Molecular Ecology (1999) 8, 289–297

Mitochondrial and ribosomal internal transcribed spacer (ITS2) diversity of the African malaria vector *Anopheles funestus*

O. MUKABAYIRE,* D. BOCCOLINI,† L. LOCHOUARN,‡ D. FONTENILLE‡ and N. J. BESANSKY*
Department of Biological Sciences, PO Box 369, 317 Galvin Life Sciences Building, University of Notre Dame, Notre Dame, IN 46556 USA, †Laboratorio di Parassitologia, Istituto Superiore di Sanità, 00161 Roma, Italy, ‡Institut Francais de Recherche Scientifique pour le Développement en Coopération (ORSTOM), Laboratoire de Zoologie Médicale de l'Institut Pasteur, BP 1386, Dakar, Sénégal

Abstract

The pattern of sequence variation in the mitochondrial DNA cytochrome *b* gene (*cyt-b*) and ribosomal DNA internal transcribed spacer 2 (ITS2) was examined in *Anopheles funestus* from Senegal and Burkina Faso in West Africa and Kenya in East Africa. From both West African countries, samples included individuals hypothesized to represent reproductively isolated taxa based upon different karyotypes and behaviours. Analysis of the *cyt-b* data revealed high haplotypic diversity (86%) and an average pairwise difference per site of 0.42%. Sequence variation was not partitioned by geographical origin or karyotype class. The most common haplotype was sampled across Africa (≈ 6000 km). Analysis of the ITS2 data revealed one of the longest spacers yet found in anophelines (≈ 704 bp). In common with other anopheline ITS2 sequences, this one had microsatellites and frequent runs of individual nucleotides. Also in common with data from other anopheline ITS2 studies, the *An. funestus* sequences were almost monomorphic, with only two rare polymorphisms detected. The results from both markers are congruent and do not support the hypothesis of reproductively isolated chromosomal taxa within *An. funestus*. Whether the lack of support by mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) sequences is a result of the recent origin of the presumptive taxa, or of the absence of barriers to gene flow, remains to be elucidated, using more rapidly evolving markers such as microsatellites.

Keywords: Anopheles funestus, chromosomal inversions, malaria vector, mtDNA, population structure, rDNA

Received 20 June 1998; revision received 25 September 1998; accepted 7 October 1998

Introduction

Across tropical Africa, three anopheline species are responsible for the majority of malaria transmission. These are *Anopheles funestus* and the two sibling species *An. gambiae* and *An. arabiensis*. Their importance as malaria vectors stems from widespread distribution, abundance, longevity and, above all, a penchant for human blood.

Behaviourally, *An. funestus* is distinguished from the other two vectors by breeding in large, permanent bodies of water, characterized by emergent vegetation, such as swamps, river edges, ditches and seepages (Gillies & De Meillon 1968). In addition, *An. funestus* is one of the most 'domestic' of African anophelines, preferring to feed and rest inside human houses for most of its adult life. Even in human dwellings shared with cattle and/or goats, most female *An. funestus* bite humans and congregate in sections occupied by humans (Gillies & De Meillon 1968). This behaviour made *An. funestus* quite vulnerable to house spraying with residual insecticides during the 1950s and 1960s (reviewed in Zahar 1984). Wherever implemented, the initial effect of house spraying was a dramatic decrease in density or total disappearance of *An. funestus* in the treated areas. In some parts of Africa, these effects were long-lasting. DDT spraying in South

Correspondence: N. J. Besansky. Fax: +01-219-6317413; E-mail: nora.j.besansky.1@nd.edu

Africa permanently eradicated *An. funestus*. *An. funestus* disappeared in the Pare area of Tanzania and the Taveta area of Kenya during the 3.5 years of dieldrin application and for at least 5 years after discontinuation of dieldrin, before becoming slowly re-established (Zahar 1984). The same pattern was observed in the Antananarivo region of Madagascar where, in 1952, *An. funestus* was considered to be eradicated. The spraying campaign ceased in 1958, *An. funestus* reappeared in 1976 and has recently been linked to a resurgence of malaria in this region (Fontenille & Rakotoarivony 1988). These observations, together with the uneven distribution of this vector before spraying (Molineaux & Gramiccia 1980), suggested that *An. funestus* may be subdivided into discrete populations.

Further evidence for population heterogeneity within and between *An. funestus* populations comes from the distribution of paracentric inversions on chromosome arms 2, 3 and 5 (Green & Hunt 1980). In Senegal, allopatric populations from the East and West are characterized by different inversion frequencies that are in Hardy–Weinberg equilibrium unless pooled between locations (L. Lochouarn, unpublished data). Samples from the western village of Dielmo revealed a monomorphic standard (uninverted) arrangement on chromosome 2 and predominantly standard arrangements for the other arms. In the eastern Kedougou region, samples were highly polymorphic on all arms. In Burkina Faso, a different pattern was found involving significant deviations from Hardy–Weinberg equilibrium for all inversions sampled from sympatric populations at each of several locations (Boccolini *et al.* 1994). Standard homokaryotypes at all four inversions were in excess, and their exclusion from each location restored the remaining samples to Hardy–Weinberg equilibrium. Moreover, the standard homokaryotypes from Burkina Faso and Senegal appeared behaviourally distinct, but in contrasting ways. Whereas they were less endophilic and anthropophilic than other karyotypes in Burkina Faso, they were more anthropophilic in Senegal. These findings have raised the possibility that *An. funestus* in West Africa includes at least two taxonomic units with different vectorial capacities (Boccolini *et al.* 1994).

Motivated by the cytological evidence, the present study sought independent molecular evidence for the existence of distinct taxonomic units within *An. funestus*. Both mitochondrial DNA (mtDNA) genes and nuclear ribosomal DNA (rDNA) have been useful targets for this purpose because of the relative rapidity with which they can reflect the effects of restricted gene flow among populations (Avise 1994). For mtDNA, this is because of its haploid maternal transmission and lack of recombination. For rDNA, organized as tandemly repeated gene copies, this is a result of concerted evolution, which homogenizes sequences at a locus within an individual and within panmictic populations. Because there is only one X-linked rDNA locus in anopheline mosquitoes examined to date (Collins *et al.* 1989; Kumar & Rai 1990), the more rapidly evolving spacer regions of the rDNA accumulate species-specific and even population-specific sequence differences that have been successfully exploited in diagnostic assays (McLain *et al.* 1989; Porter & Collins 1991; Paskewitz *et al.* 1993; Fritz *et al.* 1994; Collins & Paskewitz 1996; Cornel *et al.* 1996; Favia *et al.* 1997; Xu & Qu 1997). In this study we investigated whether variation in the cytochrome *b* gene (*cyt-b*) of mtDNA and the second internal transcribed spacer (ITS2) of nuclear rDNA from *An. funestus* is patterned according to karyotype or geographical origin.

Materials and methods

Sampling

In Burkina Faso, adult females of *Anopheles funestus* were collected indoors, by aspiration, on 16–17 November 1992 in three villages (Diarabakoko, Kiribina and Folonso) of the Banfora area (10°30' N, 4°40' W), south of BoboDioulasso (Fig. 1). The collection sites are in the southern, more humid, zone of the sub-Saharan Sudan savanna belt, characterized by a rainy season from June to October with mean annual rainfall higher than 900 mm.

In Senegal, samples were collected by pyrethrum spraying in selected bedrooms during April–July 1994 in the Sudan savanna village of Dielmo (13°45' N, 16°25' W; see Trape *et al.* 1994), and during November 1994 in the Sudan guinean zone of Kedougou (12°33' N, 12°11' W), shown in Fig. 1.

In western Kenya, gravid females were collected by aspiration from the walls or bednets inside bedrooms, in May 1987, from the villages of Asembo, Ahero and Nyakoch (McLain *et al.* 1989; Fig. 1). The samples analysed were single F_1 progeny per field-collected female.

Identification of *An. funestus* was made according to the morphological key of Gillies & De Meillon (1968). Polytene chromosomes of West African specimens were examined for chromosomal inversion polymorphisms (Green & Hunt 1980).

DNA extraction, amplification and sequencing

DNA was isolated from desiccated legs (Senegal specimens), Carnoy's-preserved carcasses without ovaries (Carnoy's solution is glacial acetic acid:ethanol (1:3 v/v); Burkina Faso specimens) and liquid nitrogen-preserved complete carcasses (Kenya specimens), according to Collins *et al.* (1987), and resuspended in 50–100 μL of water. The polymerase chain reaction (PCR) was used to

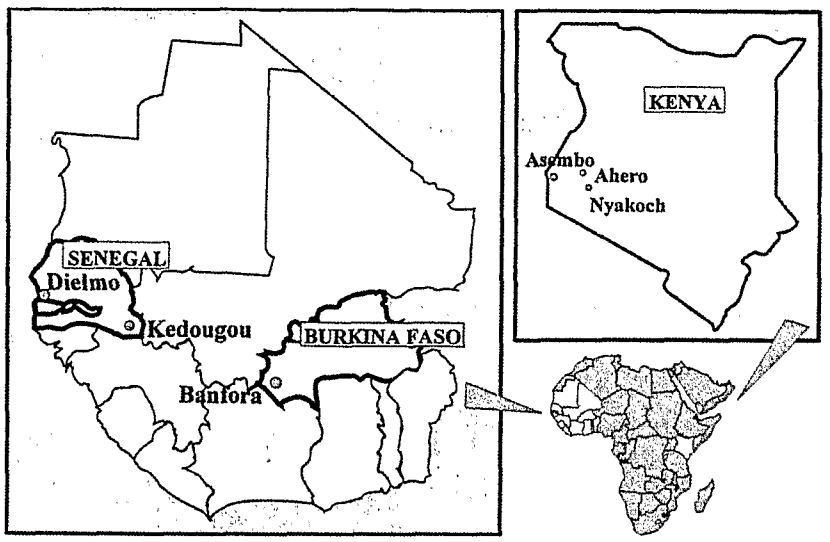

Fig. 1 Map of the *Anopheles funestus* populations sampled from West and East Africa.

amplify a 469-bp segment of the mtDNA *cyt-b* gene and the ITS2 of nuclear rDNA in a 50-μL reaction mix containing 1 μL of DNA, 50 pmol of primers, 5 μL of 10× reaction buffer containing 15 mM of $MgCl_2$, 200 μM of each dNTP (Perkin-Elmer) and 1.25 U of *Taq* polymerase (Boehringer Mannheim or GibcoBRL). The *cyt-b* segment corresponding to positions 10 821–11 290 of *An. gambiae* mtDNA (Beard *et al.* 1993) was amplified using primers *cyt-b*F and *cyt-b*R, previously described by Lyman *et al.* (1998). An 842-bp fragment containing the complete ITS2 (704 bp) was amplified using primers that anneal in the flanking 5.8S and 28S rDNA, CP-P1A and CP-P1B, respectively (Porter & Collins 1991; Wesson *et al.* 1992). These were modified by removal of the 8-bp restriction site linkers. Two additional internal primers were used(Fig. 2): ITSA-1 (5'-GAACCGCATAAATCG-CACGC-3') and ITSB-1 (5'-CGATCTCTCAACAA-CAACTC-3'). The combination of CP-P1A and ITSB-1 amplified the first 508 bp of the ITS2. For ITS2, the thermal cycling conditions were: initial denaturation at 94 °C for 1–2 min; 35 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 5–10 min. Cycling conditions for *cyt-b* were identical except that annealing was performed at 45 °C and extension was for 30 s. Both ITS2 and *cyt-b* PCR products were purified using the Wizard PCR DNA Purification System (Promega Corporation) and sequenced directly using the ABI Prism Dye Deoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems) with the same primers as employed for PCR amplification. Reaction products were purified using Centri-Sep columns (Princeton Separations) and run on a 4% acrylamide gel using an ABI 377 automatic sequencer (PE Applied Biosystems). Both strands were completely sequenced. Nonredundant sequences (haplotypes) have been assigned the GenBank accession numbers: AF062501 to AF062512.

Sequence analysis

Sequences were inspected and corrected as necessary using Sequence Navigator 1.0.1 (PE Applied Biosystems). For both genes, sequence alignment was unambiguous but was conveniently performed using the PILEUP program from GCG (Genetics Computer Group 1994). Haplotype diversity was calculated using formula 8.5 of Nei (1987). Other sequence statistics were calculated using MEGA 1.01 (Kumar *et al.* 1993) and DNASP 2.0 (Rozas & Rozas 1997).

Results

Analysis of ITS2 sequences

The *Anopheles funestus* ITS2 and flanking sequences were determined from five individuals: three from Burkina Faso (representing homokaryotypic standard and polymorphic inverted karyotypes) and two from Senegal (standard karyotypes) (Table 1). The approximate boundaries of the ITS2 (positions 45–749 in Fig. 2) were defined by comparison with previously determined anopheline 5.8S and 28S rDNA sequences (Porter & Collins 1991; Wesson *et al.* 1992; Paskewitz *et al.* 1993; Fritz *et al.* 1994; Cornel *et al.* 1996; Xu & Qu 1997). At ≈ 704 bp, the *An. funestus* ITS2 is one of the longest of any anopheline examined to date (Table 2). It is also the least GC-rich (49.1%), apparently owing to a relative paucity of C in the plus strand.

```
     5.8S                                                ↓ITS2
CON CGGACGATTA AACCCGGCCG ATGCACACAT TCTTGAGTGC CTATCAATTC CTTGATATAC  60
    .......... .......... .......... .......... .......... ..........

CON AACAAACCAA ACTTCAGGGT GGAGCGTGCC ACAATAGAAC ACTATGGCGA GCAGCCCGTC 120
    .......... .......... .......... .......... .......... ..........

CON TAGTGTCGTG GGGGAAACAC GCTTCCACAC TGTGCATAAT GGCGTGCTCG GGACCTTTGT 180
    .......... .......... .......... .......... .......... ..........
                                                (ITSA-1→)
CON TGGGACCGCA GGGCGCTGAA AGTAAAGGGG TGAACCGCAT AAATCGCACG CACGTAAACG 240
    .......... .......... .......... .......... .......... ..........

CON CGCACACACA CAAATAGAGT GAGACGTATC GTAGGATACC GCTAAGAGTA CGTTGTGAAA 300
    .......... .......... .......... .......... .......... ..........

CON CATGGGGAAA TTCAATCGAA AACCTCTTTG ATGTCCAAGA ATTCGTTGAC CGTATCCGTC 360
    .......... .......... .......... .......... .......... ..........

CON GTAATACTGG ATCAACGTGC TTGGGGGAAA ACGTCAAAGG GTTTTATAAT AGTGGTGCAT 420
    .......... .......... .......... .......... .......... ..........

CON GATTAACCCA TCGATGCCCG AGGGGAACAT GTTGTCCAAT ACAATAGTGG TGCAGTTGGC 480
    ....G..... .......... .......... .......... .......... ..........

CON TCGACATGCT CGGGGGGAGA CATCGTGGGT CCAAGTCGAC CAAGTCGACC GAGAGTTGTT 540
    .......... .......... .......... .......... .......... ..........
     (←ITSB-1)
CON GTTGAGAGAT CGAATCAAAA CGATGCCGAG CGGAACTCGT TGTCCTTATT GGAGTGATAT 600
    .......... .......... .......... .......... .......... ..........

CON TCGGACAACG TGCTCGGGGG GGCCATCGTT GATTCAAAAA TGACCGTAAA TTGCCCAATC 660
    .......... .......... .......... .......... .......... ..........

CON C--GTGTGTG TGTGTGTGAA GTGTTGTTGC GTATATATCG GTTCGCTATG CCCCGGGTTC 720
    .GT....... .......... .......... .......... .......... ..........
                                    ↓ 28S
CON GAAACGAATG GAATGTGACT GATTTTGTTG TAGGCCTCA 759
    .......... .......... .......... .........
```

Fig. 2 Nucleotide sequence of the *Anopheles funestus* ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) and flanking 5.8S and 28S regions. CON, consensus sequence. Below this, variant nucleotide positions are indicated; gaps are represented by dashes, identities by dots. The estimated start of the ITS2 and 28S regions is indicated by arrows. Sequences to which internal primers ITSA-1 and ITSB-1 anneal are underlined.

Tandem repeat motifs of dinucleotides and trinucleotides are typical of anopheline ITS2 sequences (e.g. Cornel *et al.* 1996; Xu & Qu 1997). In the *An. funestus* ITS2, the most obvious sequence features are two microsatellites, $(CA)_5$ and $(GT)_7$, starting at positions 243 and 662, respectively. An insertion of one subrepeat unit in the latter microsatellite defined one of only two polymorphisms detected in the ITS2. The $(GT)_8$ allele was shared by Burkina Faso samples from different karyotype classes, no. 149-17 and no. 152-15. Another notable feature of the *An. funestus* ITS2 is frequent runs of G and A. There were seven instances of $(G)_3$, three of $(G)_4$ and four of $(G)_{5-7}$; 13 of $(A)_3$ and four of $(A)_{4-5}$. However, no length polymorphisms were found in these runs.

With the combination CP-P1A and ITSB-1, we sequenced the first 508 bp of ITS2 from an additional 14 specimens from East and West Africa, representing homokaryotypic standard and polymorphic inverted karyotypes (Table 1). From the total sample of 19 sequences, only one polymorphic site was detected in the first two-thirds of the ITS2. This was an A→G transition at position 425 found in three specimens, designated ITS2 haplotype 2 in Tables 1 and 3. It is worth noting that although haplotype 2 was less common, it was detected in Kenya as well as Burkina Faso and in both karyotype classes.

Analysis of cyt-b *sequences*

The nucleotide sequence of 404 bp of the mtDNA *cyt-b* gene was determined from a set of 19 *An. funestus* that partially overlapped with the set used in the ITS2 analysis (Table 1). There were 13 polymorphic sites in this sample, of which 11 were singleton mutations (Table 3). All substitutions were transitions and synonymous with respect to amino acid encoded. The average number of pairwise nucleotide differences was 1.7, which corresponds to 0.42% per site. By comparison, the average level of mtDNA NADH dehydrogenase subunit 5 sequence divergence within *An. gambiae* and *An. arabiensis* populations across Africa was 0.38% and 0.46%, respectively (Besansky *et al.* 1997).

The 13 polymorphic sites defined 11 haplotypes, shown in Table 3 and Fig. 3. Of these, nine were uniquely represented (singletons). Thus, in contrast to the ITS2 data, the *cyt-b* haplotype diversity was high (86%). The most common haplotype, designated '1', was found in seven individuals, representing all sampling sites and both

Table 1 Karyotype and genotype of *Anopheles funestus* samples based on ribosomal DNA (internal transcribed spacer 2, ITS2) and mitochondrial DNA (mtDNA) (*cyt-b*) sequences

	Karyotype*	Haplotype	
Samples	arms 2/3/5	ITS2†	*cyt-b*
Burkina Faso			
149–35	IN	–	2
149–37	IN	2	4
149–36	IN	–	1
152–15	IN	1‡	–
149–21	IN	–	1
149–16	IN	–	10
152–19	IN	–	3
152–20	IN	–	2
155–44	IN	–	2
149–17	ST	1‡	–
149–19	ST	–	1
149–22	ST	1	–
149–29	ST	1	1
155–17	ST	1‡	9
155–32	ST	–	5
155–50	ST	1	6
152–21	ST	1	–
Senegal			
Dielmo			
64	ST/ST/IN	1	–
1393	ST	1‡	–
1517	ST/ST/IN	1‡	–
1538	ST	1	1
Kedougou			
441	?/IN/ST	–	1
443	IN/IN/ST	1	–
444	IN	1	–
448	IN	1	–
449	IN	1	11
Kenya			
Ahero (E)	?	2	1
Nyakoch (I)	?	2	7
Asembo (A)	?	1	8

*ST, homokaryotypic standard; IN, homokaryotypic inverted or heterokaryotypic.
†ITS2 haplotype defined by first 507 bp only.
‡Entire ITS2 sequence was determined.

karyotype classes. Because it is most common and widespread, it may be the ancestral haplotype in the sample (Castelloe & Templeton 1994). The other haplotypes can be connected to this one by one or two mutational steps, shown in the minimum spanning network of Fig. 3. There is one ambiguity in the network. As drawn, the G→A mutation at position 11 045 (Table 3) is hypothesized to be homoplasious in haplotypes 4 and 8. An alternative reconstruction requires a single G→A mutation at position 11 045 in a hypothetical (not sampled) haplotype, derived from haplotype 1, which is ancestral to both haplotypes 4 and 8.

Discussion

Until very recently, few studies have focused on the genetic structure of *Anopheles funestus* populations. However, based on two observations, the expectation in this study was to find relatively structured populations. First, the preference for breeding in patchily distributed permanent bodies of water sets *An. funestus* apart from the other principal vectors, which breed in small temporary pools that are ubiquitous during the rainy season. Second, the apparent inability of *An. funestus* to recolonize some areas treated with residual insecticides for several years following cessation of spraying suggested low dispersal ability and discontinuous populations. Therefore it was surprising that the present study found no patterns of genetic variation to corroborate this view.

For resolving intraspecific phylogeography, mtDNA has been a tool of choice (Avise 1994). The fact that little phylogeographic structure of *cyt-b* haplotypes was discernible in *An. funestus* might be an artefact of small sample size and/or insufficient resolution. Only three *cyt-b* sequences from Kenya were examined, the remainder originating from the West African countries of Senegal and Burkina Faso. In addition, all but two of the 11 haplotypes were singletons (sampled in only one individual) and therefore uninformative. Although the *cyt-b* gene has not been widely used in insects, data from other anophelines suggest it to be less variable than some other mtDNA genes, such as ND5 (Besansky *et al.* 1997). The AT composition of the sequenced fragment of *cyt-b* is high (75%), similar to that of the overall mtDNA genome (78%), but the AT bias of third codon positions, which comprise most of the polymorphic sites, is acute (93%). Clearly, the study of additional markers is necessary before any firm conclusions can be drawn about *An. funestus* phylogeography. Given the data at hand, we tentatively suggest that the high haplotype diversity (0.86) and the common presence of one haplotype (no. 1; see Fig. 3) across Africa are compatible with relatively large populations that have not experienced prolonged bottlenecks or fragmentation.

This pattern of mitochondrial variation, and the average pairwise divergence of 0.42% per site, is remarkably consistent with results, across Africa, from *An. gambiae* and *An. arabiensis*, based on mtDNA *cyt-b*, ND1 and ND5 genes (Besansky *et al.* 1997). In *An. gambiae*, the mtDNA results are in agreement with allozyme and microsatellite studies (Lehmann *et al.* 1996; Lehmann *et al.* 1997), indicating that mtDNA is unlikely to be an aberrant marker in this species. If this pattern of mtDNA variation in *An. funestus* is corroborated by further studies with this and other

Species	Length	GC%	Reference
An. gambiae complex	426–7 bp	55%	Paskewitz *et al.* (1993)
An. freeborni complex	305–310 bp	≈52%	Porter & Collins (1991)
An. occidentalis	306 bp	≈52%	Porter & Collins (1991)
An. quadrimaculatus complex	287–329 bp	55–57%	Cornel *et al.* (1996)
An. nuneztovari	363–369 bp	55–56%	Fritz *et al.* (1994)
An. punctulatus complex	≈600 bp	ND	Beebe & Saul (1995)
An. dirus A and D	710–716 bp	69%	Xu & Qu (1997)
An. flavirostris, An. filipinae	≈360	ND	Foley *et al.* (1996)
An. funestus	703–705 bp	49%	Present study

Table 2 Length and base composition of anopheline internal transcribed spacer (ITS2) sequences

markers, it suggests two things. First, active dispersal by these vectors may exceed the commonly accepted 3–5 km range (Zahar 1984; see Besansky *et al.* 1997). Second, the effect of residual insecticide spraying may not have been total eradication of local *An. funestus* populations, but rather a dramatic reduction in population density without a real bottleneck, combined with a behavioural shift to outdoor resting and biting by the survivors (see Zahar 1984).

Another surprising outcome of this study was the lack of support for reproductively isolated chromosomal forms within *An. funestus*. If *An. funestus* is composed of multiple species, the apparent conflict with the molecular evidence could be resolved if it is assumed that speciation occurred quite recently. Thus, mtDNA and ITS2 haplotypes would be shared, not because of current gene flow but because of retained ancestral polymorphism and insufficient time for diagnostic mutations to become established. It is impossible to rule out this possibility in the absence of independent evidence from additional loci.

Apparent contradictions between patterns of chromosomal and ecophenotypic vs. molecular polymorphisms have been noted in other anopheline vectors. The neotropical species *An. nuneztovari* was hypothesized to consist of two allopatric taxa distinguished by peak biting times and host preferences (anthropophilic vs. zoophilic), by egg ultrastructure (Linley *et al.* 1996) and by cytotype, as defined by fixed and polymorphic inversions (reviewed in Conn *et al.* 1998). At the molecular level, mtDNA sequences of the ND2 and ND6 genes failed to differentiate the cytotypes (Perera 1993). Although restriction fragment length polymorphism (RFLP) analysis of the complete mtDNA genome placed the cytotypes in distinctive clusters (Conn *et al.* 1998), and ITS2 sequence polymorphisms were revealed (three insertion–deletion events and one transversion; Fritz *et al.* 1994), there were no cytotype-diagnostic differences. In the *An. gambiae*

H	F	1	1	1	1	1	1	1	1	1	1	1	1	1
a	r	0	0	0	1	1	1	1	1	1	1	1	1	1
p	e	9	9	9	0	0	0	0	0	0	0	1	1	2
	q	1	3	5	2	3	3	4	4	4	9	0	9	3
No.		6	1	5	4	6	9	2	5	6	6	5	5	4
1	7	A	C	T	T	C	T	A	G	C	G	T	T	C
2	3	.	T	.	.	.	.	.	.	.	.	.	.	.
3	1	.	.	.	.	.	.	.	.	.	A	.	.	.
4	1	.	T	.	.	.	.	.	A	.	.	.	.	.
5	1	.	.	.	C	.	.	.	.	.	.	.	.	.
6	1	.	.	.	.	.	.	.	.	T	.	.	.	.
7	1	.	.	.	.	.	.	G	.	.	.	.	.	.
8	1	.	.	.	.	.	.	.	A	.	.	C	.	.
9	1	G	.	.	.	.	.	.	.	.	.	.	C	.
10	1	.	.	C	.	.	.	.	.	.	.	.	.	T
11	1	.	.	.	.	T	C	.	.	.	.	.	.	.

Table 3 Polymorphic positions* defining the mitochondrial DNA (mtDNA) *cyt-b* haplotypes of *Anopheles funestus*

*Positions numbered with respect to *An. gambiae* reference sequence (GenBank acc. no.: l20934; Beard *et al.* 1993). Dots represent identity to the first sequence listed.

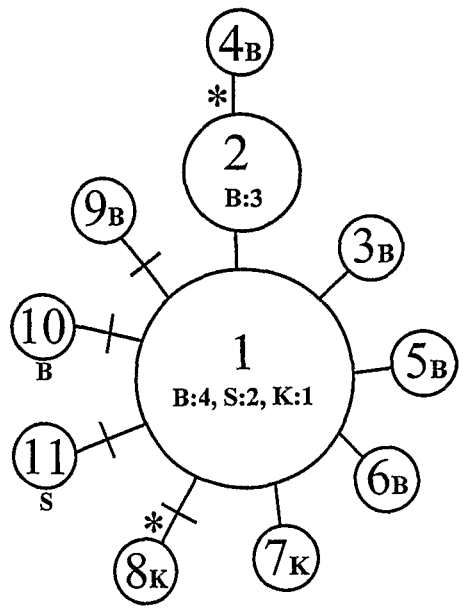

Fig. 3 Network representing minimum mutational relationships among mitochondrial *cyt-b* haplotypes. Numbered circles indicate haplotypes and letters refer to geographical origin (B, Burkina Faso; S, Senegal; K, Kenya). Circle size approximately represents haplotype frequency, which is given following the letter code if haplotype was sampled more than once. Each connecting line represents a single mutation, unless cross-hatched (= two mutations). Asterisks indicate a mutation at position 11 045 (G→A) that is homoplasious if the network accurately reflects historical relationships.

complex, *An. gambiae* and *An. arabiensis* have fixed inversion differences on the X chromosome (Coluzzi *et al.* 1979) and fixed sequence differences in the ITS2 (Paskewitz *et al.* 1993), but are indistinguishable based on mtDNA sequences of *cyt-b*, ND1 and ND5 genes and restriction mapping of the entire mtDNA genome, probably owing to small amounts of interspecific mtDNA gene flow (Besansky *et al.* 1994; Besansky *et al.* 1997; N. Besansky, P. Mehaffey & F. Collins, unpublished data). In West Africa, three ecologically distinct cytotypes of *An. gambiae* coexist: Savanna, Bamako and Mopti (Coluzzi *et al.* 1985; Toure *et al.* 1998). At the molecular level, using restriction mapping of the rDNA intergenic spacer (Favia *et al.* 1997), random amplified polymorphic DNA (RAPD)–PCR (Favia *et al.* 1994) and direct sequencing of ITS2 (O. Mukabayire, M. Coluzzi & N. Besansky, unpublished data), it has been possible to differentiate only Mopti from the other cytotypes. It is tempting to speculate that the lack of strong molecular support for within-population or even between-species heterogeneities, perceived at the behavioural and cytogenetic levels, may simply reflects a surprisingly rapid rate of change at these levels in anophelines.

An important consideration in interpreting the ITS2 sequence data is the experimental approach of determining 'consensus' sequences by direct sequencing. Implicit in this approach is the assumption that the pace of concerted evolution would be sufficiently rapid to erase mutations shared through recent common ancestry, an assumption that may be flawed on both theoretical and empirical grounds (e.g. Dover 1993 and refs. therein). It is noteworthy that *An. funestus* ITS2 electropherograms were never ambiguous, as ambiguities are expected if variant repeats are present at frequencies sufficiently high to compete with the consensus signal. This suggests that *An. funestus* ITS2 sequences are predominantly composed of one 'consensus' type, but does not rule out one or more rare variants. Analysis of rare variants requires more laborious cloning and sequencing of numerous individual repeats from each specimen, but might provide additional insights into population structure. Although direct sequencing erases information about the sequence and frequency of rare variants and therefore entails a loss of analytical power, it is unlikely to be positively misleading in the patterns it reveals.

A challenge facing future population studies of *An. funestus* is to develop molecular markers that have the potential to evolve at higher rates than rDNA ITS2 and mtDNA sequences. One candidate class of marker, microsatellites, is currently under development in our laboratories.

Acknowledgements

Mosquito collections in Burkina Faso were carried out by the entomology team of the Centre National de Lutte contre le Paludisme of Ouagadougou under the supervision of Drs Edith Sanogo and Carlo Costantini, and in Senegal with the assistance of Ibrahima Dia. We thank W. Wekesa for his contributions to the initial stages of this study. This research was supported by UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR) grants to N.J.B. and L.L. and by CNRS (Centre National de la Recherche Scientifique/France) no. 96/C/08 to D.F.

References

Avise JC (1994) *Molecular Markers, Natural History and Evolution.* Chapman and Hall, New York.

Beard CB, Hamm DM, Collins FH (1993) The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. *Insect Molecular Biology*, **2**, 103–124.

Beebe NW, Saul A (1995) Discrimination of all members of the *Anopheles punctulatus* complex by polymerase chain reaction–restriction fragment length polymorphism analysis. *American Journal of Tropical Medicine and Hygiene*, **53**, 478–481.

Besansky NJ, Lehmann T, Fahey GT *et al.* (1997) Patterns of mitochondrial variation within and between African malaria vectors, *Anopheles gambiae* and *An. arabiensis*, suggest extensive gene flow. *Genetics*, **147**, 1817–1828.

Besansky NJ, Powell JR, Caccone A, Hamm DM, Scott JA, Collins FH (1994) Molecular phylogeny of the *Anopheles gambiae* complex suggests genetic introgression between principal malaria vectors. *Proceedings of the National Academy of Sciences of the USA*, **91**, 6885–6888.

Boccolini D, Sabatini A, Sanogo E, Sagnon N, Coluzzi M, Costantini C (1994) Chromosomal and vectorial heterogeneities in *Anopheles funestus* from Burkina Faso, West Africa. *Parassitologia*, **36** (Suppl. 1), 20.

Castelloe J, Templeton AR (1994) Root probabilities for intraspecific gene trees under neutral coalescent theory. *Molecular Phylogenetics and Evolution*, **3**, 102–113.

Collins FH, Mendez MA, Rasmussen MO, Mehaffey PC, Besansky NJ, Finnerty V (1987) A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *American Journal of Tropical Medicine and Hygiene*, **37**, 37–41.

Collins FH, Paskewitz SM (1996) A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect Molecular Biology*, **5**, 1–9.

Collins FH, Paskewitz SM, Finnerty V (1989) Ribosomal RNA genes of the *Anopheles gambiae* species complex. *Advances in Disease Vector Research*, **6**, 1–28.

Coluzzi M, Petrarca V, Di Deco MA (1985) Chromosomal inversion intergradation and incipient speciation in *Anopheles gambiae*. *Bulletin Zoologia*, **52**, 45–63.

Coluzzi M, Sabatini A, Petrarca V, Di Deco MA (1979) Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **73**, 483–497.

Conn JE, Mitchell SE, Cockburn AF (1998) Mitochondrial DNA analysis of the neotropical malaria vector *Anopheles nuneztovari*. *Genome*, **41**, 313–327.

Cornel AJ, Porter CH, Collins FH (1996) Polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. *Journal of Medical Entomology*, **33**, 109–116.

Dover GA (1993) Evolution of genetic redundancy for advanced players. *Current Opinions in Genetics and Development*, **3**, 902–910.

Favia G, della Torre A, Bagayoko M, Lanfrancotti A, Sagnon N, Toure YT, Coluzzi M (1997) Molecular identification of sympatric chromosomal forms of *Anopheles gambiae* and further evidence of their reproductive isolation. *Insect Molecular Biology*, **6**, 377–383.

Favia G, Dimopoulos G, della Torre A, Toure YT, Coluzzi M, Louis C (1994) Polymorphisms detected by random PCR distinguish between different chromosomal forms of *Anopheles gambiae*. *Proceedings of the National Academy of Sciences of the USA*, **91**, 10315–10319.

Foley DH, Beebe N, Torres E, Saul A (1996) Misidentification of a Philipine malaria vector revealed by allozyme and ribosomal DNA markers. *American Journal of Tropical Medicine and Hygiene*, **54**, 46–48.

Fontenille D, Rakotoarivony I (1988) Reappearance of *Anopheles funestus* as a malaria vector in the Antananarivo region, Madagascar. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **82**, 644–645.

Fritz GN, Conn J, Cockburn A, Seawright J (1994) Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). *Molecular Biology and Evolution*, **11**, 406–416.

Genetics Computer Group (1994) Genetics Computer Group, Inc., Madison, WI.

Gillies MT, De Meillon B (1968) *The Anophelinae of Africa South of the Sahara*. South African Institute for Medical Research, Johannesburg, South Africa.

Green CA, Hunt RH (1980) Interpretations of variation in ovarian polytene chromosomes of *Anopheles funestus* Giles, *An. parensis* Gillies *and An. aruni? Genetica*, **51**, 187–195.

Kumar A, Rai KS (1990) Chromosomal localization and copy number of 18S + 28S ribosomal RNA genes in evolutionarily diverse mosquitoes (Diptera, Culicidae). *Hereditas*, **113**, 277–289.

Kumar S, Tamura K, Nei M (1993) MEGA: *Molecular Evolutionary Genetics Analysis Version* 1.01. The Pennsylvania State University, University Park, PA.

Lehmann T, Besansky NJ, Hawley WA, Fahey TG, Kamau L, Collins FH (1997) Microgeographic structure of *Anopheles gambiae* in western Kenya based on mtDNA and microsatellite loci. *Molecular Ecology*, **6**, 243–253.

Lehmann T, Hawley WA, Kamau L, Fontenille D, Simard F, Collins FH (1996) Genetic differentiation of *Anopheles gambiae* populations from East and West Africa: comparison of microsatellite and allozyme loci. *Heredity*, **77**, 192–200.

Linley JR, Lounibos LP, Conn J, Duzak D, Nishimura N (1996) A description and morphometric comparison of eggs from eight geographic populations of the South American malaria vector *Anopheles (Nyssorhynchus) nuneztovari* (Diptera: Culicidae). *Journal of the American Mosquito Control Association*, **12**, 275–292.

Lyman DF, Escalante AA, Cordon-Rosales C, Wesson DM, Dujardin J-P, Beard CB (1998) Mitochondrial DNA sequence variation among triatomine vectors of Chagas Disease. *American Journal of Tropical Medicine and Hygiene*, **in press**.

McLain DK, Collins FH, Brandling-Bennett AD, Were JB (1989) Microgeographic variation in rDNA intergenic spacers of *Anopheles gambiae* in western Kenya. *Heredity (Edinburgh)*, **62**, 257–264.

Molineaux L, Gramiccia G (1980) *The Garki Project: Research on the Epidemiology and Control of Malaria in the Sudan Savanna of West Africa*. World Health Organization, Geneva.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York, NY.

Paskewitz SM, Wesson DM, Collins FH (1993) The internal transcribed spacers of ribosomal DNA in five members of the *Anopheles gambiae* species complex. *Insect Molecular Biology*, **2**, 247–257.

Perera OP (1993) Phylogenetic analysis of two mitochondrial genes from several species of the subgenus *Nyssorhynchus* (Culicidae: Anopheles) and the development of species-specific DNA probes for their identification. PhD Thesis. University of Florida, Gainesville, Florida, pp. 110.

Porter CH, Collins FH (1991) Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). *American Journal of Tropical Medicine and Hygiene*, **45**, 271–279.

Rozas J, Rozas R (1997) DNASP version 2.0: a novel software package for extensive molecular population genetics analysis. *Computer Applications in Bioscience*, **13**, 307–311.

Toure YT, Petrarca V, Traore SF *et al.* (1998) Distribution and inversion polymorphism of chromosomally recognized taxa of the *Anopheles gambiae* complex in Mali, West Africa. *Parassitologia*, in press.

Trape JF, Rogier C, Konate L *et al.* (1994) The Dielmo project: a longitudinal study of natural malaria infection and the mechanisms of protective immunity in a community living in a holoendemic area of Senegal. *American Journal of Tropical Medicine and Hygiene*, **51**, 123–137.

Wesson DM, Porter CH, Collins FH (1992) Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae). *Molecular Phylogenetics and Evolution*, **1**, 253–269.

Xu JN, Qu FY (1997) Ribosomal DNA difference between species A and D of the *Anopheles dirus* complex of mosquitoes from China. *Medical and Veterinary Entomology*, **11**, 134–138.

Zahar AR (1984) *Vector Bionomics in the Epidemiology and Control of Malaria, Part 1*. World Health Organization, Geneva.

This work is part of an international collaboration focused on population and evolutionary genetics of African malaria vectors, involving investigators from the Universities of Notre Dame and Rome, ORSTOM and KEMRI. The long-term objective of these studies is the development of effective strategies for insecticide resistance management and vector control, including strategies based on genetic manipulation of natural vector populations.

ISSN 0962-1083

VOLUME 8
NUMBER 2
FEBRUARY
1999

MOLECULAR ECOLOGY

Published by Blackwell Science