Genomic databanks and targeted assays help characterise domestic mosquito incursions

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Abstract

Biosecurity strategies aiming to restrict the spread of invasive pests can benefit from knowing where new incursions have come from. This knowledge can be acquired using genomic databanks, where genetic variation in incursion samples is compared against variation in reference samples. Here, we use genomic databanks to investigate domestic incursions of two mosquito species in Australia. We used a deep learning method to trace a 2021 invasion of *Aedes aegypti* in Tennant Creek, Northern Territory, to Townsville, Queensland, and to trace *Ae. albopictus* incursions between several invaded and uninvaded islands in the Torres Strait. We observed high precision of tracing despite 30-70 generations separating incursions and source samples. Targeted assays also improved precision, in this case by comparing *Wolbachia* infection data to mitochondrial DNA variation in *Ae. aegypti*. For *Ae. albopictus,* incursions detected in 2021 were traced to different islands than incursions detected in 2019. Patterns of relatedness and inbreeding indicated that Tennant Creek was likely invaded by *Ae. aegypti* from one family, whereas Torres Strait incursions involved distinct kinship groups. Our results highlight the value of repeated tracing with genomic databanks that remain informative over years, and demonstrate how additional targeted assays (e.g. *Wolbachia*) can improve inferences.

Keywords: genomic databanks; incursions; deep learning; biosecurity; *Aedes* mosquito; *Wolbachia*

Introduction

Invasions of exotic pests confer a global economic burden of over $200B annually (1). These costs have risen in recent decades and are expected to rise further as invasions continue to increase in frequency worldwide (2). 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 (3). Border biosecurity operations have demonstrated success at restricting the spread of pests (4,5), however, resource limitations restrict the number of inspections on arriving vessels and the number of border sections that can be effectively monitored.

Biosecurity 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 (6) and regular inspection of known entry points (7,8). 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 (9–16), and are particularly useful for tracing samples collected beyond border biosecurity. These methods compare genome-wide genetic variation in the incursion or invasion of interest against variation across reference samples, allowing the invasion to be linked to a population or a set of related populations of origin depending on the quality of the database.

This study presents genomic tracing results for two mosquito species, the dengue mosquito *Aedes aegypti* and the tiger mosquito *Ae. albopictus.* Previous work on tracing these mosquitoes in Australia has focused on determining the country of origin of incursion samples collected at airports and seaports (13,15). In addition, some tracing of *Ae. albopictus* has also been completed within the Torres Strait Islands, Queensland, as part of a study considering population structure across this region (14).

Here we focus on new in-country detections within Australia away from entry ports where invasive samples of unknown provenance have been detected. 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 on islands where this species has been successfully eliminated (4) and where previous tracing of 2019 incursions indicated a nearby island, Keriri, as a source of incursions (14). For the *Ae. aegypti* invasion, we show how precise tracing can be achieved despite reference data being collected ≥7 years previously. We also use targeted assays for insecticide resistance and a *Wolbachia* infection to improve precision of tracing, which follow releases to establish *Wolbachia* in north eastern Australia over the last decade (17–19). For *Ae. albopictus*, we trace the 2021 incursions to a different set of islands than the source of the 2018 incursions, and we show that precision of tracing has not decreased despite frequent dispersal among

the invaded islands (14,20). Finally, by 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 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 22nd – 26th February 2021. Four adult mosquitoes from this new invasion (three females, one male) were collected from a single property at 26th February. Follow-up sampling between 9th - 11th March located an additional five adults (three females, two males) and nine immatures at four properties within ~80 m of the original detection.

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 in southeast Queensland (26.2° S) (Fig 1a). These were sampled between 2009 and 2017. We also included previously-generated sequence data from Cairns (21) and Townsville (22), sampled in 2015 and 2014 respectively. The Cairns sample included some individuals infected with *Wolbachia* from earlier releases (19) while the Townsville sample was taken before *Wolbachia* releases in this area (17).

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

Aedes albopictus incursions were detected during routine surveillance on two islands in the Torres Strait where the species has been successfully eliminated (4). Surveillance conducted in January and February 2021 detected four adult individuals from Thursday Island and three from Horn Island (Fig 1b). On each island, the incursions were not restricted to a single location. On Horn Island the incursive mosquitoes were collected from two locations ~600 m apart (Figure S1), while on Thursday Island the incursions were spread across four locations ~1 km apart (Figure S2).

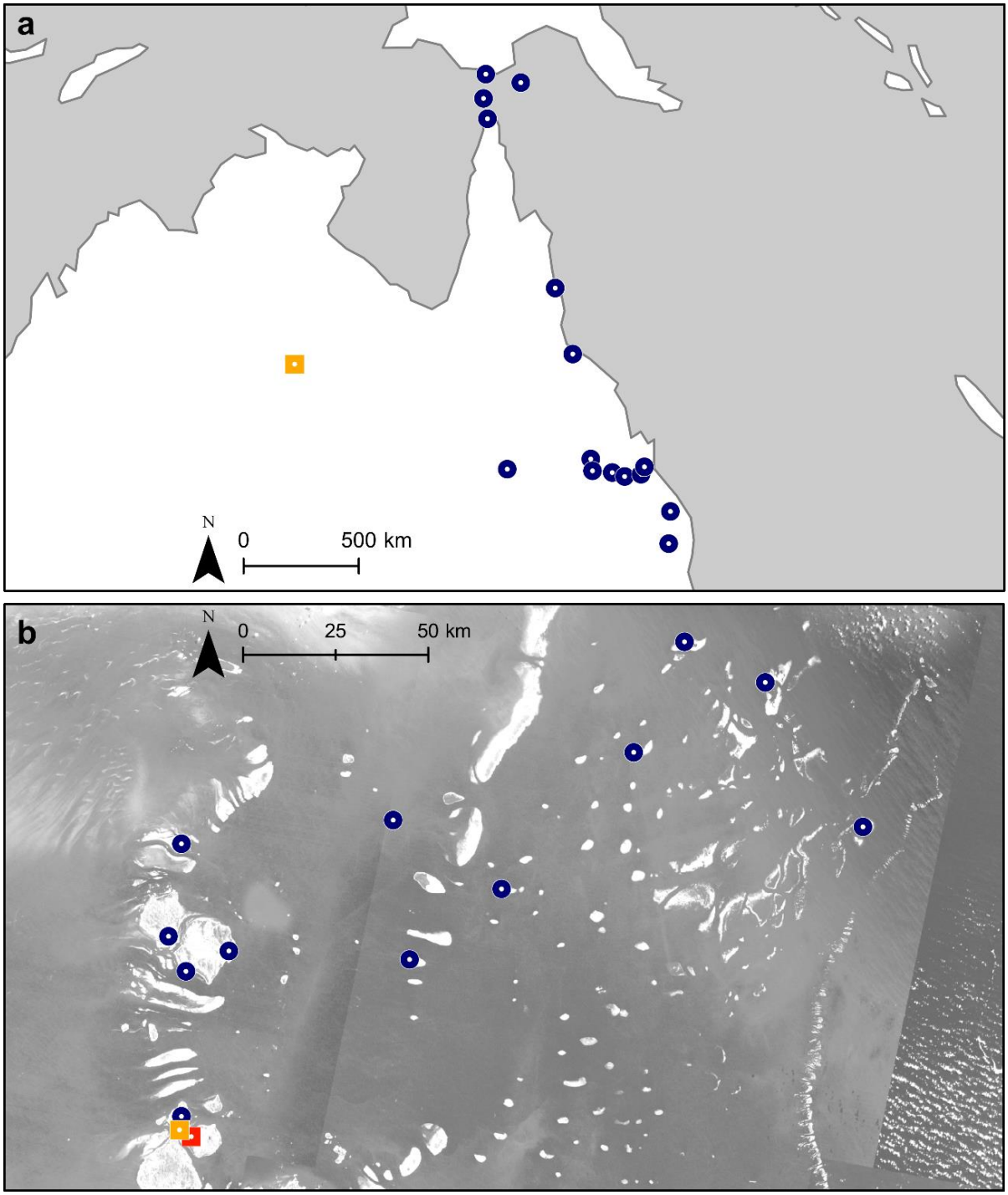

Figure 1. Locations of *Ae. aegypti* (a) and *Ae. albopictus* (b) samples. Blue circles show locations of reference samples. In (a), the orange square indicates Tennant Creek. In (b), the orange square indicates Thursday Island and the red square indicates Horn Island.

To trace the source of this invasion, we used a genomic databank containing previously generated sequence data of 240 *Ae. albopictus* from 12 Torres Strait Island communities

across 11 islands, collected in 2017 (14) (Fig 1b). Before tracing among Torres Strait Island 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 Torres Strait Island origin.

DNA sequencing and bioinformatic processing

Genomic DNA was extracted from the 98 *Ae. aegypti* and 7 *Ae. albopictus* using Qiagen DNeasy® Blood & Tissue Kits (Qiagen, Hilden, Germany). Extracted DNA was used to build double digest restriction-site associated DNA (ddRAD; (23)) sequencing libraries following the protocol of Rašić, Filipović, Weeks, & Hoffmann (2014). A technical replicate was produced of one of the Torres Strait Island incursion 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 (25) 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 (26), and the linkage-based assembly for *Ae. albopictus*, AalbF3 (27). Alignment was in Bowtie2 v2.3.4.3 (28) 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 (29) was used to further filter SNPs to retain those scored in 95% of all individuals, with a minor allele count ≥ 3 (30), 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.

Incursion tracing

i) Genome-wide genetic variation

We used Locator (31) 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 mosquito, 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 (32) 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 (33) that are common in Tennant Creek. As *Wolbachia* is coinherited with mtDNA through the maternal line (34), *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 (35), 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 (Wol+) and 87 uninfected wildtype (Wol-) individuals from Cairns, sampled in 2015 (21). We aligned demultiplexed sequences to the *Ae. aegypti* mtDNA genome assembly (36) 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 (37).

iii) Pyrethroid resistance mutations

The Tennant Creek *Ae. aegypti* were assessed for three pyrethroid resistance mutations common to *Ae. aegypti* across the Indo-Pacific (38), but not within Australia (39). For each individual, we ran three replicates of the Custom TaqMan® SNP Genotyping Assays (Life Technologies Corporation, Carlsbad, CA, USA) described in Endersby-Harshman et al. (2020), using on LightCycler II 480 (Roche, Basel, Switzerland) real time PCR machine in a 384-well format.

Genetic relatedness and inbreeding in the incursions

We investigated patterns of genetic relatedness (*k*) among pairs of individuals (dyads) in the Tennant Creek and Torres Strait incursions, and calculated Wright's inbreeding coefficient (*F*) for every individual. The patterns can help indicate whether the samples at either location had likely arrived as part of a single incursion or several distinct incursions. For instance, if several incursive individuals had arrived via the same incursion on the same vessel, dyads of these individuals should have higher *k* scores than other dyads in the

sample, as they will either be from the same family or have high genetic similarity due to their shared geographical origin. Alternatively, if the invasion was sourced from a single family and related individuals have subsequently mated together, we would expect to see higher *F* scores than in other populations. If neither of these patterns are present, it would suggest that invasive samples represent different incursions.

We used Loiselle's *k* (40) to estimate genetic relatedness, calculated in SPAGeDi (41). Loiselle's *k* describes correlations in the frequencies of homologous alleles, summarised across the genome (40). We used this estimator as it makes no assumptions regarding *F* and is suitable for markers with low polymorphism (42). We calculated *F* in VCFtools (29). As estimators of individual-level variation can be biased by uneven sample size and missing data (43), we subsampled and refiltered each databank before calculating *F.* For *Ae. aegypti*, we used the Tennant Creek (n=16), Townsville (n=16) and Cairns (n=15) samples. For *Ae. albopictus*, we used n=7 samples from each reference population and the n=7 incursion samples. We filtered to omit all sites with any missing genotypes (--max-missing 1) and to keep only sites with mean depth ≥ 20 (--min-meanDP 20).

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 S3). Rerunning Locator with reference samples outside this range omitted and 11495 SNPs indicated a likely origin of Townsville (Fig 2).

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 S4) or Townsville and the southern samples only (Fig S5). In each case, incursives were still placed around Townsville, but Townsville and Cairns were slightly harder to differentiate.

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 (F_{ST} = 0.26) than between Tennant Creek and either of the Cairns samples (both F_{ST} = 0.85) (Table 1). This indicates that no *w*Mel infection was ever present in the Tennant Creek samples or their maternal ancestors. In the pyrethroid resistance assays none of the three resistance alleles were present in the 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 3). 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 and HI-3 were also linked to the Maluilgal group, though they could not be linked to a specific island, and TI-4 was linked to a lesser extent to this group. 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.

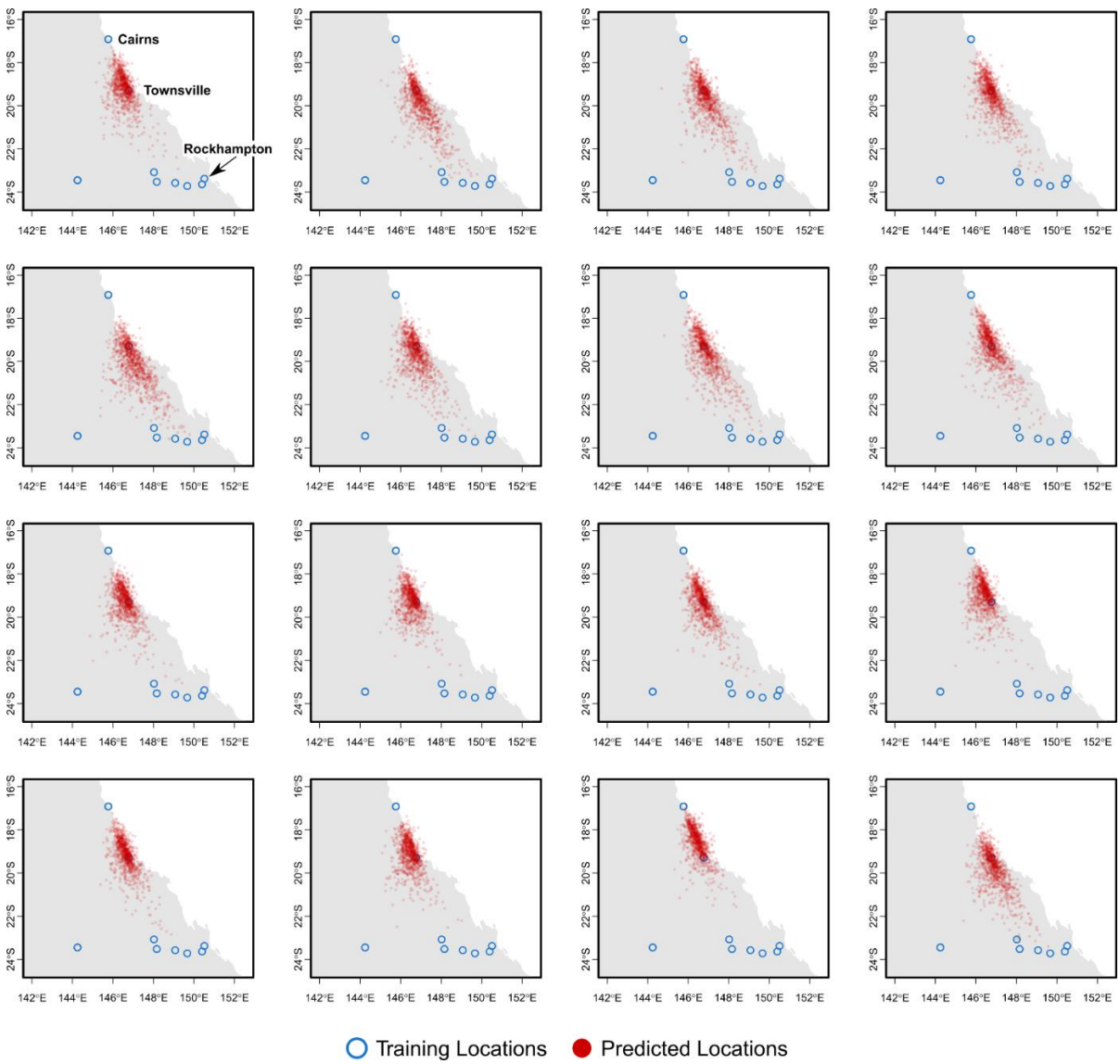

Figure 2. Origins of the 16 Tennant Creek individuals inferred from genome-wide genetic variation. Blue circles indicate reference sample locations, red circles show inferred origins from 1000 bootstrap replicate runs of Locator. Figs S3-5 show results of similar analyses using the whole dataset (S3) or different subsets of the dataset (S4-5)

Table 1. Pairwise F_{ST} of mtDNA variation among Tennant Creek, Cairns, and Townsville populations. Wol+ indicates *Wolbachia*-infected samples while Wol- are uninfected.

F_{ST}	Townsville (Wol-)	Cairns (Wol-)	Cairns (Wol+)
Tennant Creek	0.26	0.85	0.85
Townsville (Wol-)		0.87	0.87
Cairns (Wol-)			0.02

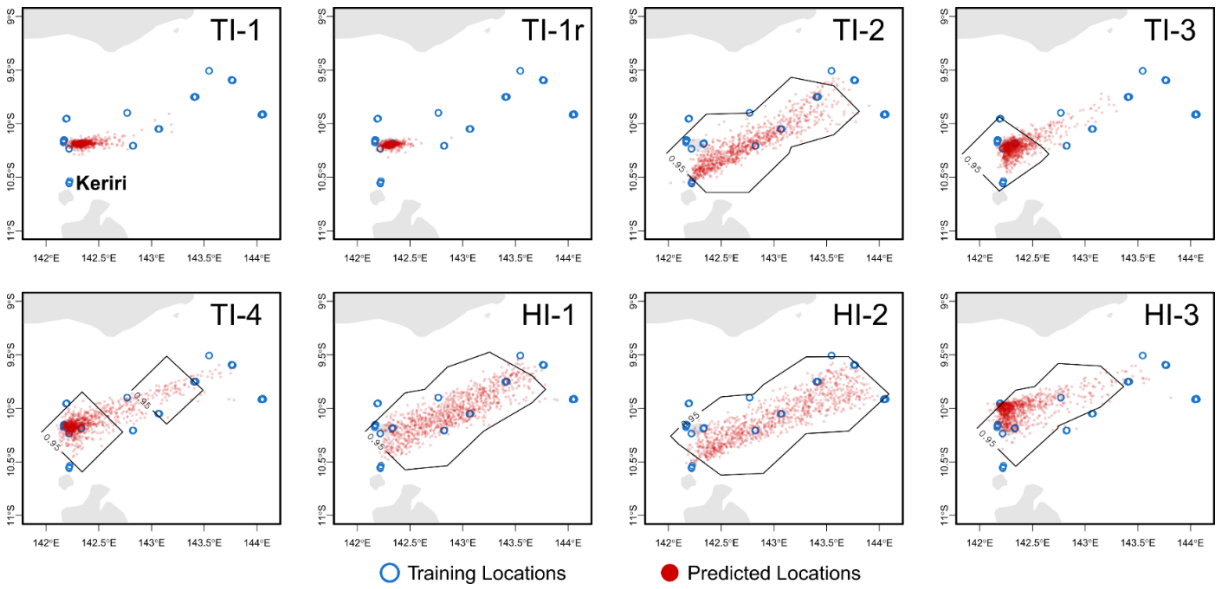

Figure 3. Origins of the 7 Torres Strait Islands individuals inferred from genome-wide genetic variation. Samples were detected on Thursday Island (TI) or Horn Island (HI), and a technical replicate was made of the TI-1 sample. Blue circles indicate reference sample locations, red circles show inferred origins from 1000 bootstrap replicate runs of Locator. Black polygons indicate 95% confidence intervals of inferences.

Genetic relatedness and inbreeding in the incursions

Fig 4 displays pairwise Loiselle's *k* scores for the Torres Strait Island (4a) and Tennant Creek (4b) dyads in rank order, and Wright's inbreeding coefficient (*F*) scores in the subsampled and refiltered datasets (10601 SNPs for *Ae. albopictus* (4c); 5073 SNPs for *Ae. aegypti* (4d)).

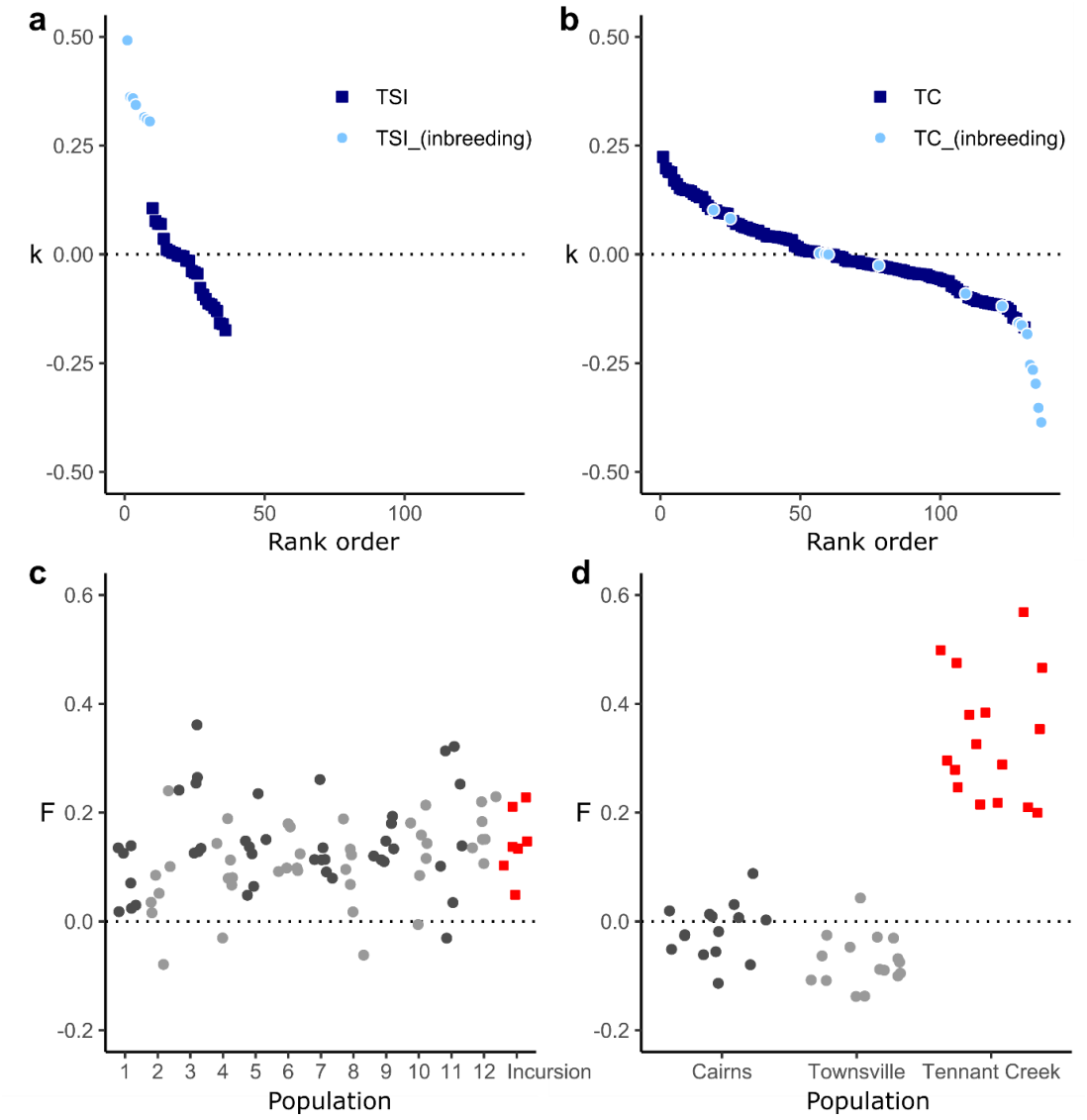

Figure 4. Genetic relatedness (*k*) and Wright's inbreeding coefficients (*F*) for *Ae. albopictus* (a,c) and *Ae. aegypti* (b,d). k scores are of dyads in the Torres Strait Islands (TSI, a) and Tennant Creek (TC, b), where dark blue squares show correlations between alleles in the chromosomes of two different individuals and light blue circles show correlations within the same individual. *F* scores are of *Ae. albopictus* (c) and *Ae. aegypti* (d), where red squares indicate invasive individuals, grey circles indicate other individuals.

Loiselle's *k* 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 relative magnitudes of inbreeding and non-inbreeding *k* scores were different in the two datasets. In the Torres Strait Islands, inbreeding *k* scores were all much higher than the *k* scores between dyads (Fig 4a). 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 Torres Strait Island 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. Instead, these results are evidence that the seven Torres Strait Island incursives each represent distinct incursions. This pattern was not observed at Tennant Creek, where inbreeding *k* scores were mostly undifferentiated from and in many cases lower than *k* scores between dyads (Fig 4b). Chromosomes within individuals are thus no more genetically similar than they are to chromosomes in other individuals. This suggests that the 16 Tennant Creek individuals are all likely to be from the same exact geographical location and are potentially all from the same family.

The above inferences were further supported by variation in *F* scores. In the Torres Strait Islands, *F* scores in incursive individuals (Fig 4c, red squares) were of similar magnitude to reference individuals, suggesting no inbreeding in incursions. In Tennant Creek, *F* scores indicated much higher inbreeding in Tennant Creek relative to Cairns and Townsville reference individuals, suggesting that the Tennant Creek invasion was sourced from a single family (Fig 4d).

Discussion

Pest genomic databanks provide an increasingly popular means of investigating invasions and incursions (9–16). The value provided by genomic 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 (44). This study has used genomic databanks to investigate new threats to biosecurity from Australian incursions 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), while none of these incursions were from a nearby island (Keriri) that had previously been identified as an incursion source. Our estimates of genetic relatedness and inbreeding showed that the Tennant Creek invasion was likely sourced from one specific location and possibly from a single family, whereas the Torres Strait incursions were all genetically distinct.

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 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, plus several generations of strong genetic drift between invasion and detection. 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 3). This is particularly noteworthy given the much finer spatial scale of the Torres Strait invasion and the low genetic differentiation among islands ($F_{ST} \approx 0.03$ (14)).

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 the *w*Mel infection, the Tennant Creek *Ae. aegypti* should be able to transmit dengue without the transmission blocking effects of *w*Mel (45).

The different patterns of genetic relatedness observed at Tennant Creek and the Torres Strait provide 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. These genetic inferences align with detection locations, where the Tennant Creek samples were collected within ~80 m of each other while the Horn and Thursday Island incursions were spread across 600 m – 1 km. Understanding the independence of incursions can be useful for building predictive models of pest incursion risk as it can indicate propagule pressure (46), though these estimates will reflect effective rather than census population sizes of incursions. The findings at Tennant Creek indicate that greater caution will be required for studies seeking to investigate new invasions using close kin dyad methodologies (47,48). While close kin dyad methods have shown promise for investigating invasions of ~100 generations age (14), 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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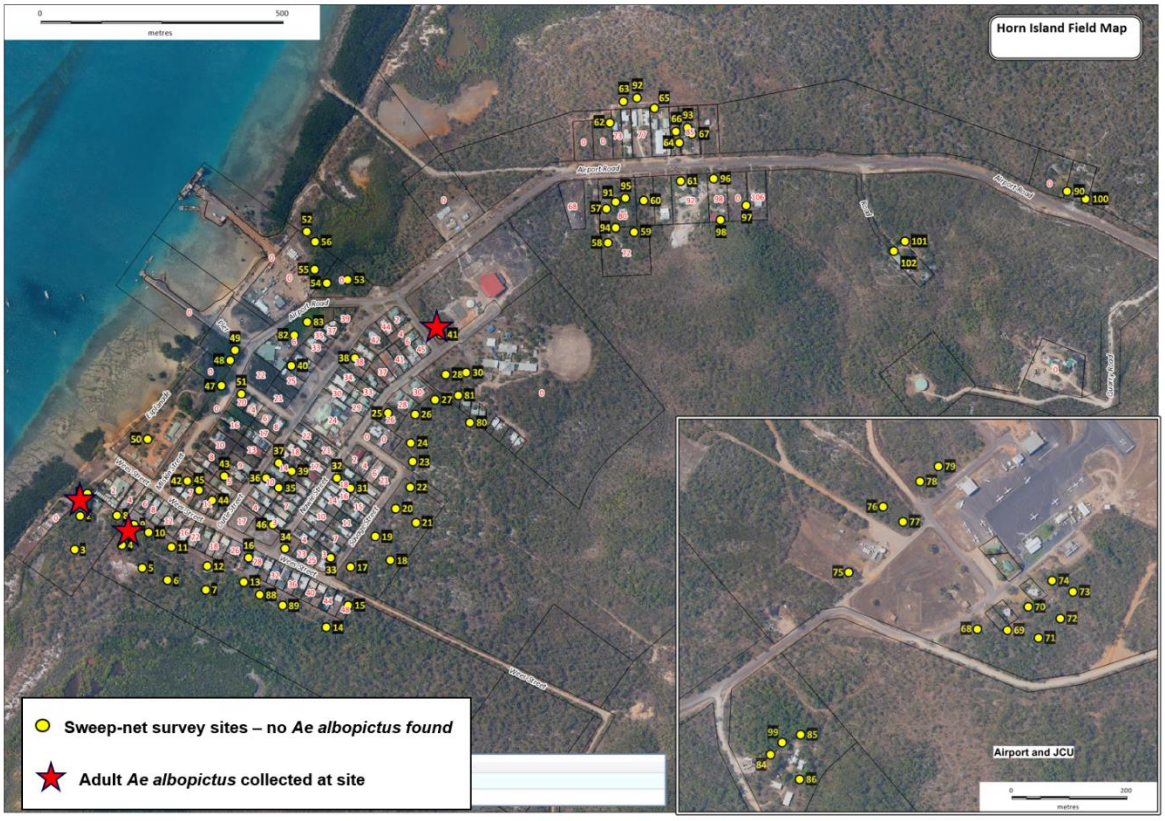

Figure S1: Locations of the three *Aedes albopictus* collected on Horn Island (red stars). Yellow circles indicate sweep-net survey sites.

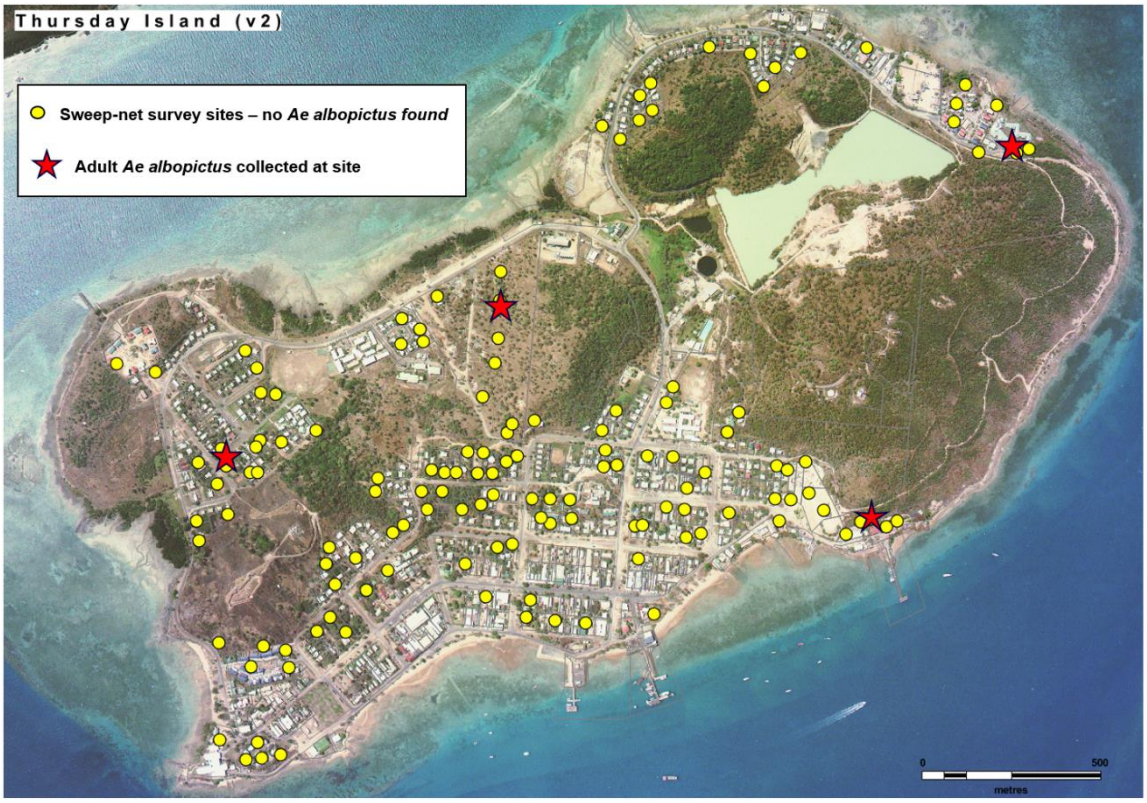

Figure S2: Locations of the four *Aedes albopictus* collected on Thursday Island (red stars). Yellow circles indicate sweep-net survey sites.

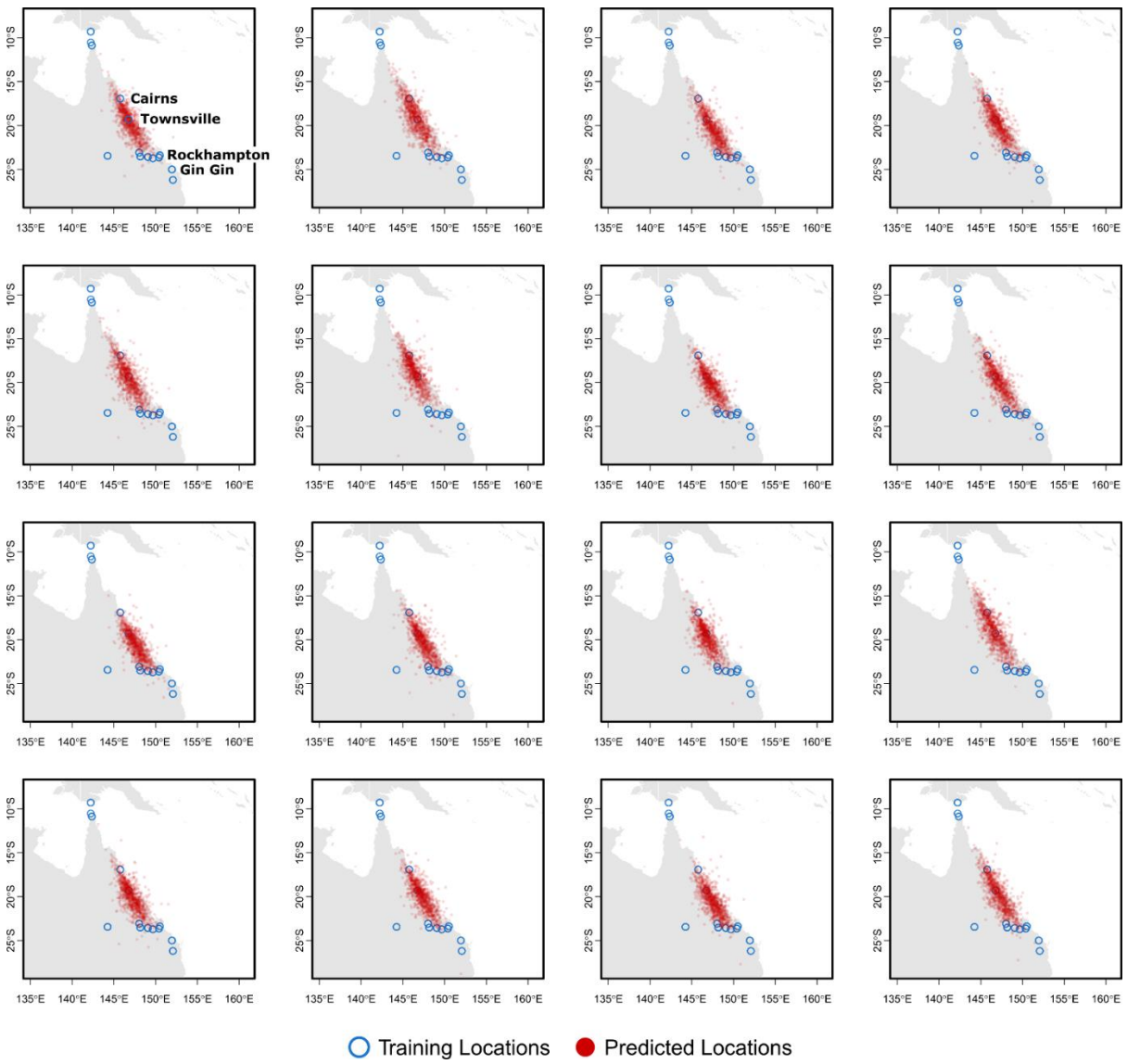

Figure S3. Tracing of the 16 Tennant Creek individuals using the entire genomic databank. Blue circles indicate reference sample locations, red circles show inferred origins from 1000 bootstrap replicate runs of Locator.

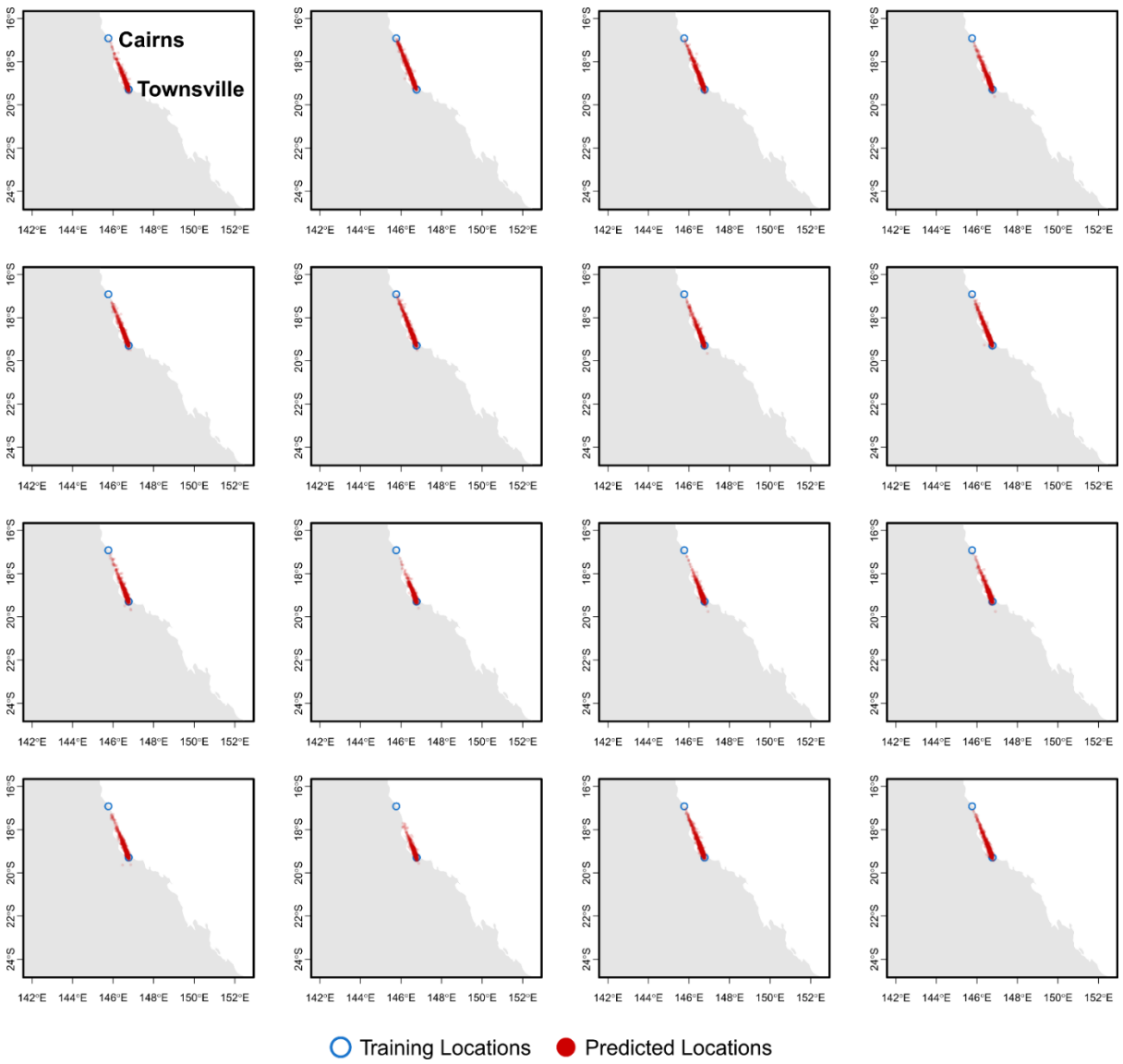

Figure S4. Tracing of the 16 Tennant Creek individuals using only Cairns and Townsville. Blue circles indicate reference sample locations, red circles show inferred origins from 1000 bootstrap replicate runs of Locator.

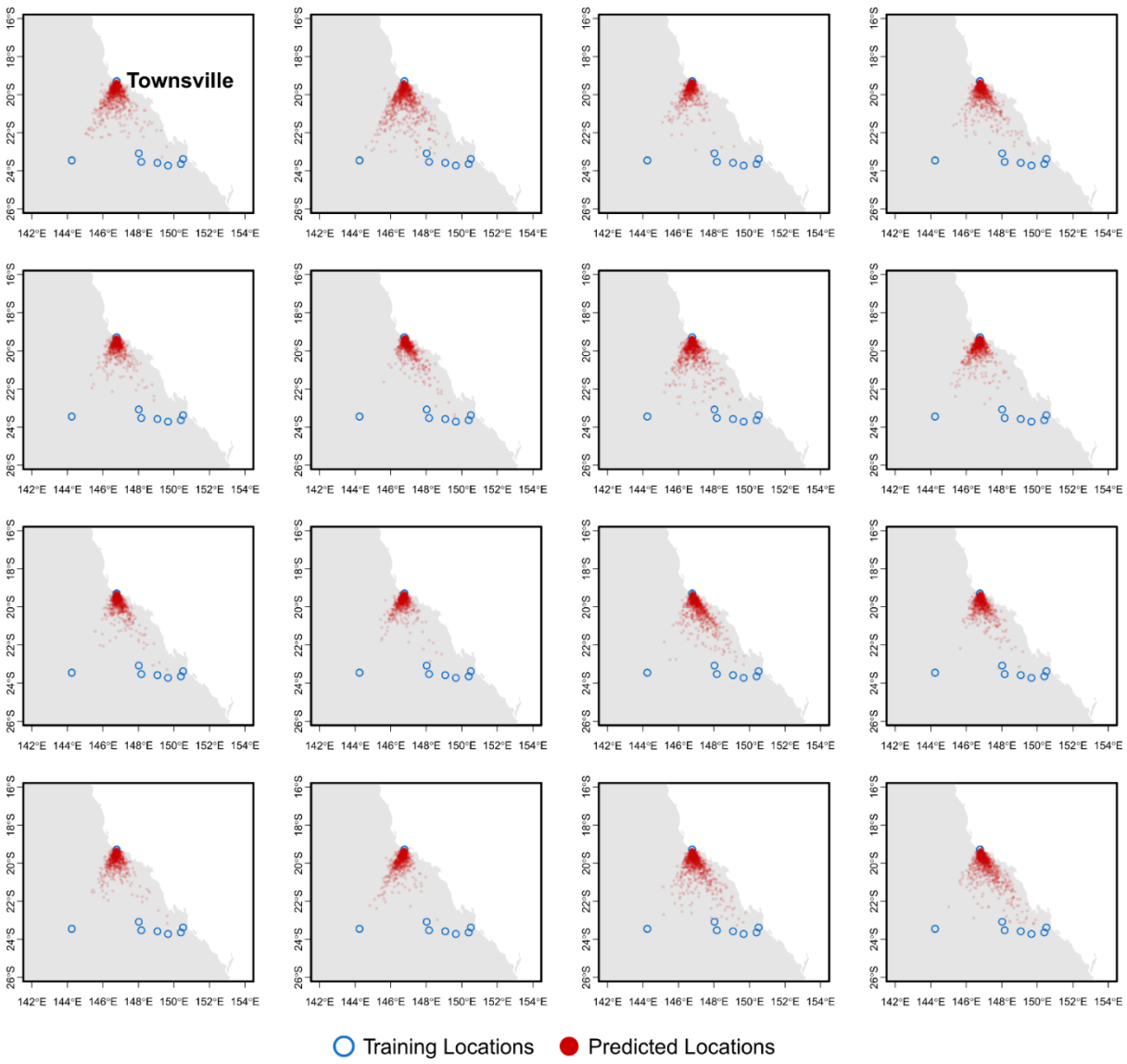

Figure S5. Tracing of the 16 Tennant Creek individuals using only Townsville and nearby populations in southern Queensland. Blue circles indicate reference sample locations, red circles show inferred origins from 1000 bootstrap replicate runs of Locator.