Distribution of the transposable element *mariner* in anopheline mosquitoes

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We have surveyed the distribution of the transposable element *mariner* using PCR in 23 species of *Anopheles* mosquitoes, including all of the most important vectors of malaria in South-east Asia. Sequencing of the nine positive species revealed elements from the irritans, mauritiana and mellifera subfamilies. These are the first data showing the presence of three subfamilies of *mariners* in anophelines. The elements we encountered are likely to be inactive, based on the presence of multiple stop codons and/or frameshifts.

Keywords: horizontal transfer, *mariner*, Thailand, transgenic mosquitoes, transposon.

Introduction

Transposable elements (TEs) are a class of dispersed, repetitive DNA sequences, named because of their ability to change chromosomal position, that are a ubiquitous feature of eukaryote genomes (Berg & Howe, 1989).

It has been suggested that the transmission of insect-borne diseases may be interrupted by replacing insect vector populations with genetically engineered individuals that are unable to transmit disease (Curtis, 1994). Transposable elements may aid this proposal in three ways. The ability of active elements to be inherited by more than the Mendelian 50% of offspring may be used to drive disease refractoriness genes into natural vector populations. Active elements may be used in the laboratory to transform the germline, as with *P* elements (Rubin & Spradling, 1982) and *mariner* in *Drosophila* (Lidholm *et al.*, 1993; Lohe & Hartl, 1996) and *Aedes* mosquitoes (Coates *et al.*, 1998). Inactive elements, whose insertion sites remain sufficiently polymorphic between different laboratory strains or natural insect populations, may be used for genetic mapping and the analysis of insect population structure.

Active elements may cause deleterious mutations upon transposition, and thus a conflict exists between TEs and the rest of the nuclear genome. Mechanisms that inactivate or eliminate TEs will be selected (Warren & Crampton, 1994), and an 'arms race' is expected to ensue. Such mechanisms are expected to suppress movement of all members of the same TE family

(Kidwell, 1992). As a result, a particular TE may not be able to transform insects that already possess that TE family (Warren & Crampton, 1994). It is therefore essential to know the native complement of TEs in natural populations of insect vectors before transformation procedures are attempted.

We have studied the distribution and evolution of the *mariner* TE in 23 species of *Anopheles* (Diptera: Culicidae) including all of the most important vectors of malaria in South-east Asia. The African malarial mosquito, *An. gambiae sensu stricto* is known to possess at least 100 copies of *mariner* in all strains examined (Robertson & Lampe, 1995; Mukabayire & Besansky, 1996) but data have not been reported for any other species of malarial vector.

The *mariner* family of TEs consists of sequences of approximately 1.3 kb, with a single open reading frame and inverted terminal repeats of 28 bp, which are features characteristic of the DNA-mediated class of transposable elements (Hartl, 1989). The first *mariner* elements to be discovered, including the autonomous element *Mos1*, were isolated from *Drosophila mauritiana* (Jacobson *et al.*, 1986). The vast majority of elements discovered so far contain multiple mutations (Maruyama & Hartl 1991a,b; Robertson & MacLeod, 1993; Robertson, 1993) and can therefore be considered to be pseudogenes. At least some nonautonomous elements are probably capable of transposition by means of *trans* complementation by autonomous elements; however, this has not been demonstrated directly.

Mariner elements have a wide phylogenetic distribution. They have been detected in a variety of arthropods

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as well as, for example, nematodes, hydras, flatworms and humans (Robertson *et al.*, 1997). In a PCR survey of 404 arthropod species, 63 were found to contain *mariner* (Robertson & MacLeod, 1993). The elements can be classified into at least five major subfamilies, which are 40–56% identical at the nucleotide level, and 16 minor subfamilies (Robertson *et al.*, 1997).

Materials and methods

Mosquito specimens

Adult female mosquitoes were collected from Thailand between November 1994 and November 1997, using mosquito landing collection techniques. Species were identified using the morphological keys of Harrison & Scanlon (1975), Peyton & Scanlon (1966) and Rattanarithikul & Green (1986). Members of the *An. dirus* complex were identified to species level by dot blot hybridization, using modifications to the methods of Panyim *et al.* (1988) and Audtho *et al.* (1995), described below.

Genomic DNA was prepared using the crude STE method of Robertson (1993). Individual insects were ground in 100 μ l of STE (50 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA), heated to 95°C for 10 min and spun at 13 000 r.p.m. for 3 min. The supernatant was removed to a fresh tube and stored at –20°C. Nylon membranes (GeneScreenPlus, Dupont, Wilmington, DL) were soaked in distilled water then in 10 $\times$ SSC solution (1.5 M NaCl, 0.15 M sodium citrate) for 15 min. The membrane was placed on a stack of 3 MM Whatman filter paper and spotted with genomic DNA, which was denatured by soaking twice in 0.5 N NaOH for 5 min, neutralized by soaking twice in 0.5 M Tris-HCl pH 7.4/1.5 M NaCl for 5 min, then blotted onto 3 MM Whatman filter paper. Membranes were incubated at 42°C for 30 min in hybridization solution (50% formamide, 5 $\times$ SSC, 5 $\times$ Denhardt solution (1% Ficoll 400, 1% polyvinylpyrrolidone, 0.5% bovine serum albumin, 50 mM EDTA, 200 mM Tris HCl pH 7.5), 20 mM Tris HCl pH 8.0, 100 μ g/ml salmon sperm DNA, 2% (SDS) and hybridized to digoxigenin-labelled species-specific probes. The probes were denatured by heating to 100°C for 10 min then snap-cooled on ice. 100–150 ng mL^{–1} of probe was added to the membrane and allowed to hybridize at 42°C for 18–24 h with gentle shaking. The membrane was washed twice with 3 $\times$ SSC for 15 min at room temperature, washed twice with 0.1 $\times$ SSC/0.1% SDS for 30 min at 65°C and blotted onto Whatman 3 MM filter paper. Probes were detected by soaking the membranes in buffer I (100 mM Tris HCl pH 7.5, 150 mM NaCl) for 1 min at room temperature, incubating with blocking solution (Boehringer Mannheim,

Mannheim, Germany), washing in buffer I and incubating with 150 mU/ml antidig-conjugate (Boehringer Mannheim, Mannheim, Germany) at room temperature for 30 min. The membrane was then washed twice with buffer I for 15 min, further equilibrated with buffer III (100 mM Tris HCl pH 8.5, 100 mM NaCl, 50 mM MgCl₂) for 2 min and incubated with substrate solution (10 ml buffer III, 45 μ l nitroblue tetrazolium chloride, 35 μ l 5-bromo-4-chloro-3-indolylphosphate) in the dark until colour developed. Development was halted by washing in distilled water for 10 min.

Polymerase chain reaction

Genomic DNA was prepared using the STE method described above. Approximately 490 bp regions of *mariner* elements were amplified by the polymerase chain reaction (PCR) using the degenerate primers MAR 124F and MAR 276R (Robertson, 1993). These primers amplify the central half of the transposase gene, representing one-third of the element. Amplifications were performed in 20 μ l volumes overlaid with one drop of mineral oil on a Hybaid OmniGene thermocycler. Each PCR included 2–20 ng of genomic DNA, 5 nmoles dNTP, 20 μ moles each primer, 50 nmoles MgCl₂ and 0.25 units of DNA polymerase using manufacturer's buffer (Promega, Madison, WI). Cycling conditions were 95°C 1 min, 50°C 1 min and 75°C 1 min for 40 cycles followed by a final extension at 75°C for 5 min. Genomic DNA from *Drosophila simulans* was included with every set of PCRs as a positive control. Products were electrophoresed through ethidium bromide-stained 1% agarose gels in 0.5 $\times$ TBE and visualized under UV light.

Sequencing

PCR products were cleaned using spin columns (Promega Wizard PCR Preps, Promega, Madison, WI) and cloned into a pGEM-T vector (Promega, Madison, WI). Plasmids were extracted (Wizard Minipreps, Promega) and sequenced in both directions using primers T7 and SP6 (AmpliTaQ DNA polymerase, FS, Applied Biosystems on an automated sequencer, ABI 377). Two clones were sequenced from each individual, except for *An. vagus*, where only one individual was sequenced.

Sequence analysis

Sequences of *mariner* obtained here were compared with those of other invertebrates of the same *mariner* subfamilies (Robertson & MacLeod, 1993; Robertson, 1993) and the active *Mos1* sequence (Jacobson *et al.*, 1986). Nucleotide sequences were translated into their presumed amino acid sequences by assuming a number

of frameshifts. Amino acid sequence and length differences were used to identify sequences to subfamily level, according to the diagnoses given in Robertson & MacLeod (1993).

Amino acid sequences were aligned using PILEUP (Genetics Computer Group, 1994) from the GCG suite of programs (Program Manual for the Wisconsin Package 1994). Phylogenetic trees were inferred by PAM 001 and neighbor-joining using programs SEQBOOT, PROTDIST, NEIGHBOR and CONSENSE from PHYLIP version 3.5c (Felsenstein, 1993) and maximum parsimony analysis using a heuristic search and treating gaps and frameshifts as missing characters using PAUP version 3.1 (Swofford, 1991). One hundred bootstrap replications were carried out. Trees were rooted using the *Bombyx mori* *Bmmar1* sequence, which represents a basal lineage of the *mariner* family (Robertson & Asplund, 1996).

Results

Table 1 shows which species of *Anopheles* were tested for the presence of *mariner* PCR products, as well as

the Subgenus, Series and Group or Complex of the species. Positive species were from the *An. maculatus* and *An. dirus* complexes, as well as *An. vagus* and *An. annularis*. Both of these complexes contain the principal malaria vectors in Thailand and elsewhere in South-east Asia. However, the malaria vector *An. minimus* s.l. appears not to possess *mariner* that can be amplified by the primers used here. All sequences obtained have been submitted to GenBank (accession number AF294472–AF294488). Note that *An. sawadwongporni* was found to have representatives from two subfamilies. We do not suppose that the *mariner* elements we have found represent all of the elements in these species; if more clones were to be sequenced, we would expect to encounter more sequence variation in many, if not all, of the species.

None of the *mariners* we encountered are likely to be autonomous. They all contain multiple stop codons and/or frameshifts. Sequences from *An. vagus* are severely truncated. The closest resemblances to the active *Mos1* sequence are in *An. dirus* D and *An. sawadwongporni*, in the mauritiana subfamily, although these are 51.7% and 82.3% divergent at the DNA level, respectively (Kimura

Table 1 Presence and absence of *mariner* PCR products in species of *Anopheles*. Absence implies that *mariner* was not detected in any of the specimens; presence implies that it was detected in at least some specimens. *N* = number of individual tested. Mosquito classification from Harbach (1994)

Species of <i>Anopheles</i>	Subgenus	Series	Group (G) or Complex (C)	<i>N</i>	Presence (+) or absence (–) of <i>mariner</i>	No. clones sequenced	Subfamily of <i>mariner</i>
<i>barbirsotris</i>	<i>Anopheles</i>	Nyssorhynchus	Barbirsotris G	12	–		
<i>hyrcanus</i> s.l.	<i>Anopheles</i>	Nyssorhynchus	Hyrcanus G	10	–		
<i>peditaeniatu</i>	<i>Anopheles</i>	Nyssorhynchus	Hyrcanus G	9	–		
<i>aconitus</i>	<i>Cellia</i>	Myzomyia	Minimus G	11	–		
<i>culicifacies</i> s.l.	<i>Cellia</i>	Myzomyia	Culicifacies C	3	–		
<i>minimus</i> s.l.	<i>Cellia</i>	Myzomyia	Minimus G	40	–		
<i>varuna</i>	<i>Cellia</i>	Myzomyia	Minimus G	1	–		
<i>dirus A</i>	<i>Cellia</i>	Neomyzomyia	Leucosphyrus G; Dirus C	9	+	2	irritans
<i>dirus C</i>	<i>Cellia</i>	Neomyzomyia	Leucosphyrus G; Dirus C	21	+	2	irritans
<i>dirus D</i>	<i>Cellia</i>	Neomyzomyia	Leucosphyrus G; Dirus C	4	+	2	mauritiana
<i>kochi</i>	<i>Cellia</i>	Neomyzomyia	—	13	–		
<i>tessellatus</i>	<i>Cellia</i>	Neomyzomyia	—	17	–		
<i>annularis</i>	<i>Cellia</i>	Neocellia	Annularis G	8	+	2	irritans
<i>dravidicus</i>	<i>Cellia</i>	Neocellia	Maculatus C	1	+	2	irritans
<i>jamesii</i>	<i>Cellia</i>	Neocellia	—	10	–		
<i>maculatus</i> s.s	<i>Cellia</i>	Neocellia	Maculatus C	40	+	2	irritans
<i>nivipes</i> s.l.	<i>Cellia</i>	Neocellia	Annularis G; Nivipes C	12	–		
<i>pseudowillmori</i>	<i>Cellia</i>	Neocellia	Maculatus C	1	–		
<i>sawadwongporni</i>	<i>Cellia</i>	Neocellia	Maculatus C	10	+	2	irritans + mauritiana
<i>splendidus</i>	<i>Cellia</i>	Neocellia	—	23	–		
<i>willmori</i>	<i>Cellia</i>	Neocellia	Maculatus C	11	+	2	irritans
<i>subpictus</i>	<i>Cellia</i>	Pyretophorus	Subpictus C	11	–		
<i>vagus</i>	<i>Cellia</i>	Pyretophorus	—	8	+	1	mellifera

2-parameter distance using a transition:transversion ratio of 1:2). As expected if the elements have not been active recently, nonsynonymous substitutions are more common than synonymous substitutions, even in the sequences that are most similar to active elements (*An. dirus* D, 82:26 and *An. sawadwongporni*, 86:40).

The neighbor-joining phylogram of the *mariner* sequences from species of *Anopheles* and other invertebrates is shown in Fig. 1. Maximum parsimony analysis supported all major branches. Bootstrap values within *mariner* subfamilies are not particularly high, as expected for highly degraded elements. The analysis does, however, clearly show that the elements encountered fall into three of the five known major subfamilies of *mariner*: mellifera, mauritiana and irritans.

Some of the relationships indicated are difficult to explain without involving horizontal transfer. For example, *mariner* sequences from the Dipteran *An. dirus* C and the Neuropteran green lacewing are very closely related (96.7% DNA identity) and yet the orders are

thought to have last shared a common ancestor approximately 265 Ma (Harland *et al.*, 1990).

Discussion

A note on species identification

Anopheles willmori has not previously been recorded from low elevations, and we therefore identify it with some caution. Species distributions are not well known, and we consider it likely that this specimen does represent *An. willmori*. However, it is also possible that it belongs to another member of the *An. maculatus* complex, or to a previously undetected member of the complex. There has been a long-standing question about the conspecificity of the chromosomal forms B, E and F of *An. maculatus*, which all finally came to be regarded as one species (Green *et al.*, 1992). It is possible that the *An. willmori* here equates to one of these forms.

Fig. 1 Neighbor-joining phylogram reconstructed using an amino acid alignment of *mariner* sequences. All sequences from *Anopheles* obtained herein; all other sequences are from the GenBank database. Bootstrap percentages greater than 40 are indicated below the branches.

Distribution of mariner

Our survey reveals that the transposable element *mariner* is common in South-east Asian *Anopheles* mosquitoes, including two of the three major malaria vector complexes: those of *An. dirus* and *An. maculatus*. The third major malaria vector, *An. minimus* s.l. does not appear to be infected, despite testing of 40 individuals. However, we did not identify this complex to species level therefore the possibility remains that some members do carry *mariner*.

Anopheles mosquitoes contain a diversity of *mariner* elements. We detected elements from three subfamilies: irritans, mauritiana and mellifera, and despite sequencing a maximum of two clones per species, we detected mariners of two different subfamilies in the genome of *An. sawadwongporni*, a member of the Maculatus Complex. This implies that many more *mariner* elements are likely to be found in these genomes. None of the elements we sequenced are likely to be autonomous, although this does not, of course, preclude the presence of autonomous elements in the genomes of these mosquitoes.

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