Genetic diversity of the dengue vector *Aedes aegypti* in Australia and implications for future surveillance and mainland incursion monitoring

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Abstract

In February 2004, the discovery of an incursion of the dengue vector *Aedes aegypti* into the town of Tennant Creek in the Northern Territory caused concern for the Northern Territory health authorities who proceeded to implement a Commonwealth-funded eradication program. To determine the origin of the incursion, we performed a genetic analysis on *Ae. aegypti* from several Queensland and overseas localities. A comparison of DNA sequences from the mitochondrial cytochrome oxidase 1 gene indicated that the incursion was probably from Cairns or Camooweal. This genetic marker was also useful in identifying a separate Townsville haplotype population and another population on Thursday Island in the Torres Strait that was genetically divergent to the mainland populations. The possible use of this marker as a surveillance tool for identifying the origins of local and overseas incursions is discussed. *Commun Dis Intell* 2005;29:299–304.

Keywords: Aedes aegypti, *mtDNA, cytochrome oxidase 1 gene, dengue, surveillance*

Introduction

Aedes aegypti is the primary vector of dengue virus. It is the only dengue vector in mainland Australia and has been responsible for outbreaks of dengue fever that reappeared in northern Queensland in the early 1980s and have continued until the present.[1,2] Historically, the distribution of *Ae. aegypti* included all mainland states and territories except Victoria and South Australia. However, in the 1950s it disappeared from Western Australia, New South Wales and the Northern Territory.[3] It maintains a strong hold in Queensland where its southern limit is Dirranbandi to Roma and west to Cloncurry and Mount Isa.[4] In February 2004, specimens of *Ae*. *aegypti* were identified in Tennant Creek in the Northern Territory.[5] This town is located on the main road links to Queensland (via the Barkly Highway) and Darwin (via the Stuart Highway) and is 670 km from Mount Isa—the nearest previously known source of *Ae*. *aegypti*.

Apart from the potential for this species to spread from Queensland into other states or territories, there is the continual threat of its introduction to Australia from overseas via international ports. Darwin alone had 13 importations of *Ae. aegypti* between 1998–2000,[6] and there have been numerous other detections by the Australian Quarantine Inspection Service (AQIS) since then, including the recent detection of an importation in February 2005 from an Indonesian fishing vessel (Whelan, unpublished data). *Aedes aegypti* is a competent traveller with three attributes that contribute to its dispersal: 1) it has a very close association with humans; 2) it readily breeds in artificial receptacles; and 3) its eggs can withstand desiccation for many months.

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The movement of this species, either within or from outside Australia, is of great concern to public health authorities and AQIS. From a surveillance and control perspective, it would be useful to know if the recent infestation at Tennant Creek originated from Queensland, or from Darwin after being imported from overseas. If it is the former, then inspections of towns along the main road, working back to Mount Isa, as the nearest probable source, will be required. If the latter, then increased surveillance and trapping in the towns from Darwin to Tennant Creek will be required. With incursions from outside of Australia, it would be relevant to know in which country the strain originated, as different geographic strains can have different colonising abilities and different competencies with regards to transmitting the dengue virus.[7–9] This situation is complicated by the fact that vessels coming to Australia may have stopped at several Asian ports where *Ae. aegypti* is endemic.

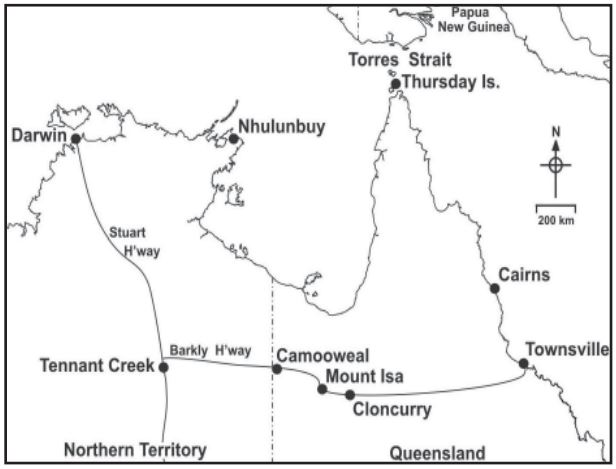
Identifying differences in mosquito strains or populations requires a DNA-based genetic marker that will be informative, will deliver an unambiguous result, will be relatively straightforward to use, and ideally, be useful in later studies of evolution or population genetics. As *Ae. aegypti* is an exotic mosquito that probably arrived in Australia during the mid-19th century,[10] a rapidly evolving genetic marker would be required to identify population variation within this species. Genetic markers based on the mitochondrial DNA (mtDNA) have been to be useful for genetic studies of other species and populations.[11,12]

The aim of this study was to assess the use of the mtDNA cytochrome oxidase 1 (CO1) gene as a genetic marker to evaluate the origin of the *Ae. aegypti* incursion into Tennant Creek. We also evaluated this marker as a potential surveillance tool for identifying populations of *Ae. aegypti* that originated from locations outside of Australia.

Method

Australian specimens of *Aedes aegypti* were collected as larvae from three different breeding sites in Tennant Creek in the Northern Territory, and from breeding sites in Cairns, Townsville and Thursday Island in Queensland. Following the discovery of *Ae. aegypti* in Tennant Creek, a container breeding survey was conducted at Camooweal located on the Barkly Highway at the Queensland-Northern Territory border, 188 km west of Mount Isa. Specimens collected during this survey were also included in this study. Specimens were also obtained from an Indonesian fishing vessel that was intercepted and inspected by AQIS approximately 1.5 km outside Melville Bay near Nhulunbuy on the north-east coast of the Northern Territory in February 2005. The ship contained *Ae. aegypti* larvae and pupal skins categorising it as a risk importation that had a potential for live adults to disperse to shore, had it not been intercepted and appropriately treated. Collection sites from within Australia are indicated in Figure 1. Specimens, collected as immature stages or from established colony material, from outside Australia were obtained from South East Asia and the south-west Pacific: Burma, Viet Nam, Thailand, Timor Leste, Papua New Guinea (PNG) and Vanuatu.

Figure 1. Northern Australia indicating *Aedes aegypti* collection sites



Mosquito DNA extraction, polymerase chain reaction amplification and DNA sequencing

Mosquitoes (partial or whole adults and larvae) were thoroughly ground in a 1.5 ml microfuge tube containing 50 µl of lysis buffer (1.0M NaCl, 0.2M sucrose, 0.1M Tris-HCl (pH 9.0), 0.05M EDTA and 0.5% SDS). Tubes were pulse microfuged to concentrate the homogenate in the bottom of the tube prior to incubation at 65° C for 30 minutes. Then 7 µl of 8.0M KAc was added to each tube; these were mixed, placed on ice for 15–30 minutes and microfuged for 15 minutes at 14,000 rpm. Supernatants were placed in a new tube to which 100 µl of 100 per cent EtOH was added and microfuged at 14,000 rpm for 15 minutes. Supernatants were removed, 100 µl of 70 per cent EtOH was added, and tubes were centrifuged again at 14,000 rpm for 5 minutes. Supernatants were again removed, tubes were air dried and resuspended in 50 µl TE containing RNase (5 µg/ml).

A 5' segment of the mtDNA CO1 gene was amplified in 25 µl volumes using a thermal cycler (DNA Engine, MJ Research Inc.). The forward primer (5'-TAGTTC CTTTAATATTAGGAGC-3') was designed to start approximately 245 bp into the CO1 5' region and the reverse primer (5'-TAATATAGCATAAATTATTCC-3') was designed back from 813 bp into the CO1 gene. The final polymerase chain reaction (PCR) mixture contained 1x *Taq* buffer II (Fisher Biotech Australia), 2.5 mM MgCl, 0.125 mM of each dNTP, 0.4 µM of

each primer, 0.5–1.0 unit of *Taq* polymerase and 5.0–10.0 ng of extracted genomic DNA (1 µl of extraction). The cycling involved an initial denaturation of 94° C for three minutes, then 35 cycles of 94° C for one minute, 50° C for one minute and 72° C for one minute with minimal transition times. The PCR products were separated by agarose gel electrophoresis (1.0%) at 100 V for 40 minutes, then visualised by staining with ethidium bromide (0.3 µg/ml) at 312 nm.

DNA sequencing and genetic analysis

Amplified products were purified using the Qiagen QIAquick PCR purification kit following their set protocol. Sequencing was performed using an ABI Big Dye™ Terminator kit (PE Biosystems) according to the manufacturer's recommendations and the same forward and reverse primers described above were used for sequencing.

The sequence alignment was performed using the PILEUP algorithm in the GCG package using default settings (Genetics Computer Group, Version 8, 1994). Genetic analyses using traditional tree-building phylogenetic methods can be inappropriate for these types of studies because they make assumptions that are invalid at the intraspecific population level.[13] Thus the analysis was performed using the TCS algorithm which estimates genealogical relationships and generates a parsimonious network.[13]

Results

Aedes aegypti genomic DNA was extracted from 46 individual specimens from Australia and various countries of South East Asia and the south-west Pacific. From these, 46 CO1 sequences were derived and aligned together and with two other *Ae. aegypti* sequences (laboratory strains originating from East and West Africa) obtained from Genbank (Table). After editing, the sequence alignment length was 503 bp and showed eight separate sequence haplotypes. All nucleotide changes occur at the third codon position. A summary of the DNA sequence variation for each haplotype (relative to haplotype 1: Tennant Creek and Cairns population, Genbank accession DQ026284) is presented in the Table along with the haplotype distributions and their frequency. Figure 2 shows a minimum parsimony network of the eight haplotypes.

The CO1 haplotypes obtained from the three separate breeding sites in Tennant Creek were the same as those found in Cairns but different to those identified from Townsville. It appears that the Tennant Creek population represents a single haplotype population (H1). The H1 haplotype from Cairns appears well dispersed as it was also found from mosquitoes collected in Viet Nam and Thailand. Haplotype H1 is one mutational step (1 nucleotide) from another well-dispersed haplotype H4, which was found in

Table. Collection sites, haplotype distribution and haplotype diversity of *Aedes aegypti* populations used in this study

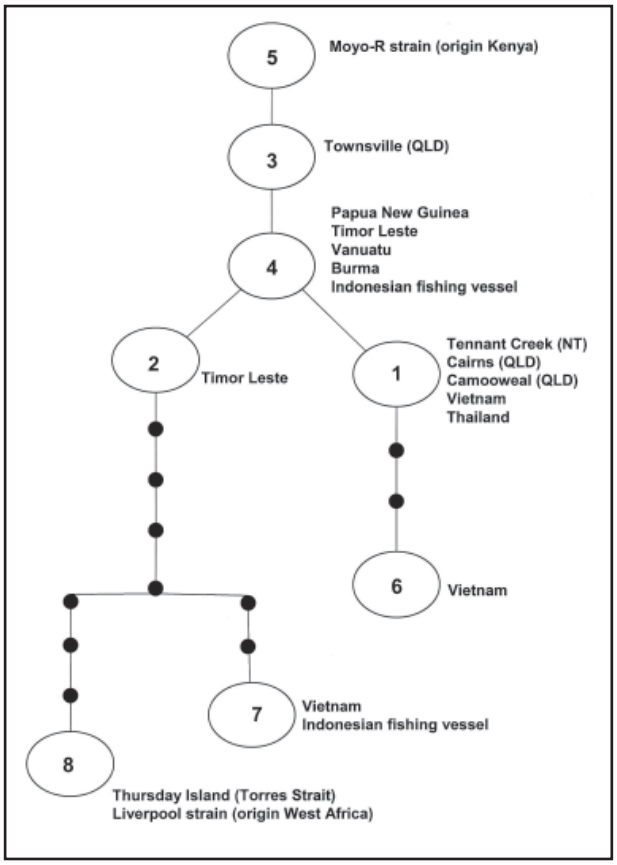
Collection site	n	CO1 haplotype	Haplotype diversity*
Cairns Qld (2 sites)†	5	H1	bp
Townsville Qld†	3	H3	112222333333344
Tennant Creek NT (3 sites)†	9	H1	46890129044568903
Camooweal Qld†	2	H1	56657984625737928
Thursday Is. Torres Strait†	3	H8	H1 GTAAACTAGTTATCACA
Indonesian fishing vessel†	5	3xH7, 2xH4	H2 ....C....
Timor Leste (3 sites)†	7	H2	H3 A..............
Thailand (Bangkok)‡	2	H1	H4 .....T..........
Viet Nam (Hanoi)‡	3	H1, H7, H6	H5 A.....C.........
Burma‡	3	H4	H6 .G...C...C.....T.
Vanuatu†	1	H4	H7 ......C.A.CGCTG.G
Papua New Guinea†	3	H4	H8 ..GGG.C.A..GCT...
MOYO-R strain (Af380835)‡	–	H5	
Liverpool strain (AY056596)‡	–	H8	

* Nucleotide changes relative to H1 (Genbank accession number DQ026284).

† Specimens collected as immature stages from breeding sites.

‡ Specimens from established colonies.

Figure 2. Mitochondrial CO1 haplotype network showing genealogical relationships



Legend: Circles represent the different CO1 sequence haplotypes with geographic regions of specimens listed. Connecting nodes represent single mutational steps between haplotypes and may be unidentified extant haplotypes.

PNG, Timor Leste, Burma, Viet Nam and Vanuatu. Haplotype H3, identified from Townsville, is also a single mutational step from the H4 haplotype, but H3 appears restricted to Townsville. The specimens from Thursday Island were all H8 and the same sequence as the Liverpool laboratory strain that was originally collected from West Africa. This Thursday Island material was considered quite divergent to the Australian mainland material with 10 mutational steps to either H1 or H3. Analysis of five specimens collected in February 2005 from the Indonesian fishing vessel revealed two separate CO1 haplotypes – three H7, and two H4 individuals. The H4 haplotype was found to be widespread, as mentioned above, while the three H7 haplotypes showed the same sequence as one specimen from Viet Nam.

Discussion

The mitochondrial DNA was selected for this study because its genome is maternally inherited through the female egg and very rarely undergoes recombination.[11] Thus it has a more linear or clonal evolution than nuclear DNA and its coding genes also display a more rapid rate of evolution, making it a useful marker for studying intraspecific population genetic variation.[11,12] The CO1 gene has been found useful for intraspecific studies of *Anopheles* and in interspecific studies of *Aedes* mosquitoes,[14,15] and for both *Anopheles* and *Culex* mosquitoes in our laboratory (Beebe, unpublished data).

We reveal for the first time that there are at least two mtDNA CO1 haplotype populations of *Ae. aegypti* on the Australian mainland (H1 from Tennant Creek, Cairns and Camooweal, and H3 from Townsville). This study suggests that the incursion into Tennant Creek was not from the military and industrial centre of Townsville, but from Cairns or Camooweal. The most likely spread was by the carriage of eggs in dry receptacles by vehicle traffic. The presence of *Ae. aegypti* at Camooweal moves the western distribution of *Ae*. *aegypti* in Queensland to the Northern Territory border. However, these conclusions should be viewed with caution as further sampling and analysis of sites within these towns will be required to determine if additional haplotypes are present.

Within Australia, the haplotype population identified on Thursday Island in the Torres Strait (H8), shows considerable genetic distance to the Australian mainland haplotypes (10 mutational steps). It is interesting to note that *Ae. aegypti* populations from Thursday Island have displayed enhanced vector competence to the dengue 2 and 4 serotypes compared to the mainland populations from Cairns and Townsville.[9] The substantial genetic distinction between the Thursday Island H8 population and the mainland Australia H1 and H3 populations may help in the understanding of the observed difference in vector competence between these different populations. It also highlights the need for state authorities and AQIS to prevent the movement of *Ae. aegypti* from the Torres Strait to mainland Australia.

Specimens of *Ae. aegypti* collected from the Indonesian fishing vessel revealed two separate haplotypes (H4 and H7). The maternal inheritance of the mitochondrial genome means that each female mosquito will only produce her own haplotype,[11] and indicates that at least two separate egg batches were laid in the receptacle on this vessel by different CO1 haplotype *Ae. aegypti* females. The origin of these haplotype populations could not be determined, as we have no samples from Indonesia for comparison. However, it is likely that these haplotypes represent Indonesian populations of *Ae*. *aegypti*.

The appearance of a divergent haplotype or lineage in the Torres Strait population may reflect the successful dispersal capabilities of this species. No one has looked at the movement of these haplotypes on a global scale. However such movement appears to be considerable, this small study has revealed, for

example, that haplotypes are shared by populations as widely dispersed as Burma and Vanuatu (H4) and Viet Nam and Australia (H1).

Each node in the network in Figure 2 may represent an extant haplotype sequence, and this study suggests that there could be 11 unidentified haplotypes that exist within this network. If we view this haplotype network, bearing in mind it is a small sampling regime, haplotypes H1 and H4 were found most frequently, were well dispersed geographically and appear embedded within the haplotype network. These factors suggest H1 and H4 may be the original (ancestral) haplotypes introduced into the Asia-Pacific region.[16] It is also interesting that the laboratory strains found in Genbank that had origins in West Africa (H8, Liverpool) and in East Africa (H5, Moyo-R, Kenya) are at the ends or tips of the network. Their positioning may indicate the breadth of genetic diversity of this species within Africa.

The dispersal and colonising ability of this species makes it a continual threat to ports in Australia and highlights the need to prevent the further westward spread from Queensland into the Northern Territory and Western Australia. We suggest it should now be a priority to screen *Ae. aegypti* populations in Australia and around our region to record and monitor the possible spread of the endemic and exotic genetic diversity of this species.

In summary, the partial sequence of the mtDNA CO1 gene from a small number of *Ae. aegypti* has enabled the identification of different genetic populations within Australia, as well as the origin of an incursion into the Northern Territory from Queensland. There was also considerable genetic difference between the mainland Australian and Thursday Island populations, which have been shown to display different vector competencies to dengue viruses.[9] Though further extensive sampling and analysis will be required to verify the robustness of this potentially useful genetic marker, this study suggests that the CO1 gene will be a practical tool to study the genetic diversity and spread of *Ae. aegypti* in Australia, as well as to monitor foreign incursions. It has a potential application in studying other species of quarantine and public health importance in Australasia such as the recent establishment of *Ochlerotatus camptorhynchus* in New Zealand, or the dispersal of *Aedes albopictus* into the Torres Strait and other areas of northern Australia.

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