Incursion pathways of the Asian tiger mosquito (*Aedes albopictus*) into Australia contrast sharply with those of the yellow fever mosquito (*Aedes aegypti*)

Running Title: Australian *Aedes albopictus* incursions

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Abstract

BACKGROUND

Understanding pest incursion pathways is critical for preventing new invasions and for stopping the transfer of alleles that reduce the efficacy of local control methods. The mosquitoes *Aedes albopictus* (Skuse) and *Ae. aegypti* (Skuse) are both highly invasive disease vectors, and through a series of ongoing international incursions are continuing to colonise new regions and spread insecticide resistance alleles among established populations. This study uses high-resolution molecular markers and a set of 241 reference genotypes to trace incursion pathways of *Ae. albopictus* into mainland Australia, where no successful invasions have yet been observed. We contrast these results with incursion pathways of *Ae. aegypti*, investigated previously.

RESULTS

Assignments successful identified China, Japan, Singapore and Taiwan as source locations. Incursion pathways of *Ae. albopictus* were entirely different to those of *Ae. aegypti*, despite broad sympatry of these species throughout the Indo-Pacific region. Incursions of *Ae. albopictus* appeared to have come predominantly along marine routes from key trading locations, while *Ae. aegypti* was mostly linked to aerial routes from tourism hotspots.

CONCLUSION

These results demonstrate how genomics can help decipher otherwise cryptic incursion pathways. The inclusion of reference genotypes from the Americas may help resolve some unsuccessful assignments. While many closely-related taxa will share common incursion pathways, this study highlights that this is not always the case, and incursion pathways of important taxa should be specifically investigated. Species differences in aerial and marine incursion rates may reflect the efficacy of ongoing control programs such as aircraft disinsection.

Keywords

Aedes albopictus, *Aedes aegypti*, biological invasions, assignment tests, genome-wide SNPs, incursion pathways

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1 Introduction

Incursions of invasive pests present a range of threats to local biodiversity, agriculture and human health.[1–3] Pest incursions into new regions threaten the colonisation of these regions,[4] while incursions into regions with established populations can lead to the introduction of advantageous alleles that reduce the efficacy of control programs.[5] Invasive pests are frequently transported as "stowaways" on trade and transport vessels,[6–8] and the distributions of these species have increased accordingly with the upsurge over recent decades in trade and transport activity.[9] Intercepting incursive material at borders can be an effective means of stopping incursions,[10] but is resource-intensive. Knowledge of incursion pathways is vital for strategic deployment of these resources, allowing inspections to focus on likely source locations[11] and common entry points.[12]

The Asian tiger mosquito (*Aedes albopictus*, Skuse) is a hyperaggressive dengue vector that has successfully invaded tropical and temperate regions worldwide from its native range in Asia.[13] It is considered one of the world's most dangerous invasive species (Global Invasive Species Database, http://www.issg.org/database/). In Australia, border security makes frequent interceptions of *Ae. albopictus* at international ports across the country.[14] While much of the Australian mainland likely presents suitable habitat for *Ae. albopictus,*[15] no successful establishment has yet been recorded. However, an invasion of *Ae. albopictus* was recently recorded in the Torres Strait Islands,[16] where its present distribution is only tens of kilometres from the Australian mainland.[14]

A recent study that traced incursion routes of the congeneric mosquito *Ae. aegypti* (Skuse) into Australia found that most (62) had come from Bali, Indonesia, with others (21) mostly assigned to other locations in Southeast Asia.[8] These assignments were derived using a reference panel of *Ae. aegypti* genotypes taken predominantly from locations with direct flights into Australia. Certain locations with direct flights and presumably large *Ae. aegypti* populations, such as Singapore, Taiwan, and Ho Chi Minh City, were not found to be source populations of any of the *Ae. aegypti*.

Although *Ae. albopictus* and *Ae. aegypti* both disperse internationally on vessels like ships and aeroplanes,[17–19] analysis of their genetic structure in the Indo-Pacific has indicated that gene flow pathways are different in these species.[20] Likewise, interceptions at New Zealand borders found that most *Ae. albopictus* were transported as larvae on ships, while most *Ae. aegypti* were transported as adults on aeroplanes.[17] One potential reason for these differences is that *Ae. albopictus* may be able to withstand longer transportation times and colder minimum temperatures by undergoing diapause,[21–23] an option unavailable to *Ae. aegypti*.[24] Diapause has also been proposed as a means by which *Ae. albopictus* has been able to colonise distant locations without undergoing 'stepping stone' colonisation of intermediate sites.[20,25–27] If gene flow proceeds along different pathways for *Ae. albopictus* and *Ae. aegypti*, incursion routes may also be different, even if both species are locally abundant and can be transported by the same vessels.

This study investigates *Ae. albopictus* incursion pathways into mainland Australia, and contrasts these with previously determined incursion pathways of *Ae. aegypti* (c.f. Schmidt et al.[8]). By genotyping *Ae. albopictus* detected at Australian borders, and comparing these genotypes with a panel of reference genotypes from across the Indo-Pacific region, the likely source population of each mosquito can be identified. Accurate identification is conditional on several factors, including sufficiently high-quality DNA of the intercepted sample, a wide geographic range of reference genotypes, and sufficient genetic differentiation among the reference genotypes. This latter factor is important to consider in assigning *Ae. albopictus*, as *Ae. albopictus* populations are typically less differentiated than those of *Ae. aegypti* [20,28], which may lead to lower precision when assigning *Ae. albopictus*. Nevertheless, the strong differentiation between Indonesian and non-Indonesian populations of *Ae. albopictus*[20] should allow for assignment between these groups, and thus determine whether *Ae. albopictus*, like *Ae. aegypti*, are transported to Australia predominantly from nearby Indonesian source populations.

2 Materials and Methods

2.1 Samples

Twenty *Ae. albopictus* were collected at Australian borders between April 4th, 2016, and December 17th, 2018. These mosquitoes, hereafter called 'incursives', were intercepted at

passenger and freight terminals at airports and seaports in Australia, with most collected at marine terminals. Table 1 contains information for each of these 20 incursives.

For the panel of reference genotypes, we used 241 *Ae. albopictus* collected from 18 locations in the Indo-Pacific region (Fig 1), with details listed in Supporting Information Appendix S1. These 18 locations were: Christmas Island, Fiji, China (Guangzhou), Japan (Matsuyama), Malaysia (Peninsula Malaysia), Mauritius, Philippines (Manila), Singapore, Sri Lanka (Colombo), Taiwan (Kaohsiung City), Thailand (Bangkok and Chiang Mai), Timor-Leste, Torres Strait Islands, Vanuatu, Vietnam (Ho Chi Minh City), and three locations in Indonesia: Bali, Bandung, and Jakarta. We considered different countries to represent different locations and aggregated mosquitoes accordingly, except in the case of Indonesia, where we treated samples from Bali, Bandung, and Jakarta as different locations. This was due to the proximity of Indonesia to Australia and the previous finding that most *Ae. aegypti* incursions into Australia were from Bali.[8]

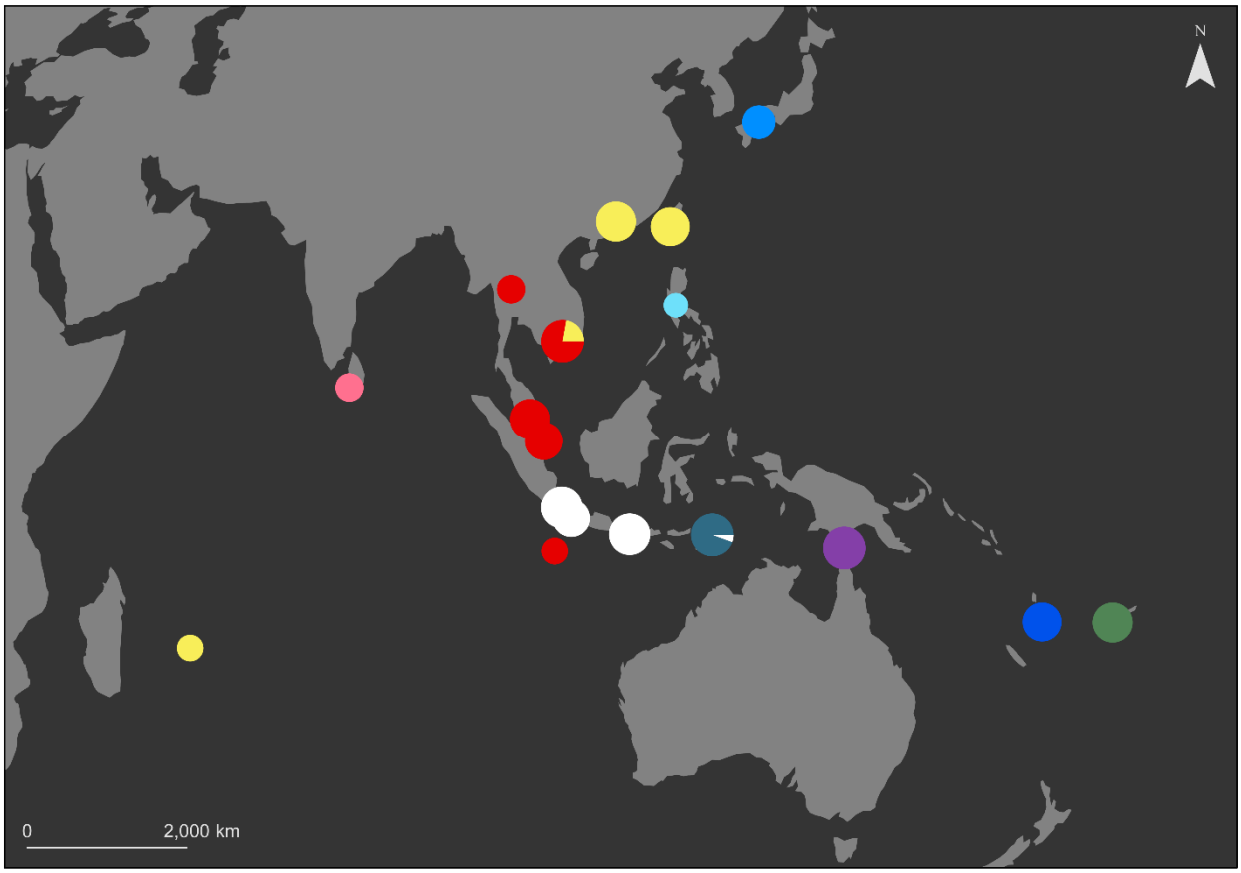

Fig 1. Reference genotypes of *Aedes albopictus*.

Pie charts are sized by the number of reference genotypes available, and colours denote clustering established during the initial discriminant analysis of principal components (DAPC; see Results).

2.2 Genotyping

DNA was extracted from incursive and reference mosquitoes using either Qiagen DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) or Roche High Pure™ PCR Template Preparation Kits (Roche Molecular Systems, Inc., Pleasanton, CA, USA), each with an RNase A treatment step.

149 We constructed double digest restriction-site associated DNA (ddRAD) libraries following
150 the ddRAD protocol for *Ae. albopictus* adapted in Schmidt et al.[29] from the protocol of Rašić,
151 Filipović, Weeks, & Hoffmann.[30] We performed initial digestions of 50 – 200 ng of genomic
152 DNA, using 10 units each of MluCI and NlaIII restriction enzymes (New England Biolabs,
153 Beverly MA, USA), NEB CutSmart buffer, and water. Digestions were run for 3 hours at 37
154 °C with no heat kill step, and the products were cleaned with paramagnetic beads. These
155 were ligated to modified Illumina P1 and P2 adapters overnight at 16 °C with 1,000 units of
156 T4 ligase (New England Biolabs, Beverly, MA, USA), followed by a 10-minute heat-
157 deactivation step at 65 °C. We performed size selection with a Pippin-Prep 2% gel cassette
158 (Sage Sciences, Beverly, MA) to retain DNA fragments of 350 – 450 bp.

159

160 Final libraries were amplified by PCR, using 1 µL of size-selected DNA, 5 µL of Phusion High
161 Fidelity 2× Master mix (New England Biolabs, Beverly MA, USA) and 2 µL of 10 µM
162 standard Illumina P1 and P2 primers. These were run for 12 PCR cycles, then cleaned and
163 concentrated using 0.8× paramagnetic beads. Each ddRAD library contained between 30
164 and 65 mosquitoes, and each was sequenced on a single sequencing lane using 100 bp
165 chemistry. Libraries were sequenced paired-end at either the Australian Genome Research
166 Facility (AGRF, Melbourne, Australia), the University of Melbourne (Pathology), or
167 GeneWiz, Inc (Suzhou, China) on either a HiSeq 2500 or a HiSeq 4000 (Illumina, California,
168 USA).

169

Raw sequence reads were processed using the Stacks 2.0 REF_MAP pipeline.[31] Reads were demultiplexed and trimmed to 80 bp length, and low-quality reads were discarded. Bowtie 2.3.4.3[32] was used to align reads to the *Aedes albopictus* reference genome (GenBank assembly accession: GCA_006496715.1) using end-to-end alignment and the --sensitive preset options. The 20 incursive individuals and 241 reference individuals were built into a Stacks loci catalog and the Stacks program POPULATIONS was used to call SNPs and export the genotypes in VCF format. Only SNPs called in 75% of individuals from each reference population and in 75% of incursives were retained (-p 19 -r 0.75). We used VCFTOOLS[33] to ensure no first-order relatives were included in the reference populations (--relatedness2; all kinship scores < 0.177).

The 20 incursive individuals and 241 reference individuals were built into a Stacks loci catalogue and the Stacks program POPULATIONS was used to call SNPs and export the genotypes in VCF format. Only SNPs called in 75% of individuals from each reference population and in 75% of incursives were retained (-p 19 -r 0.75). We used VCFTOOLS[33] to ensure no first-order relatives were included in the reference populations (--relatedness2; only including individuals with kinship scores of < 0.177).

188 ## 2.3 Population assignment

189 We used two R packages for population assignment: 'adegenet',[34] for cluster detection with
190 discriminant analysis of principal components (DAPC); and 'assignPOP',[35] for Monte Carlo
191 assignment tests using a support vector machine predictive model. Our methodology was
192 adapted from that of Schmidt et al.[8] as follows. First, we performed DAPC on the whole
193 dataset to partition the reference and incursive genotypes into broad genetic clusters,
194 which provided an initial, rough assignment for each incursive. We then analysed each
195 genetic cluster in isolation, and used assignPOP to generate assignment probabilities for
196 each incursive to each of the reference populations within the genetic cluster.

197

198 For the initial analysis with DAPC, we used all 261 genotypes. These were filtered with
199 POPULATIONS to retain SNPs scored in 75% of individuals from each reference population
200 and in 75% of incursives. The dataset was then filtered with VCFTOOLS using the following
201 settings: SNPs must be biallelic; have a minor allele count ≥ 2;[36] have a minor allele count
202 depth of 3 and a maximum of 45 ((mean depth) + 3√(mean depth));[37] and must be called
203 in at least 90% of individuals. We then used BEAGLE 4.1[38] to impute missing genotypes, with
204 10 iterations.

205

206 We used the *find.clusters* function to detect genetic clusters, with 10^9 iterations. We ran
207 cluster detection separately for each incursive, so that each incursive genotype was
208 compared with all reference genotypes and none of the other incursive genotypes. We used
209 *find.clusters* to partition the genotypes into a number of clusters (K) ranging from 1 to 20,

210 and considered the *K* with the lowest AIC as the best partitioning. Following this
211 partitioning, the dataset was divided into *K* clusters, each containing a set of reference
212 genotypes and between 0 and 20 incursives.

213

214 The genetic clusters that contained at least one incursive were then analysed
215 independently. New VCF files for each cluster were exported following the same filtering
216 steps described above. We used the assignPOP function *assign.X* to generate posterior
217 probabilities of assignment for incursives to the reference populations within their genetic
218 cluster, using a support vector machine predictive model. Following Schmidt et al.,[8] we
219 applied these posterior probabilities to calculate 'relative probabilities' of assignment,
220 defined as the probability of assignment to the most likely population divided by the
221 probability of assignment to the second most likely population. We considered incursives
222 assigned with posterior probability > 0.5 and relative probability > 3 to be well-assigned.

223

224 We performed final DAPCs using these assignments, with genotypes grouped by population
225 of origin (for reference genotypes) or population of assignment (for well-assigned
226 incursives). We omitted poorly-assigned incursives from these DAPCs. For each DAPC, we
227 used the function *xvalDapc* with 100 repetitions to determine an optimal number of
228 principal components to use, based on lowest mean squared error. DAPCs for each genetic
229 cluster were plotted to investigate relationships between reference and incursive
230 genotypes graphically.

231

We used the assignment results to compare incursion pathways of *Ae. albopictus* with those of *Ae. aegypti* established in Schmidt et al..[8] For each species, we plotted the incursion routes (source population to interception location) into Australia of all well-assigned incursives.

3 Results

After filtering, we retained 21,931 SNPs for the initial analysis with DAPC. Lowest AIC was observed at $K = 10$. Accordingly, the reference genotypes were partitioned into 10 genetic clusters (Fig 1), and the incursives were assigned to these 10 clusters one by one. Only three of the clusters were assigned incursives. Twelve of these were assigned to an "East Asian" cluster containing China, Mauritius, Taiwan, and four of the Vietnamese genotypes (Fig 1, yellow). Two were assigned to a "Southeast Asian" cluster containing *Ae. albopictus* from Christmas Island, Malaysia, Singapore, Thailand, and the remaining 14 Vietnamese genotypes (Fig 1, red). The other six incursives were assigned to a "Japanese" cluster containing the Japanese genotypes.

More precise assignments were obtained by analysing each cluster separately, using assignPOP to produce posterior and relative probabilities of assignment for each incursive. Although the Vietnamese genotypes were split between the East Asian and Southeast Asian clusters, we included all Vietnamese genotypes in analyses of each cluster. Also, as multiple

253 populations were needed for assignment tests, we included the Chinese and Taiwanese
254 genotypes in analyses of the Japanese cluster, due to their geographic proximity with Japan.
255 After filtering the East Asian cluster, there remained 14,149 SNPs for analysis. Analysis of
256 the Southeast Asian cluster was with 12,346 SNPs, and the Japanese cluster was analysed
257 with 26,980 SNPs.

258

259 Five of the 12 incursives initially assigned to the East Asian cluster were considered well-
260 assigned (posterior probability > 0.5 and relative probability > 3) (Table 1). These
261 incursives were all assigned to either China (4) or Taiwan (1), with none assigned to
262 Mauritius or Vietnam. Graphical investigation of assignments using DAPC (Fig 2) showed
263 that two of the less-confidently assigned incursives (i.e. those displayed as smaller white
264 squares) had some ambiguity of assignment between China (red circles) and Taiwan (blue
265 circles). This suggests that the genetic similarity of these two populations may make
266 precise assignments between them difficult.

267

268 The two incursives initially assigned to the Southeast Asian cluster were both assigned
269 very confidently to Singapore (both posterior probabilities > 0.9, relative probabilities >
270 11) (Table 1). DAPC of this cluster (Fig 3) indicated that the confidence of these
271 assignments was likely due in part to the clear differentiation of the Singaporean genotypes
272 (blue triangles) from those from Malaysia (yellow triangles), Thailand (green triangles),
273 and Vietnam (brown triangles), which clustered together. Of the six incursives initially
274 assigned to the Japanese cluster, two were well-assigned (Table 1). Graphical investigation

of these assignments indicated that these incursives were unlikely to have come from either China or Taiwan (Fig 4).

Aedes albopictus incursion routes into Australia were clearly different from those of *Ae. aegypti* (Fig 5, Table 2). *Aedes aegypti* incursives from international sources were assigned to Brazil (8%, not pictured in Fig 5) and three locations in the Indo-Pacific: Bali (75%), Malaysia (16%), and Thailand (1%). Although reference genotypes from these three Indo-Pacific locations were used in this study, no *Ae. albopictus* incursives were assigned to these populations. Likewise, no *Ae. aegypti* were assigned to either Singapore or Taiwan, despite available reference genotypes from these populations. Overall, most *Ae. albopictus* incursion pathways were from East Asian sources into ports in eastern Australia, particularly Brisbane. Most *Ae. aegypti* incursives were from Southeast Asia, and were detected at ports throughout Australia (but not Brisbane).

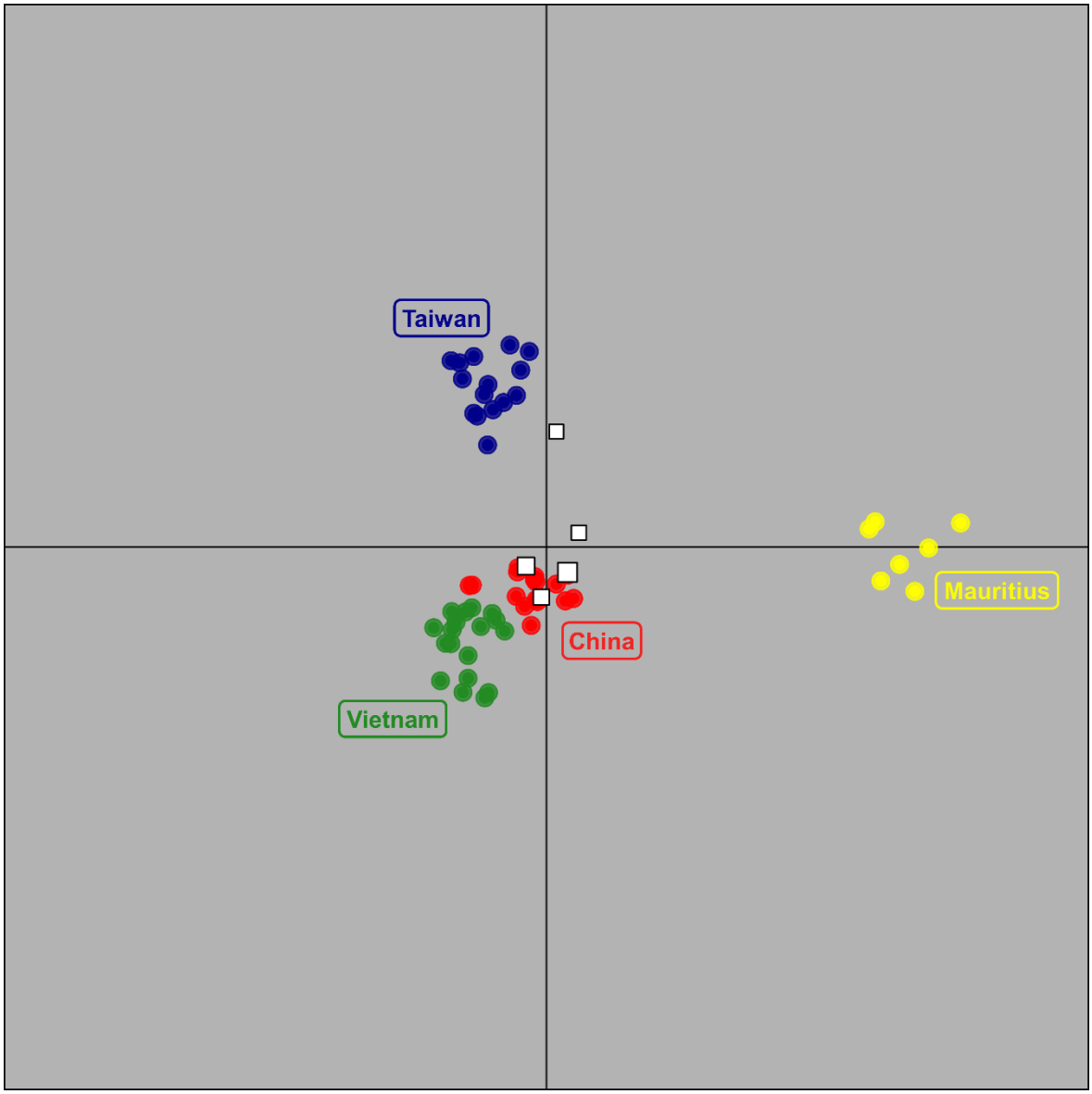

Fig 2. Discriminant analysis of principal components (DAPC) of the East Asian cluster.

White squares depict well-assigned incursive genotypes, which are sized by a logarithmic function of their relative probability of assignment. Horizontal axis describes 61.6% of total variation, vertical axis describes 26.5% of total variation.

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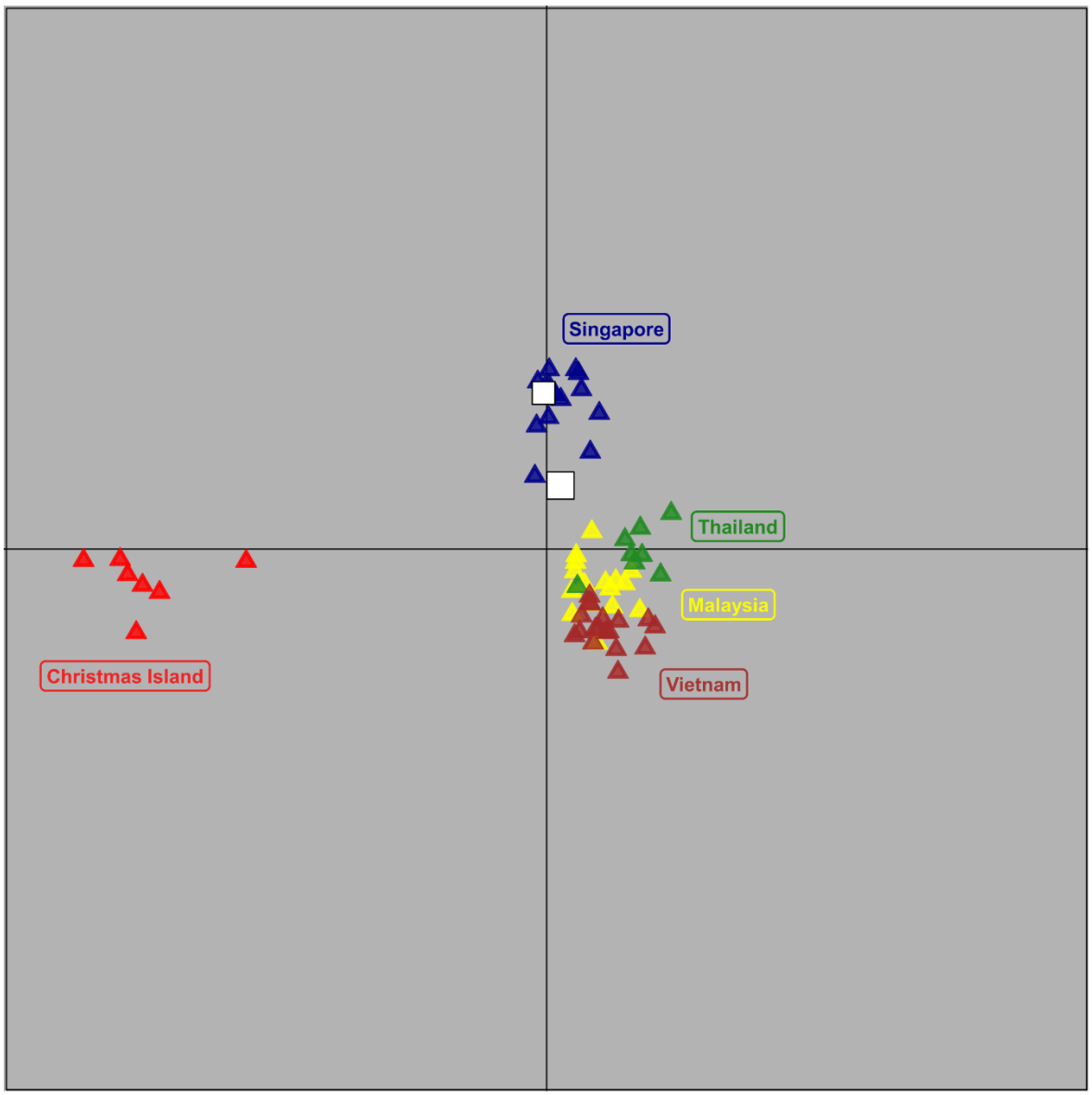

Fig 3. Discriminant analysis of principal components (DAPC) of the Southeast Asian cluster.

White squares depict well-assigned incursive genotypes, which are sized by a logarithmic function of their relative probability of assignment. Horizontal axis describes 60.3% of total variation, vertical axis describes 21.4% of total variation.

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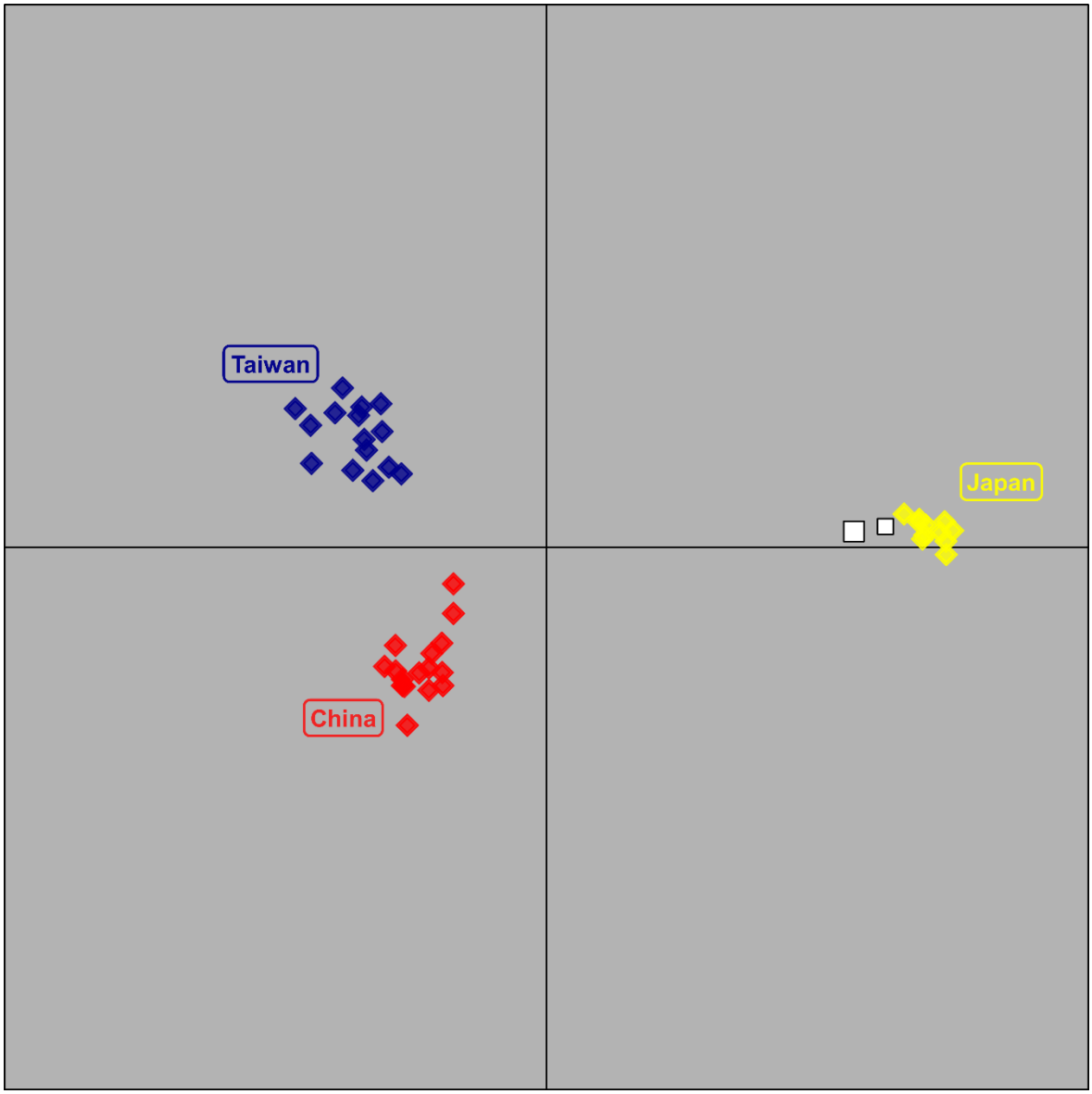

Fig 4. Discriminant analysis of principal components (DAPC) of the Japanese cluster.

White squares depict well-assigned incursive genotypes, which are sized by a logarithmic function of their relative probability of assignment. Horizontal axis describes 88.3% of total variation, vertical axis describes 11.7% of total variation.

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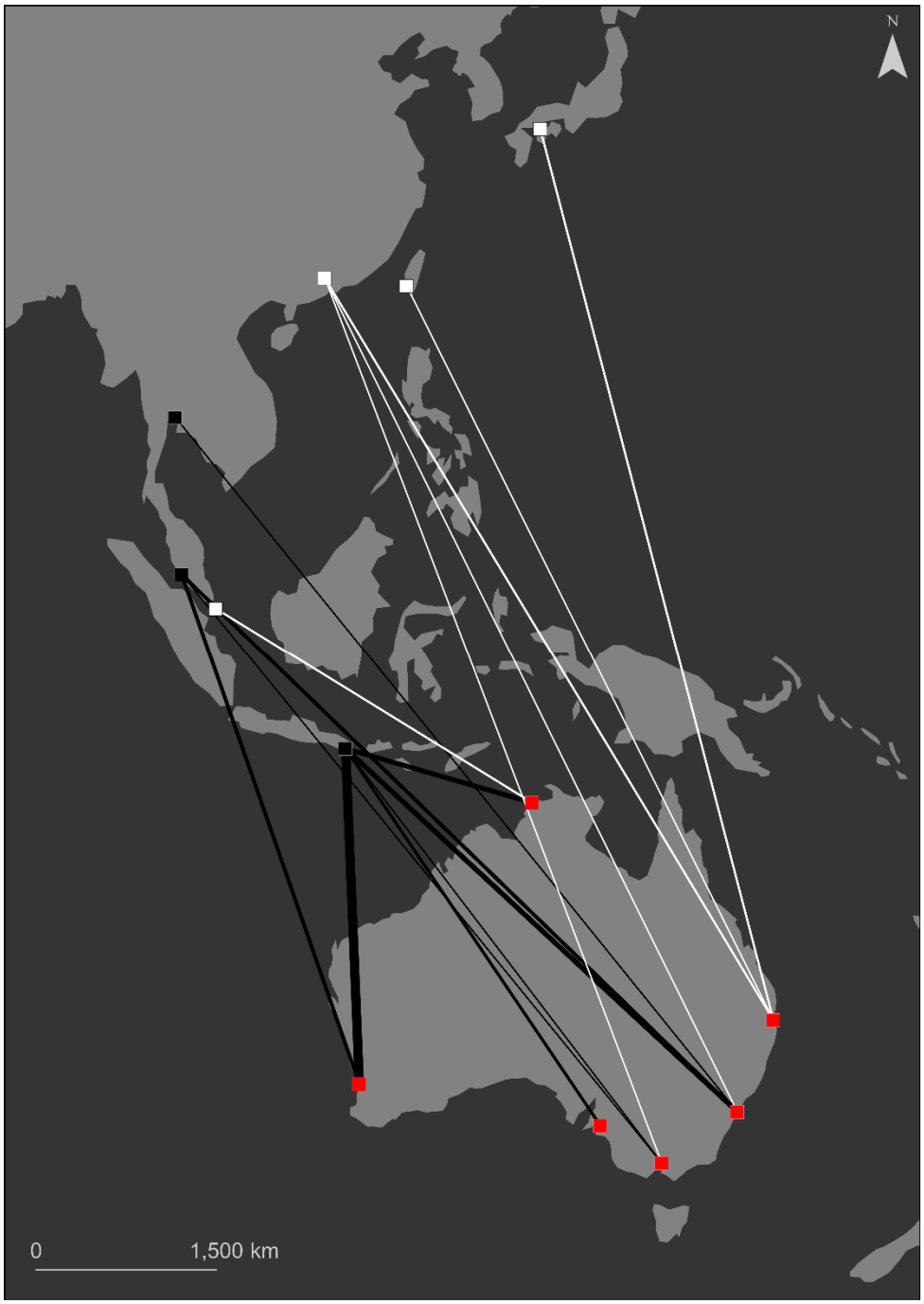

Fig 5. Indo-Pacific incursion pathways of *Aedes albopictus* (white) and *Aedes aegypti* (black).

Line thickness follows a logarithmic function of the number of incursives transported along that pathway. Not shown are seven Ae. aegypti incursives, transported into Perth, Australia, from a putative origin in the Americas.

Table 1. Detection and assignment results for the 9 well-assigned and 11 poorly-assigned *Aedes albopictus* incursives.

Well-assigned incursives are in regular typeface, poorly-assigned incursives are in italics.

Incursive	Port type	City of detection	Date of collection	Initial assignment	Final assignment	Posterior probability	Relative probability
AP01	Airport	Sydney	2016-04-30	East Asia	China	0.59	3.15
AP02	Airport	Melbourne	2018-12-17	East Asia	China	0.77	5.5
SP04	Seaport	Darwin	2017-06-05	Southeast Asia	Singapore	0.92	19.27
SP05	Seaport	Darwin	2017-06-06	Southeast Asia	Singapore	0.91	11.43
SP06	Seaport	Brisbane	2017-07-18	Japan	Japan	0.72	3.54
SP09	Seaport	Brisbane	2018-03-26	East Asia	Taiwan	0.66	3.02
SP15	Seaport	Brisbane	2018-10-24	East Asia	China	0.72	3.54
SP16	Seaport	Brisbane	2018-10-24	East Asia	China	0.66	4.18
SP18	Seaport	Brisbane	2018-10-26	Japan	Japan	0.82	6.09
SP01	*Seaport*	*Brisbane*	*2016-12-23*	*East Asia*	*n/a*	*0.62*	*2.35*
SP02	*Seaport*	*Brisbane*	*2016-12-23*	*East Asia*	*n/a*	*0.51*	*2.37*
SP03	*Seaport*	*Brisbane*	*2016-12-23*	*East Asia*	*n/a*	*0.49*	*1.45*
SP07	*Seaport*	*Brisbane*	*2017-07-18*	*Japan*	*n/a*	*0.63*	*2.49*
SP08	*Seaport*	*Brisbane*	*2017-07-18*	*East Asia*	*n/a*	*0.48*	*1.73*
SP10	*Seaport*	*Brisbane*	*2018-03-29*	*East Asia*	*n/a*	*0.59*	*2.3*
SP11	*Seaport*	*Brisbane*	*2018-05-21*	*Japan*	*n/a*	*0.5*	*1.32*
SP12	*Seaport*	*Brisbane*	*2018-05-21*	*Japan*	*n/a*	*0.5*	*1.42*
SP13	*Seaport*	*Brisbane*	*2018-05-28*	*Japan*	*n/a*	*0.52*	*1.56*
SP14	*Seaport*	*Brisbane*	*2018-05-28*	*East Asia*	*n/a*	*0.64*	*2.83*
SP17	*Seaport*	*Brisbane*	*2018-10-24*	*East Asia*	*n/a*	*0.53*	*2.48*

Table 2. Proportions of incursives assigned to each possible source population, for *Aedes albopictus* and *Aedes aegypti*.

	Aedes albopictus	*Aedes aegypti*
China	0.444	n/a
Singapore	0.222	0
Taiwan	0.111	0
Japan	0.222	n/a
Bali	0	0.747
Malaysia	0	0.157
Brazil	n/a	0.084
Thailand	0	0.012
Fiji	0	0
Sri Lanka	0	0
Vanuatu	0	0
Vietnam	0	0
Bandung	0	n/a
Christmas Island	0	n/a
Jakarta	0	n/a
Mauritius	0	n/a
Philippines	0	n/a
Timor-Leste	0	n/a
Torres Strait Islands	0	n/a
Kiribati	n/a	0
New Caledonia	n/a	0
Yogyakarta	n/a	0

302 # 4 Discussion

303 This study has identified incursion pathways of *Ae. albopictus* mosquitoes into Australia,
304 and shown that these pathways are strikingly different from those of its close relative, *Ae.*
305 *aegypti*. We identified four locations from which intercepted *Ae. albopictus* were
306 transported to Australia: three in East Asia (China, Taiwan, Japan) and one in Southeast
307 Asia (Singapore). No Australian *Ae. aegypti* incursions have been linked to any of these
308 locations, despite the presence of *Ae. aegypti* in both Taiwan and Singapore.[8] Likewise, no
309 *Ae. albopictus* in this study were found to have come from either Indonesia or Malaysia, the
310 most common source populations of *Ae. aegypti*. These results show how even
311 phylogenetically close taxa with similar capacity for active dispersal can differ greatly in
312 the dispersal pathways they take. Given that both *Ae. albopictus* and *Ae. aegypti* represent
313 dangerous pest disease vectors and quarantine risks, the work highlights the importance of
314 evaluating incursion pathways of pest species individually rather than by assuming that
315 related species will have similar incursion pathways. For Australia and other countries yet
316 to be invaded by *Ae. albopictus*, our findings should help current efforts to prevent further
317 spread of this species. Additionally, with both species carrying insecticide resistance
318 mutations,[39,40] the identification of incursion pathways can help to reduce the risk of
319 resistance alleles spreading into resident populations. For instance, in *Ae. aegypti*
320 resistance alleles have spread between geographically distant populations within the Indo-
321 Pacific region,[5] highlighting the risk involved in genetic incursions as well as species
322 incursions into areas where resistance alleles are absent [41].

323

Using the composite methodology of DAPC and assignment tests, 9 of the 20 *Ae. albopictus* were well-assigned (posterior probability > 0.5 and relative probability > 3). This proportion (45%) is lower than the proportion of well-assigned *Ae. aegypti* in Schmidt et al.[8] (76%), which is likely in part due to this study using a more conservative cut-off for assignment than used in Schmidt et al.[8] (posterior probability > 0.5 and relative probability > 2). The methodology here proved sufficient for confidently assigning among the genetically and geographically close populations of Singapore and Malaysia ($F_{ST}' = 0.0251$[20]), with the assignments to Singapore having the highest posterior and relative probabilities of all incursives (Table 1). Likewise, confident assignments were made to Japan, the source of *Ae. albopictus* incursions into Townsville, Australia, in 2012.[14] One of these incursives (SP18) was detected in a shipment of used tyres originally from Japan, providing a clear concordance between genetic inferences and observations at point of detection.

Among the poorly-assigned incursives were three *Ae. albopictus* (SP01-03) intercepted on a used power boat that was brought to Brisbane from Florida, USA, and four *Ae. albopictus* (SP11-14) found in a shipment of used tyres from the USA that transhipped through Colombia. These were initially assigned to the East Asian cluster with low confidence (Table 1). In comparison, two of three *Ae. albopictus* (SP15-17) detected in fish bins from Hong Kong and one incursive (AP01) detected on cut flowers from China were well-assigned to China, despite the high genetic similarity of Chinese and Taiwanese reference populations ($F_{ST}' = 0.0197$[20]). Considering these results, the low confidence in specific

assignments for incursives SP01-03 and SP11-14 may reflect the absence of reference genotypes from the Americas. *Aedes albopictus* in North America have genetic similarities with East Asian populations[25], and North American incursions of *Ae. albopictus* have been linked to goods transported from South China[42]. As our databank of reference genotypes grows and we are able to add populations from the Americas, we may find some of these incursives are confidently assigned to North America.

Despite the absence of American reference genotypes, the methodology used in this study is sufficient to show clear differences in incursion pathways between this species and *Ae. aegypti* (Fig 5). In contrast to *Ae. aegypti*, for which most incursives are transported to Australia from popular tourism locations like Bali, the *Ae. albopictus* incursions investigated in this study came from locations that, while popular as tourist destinations, are also major trading partners of Australia (https://dfat.gov.au/trade/resources/trade-at-a-glance/). We estimate that 90% of *Ae. albopictus* incursives came to Australia by marine routes, compared with only 5% of *Ae. aegypti*.[8] Other observations linking *Ae. albopictus* with marine incursions and *Ae. aegypti* with aerial incursions have been noted at New Zealand ports.[17] Considering the broad sympatry of these species throughout the Indo-Pacific,[43] these findings raise questions about why marine incursions are relatively rare for *Ae. aegypti* and why aerial incursions are relatively rare for *Ae. albopictus*.

The low incidence of marine incursions for *Ae. aegypti* may reflect a lack of diapause in this species.[24] By undergoing diapause, *Ae. albopictus* may be able to withstand longer

368 transportation times and colder minimum temperatures than *Ae. aegypti,*[21] which may
369 increase its likelihood of surviving marine transportation to Australia. Thus even if a vessel
370 is transporting similar numbers of *Ae. albopictus* and *Ae. aegypti* at departure, only *Ae.*
371 *albopictus* may survive long marine voyages. This is concordant with the patterns of
372 clustering observed in this study showing genetic similarities between East Asian and
373 Mauritian *Ae. albopictus* populations (Fig 1). Given the very high frequency of international
374 shipping past Mauritius[20], similarity of these locations likely reflects recent gene flow
375 associated with marine transportation. Similar patterns of genetic clustering among distant
376 populations has not been observed in *Ae. aegypti*.

377

378 A tentative explanation for the paucity of aerial incursions of *Ae. albopictus* into Australia
379 and New Zealand may relate to aircraft disinsection[44] and insecticide resistance. All
380 international flights into Australia and New Zealand undergo disinsection with synthetic
381 pyrethroids,[45] a class of insecticide to which both species have developed some resistance
382 via mutations at the *Vssc* gene.[39,40] All *Ae. aegypti* incursives into Australia and New
383 Zealand carry these resistance mutations;[8] in Bali, the most common source population for
384 *Ae. aegypti* incursions, every mosquito was homozygous for a pair of resistance mutations
385 conferring strong resistance to Type I and Type II synthetic pyrethroids. Although
386 homologous resistance mutations putatively conferring Type I pyrethroid resistance have
387 been recorded in *Ae. albopictus,*[46] there have been no reports of mutations for Type II
388 resistance and it is unclear how widely distributed the homologous mutations are in the
389 Indo-Pacific region. If *Ae. albopictus* from locations such as Bali remain susceptible to

390 certain types of pyrethroids, aircraft disinsection may be more effective at killing *Ae.*
391 *albopictus* than *Ae. aegypti*. A comprehensive geographic investigation of resistance
392 mutations in *Ae. albopictus* will be required to evaluate this hypothesis, and to test whether
393 disinsection is more effective at stopping incursions of this species than *Ae. aegypti*.

394

395 Conclusions

396 Understanding pest incursion pathways is critical for preventing new invasions and
397 stopping the transfer of alleles that reduce the efficacy of control methods. This study has
398 traced incursion pathways of *Ae. albopictus* into mainland Australia, where no successful
399 invasions have been observed. Most strikingly, we found that the source populations of *Ae.*
400 *albopictus* incursions were all different to those of the closely-related mosquito *Ae. aegypti*,
401 despite broad sympatry of these species in the Indo-Pacific region. Incursions of *Ae.*
402 *albopictus* came mostly along marine routes, from key trading locations, while *Ae. aegypti*
403 mostly came by aerial routes from tourism hotspots. These results highlight the value of
404 genomics for deciphering otherwise cryptic incursion pathways. Based on the different
405 pathways of the two species, we strongly recommend against assuming that closely-related
406 taxa will share common incursion pathways, and propose that incursion pathways of
407 important taxa should be investigated individually. Management plans for *Ae. albopictus*
408 and *Ae. aegypti* should incorporate knowledge of how insecticide resistance directs and
409 modifies incursion pathways, and how it is transmitted along incursion pathways into
410 areas where resistance alleles are absent.

411

412

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422 ## References

423 1. Gubler D and Kuno G, Dengue and dengue hemorrhagic fever: its history and
424 resurgence as a global public health problem, in Dengue and Dengue Hemorrhagic
425 Fever. CAB international, London, pp. 1–22 (1997).

426 2. Walsh JR, Carpenter SR and Van Der Zanden MJ, Invasive species triggers a massive
427 loss of ecosystem services through a trophic cascade. *Proc Natl Acad Sci* **38**: 4081-
428 4085 (2016).

429 3. Paini DR, Sheppard AW, Cook DC, De Barro PJ, Worner SP and Thomas MB, Global
430 threat to agriculture from invasive species. *Proc Natl Acad Sci* **113**: 7575-7579
431 (2016).

432 4. Hengeveld R. Dynamics of biological invasions. Chapman & Hall, London (1989).

433 5. Endersby-Harshman NM, Schmidt TL, Chung J, Rooyen A van, Weeks AR and
434 Hoffmann AA, Heterogeneous genetic invasions of three insecticide resistance
435 mutations in Indo-Pacific populations of *Aedes aegypti* (L.). *bioRxiv* DOI:
436 10.1101/768549 (2020).

437 6. Levine JM and D'Antonio CM, Forecasting biological invasions with increasing
438 international trade. *Conserv Biol* **17**: 322-326 (2003).

439 7. Westphal MI, Browne M, MacKinnon K and Noble I, The link between international
440 trade and the global distribution of invasive alien species. *Biol Invasions* **10**: 391-398
441 (2008).

442 8. Schmidt TL, van Rooyen AR, Chung J, Endersby-Harshman NM, Griffin PC, Sly A,

443 Hoffmann AA and Weeks, AR, Tracking genetic invasions: Genome-wide single
444 nucleotide polymorphisms reveal the source of pyrethroid-resistant *Aedes aegypti*
445 (yellow fever mosquito) incursions at international ports. *Evol Appl* 12:1136-1146
446 (2019).

447 9. Hulme PE, Trade, transport and trouble: managing invasive species pathways in an
448 era of globalization. *J Appl Ecol* **46**: 10-18 (2009).

449 10. Caley P, Ingram R and De Barro P. Entry of exotic insects into Australia: Does border
450 interception count match incursion risk? *Biol Invasions* **17**: 1087-1094 (2015).

451 11. Robinson A, Burgman MA and Cannon R, Allocating surveillance resources to reduce
452 ecological invasions: maximizing detections and information about the threat. *Ecol*
453 *Appl* **21**: 1410-1417 (2011).

454 12. Mehta S V., Haight RG, Homans FR, Polasky S and Venette RC, Optimal detection and
455 control strategies for invasive species management. *Ecol Econ* **61**: 237-245 (2007).

456 13. Hawley WA, The biology of *Aedes albopictus*. *J Am Mosq Control Assoc Suppl* **1**: 1-39
457 (1988).

458 14. van den Hurk AF, Nicholson J, Beebe NW, Davis J, Muzari OM, Russell RC, Devine GJ
459 and Ritchie SA, Ten years of the Tiger: *Aedes albopictus* presence in Australia since
460 its discovery in the Torres Strait in 2005. *One Heal* **2**: 19-24 (2016).

461 15. Kraemer MUG, Reiner RC, Brady OJ, Messina JP, Gilbert M, Pigott DM, Yi D, Johnson K,
462 Earl L, Marczak LB, Shirude S, Weaver ND, Bisanzio D, Perkins TA, Lai S, Lu X, Jones P,
463 Coelho GE, Carvalho RG, Bortel WV, Marsboom C, Hendrickx G, Schaffner F, Moore
464 CG, Nax HH, Bengtsson L, Wetter E, Tatem AJ, Brownstein JS, Smith DL, Lambrechts L,

465 Cauchemez S, Linard C, Faria NR, Pybus OG, Scott TW, Liu Q, Yu H, Wint GRW, Hay SI
466 and Golding N, Past and future spread of the arbovirus vectors *Aedes aegypti* and
467 *Aedes albopictus*. *Nat Microbiol* **4**: 854-863 (2019).

468 16. Ritchie SA, Moore P, Carruthers M, Williams C, Montgomery B, Foley P, Ahboo S, van
469 den Hurk AF, Lindsay MD, Cooper B, Beebe N and Russell RC, Discovery of a
470 Widespread Infestation of *Aedes albopictus* in the Torres Strait, Australia. *J Am Mosq*
471 *Control Assoc* **22**: 358-365 (2006).

472 17. Ammar SE, McIntyre M, Swan T, Kasper J, Derraik JGB, Baker MG and Hales S,
473 Intercepted Mosquitoes at New Zealand's Ports of Entry, 2001 to 2018: Current
474 Status and Future Concerns. *Trop Med Infect Dis* **4**: 101-118 (2019).

475 18. Lounibos LP, Invasions by Insect Vectors of Human Disease. *Annu Rev Entomol* **47**:
476 233-266 (2002).

477 19. Tatem AJ, Hay SI and Rogers DJ, Global traffic and disease vector dispersal. *Proc Natl*
478 *Acad Sci* **103**: 6242-6247 (2006).

479 20. SchmidtTL, Chung J, Honnen, A-C, Weeks AR and Hoffmann AA, Comparative
480 genomics of two invasive mosquitoes (*Aedes aegypti* and *Aedes albopictus*) from the
481 Indo-Pacific. *bioRxiv* DOI: 10.1101/2020.03.15.993055.

482 21. Urbanski JM, Benoit JB, Michaud MR, Denlinger DL and Armbruster P, The molecular
483 physiology of increased egg desiccation resistance during diapause in the invasive
484 mosquito, *Aedes albopictus*. *Proc R Soc B Biol Sci* **277**: 2683-2692 (2010).

485 22. Medley KA, Westby KM and Jenkins DG, Rapid local adaptation to northern winters in
486 the invasive Asian tiger mosquito *Aedes albopictus*: A moving target. *J Appl Ecol* **56**:

487 2518-2527 (2019).

488 23. Diniz DFA, De Albuquerque CMR, Oliva LO, De Melo-Santos MAV and Ayres CFJ,
489 Diapause and quiescence: Dormancy mechanisms that contribute to the geographical
490 expansion of mosquitoes and their evolutionary success. *Parasit Vectors* **10**: 310-322
491 (2017).

492 24. Mitchell C, Geographic spread of *Aedes albopictus* and potential for involvement in
493 arbovirus cycles in the Mediterranean basin. *J Vector Ecol* **20**: 44-58 (1995)

494 25. Kotsakiozi P, Richardson JB, Pichler V, Favia G, Martins AJ, Urbanelli S, Armbruster
495 PA and Caccone A, Population genomics of the Asian tiger mosquito, *Aedes*
496 *albopictus*: insights into the recent worldwide invasion. *Ecol Evol* **7**: 10143-10157
497 (2017).

498 26. Manni M, Guglielmino CR, Scolari F, Vega-Rúa A, Failloux A-B, Somboon P, Lisa A,
499 Savini G, Bonizzoni M, Gomulski LM, Malacrida AR and Gasperi G, Genetic evidence
500 for a worldwide chaotic dispersion pattern of the arbovirus vector, *Aedes albopictus*.
501 *PLoS Negl Trop Dis* **11**: e0005332 (2017).

502 27. Sherpa S, Blum MGB, Capblancq T, Cumer T, Rioux D and Després L, Unravelling the
503 invasion history of the Asian tiger mosquito in Europe. *Mol Ecol*, **28**: 2360-2377
504 (2019).

505 28. Eskildsen GA, Rovira JR, Smith O, Miller MJ, Bennett KL, McMillan WO and Loaiza J,
506 Maternal invasion history of *Aedes aegypti* and *Aedes albopictus* into the Isthmus of
507 Panama: implications for the control of emergent viral disease agents. *PLoS One* **13**:
508 e0194874 (2018).

509 29. Schmidt TL, Rašić G, Zhang D, Zheng X, Xi Z and Hoffmann AA. Genome-wide SNPs
510 reveal the drivers of gene flow in an urban population of the Asian Tiger Mosquito,
511 *Aedes albopictus*. *PLoS Negl Trop Dis* **11**: e0006009 (2017).

512 30. Rašić G, Filipović I, Weeks AR and Hoffmann AA. Genome-wide SNPs lead to strong
513 signals of geographic structure and relatedness patterns in the major arbovirus
514 vector, *Aedes aegypti*. *BMC Genomics* **15**: 275-286 (2014).

515 31. Catchen J, Hohenlohe PA, Bassham S, Amores A and Cresko WA. Stacks: an analysis
516 tool set for population genomics. *Mol Ecol* **22**: 3124-3140 (2013).

517 32. Langmead B and Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat*
518 *Methods* **9**: 357-359 (2012).

519 33. Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE,
520 Lunter G, Marth GT, Sherry ST, McVean G, Durbin R and 1000 Genomes Project
521 Analysis Group, The variant call format and VCFtools. *Bioinformatics* **27**: 2156-2158
522 (2011).

523 34. Jombart T, adegenet: a R package for the multivariate analysis of genetic markers.
524 *Bioinformatics* **24**: 1403-1405 (2008).

525 35. Chen K-Y, Marschall EA, Sovic MG, Fries AC, Gibbs HL and Ludsin SA, *assignPOP*: An R
526 package for population assignment using genetic, non-genetic, or integrated data in a
527 machine-learning framework. *Methods Ecol Evol* **9**: 439-446 (2018).

528 36. Linck E and Battey CJ, Minor allele frequency thresholds strongly affect population
529 structure inference with genomic datasets. *Mol Ecol Resour* **19**: 639-647 (2019).

530 37. Li H, Toward better understanding of artifacts in variant calling from high-coverage
531 samples. *Bioinformatics* **30**: 2843-2851 (2014).

532 38. Browning BL and Browning SR, Genotype Imputation with Millions of Reference
533 Samples. *Am J Hum Genet* **98**: 116-126 (2016).

534 39. Smith LB, Kasai S and Scott JG, Pyrethroid resistance in *Aedes aegypti* and *Aedes*
535 *albopictus*: Important mosquito vectors of human diseases. *Pestic Biochem Physiol*
536 **133**: 1-12 (2016).

537 40. Auteri M, La Russa F, Blanda V and Torina A, Insecticide Resistance Associated with
538 *kdr* Mutations in *Aedes albopictus*: An Update on Worldwide Evidences. *Biomed*
539 *Res Int* **2018**: 1-10 (2018).

540 41. Endersby-Harshman NM, Wuliandari JR, Harshman LG, Frohn V, Johnson BJ, Ritchie
541 SA and Hoffmann AA, Pyrethroid Susceptibility Has Been Maintained in the Dengue
542 Vector, *Aedes aegypti* (Diptera: Culicidae), in Queensland, Australia. *J Med Entomol*
543 **54**: 1649-1658 (2017).

544 42. Madon MB, Hazelrigg JE, Shaw MW, Kluh S and Mulla MS, Has *Aedes albopictus*
545 established in California? *J Am Mosq Control Assoc* **19**: 297-300 (2003).

546 43. Kraemer MUG, Sinka ME, Duda KA, Mylne AQN, Shearer FM, Barker CM, Moore CG,
547 Carvalho RG, Coelho GE, Bortel WV, Hendrickx G, Schaffner F, Elyazar IRF, Teng H,
548 Brady OJ, Messina JP, Pigott DM, Scott TW, Smith DL, Wint GRW, Golding N and Hay
549 SI, The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*.
550 *Elife* **4**: e08347 (2015).

551 44. IPCS, Aicraft disinsection insecticides (Environmental health criteria 243). European

552 Commission and the Policy Research Programme, Geneva (2013).

553 45. DAWR/MPI, Schedule of aircraft disinsection procedures for flights into Australia
554 and New Zealand. Department of Agriculture and Water Resources, Canberra, and
555 Ministry for Primary Industries, Wellington (2016).

556 46. Xu J, Bonizzoni M, Zhong D, Zhou G, Cai S, Li Y, Wang X, Lo E, Lee R, Sheen R, Duan J,
557 Yan G and Chen X, Multi-country Survey Revealed Prevalent and Novel F1534S
558 Mutation in Voltage-Gated Sodium Channel (VGSC) Gene in *Aedes albopictus*. *PLoS*
559 *Negl Trop Dis* **10**: e0004696 (2016).

560