Sequence analysis of mitochondrial 16S ribosomal RNA gene fragment from seven mosquito species

YOGESH S SHOUCHE* and MILIND S PATOLE

National Center for Cell Science, Pune University Campus, Pune 411 007, India

**Corresponding author (Fax, 91-20-5672259; Email, yogesh@nccs.res.in).*

Mosquitoes are vectors for the transmission of many human pathogens that include viruses, nematodes and protozoa. For the understanding of their vectorial capacity, identification of disease carrying and refractory strains is essential. Recently, molecular taxonomic techniques have been utilized for this purpose. Sequence analysis of the mitochondrial 16S rRNA gene has been used for molecular taxonomy in many insects. In this paper, we have analysed a 450 bp hypervariable region of the mitochondrial 16S rRNA gene in three major genera of mosquitoes, *Aedes*, *Anopheles* and *Culex*. The sequence was found to be unusually A + T rich and in substitutions the rate of transversions was higher than the transition rate. A phylogenetic tree was constructed with these sequences. An interesting feature of the sequences was a stretch of Ts that distinguished between *Aedes* and *Culex* on the one hand, and *Anopheles* on the other. This is the first report of mitochondrial rRNA sequences from these medically important genera of mosquitoes.

1. Introduction

Mosquitoes serve as obligate intermediate hosts for numerous diseases that collectively represent a major cause of human mortality and morbidity worldwide. There is a total of 34 genera and 3100 species of mosquitoes out of which three genera, *Anopheles*, *Aedes* and *Culex*, are the primary vectors for pathogens owing to their obligate haematophagy. The pathogens carried by mosquitoes mainly include the malaria parasite (*Plasmodium*), filaria (*Wucheria* and *Brugia*) and arboviruses. The classification of mosquitoes into the subfamilies Anopheliae and Culiciea is based on their oviposition, morphology of larvae (siphon and pamale hair) and pupae, breathing trumpet shape and size, resting angle of adults, shapes of proboscis. Taxonomically, mosquito classification based on above criteria is in a confused state. Genome organization studies have aided in understanding the systematics and evolution of mosquitoes. These studies are performed by making use of several molecular features such as DNA content, chromosomal and mitochondrial DNA organization, DNA sequences of ITS (internal transcribed spacer) and IGS (inter genic sequences) (Bensansky and Collins 1992; Hill and Crampton 1994).

Vector control remains the most successful strategy for the suppression of mosquito-borne diseases. Indiscriminate use of insecticides has resulted in the development of pesticide resistant strains and diminished the effectiveness of biopesticides (Raymond *et al* 1991; Roush 1993). The problem has become acute due to the rapid spread of *Plasmodium* strains that exhibit multiple resistance to human antimalarial drugs (Spencer 1985; Schapira *et al* 1993). An alternative strategy for vector control could be to exploit observed genetic variability in the vector populations. Not all mosquito-malaria 'pairings' are successful; interactions between the two are under genetic control (Collins *et al* 1986; Miller and Mitchell 1991; Wallis *et al* 1985; Vernick *et al* 1989). A detailed understanding of

Keywords. Mitochondrial 16S rRNA; mosquito; polymerase chain reaction; sequence analysis

Abbreviations used: ITS, Internal transcribed spacer; IGS, inter genic sequences; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA; *An. step*, *Anopheles stephensi*; *An. quad*, *Anopheles quadrimaculatus*; *Ae. aegy*, *Aedes aegypti*; *Ae. walb*, *Aedes w-albus*; *Ae. albo*, *Aedes albopictus*; *Cx. quin*, *Culex quinquifasciatus*; *Cx. trit*, *Culex tritaeniorhynchus*; *Cx. bita*, *Culex bitaeniorhynchus*.

the reasons for the failure of transmission may both highlight normal mechanisms of successful transmission and also pave the way for the novel vector control strategies. Such studies are limited due to the limited knowledge of genome structure and complexity of mosquito species (Sververson *et al* 1994). There is a need to develop molecular markers that would correspond with susceptibility or refractoriness to infection (Hill and Crampton 1994).

The mitochondrial DNA of mammals has been used for molecular evolution studies (Adoutte 1999) and recently similar techniques have been applied to insects as well (Xiang and Kocher 1991; Kambhampati 1995, Tang *et al* 1996; Tang and Unnasch 1997). Because of its high rate of evolution, mitochondrial DNA is an extremely useful molecule for high resolution analysis of evolutionary processes (Brown *et al* 1979). It has been used in the phylogenetic analysis of insects (Misof *et al* 2000), amphibians (Morita 1999) and fishes (Richards and Moore 1996). The sequence of ribosomal RNA molecules has been widely used for phylogenetic studies and sequence differences in hypervariable regions reflect strain variations. In this paper we have studied sequence variation in polymerase chain reaction (PCR) amplified 16S rRNA mitochondrial gene fragments of mosquitoes. A 500 bp fragment of mitochondrial 16S rRNA gene was amplified using universal primers and was further sequenced. The same 16S rRNA region has been used earlier for taxonomic studies on blackflies (Xiang and Kocher 1991).

2. Experimental procedures

Single larvae or adults of the mosquitoes were used for DNA extraction from *Anopheles stephensi*, *Aedes albopictus*, *Aedes aegypti*, *Culex tritaeniorhynchus* and *Culex quinquafaciatus*. For *Aedes w-albus* and *Culex bitaeniorhynchus* cell lines developed from the corresponding mosquito species were used as a source of DNA. The methods for DNA extraction were as described previously (Kshirsagar *et al* 1997, 1998). In each reaction 0·2 μM each of primer A (5′-CGCCTGTTTATCAAAAACAT-3′, nucleotide positions 13297 to 13278 with respect to *Anopheles quadrimaculatus* mitochondrial genome sequence, Mitchell *et al* 1993) and primer B (5′-CTCCGGTTT GAACTCAGATC-3′, position 12747 to 12766) were used. Amplification was carried out for 35 cycles with *Taq* polymerase (Perkin-Elmer, Branchburg, NJ, USA) using annealing temperature at 55°C for 30 s and extension at 72°C for 1 min. The PCR products were purified to remove unincorporated primers and nucleotides, either by Microcon spin columns (Amicon Inc. Beverly, MA, USA) or by PEG 8000 (20% in 2·5 M NaCl) precipitation prior to sequencing. Sequencing was carried out using a Gibco-BRL dsDNA Cycle Sequencing System (Gaithersburg, MD, USA) under conditions as specified by the company. PCR primers were used for the sequencing, and for complete sequencing of 500 bp 16S rRNA fragment, two new internal primers were designed. Each sequence was confirmed by repeating the reaction at least five times with independent PCR products. The sequences for *A. quadrimaculatus* (Mitchell *et al* 1993) and *A. albopictus* (HsuChen *et al* 1984) 16S rRNA were available in the literature. Sequence alignment was performed using CLUSTAL V (Higgins 1992). Juke-Cantor distances were calculated and a phylogenetic tree was constructed in MEGA (Kumar *et al* 1994) using the UPMGA method. Bootstrapping was done for 1000 replicates. Phylogenetic trees were also constructed using Kimura 2 parameter distances (Kimura 1980) and results were identical when both transition plus transversions or only transversions were used for the analyses.

3. Results and discussion

3.1 *PCR amplification of rRNA fragments*

PCR amplification of total mosquito DNA with universal 16S rRNA primers yielded a fragment of 500 bp. This product was directly used for sequencing after removal of unincorporated primers and dNTPs. In the manual sequencing only a total of 250 bases could be read from either end. In order to fill up the internal gap, a new set of primers was designed from the conserved regions after alignment of sequences. These were primer C: 5′-GAATGAGATATATACTGTC-3′ (position 13156 to 13140) and primer D: 5′-CTTTTTTGTCGATAAGAAC TCT-3′ (position 12852 to 12873). These primers could be used successfully for sequencing 16S rRNA gene fragments of all the mosquito species studied. With the use of this additional set of primers, complete overlapping sequence of around 450 bases was obtained.

The GeneBank accession numbers for these sequences are as follows: *A. stephensi*–AF034467, *C. tritaeniorhynchus*–AF034469, *A. w-albus*–AF034472, *C. bitaeniorhynchus*–AF034474, *A. aegypti*–AF034475, and *C. quinquifasciatus*–AF034476.

3.2 *Salient features of the 16S rRNA gene sequence of mosquitoes*

3.2a *Nucleotide base composition*: The nucleotide base composition of the sequenced rRNA segments showed that – like most other insect mitochondrial rRNA genes (Xiang and Kochar 1991; Kambhampati 1995) – the mosquito genome too has a high A + T content. It was between 75% and 78% for all the species studied in this paper.

3.2b *Transition versus transversion substitutions*: The number of differences between the sequenced fragments varied from 5 to 44 over a mean total length of 450 bases. An interesting feature of the sequence is a stretch of Ts. The length of the stretch is 7 bases in *Anopheles*, 9 in *Aedes* and *Culex* (figure 1) and 11 in moths (unpublished data). Observed nucleotide differences between each pair of mosquito species were used for calculating the frequencies of nucleotide substitutions and transition and transversion rates. The transition to transversion ratio varied from 0·143 to 0·727. The most frequent transversions were of the A-T type. Other types were very rare and accounted for only 10% of the transversion differences. Similar observations have been made in 16S rRNA genes of leafhoppers (Fang *et al* 1993), blackflies (Xiang and Kocher 1991), *Drosophila* (DeSalle 1992) and cockroaches (Kambhampati 1995). Studies on the NADH I gene of *Drosophila* (DeSalle 1992) and the cytochrome oxidase genes of ten insect orders (Liu and Beckenbach 1992) also showed A-T as the predominant type of transversion. In contrast, in primates 92% of the substitutions are transitions (Brown *et al* 1982). The difference could be due to deficient mitochondrial DNA repair mechanisms and tautomeric base pairing chemistry (Topal and Fresco 1976). Alternatively, a high A + T content could be imposing constrains on the sequence (Xiang and Kochar 1991).

The differences were not distributed evenly in the fragment but were located in particular regions. When the average trasversion frequency was calculated between two genera, it was found to be 18·3 between *Culex* and *Aedes*, 22·1 between *Culex* and *Anopheles* and 22.3 between *Anopheles* and *Aedes*. When the same transversion frequency was analysed within each species, it was the least in *Culex* (8.0) followed by *Aedes* (13·8) and *Anopheles* (18.0). This also implies that species of *Anopheles* are more distantly related to each other than species of the genera *Aedes* or *Culex*.

3.3 *Phylogenetic inferences*

Table 1 shows the distance matrix analysis for the mosquito species used in these studies. Studies on nuclear DNA content and chromosome size have shown that *Anopheles* differs significantly from other mosquito species (Rao and Rai 1990). The results from mitochondrial rRNA sequence support this observation. Also, as mentioned, species of *Anopheles* show a greater divergence among themselves than species of *Culex* or *Aedes*. Figure 2 shows the phylogenetic tree drawn from the sequence data. The grouping of genera and species in this tree is similar to the previously reported tree based on nuclear rRNA RFLPs (Rao and Rai 1990).

Classification of insect species is critical for both basic and applied research. The classification based on morphological features poses problems in the case of many groups because of their small size, morphological attributes that change as function of environment and prevalence of biotypes and species that cannot be easily differentiated by morphological criteria. There have been many attempts to use molecular taxonomy techniques to insects and these have yielded valuable results (Xiang and Kochar 1991; Fang 1993; Kambhampati 1995; Tang *et al* 1996). Heteroduplex analysis studies on mitochondrial NADH dehydrogenase subunit 4 gene of wild caught blackflies carrying *Onchocerca volvolus* infective larvae have led to the discovery of new alleles that allowed grouping of the individual flies carrying these alleles to the *Simulium damnosum s.j*. sibling species (Tang *et al* 1995).

In mosquitoes studies have been carried out using sequences of nuclear rDNA genes, internal transcribed spacers of rDNA, mitochondrial DNA and various random amplified polymorphic DNA (RAPD), and RFLP markers (Apostol 1994; Ballinger-Crabtree *et al* 1992; Black *et al* 1989; Collins 1990; Cooper *et al* 1991; Kambhampati and Rai 1991a, b; Porter and Collins 1991; Sververson *et al* 1994). These studies have unequivocally proved the

```
Cx. trit    GGATAACAGC  GTAATTTTTT  TTTAGAGTTC  ATATCGAGAA
Cx. bita    ..........  ..........  ..........  .......C..
Cx. quin    .C.CT.....  ..........  ..........  ..........
Ae. albo    ..........  ..........  ..........  T.........
Ae. walb    ..........  ..........  ..........  T......C..
Ae. aegy    ..........  ..........  ..........  ..........
An. quad    ..........  ..........  .AG.......  T......T..
An. step    .....C--A.  ..........  .AG.......  T......T..
```

Figure 1. Part of the sequence depicting a stretch of Ts in the mosquito species studied. The bases that show characteristic differences are underlined.

Table 1. Distance values for mosquito species.

	An. quad	*An. step*	*Ae. albo*	*Ae. walb*	*Ae. aegy*	*Cx. trit*	*Cx. bita*	*Cx. quin*
An. quad	0							
An. step	0·0716	0						
Ae. albo	0·0944	0·0937	0					
Ae. walb	0·0766	0·089	0·0419	0				
Ae. aegy	0·0855	0·1127	0·0586	0·043	0			
Cx. trit	0·0856	0·1005	0·0747	0·064	0·0809	0		
Cx. bita	0·0833	0·0984	0·0681	0·0596	0·0788	0·0176	0	
Cx. quinq	0·0899	0·1075	0·0877	0·0705	0·0921	0·0175	0·0307	0

S.d's for distance values were in the range of ± 0·005–0·018.

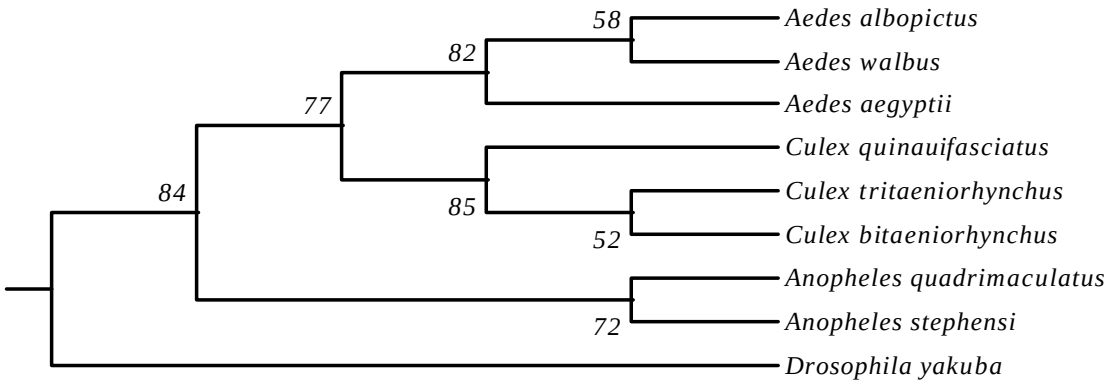

Figure 2. Phylogenetic tree constructed from the 16S rRNA sequences used in this paper. The sequences were aligned in Clustal V and the tree was constructed in molecular evolutionary genetics analysis (MEGA) using the neighbour joining method. Numbers at the nodes indicate bootstrap values. The *D. yakuba* sequence serves as an outlier.

importance and value of DNA based methods. It has been observed that though the species *Anopheles scutellaris* and *Anopheles albopictus* do not exhibit any significant morphological divergence, their mitochondrial DNA shows significant variation when analysed by RFLP. PCR amplification using random primers could differentiate between three chromosomal forms of *Anopheles gambiae* (Favia *et al* 1994). DNA fingerprinting could not only distinguish between *A. gambiae* and *Anopheles arabiensis* (Wilkerson *et al* 1993) but also between members of species complex of *A. aegypti* (Ballinger-Crabtree *et al* 1992; Kambhampati *et al* 1992).

In this work, we have extended these studies to the mitochondrial ribosomal RNA gene. Such studies have been done earlier in cockroaches (Kambhampati 1995), black flies (Xiang and Kochar 1991; Tang *et al* 1996) and other insects (Kambhampati 1995; Fang *et al* 1993; DeSalle 1992) but there are no reports of similar studies in mosquitoes. As a first step, we have analysed a hypervariable region of representative species of three important genera, *Anopheles*, *Aedes* and *Culex*. The gene fragment can be amplified from single larvae or adults and thus the technique can be used in field studies. Analysis of this and other hypervariable regions may permit an investigation of genetic relatedness of mosquito populations at the subspecific levels. The studies are being extended to different strains of species and also to include entire 12S and 16S rRNA genes of mitochondria. The analysis will be valuable in studies involving molecular taxonomy, particularly for those species that are difficult to identify using morphologic characteristics and in epidemiological research. Sequence comparisons of different geographic populations will give estimates of their genetic relatedness and provide information about vector movement. Comparison of sequences of pathogen carrying and refractory strains of the same species should provide clues about the vectorial capacity of these strains.

Acknowledgements

We acknowledge the help provided by Bioinformatics Centre, University of Pune, in the analysis of sequences.

We wish to thank Prakash and Vyankatesh for reading the manuscript and the discussions and to Supriya for providing some of the genomic DNAs used.

References

Adoutte A, Balavoine G, Lartillot N and Rosa R 1999 Animal evolution the end of the intermediate taxa?; *Trends Genet.* **15** 104–108

Apostol B L, Black W C IV, Reiter P and Miller B R 1994 Use of randomly amplified polymorphic DNA amplified by polymerase chain reaction markers to estimate the number of *Aedes aegypti* families at oviposition sites in San Juan, Puerto Rico; *Am. J. Trop. Med. Hyg.* **51** 89–97

Ballinger-Crabtree M E, Black W C IV and Miller B R 1992 Use of genetic polymorphism detected by the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) for differentiation and identification of *Aedes aegypti* subspecies and populations; *Am. J. Trop. Med. Hyg.* **47** 893–901

Bensansky N J and Collins F H 1992 The mosquito genome Organisation Evolution and Manipulation; *Parasitology Today* **8** 186–191

Black W C IV, McLain D K and Rai K S 1989 Patterns of variation in the rDNA cistron within and among world populations of a mosquito *Aedes albopictus* (Skuse); *Genetics* **121** 539–550

Brown W M, Prager E M, Wang A and Wilson A C 1982 Mitochondrial DNA sequences of primates: tempo and mode of evolution; *J. Mol. Evol.* **18** 225–239

Brown W M, Matthew G Jr and Wison A C 1979 Rapid evolution of mitochondrial DNA; *Proc. Natl. Acad. Sci. USA* **76** 1967–1971

Collins F H, Sakai R K, Vernick K D, Paskewitz S, Seeley D C, Miller L H, Collins W E, Campbell C C and Gwadz R W 1986 Genetic selection of a *Plasmodium* refractory strain of the malaria vector *Anopheles gambiae*; *Science* **234** 607–610

Collins F H, Porter C H and Stanton E Cope 1990 Comparison of rDNA and mtDNA in the sibling species *Anopheles freeborni* and *A. hermsi*; *Am. J. Trop. Med. Hyg.* **42** 417–423

Cooper L, Cooper R D and Burkot T R 1991 The *Anopheles punctulatus* complex: DNA probes for identifying the Australian species using isotopic, chromogenic and chemiluminescence detection systems; *Exp. Parasitol.* **73** 27–35

DeSalle R 1992 The phylogenetic relationships of flies in the family Drosophiladae deduced from mtDNA sequences; *Mol. Phylogenetic. Evol.* **1** 31–40

Fang Q, Black W C IV Blocker H D and Whitcomb R F 1993 A phylogeny of New World Deltocephalus like leafhopper genera based on mitochondrial 16S ribosomal DNA sequences; *Mol. Pylogenet. Evol.* **2** 119–131

Favia G, Dimopoulos G, Torre A D, Toure Y T and Coluzzi M 1994 Polymorphisms detected by random PCR distinguish between different chromosomal forms of *Anopheles gambiae*; *Proc. Natl. Acad. Sci. USA* **91** 10315–10319

Higgins D G 1992 Clustal V: improved software for multiple sequence alignments; *Comput. Appl. Biosci.* **8** 189–191

Hill S M and Crampton J M 1994 DNA based methods for the identification of insect vectors; *Ann. Trop. Med. Parasitol.* **88** 227–250

HsuChen Chuen-Chin, Kotin R M and Dubin D T 1984 Sequences of coding and flanking regions of the large ribosomal subunit RNA gene of mosquito mitochondria; *Nucleic Acids Res.* **12** 7771–7785

Kambhampati S 1995 A phylogeny of cockroaches and related insects based on DNA sequence of mitochindrial ribosomal RNA genes; *Proc. Natl. Acad. Sci. USA* **92** 2017–2020

Kambhampati S and Rai K S 1991a Mitochondrial DNA variation within and among populations of the mosquito *Aedes albopictus*; *Genome* **34** 288–292

Kambhampati S and Rai K S 1991b Variation in mitochondrial DNA of *Aedes* species (Diptera: Culicidae); *Evolution* **45** 120–129

Kambhampati S, Black W C IV and Rai K S 1992 Random Amplified Polymorphic DNA of mosquito species and populations (Diptera: Culicidae): Techniquess, Statistical Analysis, and applications; *J. Med. Entomol.* **29** 939–945

Kimura M 1980 A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences; *J. Mol. Evol.* **16** 111–120

Kshirsagar S G, Patole M S and Shouche Y S 1998 Insect cell line authentication by denaturing gradient gel electrophoresis; *In Vitro Cell. Dev. Biol. Anim.* **34** 665–667

Kshirsagar S G, Patole M S and Shouche Y S 1997 Characterization of insect cell lines: heteroduplex analysis employing a mitochondrial 16S ribosomal RNA gene fragment; *Anal. Biochem.* **253** 65–69

Kumar S, Tamura K and Nei M 1994 MEGA: Molecular Evolutionary Genetic Analysis software for microcomputers; *Comput. Appl. Biosci.* **10** 189–191

Liu H and Beckenbach A T 1992 Evolution of mitochondrial cytochrome oxidase II gene among 10 orders of insects; *Mol. Phylogenet. Evol.* **1** 41–52

Miller B R and Mitchell C J 1991 Genetic selection of a flavivirus refractory strain of the yellow fever mosquito *Aedes aegypti*; *Am. J. Trop. Med. Hyg.* **45** 399–407

Misof B, Anderson C L and Hadrys H 2000 A phylogeny of the damselfly genus calopteryx (Odonata) using mitochondrial 16S rDNA markers; *Mol. Phylogenet. Evol.* **15** 5–14

Mitchell S E, Cockburn A F and Seawright J A 1993 The mitochindrial genome of *Anopheles quadrimaculatus* species A: complete nucleotide sequence and organization; *Genome* **36** 1058–1073

Morita T 1999 Molecular phylogenetic relationships of the deep-sea fish genus Coryphaenoides (Gadiformes: Macrouridae) based on mitochondrial DNA; *Mol. Phylogenet. Evol.* **13** 447–454

Porter C H and Collins F H 1991 Species diagnostic difference in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae); *Am. J. Trop. Med. Hyg.* **45** 271–279

Rao P N and Rai K S 1990 Genome evolution in the mosquitoes and other closely related members of superfamily Culicoidea; *Heriditas* **113** 139–144

Raymond M, Callaghan A, Fort P and Pasteur N 1991 Worldwide migration of amplified insecticide resistance genes in mosquitoes; *Natrure* (*London*) **350** 151–153

Richards C M and Moore W S 1996 A phylogeny for the African treefrog family Hyperoliidae based on mitochondrial rDNA; *Mol. Phylogenet. Evol.* **5** 522–532

Roush R T 1993 Occurrence, genetics and management of insecticide resistance; *Parasitology Today* **9** 174–179

Schapira A, Beales P F and Halloran M E 1993 Malaria: living with drug resistance; *Parasitology Today* **9** 168–174

Spencer H C 1985 Drug resistant malaria – changing patterns means difficult decisions; *Trans. R. Soc. Trop. Med. Hyg.* **79** 748–758

Sververson D W, Mori, Akoi, Ying Zhang and Christensen B M 1994 The suitability of restriction fragment length polymorphism markers for evaluating genetic diversity among and synteny between mosquito species; *Am. J. Trop. Med. Hyg.* **50** 425–432

Tang J, Toe L, Back C and Unnasch T R 1995 Mitochondrial alleles of *Simulium damnosum sensu lato* infected with *Onchocerca volvulus*; *Int. J. Parasitol.* **25** 1251–1254

Tang J, Pruess K, Cupp E W and Unnasch T R 1996 Molecular phylogeny and typing of blackflies (Diptera: Simuliidae) that serve as vectors of human or bovine onchocerciasis; *Med. Vet. Entomol*. **10** 228–234

Tang J and Unnasch T R 1997 Heteroduplex analysis in medical entomology: A rapid and sensitive sequence based tool for population and phylogenetic studies; *Parasitology Today* **13** 271–274

Topal M D and Fresco J R 1976 Complementary base pairing and the origin of substitution mutations; *Nature* (*London*) **263** 285–289

Vernick K D, Collins F H and Gwadz R W 1989 A general system of resistance to malaria infection controlled in *Anopheles gambiae* controlled by two main genetic loci; *Am. J. Trop. Med. Hyg.* **40** 585–592

Wallis G P, Aitken T H, Beaty B J, Lovenz L, Amato G D and Tabachnik W J 1985 Selection for susceptibility and refractoriness of *Aedes aegypti* to oral infection with yellow fever virus; *Am. J. Trop. Med. Hyg.* **34** 1225–1231

Wilkerson R C, Parsons T J, Albright D G, Klein T A and Braun M J 1993 Random amplified polymorphic DNA (RAPD) markers readily distinguish cryptic mosquito species (Diptera: Culicidae: Anopheles); *Insect Mol. Biol.* **1** 205–211

Xiang B and Kochar T D 1991 Comparison of mitochondrial DNA sequences of seven morphospecies of black flies (Dioptera); *Genome* **34** 306–311

MS received 27 June 2000; accepted 20 September 2000

Corresponding editor: DEEPAK PENTAL