Population differentiation and Wolbachia phylogeny in mosquitoes of the Aedes scutellaris group

A. BEHBAHANI1, T. J. DUTTON1, N. DAVIES2, H. TOWNSON1 and S. P. SINKINS1

1Liverpool School of Tropical Medicine, Liverpool, U.K. and 2Richard B. Gump South Pacific Research Station, University of California Berkeley, Moorea, French Polynesia

Abstract. Mosquito species of the Aedes (Stegomyia) scutellaris (Walker) group (Diptera: Culicidae) are distributed across many islands of the South Pacific and include major regional vectors of filariasis, such as Aedes polynesiensis (Marks). Analysis of populations of Aedes polynesiensis at the extremes of its range, from Fiji and from Moorea, French Polynesia, using the rDNA ITS2 (internal transcribed spacer 2) region and six microsatellite markers showed considerable genetic differentiation between them (FST = 0.298–0.357). Phylogenetic analysis of the Wolbachia endosymbionts in three members of the complex revealed that based on the wsp gene they are all very similar and belong to the Mel subgroup of the A clade, closely related to the Wolbachia strain present in the gall wasp Callyrhytis glandium (Giraud) (Hymenoptera: Cynipidae). By contrast they are only distantly related to the A-clade Wolbachia in Aedes albopictus (Skuse), a species closely allied to the Aedes scutellaris group. There was very low differentiation between the Wolbachia in the Moorea and Fiji populations of Aedes polynesiensis.

Key words. Aedes scutellaris, Aedes polynesiensis, Wolbachia, cytoplasmic incompatibility, filariasis vectors, microsatellite, Fiji, Moorea, Polynesia.

Introduction

Aedes polynesiensis (Marks), a member of the Aedes (Stegomyia) scutellaris group (Walker), is a serious day biting pest of humans in the islands of the South Pacific (Lardeux et al., 2002) and is the major regional vector of the sub-periodic form of Wuchereria bancrofti (Cobb-bold). Several other members of the Ae. scutellaris group are listed as local or subsidiary vector species for sub-periodic Wuchereria bancrofti: Ae. cooki (Belkin), Ae. horrescens (Edwards), Ae. kesseli (Huang & Hitchcock), Ae. marshallensis (Stone & Bohart), Ae. pseudoscutellaris (Theobald), Ae. rotundac (Belkin), Ae. tabu (Ramalingam & Belkin) and Ae. tongae (Edwards) (W.H.O., 2002). Some species of the group are distributed over many islands in the South Pacific, whereas others are confined to only one. The island topography imposes geographical barriers, which will have contributed to divergence of populations and ultimately speciation (Pashley et al., 1985). Aedes polynesiensis is the most widespread species in the group and appears to have spread with human voyages (Taylor, 1998). Studies on the susceptibility of Aedes polynesiensis to Wuchereria bancrofti (Failloux et al., 1995) demonstrated that populations from different islands can vary in vector competence. Here populations at the two extremes of the range of Aedes polynesiensis in the South Pacific were compared to examine whether there is evidence for substantial genetic divergence between distant islands, using rDNA ITS2 (internal transcribed spacer 2) sequences and also recently isolated microsatellite markers.

The maternally inherited bacterium Wolbachia, which induces crossing sterility known as cytoplasmic incompatibility in mosquitoes and many other insects (O’Neill et al., 1997; Werren, 1997), occur in a number of species in the Aedes scutellaris group (Wright & Barr, 1980; Meek, 1984, 1988). Phylogenetic analysis has shown that there are two major divisions or supergroups of Wolbachia (A and B)
in arthropods (Werren et al., 1995; Zhou et al., 1998). Mosquitoes may harbour both A- and B-group Wolbachia, and superinfections with both A and B groups can occur (Sinkins et al., 1995; Zhou et al., 1998; Ruang-Areerate et al., 2003). Phylogenetic analysis of the highly variable wsp gene, a single copy gene coding for a surface protein of Wolbachia (Braig et al., 1998), has been the most commonly used gene for resolving phylogenetic relationships among Wolbachia strains (Zhou et al., 1998). Based on wsp gene sequences from different Wolbachia isolates, 12 subgroups were proposed (Zhou et al., 1998), and further groups have subsequently been added (e.g. van Meer et al., 1999).

The phylogenetic position of Wolbachia in the Ae. scutellaris group has not previously been determined. Therefore the wsp gene was used for phylogenetic reconstruction in order to compare Wolbachia between isolated populations of Ae. polynesiensis, between different species of the Ae. scutellaris group, and to determine their relationship with Wolbachia from other insect groups.

**Materials and methods**

**Mosquito specimens**

Adult Ae. polynesiensis was collected on four occasions: adults from Sigatoka (18°09’S, 177°26’E) in Viti Levu in Fiji islands in March 2000 (25 specimens) and February 2001 (28 specimens); Muñakau near Suva (18°08’S, 178°26’E) Viti Levu in Fiji islands in October 2001 (50 specimens); and Moorea (17°33’S, 149°52’W) in French Polynesia in 2003 (32 specimens). In addition, Ae. pseudoscutellaris and Ae. albopictus (Skuse) from Tamavua in Suva, Fiji and Ae. tonga from Tongatapu in Tonga island were collected in October 2001.

DNA was extracted from individual mosquitoes using the Livak buffer protocol of Collins et al. (1987) and was resuspended in 100 µl of TE buffer. Polymerase chain reaction (PCR) amplification of the wsp gene using the primers 81F/691R (Braig et al., 1998; Zhou et al., 1998) was carried out using 0.25 mM dNTPs, 2.5 mM MgCl₂, 0.2 µM primers and 0.05 U/µl Taq DNA polymerase with the following thermal cycler conditions: 95°C, 5 min (1 cycle); 95°C, 1 min; 50°C, 1 min; 72°C, 1.5 min (35 cycles); and 72°C for 10 min (1 cycle). The PCR products were purified as described and the sequences were obtained directly. A consensus sequence of two individuals from each population was generated for each species using forward and reverse primers. The rDNA gene sequences were aligned using clustal x software. Estimates of Kimura’s two-parameter distances were calculated for all pairs of the above sequences and the sequences of Ae. albopictus (Kjer et al., 1994; GenBank L22060) and Ae. flavopictus (Yamada) (Toma et al., 2002; GenBank AF353541) and neighbour-joining analyses were conducted using mega version 2.1 (Kumar et al., 2001).

PCRs were carried out on Ae. polynesiensis specimens using primers that amplified six microsatellite loci as previously described (Behbahani et al., 2004). Left primers were labelled with different dye colours supplied from Research Genetics, Inc. (Huntsville, U.S.A) as D4 (blue), D3 (green) and D2 (black). All of the Ae. polynesiensis specimens from Fiji and Moorea were analysed on a Beckmann CEQ according to the manufacturer’s protocols. Allele frequencies, observed and expected numbers of heterozygotes and the estimates of FST according to Weir & Cockerham (1984) were calculated using the GenePop software (web version 3.1c) of Raymond & Rousset (1995a). Linkage equilibrium was tested using a contingency table test for genotypic disequilibrium between pairs of populations in a locus, based upon the null hypothesis that genotypes at one locus are independent of genotypes at other loci. Calculations were performed using GenePop version 3.1, which performs a significance test using Markov chain procedures. Genetic differentiation between populations was tested using an unbiased estimate of the exact probability with Markov chain method (Raymond & Rousset, 1995b), using Genepop version 3.1. For all tests, the Markov chain was set to: Dememorization, 1000; Batches, 100; and Iterations per batch, 1000 (Raymond & Rousset, 1995a). The overall significance of multiple tests for each locus was estimated by Fisher’s combined probability test (Fisher, 1970).

**Results and discussion**

The rDNA ITS2 sequences showed numerous indels and substitutions, with lengths of 434 and 437 bp for the Moorea and Fiji strains of Ae. polynesiensis, 441 bp for Ae. pseudoscutellaris and 502 bp for Ae. tongae (GenBank AY822661–64). There were three separate indels (of 1, 11
and 16 bp) and nine substitutions between the sequenced fragments from *Ae. polynesiensis* Moorea and *Ae. polynesiensis* Fiji. A neighbour-joining (NJ) tree produced from the estimates of Kimura’s two-parameter distance values is shown in Fig. 1. Maximum parsimony analysis produced two most parsimonious trees, differing only in the position of *Ae. tongae* and *Ae. pseudoscutellaris*, with nearly the same branching order found in the NJ tree. Although more sequence data from genomic regions with fewer indels would be required for a more accurate tree, the ITS2 rDNA dataset support the taxonomic classification within the *Ae. scutellaris* (Theobald) subgenus as *Ae. pseudoscutellaris*, *Ae. polynesiensis* and *Ae. tongae* under the scutellaris group, and *Ae. albopictus* and *Ae. flavopictus* under the albopictus group (Rai *et al.*, 1982). It also demonstrates that substantial differences have accumulated between the Fiji and Moorea populations of *Ae. polynesiensis*.

All six microsatellite loci investigated were polymorphic (Fig. 2) and the observed heterozygosities ranged between 42% and 79% for the locus Ap1, between 63% and 81% for Ap2, between 0% and 46% for Ap3, between 5.3% and 75% for Ap4, between 2.2% and 50% for Ap5, and between 3.4% and 44.4% for Ap6. The test for genotypic disequilibrium between pairs of loci was not significant for each locus pair across the four populations (P-values between 0.108 and 0.993). Estimated pair wise $F_{ST}$ over all loci among the populations ranged from 0.014 (between Sigatoka 1 and Sigatoka 2) to 0.357 (between Sigatoka 1 and Moorea) (Table 2). All but the Suva and Sigatoka 2 pair were highly significant ($P < 0.001$).

The data give support to the ITS2 results in showing that populations of *Ae. polynesiensis* from Fiji show substantial genetic differentiation compared to a population from Moorea, separated by the distance of around 3460 km. It seems likely that the process of speciation has begun between these populations; colonization and crossing studies would indicate whether any reproductive barriers have arisen. Geographical isolation has probably been a very important factor in speciation within the *Ae. scutellaris* group (Dev & Rai, 1982; Meek, 1988). A lower degree of genetic differentiation of *Ae. polynesiensis* was also observed between two populations from Fiji; the microsatellite loci used therefore appear sufficiently polymorphic to allow finer-scale studies, for example to estimate the degree of migration between islands.

**Table 1.** Host insect species used in *Wolbachia* phylogenetic reconstruction; phenotype A signifies arrhenotoky, CP cyclical parthenogenesis and CI cytoplasmic incompatibility.

<table>
<thead>
<tr>
<th>Host</th>
<th>Order: family</th>
<th>Phenotype</th>
<th>Wolbachia group</th>
<th>GenBank</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes polynesiensis</em> (mosquito)</td>
<td>Diptera: Culicidae</td>
<td>CI</td>
<td>Mel</td>
<td>AY822657–8</td>
<td>This study</td>
</tr>
<tr>
<td><em>Ae. pseudoscutellaris</em></td>
<td>Diptera: Culicidae</td>
<td>CI</td>
<td>Mel</td>
<td>AY822659</td>
<td>This study</td>
</tr>
<tr>
<td><em>Ae. tongae</em></td>
<td>Diptera: Culicidae</td>
<td>CI</td>
<td>Mel</td>
<td>AY822660</td>
<td>This study</td>
</tr>
<tr>
<td><em>Ae. albopictus A</em></td>
<td>Diptera: Culicidae</td>
<td>CI</td>
<td>AlbaA</td>
<td>AF020058</td>
<td>Zhou <em>et al.</em> (1998)</td>
</tr>
<tr>
<td><em>Ae. albopictus B</em></td>
<td>Diptera: Culicidae</td>
<td>CI</td>
<td>Pip</td>
<td>AF020059</td>
<td>Zhou <em>et al.</em> (1998)</td>
</tr>
<tr>
<td><em>D. melanoagaster</em> Canton S</td>
<td>Diptera: Drosophilidae</td>
<td>CI weak</td>
<td>Mel</td>
<td>AF020063</td>
<td>Zhou <em>et al.</em> (1998)</td>
</tr>
<tr>
<td><em>Callyrhytis glandium</em> (Oak gallwasp)</td>
<td>Hymenoptera: Cynipidae</td>
<td>CP</td>
<td>Mel</td>
<td>AY95156</td>
<td>Rokas <em>et al.</em> (2002)</td>
</tr>
</tbody>
</table>

*Wolbachia*

The nucleotide sequence of a segment of 521 nucleotides of the *wsp* gene was aligned from *Wolbachia* strains of Fiji and Moorea *Ae. polynesiensis*, *Ae. pseudoscutellaris*, *Ae. tongae* and the A group *Wolbachia* of *Ae. albopictus*. There were no substitutions between *Ae. pseudoscutellaris* and *Ae. polynesiensis*-Moorea. Only one transition was observed between the *wsp* sequences of Fiji *Ae. polynesiensis* and those of *Ae. pseudoscutellaris* and Moorea *Ae. polynesiensis*. *Aedes tongae* showed five, four and four transitions with respect to the *Wolbachia* strains of Fiji and Moorea *Ae. polynesiensis* and *Ae. pseudoscutellaris*, respectively. Three most parsimonious trees were obtained by analysis of aligned *wsp* sequences, the topologies of which differed

![Fig. 1. Phylogenetic tree constructed from ITS2 (internal transcribed spacer 2) sequences in the *Aedes scutellaris* group and related species using the neighbour joining method. Numbers at the nodes indicate bootstrap values.](image-url)
from each other only with respect to small changes within the scutellaris group. A neighbour joining analysis of the same data set produced a tree (Fig. 3) that had almost identical topology to those produced by maximum parsimony analysis.

The data clearly showed that the Wolbachia in the Ae. scutellaris group members Ae. tongae, Ae. pseudoscutellaris, and Fiji and Moorea populations of Ae. polynesiensis, were very closely related to each other, but unexpectedly were not closely related to the A-group strain present in Ae. albopictus, a species that is closely allied to the Ae. scutellaris group. The Wolbachia strain present in Ae. polynesiensis,

![Fig. 2. Observed allelic frequencies (y-axes) in Aedes polynesiensis collections from Fiji and Moorea (French Polynesia) for six microsatellite loci, Ap1–6 (panels a–f, respectively). Alleles are denoted by their total size in base pairs (x-axes). The key given in (a) applies to all panels; Sigatoka and Suva are on Fiji and Moorea is in French Polynesia.](image)

**Table 2.** Microsatellite differentiation: $F_{ST}$ values between Moorea and Fiji populations of Ae. polynesiensis, upper numbers, and pairwise estimates of geographical distance (km), lower numbers (*$P$ < 0.001).

<table>
<thead>
<tr>
<th></th>
<th>Sigatoka 1</th>
<th>Sigatoka 2</th>
<th>Suva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigatoka 2</td>
<td>0.014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 km</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suva</td>
<td>0.079*</td>
<td>0.113*</td>
<td></td>
</tr>
<tr>
<td>100 km</td>
<td>100 km</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moorea</td>
<td>0.298*</td>
<td>0.299*</td>
<td>0.357*</td>
</tr>
<tr>
<td>3460 km</td>
<td>3460 km</td>
<td>3360 km</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 3. Phylogenetic tree constructed from wsp sequences using neighbour joining. Numbers at the nodes indicate bootstrap values. The B-clade Aedes albopictus B and Culex quinquefasciatus sequences served as outgroups.](image)
Ae. pseudoscutellaris group (Diptera: Culicidae) I. Crossing et al.

...can be added to the Journal of Bacteriology 19

Niv

Wolbachia Synergus gallaepomiformis gene is a considerably less sensitive marker for geographical separation of its vectors of the dengue viruses and...sequences, with identical sequences found in Drosophila melanogaster and the hymenopterans Synerus gallaepomiformis (Fonscolombe) and Callirhytis glandium (Giraud) (Rokas et al., 2002). This group of Wolbachia can be added to the AlhA, Mors, Nov, Nir, Pip, Cra, and Prep that were reported previously to infect Aedes species (Ruang-Areerate et al., 2003). Phylogenetic analysis has indicated that horizontal transmission has been a major influence of the distribution of Wolbachia in arthropods (e.g. O’Neill et al., 1992; Rousset et al., 1992; Werren et al., 1995; Vavre et al., 1999; Werren & Windsor, 2000) and the data shown here provide further evidence of the ability of Wolbachia to move horizontally between distantly related groups.

Within the Ae. scutellaris complex, there is very little variation in wsp sequences, with identical sequences found in Ae. polynesiensis from Moorea and Ae. pseudoscutellaris from Fiji. Thus it would appear that either the Wolbachia wsp gene is a considerably less sensitive marker for geographical isolation than are host nuclear microsatellites and the ITS2 gene, or alternatively that the strain of Wolbachia present has spread through the Ae. scutellaris group more recently than the divergence of their host lineages and the geographical separation of Ae. polynesiensis at the two extremes of its range. Wolbachia can spread rapidly through populations using CI (Turelli & Hoffmann, 1991; Hoffmann & Turelli, 1997), and are able to induce high levels of CI in the Ae. scutellaris group (Dutton & Sinkins, 2005). Only very low levels of migration between islands would probably be needed to sustain a spreading Wolbachia infection. It would also seem likely that Wolbachia has moved between species in the complex, possibly by introgressive hybridization; it is known for example that Ae. pseudoscutellaris and Ae. polynesiensis are partially compatible in laboratory crosses (Rozeboom & Gilford, 1954; Dev & Rai, 1982; Meek & Macdonald, 1984).

Acknowledgements

We thank Arun Raju for his assistance with mosquito collections. A.B. was supported by the Government of the Islamic Republic of Iran.

References


© 2005 The Royal Entomological Society, Medical and Veterinary Entomology, 19, 66–71.


Accepted 15 November 2004