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Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* and *An. rivulorum*, and uncover a cryptic taxon

B. J. Hackett,[1] J. Gimnig,[2] W. Guelbeogo,[3] C. Costantini,[3] L. L. Koekemoer,[4] M. Coetzee,[4] F. H. Collins[1] and N. J. Besansky[1]

[1]*Department of Biological Sciences, University of Notre Dame, Notre Dame, IN, USA,* [2]*Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA, and Kenya Medical Research Institute, Kisumu, Kenya,* [3]*Centre National de Lutte contre le Paludisme, Ouagadougou, Burkina Faso, and Istituto di Parassitologia, Università 'La Sapienza', Roma, Italy, and* [4]*Medical Entomology, Department of Tropical Diseases, School of Pathology of the South African Institute for Medical Research, and The University of the Witwatersrand, Johannesburg , South Africa*

Abstract

Differentiation among the closely related Afrotropical species comprising the Funestus Group is difficult by traditional taxonomic measures. *Anopheles rivulorum* is the second most abundant and widespread species in the Funestus Group, and is occasionally collected indoors along with the dominant member and major malaria vector, *An. funestus*. The prospect of misidentification of *An. rivulorum* as *An. funestus* prompted the development of a rapid, polymerase chain reaction (PCR)-based method for identifying these two species. The ribosomal internal transcribed spacer 2 (ITS2) was amplified from thirty-five specimens of *An. rivulorum* collected from the extremes of its range: Eastern Africa (Kenya), Southern Africa (South Africa) and Western Africa (Burkina Faso). The ITS2 region of *An. rivulorum* (≈ 380 bp) is sufficiently different in size from the ITS2 of *An. funestus* (≈ 700 bp) that these species can be distinguished by agarose gel electrophoresis of PCR products without further manipulation. Comparison of the *An. rivulorum* and *An. funestus* ITS2 nucleotide sequences revealed such extensive divergence that meaningful alignment was impossible, except for a 25 bp island near the 5′ end. Intraspecific sequence comparisons revealed no variation among *An. rivulorum* individuals collected from the same country. However, sequence divergence was 2% between specimens from South Africa and Kenya, and nearly tenfold higher (≈ 19%) between specimens from Burkina Faso and either South Africa or Kenya, an unprecedented level of intraspecific ITS2 divergence in *Anopheles*. Taken together, these data suggest that the Burkina Faso sample is not *An. rivulorum*, but rather a cryptic taxon within the Funestus Group.

Keywords: *Anopheles funestus*, *Anopheles rivulorum*, cryptic species, molecular taxonomy, ribosomal DNA.

Introduction

Although it has long been known that only some members of anopheline species complexes are important vectors of malaria, these sibling species have proven difficult if not impossible to distinguish by traditional taxonomic measures (Gillies & De Meillon, 1968; Coluzzi, 1970). This difficulty has spurred the development of molecular methods for species identification. The tandemly arrayed ribosomal RNA multigene family (rDNA) is a common target of such methods, because of useful features of its sequence organization and evolution.

Portions of the rDNA coding sequences are highly conserved even between distantly related species, allowing the application of 'universal' primers for amplification from any anopheline species. The non-coding rDNA spacer sequences, however, can be highly variable in length and sequence between closely related species. Concerted evolution acting on rDNA arrays maintains sequence homogeneity within species as it drives differentiation between species, a pattern that explains the utility of rDNA for species-diagnostic assays (Collins & Paskewitz, 1996). Ribosomal DNA-based PCR assays have been developed for many anopheline species complexes including the *Anopheles gambiae* complex, the *An. punctulatus* complex and the

Received 3 December 1999; accepted following revision 14 March 2000. Correspondence: N. J. Besansky, Department of Biological Sciences, PO Box 369, 317 Galvin Life Sciences Bldg., University of Notre Dame, Notre Dame, IN 46556–0369, USA. Tel.: + 01–219–631–9321; fax: + 01 219631 7413; e-mail: besansky.1@nd.edu

An. quadrimaculatus complex (Scott *et al.*, 1993; Beebe *et al.*, 1994; Cornel *et al.*, 1996).

Anopheles funestus Giles, along with *An. gambiae* Giles and *An. arabiensis* Patton, is one of the most important vectors of malaria in sub-Saharan Africa (Gillies & De Meillon, 1968; Zahar, 1985). *Anopheles funestus* is informally classified within the Funestus Group (Gillies & De Meillon, 1968; Harbach, 1994), a group of eight closely related species of which some are distinguishable as larvae but are difficult or impossible to distinguish in the adult stage. Unlike *An. funestus* which is abundant, widespread, and highly endophilic and anthropophilic, the other species are typically more limited in density and distribution, and mainly bite animals outdoors. However, they avidly bite humans outdoors in the absence of other hosts (Gillies & De Meillon, 1968). As these species exist sympatrically with *An. funestus* in parts of Africa, they may increase in density in response to reduced populations of *An. funestus* (Gillies & Smith, 1960) and assume a role in malaria transmission (Wilkes *et al.*, 1996).

Anopheles rivulorum Leeson, the second most abundant member of the Funestus Group, has also been shown to be a vector of human malaria in Africa, and is sympatric with *An. funestus* in the savannas of West and East Africa, including Burkina Faso and Kenya (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987; Wilkes *et al.*, 1996). These two species are easily differentiated at the larval stage (Gillies & Coetzee, 1987; Gillies & De Meillon, 1968), but characters that distinguish the adults are variable or may be lost if the specimens were not freshly collected or were damaged during handling (Wilkes *et al.*, 1996). A method of rapidly and reliably distinguishing these two species will benefit population genetic research on both species, as the most epidemiologically important segment of the population the adult females is the segment most commonly sampled.

Previously, Koekemoer *et al.* (1999) developed a polymerase chain reaction (PCR) single-strand conformation polymorphism (SSCP) assay that differentiates between four members of the Funestus Group, including *An. rivulorum* and *An. funestus*. This procedure uses primers that amplify the D3 domain in the rDNA 28S gene, but the products show no species-specific size difference when electrophoresed on an agarose gel. Discrimination between the species using this procedure depends on conformational differences due to nucleotide sequence variation, detected by eletrophoresis of denatured PCR products on a polyacrylamide gel (Koekemoer *et al.*, 1999).

The present study describes a simplified species-specific PCR assay to identify *An. funestus* and *An. rivulorum* using the second ribosomal DNA internal transcribed spacer (ITS2), and provides preliminary evidence based on ITS2 sequence heterogeneity that *An. rivulorum* may consist of at least two reproductively isolated taxa.

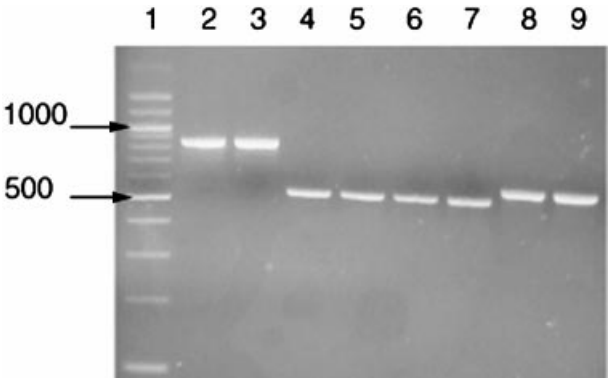

Figure 1. An ethidium bromide-stained 2% agarose gel showing the PCR products spanning the ITS2. Lane 1: 100-bp ladder; Lanes 2–3: *An. funestus* (Burkina Faso); Lanes 4–7: *An. rivulorum* (Kenya); Lanes 8–9: *An. gambiae* s.s. (M2 strain).

Results and discussion

Using a pair of PCR primers that anneal to the 5.8S and 28S coding regions of all species examined within the genus *Anopheles* (Porter & Collins, 1991), the intervening ITS2 sequence was amplified from thirty-five *An. rivulorum* specimens, including fourteen from Kenya, eight from South Africa and thirteen from Burkina Faso. Following electrophoresis of the PCR products through a 2% agarose gel (Fig. 1), a single band migrating at approximately 520 bp was visible, and this was clearly distinguishable from the corresponding PCR products from *An. funestus* (≈ 840 bp) as well as *An. gambiae* (≈ 560 bp). It therefore appeared that, based on size differences alone, a species-specific diagnostic PCR assay could identify not only *An. funestus* and *An. rivulorum*, but also could differentiate either species from *An. gambiae* and its sibling species *An. arabiensis*. Members of the *An. gambiae* complex are not difficult to distinguish morphologically from *An. funestus* and its allies, but misidentifications in the course of extensive population surveys in the field may occur, and this additional checkpoint that can be applied after a specimen has already been processed (and its morphology destroyed) is fortuitous.

Just as morphologically similar taxa are not necessarily conspecific, so identically migrating bands on gels are not necessarily identical in sequence. Therefore, both strands of the ITS2 region were directly sequenced from the PCR products of a subset of specimens identified morphologically as *An. rivulorum*. Because an unambiguous morphological identification of *An. rivulorum* can be made at the larval stage, all fourteen larval specimens from Kenya were sequenced. Although the eight South African specimens were identified as adults only by PCR-SSCP (Koekemoer *et al.*, 1999), this assay had been developed using South

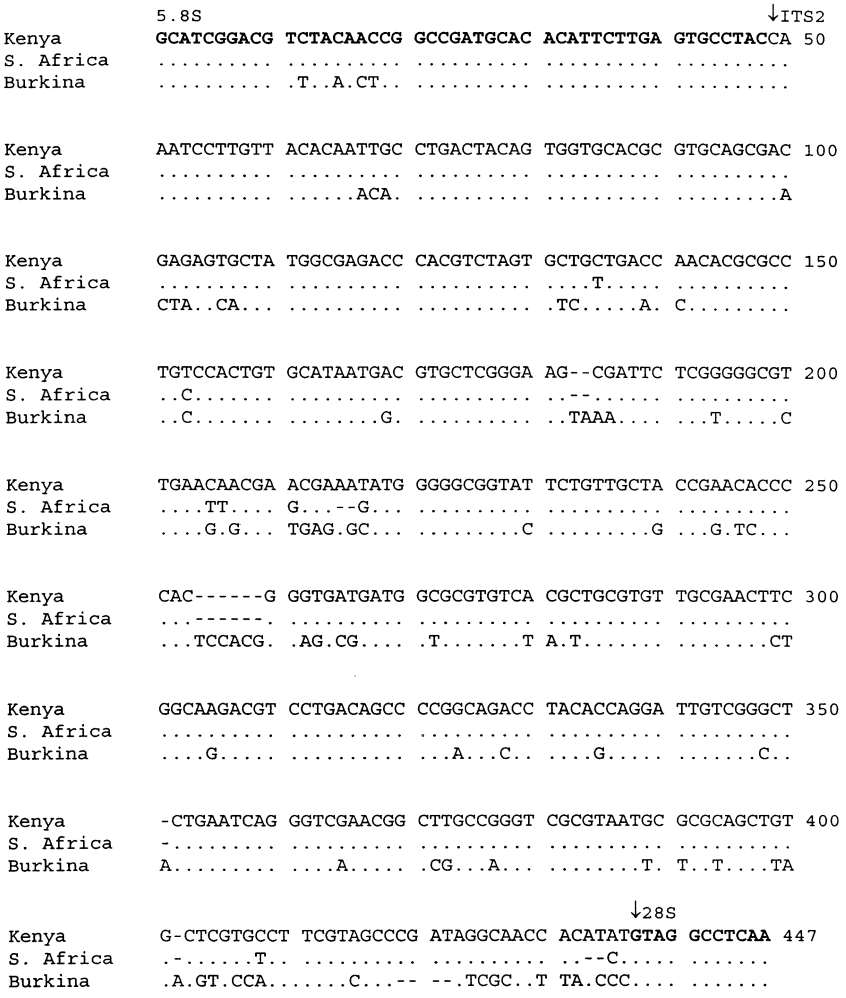

Figure 2. Alignment of *An. rivulorum* ITS2 and flanking 5.8S and 28S sequences (in bold) from Kenya (top), South Africa (abbreviated S. Africa, middle) and Burkina Faso (abbreviated Burkina, bottom). Fourteen larvae from Kenya, five adults from South Africa and five adults from Burkina Faso were sequenced. There was no interspecific variation seen within a country. Dots represent identity to the Kenyan reference sequence; dashes represent gaps introduced to maximize overall sequence similarity.

African samples that were identified morphologically as *An. rivulorum* based on egg and larval characteristics; five specimens were sequenced. Among the Burkina Faso specimens, only those adults that could be unambiguously identified morphologically as *An. rivulorum* according to the taxonomic key of Gillies & De Meillon (1968) were sequenced (i.e. wings in which two pale spots were present on the upper branch of the fifth vein, 5a, and palps in which the subapical pale band was narrower than the apical dark band). The incidence of the '2-spot' wing character is known to vary both seasonally and geographically in *An. rivulorum*. Indeed, it occurred in only eight of thirteen Burkina Faso specimens that produced the distinctive 520 bp PCR product. Although the absence of the palp and wing vein characters is not conclusive, their simultaneous presence diagnoses *An. rivulorum* according to accepted taxonomy (Gillies & De Meillon, 1968); specimens lacking both these defining characteristics were excluded from our analysis. However, the formal possibility exists that the key itself is misleading, because characters can be variable over time and space, and some Funestus Group species have received scant attention from taxonomists. According to Gillies & De Meillon (1968), adults of *An. rivulorum* and *An. brucei* Service, a morphologically similar species from West Africa, are distinctive based on the relative width of the palpal bands. However, *An. brucei* has been described only from its type locality in Nigeria and its true distribution and possible morphological variation are completely unknown.

The *An. rivulorum* ITS2 is approximately 380 bp in length (Fig. 2), and is shorter than the ITS2 of *An. funestus* and *An. gambiae* by ≈ 324 bp and ≈ 46 bp, respectively. In all cases, sequences directly determined from PCR products were free of ambiguities that would be expected if sufficient intraindividual variation were present, as was found in *An. nuneztovari* (Onyabe & Conn, 1999). This suggests that *An. rivulorum* ITS2 sequences are predominantly composed of one consensus type, as was found for *An. funestus*

Table 1. Pairwise ITS2 sequence divergence (below diagonal) and insertion–deletion differences (above diagonal) between *An. rivulorum* samples

Samples (*n*)	1	2	3
1. Kenya (14)	0.0	2.0	5.0
2. S. Africa (5)	2.1	0.0	7.0
3. Burkina (5)	19.0	18.7	0.0

previously (Mukabayire *et al.*, 1999). Furthermore, ITS2 sequences of specimens collected from one or more nearby sites within the same country were identical (Table 1).

By contrast, sequence comparisons between *An. rivulorum* sampled from different countries revealed a surprising amount of base substitution and insertion–deletion (indel) differences (Table 1 and Fig. 2). The ITS2 sequence from Kenyan specimens (378 bp) and South African specimens (374 bp) differed at eight of 374 aligned positions (2.1% divergence), excluding two 2 bp indels. Moreover, sequence comparisons between Burkina Faso ITS2 (384 bp) and either Kenya or South Africa ITS2 uncovered a level of divergence almost tenfold higher, ≈ 19% (respectively, 72/378 aligned positions excluding five indels and 70/374 aligned positions excluding seven indels).

It is also worth noting that there were 4-bp substitutions near the 3′-end of the 5.8S coding region between the Burkina Faso samples and those from the other localities. In many species of Diptera the primary 5.8S rRNA transcript is processed into a shortened 5.8S ('m5.8S') and a 2S by removal of a short spacer sequence; members of the family Culicidae are exceptional in having a single continuous 5.8S rRNA (Shimada, 1992). However, secondary structure analyses of the *An. hermsi* 5.8S and 28S by Porter & Collins (1991) predicted that the regions homologous to the m5.8S and 2S form a stable hairpin separated by a short (5 bp) loop. The corresponding regions of *An. rivulorum* rRNA also are predicted to form a stem-loop structure (data not shown). If so, the 4 bp differences between the Burkina Faso and other samples are located in this loop.

The significance of the sequence heterogeneity among *An. rivulorum* samples is best understood in the context of ITS2 polymorphism data from other anopheline species and species complexes. For example, within *An. funestus*, sequence polymorphism detected in the first 508 bp of the ITS2 among nineteen specimens from Senegal, Burkina Faso and Kenya was limited to one substitution (≈ 0.2%) (Mukabayire *et al.*, 1999). In the Afrotropical *An. gambiae* complex, intraspecific variation for the ITS2 region was 0.1–0.4% (Paskewitz *et al.*, 1993). In the Neotropical subgenus *Nyssorhynchus*, intraspecific ITS2 variation of twenty-one member species ranged from 0% to 2.8% (Manguin *et al.*, 1999). On the other hand, ITS2 sequence divergences between cryptic species range quite broadly, from a low of 0.4–1.6% in the *An. gambiae* complex (Paskewitz *et al.*, 1993) to a high of 18.5–28.7% in the North American *An. quadrimaculatus* complex (Cornel *et al.*, 1996) and 25% between the cryptic South American species *An. trinkae* and *An. dunhami* (Lounibos *et al.*, 1998). At ≈ 19%, the ITS2 sequence divergence measured between the sample from Burkina Faso and *An. rivulorum* from the other locales exceeded by at least sevenfold the levels found within other anopheline species. Indeed, it was among the highest levels of ITS2 sequence divergence measured *between* any pair of anopheline cryptic species, suggesting that the sample from Burkina Faso is an independent genetic entity whose relationship with *An. rivulorum* from other parts of Africa warrants closer examination. The possibility that this taxon may be *An. brucei* assumes that the adult characters (palpal bands) used to differentiate *An. brucei* and *An. rivulorum* are variable and that *An. brucei* occurs in Burkina Faso; otherwise, the taxon may be novel.

The high level of divergence between the ITS2 sequences of *An. rivulorum* and *An. funestus* also merits comment. These sequences are sufficiently diverged that only a small part of the sequence, an island of ≈ 26 bp at the 5′ end of the ITS2, can be aligned reliably (Fig. 3). Within this island, 25 of 26 bp (96%) were identical. Curiously, BLAST searches using the Kenyan *An. rivulorum* ITS2 sequence against sequences in GenBank revealed a very similar island (22/26, or ≈ 85%) at the 5′ end of the *An. gambiae* ITS2 (Fig. 3), but no other matches to other regions nor to other anopheline ITS2 sequences in the database. It is tempting to speculate that this region may be important in maintaining secondary folding structure of the ITS2. However, outside of this island, no significant sequence similarity between *An. rivulorum* (Kenya) and *An. funestus* was apparent.

That *An. rivulorum* and *An. funestus* may be differentiated morphologically at several developmental stages is reflected in their classification by mosquito systematists

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An.  rivulorum 151 TGTCCACTGT GCATAATGAC GTGCTCGGGA AGCGATTCTC 190
An.  funestus  144 .CCA...... ........G. .......... CCTTTG.TGG 183
An.  gambiae   629 C.GT.G.... .......... .....T..TC CC..TC.GCG 668
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Figure 3. Island of ITS2 sequence similarity shared between *An. rivulorum*, *An. funestus*, and *An. gambiae*. Dots represent identity to the *An. rivulorum* sequence. Numbering of the *An. funestus* sequence refers to Fig. 2 of Mukabayire *et al.* (1999; AF062512); numbering of the *An. gambiae* sequence refers to Fig. 1 of Paskewitz *et al.* (1993; X67157).

as a Group, as opposed to a species complex. The only other study that has compared ITS2 sequences among anophelines at the Group level of is that of J. Danoff-Burg and J. Conn (pers. comm.), who examined the Oswaldoi Group in addition to other species within the subgenus *Nyssorhynchus*. Although there were regions that were difficult to align, portions of the ITS2 could be aligned even among members of different Groups within *Nyssorhynchus*. Excluding gaps, the average pairwise distance between ITS2 sequences of *An. oswaldoi*, *An. nuneztovari* and *An. strodei* was 15% across ≈ 360 aligned sites (J. Danoff-Burg and J. Conn, unpublished). These data suggest that *An. rivulorum* and *An. funestus* are more distantly related evolutionarily than members of the Oswaldoi Group.

Species-specific differences in vectorial capacity between anophelines has proven crucial in understanding patterns of malaria transmission. Although *An. rivulorum* is not normally an important vector, the fact that it may naturally transmit *Plasmodium falciparum* and may be confused morphologically with *An. funestus* (Wilkes *et al*., 1996) suggested the need for a rapid and robust method of species identification. The method developed here appears suitable for separating *An. funestus* from *An. rivulorum* and *An. gambiae s.l.* It also unexpectedly revealed a novel cryptic taxon, possibly *An. brucei*, in Burkina Faso whose relationship to *An. rivulorum* and other members of the Funestus Group requires further research using both morphology of the immature stages and independent molecular markers. Currently, we are examining other members of the Funestus Group to explore the feasibility of expanding the ITS-based PCR assay to include all members.

Experimental procedures

Sampling

In Kenya, larval *An. rivulorum* were collected in June of 1998 from Asembo Bay. The specimens were identified according to the morphological key of Gillies & De Meillon (1968), preserved in 70% ethanol and stored at −80 °C. In South Africa, adult *An. rivulorum* were collected from pit shelters in May of 1999 from Thomo Village, Northern Province. The specimens were identified using the PCR-SSCP assay of Koekemoer *et al.* (1999) and were preserved desiccated on silica gel. In Burkina Faso, adult Funestus Group specimens were collected from pit shelters in September of 1999 from Kuiti and Koubri villages near Ouagadougou, and preserved desiccated in silica gel.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from individual mosquitoes according to Collins *et al.* (1987), and resuspended in 100 µl of TE. The rDNA ITS2 was amplified using primers that anneal in the flanking 5.8S and 28S rDNA, CP-P1A and CP-P1B (Porter & Collins, 1991), as described in Mukabayire *et al.* (1999). The ITS2 region was amplified in a 50-µl reaction containing 1 µl of DNA, 50 pmol of primers, 1.5 mM $MgCl_2$, 200 µM of each dNTP, and 1.25 U of *Taq* polymerase. The thermal cycling conditions were: initial denaturation at 94 °C for 2 min; thirty-five cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 15 s, extension at 72 °C for 45 s; and a final extension at 72 °C for 10 min. A 5-µl portion of the PCR product was electrophoresed on a 2% agarose gel; the remainder was purified using the Stratagene PCR purification kit and directly cycle-sequenced using the ABI Prism Dye Deoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems) and the same primers used for PCR amplification. Reaction products were purified by ethanol precipitation and run on a 6.25% acrylamide gel using an ABI 377 automatic sequencer (PE Applied Biosystems). Both strands were completely sequenced and assigned GenBank accession numbers AF180524 (Kenya), AF210724 (South Africa) and AF210725 (Burkina Faso). Sequences were analysed using programs in GCG 9.1 (Genetics Computer Group, 1997).

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