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Ribosomal DNA spacer genotypes of the *Anopheles bancroftii* group (Diptera: Culicidae) from Australia and Papua New Guinea

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Abstract

Mosquitoes of the *Anopheles bancroftii* group collected from Northern Australia and Papua New Guinea (PNG) were investigated for sequence variation within the ribosomal DNA ITS2. Wing fringe morphology originally used to identify members of this group was compared to genotypes identified by restriction fragment length polymorphism analysis (RFLP) and heteroduplex analysis (HDA) of the rDNA ITS2. Members of this group separated into four RFLP genotypes (A, B, C and D) with some genotypes displaying wing fringe polymorphisms. Heteroduplex analysis of the ITS2 within and between populations identified genotype A as containing two geographically separate ITS2 sequences: A1 from the Northern Territory of Australia and A2 from Queensland and the Western Province of PNG. Genotypes B and C and genotypes C and D were found sympatric and appeared to be evolving independently suggesting the possibility of cryptic species. Genotype C contained two ITS2 sequence types within the genome.

Keywords: *Anopheles bancroftii*, cryptic species, mosquito, ITS2, rDNA.

Introduction

The *Anopheles bancroftii* group lies within the *Myzorhynchus* Series, Laticorn Section of the Subgenus *Anopheles*. The group consists of two species, *Anopheles bancroftii* Giles and *Anopheles pseudobarbirostris* Ludlow (Reid & Knight, 1961). The two species can only be separated in the adult female by pale patches on the wing fringe. However, this character appears to be quite variable and although the two species are believed to occur in Northern Australia and New Guinea there is considerable confusion as to their exact distribution. *Anopheles bancroftii* has been incriminated as a vector of malaria in Irian Jaya (Lee *et al.*, 1987) and in Papua New Guinea (PNG) (J. Hii, pers. comm.) and it has been incriminated as a vector of the filarial worm *Wuchereria bancrofti* (Lee *et al.*, 1987). However, because the morphological characters separating *An. bancroftii* and *An. pseudobarbirostris* are so variable, it is difficult to determine which, if not both, of these species are vectors of these parasites (Lee *et al.*, 1987). This situation highlights the need to accurately identify the potential vector species in order to validate epidemiological studies and to define behavioural and ecological characteristics that would be essential in the planning of control strategies. The range of these two species covers a number of different climate types such as the wet/dry monsoonal tropics of Northern Australia to the continuous wet tropics of New Guinea. Thus there is the possibility that a number of cryptic species may have evolved.

Recent advances in DNA based technology has made available a wide range of molecular characteristics for taxonomic and systematic studies of mosquitoes. One region of the anopheline mosquito genome that has received particular attention is the ribosomal DNA (Collins & Paskewitz, 1996). The rDNA consists of tandemly repeated transcriptional units composed of structural genes (28S, 5.8S and 18S), intergenic spacers (IGS) and two internal transcribed spacers (ITS1 and ITS2). The rDNA gene family appears to occur mostly at a single haploid locus in mosquitoes (Kumar & Rai, 1990; Collins & Paskewitz, 1996) and shows sequence similarity within copies of the rDNA unit among individuals in an interbreeding population or species (Dover, 1982). The spread of variant sequences through the rDNA array is called homogenization and may eventually lead to fixation of these sequences within an interbreeding population. These processes are dependent on the rates of turnover within and between chromosomes.

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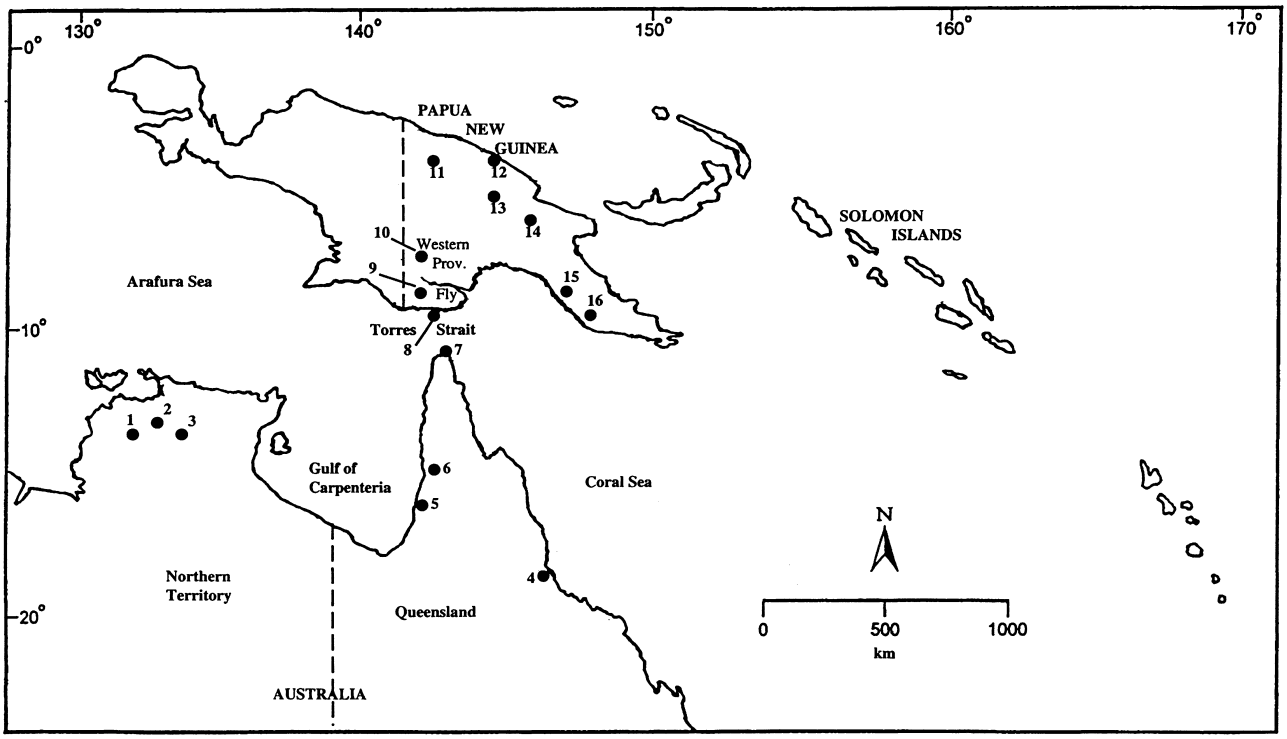

Figure 1. Map of the south-west Pacific showing collection sites for the members of the *An. bancroftii* group.

Variant repeats are either removed from the rDNA array or homogenized through a process of concerted evolution that involves a number of DNA replication and repair mechanisms, such as gene conversion, gene amplification and unequal crossover between repeated units (Dover, 1982; Schlotterer & Tautz, 1994).

The ITS2 region has been useful for the identification of closely related anopheline mosquitoes (Porter & Collins, 1991; Fritz *et al.*, 1994; Beebe & Saul, 1995; Cornel *et al.*, 1996; Walton *et al.*, 1999). Amplification of the ITS2 spacer is quite simple using primers that anneal to the conserved 18S and 5.8S genes. In some cases restriction analyses of the ITS2 region is sufficient to generate species-specific restriction fragment length polymorphism (RFLP) profiles for closely related mosquito groups such as the *An. punctulatus* group (Beebe & Saul, 1995; Beebe *et al.*, 1999). In other anopheline mosquitoes, species-specific sequence variation has been used to develop diagnostic PCR assays for species identification (Porter & Collins, 1991; Walton *et al.*, 1999), particularly for separating morphologically similar or identical species. In the examples above, species taxa had already been identified prior to the development of the diagonstic tool. In this paper we use PCR-RFLP and heteroduplex analyses of the rDNA ITS2 region to follow the population structure of the current morphological species in the *An. bancroftii* group from Australia and PNG, and determine the practicality of using this region to study the intraspecific population structure of these mosquitoes.

Results

Mosquitoes morphologically identified as members of the *An. bancroftii* group obtained from collections in Australia and PNG (Fig. 1) were subjected to genetic analysis using a PCR-RFLP technique to assess the presence or absence of restriction sites and sequence insertion/deletions. The PCR products were then subjected to heteroduplex analysis (HDA) to determine lower-level sequence variations in the ITS2 within the rDNA array and to detect within and between population sequence diversity. Four separate PCR-RFLP profiles were identified and were designated genotype A, B, C and D. Genotype A occurred in Northern Australia and the Western Province of PNG (sites 1–10). This genotype displayed three main bands at 180, 120 and 100 bp (Fig. 2). Several different wing fringe patterns were identified from this genotype including *An. bancroftii*, *An. pseudobarbirostris* and *Anopheles barbirostris* van der Wulp and some that were new undescribed fringe patterns (Table 1).

Mosquitoes from sites 11–16 in PNG displayed three different RFLP profiles constituting genotype B (bands at 180 and 100), C (bands at 260, 180, 120 and 100 bp) and D (one band at 450 bp) (Fig. 2). At sites 11–13 genotype C and D occurred together and at sites 15 and 16 genotype C and B occurred together (Table 1). Again, there was no correlation between wing fringe patterns and genotype status. The banding profile of genotype C appeared the same

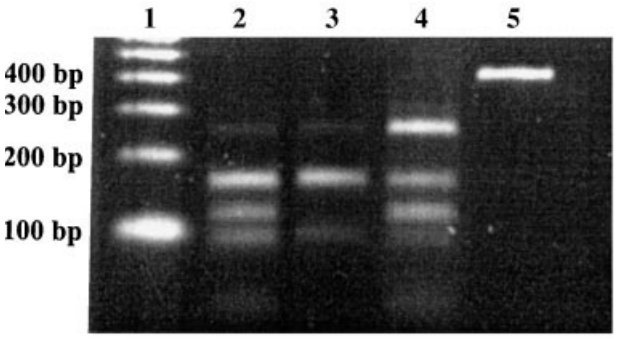

Figure 2. Analysis of the ITS2 PCR product by *Msp*I restriction digest and electrophoresis through a 3.0% agarose gel. Lane 1 is a 100 bp ladder; lane 2 is the profile of genotype A; lane 3 is genotype B; lane 4 is genotype C and contains two different ITS2 sequences; lane 5 is genotype D and contains no *Msp*I restriction site.

Table 1. *Anopheles bancroftii* and *An. pseudobarbirostris* collection summary showing species identifications based on morphology and ITS2 PCR-RFLP genotype identification.

Collection site	*n*	Wing fringe morphology*	ITS2 Genotype
1	23	both	A1
2	14	*An. bancroftii*†	A1
3	33	*An. bancroftii*†	A1
4	34	*An. bancroftii*†	A2
5	22	–	A2
6	5	–	A2
7	5	–	A2
8	12	both	A2
9	1	*An. bancroftii*	A2
10	25	both	A2
11	2	*An. bancroftii*	C/D
12	6/3	*An. pseudobarbirostris*	C/D
13	8/9	*An. pseudobarbirostris*	C/D
14	3	–	D
15	2/3	*An. bancroftii*	C/B
16	2/3	both	C/B

†Collections also contained individuals with undescribed wing fringe patterns.
*Damaged wing fringes prevented identification of many individuals.

in all individuals and indicated the presence of multiple ITS2 sequences. Subsequent HDA of this genotype confirmed the presence of two ITS2 sequence copies in the mosquito genome (Fig. 3), which after cloning and RFLP analysis revealed one sequence (C1) as giving bands at 280 bp and 120 bp, while the other (C2) showed an identical profile to that of genotype A with bands at 180 bp, 120 bp and 100 bp (data not shown).

To detect sequence variation within and between collection sites, heteroduplex analysis was performed; i.e. PCR products within and between sites were mixed together, denatured and electrophoresed in an acrylamide gel. Heteroduplexes occurred when PCR products of the A

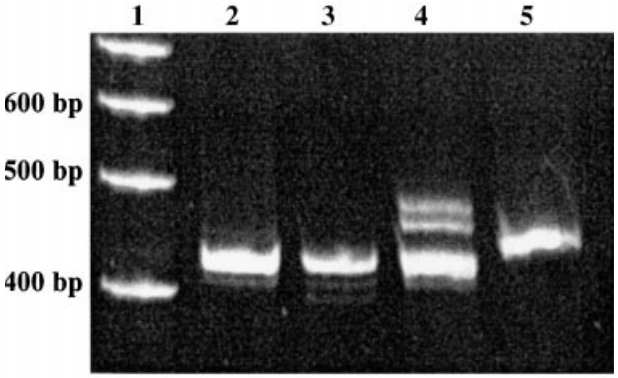

Figure 3. The four genotypes identified by PCR-RFLP analysis electrophoresed on a 7.0% acrylamide gel, which is sensitive to double stranded secondary structure. Lane 1 is the 100 bp ladder; lanes 2 and 3 are genotypes A and B, respectively, showing a single band for the amplified ITS2 (homogenized ITS2 sequence or homoduplex). Lane 4 is genotype C showing both homoduplex (bottom band) and heteroduplex products (sequence mispairing alters the secondary structure of duplex and retards migration). Lane 5 is genotype D and appears to migrate slower due to secondary structure rather than sequence length.

genotype from the Northern Territory (sites 1–3) were mixed with PCR products individuals collected from Queensland/Western Province (sites 4–10). Mosquitoes from within these regions did not show detectable sequence variants using HDA. Thus it appears that within the Genotype A PCR profile two geographically restricted genetic variants exist; a Northern Territory variant and a Queensland/Western Province variant. These two sequence variants were subsequently designated A1 (Northern Territory) and A2 (Queensland and Western Province of PNG).

The ITS2 region was sequenced for all the observed genotypes and the resulting six sequences were deposited in GenBank (AF203378-83). An alignment of these sequences is shown in Fig. 4. Genotype D exhibited the most sequence variation with forty-six variant nucleotides, which included a 12 bp insertion that was also present in both the C1 and C2 sequences. The sequence differences in D may be responsible for structural changes in the DNA duplex causing slower migration through the acrylamide gel (Fig. 4, lane 5). Genotypes A1 and A2 (identified through HDA analysis) differed by eight nucleotides (1.9%). Genotype C contained two ITS2 sequences (C1 and C2; Fig. 4, lane 4) that diverged by four nucleotides (1.0%). Genotype B differed by only three nucleotides from the other sequences, one of which was at position 299 where the nucleotide change prevented restriction by *Msp*I and was thus diagnostic in the PCR-RFLP analysis.

Excluding genotype D, the variation between the other sequences constituted 3.5% of the ITS2 region, and 11.6% when including genotype D. The majority of this sequence variation occurred at the 3′ end of the spacer, complementing previous observations by Beebe *et al.* (1999).

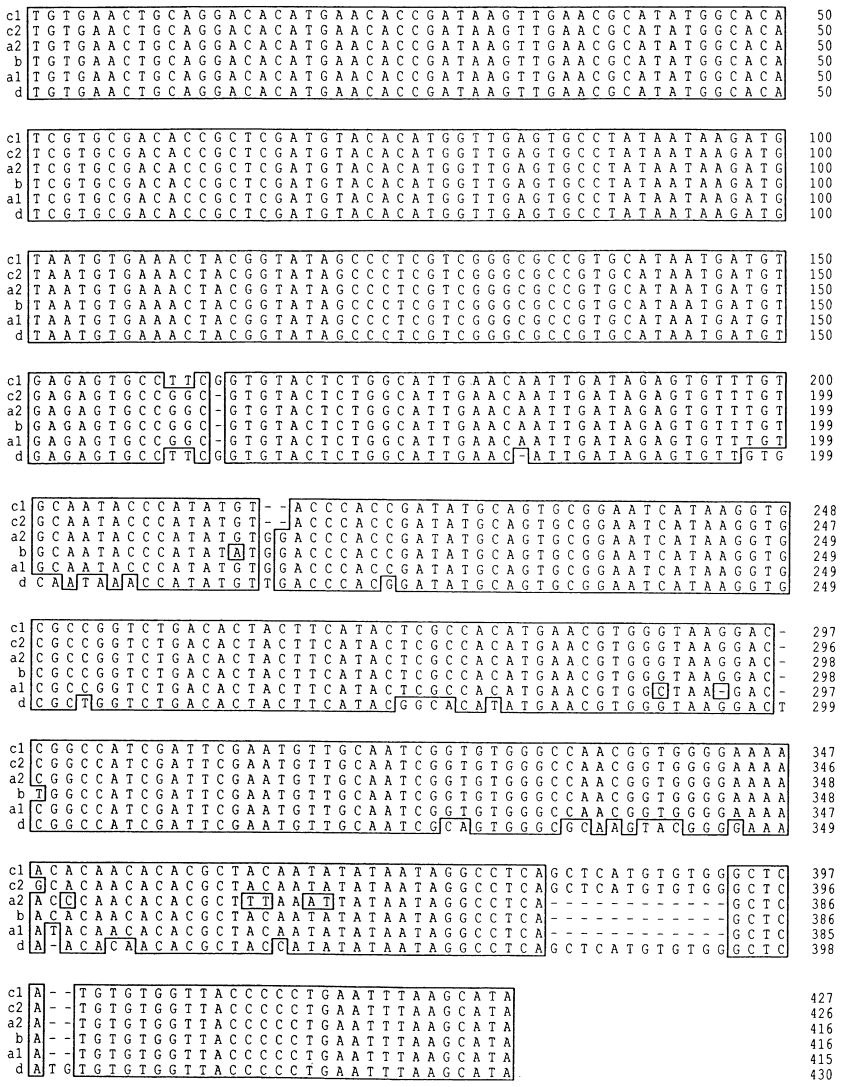

Figure 4. DNA sequence alignment of the ITS2 sequences identified from the *An. bancroftii* genotypes from Australia and PNG. Regions of sequence variation are boxed. The sequences of C1 and C2 represent the two ITS2 sequences identified from genotype C.

Discussion

There appeared to be no correlation in the wing fringe characteristics previously used to identify *An. bancroftii* and *An. pseudobarbirostris* with any of these identified genotypes. Moreover, there were often individual mosquitoes that had wing fringes unlike either of these species. These results would question the validity of using this morphological character to identify adult females of these two species.

The independent evolution of these rDNA gene families to detectable genotypes and the sympatric occurrence of these genotypes appear to suggest that a number of these genotypes exhibit natural mating barriers and thus possibly represent separate species (Paterson, 1985). Genotypes D and C occurred together at sites 11–13, while, genotypes B and C occurred together at sites 15 and 16. Evidence for any natural hybridization between these genotypes may possibly manifest as combinations of PCR-RFLP profiles in individual mosquitoes in a population, possibly in a similar manner to that observed in genotype C. Genotype C exists over a relatively large geographical range in PNG (sites 11–13, 15 and 16) and has maintained two divergent paralogues (C1 and C2) throughout this range without any detectable sequence homogenization.

Divergent paralogues in the ITS are not unusual and have been observed in other interbreeding mosquito populations, such as the south-west Pacific malaria vector *Anopheles farauti* s.s. (Beebe *et al.*, 2000). Moreover, one population of *An. farauti* s.s. had been an inbreeding colony for more than 600 generations and had maintained two separate ITS2 sequences. The presence of the C genotype at sites 11–13, 15 and 16 in PNG is interesting and may indicate this genotypes inability to homogenize these two sequences. Both these cases imply that either the rDNA array exists at multiple loci or that the architecture or position of the rDNA array on the chromosome obstructs or

retards the homogenizing machinery involved in the concerted evolution of this gene family. The possibility of pseudogenes existing outside the array is possible, however the absence of an increased substitution rate in either C1 or C2 suggests both sequences remain under functional constraints (Buckler *et al*., 1997).

Genotypes A1 and A2 have not yet been identified together or with genotypes B, C or D, preventing any conclusions on their potential species status. Moreover, identification of hybridization between A1, A2 and B could not be determined by the PCR-RFLP analysis, but may be resolved by HDA followed by sequencing, or more simply by the development of ITS2 sequence specific primers for allele specific PCR such as that described by Walton *et al*. (1999) for the *An. gambiae* complex. Sequence analysis of the ITS2 does indicate that equivalent genetic distances do exist between A1 and A2 as compared to the B and C genotypes. It is probably premature at present to suggest species status for these genotypes, especially as the distribution of this group extends through Irian Jaya to the islands of the Moluccas. Further genetic studies with larger sample sizes over a wider geographical distribution are now required to determine the species status and distribution of this group.

Sequence variation in the rDNA itself cannot reveal mating barriers, however sequence variation in the context of the homogenizing machinery of the rDNA may be useful in identifying these. If separate ITS2 sequence genotypes identified using either RFLP, HDA or allele specific PCR analysis are found sympatric and no individuals are present which contain ITS2 sequences from these distinct genotypes, it may be possible view these ITS2 genotype populations as having natural mating barriers. Conformation at more than one field collection site would reinforce this conclusion. Further support could also come from running parallel studies on a separate variable marker such as one from the mitochondria. If homogenization of rDNA sequence variants is not fast or immediate, rDNA sequence variants may remain in a population for a period of time such as that observed in *An. farauti* s.s. (Beebe *et al*., 2000). It would appear, however, that the ITS2 sequence paralogues of genotype C (C1 and C2) are not undergoing any observable homogenization and have possibly become genetically fixed, because the relative abundance of the bands in the RFLP profile of this genotype appear similar in all individuals examined from the five different collection sites.

Genotype biogeography and biology

North and east of the Fly River, within the Western Province of PNG, there is a region of marked climate change separating the continual wet, non-seasonal climate of northern and eastern PNG and the distinctly seasonal wet/dry monsoonal conditions of the Southern Western Province, and in particular, northern Australia, with its quite severe dry seasons (McAlpine *et al*., 1983). Genotypes A1 and A2 may represent forms adapted to the drier monsoonal climate because they do not appear in the wetter parts of PNG with the northern limit of A2 being the northern limit of this monsoonal influence (sites 9 and 10). Conversely, genotypes B, C and D appear confined to the continual wet climatic regions of PNG, having failed to adapt to the drier southern regions.

Within northern Australia the southern distribution of genotypes A1 and A2 appears to be the 1000 mm isohyet (Cooper *et al*., 1996). This may explain the existence of the A1 Northern Territory genotype and the A2 Queensland/Western Province genotype, because the Gulf of Carpenteria, a region of below 1000 mm rainfall, separates these two populations and may act as a barrier to gene flow, allowing the independent evolution of the two populations into different genotypes. These two regions were joined as recently as 8000–14 000 years ago, when a series of ice ages and associated low sea levels created a continuous landmass joining northern Australia with New Guinea (Kikkawa *et al*., 1981). Since this time rising sea levels have created the Arafura Sea, the Gulf of Carpentaria and the Torres Strait. Therefore, it is feasible that the A1 and A2 genotypes diverged less than 14 000 years ago. Although members of the *An. bancroftii* group are known to occur on a number of the islands in the Torres Strait (Booth, 1988), this region may also act as a barrier to gene flow between the Queensland and PNG populations. However, this was not detected, possibly due to the more recent occurrence of lower sea levels resulting in a land bridge joining these two landmasses.

The presence of the A1 and A2 genotypes within Australia may also help explain a behavioural difference noted in the *An. bancroftii* mosquitoes from these two regions. In the Northern Territory, *An. bancroftii* readily and aggressively bites humans and, during the late wet and early dry seasons, this mosquito is considered a major pest species. However, in Queensland, although populations of *An. bancroftii* will feed on humans, they have been found to be indifferent to humans in many areas, and appear to be more zoophilic (Lee & Woodhill, 1944).

Four genotypes described here can be readily identified by what is now a routinely used PCR-RFLP procedure. In this case the process is facilitated by the fact that only one enzyme is needed to generate fragments that will separate all four genotypes. Moreover, these PCR priming regions are highly conserved so as to amplify across the mosquito genus with the consequence that all mosquitoes analysed will generate an amplified product. Thus, anomalies such as the occurrence of new species or genotypes can be quickly resolved, enabling studies into their distribution, biology and behaviour to proceed in order to understand their role in the transmission of malaria and filariasis.

Further studies are now required to verify the species status of these genotypes and study the relationship of this group with the closely related *An. barbirostris* group

from Indonesia and the Orient. The members of the *An. barbirostris* group (*An. barbirostris* and *An. vanus*) are closely related to the *An. bancroftii* group and also rely on the use of wing fringe morphology to identify their members. It is possible that this morphological characteristic may also be unreliable for the *An. barbirostris* group as it has been found here with the *An. bancroftii* group.

Experimental procedures

Mosquito material

Mosquitoes were collected as larvae or as adults from sites in northern Australia and PNG (Fig. 1, Table 1). The larvae were reared through to adults where possible. Adults were preserved and stored in the field by freezing (–70 °C) or by desiccation on silica gel; larvae were stored in 100% alcohol. Material was identified as belonging to the *An. bancroftii* group using the morphological keys of Lee *et al.* (1987); the colouration of the wing fringe was scored for *An. bancroftii*, *An. pseudobarbirostris* or other species according to those proposed by Lee & Woodhill (1944). For genetic analysis genomic DNA was extracted from whole or partial mosquitoes using methods described in Black & Munsterman (1996).

PCR-RFLP analysis

All polymerase chain reactions were carried out in 0.5 ml microfuge tubes in a 25 μl volume on a Hybaid Omni Gene Thermocycler. The final PCR mixture contained 1–10 ng of template DNA, 20 pM of each primer, 1.25 mM $MgCl_2$, 2.5 mM of each dNTP, 5.0% DMSO, 1 × *Taq* reaction buffer (Biotech) and 1.0 unit of *Taq* DNA polymerase. The primers used for ITS2 amplification were those used in Beebe & Saul (1995): ITS2A-5′-TGTGAACTGCAGGACACAT-3′; ITS2B-5′-TATGCTTAAATTCAGGGGGT-3′. Reaction cycles involved an initial denaturation at 94 °C for 4 min followed by thirty-four cycles of 94 °C for 1 min, 51 °C for 2 min and 72 °C for 1 min.

Restriction endonuclease digestion was carried out in a 0.5 ml microfuge tube containing 3 μl of PCR product, to which was added 3.0 μl of a stock solution containing 2 × *Msp*I Buffer and 1 U of *Msp*I per reaction (NEB II, New England Biolabs, New England) to give a final volume of 6.0 μl. The mixture was incubated in 37 °C for 1 h. The digested product was run on a 3.0% agarose gel at 60 V for 60 min for RFLP identification. The gel was stained with ethidium bromide (5 μg/ml) for 15 min and viewed on an ultraviolet transilluminator at 312 nm.

ITS2 heteroduplex analysis (HDA)

Analysis of ITS2 polymorphism between and within mosquito populations required 3 μl of PCR product from two separate mosquitoes: these were mixed in a 0.5 ml microfuge tube, overlaid with a drop of oil and subjected to 95 °C heat for 5 min in a heating block. Tubes were gradually brought down to room temperature by switching the heating block off (approximately 15 min). Renatured products were electrophoresed on an 7.0% non-denaturing polyacrylamide gel (NOVEX) containing 10% glycerol at 200 V for 2.0 h. The gel rig was placed in an ice water bath, to prevent heating of the gel and denaturation of double stranded duplexes. After electrophoresis, the gel was stained with EtBr (5 μg/ml) for 2 min and visualized at 312 nm. Analysis of ITS2 polymorphism from single mosquitoes (sequence variants within the rDNA array) involved running 3 μl of the PCR product mixed with loading buffer on a 7.0% acrylamide gel as mentioned above.

Cloning the ITS2 sequences from genotype C

The ITS2 amplification products from a mosquito identified as having multiple ITS2 sequences (genotype C) were ligated into a pGem-T vector according to the manufacturers recommendation (Promega). The ligation products were then transformed into *Escherichia coli* (DH5 alpha). Positive colonies were selected with a sterile pipette tip and reamplified by dipping the tip into the reaction mixture and cycled as above for twenty-five cycles. Amplified ITS2 products were then subjected to restriction enzyme digestion to determine the different banding profiles. The appropriate clones were then prepared for sequencing.

Sequencing

The ITS2 PCR products from a single individual representing genotypes A1, A2, B and D were purified for direct sequencing using Qiagen PCR purification chromotography following the manufacture's directions. The cloned C1 and C2 sequences were also PCR purified as above and all purified products were sequenced using a 373 ABI automated sequencer and were analysed using the Australian National Genomic Information Service (ANGIS, http://www.angis.org.au/). Sequence alignments were carried out with the GCG (Version 5) PILEUP program using the default settings. The output file was then subjected to the PRETTYPLOT program.

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