucheti s.l.* close to the type localities of *An. m. nigeriensis* (Nigeria, Akaka) and *An. m. bervoetsi* (DRC, Tsakalakuku) differed clearly from that of *An. moucheti s.l.* from Cameroon and Uganda. As outlined above, there was an unexpected nucleotide difference between *An. m. bervoetsi*, *An. m. moucheti* and *An. m. nigeriensis*, despite the geographical proximity to type locations in Nigeria and Cameroon. The three markers showed a high between-subspecies divergence, ranging from 3% to 16.6%, and an intra-subspecies divergence of under 0.3%. In other Anopheles species complexes, the level of interspecific nucleotide sequence divergence ranges from 0.4% to 1.6% among the *An. gambiae* complex (Paskewitz & Collins, 1990) and 18.5% to 28.7% among members of the *Anopheles quadrimaculatus* Say complex (Cornel & Collins, 1996).

Polymerase chain reaction is now routinely used to identify members of Anopheles complexes (Scott *et al.*, 1993; Cohuet *et al.*, 2003; Kengne *et al.*, 2003). Materials from any life stage, sex, body part, and from fresh or dried specimens, can be successfully identified using this procedure. With the molecular assay reported in this study, we were able to differentiate *An. m. nigeriensis* from *An. m. bervoetsi* and *An. m. moucheti* without ambiguity. The results obtained on specimens collected in Nigeria and the DRC are in agreement with morphological features described by D'Haenens (1961) and Gillies & De Meillon (1968). The present study shows the utility of applying a molecular approach to the identification of species or subspecies that are difficult to separate morphologically. Studies are underway to

assess the distribution and biology of these subtypes in equatorial Africa. One important aspect that needs more extensive study in the near future concerns the demonstration of reproductive isolation among subspecies and the process of speciation that has occurred among the various members of the complex.

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