

SPECIES IDENTIFICATION WITHIN THE *ANOPHELES FUNESTUS* GROUP OF MALARIA VECTORS IN CAMEROON AND EVIDENCE FOR A NEW SPECIES

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Abstract. *Anopheles funestus* is a major vector of malaria in Africa. It belongs to a group of sibling species that can be identified morphologically only at certain stages of their development. A diagnostic polymerase chain reaction (PCR)-based tool made it possible to differentiate five species of the group. The assay seems to be applicable over all their distribution area for four of these species: *An. funestus*, *An. lesoni*, *An. parensis*, and *An. vaneedenip*. The fifth species, *An. rivulorum*, is the second most abundant species of the group and can be mistaken at its adult stage for the major vector of malaria *An. funestus*. Molecular and morphologic observations of specimens identified as *An. rivulorum* from Cameroon and South Africa showed that they belong to two different taxa. The species identified in Cameroon, and named here *An. rivulorum*-like, might extend to western Africa and central Africa. The species-specific PCR assay is supplemented by a primer specific to *An. rivulorum*-like and thus makes it possible to differentiate the five species of the *An. funestus* group and the newly defined taxon.

INTRODUCTION

Anopheles funestus Giles, known for its major role in the transmission of human malaria,¹ belongs to a group of nine species (*An. funestus* s.s., *Anopheles rivulorum* Leeson, *An. lesoni* Evans, *Anopheles vaneedeni* Gillies & Coetzee, *An. parensis* Gillies, *An. confusus* Evans & Leeson, *An. aruni* Sobti, *An. fuscivenosus* Leeson, and *An. brucei* Service) that are morphologically very similar and can only be distinguished at specific stages of their development.^{1,2} Their biology and vectorial capacity is highly contrasting. The mainly endophilic and anthropophilic *An. funestus* is considered a major human malaria vector in Africa. *Anopheles rivulorum* is primarily zoophilic but was found infected with *Plasmodium falciparum* in Tanzania and can transmit human malaria.³ *Anopheles vaneedeni* has been infected in the laboratory with *P. falciparum*, but its vectorial role has never been demonstrated in a natural environment.⁴ The other members of the *An. funestus* group are mainly zoophilic and do not seem to be involved in the transmission of malaria.

Anopheles funestus, *An. rivulorum*, and *An. lesoni* have a wide geographic distribution, extending throughout sub-Saharan Africa. The other members of the group are more localized or their true distribution is largely unknown: *An. parensis* and *An. confusus* are found in eastern Africa, *An. vaneedeni* in the northern areas of South Africa, *An. aruni* in Zanzibar, *An. fuscivenosus* in Zimbabwe, and *An. brucei* in Nigeria.^{1,2}

The difficulty in differentiating these species morphologically has led to the search for a simple and rapid method for identifying members of the *An. funestus* group. Cytogenetic studies distinguished six species of the group,^{5,6} but this technique is highly sex- and stage-specific and only half-gravid females or fourth instar larvae can be identified. Molecular methods have been reported, but these too have had limitations. The polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) assay was able to identify four members of the *An. funestus* group.⁷ A PCR-based test was then developed to distinguish *An. funestus* from *An. rivulorum*.⁸ Recently, Koekemoer and others⁹ have developed a

species-specific PCR assay able to rapidly identify five of the most commonly found members of the *An. funestus* group: *An. funestus*, *An. rivulorum*, *An. lesoni*, *An. parensis*, and *An. vaneedeni*.

This diagnostic PCR is based on interspecies variations in the internal transcribed spacer 2 (ITS2) region of the ribosomal DNA (rDNA). However, ITS2 sequences used to develop the species-specific primers were obtained from field mosquitoes collected only in southern and eastern Africa. For species whose distribution extends far beyond the sampling zone, such as *An. funestus*, *An. rivulorum*, and *An. lesoni*, validation of the assay is highly recommended using specimens from other African areas.

Anopheles funestus specimens collected in Burkina Faso, Senegal, Kenya,¹⁰ and Madagascar⁹ exhibited highly conserved ITS2 sequences and were thus correctly identified using the PCR protocol reported by Koekemoer and others.⁹ Conversely, Hackett and others⁸ provided evidence for high amounts of sequence divergence between *An. rivulorum* specimens from western and southeastern Africa, suggesting cryptic speciation. Furthermore, to date, only *An. lesoni* specimens from southern Africa have been investigated, although this species is widespread throughout the continent.

In this paper, we present data on the *An. funestus* group from Cameroon in central Africa, in particular on species identified as *An. rivulorum* and *An. lesoni* that are commonly found in sympatry with *An. funestus* in certain parts of this country.

MATERIALS AND METHODS

Mosquito sampling and morphologic identification. Collections were made in northern Cameroon in December 2001 and January 2002, and in South Africa between 1997 and 2002. Anopheline larvae were collected in the tributaries of the Benoue River where Mouchet and Gariou¹¹ reported the presence of *An. rivulorum* and *An. lesoni*.

Morphologic identification was conducted on live specimens. Larvae identified morphologically as *An. lesoni* were preserved in 70% ethanol. A sample of the larvae identified

as *An. rivulorum* were reared to adulthood in our insectarium. Emerging adults were preserved and mounted on insect pins. Before mounting, 2–3 legs of these adults were removed, preserved in 70% ethanol, and stored at -20°C .

Adult mosquitoes were collected by indoor-spraying with insecticides in houses close to the rivers where larval collections were undertaken. Members of the *An. funestus* group were isolated from other anophelines according to the morphologic identification keys of Gillies and De Meillon and Gillies and Coetzee^{1,2} and preserved in dry form in tubes with desiccant and stored at -20°C . Control specimens of *An. rivulorum*, *An. vaneedeni*, *An. parensis*, and *An. lesoni* were collected in South Africa. They were identified morphologically and by SSCP.⁷

Extraction of DNA, PCR amplification, and sequencing of DNA. Genomic DNA, extracted from the last abdominal segment of larvae or from 1–2 adult legs following a slightly modified version of the protocol of Cornel and others,¹² was resuspended in sterile water. The ITS2 and D3 regions of the rDNA were amplified from approximately 20 ng of template DNA in 50- μL reaction mixture containing 5 μL of 10 \times reaction buffer (Qiagen, Courtaboeuf, France), 1.5 mM MgCl_2 , 200 μM each deoxynucleotide triphosphate (Eurogentec, Herstal, Belgium), 0.5 units of *Taq* DNA polymerase (Qiagen), and 20 pmol each of forward and reverse primers.

Consensus ITS2a and ITS2b primers¹³ were used to amplify the ITS2 region. Primers D3a and D3b^{7,14} were used to amplify the variable D3 domain of the 28S rDNA subunit. The sequences of these primers are ITS2a: 5'-TGTGAACTG-CAGGACACAT-3' (forward), ITS2b: 5'-TATGCT-TAAATTCAGGGGGT3' (reverse), D3a: 5'-GACCCGTCTTGAAACACGGA-3' (forward), and D3b: 5'-TCGGAAGGAACCAGCTACTA-3' (reverse).

The PCR conditions included an initial denaturation step at 94°C for three minutes, followed by 36 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension step at 72°C for 10 minutes. After amplification, 5 μL of the PCR products were analyzed by electrophoresis on 1.5% agarose gels containing 0.5 $\mu\text{g}/\text{mL}$ of ethidium bromide. Bands were revealed and photographed under ultraviolet light. After electrophoresis, the remaining PCR products were used for sequencing in both directions with the previous forward and reverse primers. Using CAPTM (InfobioGen, Evry, France), we compared the sequences of complementary strands for each sample. Multiple alignment was performed using CLUSTLAWTM and EDTLANTM (available at <http://infobiogen.fr>). Genetic distances between haplotypes¹⁵ were computed using Molecular Evolutionary Genetics Analysis 2 (MEGA2)¹⁶ software under the pairwise deletion option and using the Kimura 2-parameters¹⁷ correction to take into account multiple hits.

RESULTS

Characterization of field specimens collected in Cameroon. Five larvae were identified morphologically as *An. lesoni*. They were collected in December 2001 in three different rivers that cross the track passing through Poli at the level of the Wante, Gombo, and Dakidongo villages ($8^{\circ}28'\text{N}$, $13^{\circ}18'\text{E}$; $8^{\circ}30'\text{N}$, $13^{\circ}70'\text{E}$; and $8^{\circ}30'\text{N}$, $13^{\circ}60'\text{E}$, respectively). These brooks are non-permanent and were flowing gently at

the time of collection, at the beginning of the dry season. The larvae were found at the edges of the brooks, in dense standing vegetation. Some larvae of *An. funestus* were also found in these breeding sites. Their identification was first based on morphology, then confirmed by the species-specific PCR developed by Koekemoer and others,⁹ thus providing evidence for accuracy of this PCR assay in identifying *An. funestus* and *An. lesoni* from Cameroon.

According to Gillies and De Meillon,² *An. rivulorum* is morphologically very close to *An. brucei* at the adult stage and both species are identical at the larval stage. Approximately 30 larvae were identified morphologically as *An. rivulorum-brucei*. They were found in December 2001 and January 2002 in the Boki River, at its point of crossing with the road leading from Ngaoundere to Garoua ($8^{\circ}42'\text{N}$, $13^{\circ}33'\text{E}$). These larvae were breeding at the edge of the river where the water was flowing very gently in short but dense emerging vegetation. *Anopheles funestus* larvae were also found at the same place. Eight larvae were preserved in alcohol while others were reared to adulthood. Among them, 11 reached the adult stage including seven females and four males. The main morphologic features of these specimens were compared with reference characteristics for *An. rivulorum* and *An. brucei*. All specimens fitted the morphologic description of *An. rivulorum* given by Gillies and De Meillon,² except for the coloration of thoracic integument that was gray instead of orange-brown. Two morphologic variants were observed, similar to *An. rivulorum* with one or two pale spots on the upper branch of the fifth vein of wings. Thus, these specimens were distinct from *An. brucei*,¹⁸ and had one morphologic feature different from *An. rivulorum*.

When subjected to the PCR test of Koekemoer and others,⁹ all of these specimens showed weak and non-reproducible signals of the expected size, suggesting possible mutations in the region of primer annealing.

Analysis of the ITS2 sequences. Sequencing of the ITS2 region was carried out in 15 *An. rivulorum* specimens (5 larvae and 10 adults) and two *An. lesoni* larvae from Cameroon. Boundaries of the ITS2 region were identified through sequence comparison with previously determined anopheline 5.8S and 28S rDNA.^{12,19–23}

The ITS2 sequences were aligned with other sequences available on GeneBank: specimens morphologically identified as *An. rivulorum* from Burkina Faso, *An. rivulorum* from Kenya and South Africa (AF210725, AF180524, and AF2107724),⁸ *An. funestus* (AF062512),¹⁰ *An. lesoni* from South Africa (AY035719),⁹ *An. parensis* (AY035720),⁹ *An. vaneedeni* (AY035718),⁹ *An. minimus* A (AF230461),²⁴ and *An. moucheti* (AJ430581) (Kengne P, unpublished data).

No polymorphism was detected between both *An. lesoni* specimens from Cameroon (Figure 1). Only one single nucleotide substitution was detected by comparison with the consensus sequence for *An. lesoni* from South Africa (AY035719).⁹ This is a G to T transition in position 175, therefore located outside of the DNA region complementary to the *An. lesoni*-specific primer (Figure 1). Therefore, this point mutation did not prevent correct molecular identification of *An. lesoni* from Cameroon.

Alignment of ITS2 sequences of *An. rivulorum* specimens is shown in Figure 2. Nucleotide diversity was low ($\pi = 0.003$, $\text{SE} = 0.002$) among specimens from Cameroon ($n = 15$). The consensus sequence obtained from this alignment perfectly

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      10      20      30      40      50
1 CAATTCCTTGTACACAATAATCTAACTACATGGCGCCCGTGTACAGACG
2 .....
3 .....
      60      70      80      90     100
1 GCATCATGGCGAGCAGCCCCGCTCTGATGTTGCTGAATGAACACGTGAGC
2 .....
3 .....

      110     120     130     140     150
1 GCACTGTGCATAATAGCGTGCAAGGCTCGTCTCCTACCGGGACCTTGGGC
2 .....
3 .....

      160     170     180     190     200
1 GCTGAAAGGTAAGGCAGTACAGTGGCACTGTACTTAGCGGGGTGCAGCG
2 .....
3 .....

      210     220     230     240     250
1 TCAAGTCGCACGGGACGAACCTTCGGCTATGGACGACCTGAGATACCCGGC
2 .....
3 .....

      260     270     280     290     300
1 AGCCTACTAACCCAGGCTAGTCGATCCGGTTCAGGGGTTACGAATCAT
2 .....
3 .....

      310     320     330     340     350
1 CCGCGCAGTCTGTAAACGCGTGACGACCCATACGGTGGGCCCTTGTGTC
2 .....
3 .....

      360
1 ATGAAATCCTACCTAA
2 .....
3 .....

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FIGURE 1. Alignment of the internal transcribed spacer 2 (ITS2) sequences of *Anopheles lesoni*. 1 = consensus sequence for South African *An. lesoni*;⁹ 2 and 3 = *An. lesoni* larvae from Cameroon. The **arrow** shows the position of the hybridization zone of the primer LEE (i.e., between positions 25 and 44). The size of the ITS2 region was 367 basepairs.

matched the one obtained by Hackett and others⁸ with specimens from Burkina Faso.

Genetic distance between *An. rivulorum* specimens from western and central Africa (Burkina Faso–Cameroon: $d = 0$) on the one hand and the specimens from southern and eastern Africa (South Africa–Kenya: $d = 0.008$, $SE = 0.008$) on the other hand showed divergence rates were at a common level of intraspecific variability as observed in other anophelines.^{21,25} However, the average distance between consensus sequences from western–central and southern–eastern groups was 0.145 ($SE = 0.039$), a value in the range of interspecies divergence in the *An. funestus* group (Table 1) and other anophelines.^{9,12,25,26} Such level of divergence could not be attributed to geographic distance alone. Indeed, estimates of genetic distances were at least 18 times higher between Kenya and Cameroon than between Kenya and South Africa, despite comparable geographic distances between them ($\approx 3,000$ km). Therefore, these results provide support for the hypothesis of cryptic speciation within *An. rivulorum*.⁸

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      10      20      30      40      50
1-12 AAATCCTTGTACACAAACACCTGACTACAGTGGTGCACCGTGCAGC-G 49
13 .....C. 50
14 ..... 49
15 .....C. 50
16 ..... 49
17 .....TTG. 49
18 .....TTG. 49

      60      70      80      90     100
1-12 AACTAAGCACTATGGCGAGACCCACGTCTAGTGTGCTGAACACACGGC 99
13 ..... 100
14 ..... 99
15 ..... 100
16 ..... 99
17 .CGAG..TG.....CT....C.A..... 99
18 .CGAG..TG.....CT.T...C.A..... 99

      110     120     130     140     150
1-12 CCTGCCCACTGTGCATAAATGGCGTCTCGGGAAGTAAAATCTCGTGGC 149
13 ..... 150
14 ..... 149
15 ..... 150
16 ..... 149
17 ...T.....A.....CG--..G... 147
18 .....A.....CG--..G... 147

      160     170     180     190     200
1-12 GCTGAAGAGCGATGAGAGCATGGGGCGGTACTCTGTTGCTGCCGGATCC 199
13 ..... 200
14 ..... 199
15 ..... 200
16 ..... 199
17 .T...C.A...ACGA.AT.....T.....A...A--- 194
18 .T...TTA...GCGAGAT--.....T.....A...A--- 192

      210     220     230     240     250
1-12 CCCACTCCACGGGAGGCGGATGGTGCCTGTCTAGTTGGCTGTTGCGAACT 249
13 ..... 250
14 ..... 249
15 ..... 250
16 ..... 249
17 --.C.....T.AT.-...C.....AC.C..... 241
18 .C.....T.AT.-...C.....AC.C..... 239

      260     270     280     290     300
1-12 CTGGCAGGACGTCTGACAGCCCCGACAGCCCTACAGCAGGATGTCGGC 299
13 ..... 300
14 ..... 299
15 ..... 300
16 ..... 299
17 TC...A.....G...A.....C.....G 291
18 TC...A.....G...A.....C.....G 289

      310     320     330     340     350
1-12 CTAAGTAAATCAGGGTCAAACGGCCCGCAGGTGCGGTAATTCCTGATGCT 349
13 ..... 350
14 ..... 349
15 ..... 350
16 ..... 349
17 ..G.....TT..G.....G.G..C... 340
18 ..G.....TT..G.....G.G..C... 338

      360     370     380     390
1-12 -TAGACGTGCCACTTCGTCGCCATCGCACTTAACCC----- 384
13 ..... 385
14 -.A..... 384
15 -.T.....T..... 385
16 ..... 384
17 G.GCT.....A...-CGAT.GGC...ACATAT 378
18 G.GCT.....A...-CGAT.GGC...ACCT-- 374

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FIGURE 2. Alignment of internal transcribed spacer (ITS2) sequences of *Anopheles rivulorum*. 1–15 = *An. rivulorum* from Cameroon; 16 = consensus sequence of *An. rivulorum* from Burkina Faso;⁸ 17 = consensus sequence of *An. rivulorum* from Kenya;⁸ 18 = consensus sequence of *An. rivulorum* from South Africa.⁸ The **arrows** show the regions of annealing of primers RIV and RIVLIKE. The size of the ITS2 region was 384 basepairs for *An. rivulorum* from Cameroon and Burkina Faso and 375 basepairs for *An. rivulorum* from South Africa and Kenya.

TABLE 1

Pairwise nucleotide distances between species in the D3 (below the diagonal) and the internal transcribed spacer 2 (above the diagonal) regions in *Anopheles* mosquitoes*

	<i>An. rivulorum</i> -like	<i>An. rivulorum</i>	<i>An. lesoni</i>	<i>An. parensis</i>	<i>An. vaneedeni</i>	<i>An. funestus</i>	<i>An. minimus</i>	<i>An. moucheti</i>
<i>An. rivulorum</i> -like	–	0.145 (0.039)	0.430 (0.084)	0.628 (0.110)	0.564 (0.100)	0.492 (0.089)	0.421 (0.081)	0.886 (0.206)
<i>An. rivulorum</i>	0.096 (0.033)	–	0.500 (0.102)	0.529 (0.097)	0.474 (0.092)	0.391 (0.076)	0.431 (0.084)	0.903 (0.219)
<i>An. lesoni</i>	0.087 (0.034)	0.074 (0.030)	–	0.681 (0.130)	0.578 (0.102)	0.554 (0.101)	0.084 (0.027)	0.821 (0.162)
<i>An. parensis</i>	0.126 (0.041)	0.140 (0.043)	0.117 (0.041)	–	0.388 (0.068)	0.385 (0.070)	0.702 (0.127)	1.460 (0.592)
<i>An. vaneedeni</i>	0.128 (0.043)	0.142 (0.044)	0.118 (0.041)	0.011 (0.012)	–	0.123 (0.026)	0.582 (0.100)	1.515 (0.673)
<i>An. funestus</i>	0.157 (0.048)	0.157 (0.047)	0.147 (0.047)	0.036 (0.020)	0.048 (0.024)	–	0.559 (0.100)	1.313 (0.491)
<i>An. minimus</i>	0.114 (0.039)	0.074 (0.031)	0.024 (0.016)	0.117 (0.041)	0.118 (0.041)	0.147 (0.047)	–	0.832 (0.157)
<i>An. moucheti</i>	0.084 (0.032)	0.059 (0.027)	0.061 (0.027)	0.144 (0.046)	0.145 (0.046)	0.131 (0.044)	0.061 (0.028)	–

* Values in parentheses are standard errors estimated by bootstrap analysis.

For this reason, we propose to provisionally refer to the taxon from western–central Africa as “*An. rivulorum*-like.”

Analysis of the D3 region. The rDNA 28S D3 domain was sequenced in three *An. rivulorum*-like and three *An. lesoni* specimens from Cameroon, two *An. rivulorum*, two *An. lesoni*, and one *An. parensis* specimens from South Africa. Original sequences of the D3 region have been deposited in the GenBank database under the following accession numbers: AY257553 for *An. lesoni*, AY256345 for *An. rivulorum*, AY256346 *An. rivulorum*-like, and AY256347 for *An. parensis*. The D3 sequences available on GenBank for *An. vaneedeni* (AF007095), *An. funestus* (AF007094),²⁷ *An. minimus* A (AF416782), and *An. moucheti* (Kengne P, unpublished data) were included in the analysis. Nucleotide variability within the species *An. lesoni*, including specimens from South Africa and Cameroon, is nil.

Sequences in the taxa *An. rivulorum* and *An. rivulorum*-like showed very weak intragroup variability ($\pi = 0$ and $\pi = 0.011$, SE = 0.011, respectively). Genetic distances between recognized species ranged from 0.011, SE = 0.011 between *An. vaneedeni* and *An. parensis* to 0.157, SE = 0.047 between *An. rivulorum* and *An. funestus* (Table 1). The genetic distance separating *An. rivulorum* and *An. rivulorum*-like was 0.096, SE = 0.033, therefore strengthening the ITS2 findings.

Improving the PCR-based diagnostic assay. The set of species-specific primers defined by Koekemoer and others⁹ was supplemented with an additional primer allowing specific identification of the new taxon we described earlier in this report. This new primer was named RIVLIKE (Figure 2). Its sequence was 5'-CCG CCT CCC GTG GAG TGG GGG-3' with a melting temperature (T_m) of 60.7°C. It anneals in a region of the ITS2 sequence where 8 of 21 nucleotides allow distinction between both *An. rivulorum*, thus ensuring its specificity. The amplified PCR fragment characteristic of *An. rivulorum*-like is 313 basepairs long, allowing easy identification on regular agarose gels (Figure 3). The primers and the sizes of the diagnostic PCR products are summarized in Table 2.

With this additional primer, the PCR conditions were slightly modified compared with those recommended by Koekemoer and others⁹: final reaction volume was 25 μ L containing 2.5 μ L of 10 \times buffer including 15 mM MgCl₂, 5 pmol of each primer, 200 μ M of each dNTP, and 0.5 units of *Taq* polymerase unit. Amplification started with an initial denaturation step at 94°C for two minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, and elongation at 72°C for 40 seconds, with a final extension step at 72°C for five minutes. The PCR

products were loaded onto regular 2.5% agarose gels, electrophoresis was done at 120 V/250 mA for one hour, and diagnostic bands were visualized after staining with ethidium bromide.

The RIV primer, specific for *An. rivulorum* from South Africa and Kenya, anneals in a region where three mismatches hamper reliable amplification of the DNA from *An. rivulorum*-like specimens from Cameroon and Burkina Faso (Figure 2). Our updated PCR protocol showed that specimens of *An. rivulorum*-like from Cameroon consistently had a single band at 313 basepairs (Figure 3), while control *An. rivulorum* specimens from South Africa had the expected band at 411 basepairs. Thus, the PCR protocol described in this paper allows clear distinction between *An. rivulorum* from southeastern Africa and *An. rivulorum*-like from west-central Africa. Reliable and reproducible identification of *An. lesoni*, *An. parensis*, *An. vaneedeni*, and *An. funestus* specimens was also achieved, demonstrating that addition of the RIVLIKE primer and other modification we added to the protocol did not alter the sensitivity nor specificity of the assay.

Hybrid specimens were mimicked by mixing equal amounts of DNA from all possible combinations prior to amplification. Two bands of the expected sizes were obtained in all cases (Figure 4).

Implementation of the assay. A total of 45 adult females of the *An. funestus* group collected resting indoors in the vicinity of the prospected breeding sites were identified using the species-specific PCR described in this paper: 42 belonged to the species *An. funestus* and 3 were *An. lesoni*. The preva-

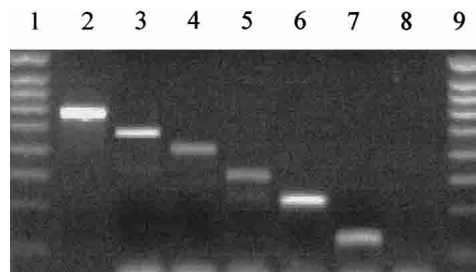


FIGURE 3. Amplified fragments using the species-specific polymerase chain reaction for the identification of members of the *Anopheles funestus* group. Lanes 1 and 9, 100-basepair DNA size marker ladder; lane 2, *An. vaneedeni*; lane 3, *An. funestus*; lane 4, *An. rivulorum*; lane 5, *An. rivulorum*-like; lane 6, *An. parensis*; lane 7, *An. lesoni*; lane 8, negative control. The sizes of the fragments of the ladder are 1,000, 800, 700, 600, 500, 400, 300, 200, and 100 basepairs.

TABLE 2

Polymerase chain reaction (PCR) primers and sizes of the amplified products for species diagnostic within the *Anopheles funestus* group*

Primers	Sequences (5' → 3')	Tm (°C)	Identified species	Size of the PCR product (bp)
UV	TGT GAA CTG CAG GAC ACA T	55.3	—	—
FUN	GCA TCG ATG GGT TAA TCA TG	52.4	<i>An. funestus</i>	505
VAN	TGT CGA CTT GGT AGC CGA AC	58.0	<i>An. vaneedeni</i>	587
RIV	CAA GCC GTT CGA CCC TGA TT	58.8	<i>An. rivulorum</i>	411
PAR	TGC GGT CCC AAG CTA GGT TC	60.5	<i>An. parensis</i>	252
LEES	TAC ACG GGC GCC ATG TAG TT	60.2	<i>An. lesoni</i>	146
RIVLIKE	CCG CCT CCC GTG GAG TGG GGG	60.7	<i>An. rivulorum-like</i>	313

* Tm = melting temperature; bp = basepairs.

lence of *An. funestus* in this sample is consistent with its well-known endophilic behavior. The presence of several *An. lesoni* females shows that this species, which is primarily exophilic, could also rest inside human dwellings. This had been reported earlier in a province of South Africa by Hargreaves and others.²⁸ The ITS2 region of the three adult *An. lesoni* were sequenced and were perfectly identical to those of the larvae. During this study, no adult *An. rivulorum*-like or *An. rivulorum* was collected inside houses.

DISCUSSION

In the present paper, based on morphologic and molecular data, we highlight the existence of a new taxon within the *An. funestus* group. Careful screening of sequence variation in specimens collected in Cameroon confirmed earlier findings of genetic heterogeneity within *An. rivulorum*.⁸ We thus complemented the assay with an additional primer, RIVLIKE, that allows one to distinguish the type species *An. rivulorum* originally described from Zimbabwe and Kenya^{29–31} from the west/central African taxonomic unit we refer to as “*An. rivulorum*-like.” Slight modifications of the protocol did not alter the sensitivity or specificity of the assay in identifying other members of the group.

The possibility that the cryptic taxon corresponded to *An. brucei* was discarded by morphologic observations that clearly showed discriminating criteria. *Anopheles brucei* was described only from its type locality (Lokoja in northern Nigeria) from a very limited number of specimens (six adults and

three 3 larvae),¹⁸ which are kept at the British Natural History Museum in London. Nothing is known to date about its geographic distribution and we do not envision processing archived specimens for genetic analysis. Additional collections from the type locality of *An. brucei* are needed to determine the taxonomic status of this species and assess in more detail its relationship to *An. rivulorum*-like.

Koekemer and others⁹ developed their assay from a limited number of specimens originating from a very restricted part of the species ranges. It was therefore necessary to assess accuracy of the primers for reliable amplification of the DNA of specimens from other areas in Africa. *Anopheles funestus* and *An. lesoni* specimens collected in Cameroon were correctly identified at different life stages, suggesting that the assay can probably be generalized to the whole geographic range of both species.

Anopheles lesoni has been classified in the *An. funestus* group based on morphologic and geographic criteria,² but some observations of its polytene chromosomes led Green⁵ to group *An. lesoni* with the Asian *An. fluviatilis/culicifacies* species (*An. minimus* was not included in this study). Our results are consistent with these findings and it is noteworthy that estimates of genetic distances between *An. lesoni* and *An. minimus* are significantly lower than those between *An. lesoni* and any other species of the *An. funestus* group (Table 1). To date, the classification of *An. lesoni* is still a moot point and further studies are needed to settle relationships between the African *An. funestus* and the Asian *An. minimus* groups.

All D3 and ITS2 sequences gathered so far for *An. lesoni* are roughly identical, both at a locale geographic scale (e.g., larvae from a single breeding site) or when populations are sampled across wide distances (i.e., between Cameroon and South Africa). Similar patterns were found within *An. funestus*, and were thoroughly discussed by Mukabayire and others.¹⁰ Clearly, genetic homogeneity in the ITS2 region seems to be the rule within species of the *An. funestus* group, providing support for 1) sensitivity and specificity of a diagnostic PCR assay based on such markers and 2) reliable use of this molecular tool throughout the species range. Furthermore, this gives weight to the statement that *An. rivulorum* is actually a mixture of at least two different taxonomic units.

Studies of the biology, ecology, and eventually epidemiologic importance as a vector of human malaria of the various members of the *An. funestus* group will be facilitated by their straightforward identification at all stages of development. Reliable species identification is indeed important to determine areas of sympatry and assess the relative role played by each species in the transmission of *Plasmodium*. Further-

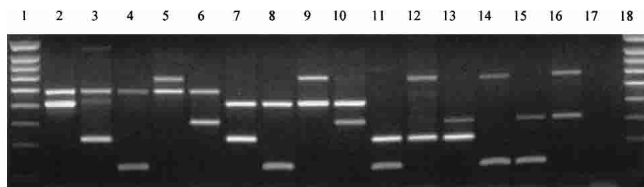


FIGURE 4. Amplification of hybrid DNA between members of the *Anopheles funestus* group. Lanes 1 and 18, 100-basepair DNA size marker; lane 2, *An. funestus* and *An. rivulorum*; lane 3, *An. funestus* and *An. parensis*; lane 4, *An. funestus* and *An. lesoni*; lane 5, *An. funestus* and *An. vaneedeni*; lane 6, *An. funestus* and *An. rivulorum*-like; lane 7, *An. rivulorum* and *An. parensis*; lane 8, *An. rivulorum* and *An. lesoni*; lane 9, *An. rivulorum* and *An. vaneedeni*; lane 10, *An. rivulorum* and *An. rivulorum*-like; lane 11, *An. parensis* and *An. lesoni*; lane 12, *An. parensis* and *An. vaneedeni*; lane 13, *An. parensis* and *An. rivulorum*-like; lane 14, *An. lesoni* and *An. vaneedeni*; lane 15, *An. lesoni* and *An. rivulorum*-like; lane 16, *An. vaneedeni* and *An. rivulorum*-like; lane 17, negative control. The sizes of the fragments of the ladder are 1,000, 800, 700, 600, 500, 400, 300, 200, and 100 basepairs.

more, availability of such diagnostic tools will improve our ability to evaluate efficacy of vector control measures implemented in areas where several species of the *An. funestus* group are present, a situation that could mislead evaluation of control programs.⁴

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