# Genomic databanks and targeted assays help characterise mosquito incursions within Australia

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## Abstract

Biosecurity strategies that aim to restrict the spread of invasive pests can profit greatly from knowledge of where new incursions have come from. This knowledge is often difficult to acquire, particularly for incursions that are detected outside their vessel of entry. Genomic databanks have shown recent efficacy for determining origins of individual incursions of pests such as mosquitoes and fruit flies, where genome-wide genetic variation in incursion samples is compared against variation in reference samples. This paper uses genomic databanks to trace and genetically characterise incursions of two species of mosquito (*Aedes aegypti* and *Ae. albopictus*) within Australia. We built an *Ae. aegypti* databank of samples from across its Australian range, and used this to trace a 2021 invasion of Tennant Creek, Northern Territory, to Townsville, Queensland. We used targeted assays to show the invasion did not carry a *Wolbachia* infection, but had similar mtDNA to wildtype Townsville samples, and thus concluded that the invasion was sourced from an uninfected area within or near Townsville, where the *Wolbachia* infection has been widespread since 2018. For *Ae. albopictus*, we traced incursions into the southern Torres Strait Islands from invaded islands, and show how origins of incursions collected in 2021 differed from those collected in 2019. Comparing patterns of genetic relatedness in both species indicated that Tennant Creek was likely invaded by mosquitoes from the same geographical location and are potentially all from the same family, whereas the Torres Strait incursions were all from distinct kinship groups. Our results highlight the value of repeated tracing over years and the utility of targeted assays to improve accuracy of inferences. The specificity of tracing to populations sampled >8 years previously suggest that genomic databanks can remain informative for tracing over years.

# Introduction

Invasions of exotic pests have a global economic burden estimated at over $200B annually (Diagne et al., 2021). These costs have risen in recent decades and are expected to rise further. For invertebrates, most invasions establish via accidental transportation on ships, planes, or land vehicles, and are typically hard to eradicate with insecticides due to resistance that is either present on arrival or evolved shortly afterward (Gao et al., 2021). Border biosecurity operations have demonstrated success at restricting the spread of pests (Caley, Ingram, & De Barro, 2015; Muzari et al., 2017), however, resource limitations restrict the number of inspections on arriving vessels or the number of border sections that can be monitored.

These operations can be greatly assisted by knowledge of incursion pathways, as this allows for more frequent inspection of goods and conveyances from likely source locations (Robinson, Burgman, & Cannon, 2011) and regular inspection of known entry points (Mehta, Haight, Homans, Polasky, & Venette, 2007; Myers, Simberloff, Kuris, & Carey, 2000). While pathways may be easily inferred if incursions are detected on their vessel of entry, incursions that are detected later pose a greater challenge. Recent work using genomic databanks to trace incursions or recently established invasions indicates that these methods can reliably infer the most likely origin out of a set of reference populations (Chen et al., 2021; Gloria-Soria et al., 2018; Kelly et al., 2021; Popa-Báez et al., 2021; Schmidt et al., 2020, 2021, 2019; Sherpa et al., 2019), and are particularly suitable for tracing samples collected beyond border biosecurity. These methods compare genome-wide genetic variation in the incursion or invasion of interest against variation in a set of reference samples, and infer one population or set of related populations as being the most likely origin.

This study presents genomic tracing results for two mosquito species, the dengue mosquito *Aedes aegypti* and the tiger mosquito *Ae. albopictus*, where invasive samples of unknown provenance have been detected in Australia and traced to established Australian populations. For *Ae. aegypti*, we trace an invasion of Tennant Creek, Northern Territory, that was detected in early 2021. For *Ae. albopictus*, we trace a set of new incursions detected in the Torres Strait Islands, Queensland, on islands where this species has been successfully eliminated (Muzari et al., 2017) and where previous tracing in 2018 has indicated a nearby island, Keriri, as a source of incursions (Schmidt et al., 2021). For the *Ae. aegypti* invasion, we show how precise tracing can be achieved despite reference samples being ≥8 years old. We also use targeted assays for insecticide resistance and the *w*Mel *Wolbachia* infection to improve precision of tracing. For *Ae. albopictus*, we trace the 2021 incursions to a different set of islands than the 2018 incursions, and show how precision of tracing has not decreased despite frequent dispersal among the invaded islands (Maynard et al., 2017; Schmidt et al., 2021). Finally, comparing genetic relatedness among samples in both species, we find evidence that the *Ae. aegypti* invasion was likely sourced from a single

geographical location and potentially from a single family, while the *Ae. albopictus* incursions were all from different family groups.

# Materials and Methods

## Mosquito sample collection

### *Aedes aegypti* invasion of Tennant Creek, NT, Australia

*Aedes aegypti* was detected in Tennant Creek during routine surveillance between 22$^{nd}$ – 26$^{th}$ February 2021. Four samples from this new invasion were collected from a single property at 26$^{th}$ February, and follow-up sampling between 9$^{th}$ - 11$^{th}$ March located 14 additional samples at four properties within ~80 m of the original detection. The 18 invasive samples included adults and immatures.

To trace the source of this invasion, we built a genomic databank from 80 *Ae. aegypti* sampled from 11 locations across its Australian distribution in Queensland, from the northern islands of the Torres Strait (9.3° S) to Goomeri (26.2° S). These were sampled between 2009 and 2017. We also included previously-generated sequence data from Cairns (Schmidt, Filipović, Hoffmann, & Rašić, 2018) and Townsville (Rašić et al., 2016), sampled in 2015 and 2014 respectively. The Cairns sample included some individuals infected with *Wolbachia* from earlier releases (Schmidt et al., 2017) while the Townsville sample was taken before *Wolbachia* releases in this area (O'Neill et al., 2018).

### *Aedes albopictus* incursions in the Torres Strait Islands, QLD, Australia

*Aedes albopictus* was detected below the Torres Strait *cordon sanitaire* during routine surveillance in January and February 2021. This included four adult individuals from Thursday Island and three from Horn Island.

To trace the source of this invasion, we used a genomic databank containing previously-generated sequence data of 240 *Ae. albopictus* from 13 Torres Strait Island (TSI) communities across 12 islands, collected in 2017 (Schmidt et al., 2021). Before tracing among TSI locations, we checked to ensure that the sample was not from either Papua New Guinea or Indonesia using the same methods as for other tracing, and found that they all had a TSI origin.

## DNA sequencing and bioinformatic processing

Genomic DNA was extracted from the 98 *Ae. aegypti* and 7 *Ae. albopictus* using Qiagen DNeasy kits. Extracted DNA was used to build double digest restriction-site associated DNA (ddRAD; (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012)) sequencing libraries following

the protocol of Rašić, Filipović, Weeks, & Hoffmann (2014). A technical replicate was produced of one of the TSI samples from Thursday Island, TI-1, which was replicated through the entire protocol after DNA extraction. Libraries were individually barcoded, then sequenced on a HiSeq 4000 using 150 bp chemistry.

New and old sequence data were combined for each species, and run through the same bioinformatic pipeline. We used the Stacks v2.54 (Rochette, Rivera-Colón, & Catchen, 2019) program process_radtags to demultiplex sequences, trim them to 140 bp and remove sequences with Phred scores below 20. As the Cairns and Townsville libraries were produced using 100 bp chemistry, we trimmed these to 80 bp. Sequences were aligned to the nuclear genome assembly for *Ae. aegypti*, AaegL5 (Matthews et al., 2018), and the linkage-based assembly for *Ae. albopictus*, AalbF3 (Boyle et al., 2021). Alignment was in Bowtie2 v2.3.4.3 (Langmead & Salzberg, 2012) using –very-sensitive settings. Sequences were built into catalogs for each species using the Stacks program ref_map.

The Stacks program populations was used to export VCF files containing SNP genotypes for all individuals in each catalog, filtering to retain SNPs called in at least 75% of individuals from each population. VCFtools v0.1.16 (Danecek et al., 2011) was used to further filter SNPs to retain those scored in 95% of all individuals, with a minor allele count ≥ 3 (Linck & Battey, 2019), and at least 25 kbp from other SNPs. Two of the 18 Tennant Creek *Ae. aegypti* had too high missing data for genomic analysis and were omitted, all other individuals were retained.

## Incursion tracing

### i) Genome-wide genetic variation

We used Locator (Battey, Ralph, & Kern, 2020) to infer the origin of the 16 invasive *Ae. aegypti* and the 8 incursive *Ae. albopictus* (including the technical replicate). Locator is a deep learning method that uses individual genotypes of known geographical location (i.e. the reference individuals) to predict the locations of a set of individuals of unknown location (i.e. the incursive mosquitoes). For each incursive, we set Locator to provide a point estimate of its origin and 1,000 bootstrap subsamples to indicate confidence around these estimates.

### ii) *Wolbachia* infection

The Tennant Creek *Ae. aegypti* were assessed for infection of *w*Mel *Wolbachia*, which are present at high frequency in *Ae. aegypti* populations in Cairns, Townsville, and several other locations in northern Queensland, but not in central Queensland populations. *Wolbachia* infection was assessed using a high-throughput PCR assay (Lee, White, Weeks, Hoffmann, & Endersby, 2012) which was run twice on all 18 samples.

In the case of uninfected samples, we also considered the possibility that the *w*Mel infection was initially present but then lost from the invasive population due to the vulnerability of this *Wolbachia* strain to high temperatures (Ross et al., 2017) that are common in Tennant Creek. As *Wolbachia* is coinherited with mtDNA through the maternal line (Hoffmann & Turelli, 1997), *w*Mel-infected individuals will all have inherited the same mtDNA haplotype from the same common ancestor within the past ~10 years. This mtDNA haplotype will be retained even if *Wolbachia* has been lost, and its presence would be revealed by high genetic similarity in mtDNA between the Tennant Creek *Ae. aegypti* and samples infected with *w*Mel.

To test this hypothesis, we looked at mtDNA variation among our *Ae. aegypti* samples alongside variation from 38 *w*Mel-infected and 87 uninfected wildtype (WT) individuals from Cairns, sampled in 2015 (Schmidt et al., 2018). We aligned demultiplexed sequences to the *Ae. aegypti* mtDNA genome assembly (Behura et al., 2011) and called mtDNA SNPs using Stacks. We used VCFtools to output mtDNA genotypes for all individuals with minimum depth of 5, omitting SNPs with any heterozygous genotypes which could represent NUMTs (Hlaing et al., 2009).

### iii) Pyrethroid resistance mutations

The Tennant Creek *Ae. aegypti* were assessed for three pyrethroid resistance mutations common to *Ae. aegypti* across the Indo-Pacific (Endersby-Harshman et al., 2020) but not within Australia (Endersby-Harshman et al., 2017). For each individual, we ran three replicates of the custom TaqMan® SNP Genotyping Assays (Life Technologies) described in Endersby-Harshman et al. (2020), using on LightCycler II 480 (Roche) real time PCR machine in a 384-well format.

## Genetic relatedness of the incursions

We investigated patterns of genetic relatedness among pairs of individuals (dyads) in the Tennant Creek and Torres Strait incursions. The patterns would help indicate whether the samples at either location had likely arrived as part of a single incursion or several distinct incursions. If multiple incursive individuals had arrived via the same incursion (e.g. on the same vessel), this could be revealed in relatedness estimates showing that they are more closely related than other dyads. Also, if individuals are inbred as a result of the invasion being sourced from a single family, this could produce a pattern where coefficients of individual inbreeding are indistinguishable from other kinship coefficients. If neither of these patterns are present, this suggest that invasive samples are from different incursions.

We tested three relatedness estimators for their appropriate use in characterising genetic patterns in the two incursion samples. These were: Loiselle's *k* (Loiselle, Sork, Nason, &

Graham, 1995), calculated with SPAGeDi (Hardy & Vekemans, 2002); KING (Manichaikul et al., 2010), calculated with VCFtools; and PC-Relate (Conomos, Reiner, Weir, & Thornton, 2016). While these estimators are all appropriate under a range of conditions, here the small sample sizes (8 – 16) and potentially extremely high levels of inbreeding may make accurately estimating genetic relatedness difficult. We considered an estimator appropriate based on its accuracy in classifying the two TSI technical replicates as genetically identical.

# Results

## Incursion tracing

### *Aedes aegypti* invasion of Tennant Creek, NT, Australia

Genomic tracing with Locator was run sequentially on different subsets of samples. An initial run with all 112 reference samples and 11866 SNPs indicated all 16 samples had a likely origin between Cairns (16.9° S) and Rockhampton (23.4° S) (Fig S1). Rerunning Locator with reference samples outside this range omitted and 11495 SNPs indicated a likely origin of Townsville (Fig 1).

We checked whether this assignment to origin was biased by Townsville's geographically central location by rerunning Locator using either Townsville and Cairns only (Fig S2) or Townsville and the southern samples only (Fig S3). In each case, incursives were still placed around Townsville but Townsville and Cairns were slightly harder to differentiate between.

Assays for *w*Mel *Wolbachia* indicated none of the Tennant Creek samples were infected with *w*Mel. Analysis of 116 mitochondrial SNPs showed that pairwise $F_{ST}$ between Tennant Creek and Townsville was lower (0.26) than between Tennant Creek and either Cairns sample (Table 1). This indicates that no *w*Mel infection was ever present in the Tennant Creek samples or their maternal ancestors. Pyrethroid resistance assays found none of the three resistance alleles were present in Tennant Creek samples.

### *Aedes albopictus* incursions in the Torres Strait Islands, QLD, Australia

Locator, run using 2403 filtered SNPs, indicated several of the incursives had an origin in the Maluilgal (Western) island group (Fig 2). These included the TI-1 sample and its technical replicate TI-1r, which were both traced specifically to Moa Island with very low variation among the bootstrap samples. TI-3, TI-4, and HI-3 were also linked to the Maluilgal group, though could not be linked to a specific island. The remaining incursives could not be placed with any specificity. Unlike a previous sample from 2019, none of the 2021 incursives were clearly linked to Keriri in the Kaiwalagal (Inner) island group.

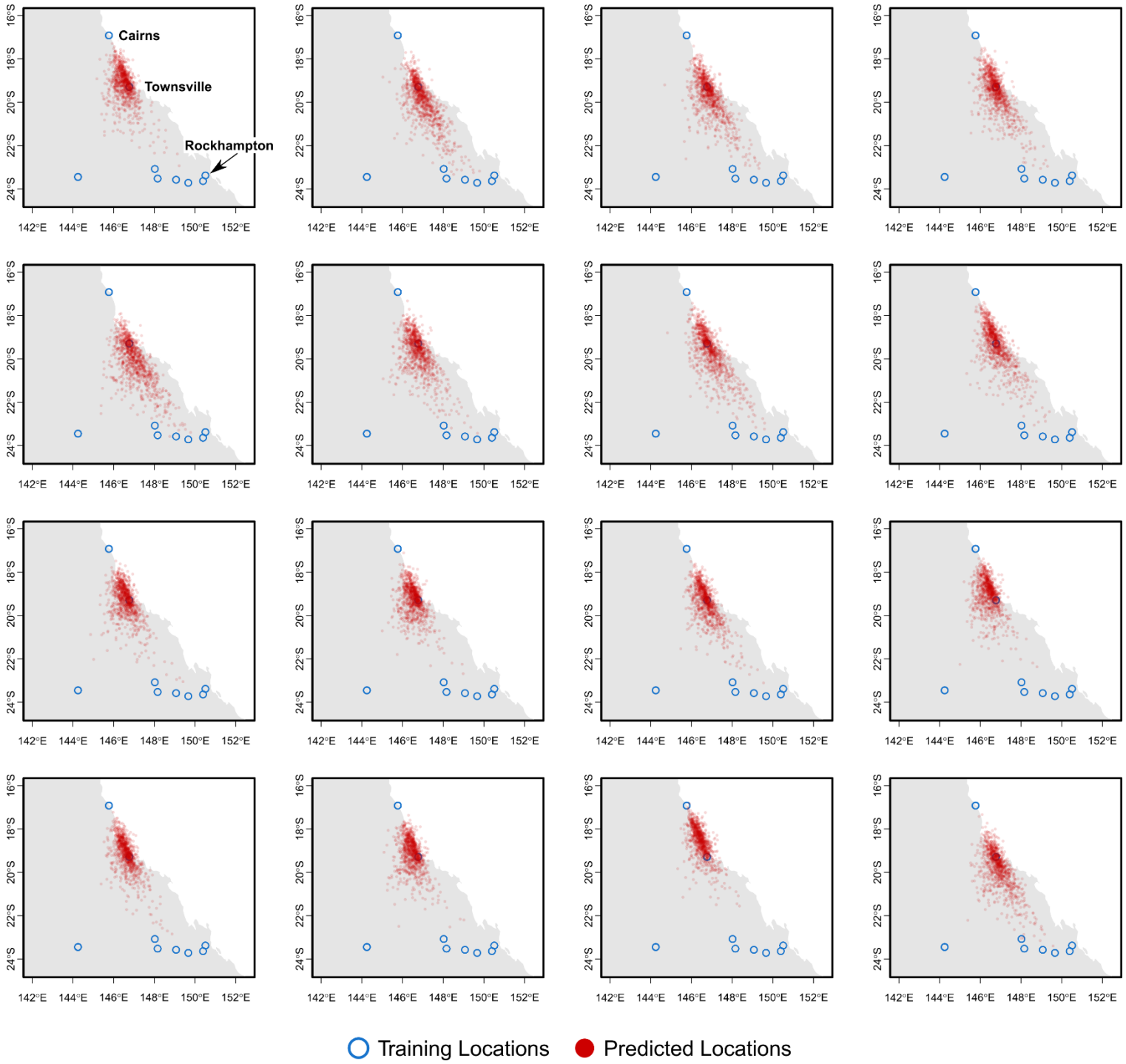


Figure 1. Inferred origins of the 16 Tennant Creek individuals from genome-wide genetic variation. Blue circles indicate reference sample locations, red circles show inferred origins from 1000 bootstrap replicate runs of Locator. Figs S1-3 show results of similar analyses using the whole dataset (S1) or different subsets of the dataset (S2-3)

Table 1. Pairwise $F_{ST}$ of mtDNA variation among Tennant Creek, Cairns, and Townsville populations.

| $F_{ST}$ | Townsville WT | Cairns WT | *w*Mel Cairns |
|---|---|---|---|
| Tennant Creek | 0.26 | 0.85 | 0.85 |
| Townsville | | 0.87 | 0.87 |
| Cairns WT | | | 0.02 |

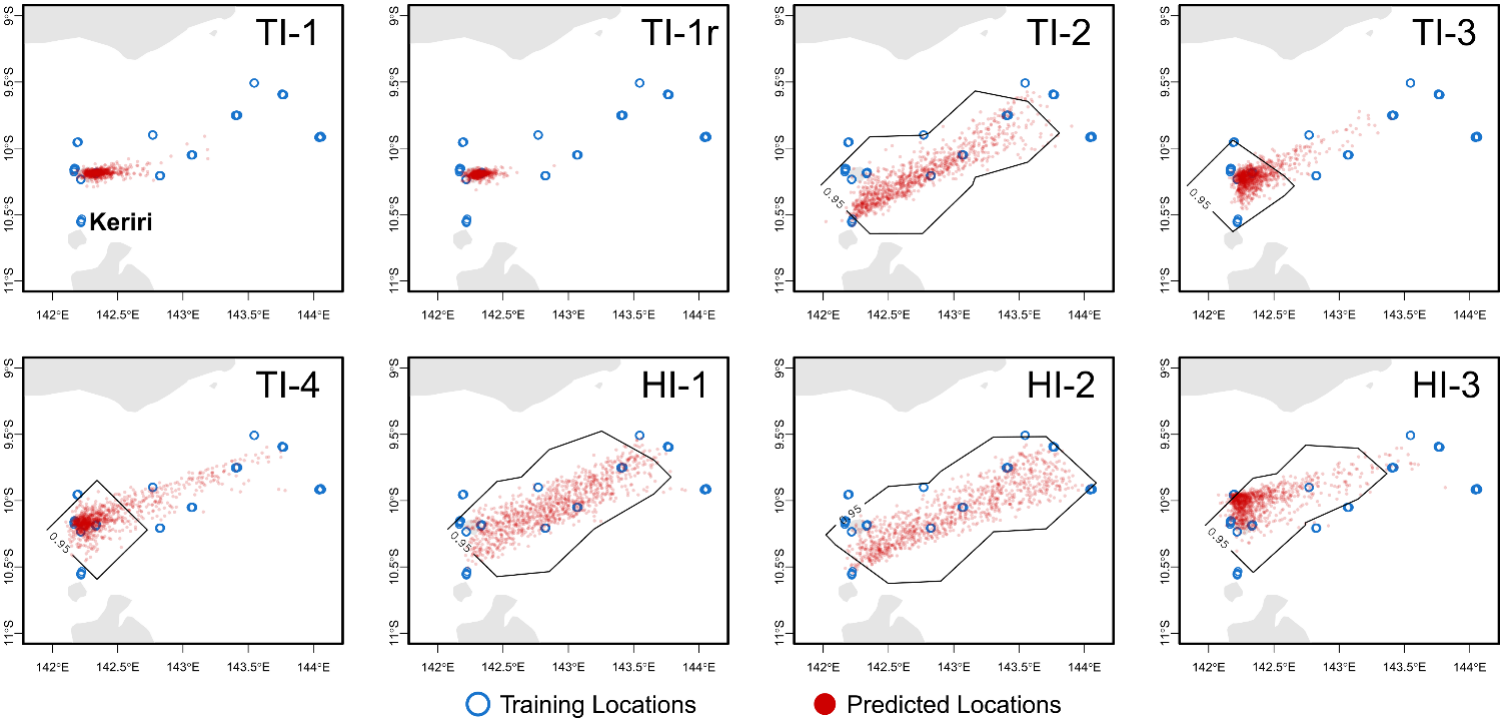


Figure 2. Inferred origins of the 7 Torres Strait Islands individuals from genome-wide genetic variation. Samples were detected on Thursday Island (TI) or Horn Island (HI), and a technical replicate was made of the TI-1 sample. Blue circles indicate reference sample locations, red circles show inferred origins from 1000 bootstrap replicate runs of Locator.

## Genetic relatedness of the incursions

Both PC-Relate and KING assigned a negative kinship coefficient to the two TSI technical replicates, as well as all other dyads. Loiselle's *k* assigned the replicates a coefficient of *k* = 0.32, suggestive of closely-related, first-order kin. As this was the closest approximation of their true identical relationship, we used Loiselle's *k* for describing genetic relatedness. *k* scores describe correlations in the frequencies of homologous alleles, summarised across the genome (Loiselle et al., 1995).

Fig X displays pairwise *k* scores for Tennant Creek (TC) and Torres Strait Island (TSI) in rank order. Scores represent either correlations between alleles in the chromosomes of two different individuals (dark blue squares) or the same individual (light blue circles; "inbreeding"). The inbreeding *k* scores of the two TSI replicates were almost identical to the *k* score estimated from treating them as two individuals (orange square), further validating Loiselle's *k* for this analysis.

The inbreeding *k* scores were distributed very differently between the two datasets. In the TSI, inbreeding *k* scores were all much higher than the *k* scores between dyads. This pattern means that the two chromosomes within each individual are more genetically similar to each other than to chromosomes found in other individuals. The large gap between the inbreeding and dyad scores suggests that none of the TSI dyads are likely to be from the same family, as dyads from the same family would have chromosomes with high genetic similarity via identity by descent. These dyads would thus have higher *k* scores than the other, unrelated dyads. Instead, these results are evidence that the seven TSI incursives each represent distinct incursions.

This pattern was not observed at TC, where inbreeding *k* scores were mostly undifferentiated from and in many cases lower than *k* scores between dyads. Chromosomes within individuals are thus no more genetically similar than they are to chromosomes in other individuals. Supp Text S1 details an analysis of an *Ae. aegypti* population sampled over 0.8 $km^2$, showing that isolation by distance will lead to higher inbreeding *k* scores than *k* scores between dyads even at fine scales. This suggests that the 16 TC individuals are all likely to be from the same precise geographical location and are potentially all from the same family. This strongly suggests than the TC invasion had a single geographical source.

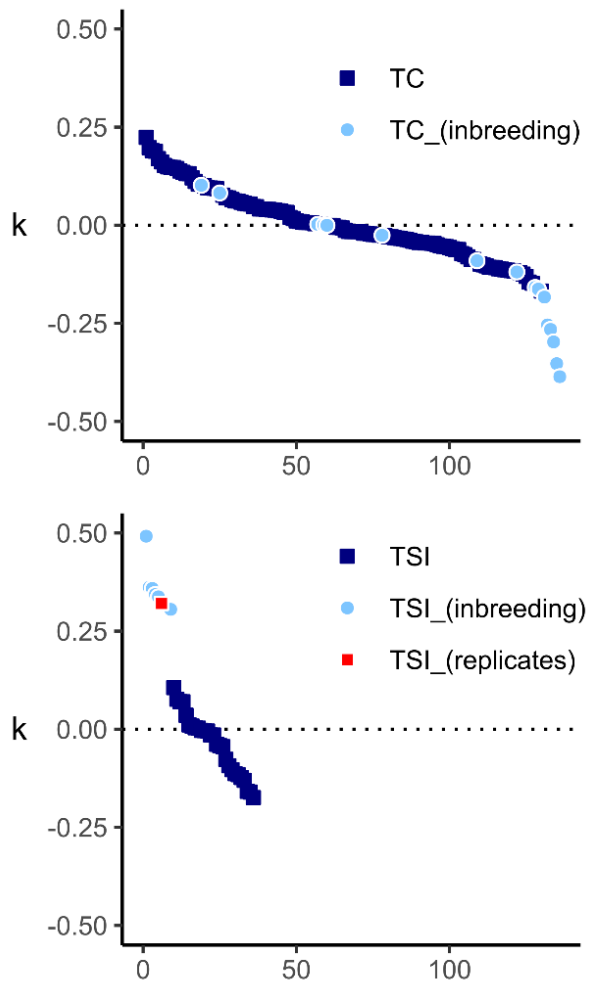


Figure 3. Genetic relatedness of all dyads within Tennant Creek (TC, top) and within the Torres Strait Islands (TSI, bottom), using Loiselle's *k*. Dark blue squares show correlations between alleles in the chromosomes of two different individuals and light blue circles show correlations within the same individual ("inbreeding"). The orange square represents the inbreeding *k* scores of the two TSI replicates.

## Discussion

Pest genomic databanks have become popular for answering questions around invasions and incursions (Chen et al., 2021; Gloria-Soria et al., 2018; Kelly et al., 2021; Popa-Báez et al., 2021; Schmidt et al., 2020, 2021, 2019; Sherpa et al., 2019). The value provided by these databanks should continue to rise as costs of generating genome-scale data from large numbers of pests continue to decrease and as new genomic methods appropriate for pests are developed (North, McGaughran, & Jiggins, 2021). This study presents the use of two genomic databanks to solve emerging problems for biosecurity and human health, which

concern new Australian incursions and invasions of the mosquitoes *Ae. aegypti* and *Ae. albopictus*. We were able to infer that the *Ae. aegypti* invasion of Tennant Creek, Northern Territory came from a location near or within Townsville, Queensland that did not have a *Wolbachia* infection at fixation. We also traced one of the seven *Ae. albopictus* incursions into the southern Torres Strait Islands to a specific island and three others to a specific island group (Maluilgal). Our estimates of genetic relatedness showed that the Tennant Creek invasion was likely sourced from one specific location and possibly a single set of close relatives, whereas the absence of close relatives in the Torres Strait incursives suggests these were all from distinct incursions.

As well as helping solve these specific biosecurity issues, this study helps clarify some ideas around the use of genomic databanks in ongoing pest management. In order for databanks to provide value for money they should be reusable for tracing over years or decades. However, effects of genetic drift and dispersal will ensure that reference samples in the databank will become incrementally less representative of the current populations with each passing generation. Our analysis of *Ae. aegypti* showed that the 2021 Tennant Creek invasion samples, which themselves may have been subject to strong genetic drift in the generations between invasion and detection, could be traced to the Townsville genetic background represented by samples collected in January 2014. Assuming 10 generations/year for *Ae. aegypti*, the Tennant Creek and Townsville samples should be separated by ~70 generations of evolution. The databank of Torres Strait *Ae. albopictus* showed similar robustness in tracing, wherein incursions sampled 3 years (~30 generations) after the reference samples could in one case be traced to a specific island with very high confidence (TI-1 and TI-1r, Fig 2). This is particularly impressive given the much finer spatial scale of the Torres Strait invasion and the low genetic differentiation among islands ($F_{ST} \approx 0.03$ (Schmidt et al., 2021)).

Using targeted assays for *Wolbachia* infection and pyrethroid resistance, combined with mtDNA data from the databank, we were able to trace the Tennant Creek *Ae. aegypti* with greater specificity than using nuclear genomic data alone. Importantly, these findings indicate that the invasion may have been sourced from an unsampled location near to Townsville, or from a region of outer Townsville where the *w*Mel infection is uncommon. These assays also indicate that the invasion may pose a greater biosecurity threat than otherwise, as without *w*Mel infection the Tennant Creek invasion should be able to transmit dengue without the transmission blocking effects of *w*Mel (Walker et al., 2011).

The different patterns of genetic relatedness observed at Tennant Creek and the Torres Strait provides a useful indication of the genetic characteristics of each invasive system, in that we can infer the incursions into the southern Torres Strait Islands are independent from each other, whereas the invasion of Tennant Creek is from a single source. This knowledge can be useful for building predictive models of pest incursion risk as it can help estimate propagule pressure (Camac, Baumgartner, Hester, Subasinghe, & Collins, 2021), though these estimates will reflect effective rather than census population sizes of incursions. The

findings at Tennant Creek pose some additional questions for studies seeking to investigate new invasions using close kin dyad methodologies (Jasper, Hoffmann, & Schmidt, 2021; Waples & Feutry, 2021). While close kin dyad methods have shown promise for investigating invasions of ~100 generations age (Schmidt et al., 2021), close kin methods should be applied cautiously to invasions that are very new or that are sourced from a small genetic pool.

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