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

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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 *Ae. 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 ($F_{ST} = 0.298$–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 *Ae. scutellaris* group. There was very low differentiation between the *Wolbachia* in the Moorea and Fiji populations of *Ae. 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* (Cobbold). 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. rotumae* (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 *Ae. 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 *Ae. 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 *Ae. 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)

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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); Muanikau 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 $MgCl_2$, 0.2 µM primers and 0.05 U/µl *Taq* DNA polymerase with the following thermal cycler conditions: 95°C, 5 min (1 cycle); 94°C, 1 min; 55°C, 1 min; 72°C, 1 min (35 cycles); and 72°C for 10 min (1 cycle). The PCR products were ligated into pGEM-T (Promega Ltd, Madison, WI), used for transformation of JM109 cells (Promega), selected and purified with Qiaprep columns (Qiagen UK Ltd, Crawley, U.K.). The sequences of one or two specimens from each species were obtained on a Beckman CEQ sequencer (Coulter UK Ltd, High Wycombe, U.K.). The sequences used for reconstructing a phylogenetic tree consisted of 16 *wsp* sequences (Table 1): four from mosquitoes in this study, nine from Zhou *et al*. (1998), two from Rokas *et al*. (2002) and one from Ruang-Areerate *et al*. (2003). Partial *wsp* gene sequences were aligned using Clustal X software. Maximum parsimony and neighbour-joining phylogenetic tree analyses were conducted using MEGA version 2.1 (Kumar *et al*., 2001).

The rDNA ITS2 regions were amplified using conserved primers from the 5.8S and 28S coding regions: 5.8S (forward) ATCACTCGGCTCATGGATCG and 28S (reverse) ATGCTTAAATTTAGGGGGTAGTC) (Paskewitz & Collins, 1990; Collins & Paskewitz, 1996). The PCR conditions were: 1 × reaction buffer; 0.2 mM dNTPs mix; 2.5 mM $MgCl_2$; 0.2 µM forward primer; 0.2 µM reverse primer; 0.04 µ/µl *Taq* DNA polymerase; nuclease-free water and template DNA. PCR was performed in 50 µl total volume under 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 F_{ST} 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

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

Host	Order: family	Phenotype	*Wolbachia* group	GenBank	Reference
Aedes polynesiensis (mosquito)	Diptera: Culicidae	CI	Mel	AY822657–8	This study
Ae. pseudoscutellaris	Diptera: Culicidae	CI	Mel	AY822659	This study
Ae. tongae	Diptera: Culicidae	CI	Mel	AY822660	This study
Ae. albopictus A	Diptera: Culicidae	CI	AlbaA	AF020058	Zhou *et al.* (1998)
Ae. albopictus B	Diptera: Culicidae	CI	Pip	AF020059	Zhou *et al.* (1998)
Armigeres subalbatus (mosquito)	Diptera: Culicidae	CI	Sub	AF317488	Ruang-Areerate *et al.* (2003)
Culex quinquefasciatus (mosquito)	Diptera: Culicidae	CI	Pip	AF020060	Zhou *et al.* (1998)
Drosophila auraria	Diptera: Drosophilidae	CI	Riv	AF020062	Zhou *et al.* (1998)
D. melanogaster Aubiry	Diptera: Drosophilidae	CI weak	Mel	AF020063	Zhou *et al.* (1998)
D .melanogaster Canton S	Diptera: Drosophilidae	None	Mel	AF020065	Zhou *et al.* (1998)
Glossina centralis (Tsetse fly)	Diptera: Glossinidae	?	Mors	AF020078	Zhou *et al.* (1998)
G. morsitans	Diptera: Glossinidae	?	Mors	AF020079	Zhou *et al.* (1998)
Nasonia vitripennis (parasitoid wasp)	Hymenoptera: Pteromalidae	CI	Mors	AF020081	Zhou *et al.* (1998)
Synergus gallaepomiformis (Oak gallwasp)	Hymenoptera: Cynipidae	A	Mel	AY095155	Rokas *et al.* (2002)
Callyrhytis glandium (Oak gallwasp)	Hymenoptera: Cynipidae	CP	Mel	AY095156	Rokas *et al.* (2002)

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. stegomyia* (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 ($P < 0.001$).

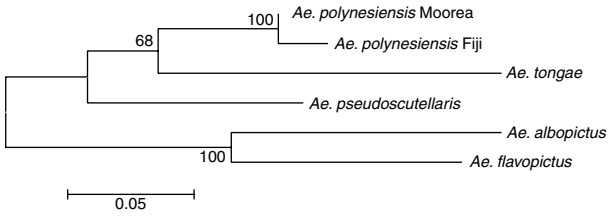

Fig. 1. Phylogenetic tree constructed from ITS2 (internal transcribed spacer 2) sequences in the *Aedes scutellaris* group and related species using the neighbour joining method. Numbers at the nodes indicate bootstrap values.

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.

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

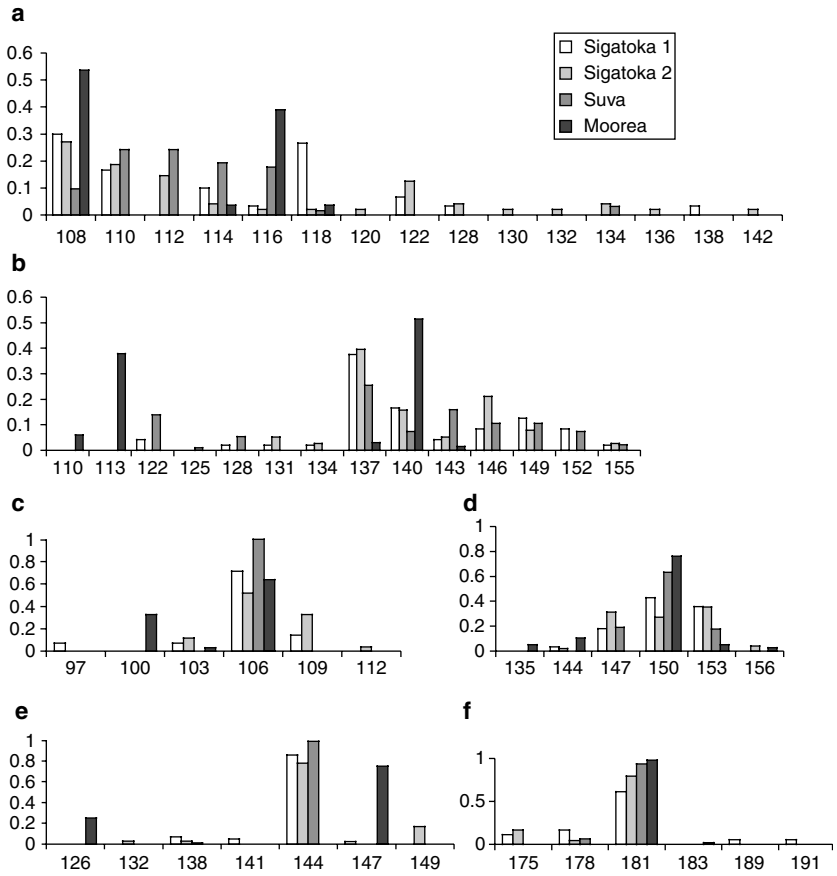

Fig. 2. Observed allelic frequencies (*y*-axes) in *Aedes polynesiensis* collections from Fiji and Moorea (French Polynesia) for six microsatellite loci, *Ap*1–6 (panels a–f, respectively). Alleles are denoted by their total size in base pairs (*x*-axes). The key given in (a) applies to all panels; Sigatoka and Suva are on Fiji and Moorea is in French Polynesia.

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*,

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 (*$P < 0.001$).

	Sigatoka 1	Sigatoka 2	Suva
Sigatoka 2	0.014		
	0 km		
Suva	0.079*	0.113*	
	100 km	100 km	
Moorea	0.298*	0.299*	0.357*
	3460 km	3460 km	3360 km

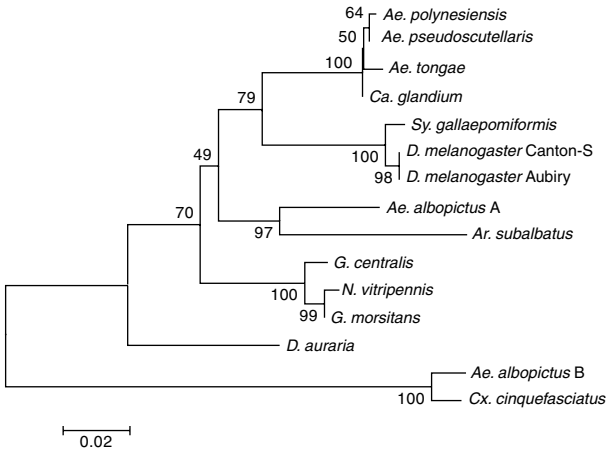

Fig. 3. Phylogenetic tree constructed from *wsp* sequences using neighbour joining. Numbers at the nodes indicate bootstrap values. The B-clade *Aedes albopictus B* and *Culex quinquefasciatus* sequences served as outgroups.

Ae. tongae and *Ae. pseudoscutellaris* was named *w*Scu and fell within the *Mel* subgroup, which consists of the *Wolbachia* strains of *Drosophila melanogaster* and the hymenopterans *Synergus gallaepomiformis* (Fonscolombe) and *Callyrhytis glandium* (Giraud) (Rokas *et al*., 2002). In fact *w*Scu shares around 99.6% identity with the *Wolbachia* strain isolated from the gallwasp *C.glandium*, which shows cyclic parthenogenesis (Rokas *et al*., 2002). This group of *Wolbachia* can be added to the *AlbA*, *Mors*, *Nov*, *Niv*, *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).

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