Article Type: Letter

Article Title: Lineage maintenance due to assortative mating but not competitive exclusion in a sympatric sibling species pair

Mitchell Irvine[1]*, Zachary Stewart[1], Nagalingam Kumaran[2], Chapa G. Manawaduge[1], Solomon Balagawi[3], Brendan Missenden[4], Melissa Starkie[4], Anthony R. Clarke[1], David Hurwood[1], and Peter Prentis[1]

Author Information

* Correspondence: mitchell.irvine@hdr.qut.edu.au

Mitchell Irvine (ORCID ID – 0009-0004-2338-8463)

[1]School of Biological and Environmental Sciences, Queensland University of Technology, GPO Box 2434, Brisbane, Qld, Australia

[2]Commonwealth Scientific and Industrial Research Organization (CSIRO), Ecosciences Precinct, Dutton Park, Queensland, Australia.

[3]Horticulture and Forestry Science, Department of Agriculture and Fisheries, P.O. Box 652, Cairns, Queensland, Australia.

[4]Department of Agriculture and Fisheries, Ecosciences Precinct, Dutton Park, Queensland, Australia

Running Title: Ecological differentiation absent in sympatric species pair

Abstract Word Count: 223

Word Count: 5,176 (excl. abstract, lay summary, acknowledgements, author contributions, data accessibility and references)

Abstract

Lineage theory suggests that the development of traits associated with reproductive isolation coupled with traits associated with ecological differentiation are essential for the maintenance of sympatric lineages. In the absence of one of these elements, lineage boundaries are expected to breakdown. The tephritid fruit fly species pair, *Bactrocera tryoni* and *B. neohumeralis* have significant overlap in geographic range and host use, with time of day of male mating the only known difference in their mating systems. Using this system, we tested the relative importance of ecological differentiation versus reproductive isolation in sympatric lineage maintenance.

Genome-wide SNP analyses found strong genetic differentiation between the species with no evidence for hybridization in the field. Most outlier SNPs were restricted to narrow regions towards the centromeres and telomeres of chromosomes. Enrichment of annotation terms indicated an overabundance of genes with the 'abnormal neuroanatomy' term. Terms of interest associated with sleep and circadian rhythm, potentially important to the allochronic reproductive barrier, were non-enriched. Ecological data found no evidence for ecological differentiation or competitive displacement between the two species based on significant positive correlations between species numbers trapped at different times of the year, trapped in different habitats within a region, or when reared from fruit.

Our study highlights the significance of assortative mating over ecological differentiation for sympatric lineage maintenance of the *B. tryoni*/*B. neohumeralis* sibling pair.

Keywords

Bactrocera tryoni, *Bactrocera neohumeralis*, lineage maintenance, coexistence, Tephritidae, competitive displacement, recombination

Lay Summary

The combination of differential adaptation to ecological niches and the development of reproductive barriers are considered necessary for maintaining co-existing lineages. Without these two attributes, traditional lineage theory predicts that co-existence will not be sustainable and will end in the extinction of one lineage through competitive exclusion or the breakdown of lineages boundaries leading to a single hybrid lineage. When both attributes are present, ecological differentiation acts to minimize competitive pressures between lineages while reproductive barriers prevent hybridization that often leads to the dissolution of lineage boundaries. The closely related Tephritid fruit fly species pair, *Bactrocera tryoni* and *B. neohumeralis*, demonstrate significant overlap in distribution and host utilization. A difference in male mating times is the only known reproductive barrier preventing hybridization between the pair. Using a combination of ecological (seasonal abundance, host use and habitat use) and genomic data, we tested the differing roles of competition and assortative mating on sympatric lineage maintenance in this species pair.

Ecological data demonstrated strong correlation in seasonal abundance, habitat use and host use between the species and provided no evidence for ecological differentiation or competitive pressure between the species. In contrast, genome-wide SNPs showed strong genetic differentiation between species. Additionally, nineteen genes associated with abnormal neurology and circadian rhythm were identified, some of which may be involved in maintaining reproductive isolation between the species.

Despite the lack of ecological differentiation, genomic data shows little evidence of hybridization occurring between these species. Instead, the chromosomal positioning of genes

associated with reproductive barriers appears to be sufficient in maintaining lineage boundaries.

Introduction

Species are increasingly defined as separately evolving lineages in order to overcome incompatibilities between alternative species concepts (herein referred to as lineage theory) (de Queiroz, 1999, 2005; Fišer et al., 2018; Mayden, 1997; Shirley et al., 2014). If lineages remain separate, then attributes used to define a species, such as unique mate recognition, distinct phenotypes and adaptation to specific habitats, may (or may not) emerge through mutation, selection, genetic drift, or pleiotropic change (de Queiroz, 2005, 2007). However, while the utility of lineage theory may be increasing, the questions faced by evolutionary biologists of what drives lineage divergence and then keeps lineages separate in sympatry, remain (Martin & Richards, 2019; Saxenhofer et al., 2019).

Central to our understanding of lineage maintenance, particularly between sympatric or parapatric lineages, are the opposing roles of natural selection and recombination on the formation or dissolution of lineage boundaries (Felsenstein, 1981). Natural selection promotes the development of reproductive isolation through the formation of locally adapted gene complexes; while free recombination acts to dissolve them through breaking down the associations of such complexes (Felsenstein, 1981; Hey, 2001; Schluter & Rieseberg, 2022). A reduction in recombination rates is considered helpful for lineage boundaries to persist and strengthen over time (Butlin, 2005). Traits associated with reproductive isolation, such as those involved in assortative mating, are considered effective mechanisms for constraining gene flow and are essential for sympatric lineage divergence when coupled with the

development of traits associated with local adaptation (Ortiz-Barrientos et al., 2016; Schluter & Rieseberg, 2022).

On an ecological scale, the persistence of two lineages in sympatry depends on two criteria; their ability to overcome the effects of hybridization through the development of reproductive isolation, and the development of niche partitioning to avoid inter-lineage competition asymmetries (Coyne & Orr, 2004; Felsenstein, 1981; Weber & Strauss, 2016). Without meeting these criteria, theory suggests that lineages will likely dissolve through hybridization or cease to exist in sympatry through competitive displacement (Germain et al., 2021; Weber & Strauss, 2016).

The Australian frugivorous tephritids (Diptera: Tephritidae), *Bactrocera tryoni* (Froggatt) and *Bactrocera neohumeralis* (Hardy) [earlier taxonomic names *Dacus tryoni* and *D. neohumeralis*], are a fruit fly sibling pair which has been studied in terms of their speciation histories and genetic stability for over 60 years (Birch, 1961; Gilchrist et al., 2005; Gilchrist & Ling, 2006; Gilchrist et al., 2014; Lewontin & Birch, 1966; Meats et al., 2003; Pike, 2004; Raphael et al., 2019; Smith, 1979; Yeap et al., 2020). Genetically, *B. tryoni* and *B. neohumeralis* are very similar (Gilchrist et al., 2014; Morrow et al., 2000; Wang et al., 2003), with the sharing of polymorphisms in coding and non-coding regions indicating continued genetic exchange or recent separation (Gilchrist et al., 2005; Gilchrist & Ling, 2006; Wang et al., 2003). The species show only minor variation in their morphology; the humeral calli, located on the ‘shoulders’ of the fly, are bright yellow in *B. tryoni* and brown in *B. neohumeralis* (Drew, 1989). While intermediate colour variants are well documented, genetic analysis using microsatellites suggest that intermediates represent intraspecific phenotypic variation, rather than hybrid individuals (Gilchrist & Ling, 2006; Pike, 2004; Wolda, 1967). While *B. tryoni* is typically more abundant than *B. neohumeralis*, the geographic ranges of the

two species extensively overlap along the east coast of Australia;, with the endemic range of *B. neohumeralis* being entirely encompassed by the range of *B. tryoni* except for a few islands in the Torres Strait, north of Queensland (Fig. 1a,b) (Osborne et al., 1997). Both *B. tryoni* and *B. neohumeralis* are highly polyphagous, using fruit from multiple plant families for breeding, with host utilization data [presented as 'simple' lists of host fruit that either species have been reared from at least once with little or no further information] showing significant overlap between the pair (Fig. 1c) (Hancock et al., 2000; Leblanc et al., 2012; QDPC, 2021).

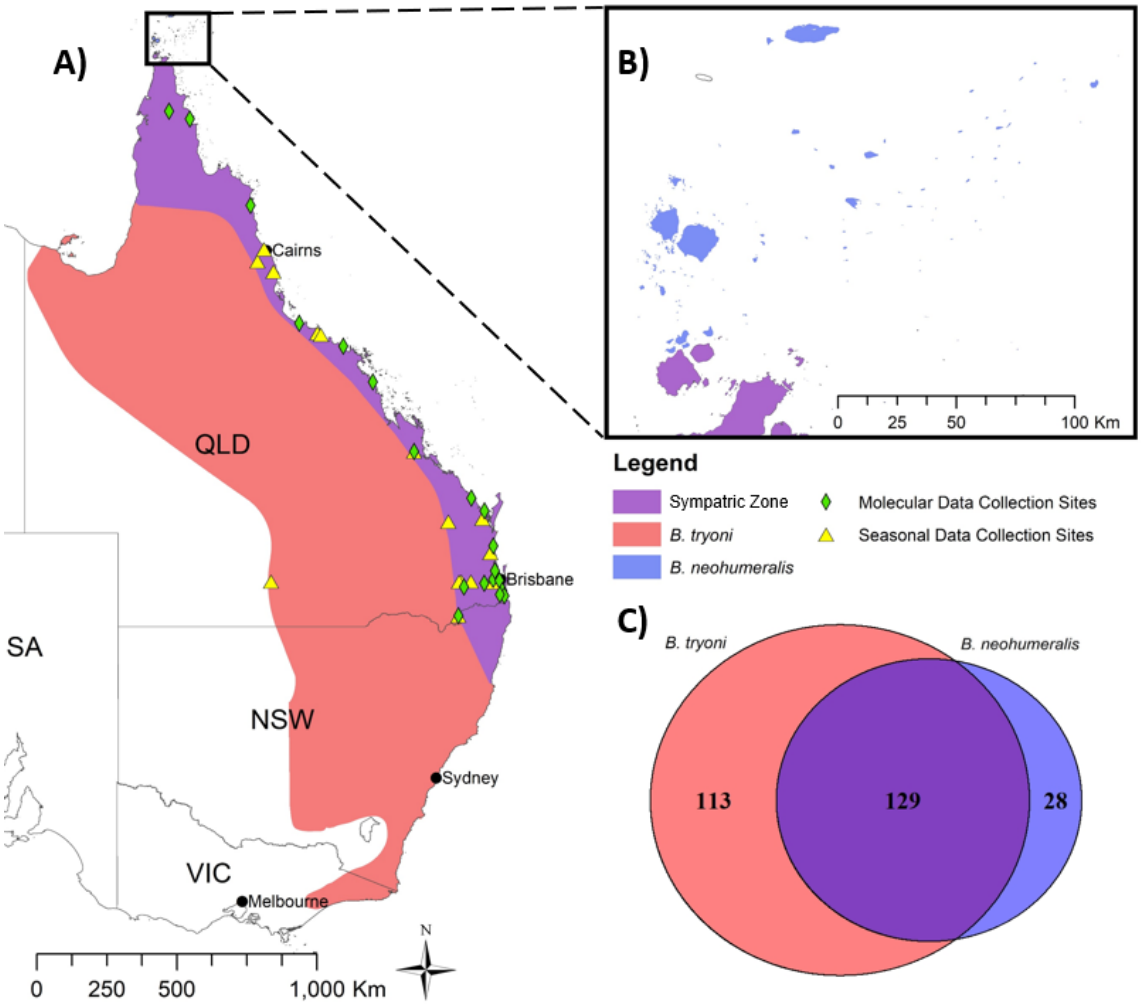

Figure 1. A) Distribution of *Bactrocera tryoni* and *B. neohumeralis* along the east coast of Australia. B) Distribution of both species in the Cape York Peninsula and Torres Strait Islands. C) Host usage overlap between species. Numbers in circles represent the known number of host species utilized. Range and host data gathered from Hancock et al. (2000); Leblanc et al. (2012); QDPC (2021). Note: Recent expansion of *B. tryoni* into central and western Victoria is not shown because it is still under regulatory control in many places and is not associated with the species' evolutionary history.

So closely related are *B. tryoni* and *B. neohumeralis* that only one known trait unambiguously defines them as separate biological species: their time of mating (Birch & Vogt, 1970; Lewontin & Birch, 1966; Vogt, 1977). *Bactrocera neohumeralis* mates during the middle of the day at high light intensity over a mating window of three to seven hours, while *B. tryoni* initiates mating during dusk at low light intensity in a mating window of approximately one hour (Ekanayake et al., 2017). This difference in mating time is the only known mechanism maintaining reproductive isolation between the species (Birch & Vogt, 1970; Lewontin & Birch, 1966; Meats et al., 2003; Raphael et al., 2019; Smith, 1979), with other components of their mating system not known to be different (Bellas & Fletcher, 1979; Ekanayake et al., 2017; Mankin et al., 2008), including an absence of post-zygotic barriers (Yeap et al., 2020). Female *B. neohumeralis* will, like *B. tryoni*, also mate at dusk, at which time *B. tryoni* males do not discriminate between females of either species(Yeap et al., 2020). This caused Yeap et al (2020) to conclude "*the mating time difference may be a weaker reproductive isolating barrier than once assumed*".

If mating time is a weak reproductive barrier between the two species, then other factors must be playing a role in maintaining separate lineages; for example, genomic incompatibility (Dobzhansky, 1936; Turelli et al., 2001). Additionally, for herbivorous insects such as fruit flies, lineage divergence is largely considered to be driven by host switching and host specialization that reduces the effects of competition and natural enemies (Gavrilets & Losos, 2009; Janz & Nylin, 2008; Jeffries & Lawton, 1984; Schluter, 2000; Yoder et al., 2010) and so ecological factors also need to be considered when investigating lineage maintenance. Conversely the lineages may not be evolutionarily stable, in which case evidence of gene flow should be expected from sympatric field collections of the two taxa.

In this study, we conducted a comprehensive investigation of how *B. tryoni* and *B. neohumeralis* lineages have been maintained in sympatry through dense geographic sampling of nuclear genomes and structured field sampling of changes in temporal population abundance and habitat- and host-usage. We assessed the extent of genetic differentiation and identified putative candidate genes associated with reproductive isolation between the two species. Using the field data, we conducted correlation analyses on seasonal abundance, host use and habitat utilization for evidence of competitive displacement between the two species. Shifts in any or all of these three ecological attributes have been previously associated with competition between fruit fly species (Clarke & Measham, 2022).

Methods

Molecular data sample collection

Individuals of *B. tryoni* and *B. neohumeralis* were collected in December 2020 and December 2021, using cue lure baited Steiner (Steiner, 1957), Lynfield (Cowley et al., 1990) or Paton traps (Plant Health Australia, 2023). These traps were deployed by Queensland Department of Agriculture and Fisheries (QDAF) as part of ongoing Biosecurity Queensland (BQ) surveillance. A subsample of the trapping sites were selected from the existing network to represent the geographic range of both species (Fig. 1). Up to 10 individuals of each species were collected per site with a total of 81 *B. tryoni* and 105 *B. neohumeralis* individuals collected from 19 sites. Detailed trapping records are included in Table S1. Identity of the samples were confirmed following the descriptions in fruit fly identification handbooks (Drew, 1989; Plant Health Australia, 2018), and the samples were stored at -20°C in 100% ethanol.

Extraction and sequencing

DNA was extracted using the QIAGEN DNeasy® Blood & Tissue Kit following the manufacturer's protocol. Samples were screened for quality and quantity, using both gel electrophoresis and Qubit assay, and then sent to Diversity Arrays Technology Pty Ltd, Canberra (DArT P/L), for DArTseq high-density genotyping (Kilian et al., 2012). A PstI/SphI, restriction enzyme combination was used by DArT P/L, and the fragments (up to 90bp) were sequenced on an Illumina Hiseq2500 as single end reads.

Data sourcing

All relevant analyses made use of the *B. tryoni* genome assembly (Genbank accession = GCF_016617805.1).

Read alignment and SNP calling

Reads were demultiplexed using process_radtags in Stacks v2.60 (Rochette et al., 2019). Read mapping used BWA-MEM (Li, 2013) to produce a SAM alignment file with appropriate read group specification for each sample. Subsequently, SAMtools (Li et al., 2009) produced sorted and indexed BAM alignment files. Freebayes v1.3.6 (Garrison & Marth, 2012) was used in an iterative process to predict single nucleotide polymorphisms (SNPs) in all samples. Firstly, each sample had SNP calling performed individually using freebayes; output Variant Call Format (VCF) files were left-aligned using BCFtools (Danecek et al., 2021) and block substitutions were decomposed using vt (Tan et al., 2015). Resulting VCF files were merged using BCFtools, and this VCF file was used as input for a second round of SNP calling using freebayes (*-@ file.vcf --only-use-input-alleles*) to ensure all samples were genotyped at the same locations. The variant calls were filtered using a method based upon James et al. (2021) wherein SNPs were removed if they had missing data in > 50% of the population, had a quality score < 30, a depth < 3 read alignments, or a minor allele count < 1; we also filtered SNPs with a minor allele frequency < 5%.

Population structure

To investigate whether admixture was occurring between the two species, we used fastSTRUCTURE v1.0 (Raj et al., 2014). A simple prior of K=1-10 was used, after which the chooseK.py utility program provided with fastSTRUCTURE was used to assess the optimal number of genetic clusters to explain the structure among species and populations. Plots were generated using pophelper v2.3.1 (Francis, 2017) in R.

Additionally, we ran a Principal Component Analysis (PCA) to provide further visualization of the variations within and between the two species, using the SNPRelate v1.24.0 (Zheng et al., 2012) package in R. The linkage disequilibrium-based SNP pruning function provided with this software was run with default parameters to mitigate any bias associated with linked SNP clusters upon the downstream PCA. We similarly performed this SNP pruning prior to calculation of IBS proportions for samples using SNPRelate.

Mean F_{ST} values (Weir & Cockerham, 1984) were calculated both within and between the two species using VCFtools (Danecek et al., 2011). Within species comparisons were conducted for each species separately and samples from each collection site were considered as separate populations; for between species comparison, the samples were grouped by species and entered as two separate populations.

Outlier SNPs and gene candidates

Variant predictions were converted from VCF to GESTE format using PGDSpider v2.1.1.5 (Lischer & Excoffier, 2012), then outlier SNPs were predicted using Bayescan v2.1 (Foll & Gaggiotti, 2008) with default parameters. Genes containing outlier SNPs within their gene model (exons and/or introns), as well as genes that were the nearest to an intergenic outlier SNP were identified using a custom script.

Candidate genes were queried against the *Drosophila melanogaster* gene models (Larkin et al., 2021)(FlyBase release FB2023_01) using MMseqs2, and the FlyBase automated gene summaries of the best hit were parsed for functional and phenotypic annotations which we attributed to our sequences if a minimum E-value of $1e^{-10}$ was achieved.

Seasonal abundance

A historical dataset (May, 1961) was used for the comparison of temporal abundance of *B. neohumeralis* with *B. tryoni*. This dataset has been previously used to report *B. tryoni* [but not *B. neohumeralis*] seasonal phenology (Muthuthantri et al., 2010), and so the phenology patterns *per se*, are not explored here. Rather, we focus on evidence for temporal niche segregation in *B. neohumeralis* and *B. tryoni* through paired t-tests and Pearson correlation analysis using the R package ggpubr (Kassambara, 2023). In Africa, a shift in seasonal abundance to minimize an overlap in host usage (i.e. temporal niche displacement) has been reported as the outcome of competition between the native mango fruit fly (*Ceratitis cosyra* (Walker)) and invasive Oriental fruit fly (*Bactrocera dorsalis* (Hendel))(Vayssières et al., 2015). Therefore, we predict that if there is competition-driven temporal niche displacement between the two species, then there should be no, or even negative correlation between their seasonal abundances. We interpret significant positive correlation between their populations as a lack of evidence of temporal niche displacement.

Detailed methodology on the fly trapping dataset is described elsewhere (May, 1961; Muthuthantri et al., 2010). In summary, 15 trapping sites across Queensland, Australia, were used, ranging from temperate Stanthorpe in the south to tropical Cairns, ~1500km to the north (See Table S5). Fly trapping was carried out for up to six years per location using a fruit-based liquid lure trap (May & Caldwell, 1944). At each location ten traps were maintained,

with traps cleared weekly. The data in May (1961) vary between sites in how they are presented, and modifications were needed in order to be able to directly compare *B. tryoni* and *B. neohumeralis* populations. Six sites were excluded as seasonal data were not available (see Table S5 for details). The t-tests and correlation analyses between *B. tryoni* and *B. neohumeralis* populations was conducted on a site-by-site basis (i.e., data were never collated across sites), so the different manipulations performed on the site-specific data sets did not affect results.

Habitat use

This study compared abundance of *B. tryoni* and *B. neohumeralis* across different human-defined habitat types to determine habitat use patterns. Six habitat types, grassland, suburbia, horticultural farming, mixed sugarcane and other crops(mixed farming hereafter), dry forest, and wet forest were chosen for the study in the Bundaberg region of Queensland (~350 km north of Brisbane). Specific descriptions of each habitat type are outlined in Table S7. For each habitat type there were nine replicate fruit fly traps. Trapping commenced in September 2010 (spring) and was completed in March 2011 using cuelure baited modified Steiner traps (Drew et al., 1982). The trapping period covers the period of peak yearly activity for *B. tryoni* (Clarke et al., 2022). The trapping program involved setting up the traps on a monthly basis at each trap site, removing the traps at each site three days after setting up and replacing the traps with fresh wicks and attractants in the following month. Flies collected over the three days in each month were counted and identified to species level using a fruit fly identification guide (Drew, 1989). Paired t-tests and Pearson correlation analyses on the total trapping dataset for a habitat type were performed to compare the relationship in abundance of the two different species at that habitat type. Examples of spatial displacement of competing fruit flies are presented in Duyck et al. (2004).

Host fruit use

Fruits were collected from the QDAF Maroochy Research Facility, Nambour, Queensland (~100 km north of Brisbane) from separate orchard blocks that consisted of either mixed tropical fruit varieties, stone fruit or mangoes, as well as some isolated trees. Small numbers of ripe or mature green fruit were sampled on a weekly or fortnightly basis between December 2012 and October 2015. Fruit were selected from a number of trees within the orchard block and picked from various heights and aspects to obtain a random sample. Fruits collected were: white sapote (*Casimiroa edulis*), mulberry (*Morus nigra*), peach (*Prunus persica*), plum (*Prunus domestica*), carambola (*Averrhoa carambola*), nectarine (*Prunus persica*), guava (*Psidium guajava*), sapodilla (*Manilkara zapota*), *Syzigium* spp, grumichama (*Eugenia brasiliensis*), hog plum (*Spondias mombin*), jabotica (*Plinia cauliflora*), white mulberry (*Morus alba*), avocado (*Persea americana*), black sapote (*Diospyros nigra*), longan (*Dimocarpus longan*), mango (*Mangifera indica*) and cashew (*Anacardium occidentale*).

Fruit were placed in paper bags and transported to the QDAF Brisbane laboratories. Fruit were counted and weighed and then placed on gauzed plastic containers over vermiculite, in plastic boxes with gauzed lids to allow ventilation. Boxes were held in a Controlled Environment Room (26°C and 70%RH) to allow insects to develop through to the pupal stage. The vermiculite was sieved weekly until all insects had exited fruit and pupated, and fruit was inspected before being discarded. Fruit fly pupae that were reared out of fruit samples were placed into small plastic boxes with gauzed lids containing vermiculite. Once adult fruit flies had emerged from pupation, they were identified to species level using morphological taxonomic characters. The abundance of the two fly species from a host was compared using a paired t-test and Pearson correlation analysis. Changes in fruit fly host

usage patterns due to competition are well documented in the fruit fly literature (Charlery de la Masselière et al., 2017; Hassani et al., 2022).

Results

Molecular Data

Read processing, alignment and SNP calling

After demultiplexing samples, we obtained between 2,596,430 and 5,228,647 high-quality sequence reads for each sample. Alignment to the *B. tryoni* genome assembly using BWA-MEM indicated that most samples had high mapping rates (median alignment percentage = 74.98%), with a small number of samples having low sequence alignment (minimum alignment percentage = 0.38%); see Table S2 and Table S3 for full details of read counts and alignment percentages. 28,334 SNPs were identified using Freebayes from the two species that met our threshold for inclusion.

Populations cluster by species and not geography

We found that populations clustered based on species and not geography in all analyses. PCA (Fig. 2) and fastSTRUCTURE (Fig. 3) both resolved species-specific groups, demonstrating clear genetic delineation of the two species and an absence of intermediate genotypes between the species. Several *B. neohumeralis* samples (genotype codes 526, 528, 529, 530, and 531 from the NS location) appear to be outliers based on PCA (See Fig. S1), but one sample from the same location (527) grouped with the majority of *B. neohumeralis* samples in ordination space. Additionally, the chooseK.py function of fastSTRUCTURE predicted three population clusters, with the outlier samples from the PCA representing the third cluster (See Fig. S2). These outlier samples had poor genotype calling rates (min = 27.9%, max = 32.3%) compared to sample 527 (91.3%) and most other samples (median = 89.1%). As such, we believe these outliers to result from sequencing issues and were excluded from further analysis.

Patterns of differentiation among populations (F_{ST}) were also lower between populations within a species (*B. neohumeralis* = 0.0025796 when ignoring outlier samples, *B. tryoni* = 0.0036269) and two orders of magnitude higher between species (0.10843). Identity-By-State (IBS) analysis also provided insight into the genetic homogeneity of the two species, with *B. tryoni* having a mean value of 0.797 and *B. neohumeralis* having a mean value of 0.790 (species with a value closer to 1 are more identical) suggesting that both species are approximately equally homogenous.

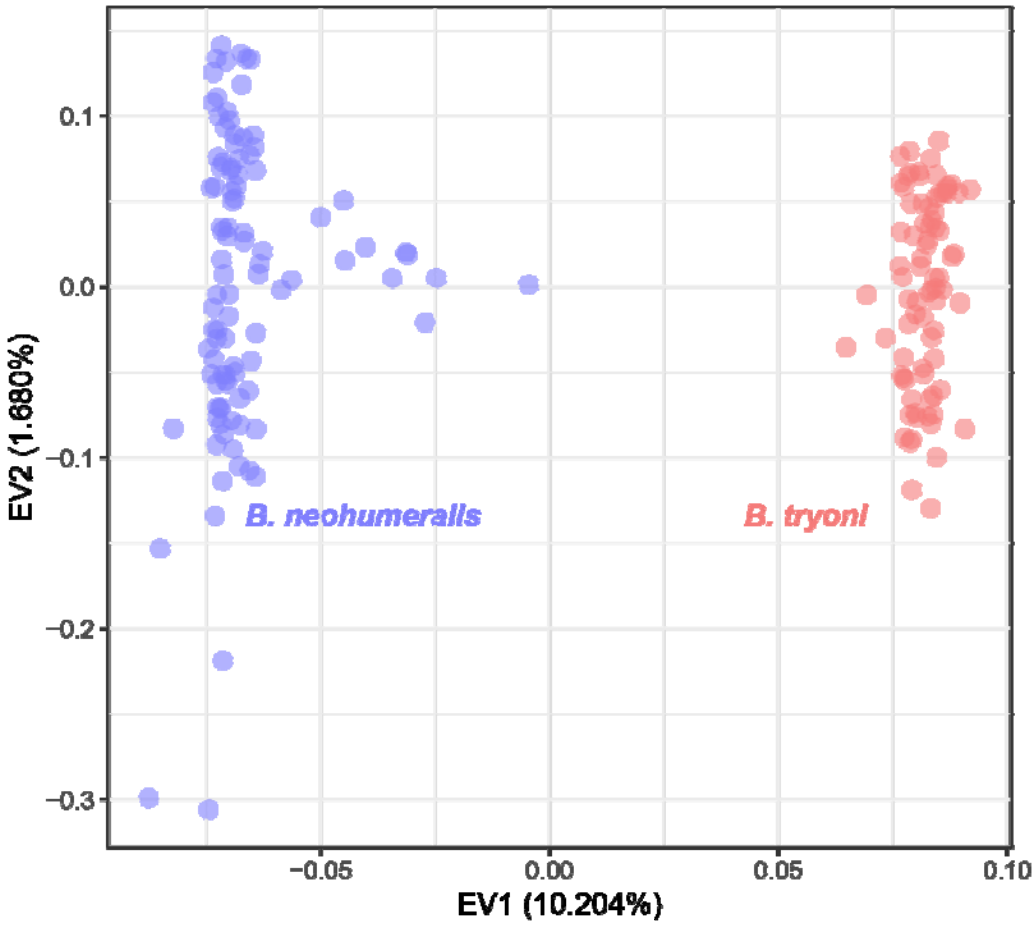

Figure 2. Principal component analysis (PCA) of single nucleotide polymorphisms (SNPs) *Bactrocera neohumeralis* and *B. tryoni*, with the values from the first two eigenvectors

plotted for each individual. Eigenvectors one (horizontal) and two (vertical) explain 10.204% and 1.68% of the variance in the data, respectively.

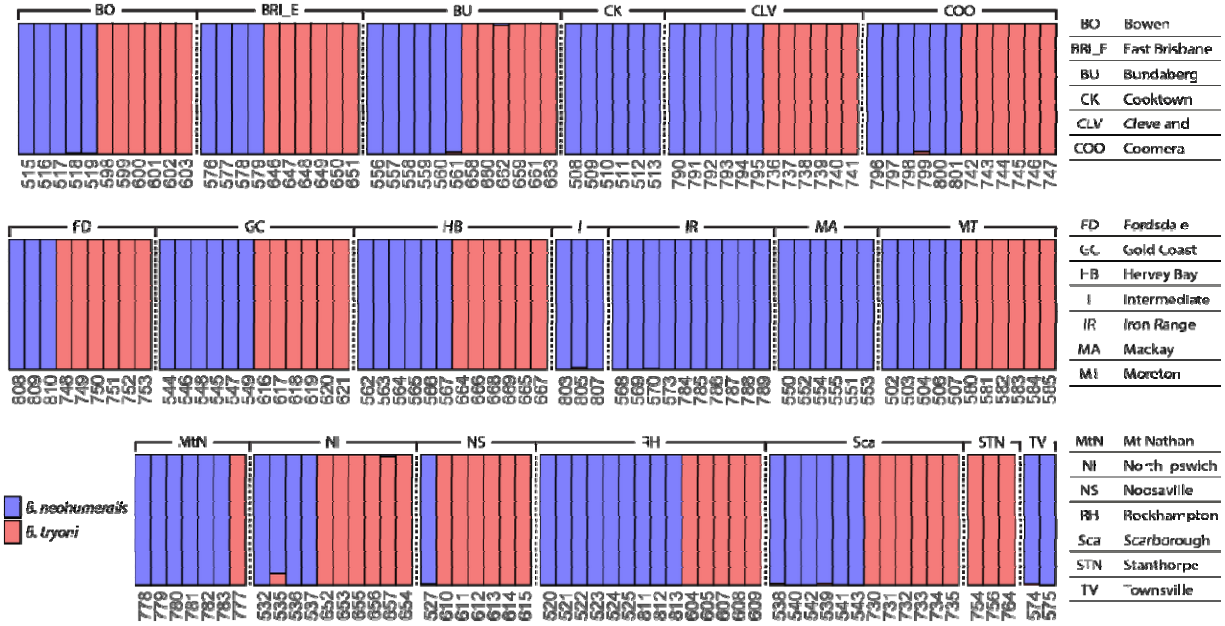

Figure 3. fastSTRUCTURE results at cluster size (K) equals two for single nucleotide polymorphisms (SNPs) for *Bactrocera neohumeralis* and *B. tryoni* grouped by collection site. Red and blue colouration of the bars indicates the inferred population admixture proportions as a percentage (numbers not shown). Three-digit numbers along the bottoms of each row of bars are genotype ID codes. The intermediate grouping (I) includes samples with an intermediate phenotype. All three intermediate samples (genotypes 803, 805 and 807) presented as *B. neohumeralis* genotypes.

Outlier loci are clustered in specific chromosome segments between the species

Using outlier detection in BayeScan, we found 357 of the 28,334 SNPs (1.26%) displayed a significantly elevated level of differentiation between the species. The distribution of outlier SNPs along autosomal chromosomes (Fig. 4, NC_ prefix) was largely restricted to ~ 16 narrow genomic regions. These narrow genomic regions with outlier loci were not uniformly

distributed across the five autosomal chromosomes with 10 occurring on chromosome ends and two near the centre of chromosomes. A relatively small number of outlier SNPs were mapped to 'unplaced contigs' (See Fig. S3).

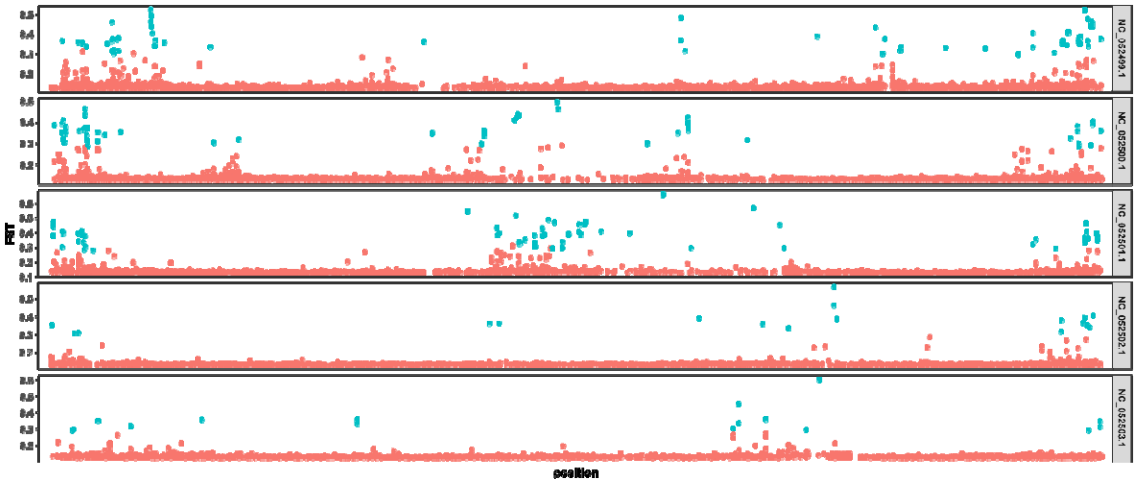

Figure 4. Outlier plot representation of SNPs for *B. neohumeralis* and *B. tryoni* identified on the five autosomal chromosomes. F_{ST} values for each SNP were computed and are plotted as the Y-axis value (left) for each chromosome, with the X-axis representing the position in base pairs along each chromosome (scaled proportionately); these chromosomes and contigs are shown as separate rows in the figure, with their NCBI sequence identifier shown to the right. Outlier SNPs are highlighted in blue.

We investigated genes that contained outlier SNPs or were in close proximity to outlier SNPs, resulting in the identification of 266 genes. Enrichment of annotation terms indicates an overabundance of genes with the 'abnormal neuroanatomy' term (n=11 genes, p= 0.0164) within these candidate genes. Several neurology-related terms that were not enriched but still of interest included 'nervous system development', 'central nervous system development', 'sensory system', and 'sensory organ development'. Additional non-enriched terms of interest included sleep and circadian rhythm-related terms, including: 'abnormal sleep', 'circadian

rhythm', 'abnormal circadian rhythm', and 'abnormal circadian behavior'. Inositol-related terms were statistically enriched, that included 'inositol biosynthetic process' (n=1 gene, p= 0.0115) and 'inositol-1,4,5-trisphosphate 3-kinase activity' (n=1 gene, p= 0.0467). The 19 genes annotated with these terms are shown in Table S4.

Ecological Data

No differences in seasonal activity between the species

Trap data collected over multiple years and sites showed no difference in seasonal activity between *B. neohumeralis* and *B. tryoni* (Fig. 5 & Fig. S3). For any given sampling event there were often significantly more *B. tryoni* than *B. neohumeralis* trapped (See Table S5), but the abundance of both species over time were highly linearly correlated across nine sites with correlation values ranging from: $r = 0.776$ (Ayr, $p < 0.001$) to $r = 0.993$ (Maryborough, $p < 0.001$) (See Table S6). South Johnstone was the only site with a non-significant result ($r = 0.926$; $p = 0.074$) however, limited collections were recorded at this site ($n = 4$), providing insufficient statistical power to detect an association.

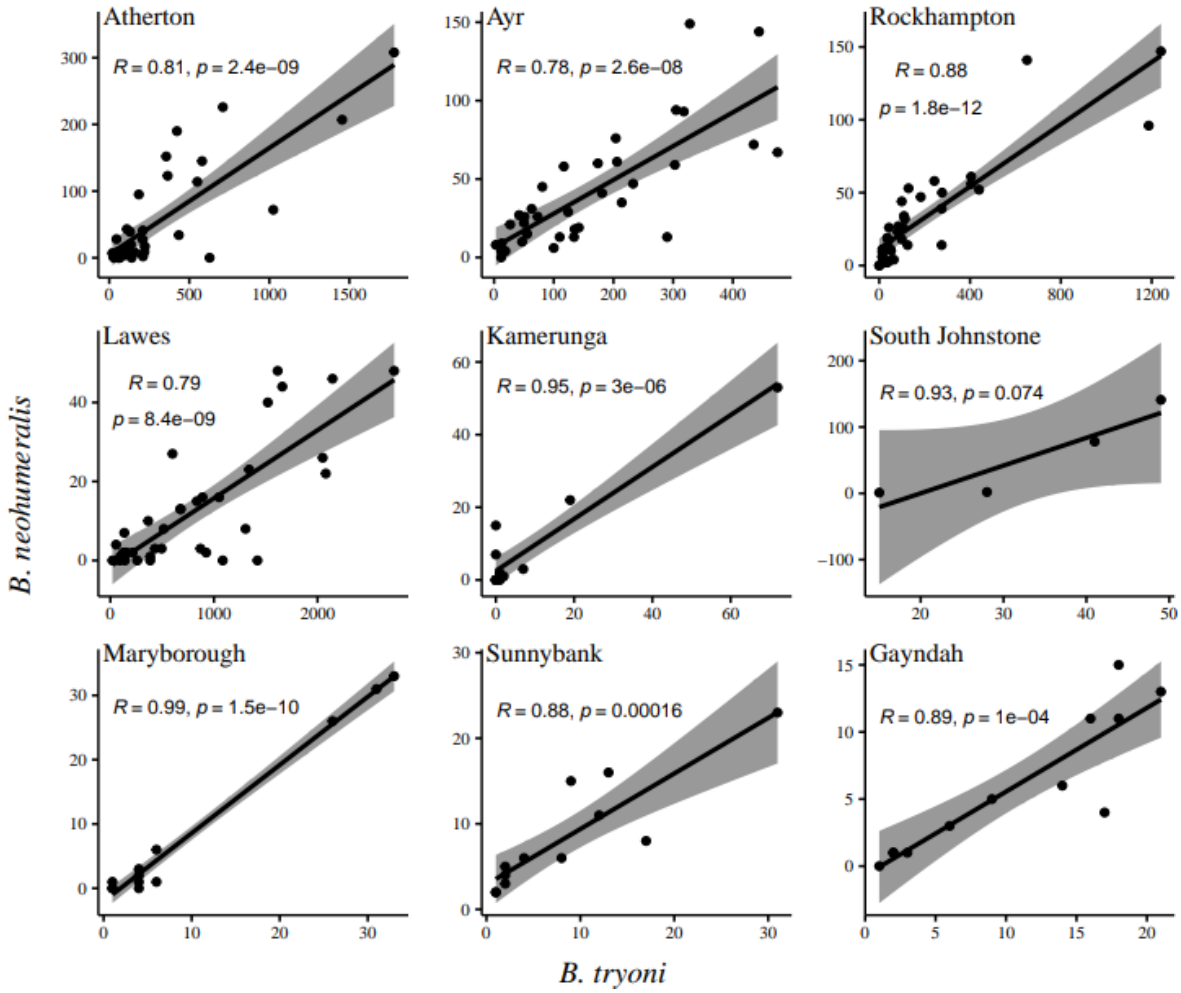

Figure 5. Correlation of seasonal abundance of *Bactrocera tryoni* and *B. neohumeralis* at multiple locations in Queensland, Australia. Scatterplots were developed using monthly mean of trap catches over 2 - 7 years. Shaded areas represent the 95% confidence intervals. Data from May (1961).

No differences in habitat use between the species

There were significant positive correlations between *B. tryoni* and *B. neohumeralis* abundances in their habitat use (Fig. 6). Within a habitat type, there was a significant correlation in the two species monthly abundances for all habitat types, with the correlation

ranging from r = 0.54 (dry forest, p <0.001) to r = 0.90 (mixed farming, p < 0.001) (See Table S8).

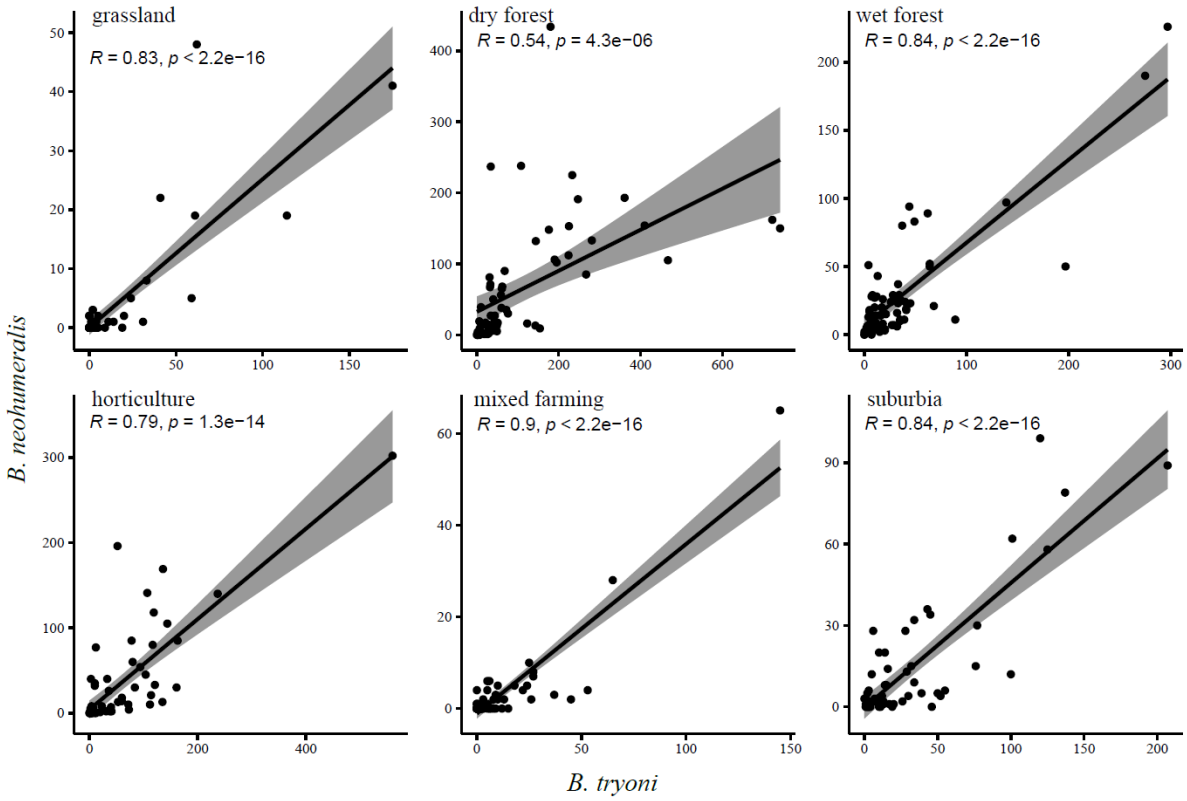

Figure 6. Correlation in habitat use of *Bactrocera tryoni* and *B. neohumeralis* in different human-defined habitat types within the Bundaberg district of Queensland. Each data point is the adult trap catch of both species for a trap run for three consecutive days. For each habitat type, nine traps were run per month for seven months. Shaded areas represent the 95% confidence intervals.

Limited differences in host use between species

A total of 1,607 fruits were sampled from the field and the emergence of *B. tryoni* and *B. neohumeralis* adults counted. For white sapote, mulberry, peach, plum, carambola and nectarine, sufficient samples were collected to carry out formal analysis. Highly significant

correlations between the number of *B. tryoni* and *B. neohumeralis* adults reared from each of these fruit types were found (correlation ranging from r = 0.904, p <0.001 for white sapote to r = 0.952, p < 0.001 for peach) (Fig. 7 and Table S9). For guava, two collections occurred: a single *B. tryoni* and no *B. neohumeralis* emerged from the September 2013 collection while 1609 *B. tryoni* and 1353 *B. neohumeralis* emerged from the October 2013 collection; for feijoa a single collection occurred with equal numbers [71 flies each] of *B. tryoni* and *B. neohumeralis* emerging. For sapodilla, *Syzigium* spp., grumichama, hog plum, jabotica, white mulberry, avocado, black sapote, longan, mango and cashew, *B. tryoni* but no *B. neohumeralis* emerged.

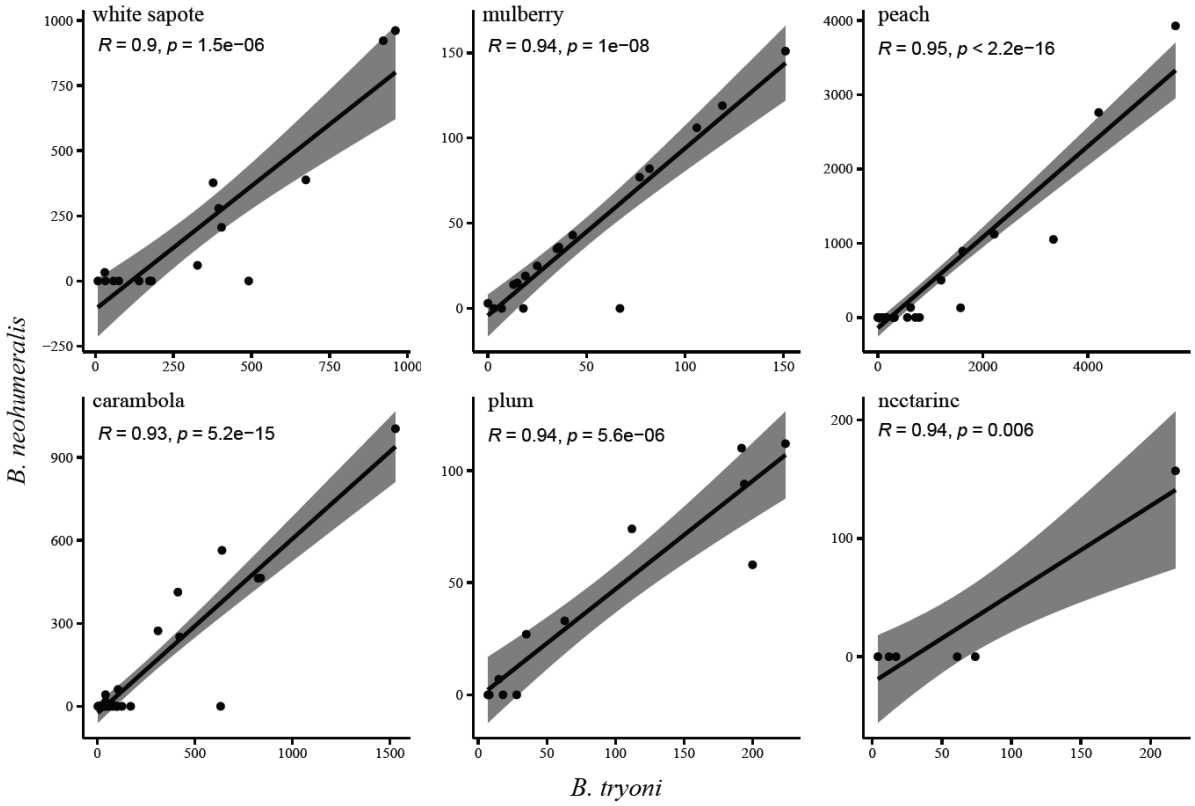

Figure 7. Correlation in host use of *Bactrocera tryoni* and *B. neohumeralis*. Each data point is the number of adult flies reared from an individual collection of multiple fruit pieces from a

research orchard at Nambour, Queensland. Shaded areas represent the 95% confidence intervals.

Discussion

Assortative mating and reduced recombination in the absence of local niche adaptation

Genetic comparisons of *B. tryoni* and *B. neohumeralis* individuals collected throughout their overlapping range showed no evidence of hybridization between the species. This was despite samples of each species being collected from the same traps, i.e., in immediate sympatry. Several intermediate phenotypes were collected; however, these samples presented strongly as *B. neohumeralis* genotypes with no indication of hybridization. This is consistent with the findings of prior studies of the species pair and supports the theory that strong reproductive barriers exist in non-laboratory conditions (Gilchrist & Ling, 2006; Pike, 2004; Yeap et al., 2020). The lack of evidence of hybridization identified from natural populations in this study, along with asymmetric hybridization observed in a prior laboratory-based study (Yeap et al., 2020), suggest that assortative mating is the most prevalent form of reproductive isolation responsible for maintaining species boundaries between the pair, however, we cannot confidently exclude the existence of hybrid selection in natural environments.

The linkage of genes involved in assortative mating with those associated with habitat preference and performance is considered necessary for sympatric speciation (Ortíz-barrientos et al., 2002; Yeaman, 2013) and has been revealed to have involvement in host switching and specialization of phytophagous insects such as apple maggot flies and pea aphids (Feder et al., 2003; Hawthorne & Via, 2001). In these studies, the genes involved appear to map tightly together along narrow regions of the chromosome that is consistent with the patterns observed in our data. These tightly mapped genes have been theorized as a mechanism for speciation in

the presence of gene flow and are generally considered to occur through chromosomal rearrangements, such as inversions, where the genes are brought into physical linkage (Ortíz-barrientos et al., 2002; Yeaman, 2013). A small region of modifier alleles can also act to reduce recombination between populations in the absence of chromosomal rearrangements (Butlin, 2005; Ortiz-Barrientos et al., 2016). Regardless of the mechanism causing genetic variation, the regions of differentiation are expected to involve locally adapted alleles that are linked strongly with modifier alleles which reduce recombination between populations depending on the strength of the linkage between the two loci (Felsenstein, 1981; Ortiz-Barrientos et al., 2016). In our data, while genes related to mating behavior and circadian rhythm were not enriched, several have been identified in outlier regions that we propose are responsible for maintaining separation between the two species and forming the time-of-day premating reproductive barrier through assortative mating.

Further, the positioning of these genes in narrow regions along the chromosome may have had the greatest influence on reducing recombination. Most outlier SNPs observed in our data were largely restricted to narrow regions located towards the telomeres or centromeres of chromosomes (Fig. 4). Reduced recombination has been observed in these regions in several taxa, including *Drosophila*, mice, rabbits, and humans (Carneiro et al., 2009; Carpenter, 1979; Lindsley et al., 1977; Nachman & Churchill, 1996; Ortíz-barrientos et al., 2002; Payseur & Nachman, 2000). The combination of genes associated with assortative mating, and their chromosomal positioning appear to have suppressed recombination, preventing gene flow between species. However, there is uncertainty as to whether these genes associated with assortative mating are linked to locally adapted genes, given an apparent lack of ecological niche differentiation between the species.

A lack of competitive displacement still allows co-existence

Competitive displacement has long been suggested as an important mechanism facilitating divergence and speciation (Rice & Salt, 1988; Thoday & Gibson, 1962; Via, 2001). The ability of two nascent lineages to coexist by minimizing competition, or by utilizing non-competitive ecological space, should be considered central to early lineage divergence and persistence (Germain et al., 2021). Where two herbivore populations share resources, then displacement can occur in time (i.e. seasonal activity), location or host usage (Clarke & Measham, 2022; Facon et al., 2021). However, our data show no evidence for this in the *B. tryoni*/*B. neohumeralis* sibling pair. Both species were active at the same time of the year and in the same habitats within a landscape. *Bactrocera tryoni* was recovered from a greater range of host fruits than *B. neohumeralis* and that may be seen as evidence for competitive displacement (i.e. competitive advantage by *B. neohumeralis* causing *B. tryoni* to use novel hosts), but we consider it more likely that this pattern simply reflects *B. tryoni*'s greater abundance in the environment and so greater likelihood of being sampled, rather than a competition avoidance mechanism. This is because when both species were reared from a fruit, their numbers were closely correlated. In cases where competition between fruit flies has been known to cause changes in field host use patterns, one species will dominate in a given host, pushing a second species to host fruits that the first only irregularly uses (Charlery de la Masselière et al., 2017; Hassani et al., 2022): we did not observe this pattern. The positive correlation in the number of adults of both species reared from fruit also offers field data supporting the work of Kay et al. (2024), who in laboratory studies found no interspecific larval competition between the species.

Ecological traits typically associated with competition driven divergence between fruit fly species (i.e. differential host use, habitat usage and seasonal abundance (Clarke & Measham, 2022; Duyck et al., 2004; Inskeep et al., 2021)) do not appear to differ between our study

taxa. Thus, in terms of lineage maintenance, difference in mating time remains the only known distinction between the species pair. It is very difficult to conclusively state whether this reproductive barrier was initially driven by selection or drift, versus some competitive avoidance mechanism(s) which occurred in the past but is not currently observable because the competitive problem has been resolved over evolutionary time (i.e. the problem of the ghost of competition past (Connell, 1980)). Irrespective of the factors underlying the origin of the two lineages, our data suggests that currently, selection against hybridisation is acting to maintain the time-of-day mating differences that allows these to lineages to persist in sympatry.

Our study provides a comprehensive comparison of both molecular and ecological data, between two sympatric sister taxa. High levels of niche conservatism and a lack of competitive displacement suggests that the formation and persistence of these taxa occurred in the absence of traditionally observed ecological selection pressures. Further, the positioning of genes associated with assortative mating in tightly linked sites within chromosomal telomeres and centromeres appears to have sufficiently reduced geneflow between the species to maintain the lineage boundaries in sympatry, suggesting that reproductive isolation had a significant influence on lineage maintenance.

Author contributions

A.R.C. and P.P. conceived the study. N.K., C.G.M., S.B. and, B.M. conducted field work and collected samples for molecular and ecological analysis. M.I., N.K., A.R.C. and, P.P. designed, collated, and analyzed ecological data. Z.S. and P.P. designed and performed bioinformatics and SNP analysis. M.I., Z.S., P.P. collated analyzed molecular data. M.I., Z.S.,

N.K., C.G.M., M.S., A.R.C., D.H. and P.P. contributed to the writing of the manuscript. All authors have read and approved the final version.

Acknowledgements

We thank J. Ryan for assisting with generating the distribution map, E. Vueti who carried out much of the Bundaberg sampling as part of an uncompleted postgraduate study, and L. Senior (QDAF) for her assistance with rearing flies from fruit that provided data for the host utilization study. We also thank The Southern Downs Regional Council, The Granite Belt Growers Association, B. Doohan, The Greenhoose, Lockhart River, D. Masters, J. Kerr and J. Whistler for their assistance in collecting samples for the molecular analysis.

This research was partially funded by the ARC ITTC project IC150100026, Centre for Fruit Fly Biosecurity Innovation awarded to A.R.C. and P.P.

The authors declare no conflicts of interest.

Information on Data Deposition

BioProject PRJNA1008389, Zachary Stewart, 2023, DArTseq of *Bactrocera neohumeralis* and *tryoni* (Queensland fruit fly / Qfly), NCBI SRA, BioProject PRJNA1008389.

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