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A New Species Concealed by *Anopheles funestus* Giles, a Major Malaria Vector in Africa

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Abstract. The major malaria vector *Anopheles funestus* belongs to a group of morphologically similar species that are commonly distinguished from one another through the use of chromosomal and molecular techniques. Indoor resting collections of mosquitoes from Malawi were initially identified as *An. funestus* by morphology, but failed to have this confirmed by the species-specific polymerase chain reaction assay. Sequence analysis of the internal transcribed spacer region 2 identified variations within the *An. funestus*-specific primer binding site and showed a sequence variation of 4.5% compared with *An. funestus*. Domain 3 analysis showed sequence variation of 1.5% from *An. funestus*. Cytogenetic analysis of the polytene chromosome banding arrangements showed that the specimens were homosequential with *An. funestus*, with fixed inverted arrangements of the 3a, 3b, and 5a inversions commonly polymorphic in *An. funestus*. The chromosomes of hybrid females showed levels of asynapsis typical of inter-species crosses. These molecular and cytogenetic observations support the conclusion that this Malawi population is a new species and it has provisionally been named *An. funestus*-like.

INTRODUCTION

The *Anopheles funestus* Giles group consists of nine African species,[1–3] of which five belong to the Funestus subgroup on the basis of phylogenetic analyses: *An. funestus*, *An. vaneedeni* Gillies and Coetzee, *An. parensis* Gillies, *An. aruni* Sobti, and *An. confusus* Evans and Leeson.[3,4] The remaining members of the group belong to the Rivulorum subgroup: *An. brucei* Service, *An. fuscivenosus* Leeson, *An. rivulorum* Leeson, and the *An. rivulorum*-like species from west Africa.[3,5–7] All the species are morphologically similar at the adult stage and species identification at the egg and larval stages is possible only for *An. confusus*.

Although the members of the *An. funestus* group may be similar in morphology, their efficiencies as malaria vectors vary greatly. Because of its highly anthropophilic and endophilic nature, *An. funestus* s.s. is one of the primary vectors of malaria in sub-Saharan Africa.[1] *Anopheles rivulorum* has only once been implicated in malaria transmission in Tanzania[8] but generally elects to blood feed on domestic animals rather than humans. The remaining members of the *An. funestus* group have never been shown to be malaria vectors in nature,[1,2] although *An. vaneedeni* was infected experimentally in the laboratory.[9]

Because of the different vectorial capacities, biting and resting behaviors and the close morphologic similarity of members of the *An. funestus* group, accurate identification of field-caught material is critical for vector control programs. Early identification methods relied solely on morphologic characters that detailed minor differences between the members of the group.[1,2,10–12] This process of identification relies on the availability of multiple life stages and requires a high level of expertise because the characteristics used are frequently overlapping and only two of the species can be unequivocally identified using morphology.

The success of cytogenetics to elucidate the members of the *An. gambiae* complex led to similar studies on the *An. funestus* group. Analysis of the banding arrangements of giant polytene chromosomes distinguished *An. parensis* from *An. funestus*.[13] However, *An. vaneedeni* (formerly *An. aruni*?) had homosequential chromosomal banding patterns with *An. funestus*.[13] Cross-mating studies between *An. vaneedeni* and *An. funestus* produced sterile male hybrids and asynapsis of the hybrid polytene chromosomes,[13] thereby confirming the specific status of *An. vaneedeni*. More recent cytogenetic studies in west Africa have shown clear evidence of genetic differentiation in sympatric populations of *An. funestus*, indicating that this taxon may consist of a complex of cryptic species.[14,15] The need for simpler identification methods resulted in the development of a multiplex polymerase chain reaction (PCR) assay[16] targeting the variable internal transcribed spacer region 2 (ITS2) regions for the identification of the four most common species of the group: *An. funestus*, *An. vaneedeni*, *An. parensis*, and *An. rivulorum*.

In this paper, we report evidence of a new species of *Anopheles* mosquito from Malawi, which occurs in sympatry with *An. funestus* and shows morphologic overlap with this species and cannot be identified using the standard multiplex PCR assay.

MATERIALS AND METHODS

Study site and collection method. Anopheline collections were carried out in December 2007 in rural villages around Karonga in northern Malawi (10°18.627′S, 34°07.901′E), close to the shoreline of Lake Malawi. The collections were predominantly indoor resting catches with the exception of samples that were collected from tyres stacked at the entrance

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to a reed hut. The adult specimens were identified as members of the *An. funestus* group, according to morphologic keys.[1,2] Male specimens were dry-preserved on silica and the females were transported alive to the insectaries of the Vector Control Reference Unit, National Institute for Communicable Diseases (Johannesburg, South Africa).

Laboratory rearing of wild-caught material. A total of 63 *An. funestus* females were placed in glass vials for egg laying. They were maintained on 10% sucrose and offered a blood meal every alternate day. After egg laying, females that survived were offered a blood meal and kept until half gravid for chromosomal studies. The morphology of the egg batches confirmed that the samples belonged to the *An. funestus* subgroup.[1] Each egg batch was treated as an individual family and the resulting F_1 progeny were reared to adulthood and used for molecular identifications and cross-mating experiments.

Molecular identification of field material. DNA was extracted[17] from an individual adult from each family. The multiplex PCR for the identification of *An. funestus*, *An. rivulorum*, *An. vaneedeni*, *An. parensis*, and *An. leesoni*[16] was performed with all DNA samples. Each sample was tested 2–3 times to ensure accuracy of identification. A negative control containing no DNA and positive controls of *An. funestus* from laboratory colony material and *An. leesoni* from a previous wild collection were included in each PCR.

Sequencing and primer design for unidentified Malawi samples. A segment of the ITS2 region of four unidentified Malawian (MalaF) samples and an *An. funestus* s.s. control were amplified using primers ITS2A (5′-TGT GAA CTG CAG GAC ACA T- 3′) and ITS2B (5′-TAT GCT TAA ATT CAG GGG GT-3′).[16] The 25-µL PCR mixture contained 50 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1.25 units of *Taq* DNA polymerase, and 1 µL of DNA.[5] The cycling conditions were initial denaturation at 94°C for 2 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 40 seconds; and a final extension at 72°C for 10 minutes. The resulting amplicons were subjected to electrophoresis on a 1.8% low-melting temperature Tris-acetate-EDTA (TAE) agarose gel stained with ethidium bromide. Amplicons (approximately 850 basepairs) were excised and cleaned using the Qiaquick Gel Extraction kit (catalog no. 28704; Qiagen, Valencia, CA) and sequenced with the ITS2A and ITS2B primers. Direct sequencing of the PCR products was performed by Inqaba Biotechnical Industries (Pretoria, South Africa). Sequencing was carried out for both strands using the above primers. Sequence alignment and analysis was carried out using (Lasergene version 6; DNAStar; Madison, WI). A consensus sequence was created for the four MalaF samples and aligned to the *An. funestus* s.s. control sequence. Primer annealing sites specific to the MalaF samples were identified and new primers were designed to yield an amplicon with a different size to the amplicon yielded for *An. funestus* s.s. in the species-specific PCR.[16]

Domain 3 (D3) of the 28s rDNA gene was amplified for the same MalaF samples and *An. funestus* s.s. control used for the ITS2 sequencing. The primers D3A (5′-GAC CCG TCT TGA AAC ACG GA-3′) and D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) were used for amplification.[18] Each reaction was carried out in a volume of 25 µL that contained 25 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 2 units of *Taq* DNA polymerase, and 1 µL of DNA. The cycling conditions were initial denaturation at 94°C for 3 minutes; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 40 seconds, extension at 72°C for 40 seconds; and a final extension at 72°C for 10 minutes. The resulting amplicons were subjected to electrophoresis and sequenced as for the ITS2 amplicons. Sequencing was carried out for both strands using the D3A and D3B primers. Sequence alignment and analysis was carried out using Lasergene version 6. A consensus sequence was created for the four MalaF samples and this was then aligned to the *An. funestus* s.s. control sequence.

Application of the MalaF-specific PCR primer. Two potential primers (MalaFA: 5′-CCT GCG TCC CAA GGT T-3′; MalaFB: 5′-GTT TTC AAT TGA ATT CAC CAT T-3′) were individually tested for their efficiency in the species-specific PCR[16] for the identification of *An. funestus* group members. Each of the newly designed primers was included in the reaction mixture and the products were subjected to electrophoresis on a 3% TBE agarose gel stained with ethidium bromide. All 61 of the unidentifiable *An. funestus*-like samples were tested with the MalaFB primer. Any samples that failed to amplify with the new MalaFB primer were confirmed for the presence of nucleic acids by using a nanodrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). To confirm the presence of DNA in these samples, they were subjected to a PCR assay designed to detect, but not distinguish, members of the Funestus subgroup. The primers used were: UF (5′-TGT GAA CTG CAG GAC ACA T-3′) and LRev (5′-CCA AGC ACG TTG ATC CAG TAT TAC-3′). Each 25-µL PCR reaction mixture contained 6.6 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 unit of *Taq* DNA polymerase, and 1 µL of DNA. The cycling conditions were initial denaturation at 94°C for 2 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, extension at 72°C for 30 seconds; and a final extension at 72°C for 10 minutes. The resulting amplicons were subjected to electrophoresis on a 2.5% TAE gel stained with ethidium bromide. The presence of a product (approximately 440 basepairs) confirmed the presence of DNA for these samples.

Cytogenetics. Half-gravid wild females and F_1 progeny from the MalaF × *An. funestus* s.s. (Fumoz) crosses were dissected and their ovaries prepared for cytogenetic analysis.[13,19] The banding patterns of the polytene chromosomes were compared by chromosomal analyses with other members of the *An. funestus* group.[7,13] Slides were viewed and photographed under phase contrast microscopy. The arm nomenclature follows that of Green,[7] Green and Hunt, [13] Lochouarn and others,[14] and Costantini and others.[15]

Cross-mating studies. Virgin F_1 females from the MalaF families were cross-mated with males from a laboratory colony of *An. funestus* s.s. (Fumoz). Their hybrid F_1 female progeny were given a blood meal and ovaries were dissected at the half gravid stage. Polytene chromosomes were prepared as above and checked for asynapsis.[20]

The testes of eight hybrid males from the cross-mating were dissected and viewed by phase contrast microscopy. The gross morphology of each pair of testes was examined, after which the testes were squashed to release the spermatozoa. The morphology of the spermatozoa was examined for signs of infertility. The reciprocal cross (MalaF males × Fumoz females) was also attempted.

Enzyme-linked immunosorbent assay for sporozoite detection. The heads and thoraces of the entire group (n = 63)

of wild-caught MalaF females were tested for the presence of *Plasmodium falciparum* circumsporozoite protein. The sandwich enzyme-linked immunosorbent assay (ELISA) technique[21] was used. The ELISA plates were read and analyzed using a Multiskan Ascent plate reader (Thermo Electron Corporation, Shanghai, China).

RESULTS

Species identification of field material. Of the 63 wild females that were brought back to the laboratory for egg laying, only two specimens could be positively identified using the multiplex PCR assay[16] and these were both *An. rivulorum*, confirmed by morphology. The remaining 61 samples repeatedly failed to amplify any PCR product using the species-specific primers.

Sequence analysis, primer design, and application. The ITS2 region of four of the unidentifiable MalaF samples (735 basepairs) and an *An. funestus* control (736 basepairs) were sequenced and aligned (Figure 1). The sequence data for the unidentifiable MalaF samples showed a three-basepair deletion and a T to C transition within the *An. funestus* specific primer binding site. The MalaF consensus sequence (GenBank accession no. FJ438963) showed a 4.5% (33 of 740 basepairs) difference in sequence to the *An. funestus* control, including insertions and deletions.

Primers were designed to anneal to the two most variable regions in the MalaF consensus sequence (Figure 1). Both of the MalaF specific primers were tested on MalaF samples prior to being combined into the species identification PCR cocktail.[16] The MalaFA primer was inconsistent and did not always result in an amplicon although the same template sample was used. The MalaFB primer consistently gave good amplicon yield on the same, as well as different, MalaF template samples. The MalaFB primer, when combined in the species cocktail PCR, resulted in good amplicon yield with a product of 390 basepairs (Figure 2). The MalaFB primer has been tested on the 61 *An. funestus*-like samples and amplicons were obtained for 54 (88.5%) of the samples. The remaining seven *An. funestus*–like samples failed to amplify. The presence of DNA in these samples was confirmed using a Funestus subgroup–specific PCR and spectrophotometry.

The D3 sequence data for the MalaF samples showed a five-basepair (GenBank accession no. FJ843022) change from the *An. funestus* control sequence (Figure 3). This change includes a two-basepair deletion, two base transversions and a single

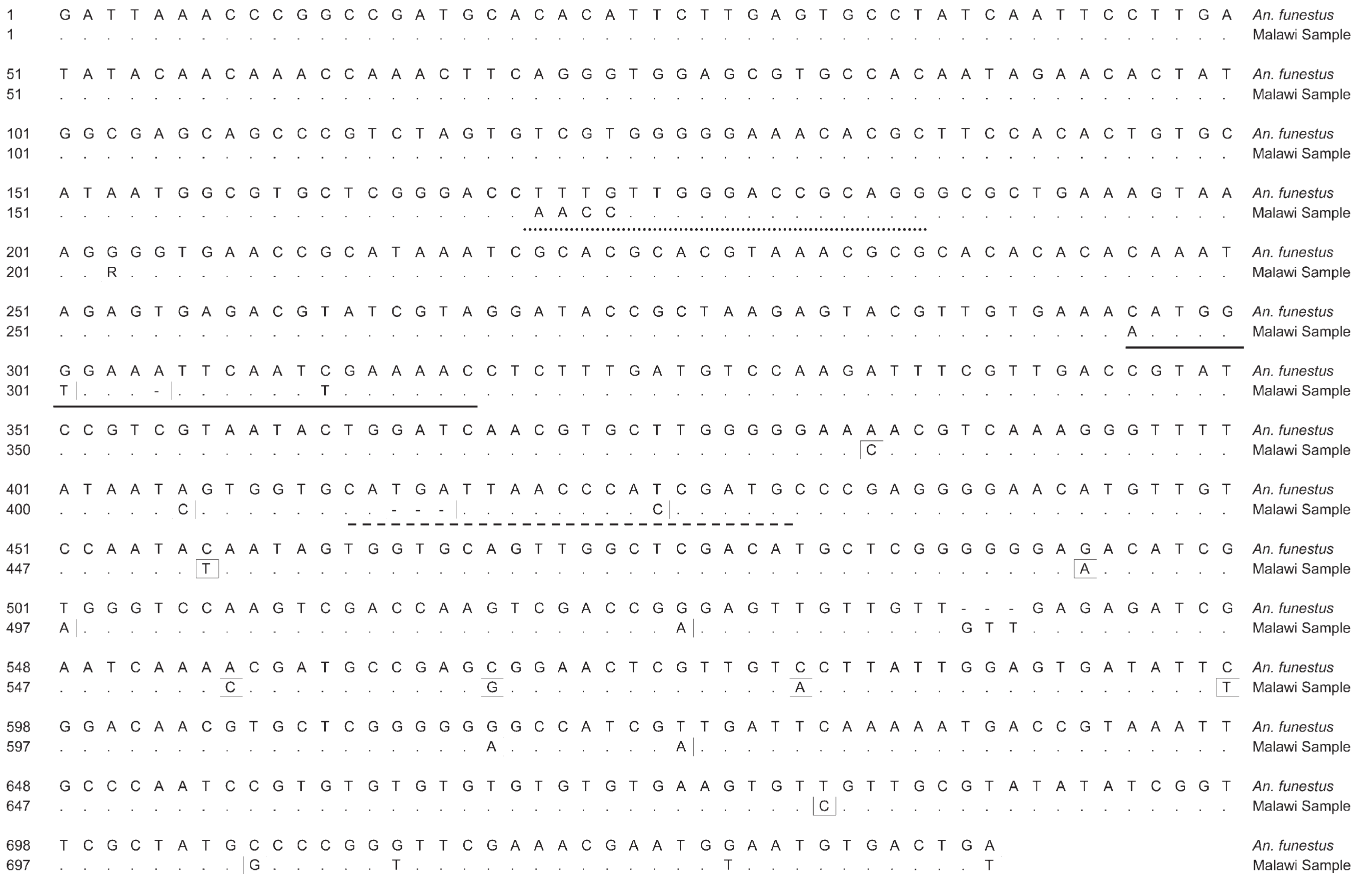

FIGURE 1. Sequence alignment of the internal transcribed spacer region 2 of the unidentifiable Malawian (MalaF) samples and *Anopheles funestus*. Blocks highlight sequence variation. – indicates deletions.

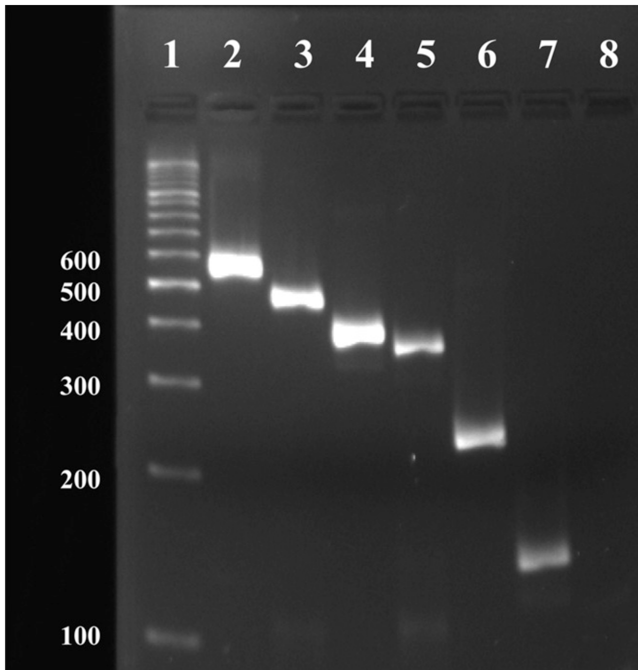

Figure 2. Primer MalaFB included in the polymerase chain reaction (PCR) cocktail mixture for *Anopheles funestus* group identifications. Lane 1, 100-basepair DNA ladder; lanes 2–4, positive controls: *An. vaneedeni*, *An. funestus*, and *An. rivulorum*, respectively; lane 5, MalaF sample; lanes 6 and 7, positive controls: *An. parensis* and *An. leesoni*, respectively; lane 8, PCR negative control.

base transition, which translates into 1.5% (5 of 330 basepairs) sequence variation from the *An. funestus* control.

Cytogenetics. Chromosomes from the wild MalaF females displayed homosequential banding arrangements with *An. funestus*. Autosomal inversions 3a, 3b, and 5a that are common as polymorphisms in *An. funestus* were each fixed in their inverted (theoretically derived) arrangements in the MalaF sample. A single, rare, polymorphic inversion was seen on autosome arm 2 from one female.

Cross-mating studies. Cross-mating between F_1 MalaF females and laboratory-reared *An. funestus* males was successful and resulted in hybrid progeny. The polytene chromosomes obtained from the hybrid females displayed asynapsis between homologous chromosomes (Figure 4). Examination of the testes of the hybrid males by microscopy showed fully developed testes that appeared normal in gross morphology. Although the head region of the spermatozoa appeared to be slightly narrower than expected, we could not carry out back-crosses to laboratory *An. funestus* to determine their viability and fertility because of insufficient material.

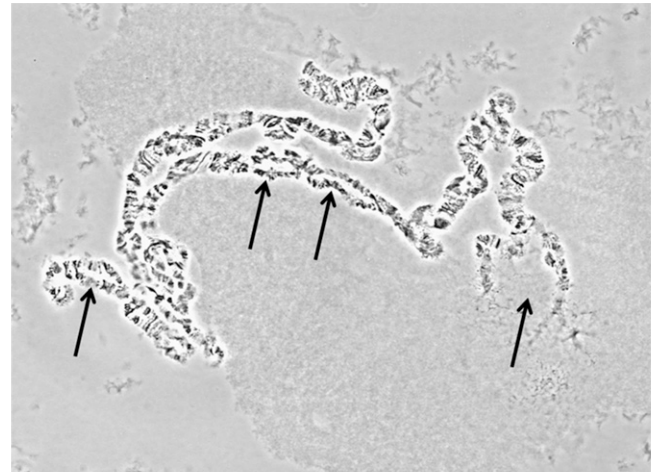

Figure 4. Asynapsis in hybrid polytene chromosomes indicated by arrows.

The reciprocal crosses of MalaF males to laboratory reared *An. funestus* females resulted in numerous egg batches (> 900 eggs). Only two of these eggs hatched, yielding a hatch rate of less than 0.2%. The adults that emerged were both females and died prematurely before taking a blood-meal. Thus, no further studies could be carried out.

ELISA for sporozoite detection. All of the wild-caught females that were brought back to the laboratory for rearing were tested for the presence of *P. falciparum* and were negative.

DISCUSSION

The Malawian malaria vectors are predominantly *An. gambiae*, *An. arabiensis*, and *An. funestus* (Chiphwanya J, unpublished data). A previous collection of *An. funestus* carried out in Karonga in August 2007 resulted in 80% failure to identify the specimens using the multiplex PCR assay.[16] The identification of the specimens collected for this study again failed to give amplicons for 61 of 63 specimens collected although these samples were identified morphologically as the *An. funestus*

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1   TTATAACCATTAAACCCACAGGCGAAGACAACTCGATTGTCACGG  An. funestus
1   .............................................  Malawi Sample

46  GATTACGGGCACGGATAGGTGGCGCAAGCCCCTTATAGAACCGAG  An. funestus
46  ................G.--.............A...T.......  Malawi Sample

91  CCCCTCCATCCCAGGGTGCTCCGTCACGGGTGCTTGCACCCAGCG  An. funestus
89  .............................................  Malawi Sample
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Figure 3. Sequence alignment of the partial D3 region of the unidentifiable Malawian (MalaF) samples (133 basepairs) and *Anopheles funestus* (135 basepairs). Blocks highlight sequence variation. – indicates deletions. The forward D3A primer binds approximately 90 basepairs upstream from the start of this sequence.

group.[1,2] The failure of primer annealing in the PCR assay is similar to observations for *An. rivulorum*-like in Cameroon.[6]

Sequencing of the ITS2 region of four of the unidentified samples showed the presence of a three-basepair deletion and a T to C transition in the *An. funestus*-specific primer site. This change in sequence resulted in the species-specific primer not annealing and subsequent failure to amplify the region. Further analysis of this ITS2 region showed a high level of sequence variation (4.5%) when compared with the *An. funestus* control.

The D3 sequence analysis of the MalaF specimens showed a 1.5% variation compared with the *An. funestus* control, which for this gene region, is significant. Differences as small as 2–3 basepairs within the D3 region have been used to differentiate the members of the *An. fluviatilis* complex;[22] *An. minimus* species A and C differ by five subsitutions.[23] Combined, the levels of variation seen in the MalaF specimens are significant. Levels of inter-specific sequence divergence can range from lows of 0.4% to 1.6% differences, as in the *An. gambiae* complex,[24] to 19% as observed between *An. rivulorum* in eastern and southern Africa and *An. rivulorum*-like in west and central Africa.[5]

A PCR primer (MalaFB) specific to the ITS2 region of the unidentified samples was designed and effectively used to amplify this region. However, this amplicon (approximately 390 basepairs) is too close in size to that of *An. rivulorum* (approximately 411 basepairs) to be incorporated into the multiplex PCR mixture, as seen by the species product ladder in Figure 2. The optimum difference in amplicon size for easy visualization on an agarose gel is approximately 50 basepairs, a criterion used in the design of the species-specific multiplex PCR assay.[16] However, the MalaFB primer does enable identification of our unidentified specimens where the initial multiplex PCR fails. We are currently testing the new primer on field-caught material from other regions that have failed to amplify using the *An. funestus* multiplex PCR. The seven MalaF samples that failed to amplify with the MalaFB primer are undergoing further molecular analysis. Because DNA extractions are routinely carried out with positive controls from insectary material, the quality of the DNA can be ensured. The DNA integrity of these samples was tested using primers known to amplify the members of the Funestus subgroup, and the presence of amplicons confirms that the failure to amplify using the MalaFB primer was not caused by DNA degradation.

Prior to the advent of DNA-based technologies, salivary gland and ovarian polytene chromosomes were used to distinguish members of sibling species complexes. Members of the European *An. maculipennis* complex were distinguished by the chromosomal banding patterns seen in salivary gland polytene chromosomes.[25,26] This success in cytotaxonomy was quickly followed by the cytogenetic description of the members of the *An. gambiae* complex.[27–29] The chromosomal banding patterns of the Malawian specimens displayed homosequential banding arrangements with *An. funestus*, but were fixed for the inverted arrangements 3a, 3b, and 5a, which are commonly polymorphic in *An. funestus*. Although *An. vaneedeni* also has homosequential chromosomes with *An. funestus*,[13] the fixed inverted arrangements on arms 3 and 5 of MalaF distinguish it from *An. vaneedeni.* Even though cytogenetic studies on west African populations of *An. funestus* have provided evidence of species differentiation,[14,15] these investigators did not undertake cross-mating studies because of lack of colonized *An. funestus* at that time.

Species-crossing experiments have been widely used to prove the distinction of sibling species within anopheline complexes (*An. maculatus* form K,[30] *An. pseudopunctipennis* species C,[31] *An. minimus* species E,[32] *An. quadrimaculatus* types A and B,[33] and *An. annulipes* species A and G[34]) with hybrids being scored for asynapsis between homologous chromosomes and hybrid infertility. In the present study, the hybrid chromosomes resulting from the MalaF females × *An. funestus* males showed consistent asynapsis between homologous chromosomes, typical of inter-species crosses.[13,20,35] The male hybrids appeared to have normal testis morphology with the possible exception that the head region of the spermatozoa appeared narrower. Unfortunately, the effect of this narrower morphology in terms of male fertility is unknown because we were unable to carry out back crosses. Eggs were produced from the reciprocal crosses, but their viability was extremely low (< 0.2% hatch rate), suggesting a genetic discontinuity between the parental samples.

On the basis of the combined molecular, cytogenetic, and cross-mating evidence, we conclude that the Malawi population is a new member of the *An. funestus* subgroup. We provisionally designate it *An. funestus*-like until a formal description is published. Further molecular investigations are needed to determine how this new species impacts on the variation seen in restriction fragment length polymorphism[36] and mitochondrial DNA[37] analyses of *An. funestus* populations from the southern African region.

Further investigations into the biology of this new species are also required. Although none of the 61 specimens examined for malaria parasite infection during this study were positive for *P. falciparum*, the fact that these mosquitoes are common inside houses makes them potential vectors. Collections at different times of the year are needed to clarify their vector status and to provide data on the interactions between this new species and *An. funestus* s.s. in areas where they occur in sympatry.

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