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Strong genetic differentiation but limited niche partitioning in a sympatric species pair separated by an allochronic reproductive barrier

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Abstract

In the absence of differential adaptation to ecological niche and the development of reproductive barriers, boundaries between coexisting species are expected to breakdown. The fruit fly species pair, *Bactrocera tryoni* and *B. neohumeralis*, have significant overlap in geographic range and host use, with mating time the only confirmed difference in their mating systems. Using a combination of ecological and genomic data, we tested the roles of competition and reproductive isolation on the maintenance of species boundaries in this pair. Genome-wide SNP analyses found strong genetic differentiation between the species with no evidence for hybridization. Most outlier SNPs were restricted to narrow regions towards the centromeres and telomeres of chromosomes, while high nucleotide diversity rates were observed in both species. Enrichment of annotation terms indicated an overabundance of genes with the 'abnormal neuroanatomy' term, but non-enrichment of terms associated with sleep and circadian rhythm. Field data found minimal evidence for ecological differentiation based on significant positive temporal, spatial and hostuse correlations between the species. However, *B. tryoni* appears less sensitive to low humidity than *B. neohumeralis* and used hosts which were not used by *B. neohumeralis* suggesting subtle microecological variation between species contributing to reproductive isolation. The simultaneous comparisons of molecular and ecological data in our study have provided a more nuanced understanding of how species boundaries have been maintained in this sibling species pair. Our analyses highlight the importance of genetic divergence for their maintenance in sympatry, but less for a role of competitive displacement or other ecological adaptation.

KEYWORDS
assortative mating, *Bactrocera*, coexistence, fruit fly, genomic divergence, niche partitioning, speciation, Tephritidae

INTRODUCTION

Understanding the ecological and genetic basis of speciation and species maintenance is a fundamental element of evolutionary biology. In geographically isolated populations an absence of gene flow between incipient species is expected to result in genetic divergence and species maintenance via both selection and drift (Feder et al., 2013). However, when two populations experience gene flow through primary or secondary contact, reproductive isolation is considered necessary for species maintenance (Coyne & Orr, 2004).

Central to our understanding of speciation in the presence of gene flow are the opposing roles of natural selection and recombination on the formation or dissolution of species boundaries (Felsenstein, 1981). Natural selection promotes the development of reproductive isolation between populations 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 species 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 species divergence when coupled with the development of traits associated with local adaptation (Ortiz-Barrientos et al., 2016; Schluter & Rieseberg, 2022).

Additional to the development of reproductive isolation to overcome the effects of recombination, the persistence of two species in sympatry also depends on the development of niche partitioning to avoid inter-species competition asymmetries (Coyne & Orr, 2004; Felsenstein, 1981; Weber & Strauss, 2016). Without meeting these two criteria, theory suggests that species will likely dissolve through hybridization or cease to exist in sympatry through competitive displacement (Germain et al., 2021; Weber & Strauss, 2016). The coexistence of ecologically equivalent species may occur long term where sexual selection exists, provided the local carrying capacity is sufficient to maintain both populations (M'Gonigle et al., 2012).

The Australian frugivorous tephritids (Diptera: Tephritidae), *Bactrocera tryoni* (Froggatt) and *Bactrocera neohumeralis* (Hardy), are a fruit fly sibling pair studied for their speciation histories and genetic stability for over 60 years (Birch, 1961; Gilchrist et al., 2005; Gilchrist et al., 2014; Gilchrist & Ling, 2006; 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, with shared polymorphisms in coding and non-coding regions based on mitochondrial and nuclear genes (Morrow et al., 2000), microsatellites (Wang et al., 2003) and genomic data (Gilchrist et al., 2014), 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) (Figure 1d,e). While intermediate colour variants are documented, genetic analysis using microsatellites suggests 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* entirely encompassed by the range of *B. tryoni* except for a few islands in the Torres Strait, between the Queensland (QLD) mainland and Papua New Guinea (Figure 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 (Figure 1c) (Hancock et al., 2000; Leblanc et al., 2012; QDPC, 2021). The only two prior ecological studies which have directly compared the pair in the field found their seasonal abundances to be highly correlated (Drew et al., 1984) and no evidence for competitive displacement in their host use (Gibbs, 1967).

So closely related are *B. tryoni* and *B. neohumeralis* that only one known trait is considered to define 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 7 h, while *B. tryoni* initiates mating during dusk at low light intensity in a mating window of approximately 1 h (Ekanayake et al., 2017; Pike et al., 2003). This difference in mating time is the only confirmed mechanism maintaining reproductive isolation between the species (Birch & Vogt, 1970; Lewontin & Birch, 1966; Meats et al., 2003; Raphael et al., 2019; Smith, 1979), with nearly all other components of their mating system not known to be different (Ekanayake et al., 2017; Mankin et al., 2008). An exception occurs in the pheromones of the two species. Contrary to an earlier study (Bellas & Fletcher, 1979), qualitative and quantitative chemical differences in the male pheromones of *B. tryoni* and *B. neohumeralis* have now been reported (Castro-Vargas et al., 2024), with the species' peak times of pheromone release matching their respective peak mating times (Castro-Vargas et al., 2025). It is thus probable that pheromones may also play a role in the premating isolation between the species, although the functionality of the detected differences has not yet been tested. If the pheromone differences are found to have a functional effect, they will still be confounded with the allochronic difference (Castro-Vargas et al., 2025). Post-zygotic barriers are not known to occur between the species, with both F1 (Pike & Meats, 2002; Smith, 1979) and multigenerational hybrids (Pike et al., 2003; Yeap et al., 2020) readily generated in the laboratory. Pike et al. (2003) tested the fate of hybrids between the two species and found that from the F1 hybrid generation, it took three generations of backcrosses to the parental *B. neohumeralis* population before the day mating pattern was fully regained. Rather, nearly all F1 hybrids and subsequent backcrosses (with either parental species) resulted in the dusk mating phenotype, leading the authors to consider that the largely unidirectional selection for dusk mating led to the continued maintenance of the two species in the face of possible field hybridization. However, female *B. neohumeralis* will, like *B. tryoni*, also

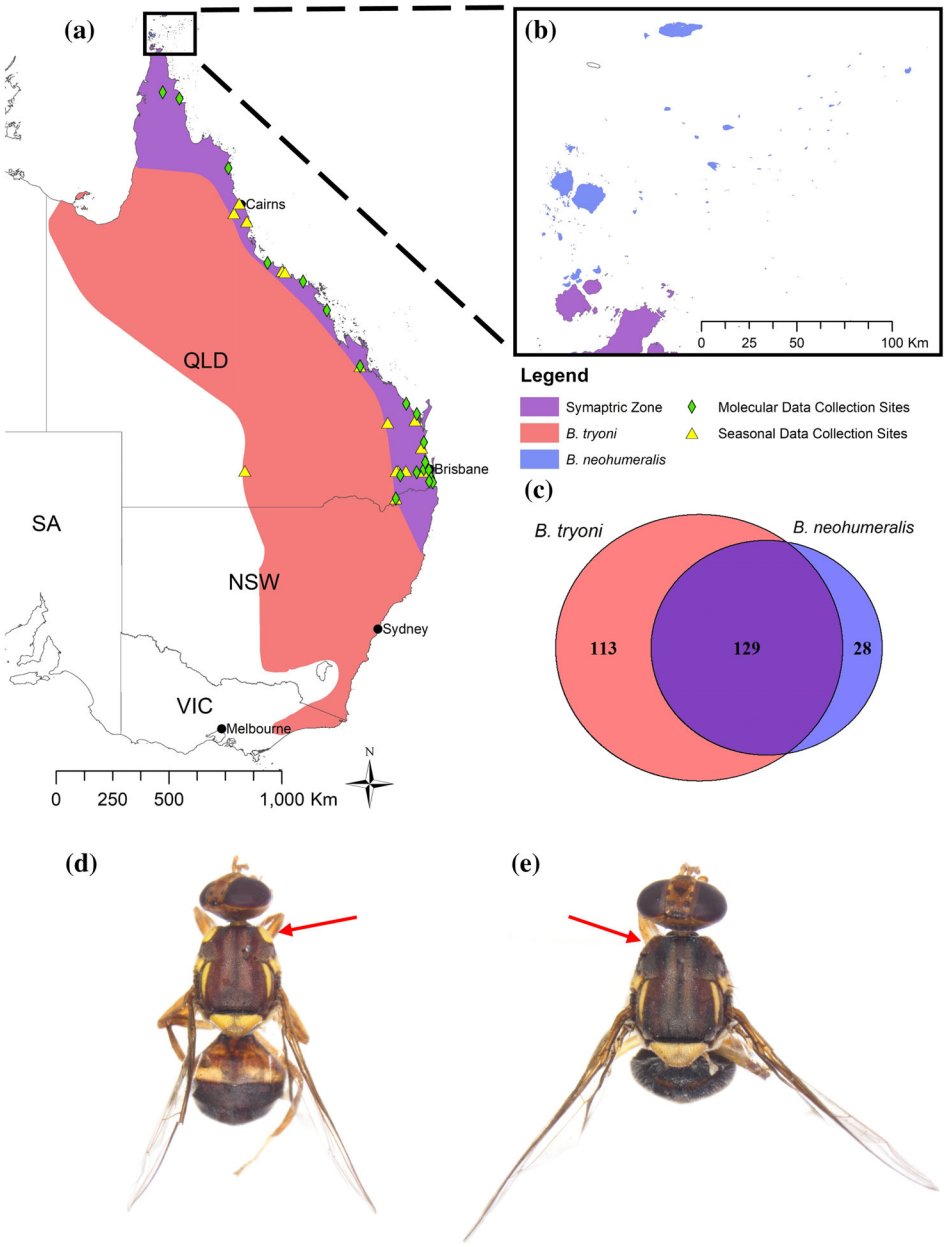

FIGURE 1 (a) Distribution of *Bactrocera tryoni* and *B. neohumeralis* along the east coast of Australia. (b) Distribution of both species in the Cape York Peninsula and Torres Strait Islands. (c) Host usage overlap between species. Numbers in circles represent the known number of host species utilized. Range and host data gathered from Hancock et al. (2000); Leblanc et al. (2012); QDPC (2021). Differences in humeral calli coloration between (d) *B. tryoni* and E) *B. neohumeralis*. Recent range expansion during the past 20 years of *B. tryoni* into central and western Victoria is not shown because it is not associated with the species' evolutionary history.

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 species maintenance; for example, genomic incompatibility (Dobzhansky, 1936; Turelli et al., 2001). Additionally, for herbivorous insects such as fruit flies, species 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). Thus, ecological factors also need to be considered when investigating the maintenance of species boundaries in this sibling pair. Conversely, the two species 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 the *B. tryoni* and *B. neohumeralis* species lineages have diverged and been maintained in apparent sympatry. We did this through dense

geographic sampling of nuclear genomes, and structured field sampling of temporal population abundance and habitat and host usage. We assessed the extent of ongoing gene flow and genetic differentiation between the species and identified putative candidate genes associated with differential mating times. Using the field data, we compared seasonal abundances, host usage and habitat utilization of the two species to determine if there may be ecological factors that contribute to cumulative reproductive isolation (Germain et al., 2021; Sobel, 2014) between the two species and, particularly, to document if there is evidence of competitive displacement between these sympatric species. Shifts in any or all of seasonal abundance, host usage and habitat utilization 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 Primary Industries (QDPI) as part of ongoing Biosecurity Queensland (BQ) surveillance. A subsample of the trapping sites was selected from the existing network to represent the geographic range of both species (Figure 1a). 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 Supplementary Table S1. Identity of the samples was confirmed following the descriptions in fruit fly identification handbooks (Drew, 1989; Plant Health Australia, 2018), and 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 and samples were screened for quality and quantity, using both gel electrophoresis and Qubit assay. The *B. tryoni* and *B. neohumeralis* samples were 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 90 bp) were sequenced on an Illumina Hiseq2500 as single end reads.

Read alignment and SNP calling

Reads were demultiplexed using process_radtags in Stacks v2.60 (Rochette et al., 2019). Raw reads for all species had adapters and low-quality ends trimmed with bbduk (Bushnell, n.d.) with settings ktrim = r k = 23 mink = 11 hdist = 1 qtrim = rl trimq = 5 minlength = 25 tpe tbo. FASTQC was used to ensure trimming effectiveness (Andrews, 2010). Trimmed reads were mapped with BWA-MEM (Li, 2013) to the *B. tryoni* genome assembly (Genbank accession = GCF_016617805.1) 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. Variant calling made use of freebayes v1.3.6 (Garrison & Marth, 2012) 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 normalized using BCFtools (Danecek et al., 2021) and had block substitutions decomposed using vt decompose_blocksub (Tan et al., 2015). Resulting VCF files were merged using BCFtools, and this VCF file was then 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 out SNPs with a minor allele frequency <5%.

As detailed later, we made use of pixy v1.2.11.beta1 (Korunes & Samuk, 2021) to compute several population genetics statistics. Analysis with pixy requires both variant and invariant sites to be called. To merge invariant site calls into the results already obtained by freebayes, we first called variant and invariant sites using the BCFtools mpileup and call functions, with subsequent variant normalization achieved with the norm function. Block substitutions were then decomposed using vt (Tan et al., 2015). A custom script (merge_invariant_sites.py from https://github.com/zkstewart/Various_scripts) identified invariant site predictions which did not overlap with variant calls previously made by freebayes and merged these sites into the existing freebayes VCF. These results were only used for the analysis by pixy; performing this process allowed us to make use of a consistent set of variant calls across our analyses despite the otherwise lack of compatibility between our original variant calling with freebayes and the requirements of pixy.

Population structure and genetic differentiation

Comparative analyses between the existing *B. tryoni* (Genbank accession = GCF_016617805.1) and *B. neohumeralis* (Genbank accession = GCF_024586455.1) genome assemblies are not included in this study. A synteny analysis comparing the genomes detected some significant mapping differences between the pair which we considered could obscure biological differences. The synteny analysis is available upon request from the senior author.

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 programme 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 the pophelper v2.3.1 package (Francis, 2017) in R.

Additionally, we ran a principal component analysis (PCA) to provide further visualization of the variation 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 (i.e., snpgdsLDpruning) was used to filter SNPs with linkage disequilibrium values above 0.2 to mitigate any bias associated with linked SNP clusters upon the downstream PCA. We similarly performed this SNP pruning prior to calculation of isolation by state (IBS) proportions for samples using SNPRelate.

To complement the PCA and STRUCTURE results, relatedness analyses were performed using the EMIBD9 (Wang, 2022) and rrBLUP (Endelman, 2011) packages. Each analysis measures relatedness using different methods, and both were included to ensure the results are reliable. Variants were filtered in R (R Core Team, 2023) using the dartRverse package (Gruber et al., 2018; Mijangos et al., 2022) to remove those with a callrate lower than 95%, those deviating from Hardy–Weinberg Equilibrium to a significance threshold of 0.05, and those with linkage disequilibrium values above 0.2. The remaining variants were used as input for the EMIBD9 and rrBLUP packages to estimate the relatedness of samples in a pairwise manner.

Phylogenetic tree reconstruction and introgression analysis

Rates of introgression were calculated using Patterson's D-statistic (Green et al., 2010) and f_4- ratio (Patterson et al., 2012; Reich et al., 2010) to quantify levels of historical gene flow between species, as implemented in Dsuite v0.5 (Malinsky et al., 2021). This analysis required us to generate a new set of variant calls with inclusion of additional taxa to serve as an outgroup and to form species trios.

Bactrocera oleae (Rossi), a more distantly related species of *Bactrocera*, was chosen as the outgroup for the analysis. We obtained raw sequence data for *B. oleae* from the NCBI SRA (accession SRR8788622). Samples of *Bactrocera curvipennis* (Froggatt), a closely related yet geographically isolated species to both *B. tryoni* and *B. neohumeralis*, were included to estimate introgression between species trios. Nine samples of *B. curvipennis*, collected from New Caledonia in the South Pacific, were provided by QDPI. DNA was extracted using the methodology detailed above and library preparation was conducted using the Integrated DNA Technologies (IDT) xGen™ DNA enzymatic fragmentation library preparation kit. Whole genome sequencing was conducted with Illumina NextSeq using a P2 300 flow cell.

In addition to these two new species inclusions, we selected a single individual collection site for *B. tryoni* and *B. neohumeralis* from our existing dataset to estimate levels of introgression between locations; for each site and species, we chose the sample which had the fewest missing sites in the previous set of variant calls. Variant calling took place using the BCFtools mpileup and call functions, with subsequent normalization and block substitution occurring as described previously. Filtering was as previously described except that we chose to retain only variants which had successful genotype calling in ≥80% of samples. More stringent filtering was applied here to reduce the influence of missing data on the results of Dsuite. The filtered variant calls were used as input to IQ-TREE 2 (Minh et al., 2020) with polymorphism-aware phylogenetic models (PoMo); ultrafast bootstrapping was performed with 1000 replicates, and *B. oleae* was specified as the outgroup species. Tree visualizations were produced with all samples present, as well as after pruning several samples which were identified as putative outliers (as detailed later in our Results) whose clade was otherwise supported with 100% bootstrapping support, indicating that their absence in the results visualization would have no untoward influence on the rest of the tree's groupings.

We then calculated the D-statistic and f_4-ratios for trio combinations using the Dtrios command in Dsuite v0.5 (Malinsky et al., 2021) and ran the *-ABBAclustering* command by Koppetsch et al. (2024) to disentangle introgressed alleles from homoplasies. The *-ABBAclustering* script runs two clustering analyses; (i) a 'sensitive' test which has greater statistical power but is susceptible to false positives when mutation rates vary along the genome, and (ii) a 'robust' test which can overcome issues with mutation rate variation but has lower statistical power. Where the clustering analysis for a trio combination is non-significant, excess allele sharing in the ABBA arrangement is likely due to an accumulation of homoplasies as opposed to a signature of introgression (Koppetsch et al., 2024). The p-values for the D-statistic and both clustering analyses were corrected using the Bonferroni correction and Benjamini & Hochberg (BH) (Benjamini & Hochberg, 1995) correction methods.

Population genetics statistics and outlier SNPs

Using the original set of variant calls made by freebayes, mean F_{ST} (θ) values (Weir & Cockerham, 1984) were calculated both within and between the two *Bactrocera* species using VCFtools (Danecek et al., 2011). Within species comparisons were conducted for each species separately, and samples from each collection site were considered separate populations. For between species comparison, the samples were grouped by species and entered as two separate populations.

Additional population statistics were calculated to estimate levels of relative genetic differentiation between species (F_{ST}), absolute genetic divergence between species (d_{XY}) and nucleotide diversity within species (π). These statistics were calculated within non-overlapping windows of 50 Kbp by pixy using the variant and invariant site calls. Statistics were visualized along each chromosome to identify patterns of differentiation within the chromosomal landscape.

The recombination rate along each chromosome was approximated using a recurrent neural network as implemented by ReLERNN (Adrion et al., 2020). To facilitate this, the original set of variant calls made by freebayes was filtered to retain only biallelic SNPs, after which each species' data was processed using ReLERNN_SIMULATE to generate training and validation data (with default settings), ReLERNN_TRAIN to train the neural network (with -nEpochs 100 and

-nValSteps 20), ReLERNN_PREDICT to predict per-base recombination rate and ReLERNN_BSCORRECT to perform parametric bootstrapping and estimate 95% confidence intervals.

The freebayes 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. Using a custom script, RefSeq gene annotations for the *B. tryoni* assembly were assessed to identify protein-coding genes that contained outlier SNPs (i.e., within their exons and/or introns), and to identify the genes that were nearest to an intergenic outlier SNP within a maximum distance of 50 kbp.

Genes which contained or were proximal to outlier SNPs were queried against the *Drosophila melanogaster* gene models (Larkin et al., 2021) (FlyBase release FB2023_01) using MMseqs2. The gene name and FlyBase automated gene summaries of the best hit were associated to the query sequence if an E-value ≤$1e^{-10}$ was achieved. From these results, we specifically searched for genes that had been previously associated with differences in time-of-day mating and behavioural activities between the species. These genes were *Cryptochrome* (*cry*) (An et al., 2004), *Kismet* (*kis*), *Neuroligin 4* (*NLg4*), *Adenylate cyclase 3* (*AC3*) and *Vascular glutamate transporter* (*VGLUT*) (Raphael et al., 2019). Aside from this, we also sought to obtain information regarding other genes that had not been previously associated with differences in mating times. To enable this, the FlyBase summaries for all genes were manually parsed to identify functional and phenotypic annotations. Gene Ontologies (GOs) were also associated with each gene by first querying their protein sequence against the UniRef90 database (release 2023_02) (Suzek et al., 2007); where a UniRef match with GO annotations was found with and E-value of ≤ $1e^{-5}$, we associated these GO annotations to the *B. tryoni* gene. A functional enrichment analysis of the FlyBase and GO annotations was conducted with GOseq (Young et al., 2010) in R to identify over/under represented functional annotations.

Ecological differentiation

Seasonal abundance

A shift in seasonal population peaks to minimize overlap in host usage (i.e. temporal niche displacement) has been reported as the outcome of competition between native and invasive fruit flies (Vayssières et al., 2015). With this background, we interpret significant positive population correlations between our target species as a lack of evidence for temporal niche displacement, and no or negative population correlations as evidence for niche displacement.

A historical dataset (May, 1961) was used for the comparison of the temporal abundances of *B. neohumeralis* and *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 conducted in R (R Core Team, 2023). 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 southern Queensland to tropical Cairns ~1500 km to the north (see Figure 1a and Supplementary Table S5). Trapping was carried out for up to 6 years per location using a fruit-based liquid lure trap (May & Caldwell, 1944). Ten traps were maintained at each location, with traps cleared weekly. The data in May (1961) vary between sites in how they are presented, and modifications were needed in order to directly compare *B. tryoni* and *B. neohumeralis* populations. The *t*-tests and correlation analyses between *B. tryoni* and *B. neohumeralis* were conducted on a site-by-site basis (i.e., data were never collated across sites), so the different manipulations performed on the site-specific datasets did not affect results. Six sites were excluded as seasonal data were not available (see Supplementary Table S5 for details).

Given the variability in how data from different locations are presented in the historical dataset, a trapping dataset collected as part of a recent landscape genetics study (Ryan et al., 2025) was also used to assess seasonal abundance on a fine scale. The *t*-tests and correlation analyses between *B. tryoni* and *B. neohumeralis* populations were conducted for each of six bi-monthly collections. Detailed sampling techniques are presented in Ryan et al. (2025) with trap abundance data being used for both the abundance correlation analysis and the landscape habitat use analyses. In brief, samples were collected from 25 locations in the Wide Bay-Burnett Region of Queensland (see Supplementary Figure S1) in April 2021, August 2021, October 2021, December 2021, February 2022 and April 2022.

Landscape habitat use

Differences in habitat use may occur between fruit fly species due to local adaptation (Finnie et al., 2021) or through competition-driven spatial displacement (Duyck et al., 2004). We used the data collected for the fine scale seasonal abundance analysis (above) to also compare the abundance of *B. tryoni* and *B. neohumeralis* across different human-defined habitat types to determine habitat use patterns. Four habitat types, agriculture area, *Eucalyptus* woodland, rainforest and residential area, were chosen for the study within the Wide Bay-Burnett region of Queensland (~350 km north of Brisbane). Details on data collection are located in Supplementary Methods 2 and Ryan et al. (2025). 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.

In addition to the *t*-test and correlation analyses, linear mixed modelling (LMM) was used to investigate patterns in *B. neohumeralis* abundance across the study area (Figure 2) to complement the study of Ryan et al. (2025) which investigated environmental predictors of *B. tryoni* abundance. The LMM analysis used the same statistical methodology and landscape predictor variables as used in Ryan et al. (2025). The R package lme4 version 1.1–30 (Bates et al., 2015) was

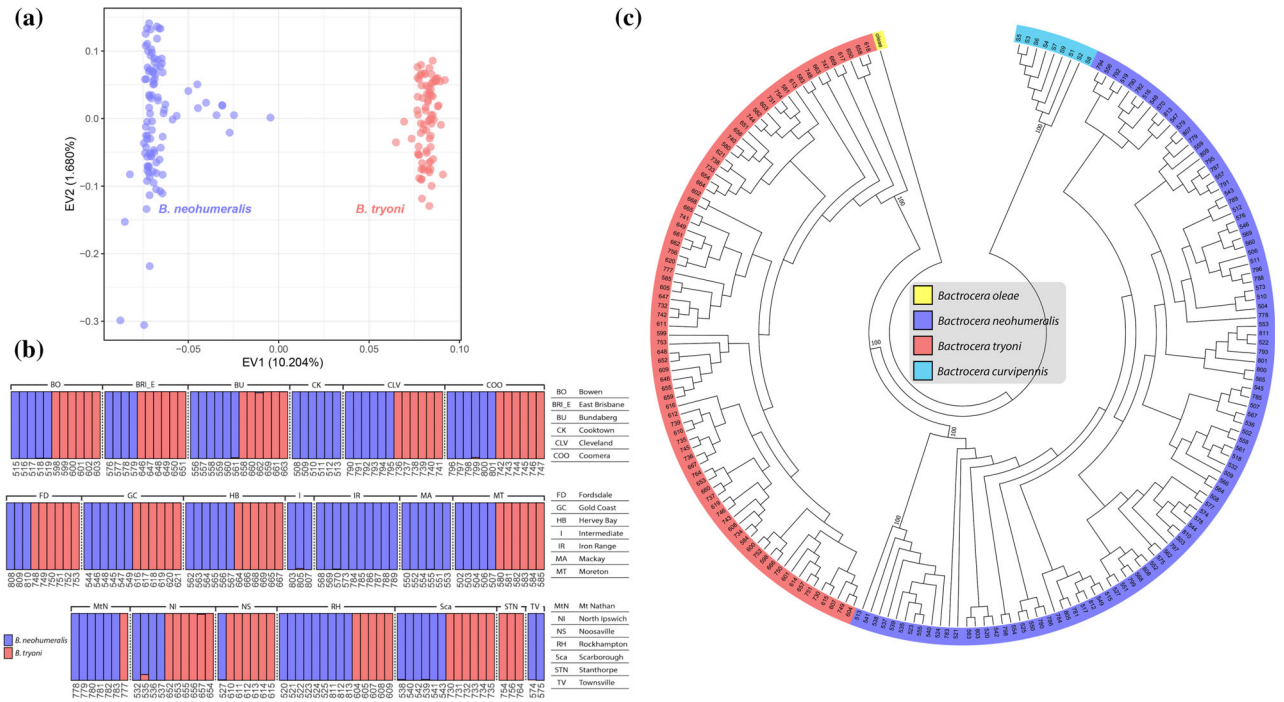

FIGURE 2 (a) Principal component analysis (PCA) of single nucleotide polymorphisms (SNPs) for *Bactrocera neohumeralis* and *B. tryoni*, with the values from the first two eigenvectors plotted for each individual. Eigenvectors one (horizontal) and two (vertical) explain 10.204% and 1.680% of the variance in the data, respectively. (b) 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. (c) Maximum Likelihood tree for *B. tryoni*, *B. neohumeralis*, *B. curvipennis* and *B. oleae* as the outgroup estimated using polymorphism-aware phylogenetic models (PoMo) with ultrafast bootstrapping performed with 1000 replicates. Bootstrap values are presented at the major clade nodes.

used to create our LMMs and the 'dredge()' function implemented in the R package MuMIn version 1.47.1 (Barton, 2022) to assess all combinations of predictor variables, using sampling time as a random variable. Following dredging, LMMs with an ΔAICc <2 were used to perform model averaging and determine for each landscape predictor: linear coefficient, standard error, statistical significance and upper and lower 95% confidence intervals. The predictor variables included percentage coverage of five landscape categories (grazing area, residential area, *Eucalyptus* woodland, rainforest and agricultural production) within a 1 km radius of each sampling site, as well as latitude, longitude and distance from each sampling site to the nearest perennial water source. Additionally, the Empirical Bayesian Kriging (Geostastical Analyst Tools) tool was implemented in ArcGIS Pro v3.4 to interpolate *B. neohumeralis* abundance across the study area to visualize spatially explicit patterns of abundance.

Host fruit use

Changes in fruit fly host usage patterns due to interspecific competition are well documented in the fruit fly literature (Charlery de la Masselière et al., 2017; Hassani et al., 2022). Here we tested rates of host utilization on 21 species or varieties of host fruit for both *B. tryoni* and *B. neohumeralis*. Fruits were collected from the QDPI 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 November 2015. Fruits 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*), *Syzygium* spp., grumichama (*Eugenia brasiliensis*), hog plum (*Spondias mombin*), jaboticaba (*Plinia cauliflora*), avocado (*Persea americana*), mango (*Mangifera indica*), mandarin (*Citrus reticulata*), wampi (*Clausena lansium*), star apple (*Chrysophyllum cainito*), prickly pear (*Opuntia vulgaris*), miracle fruit (*Synsepalum dulcificum*), feijoa (*Acca sellowiana*) and cashew (*Anacardium occidentale*).

Fruits were placed in paper bags and transported to the QDPI Brisbane laboratories. Fruits 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 were 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 abundances of the two fly species from a host were compared using a paired *t*-test and Pearson correlation analysis. A chi-squared test for independence and likelihood ratio chi-squared test (G-test) were also conducted using the *Desctools* package in R (Signorell, 2025) to test for differential host utilization between fly species. To avoid violating the parameters of each test, host fruit in which less than five flies from either species had been collected were grouped.

RESULTS

Molecular data

Read processing, alignment and SNP calling

After demultiplexing samples, between 2,596,430 and 5,228,647 high-quality sequence reads were obtained 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 Supplementary Tables S2 and S3 for full details of read counts and alignment percentages. 28,334 SNPs were identified using freebayes from the two species that met all predetermined threshold criteria for inclusion.

Populations cluster by species and not geography

We found that populations clustered based on species and not geography in all analyses. PCA (Figure 2a) and fastSTRUCTURE (Figure 2b) 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 Supplementary Figure S2), 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 Supplementary Figure S3). 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 excluded them from further analysis.

Relatedness of individuals

After linkage disequilibrium filtering, 3710 variants remained for use in the relatedness analyses. The EMIBD9 estimates of relatedness (i.e., Identity by Descent) for the sampled individuals are presented in Supplementary Figure S4A. Notably, each species is largely segregated into distinct groups showing increased relatedness values within species groups compared to between species groups where values approached zero. Three samples (genotype codes 662, 787 and 788) appear as an outgroup relative to the other samples and show elevated levels of relatedness both within the same species and between species. While the levels of relatedness are elevated in these samples, they still present higher levels of relatedness within species groups compared to between species groups.

The rrBLUP estimates of relatedness are presented in Supplementary Figure S4B. These results corroborate the trends observed in the results of EMIBD9, although genotype codes 662, 787 and 788 do not appear to be outliers in this analysis. Instead, all species only show relatedness to other members of their species group, with negative values obtained when compared to the opposite species, suggesting non-random mating between the species groups. We expect the minor differences in results to be due to differences in methodology between the two packages.

Phylogenetic tree reconstruction and introgression analysis

Phylogenetic analysis recovered each species as a strongly supported monophyletic group with maximal bootstrap values (100%) (Figure 2c). The Dtrios analyses estimated D-statistic and f4-ratio values for 7770 trio combinations, of which 1467 produced significant D-statistic values after applying the BH correction method, while only 512 trios produced significant D-statistic values after applying the Bonferroni correction method. However, the *-ABBAclustering* analysis failed to detect any significant clustering in all trio combinations after applying either the BH or Bonferroni correction methods. These results suggest that shared alleles are more likely a consequence of incomplete lineage sorting (ILS) coupled with an accumulation of homoplasies, as opposed to true signatures of historical introgression.

Population genetics statistics, recombination rates and structure along chromosomes

Patterns of differentiation among populations (F_{ST}) were 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). Using outlier detection in BayeScan, 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 (See Figure 3 and Supplementary Figures S5–S8) was largely restricted to ~16 narrow genomic regions. Elevated windowed F_{ST} and d_{XY} values

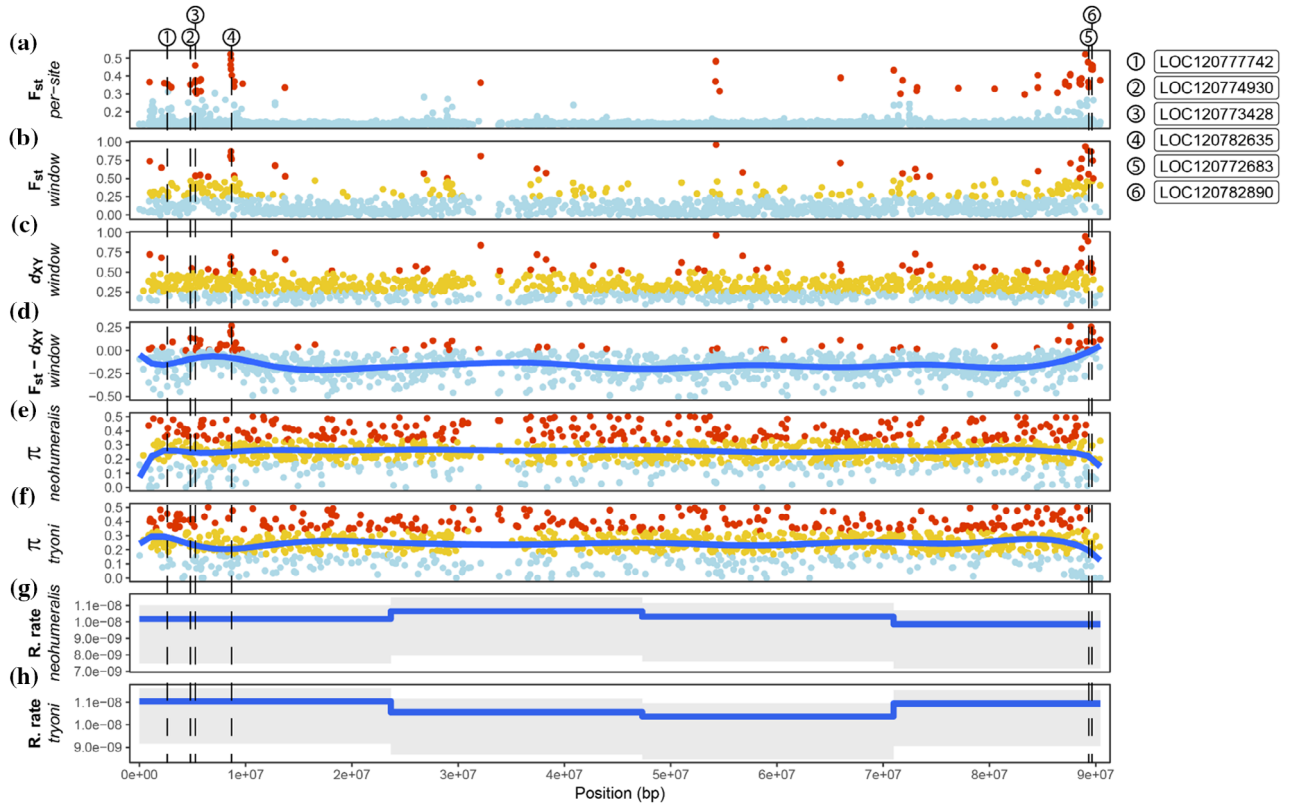

FIGURE 3 Population genetics statistics calculated for chromosome 1 labelled as NC_052499.1. Where indicated on the Y-axis that a statistic is windowed, values have been obtained through averaging over window lengths of 50 Kbp. Each statistic is presented along the length of the chromosome where the position labelled as 1e+07 equates to 10 Mbp. Candidate genes potentially involved in the differential time-day-mating between the two species are indicated as gapped vertical lines denoted by a number which relates to the locus identifiers shown in the upper right corner of the figure (See Supplementary Table S4 for candidate gene details). For subfigures b and c, colours are depicted according to values being in the range of: 0 to 0.25 is light blue; >0.25 to 0.5 is yellow; >0.5 to 1 is red. For subfigures e and f, colours are depicted according to values being in the range of: 0 to 0.2 is light blue; >0.2 to 0.35 is yellow; >0.35 to 1 is red. (a) F_{ST} calculated for each variant is indicated, with outlier variants coloured in red and non-outliers as light blue. (b) F_{ST} calculated in windows inclusive of invariant sites. (c) d_{XY} is calculated in windows inclusive of invariant sites. (d) The values shown in subfigures b and c are subtracted to depict the difference between F_{ST} and d_{XY} values in each windowed region with the trendline in blue. (e) Nucleotide diversity (π) is calculated in windows inclusive of invariant sites for *Bactrocera neohumeralis* with the trendline in blue. (f) As above but showing *B. tryoni's* nucleotide diversity. (g) The recombination rate as predicted by ReLERNN for *B. neohumeralis* is indicated with grey shading showing the 95% confidence interval. (h) As above but showing *B. tryoni's* recombination rate.

calculated using pixy were also clustered in similar regions to those observed for the outlier SNPs. 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 Supplementary Figure S9). 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 similar), suggesting that both species are approximately equally homogenous.

Mean nucleotide diversity estimates (π) produced similar results for each species (*B. tryoni* = 0.243962, *B. neohumeralis* = 0.242033). Nucleotide diversity estimates for each species were homogeneously distributed along the chromosomes with no substantial clustering of elevated or lower rates observed (See Figure 3 and Supplementary Figures S5–S8).

Overall, recombination rates within each species were low, with *B. tryoni* having a mean value of 9.02×10^{-9} per base and *B. neohumeralis* having a mean value of 9.78×10^{-9} per base. Recombination rates were overall consistently distributed along each chromosome, with minor decreases observed towards the telomeres and the chromosomal centres, with eight regions of decreased recombination rates occurring for each species (See Figure 3 and Supplementary Figures S5–S8). In each instance, these regions included clustered regions of elevated F_{ST} and d_{XY} values; however, not all clustered regions of elevated F_{ST} and d_{XY} occurred in regions with lower recombination rates.

Outlier loci are clustered in specific chromosome segments between the species

Genes that contained outlier SNPs or were proximal to outlier SNPs were investigated, resulting in the identification of 266 genes. Of

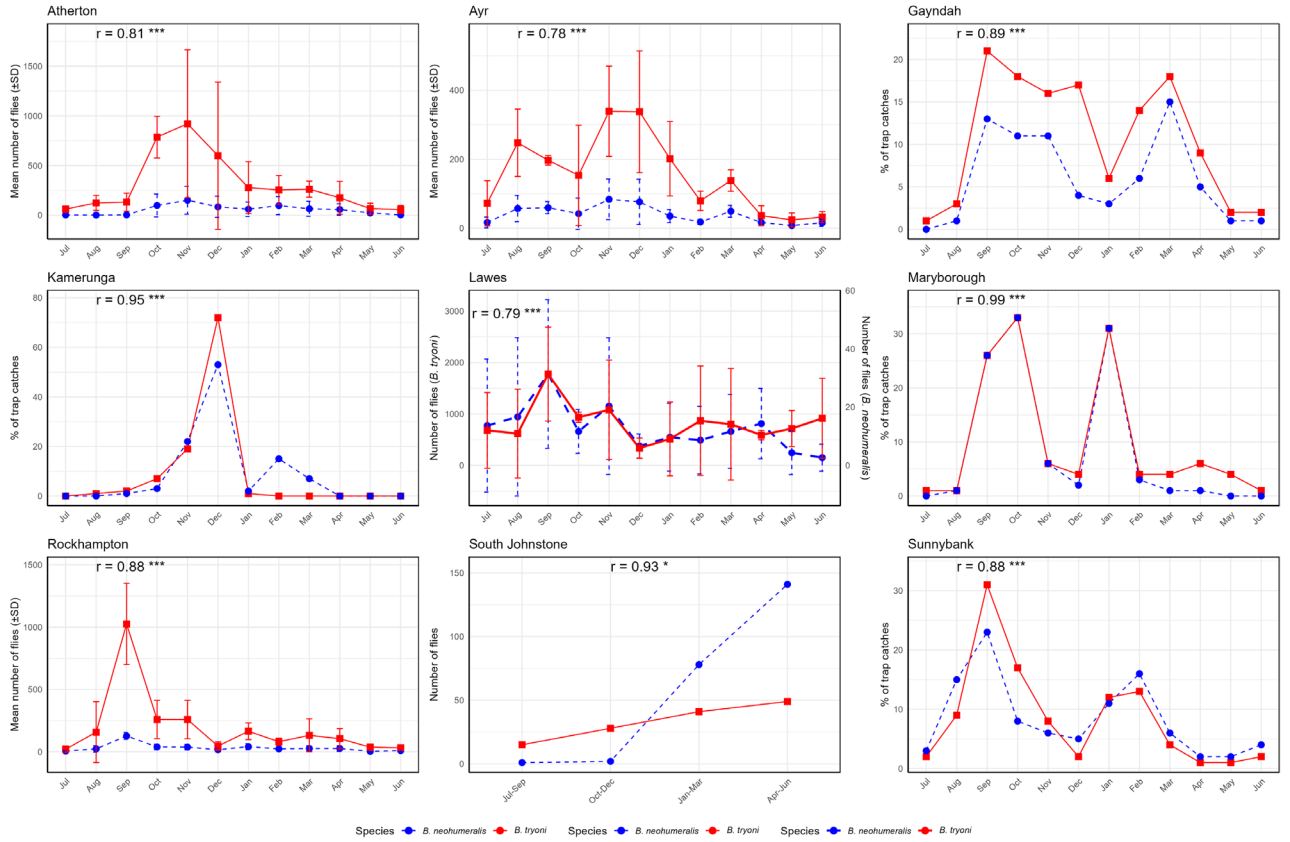

FIGURE 4 Seasonal abundance of *Bactrocera tryoni* and *B. neohumeralis* at eight sites across Queensland, Australia. Data from May (1961). Significance of correlations: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

the five genes previously associated with differential time-of-day mating, only NLg4 occurred in a region containing outlier SNPs (*B. tryoni* homologue = LOC120773428) (Figure 3). 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 other neurology-related terms that were not statistically enriched but were found among the 266 genes of interest included 'nervous system development', 'central nervous system development', 'sensory system' and 'sensory organ development'. Additional terms which were not statistically enriched but identified among candidate genes included sleep and circadian rhythm-related terms such as: 'abnormal sleep', 'circadian rhythm', 'abnormal circadian rhythm' and 'abnormal circadian behaviour'. Inositol-related terms were statistically enriched, which included 'inositol biosynthetic process' ($n = 1$ gene, $p = 0.0115$) and 'inositol-1,4,5-trisphosphate 3-kinase activity' ($n = 1$ gene, $p = 0.0467$); although these results were statistically significant, the low number of observations ($n = 1$) indicates low statistical power. Based on assessment of these results, we selected 19 genes as potentially important candidates for the understanding of differences between *B. tryoni* and *B. neohumeralis*, with these results being shown in Supplementary Table S4.

Ecological analyses

Seasonal abundance

Historical trapping: Broad scale, historical trap data collected over multiple years and sites throughout the sympatric range showed highly correlated patterns in seasonal activity between *B. neohumeralis* and *B. tryoni* (correlation values ranging from $r = 0.61$) (Gayndah, $p = < 0.05$) to $r = 0.95$ (Maryborough, $p < 0.001$) (Supplementary Table S6, Figure 4). For any given sampling event, there were significantly more *B. tryoni* than *B. neohumeralis* trapped in Atherton, Ayr, Lawes and Rockhampton, while the number of flies of each species trapped was not significantly different in the other locations (Supplementary Tables S5 and S6).

Modern trapping: Data collected in the Wide Bay-Burnett region over a 12-month period found the abundance of both species across sites significantly correlated for five of the six collection periods; the exception being the December sampling ($r = 0.12$, $p = 0.57$) (Supplementary Figure S10, and Tables S7 and S8). More *B. tryoni* than *B. neohumeralis* were collected for all sampling events, except for the April 2022 sampling, when there was no significant difference in the abundances of the two species (Supplementary Table S7).

Abundance across habitat types

There were significant correlations observed between *B. tryoni* and *B. neohumeralis* abundances in all habitat types (Supplementary Figure S11). There were often more *B. tryoni* than *B. neohumeralis* collected in *Eucalyptus* and residential habitats, but no significant differences in numbers collected in agriculture and rainforest habitats (Supplementary Table S9).

The LMM analysis for *B. neohumeralis* involved a total of 512 combinations of landscape predictors. Model averaging was performed using LMMs with ΔAICc <2 from the top model (Supplementary Table S10). The top performing models retained eight predictors (Supplementary Table S10) from the nine included in the analysis, with only elevation not included in one of the top performing models. The top performing model was a combination of rainforest area, *Eucalyptus* woodland, latitude and longitude, with each predictor positively correlated with *B. neohumeralis* abundance. The 13 other top performing LMMs also all included rainforest area as a predictor. No other predictor was included in all the top performing LMMs. Following model averaging only rainforest area was a statistically significant predictor of *B. neohumeralis* abundance (Supplementary Table S11) and was positively correlated. This pattern can be observed in Supplementary Figure S12 (Ai, ii, v, vi, vii), where the high levels of abundance observed correlate with rainforests. In contrast, Ryan et al. (2025) found grazing area was the only significant predictor of *B. tryoni* abundance and was negatively correlated (Supplementary Figure S12B.viii, and Supplementary Tables S12 and S13).

Differences in host use between species

A total of 15,308 fruits with a combined weight of 845 kg was 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 comparative analyses. 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 $r = 0.88$, $p < 0.05$ for plum to $r = 0.99$, $p < 0.001$ for white sapote) (Supplementary Figure S13 and Table S14). When accounting for all fruit collected (i.e. inclusive of samplings yielding low or zero fly numbers of one or either species), host utilization patterns were significantly different between species ($\chi^2 = 852.84$, df = 7, $p < 0.001$, G-statistic = 509.68, df = 7, $p < 0.001$) with *B. tryoni* utilizing more host species and varieties than *B. neohumeralis* (Table 1).

TABLE 1 Host fruit collection data.

		B. Tryoni			*B. Neohumeralis*		
Host fruit	No. of fruit collection months	Mean monthly abundance	SD	Total abundance	Mean monthly abundance	SD	Total abundance
Avocado	4	2	4	8	0	-	0
Carambola	21	77.86	192.47	1635	4.14	13.25	87
Cashew	1	23	-	23	0	-	0
Feijoa	1	14	-	14	31	NA	31
Grumichama	3	7	12.12	21	0	-	0
Guava	2	66.5	94.05	133	4	5.66	8
Hog plum	3	70.33	117.52	211	0	-	0
Jaboticaba	7	0.86	1.57	6	0	-	0
Mandarin	5	0	–	0	0	-	0
Mango	7	20.43	39.89	143	0	-	0
Miracle fruit	1	0	–	0	0	-	0
Mulberry	8	47.12	94.74	377	14.12	26.86	113
Nectarine	6	36.5	59.09	219	0.33	0.82	2
Peach	13	837.92	2034.66	10,893	40.15	96.49	522
Plum	7	44.29	59.43	310	10.57	15.36	74
Prickly pear	1	0	-	0	0	-	0
Sapodilla	12	7	14.14	84	0	-	0
Star apple	1	0	-	0	0	-	0
Syzygium sp.	7	66.86	69.36	468	0	-	0
Wampi	3	9.67	14.15	29	1.33	2.31	4
White Sapote	10	94.2	177.18	942	8.1	19	81

DISCUSSION

Bactrocera tryoni and *B. neohumeralis* are a genetically closely related sibling pair for which the mechanism(s) of initial divergence and species maintenance are either unknown or unclear. Our combined genetic and ecological study confirms earlier studies (Birch & Vogt, 1970; Vogt, 1977; Wolda, 1967) that hybridization does not occur in the field and so ongoing species maintenance can be attributed to reproductive barriers. However, contrary to expectations from niche theory (Coyne & Orr, 2004; Felsenstein, 1981; Weber & Strauss, 2016), this genetic maintenance was not found to be accompanied by obvious spatial or temporal ecological divergence, which would be expected in two sympatric species with overlapping host usage (Germain et al., 2021). The following discussion focuses on the potential drivers for both initial divergence and maintenance in these two species.

Genetic results

Species maintenance

Genetic comparisons of *B. tryoni* and *B. neohumeralis* individuals collected throughout the overlapping range showed no evidence of hybridization or introgression between the species. This was despite samples of each species regularly 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 natural conditions (Gilchrist & Ling, 2006; Pike, 2004; Yeap et al., 2020). The lack of evidence of hybridization identified from natural populations in this study is consistent with the results of a recent diagnostic tool development study where no hybrids were identified from *B. tryoni* ($n = 430$) and *B. neohumeralis* ($n = 99$) samples collected across >40 collection sites throughout their overlapping range (Starkie et al., 2023). These results, along with asymmetric hybridization observed in a prior laboratory-based study (Yeap et al., 2020), suggest that differential time-of-day-mating is an effective form of reproductive isolation for maintaining species boundaries between the pair. Diel mating differences have been attributed to reproductive barriers between populations of rice stem borer (Quan et al., 2017) and fall army worm (Fiteni et al., 2022; Tessnow et al., 2022), however, host differentiation also appears to contribute substantially to overall reproductive isolation with specialist populations forming on different host species in these system. The cumulative contribution of other reproductive isolating mechanisms in our species is discussed further below.

Of the genes previously identified as being associated with differences in time-of-day mating between our species (An et al., 2004; Raphael et al., 2019), only Nlg4 appeared within the tightly clustered regions of high genetic differentiation. In *Drosophila*, Nlg4 plays a critical role in sleep regulation (Li et al., 2013). In *B. tryoni*, Nlg4 transcripts appear in greater abundance at night compared to day and may be associated with circadian patterns (Raphael et al., 2019). A further 18 candidate genes were also located within these regions and may contribute to maintaining separation between the two species and forming the time-of-day premating reproductive barrier through assortative mating.

We acknowledge that our analyses are not capable of establishing a causal link between these candidate genes and reproductive isolation, nor can we discount the role other previously identified candidate genes may still have in maintaining species boundaries despite not appearing in outlier SNP regions. However, we offer 19 candidate genes that warrant further investigation to determine whether they are indeed associated with reproductive isolation or are linked to locally adapted genes, given the limited ecological niche differentiation between the species as discussed below.

Genetically similar but not genetically identical

Similar levels of nucleotide diversity (π) were estimated for *B. tryoni* (0.243962) and *B. neohumeralis* (0.242033). These values are consistent with prior studies comparing the nucleotide diversity of native (0.21552–0.22575) and invasive populations (0.19929–0.2199) of *B. tryoni* throughout Australia and the South Pacific (Parvizi et al., 2024), but substantially higher than those observed in other widespread *Bactrocera* species (e.g. *B. dorsalis*—0.00148-0.0183) (Deschepper et al., 2023). High effective population sizes and high genetic diversity have previously been observed in *B. tryoni* (Popa-Báez et al., 2020; Ryan et al., 2025) which may account for the elevated levels of nucleotide diversity we observed in both species. Low chromosome-wide recombination rates may also explain high nucleotide diversity as genetic mutations, typically purged through recombination (Kondrashov, 1994), are able to accumulate. Furthermore, low recombination rates may allow for the preservation of associations between linked alleles (Ortiz-Barrientos et al., 2016; Otto, 2009). Yeap et al. (2020) observed a persistence in linkage between two *B. tryoni*—*B. neohumeralis* hybrid phenotypes involving humeral calli colour and time-of-day mating over 25 generations of hybrid backcrossing (without deliberate selection) despite a predicted linkage decay of 98.3% over 24 generations. The authors postulated that a reduction in recombination rates as a result of chromosomal rearrangements likely contributed to the persistence of linkage, while also suggesting that an accumulation of nucleotide differences may have been a factor. Low recombination rates have been observed in a number of Diptera (Cladera, 1981; Foster et al., 1980; Rössler, 1982) which can be attributed to an absence (or scarcity) of recombination in males (Zhao et al., 2003).

The Dsuite analysis failed to detect signatures of introgression among all trio combinations generated, instead suggesting that shared alleles persisted through ILS. While such strong results suggest an absence of ongoing gene flow between the species, we acknowledge that low recombination rates, resulting in high levels of nucleotide diversity in both species, may mask weaker signals of introgression through the accumulation of homoplasies in both the ABBA and

BABA arrangements. Further investigation into the extent to which low recombination rates have influenced nucleotide diversity, particularly in allowing the accumulation of minor deleterious alleles within these species, is needed.

Initial lineage divergence

Tightly clustered regions of elevated genetic differentiation appear to occur in regions with further reduced recombination along chromosomes 2–5 (See Figure 3 and Supplementary Figures S5–S8). These regions are consistent with models of hybridizing species where the linkage of genes involved in assortative mating with those associated with habitat preference and performance is considered necessary for speciation with gene flow (Felsenstein, 1981; Ortíz-Barrientos et al., 2002; Yeaman, 2013). The formation of the tightly clustered regions of genetic differentiation observed in our data may have been associated with the initial divergence of the two species if speciation occurred in sympatry. However, based on the high levels of nucleotide diversity observed, it is perhaps more likely that these tightly clustered regions were important in maintaining species boundaries during the early periods of secondary contact after the species diverged in allopatry. If reproductive barriers were porous during secondary contact, the strong linkage of locally adapted alleles and genes associated with assortative mating would have likely strengthened the reproductive barriers between the two species, limiting the homogenizing effect of hybridization (Felsenstein, 1981; Ortíz-Barrientos et al., 2002).

In addition to those clustered regions with reduced rates of recombination, regions of elevated genetic diversity without a reduction in recombination rates are present. Elevated genetic diversity without reduced rates of recombination is consistent with the patterns expected under the model of divergence after speciation where populations experience a period of geographic isolation allowing divergence to occur in the absence of gene flow (Cruickshank & Hahn, 2014). Under this model, differential selective pressures drive divergence between the species through selective sweeps and the absence of gene flow allows them to persist without a reduction in recombination. The level of absolute genetic divergence (d_{XY}) in relation to relative genetic differentiation (F_{ST}) could provide insight into the timing of these selective sweeps relative to the completion of speciation between taxa. Where both statistics are elevated, the selective sweep likely occurred soon after speciation. In contrast, regions with elevated F_{ST} and non-elevated d_{XY} could suggest a more recent selective sweep (Cruickshank & Hahn, 2014; Ravinet et al., 2017).

Consistently high levels of nucleotide diversity along each of the chromosomes coupled with predominantly low F_{ST} and d_{XY} values could be an indicator as to how initial divergence between these species occurred. Under a scenario of speciation with gene flow or allopatric speciation involving a small founder population, a genetic bottleneck is expected to reduce the nucleotide diversity of the population expanding into the novel environment (Coyne & Orr, 2004). The low recombination rates observed in *B. tryoni* and *B. neohumeralis* may have allowed for the accumulation of high nucleotide diversity within each species post speciation; however, one would expect to see elevated F_{ST} and d_{XY} values across the chromosome as a result, which is not evident in our data. An alternative explanation is that a substantial vicariance event separated the ancestral species into two large populations, allowing speciation to occur in the absence of gene flow. During the period of geographic isolation, differential selection pressure would have led to the differences in the time-of-day mating between the species, limiting hybridization upon secondary contact (Coyne & Orr, 2004; Mayr, 1957). However, what that differential selection pressure may have resulted from is not obvious, nor is it obvious how such a dramatic difference in mating time would have spread through a large founder population. Thus, while the most parsimonious explanation for the genetic data is initial vicariance of two large founder populations, what then followed to lead to lineage divergence remains unclear.

Ecological results

Evidence for competitive ecological divergence

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 species to coexist by minimizing competition, or by utilizing non-competitive ecological space, should be considered central to early species 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, the data presented here show little 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* resulting in *B. tryoni* adapting to novel hosts). However, we consider this unlikely. This is because when both species were reared from the same 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); this pattern was not observed in our data. The positive correlation in the number of adults of both species 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. Rather, our data suggests that the host range of *B. neohumeralis* is simply a subset of the host range of *B. tryoni*. In this host use mirrors geographic range, where both the geographic and host ranges of *B. neohumeralis* are subsets of the respective ranges in *B. tryoni*.

While minimal ecological differences were found between the two species, similar to the results of Drew et al. (1984) and

Gibbs (1967), the species are not ecologically identical. The results of our landscape modelling for *B. neohumeralis*, compared with those of Ryan et al. (2025) for *B. tryoni*, indicate minor differences in habitat preference. We found that increasing abundance of *B. neohumeralis* was best predicted by percentage of local rainforest in the immediate landscape, while decreasing abundance of *B. tryoni* was affected by increasing amounts of cleared grazing area. Demonstrated at a regional level, the pattern of *B. neohumeralis* being more influenced by the availability of humid (i.e. rainforest) habitat than *B. tryoni* is also found at larger geographic scales. Sultana et al. (2020), using Maximum Entropy (Maxent) modelling, found *B. neohumeralis* distribution across its range was best predicted by precipitation in the wettest month, while *B. tryoni* distribution was best predicted by mean temperatures. How much this subtle variance in habitat preference reflects differences in the initial founding populations, versus subsequent local adaptation, remains unknown. However, the divergence in the genomes of the two species strongly suggests at least some post-vicariance local adaptation has occurred.

Accumulative reproductive isolation in nature

Our data confirm that, in nature, reproductive isolation between *B. tryoni* and *B. neohumeralis* is sufficient to prevent gene flow between the species. The diel time-of-day mating differences are the greatest known inhibitor to gene flow between the pair in sympatry; however, additional factors may have a cumulative effect (Sobel & Chen, 2014) on the overall reproductive isolation in nature. While mating time may be a 'leaky' isolating mechanism in cage studies (Pike & Meats, 2002; Yeap et al., 2020), minor differences in landscape usage may aid in limiting gene flow when in sympatry. Notably, we (and others) have failed to identify the full mating system of the two species. *Bactrocera tryoni* aggregates at dusk to mate (Tychsen, 1978) and likely landmarks in tall trees (Ekanayake et al., 2017). However, both Pike and Meats (2002) and Ekanayake et al. (2017) have suggested that, due to its daytime mating, *B. neohumeralis* may have a quite different mating system. If so, and if this leads to physical as well as temporal isolation between the species, then this will cumulatively add to the reproductive isolation between the species in sympatry. Finally, while our study was unable to detect evidence of hybridization between the species, we are unable to definitively exclude selection against hybrid offspring in natural settings as a post-zygotic barrier. Although laboratory hybridization studies suggest no breakdown in hybrid fitness (Yeap et al., 2020), strong selection against hybrid offspring may still occur in natural settings, contributing to cumulative reproductive isolation. Further investigations into the drivers of the differential mating times, the strength of local ecogeographic isolation and the potential for hybrid inviability in natural conditions will all help characterize the full suite of reproductive barriers between this species pair and provide greater understanding as to how these closely related species have maintained species boundaries with minimal ecological differentiation.

AUTHOR CONTRIBUTIONS

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

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw sequence reads are deposited in the SRA (https://www.ncbi.nlm.nih.gov/) (Bioproject PRJNA1008389, Biosample accession numbers SAMN37118180 to SAMN37118365). Individual genotype data are available on DataDryad https://doi.org/10.5061/dryad.79cnp5j66 (Irvine et al., 2026). NCBI Biosample accession numbers are provided in Supplementary Tables S2 and S3.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Collection sites throughout the Wide Bay-Burnett region of South East Queensland for the fine scale seasonal abundance analysis.

Figure S2. Principal component analysis (PCA) of single nucleotide polymorphisms (SNPs) *Bactrocera neohumeralis* and *B. tryoni*, including outlier samples, with the values from the first two eigenvectors plotted for each sample.

Figure S3. fastSTRUCTURE results at cluster size (K) equals three for single nucleotide polymorphisms (SNPs) for *Bactrocera neohumeralis* and *B. tryoni*.

Figure S4. (a) Heatmap generated using the results of EMIBD9 assessing the relatedness of sampled individuals via their Identity by Descent (IBD) coefficients. (b) Plot generated using the results of rrBLUP assessing the estimated relatedness of sampled individuals via an additive relationship matrix.

Figure S5. Population genetics statistics calculated for chromosome 2 labelled as NC_052500.1.

Figure S6. Population genetics statistics calculated for chromosome 3 labelled as NC_052501.1.

Figure S7. Population genetics statistics calculated for chromosome 4 labelled as NC_052502.1.

Figure S8. Population genetics statistics calculated for chromosome 5 labelled as NC_052503.1.

Figure S9. Unplaced contigs positioned and scaled along the X-axis in proportion to the five autosomal chromosomes displayed in Figure 4.

Figure S10. Correlation of fine scale seasonal abundance of *Bactrocera tryoni* and *B. neohumeralis* at multiple locations in the Wide Bay-Burnett region of Southeast Queensland.

Figure S11. *Bactrocera tryoni* and *B. neohumeralis* abundance in different habitat types across the Wide Bay-Burnett region of South East Queensland, Australia.

Figure S12. (a) Empirical Bayesian kriging of *Bactrocera neohumeralis* abundance during each sampling period. (b) Empirical Bayesian kriging of *B. tryoni* abundance during each sampling period.

Figure S13. Host fruit usage on fruits where both *B. tryoni* and *B. neohumeralis* emerged including correlation coefficient statistics.

Table S1. Trapping records from 19 collection sites for molecular data analysis.

Table S2. Read counts obtained for *Bactrocera neohumeralis* samples after demultiplexing with process_radtags.

Table S3. Read counts obtained for *Bactrocera tryoni* samples after demultiplexing with process_radtags.

Table S4. A selection of candidate genes containing or in near proximity to outlier SNPs.

Table S5. Trap catches of *B. tryoni*: *B. neohumeralis* for seasonal activity in various locations in Queensland, Australia.

Table S6. Results for the broad scale seasonal abundance data *t*-test and correlation analyses between *B. tryoni* and *B. neohumeralis*.

Table S7. Results for the fine scale seasonal abundance data *t*-test and correlation analyses between *B. tryoni* and *B. neohumeralis*.

Table S8. Trap catches of *B. tryoni*: *B. neohumeralis* for fine scale seasonal activity and habitat use in various locations in the Wide-Bay/Burnett region of Queensland, Australia.

Table S9. Results for the habitat use data *t*-test and correlation analyses between *B. tryoni* and *B. neohumeralis*.

Table S10. Model support for top performing linear mixed models (LMMs) correlating *Bactrocera neohumeralis* abundance with landscape predictors after model averaging.

Table S11. Statistical support for each landscape predictor following model averaging.

Table S12. Top five performing linear mixed models (LMMs) correlating *B. tryoni* abundance with landscape predictors.

Table S13. Results of model averaging for the top five performing linear mixed models (LMMs).

Table S14. Results for the host use data *t*-test and correlation analyses between *B. tryoni* and *B. neohumeralis*.

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