Tracing the Tiger: Population Genetics Provides Valuable Insights into the *Aedes (Stegomyia) albopictus* Invasion of the Australasian Region

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Abstract

Background: The range of the Asian tiger mosquito *Aedes albopictus* is expanding globally, raising the threat of emerging and re-emerging arbovirus transmission risks including dengue and chikungunya. Its detection in Papua New Guinea’s (PNG) southern Fly River coastal region in 1988 and 1992 placed it 150 km from mainland Australia. However, it was not until 12 years later that it appeared on the Torres Strait Islands. We hypothesized that the extant PNG population expanded into the Torres Straits as an indirect effect of drought-proofing the southern Fly River coastal villages in response to El Nino-driven climate variability in the region (via the rollout of rainwater tanks and water storage containers).

Methodology/Principal Findings: Examination of the mosquito’s mitochondrial DNA cytochrome oxidase I (COI) sequences and 13 novel nuclear microsatellites revealed evidence of substantial intermixing between PNG’s southern Fly region and Torres Strait Island populations essentially compromising any island eradication attempts due to potential of reintroduction. However, two genetically distinct populations were identified in this region comprising the historically extant PNG populations and the exotic introduced population. Both COI sequence data and microsatellites showed the introduced population to have genetic affinities to populations from Timor Leste and Jakarta in the Indonesian region.

Conclusions/Significance: The *Ae. albopictus* invasion into the Australian region was not a range expansion out of PNG as suspected, but founded by other, genetically distinct population(s), with strong genetic affinities to populations sampled from the Indonesian region. We now suspect that the introduction of *Ae. albopictus* into the Australian region was driven by widespread illegal fishing activity originating from the Indonesian region during this period. Human sea traffic is apparently shutting this mosquito between islands in the Torres Strait and the southern PNG mainland and this extensive movement may well compromise *Ae. albopictus* eradication attempts in this region.

Introduction

The Asian tiger mosquito *Aedes (Stegomyia) albopictus*, originally described by Skuse from Calcutta, India, in 1894, is considered native to the Southeast Asian region where the larvae are often found in forest tree holes — a characteristic that assists its current global expansion via rapid adaptation to human-made container habitats [1,2,3]. This global expansion is also driven by human behavior, often facilitated by the transport of used tyres that contain desiccation-resistant eggs or, in some cases, by the movement of the containers themselves [4,5]. Prior to the 1980s, *Ae. albopictus* had spread to several islands in the Indian Ocean, as well as to the Hawaiian Islands in the Pacific [6]. It was discovered in Albania in Europe in 1979 [7], and has also established in both North [8] and South America [9], in Africa in 1992 [10], and in southern Europe [11]. It is currently expanding into over 20 European countries [2].

Alongside this species’ global expansion, its status as a vector of human pathogens is also of increasing concern. As a laboratory vector of over 25 arboviruses, its role in arbovirus transmission cycles has mostly been secondary to other incriminated vectors [12,13,14]. In the absence of the primary dengue vector, *Aedes (Stegomyia) aegypti*, *Ae. albopictus* has been the epidemic vector of dengue viruses in Hawai‘i, Macao and China [14,15,16,17].
Author Summary

The range of the Asian tiger mosquito Aedes albopictus is expanding globally, raising the threat of emerging and re-emerging arbovirus transmission risks, including chikungunya and dengue. Detection of Aedes albopictus in southern Papua New Guinea (PNG) in 1988 and 1992 placed it 150 km from mainland Australia. In 2004–05 it reached the islands in Australia’s Torres Strait that separate the mainland from PNG. Suspecting a range expansion from PNG driven by human adaptation to climate variability, we employed population genetics methodologies to investigate possible origins of the introduced population, as well as population structure of previously existing populations from New Guinea. Mitochondrial cytochrome oxidase I sequences and 13 novel microsatellite markers revealed a clear genetic distinction between regional populations in PNG and the newly introduced population in the Torres Strait and Fly Region. The closest genetic relative to the introduced population was found in the Indonesian region to the west and it is now suspected that this species may have been brought into the Torres Strait by sea vessels involved in extensive illegal fishing activities during this period.

The discovery of Aedes albopictus in the Torres Strait in the mid-2000s led to the question of whether recent adaptation to climatic variability had played a role in its expansion—as we had suggested in earlier work on other container inhabiting Aedes species in this region [30]. The 1997–98 El Niño conditions contributed to the worst drought in PNG for 100 years: traditional groundwater supplies were greatly affected, either drying up or becoming contaminated [31]. As local springs and streams dried up, it became necessary for villages to store water in large containers including 220 L (44 gal) drums and rainwater tanks—both of which provide highly productive larval sites for container-inhabiting mosquito species [32]. As part of an international aid response, AusAID funded and transported 9,000 L polypropylene rainwater tanks and 200 L water containers to the southern Fly River region villages immediately adjacent to the Torres Strait in a project completed in 2002 [33,34]. This human adaptation to climate variability may have provided abundant productive larval sources for the population of Aedes albopictus already present in PNG, leading to its rapid population expansion and a subsequent spillover into the Torres Strait Islands. Once Aedes albopictus was established on the islands, the continuous human ocean traffic would have rapidly shuttled mosquitoes through the region. Thus our working hypothesis was that climate variability driven by the 1997–98 El Niño resulted in water storage management changes in PNG’s southern Fly River coastal villages and was indirectly responsible for the invasion of a local Aedes albopictus population through the Torres Strait Islands.

In this study, we use extensive regional mosquito collections and population genetics methodologies to investigate the origins and dynamics of the introduction of Aedes albopictus into and through the Torres Strait islands, as well as the population structure of this species throughout PNG. The maternally inherited mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) is used as both a population genetics marker and a proxy for female movement between islands and southern PNG villages. The rationale here is that the DNA sequence of each female is (barring mutation) identical to that of her offspring, providing insights into the dynamics and diversity of the females’ contribution to each population. This proxy would ultimately be an underestimate of movement as different females of the same sequence cannot be distinguished. Additionally, we developed and ran 13 microsatellites markers that permitted the evaluation of the nuclear background of these mosquitoes.

Materials and Methods

Mosquito samples

Container-inhabiting mosquitoes were collected from throughout the Torres Strait and PNG’s southern Fly River region villages by Queensland Health between 2004 and 2010 (Tables 1 and 2, and Figure 1). Populations of Aedes albopictus collected in 1992 from Daru Island (northeastern Torres Straits) and the Kiunga Port area in Western Province were provided by the Australian Defence Force. When samples were collected from private residences, permission was granted prior to entry. In most cases larvae were sampled from different containers at each location and preserved in 70% ethanol, and in some cases adults were collected. In many cases only a few individuals were collected at each location in order to reduce the chance of sampling siblings (as larvae in the same container). Larvae were initially identified as Aedes albopictus using the morphological keys of [35] and then by either real-time PCR assays [36] or by a PCR-restriction digest procedure [28] to distinguish them from endemic members of the Aedes (Stegomyia) scutellaris taxonomic group.
mtDNA sequencing and analyses

Specimens identified as *Ae. albopictus* had genomic DNA extracted using a salt extraction method [37]. For PCR amplification of a 445 bp (final edited product size) region of the mtDNA cytochrome oxidase 1 (COI), the forward primer 5′ GAY CCT GGT ATA TTT ATT GG 3′ and reverse primer 5′AAT TAA AAT ATA AAC TTC TGG were modified from [38]. The reaction was carried out in 0.2 ml well PCR plates (Astral Scientific) using 25 μl final volume and oil overlay (single drop). Final PCR mixture contained 16.6 mM [NH₄]₂SO₄, 67 mM Tris-HCl pH 8.8 (at 25 °C), 0.45% Triton X-100, 0.2 mg/ml gelatin, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.4 μM of each primer. One unit of Taq polymerase (Bioline) and 2–10 ng of purified genomic DNA (1 μl of gDNA) were used per reaction. Cycling (MJ research PTC200 or a BioRad C-1000 thermal cycler) was 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 40 °C for 1 min, and 72 °C for 1 min using minimum transition times between steps. The PCR products were visualized on a 1% agarose gel containing 0.5 μg/ml ethidium bromide and visualized at 312 nm. PCR product purification was via QIAquick (Qiagen) PCR purification columns using manufactures recommendations.

All sequences were edited and aligned using the Geneious software [39]. To examine phylogeographic relationships, we constructed maximum parsimony haplotype networks in TCS 1.21 [40] under a 95% connection limit. Pair-wise $F_{ST}$ values were estimated in Arlequin version 3.5 (distance method) [41] to assess levels of differentiation between the regions for the COI locus: regions were designated Torres Strait Islands (excluding Daru Island), southern Fly region, Daru Island, Kiunga, Port Moresby, Madang/Lae Region, Timor Leste and Jakarta. The significance levels of $F_{ST}$ comparisons were assessed using permutation tests (1,023 permutations per comparison), also implemented in Arlequin. DnaSP 5 [42] was used to estimate haplotype diversity and nucleotide diversity within regions. We performed Tajima’s D and Fu’s Fs tests of neutrality for the COI data per population in the program Arlequin.

Microsatellite development and population genetics analyses

Candidate microsatellite markers were isolated from Roche GS FLX 454 sequencing data (1/16 plate - 25,000 reads at ~400 bp length) generated from genomic DNA of *Ae. albopictus* and performed by Macrogen (Korea). To design primers for micro-
we ran the resultant data through the program msatcommander [43]. We used this program to find primers for dinucleotide, trinucleotide and tetranucleotide repeats, and allowed the program to design primers with a melting temperature in the range of 50–62°C with a GC content between 30 and 70 percent. Long polynucleotide repeats (>5 bp) within sequences to be amplified were avoided and duplicate markers (i.e. primers designed for sequence analogues) were excluded. Screening of candidate markers involved PCR amplification of a subset of samples using standard primers and visualization of products on 1% ethidium bromide stained agarose gels. Positive product primer sets were re-amplified with M13 labeled forward primers and dyes (VIC, FAM, PET and NED) and standard reverse primers. The final PCR mixture contained 1× MyTaq buffer (Bioline)(containing pre-optimized concentrations of MgCl₂ and dNTPs), 0.4 μM of each primer, 0.5–1.0 unit of MyTaq polymerase (Bioline) and 5.0–10.0 ng of extracted genomic DNA (1 μl of extraction). The cycling involved an initial denaturation of 95°C for 3 min, then 13 cycles of 95°C for 30 s, 56°C for 40 s with a gradient decrease of 0.5°C/cycle, and 72°C for 30 s, followed by 25 cycles of 95°C for 30 s, 50°C for 40 s and 72°C for 30 s, and a final 72°C for 5 min using minimum transition times. M13 labeled products for 13 microsatellite markers (see Table 3 for details) that generated clean peaks and that amplified consistently were purified using ExoSap (Antarctic phosphatase and Exonuclease I-New England Biolab) and were sent to Macrogen (Macrogen, Geumchun-gu, Seoul, Korea) for genotyping. We attempted to genotype 199 individuals sampled from the Torres Strait Islands, New Guinea, Timor Leste and Jakarta (see Table 2 for sampling information).

Alleles for each marker were scored manually in the program GeneMarker [44]. We checked for the possible presence of null alleles for each marker at a population level (based on the regions: Torres Strait, Fly Region, Daru, Kiunga, Madang, Port Moresby, Timor Leste and Jakarta) using the program MICRO-CHECKER [45]. Using these same population definitions, we checked for HWE, as well as calculating observed (Ho) and expected (He) heterozygosity in the program GenAlEx, v6 [46] and the program GenoDive [47] was used to calculate Fis for each population (Table 4 contains details of Null Alleles, HWE, etc).

The Bayesian program STRUCTURE, v. 2.3.2 [48,49], was used to infer the most likely number of genetically distinct groups

Table 1. Summary of mtDNA COI study collection sites and haplotype distribution.

| Region                  | Site         | Year   | Haplotypes: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | Totals |
|-------------------------|-------------|--------|-------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|------|
| PNG Southern Fly        | Buzi        | 2007   |             | 1 |   |   |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Ber         | 2007   |             | 3 | 4 |   |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Sigabaduru  | 2007   |             | 7 | 1 |   |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Mabaudian   | 2007   |             | 7 | 6 | 7 |   |   |   |   |    |    |    |    |    |    |    |    |     |
|                         | Ture Ture   | 2007   |             | 5 | 1 |   |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Katatai     | 2007   |             | 3 | 2 | 1 |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Dorogori    | 2007   |             | 1 | 2 | 1 |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Papua       | 2007   |             | 5 | 3 | 4 |   |   |   |   |   |    |    |    |    |    |    |    |     |
| Torres Strait Nth       | Eurb        | 2006   |             | 3 | 2 | 1 | 4 |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Ugar        | 2006   |             | 8 | 1 |   |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Masig       | 2006, 2007 |           | 4 | 4 | 2 |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Mer         | 2007   |             | 2 |   |   |   |   |   |   |   |    |    |    |    |    |    |    |     |
| Torres Strait Central   | Poruma      | 2007   |             | 8 | 1 |   |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Mabuiag     | 2007   |             | 1 | 2 | 3 |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Badu        | 2007   |             | 2 | 1 | 4 | 1 |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Moa         | 2007   |             | 1 | 7 |   |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Warraber    | 2007   |             | 2 | 2 | 6 |   |   |   |   |   |    |    |    |    |    |    |    |     |
| Torres Strait South     | Waiben      | 2010   |             | 4 |   |   |   |   |   |   |   |    |    |    |    |    |    |    |     |
|                         | Ngarupai    | 2010   |             | 1 | 16 | 1 | 6 | 1 | 2 | 27 |   |    |    |    |    |    |    |    |     |
|                         | Muralag     | 2010   |             | 2 | 1 | 3 |   |   |   |   |   |    |    |    |    |    |    |    |     |
| Cape York               | New Marpoon | 2010   |             | 2 | 2 |   |   |   |   |   |   |    |    |    |    |    |    |    |     |
| PNG                     | Daru        | 1992, 2008 |           | 29 | 1 | 1 | 2 |   |   |   | 1 |   |    |    |    |    |    |    | 35 |
| PNG                     | Kiunga      | 1992   |             | 34 | 1 |   |   |   |   |   |   |    |    |    |    |    |    |    |    | 38 |
| PNG                     | Port Moresby| 1997, 1998 |           | 41 | 1 | 12 | 1 | 3 |   |   |   |    |    |    |    |    |    |    | 58 |
| PNG                     | Madang/Lae  | 2011/1996 |           | 29 |   |   |   |   |   |   |   | 10 |    |    |    |    |    |    | 39 |
| Indonesia               | Timor Leste | 2001   |             | 23 | 1 |   |   |   |   |   |   |    |    |    |    |    |    |    |    | 17 |
| Indonesia               | Jakarta     | 2011   |             | 7 |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    | 8 |
| Totals                  |             |        |             | 134 | 2 | 1 | 1 | 2 | 94 | 1 | 1 | 1 | 20 | 70 | 21 | 1 | 5 | 22 | 1 | 377 |

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(K) in the region sampled, based on the microsatellite data. STRUCTURE was run using the admixture model, and using sampling locations as priors. Including information on sampling locations in STRUCTURE analyses has been shown to be useful for detecting subtle genetic structure, without detecting structure that is not present [48]. Due to the potential presence of null alleles at a number of markers in some populations, we used a dominant marker model in STRUCTURE (as recommended in the user manual). STRUCTURE was run for five iterations of K = 2 to K = 8, for a total of 1 million generations per iteration with a burn-in of 200 000 iterations. STRUCTUREHARVESTER, a program that implements the Evanno et al. Delta K method [50,51], was then used to infer the most likely value of K and CLUMPP v. 1.1.2 [52] was used to average the results of the replicates for K = 5 (the most likely value based on the Delta K method). We used the Greedy algorithm in CLUMPP with 1000 repeats. The output from CLUMPP was run through DISTUCT [53], which provides more flexibility in generating figures than STRUCTURE. Additionally, the program GENETIX v. 4.05 [54] was used to perform a Factorial Correspondence Analysis (FCA). FSTAT v2.9.3 [55] was used to test for linkage disequilibrium between loci, and finally Arlequin v.3.5 [41] was used to estimate pair-wise FST values between the eight populations defined above.

**Results**

**Mitochondrial DNA**

A total of 16 mtDNA COI haplotypes were identified from 377 individuals (haplotype diversity Hd = 0.769) collected throughout the southern Fly River villages, the Torres Strait islands, north and south PNG, Timor Leste and Jakarta – 10 of these haplotypes were present in the Torres Strait Islands and southern Fly River region (see Table 1 and Fig. 1 for details on collections and mtDNA haplotype occurrence). All DNA sequences are available through GenBank (KC572145 - KC572496, KF042861-KF042885) and all tests of neutrality (Tajima’s D and Fu’s Fs) were non-significant. Haplotype diversity varied for each region with the Torres Strait Islands (122 individuals, 10 haplotypes, Hd = 0.801) and southern Fly River region (60 individuals, 6 haplotypes, Hd = 0.649) having substantially higher haplotype diversities than the PNG populations from Daru Island (35 individuals, 6 haplotypes, Hd = 0.316), Kiunga (38 individuals, 3 haplotypes, Hd = 0.198), Port Moresby (58 individuals, 5 haplotypes, Hd = 0.462) and Madang/Lae Region (39 individuals, 2 haplotypes, Hd = 0.391) as well as those from Timor Leste (17 individuals, 2 haplotypes, Hd = 0.118) and Jakarta (8 individuals, 2 haplotypes, Hd = 0.250).

The COI haplotype network (Figure 2) suggests that there is some mitochondrial genetic structure between regions, with one of the most common haplotypes (H1) sampled found predominantly in the populations extant in PNG (the Madang/Lae region, Port Moresby, Kiunga and Daru Island – with Daru collections from both 1988 and 2008), and only being sampled once (in one individual) in the Torres Strait Islands.
### Table 4. Characteristics of microsatellite markers per population.

| Locus   | Torres Strait (n = 60) | Fly Region PNG (n = 12) | Daru (n = 26) | Klungu (n = 20) |
|---------|------------------------|-------------------------|--------------|----------------|
|         | HWE, Null, % missing   | [Na, Fis] (Ho, He)      | HWE, Null, % missing | [Na, Fis] (Ho, He) |
|         | y, n, 0                | [5, 0.505] (0.233, 0.465) | y, n, 8      | [5, 0.623] (0.182, 0.446) |
|         | y, n, 5                | [8, 0.491] (0.368, 0.700) | y, n, 0      | [6, 0.675] (0.525, 0.715) |
|         | y, n, 0                | [7, 0.522] (0.300, 0.661) | y, n, 0      | [5, 0.166] (0.538, 0.631) |
|         | y, n, 10               | [12, 0.083] (0.630, 0.680) | y, n, 16     | [6, 0.512] (0.364, 0.720) |
|         | y, n, 22               | [6, 0.716] (0.213, 0.737) | y, n, 50     | [4, 0.792] (0.167, 0.681) |
|         | y, n, 0                | [13, 0.421] (0.483, 0.825) | y, n, 8      | [7, 0.459] (0.545, 0.785) |
|         | y, n, 22               | [9, 0.650] (0.277, 0.776) | y, n, 17     | [7, 0.744] (0.200, 0.805) |
|         | y, n, 0                | [5, 0.166] (0.517, 0.614) | y, n, 8      | [7, 0.221] (0.545, 0.661) |
|         | y, n, 0                | [7, 0.093] (0.683, 0.747) | y, n, 8      | [4, 0.277] (0.545, 0.711) |
|         | y, n, 0                | [6, 0.425] (0.345, 0.593) | y, n, 8      | [4, 0.469] (0.273, 0.479) |
|         | y, n, 0                | [7, 0.277] (0.485, 0.657) | y, n, 17     | [5, 0.091] (0.800, 0.700) |
|         | y, n, 2                | [9, 0.469] (0.424, 0.781) | y, n, 0      | [9, 0.458] (0.444, 0.806) |
|         | y, n, 12               | [9, 0.014] (0.667, 0.765) | y, n, 4      | [8, 0.212] (0.643, 0.798) |
|         | y, n, 0                | [10, 0.171] (0.667, 0.790) | y, n, 0      | [8, 0.076] (0.655, 0.696) |
|         | y, n, 3                | [9, 0.581] (0.313, 0.728) | y, n, 0      | [6, 0.647] (0.276, 0.759) |
|         | y, n, 0                | [5, 0.153] (0.606, 0.703) | y, n, 0      | [6, 0.090] (0.679, 0.732) |
|         | y, n, 22               | [4, 0.051] (0.273, 0.534) | y, n, 4      | [6, 0.265] (0.500, 0.665) |
|         | y, n, 0                | [7, 0.070] (0.630, 0.673) | y, n, 0      | [5, 0.190] (0.552, 0.667) |
|         | y, n, 0                | [5, 0.098] (0.485, 0.528) | y, n, 4      | [6, 0.265] (0.500, 0.665) |
|         | y, n, 0                | [5, 0.120] (0.758, 0.668) | y, n, 0      | [5, 0.073] (0.724, 0.766) |
|         | y, n, 10               | [7, 0.445] (0.333, 0.587) | y, n, 10     | [5, 0.632] (0.269, 0.709) |
|         | y, n, 0                | [5, 0.085] (0.600, 0.620) | y, n, 0      | [4, 0.090] (0.778, 0.735) |

**Table Notes:**

- HWE: Hardy-Weinberg equilibrium.
- Null: null alleles.
- %missing: percentage of missing data.
- [Na, Fis] (Ho, He): [Nei's gene diversity, fixation index] (Heterozygosity, Expected heterozygosity).

**Population Abbreviations:**

- Torres Strait: Torres Strait (n = 60)
- Fly Region PNG: Fly Region PNG (n = 12)
- Daru: Daru (n = 26)
- Klungu: Klungu (n = 20)
- Madang: Madang (n = 33)
- Port Moresby: Port Moresby (n = 29)
- Timor Leste: Timor Leste (n = 10)
- Jakarta: Jakarta (n = 9)

**References:**

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common PNG haplotype (H6) was sampled at relatively high frequency in the Torres Strait. Two other haplotypes (H11 and H12) that were sampled relatively commonly in both the Torres Strait and in the Fly Region of PNG were also sampled in Daru. Haplotype 11 was also the most predominant haplotype sampled in both of the Indonesian populations (Jakarta and Timor Leste), with Timor Leste also sharing H12 and Jakarta possessing one other private haplotype (H16). This suggests that there is a close affinity between Indonesian populations and those found in the Fly Region of PNG as well as those in the Torres Strait. Six private haplotypes were sampled in the Fly Region/Torres Strait (H7, H9, H10, H13, H14, H15), three of which were only found singly in Torres Strait (H7, H9, H13) and there were 5 private haplotypes sampled in PNG; H2, H4 and H5 found only in Daru; with H3 and H8 found only in Port Moresby.

Mitochondrial COI pair-wise $F_{ST}$ relationships and significance comparisons supported the presence of structure between populations (see Table 5). Again the PNG Southern Fly Region and Torres Strait populations appeared highly distinct from the PNG populations with high and significant $F_{ST}$ values between the populations (roughly between 0.4 to 0.5). The $F_{ST}$ value between Torres Strait and Fly Region populations is significant but small (0.043), and most comparisons between PNG populations are non-significant (all $F_{ST} < 0.1$). Indonesian populations are not significantly differentiated from each other but have significant $F_{ST}$ values in all other comparisons, with the Torres Strait populations being the most closely related to them, followed by the Fly Region and then by PNG populations.

### Microsatellites

A total of 199 individuals were genotyped for the 13 microsatellites (see Table 2 for mosquito sampling). Putative null alleles were found at some loci in some populations and tests for Hardy Weinberg equilibrium revealed that some loci did not meet the expectations of this model (see Table 4 for detailed information), but no evidence of linkage disequilibrium between loci was found. The overall number of alleles per locus ranged from 8 to 17 (Table 3). The inbreeding coefficients ($F_{IS}$) of the majority of loci were positive, and observed heterozygosity was less than expected heterozygosity in most cases, indicating an excess of homozygote genotypes at most loci (see Table 4). Although $F_{ST}$ values are generally smaller for the microsatellite data than for the mitochondrial data (Tables 5 & 6), all microsatellite based pair-wise $F_{ST}$ values between populations were significant, with the exception of the Torres Strait – Fly Region comparison ($F_{ST} = 0.00421$, Table 6). Low $F_{ST}$ values were found between

| Table 5. | Pairwise COI $F_{ST}$ values (bold = non-significant). |
| --- | --- |
| | Torres Strait | Fly Region | Daru | Kiunga | Port Moresby | Madang | Timor Leste |
|---|---|---|---|---|---|---|---|
| Fly Region | 0.04294 | | | | | | |
| Daru | 0.46207 | 0.46889 | | | | | |
| Kiunga | 0.47707 | 0.50317 | $-0.00658$ | | | | |
| Port Moresby | 0.44540 | 0.43966 | 0.02975 | 0.04464 | | | |
| Madang | 0.46563 | 0.47669 | 0.03726 | 0.06915 | $-0.01029$ | | |
| Timor Leste | 0.12338 | 0.30382 | 0.81124 | 0.86803 | 0.80658 | 0.89127 | |
| Jakarta | 0.09859 | 0.26626 | 0.77880 | 0.84574 | 0.78493 | 0.87307 | 0.02812 |

Figure 2. Mitochondrial COI haplotype network showing the 16 haplotypes identified throughout PNG and the Australian region. Haplotypes are colored by region and the size of their circle is proportional to number of individuals showing that haplotype sequence with each connection a single mutational step.

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PNG populations, as well as between Torres Strait/Fly Region populations and those from Indonesia, providing further evidence of close affinities between these populations.

The most likely number of genetic clusters \( K \) inferred by STRUCTURE HARVESTER was \( K = 5 \) \cite{50,51}. The bar plot generated in STRUCTURE (Fig. 3) suggests five populations (Figure 3) with three historically extant populations within PNG that may have experienced various levels of admixture, and one distinct population encompassing the Torres Strait Islands and the southern Fly River region (purple). An additional population was found in Indonesia (pink), and bar-plots suggest some similarity of these populations to some individuals in the Torres Strait and Fly Region. The populations from Daru Island (collected in 1992 and 2008), which sits geographically adjacent to the southern Fly River coastal region, are clearly differentiated from the introduced populations, with all individuals from these regions being assigned with high probability to a single population (green). Samples from Kiunga are assigned with high probability to a distinct cluster (red) to which individuals from Port Moresby are also partially assigned, although these (Port Moresby) individuals are also assigned to another cluster (yellow) with higher probability. Individuals from Madang in northern PNG are assigned with highest probability to the green cluster (mostly found in Daru) and with a lower probability to the yellow cluster (mostly found in Port Moresby).

The factorial correspondence analysis performed in GENETIX (Figure 4) supports the results of the STRUCTURE analysis. It clearly shows the close relationship between individuals from Daru Island and Madang, as well as between individuals from Port Moresby and Kiunga. Additionally, the introduced populations from the Torres Strait Islands and the southern Fly region are closely associated. The population with the greatest genetic affinities to the introduced population based on the FCA is Timor Leste, suggesting that the source of the introduction was more likely from the Indonesian region (where \textit{Ae. albopictus} is common) than from the extant PNG populations, as had been previously hypothesized. The Jakarta population is relatively isolated on the FCA plot.

**Discussion**

Detection of \textit{Ae. albopictus} in southern Papua New Guinea (PNG) in 1988 and 1992 placed it only 150 km across the Torres Strait from mainland Australia. In 2004–05 it appeared on the Torres Strait Islands and we initially suspected a range expansion from PNG potentially driven by human adaptation to climate variabil-

**Table 6.** Pairwise \( F_{ST} \) msats: 13 loci (bold = non-significant).

|                  | Torres Strait | Fly Region | Daru | Kiunga | Madang | Port Moresby | Timor Leste | Jakarta |
|------------------|---------------|------------|------|--------|--------|--------------|-------------|---------|
| **Fly Region**   | 0.00421       |            |      |        |        |              |             |         |
| Daru             | 0.15061       | 0.13217    |      |        |        |              |             |         |
| Kiunga           | 0.14283       | 0.16632    | 0.07760 |        |        |              |             |         |
| Madang           | 0.15134       | 0.13189    | 0.03805 | 0.07871 |        |              |             |         |
| Port Moresby     | 0.12791       | 0.11830    | 0.09477 | 0.05612 | 0.08168 |              |             |         |
| Timor Leste      | 0.10243       | 0.07446    | 0.15200 | 0.11821 | 0.15745 | 0.08435      |             |         |
| Jakarta          | 0.13908       | 0.12848    | 0.25316 | 0.26416 | 0.24757 | 0.20855      | 0.11396     |         |

Figure 3. Panel A: Microsatellite Bayesian Structure Plot for \textit{Ae. albopictus}. Thirteen microsatellite markers were run on 199 \textit{Ae. albopictus} individuals assuming five populations \((K = 5)\). Each bar represents an individual with the color of the bar the probability of the individual belonging to a genetic population or cluster. Panel B: The structure plot results \((K = 5)\) integrated into a map of the region with the locations of each population shown.

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ity. The AusAID funded drought-proofing expansion of rainwater tanks and 200 L water containers into the southern Fly River region villages immediately adjacent to the Torres Strait was completed in 2002 as a response to climate variability in the region [33,34]. Thus it was reasonable to hypothesize that the population historically extant in PNG had undergone a range expansion, initially into the southern Fly River region and subsequently into the Torres Strait Islands. The introduction of the species into the Torres Strait Islands was traced back to 2004 [29], at a date that appeared to correlate with the change in water management which had occurred a few years earlier.

Initially the mtCOI suggested that two genetically distinct populations were present in this region, providing the first piece of evidence that the invading population may not have originated from the population previously extant in PNG. Shared haplotypes between the southern Fly Region, the Torres Strait Islands and Indonesian populations provided the first clue as to where the invading population may have originated. Despite haplotype diversity being biased by the larval sampling method (which favors the collection of siblings of the same haplotype), the Torres Strait Island populations revealed four more haplotypes (a total of 10, $Hd = 0.301$) than the southern Fly River (6 haplotypes, $Hd = 0.649$). This difference in haplotype diversity may suggest that the initial introduction into the region started in the Torres Strait Islands from the Indonesian region, however more COI sequencing from the southern Fly region may be needed to clarify whether or not this is the case. Interestingly however, genetic diversity appears higher in the Torres Strait and Fly Region than in the Indonesian populations, possibly suggesting multiple introductions of closely related populations from different parts of Indonesia, or that the founding population was more genetically diverse than those sampled from the Indonesian region.

The mitochondrial DNA was informative at another level with the discovery that multiple *Ae. albopictus* mtDNA haplotypes (representing different females contributing to the population) were moving between islands. This suggests that attempts to eradicate the species from individual islands would likely be unsuccessful given the high potential for re-introductions. Indeed, this information has assisted Queensland Health – the regional state health authority – in its decision to move from the island

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**Figure 4. Factorial Correspondence Analysis (FCA) of 199 *Ae. albopictus* individuals.** Each point represents one individual assessed for 13 microsatellites with their relative proximity to each other on the graph representing genetic relatedness. Panels A and B represent two perspectives of the graph. doi:10.1371/journal.pntd.0002361.g004
eradication program implemented in 2006 to a cordon sanitaire in 2008, whereby surveillance and control was focused on the inner Torres Strait islands of Waiben, Muralug and Ngrupai (Thursday, Prince of Wales and Horn islands) adjacent to mainland Australia. In particular, Muralug and Ngrupai act as the major regional transport hubs and are thus the most likely staging point for species’ introduction onto the Australian mainland. This cordon sanitaire was breached in 2009 and *Ae. albopictus* now exists on Waiben and Ngrupia, less than 30 km from Australia’s Cape York Peninsula. In 2010, *Aedes albopictus* larvae were collected from New Marpoon on mainland Australia’s Cape York although no other individuals have been collected in this area since.

The 13 microsatellite loci reaffirmed the findings of the mitochondrial data that the introduced population was genetically distinct to the populations already present in PNG. As microsatellites evolve more rapidly than mitochondrial sequence data, they were more informative, resolving five genetically distinct populations in total with three historically extant populations in PNG that have experienced various levels of admixture and one distinct population encompassing the Torres Strait Islands and the southern Fly River region—the introduced population. Samples from Madang in northern PNG, as well as from Port Moresby on the southern Papuan Peninsula, appear to be admixtures, with individuals from Port Moresby being more similar to samples from Kiunga, and individuals from Madang more similar to the Daru Island population (Figures 3 and 4). Although Daru Island is proximal to the introduced population, material collected there on two separate occasions (1992 and 2008) was assigned with high probability to a separate population with close genetic affinities to the Madang material. Interestingly, the material collected from the Indonesian region (Timor Leste and Jakarta) forms a distinct population with apparent genetic affinities to the introduced population in the Torres Strait and southern Fly River Region. Timor Leste revealed the highest genetic affinities in the FCA analysis and there are records of *Ae. albopictus* being present in Timor Leste dating to the 1920s [23]. Thus, our combined data strongly suggests that the introduction of *Ae. albopictus* into the Torres Strait and southern Fly River region came from the Indonesian region.

Since it now appears highly unlikely that the introduced population originated from PNG but rather came from the Indonesian region (west of the Torres Strait), it is conceivable that the introduction was driven by foreign fishing vessels that travelled from the Indonesian region, harboring *Ae. albopictus* specimens which then infested the islands of the Torres Strait and/or the southern Fly region. Indonesian/Macassan visitations to the coastline of northern Australia have a long history predating European settlement, and Indonesian fishermen have been known to illegally enter Australian waters more recently to fish for shark fin (Walker, J. Pers. Com.). This activity is reported to have peaked between 2005–07, during which time several hundred landings occurred where fuel, water, shark fin, fishing nets and lines were often caught. Many of these landings were associated with well-established camps that received multiple visits. Uninhabited islands in the Torres Strait were one focus for these activities and evidence gathered from apprehended vessels indicates that most shark boats (Type III - highly mobile, motorized vessels) carried large open-topped water drums of which a significant proportion harboured *Ae. aegypti* and *Ae. albopictus* larvae. There have been documented collections of various *Aedes* (Stegomyia) species, including *Ae. albopictus*, from illegal fishing vessels that were intercepted at the port of Darwin in the Northern Territory that clearly indicate that the mosquitoes’ survival in smaller vessels is possible [56,57].

With regard to the expansion and movement of *Ae. albopictus* in this region, populations on Daru Island (which adjoins the Torres Strait) appear to have been unaffected by this exotic invasion up until 2008 and follow-up collections are now warranted to determine if the introduced population has established there since. Despite Daru Island sitting geographically adjacent to the southern Fly River coastal region, its *Ae. albopictus* population seems to be clearly isolated from the introduced population. The occurrence of two genetically distinct populations adjacent to each other in the northern Torres Strait/PNG region can be best explained by their different jurisdictions. While the Torres Strait operates as part of Australia, Daru Island is politically part of PNG and its function as the international customs clearance station into Western Province means that it encounters different incoming and outgoing transport movements.

The movement of *Ae. albopictus* appears to be extensive in the Australasian region, particularly where the Torres Strait Islands’ junction separates New Guinea and mainland Australia. With the maximum reported flight range of *Ae. albopictus* being 1 km [58], movement between the Torres Strait’s islands has most likely taken place via human-mediated transport. Australia has an oceanic border with PNG and the Torres Straits, but unlike most other international countries, it has no clearly marked frontier with border policing or customs controls. Thus relatively free movement occurs between PNG and the Torres Straits with approximately 5,000 international shipping movements per year [59], and countless domestic movements that would likely shuttle *Ae. albopictus* between the islands and back forth from the southern fly region’s villages. This movement is sanctioned under The Torres Strait Treaty (Miscellaneous Amendments) Act 1984 that allows for cross-border movement for trade, fishing, and family gatherings or for seeking medical attention without the need for customs protocols [60]—all of which may compromise quarantine in the region. The primary mode of transport between PNG villages and the Torres Strait Islands is via open-topped, outboard-motor-powered boats. In addition to sheltered harborage sites for adult mosquitoes, these vessels contain fresh water—both within drums for human consumption, and where rainwater has collected—which in turn provides oviposition sites and larval habitats for container-inhabiting species such as *Ae. albopictus*.

Given its current location, its mobility and its phenotypic fondness for containers, *Ae. albopictus* is more than likely to arrive in a town or city on the Australian mainland via human transport. Due to the intrinsic ecological plasticity in both larval habitat (both natural and human-made) and host feeding patterns [3], it will likely be able to move between urban and sylvan habitat, and control will be extremely challenging once it enters the latter. Its effect on native virus vector systems in Australia represents an unknown risk both to humans and to native and domestic animals. However, its cool climate tolerant biology and plasticity will certainly present new risks for dengue and chikungunya transmission in summer throughout most of the Australian region [27,30].

*Aedes albopictus*’s peculiar biology permits its container inhabiting ecological niche to once again facilitate its global expansion. It is important to consider it’s potential to rapidly exploit the outcomes of any socio-economic or policy-driven interventions, such as the recent and dramatic expansion of domestic rainwater tanks throughout Australian urban regions as a drought-proofing adaptation to observed and forecasted climate change [30]. Although this adaptation did not ultimately explain the range expansion of the species on Australia’s northern doorstep, it will nonetheless provide a valuable niche in the landscape, which may augment this vector’s existence across Australia’s urban regions.

*Ae. aegypti* and *Ae. albopictus* are potential vectors for the expanding global dengue and chikungunya transmission threat in Australasia.
considering its eventual arrival, the public health risks associated with arboviruses meet the possibility of substantial daytime nuisance biting that will also negatively impact Australia’s urban alfresco lifestyle.

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Author Contributions

Conceived and designed the experiments: NWB SAR RCR AFvdH. Performed the experiments: NWB LA LAH. Analyzed the data: NWB LA. Contributed reagents/materials/analysis tools: RDC JBD GH LJR RCR NFI DS AFvdH. Wrote the paper: NWB LA SAR AFvdH.

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References

1. Benedict MQ, Levine RS, Hawley WA, Lounibos LP (2007) Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector Borne Zoonotic Dis 7: 39–48.
2. Medlock JM, Hansford KM, Schaffner F, Versteirt V, Hendrickx G, et al. (2012) A review of the invasive mosquitoes in europe: ecology, public health risks, and control options. Vector Borne and Zoonotic Diseases 12: 435–447.
3. Hawley WA (1988) The biology of *Aedes albopictus*. J Am Mosq Cont Assoc 4: 1–40.
4. Scholer RJ, Takken W, Knols BG (2007) Infection of adult *Aedes aegypti* and *Aedes albopictus* mosquitoes with the entomopathogenic fungus Metarhizium anisopliae. Acta Tropica 102: 151–158.
5. Reiter P, Sprenger D (1987) The used tire trade: a mechanism for the worldwide dispersal of container breeding mosquitoes. J Am Mosq Cont Assoc 3: 494–501.
6. Estrada-Franco EG, Graig GB (1993) Biology, Disease Relationship, and Control of *Aedes albopictus*. Washington. 1–49 p.
7. Adhami J, Reiter P (1998) Introduction and establishment of *Aedes (Stegomyia) albopictus* skuse (Diptera: Culicidae) in Alabama. J Am Mosq Cont Assn 14: 340–343.
8. Sprenger D, Wuithayanong T (1996) The discovery and distribution of *Aedes albopictus* in Harris County, Texas. J Am Mosq Cont Assoc 2: 217–219.
9. Forattini OP (1996) Identification of *Aedes (Stegomyia) albopictus* (Skuse) in Brazil. Rev Saude Publica 20: 244–245.
10. Savage HM, Eade VL, Nwanoco AK, Spiegel R, Miller BR (1992) First record of breeding populations of *Aedes albopictus* in continental Africa: implications for arboviral transmission. J Am Mosq Cont Assn 8: 101–103.
11. Sabatini A, Raineri V, Trovato G, Coluzzi M (1990) Invasion by *Aedes aegypti* in the Solomon and Santa Cruz islands, South Pacific. Trans Roy Soc Trop Med Hyg 74: 288–295.
12. Nicholson JN, Ritchie S, Zalucki M, van den Hurk A (2009) Potential. PLoS Negl Trop Dis 3: e249.
13. Allen BJ, Bourke EM (1997) Report of an assessment of the impact of frost and drought in Papua New Guinea. Port Moresby: Australian Agency for International Development. pp. 1–21.
14. Hill LA, Davis JB, Hughson G, Whelan PL, Smith GA, et al. (2008) Rapid identification of *Aedes albopictus*, *Aedes scutellaris*, and *Aedes aegypti* life stages using real-time polymerase chain reaction assays. J Troop Med Hyg 79: 866–875.
15. Beebe NW, van den Hurk AF, Chapman H, Frances SP, Williams CR, et al. (2002) Development and evaluation of a species diagnostic polymerase chain reaction-restriction fragment-length polymorphism procedure for cryptic members of the *Caedes* subgenus (Diptera: Culicidae) subgroup in Australia and the southwestern Pacific. J Med Entomol 39: 362–369.
16. Kariohampa S, Smith PT (1996) PCR primers for the amplification of four distinct mitochondrial gene segments. Insect molecular biology 4: 233–236.
17. Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, et al. (2011) Genious v5.4.
18. Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate genealogies. Mol Ecol 9: 1325–1329.
19. Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10: 564–567.
20. Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.
21. Faircloth BC (2008) MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. Mol Ecol Res 8: 92–94.
22. Holland MM, Parson W (2011) GeneMarker®/R HGD: A reliable software tool for the analysis of forensic STR data. Journal of forensic sciences 56: 29–35.
23. Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4: 535–538.
24. Peakall R, Smouse PE (2006) GENALEX, 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6: 281–293.
25. Meirmans PG, Van Tienderen PH (2004) GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of sexual organisms. Molecular Ecology Notes 4: 792–794.
26. Hubisz MJ, Falush D, Stephens M, Pritchard JK (2009) Inferring weak population structure with the assistance of sample group information. Molecular ecology resources 9: 1322–1326.
27. Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945–959.
28. Earl DA, Vangel BS (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359–361.
51. Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14: 2611–2620.
52. Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure Bioinformatics 23: 1801–1806.
53. Rosenberg NA (2004) DISTRACT: a program for the graphical display of population structure. Molecular Ecology Notes 4: 137–138.
54. Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (1996–2004) GENETIX 4.05, Windows TM software for population genetics. Montpellier (France): Laboratory Genome, Populations, Interactions, The Montpellier 2 University.
55. Goudet J (1995) FSTAT (Version 1.2): A computer program to calculate F-statistics. Journal of Heredity 86: 485–486.
56. Whelan P, Hayes G, Tucker G, Carter J, Wilson A, et al. (2003) The detection of exotic mosquitoes in the Northern Territory of Australia. Arbovirus Res Aust 8: 395–404.
57. Whelan PL, Tucker G (1998) Exotic Aedes surveillance and exclusion from the Northern Territory of Australia. Bull Mosq Cont Assoc Aust 10: 85–99.
58. Maciel-de-Freitas R, Neto RB, Goncalves JM, Codeco CT, Lourenco-de-Oliveira R (2006) Movement of dengue vectors between the human modified environment and an urban forest in Rio de Janeiro. J Med Entomol 43: 1112–1120.
59. McFarlane J (1998) Torres Strait: Policing the Open Border. Canberra: Australian Institute of Criminology. 1–6 p.
60. Arthur WS (2001) Autonomy and identity in Torres Strait, a borderline case? Journal of Pacific History 36: 215–224.