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ORIGINAL ARTICLE

A novel diagnostic gene region for distinguishing between two pest fruit flies: *Bactrocera tryoni* (Froggatt) and *Bactrocera neohumeralis* (Hardy) (Diptera: Tephritidae)

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Abstract *Bactrocera tryoni* and *Bactrocera neohumeralis* are morphologically similar sibling pest fruit fly species that possess different biological attributes, geographic distributions, and host ranges. The need to differentiate between the two species is critical for accurate pest status assessment, management, biosecurity, and maintenance of reference colonies. While morphologically similar, adults may be separated based on subtle characters; however, some characters exhibit intraspecific variability, creating overlap between the two species. Additionally, there is currently no single molecular marker or rapid diagnostic assay that can reliably distinguish between *B. neohumeralis* and *B. tryoni*; therefore, ambiguous samples remain undiagnosed. Here we report the first molecular marker that can consistently distinguish between *B. tryoni* and *B. neohumeralis*. Our diagnostic region consists of two adjacent single nucleotide polymorphisms (SNPs) within the *pangolin* (*pan*) gene region. We confirmed the genotypes of each species are consistent across their distributional range, then developed a tetra-primer amplification refractory mutation system (ARMS) PCR assay for rapid diagnosis of the species. The assay utilizes four primers in multiplex, with two outer universal primers, and two internal primers: one designed to target two adjacent SNPs (AA) present in *B. tryoni* and the other targeting adjacent SNPs present in *B. neohumeralis* (GG). The assay accurately discriminates between the two species, but their SNP genotypes are shared with other nontarget tephritid fruit fly species. Therefore, this assay is most suited to adult diagnostics where species confirmation is necessary in determining ambiguous surveillance trap catches; maintaining pure colony lines; and in Sterile Insect Technique management responses.

Key words ARMS-PCR; colony; molecular diagnostics; pangolin; tetra-primer

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Introduction

Morphologically cryptic species exist across many branches of the tree of life (Barr & McPheron, 2006; Suatoni *et al.*, 2006; Cai *et al.*, 2020). The advent of molecular techniques has aided in resolving and defining the species status of many members of cryptic complexes

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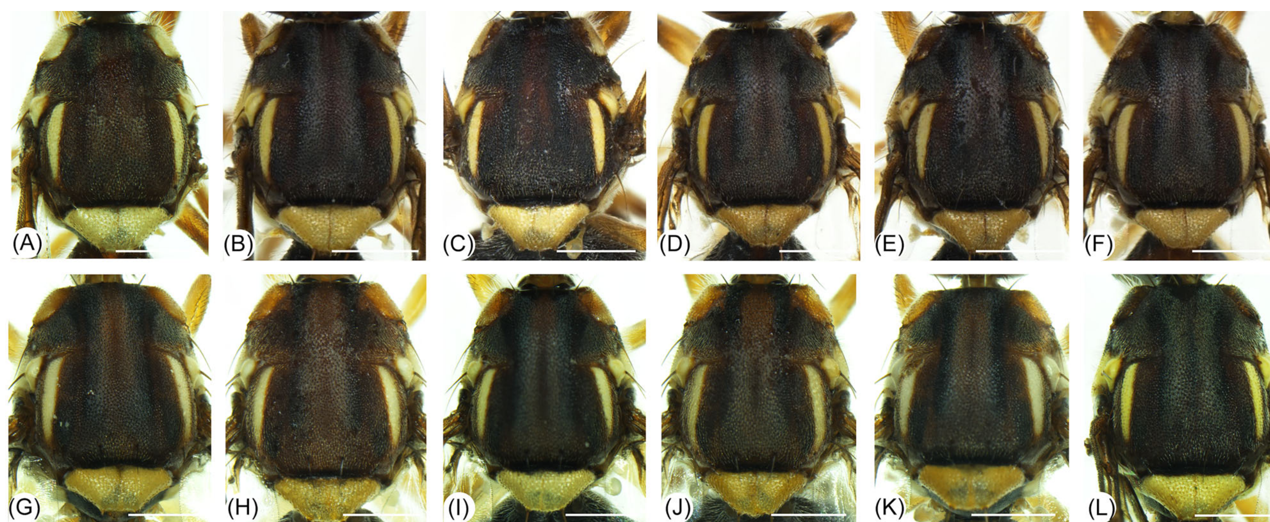

Fig. 1 Variation in postpronotal lobes observed across *Bactrocera tryoni* and *Bactrocera neohumeralis*. A: Typical *Bactrocera tryoni* coloration; B−F: wild caught morphological *Bactrocera tryoni* and *Bactrocera neohumeralis* intermediates; G−K: *Bactrocera neohumeralis* colony flies with intermediate postpronotal lobe coloration; and L: typical *Bactrocera neohumeralis* coloration. Scale bar = 500 μm.

(Bucciarelli *et al.*, 2002; Hebert *et al.*, 2004) as well as their diagnosis, but others remain unresolved. One group with many unresolved and cryptic species is the Dacini fruit flies (Diptera: Tephritidae: Dacinae), a tribe containing over 900 recognized species (Doorenweerd *et al.*, 2018).

Within the Dacini, one particularly difficult group to define is the *Bactrocera tryoni* (Froggatt) species complex (Clarke *et al.*, 2011). Taxonomically, the *B. tryoni* complex was considered to consist of Northern Territory fruit fly *B. aquilonis* (May), the Queensland fruit fly *B. tryoni*, the Lesser Queensland fruit fly *B. neohumeralis* (Hardy), and *B. melas* (Perkins & May) (Drew, 1989). Subsequent multilocus phylogenetic evidence resolves *B. mutabilis* (May), *B. ustulata* Drew, *B. curvipennis* (Froggatt), and *B. erubescentis* (Drew & Hancock) within the complex (Starkie *et al.*, 2022). These additional four species can easily be identified using morphological and molecular methods (Plant Health Australia, 2018b; Starkie *et al.*, 2022), while the original four species are sufficiently similar in morphological appearance that identification can be problematic including between *B. tryoni* and *B. neohumeralis*.

Bactrocera tryoni and *B. neohumeralis* are two of Australia's most economically important fruit fly pests (Hancock *et al.*, 2000; Plant Health Australia, 2018a). Both are of international trade concern (Biosecurity New Zealand, 2023), although generally no distinction is made between them for management purposes (Haynes & Dominiak, 2018), despite recognized biological differences (Clarke *et al.*, 2011). Distinguishing *B. tryoni* from *B. neohumeralis* as adults can be difficult due to a high degree of shared morphological characters. Coloration of the postpronotal lobes is used as the primary character to diagnose them, which are described as yellow in *B. tryoni* and dark-brown in *B. neohumeralis* (Drew, 1989). Variability in the postpronotal lobes however, has been observed in laboratory (Leach pers. comm) and wild populations (Wolda, 1967; Gibbs, 1968; Gilchrist & Ling, 2006). Such variation includes but is not limited to orange "in-between" coloration; yellow with dark-brown spots; and 50% yellow/brown coloration (see Fig. 1), referred to as "intermediates" from here on. Many have investigated the cause of intermediate phenotypes (Wolda, 1967; Birch & Vogt, 1970; Pike, 2004), with two main hypotheses: (i) they are the result of hybridization (Lewontin & Birch, 1966), or (ii) are the product of naturally occurring, overlapping, intraspecific variation (Gilchrist & Ling, 2006). Confounding this further, discoloration from exposure to harsh environmental conditions in field traps may result in morphological damage and decay, which can affect the color of the postpronotal lobes, further impacting species diagnostics (de Lillo *et al.*, 2010). Morphology alone (especially color) is therefore insufficient for confident diagnoses of this species pair.

Modern species diagnostics is usually reliant on molecular approaches when morphological characters are unavailable (Piper *et al.*, 2019), and at present, there are

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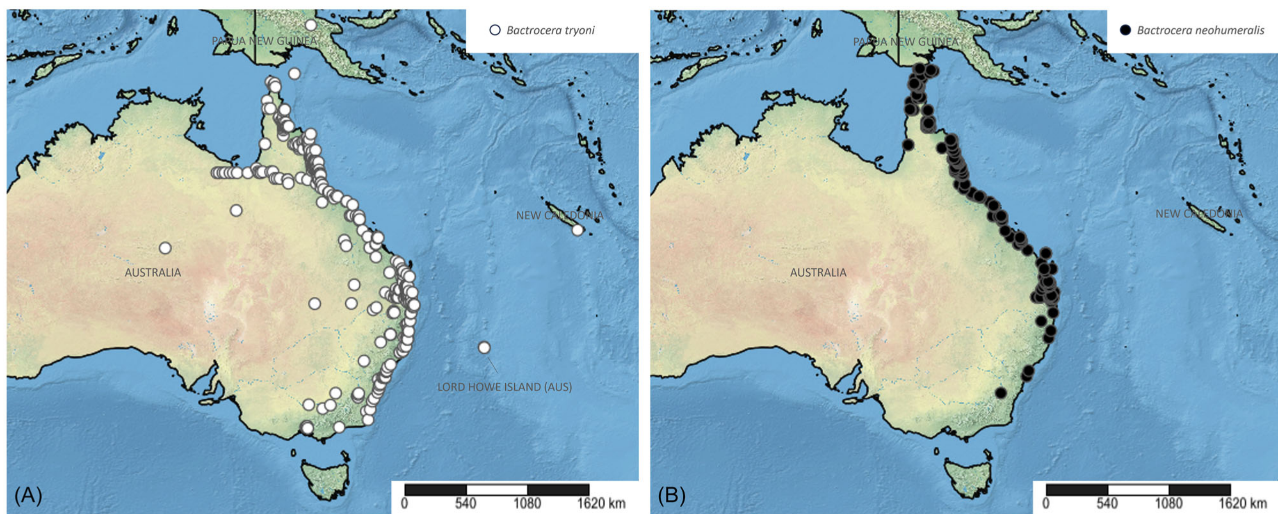

Fig. 2 Distribution of (A) *Bactrocera tryoni* and (B) *Bactrocera neohumeralis* plotted using filtered data obtained from Atlas of Living Australia and data from the Queensland Primary Industries Insect Collection (QDPC) (Creative Commons 2.5).

no single locus diagnostic markers that can either discriminate between *B. tryoni* and *B. neohumeralis* or be adapted for quarantine or surveillance diagnostics. The more complex, time consuming multilocus simple sequence repeats (SSR) method (Wang *et al.*, 2003), does distinguish these species, but other approaches successfully used for *Bactrocera* such as PCR-RFLP of rDNA (Armstrong *et al.*, 1997), the standard DNA barcoding marker COI (Blacket *et al.*, 2012; Blacket *et al.*, 2020), or novel single-copy loci specifically developed for fruit fly diagnostics (Krosch *et al.*, 2019; Plant Health Australia, 2020) were unable to separate them. Morrow *et al.* (2000) reported minor fixed differences between the two species at a multicopy locus, the internal transcribed spacer region 2 (ITS2). However, our further investigation found this region unsuitable for assay development due to nonspecific amplification (data not shown), prompting investigation for alternative informative genetic markers.

Despite the demonstrated morphological and genetic similarities, there are behavioral and biological differences between *B. tryoni* and *B. neohumeralis*, which support their status as discrete species. One key biological distinction is that *B. tryoni* mates at dusk, while *B. neohumeralis* mates during a period of several hours during the day, maintaining reproductive isolation despite their ability to hybridize in the lab (Pike & Meats, 2002). Although the two species share 137 host species, *B. tryoni* has been recorded from a total of 243 hosts, compared to *B. neohumeralis* which has been recorded from 166 hosts (Hancock *et al.*, 2000). In addition, *B. tryoni* and *B. neohumeralis* overlap in their distribution range (Fig. 2); however, *B. tryoni* is far more widespread, found across northern and eastern Australia, including drier inland sites (Dominiak & Daniels, 2012) and is also established in New Caledonia, French Polynesia and Pitcairn Islands (Leblanc *et al.*, 2012; Duyck *et al.*, 2022).

The lack of reliable morphological and molecular diagnostic characters currently impacts several aspects of fruit fly research, monitoring, and pest management, all of which could be overcome through the identification of a reliable diagnostic marker and development of a rapid screening method. For example, government and research organizations maintain reference colonies for fundamental research activities which are required to be pure colonies of the target pest (FAO/IPPC, 2007), with taxonomic identification performed on each colony and voucher specimens preserved (FAO/APPPC, 2004; NAPPO, 2011; FAO/IPPC, 2016). Further, research on host suitability and stress tolerance informs the protocols for treatment and export of produce to overseas markets (Heather & Hall, 2008; Leach, 2019). Colonies are also maintained for suppression of *B. tryoni* incursions in pest-free regions through the release of sterilized males, referred to as the Sterile Insect Technique (SIT) (Klassen & Vreysen, 2021). If a reference colony is found to be contaminated by another species or, in the case of *B. neohumeralis* or *B. tryoni*, the presence of ambiguous morphological individuals, in the absence of a reliable diagnostic assay, the entire colony must be destroyed (P. Leach pers. comm) and rebuilt to avoid developing methods for

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nontarget species. Likewise, maintaining pure colonies for the generation and release of sterilized males is of utmost importance to ensure the correct species are released to mate with target populations in the wild. Finally, routine surveillance activities would also benefit from accurate diagnosis of ambiguous adults, whether encountered in a trap, or reared from infested fruit, to better inform distribution and host use records, or, in the event of an incursion, allow for a targeted response strategy.

The aims of this study were, therefore to (i) identify a gene region capable of distinguishing between *B. tryoni* and *B. neohumeralis*; (ii) develop a tetra-primer amplification refractory mutation system (ARMS) PCR assay using the identified region for rapid discrimination of *B. tryoni* from *B. neohumeralis*; (iii) evaluate the gene region and diagnostic assay against other closely related species within the greater *B. tryoni* species complex; and (iv) explore the limits of this assay and gene region for other difficult species groups, such as the *B. dorsalis* (Hendel) complex.

Materials and methods

Identifying diagnostic gene regions using genotype by sequencing (GBS)

Cue-lure trapped flies were collected from three regions at the following time periods: April 2013 in northern and southern Queensland; November−December 2013 in southern Queensland; and February−March 2014 in northern Queensland and New South Wales. Species were identified using the keys provided by Drew (1989), White & Elson-Harris (1992), and the online LUCID key by Plant Health Australia (2018c). Collection details of samples can be found in the supporting information. High-resolution voucher photographs were taken of all specimens used for DNA extractions using an Olympus SC20 camera mounted onto an Olympus SZX12 stereo microscope and processed using cellSens Dimension 1.8 (Olympus).

Of > 1300 flies collected, extraction of high-quality DNA from whole *B. tryoni* ($n = 118$) and *B. neohumeralis* ($n = 72$) flies used for GBS, plus additional samples used below for subsequent preliminary primer testing, was carried out as per Patrick *et al.* (2016). Following initial 1% gel electrophoresis assessment to confirm presence of high molecular weight DNA (> 20 kb) and 50–100 ng/mL, accurate sizing and quantitation was carried out using an Agilent 2100 Bioanalyzer. Restriction enzyme optimization tests for the GBS resulted in *Eco*T22I being chosen to produce the genome subsets rather than *Pst*I which produced sequencing libraries containing highly repetitive DNA. The GBS was carried out on the Illumina sequencing platform at Cornell University, Institute for Genomic Diversity, NY, USA as per the methods described in Elshire *et al.* (2011). The resulting data were analyzed using the TASSEL-GBS pipeline (Glaubitz *et al.*, 2014); first, raw sequences were trimmed to the same length (64 bp) and identical reads were collapsed into unique sequence "tags," retaining only those with >3 supporting reads across the whole dataset. The unique tags were then aligned to assembly 2.2 of the *B. tryoni* reference genome (NCBI BioProject Accession PRJNA241080) using BWA-aln v0.7.12 (Li & Durbin, 2009) with default parameters, to obtain their genomic coordinates. SNPs were identified within all successfully aligned tags using the Discovery SNP Caller in TASSEL-GBS, then quality filtered according to Elshire *et al.* (2011), retaining only biallelic SNPs with a minor allele frequency (MAF) >1% and <90% missing data. The positions of those representing major alleles, that is, SNPs differing between the species with a frequency of one and having been counted in TASSEL-GBS as occurring in all samples of one species while not occurring at all in the other, were visualized against the *B. tryoni* reference genome in Integrative Genomics Viewer (Robinson *et al.*, 2011; Thorvaldsdottir *et al.*, 2013). Sequences flanking those positions were used to design primers by eye. Preliminary confirmation of primer suitability was achieved using both a subset of specimens from the original GBS analysis (*B. tryoni* $n = 6$ and *B. neohumeralis* $n = 10$) and additional specimens of each species (*B. tryoni* $n = 7$ and *B. neohumeralis* $n = 5$). PCR conditions are outlined below. Amplicons were Sanger sequenced at the Bio-Protection Research Centre Sequencing Facility, Lincoln University, New Zealand. Based on the consistency of the SNP mutation and strength of amplification, only one primer pair was selected for further species screening (see below), renamed here as Outer-F and Outer-R (Table 1).

Genomic location of putative diagnostic SNPs and consistency across species and geographic populations

To further assess the consistency of the GBS-identified adjacent 2 bp SNPs, a combination of existing high-throughput sequencing (HTS) reads from Piper (2021) and a newly generated Sanger sequence dataset (total of 200 *B. tryoni* from 47 geographic locations and 35 *B. neohumeralis* from eight geographic locations) was used. Specimens also included additional species of

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Table 1 Nucleotide sequence and predicted anneal temperature of primers designed in this study.

Primer name	Sequence (5'–3')	Tm (°C)
Outer-F	TTGGCCACACATATCGCACT	57.1
Outer-R	CGGTTATACCCAAACTGCTTCCT	57.0
Inner-Btry-F†	TATGCACGTACTTGCAGTTTAA	52.5
Inner-Bneo-R†	CCCGAAAATTCCTAAAAACC	49.5

†Underlined region indicates the 2 bp adjacent diagnostic SNPs.

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XM_040111650.1 B_tryoni_protein_pangolin   TTGGCCACACATATCGCACTATTCAACAATTCGATGACATACACATACATATATGAGTATACACACATATGCATGCTTGTATGCACGTACTTGCAGTTTAATTTTTAGGAATTTTCGGGTTTTTTAAGGAAGCAGTTTGGGTATAACCG
Outer-F                                     TTGGCCACACATATCGCACT
Outer-R                                     CGGTTATACCCAAACTGCTTCCT
Inner-Btry-F                                TATGCACGTACTTGCAGTTTAA
Inner-Bneo-R                                CCAAAAATCCTTAAAAGCCC
B_tryoni_TRY010                             ---------------------------------------------------------TATACACACATATGCATGCTTGTATGCACGTACTTGCAGTTTAATTTTTAGGAATTTTCGGGTTTTTTAAGGAAGCAGTTTGGGTATAACCG
B_tryoni_TRY007                             ---------------------------------------------TACATATATGAGTATACACACATATGCATGCTTGTATGCACGTACTTGCAGTTTAATTTTTAGGAATTTTCGGGTTTTTTAAGGAAGCAGTTTGGGTATAACCG
B_tryoni_TRY008                             ---------------------------------------------TACATATATGAGTATACACACATATGCATGCTTGTATGCACGTACTTGCAGTTTAATTTTTAGGAATTTTCGGGTTTTTTAAGGAAGCAGTTTGGGTATAACCG
B_tryoni_TRY3                               ---------------------------------------------TACATATATGAGTATACACACATATGCATGCTTGTATGCACGTACTTGCAGTTTAATTTTTAGGAATTTTCGGGTTTTTTAAGGAAGCAGTTTGGGTATAACCG
B_neohumeralis_NEO006                       ---------------------------------------------TACATATATGAGTATACACACATATGCATGCTTGTATGCACGTACTTGCAGTTTGGTTTTTAGGAATTTTCGGGTTTTTTAAGGAAGCAGTTTGGGTATAACCG
B_neohumeralis_NEO007                       ---------------------------------------------TACATATATGAGTATACACACATATGCATGCTTGTATGCACGTACTTGCAGTTTGGTTTTTAGGAATTTTCGGGTTTTTTAAGGAAGCAGTTTGGGTATAACCG
B_neohumeralis_NEO008                       ---------------------------------------------TACATATATGAGTATACACACATATGCATGCTTGTATGCACGTACTTGCAGTTTGGTTTTTAGGAATTTTCGGGTTTTTTAAGGAAGCAGTTTGGGTATAACCG
B_neohumeralis_NEO009                       ---------------------------------------------TACATATATGAGTATACACACATATGCATGCTTGTATGCACGTACTTGCAGTTTGGTTTTTAGGAATTTTCGGGTTTTTTAAGGAAGCAGTTTGGGTATAACCG
```

Fig. 3 Partial *pan* gene sequence showing direction of two outer (Outer-F and Outer-R) and two inner (Inner-Btry-F and Inner-Bneo-R) primers of the tetra-primer ARMS-PCR assay designed to target *Bactrocera tryoni* (69 bp) and *Bactrocera neohumeralis* (108 bp). Total (outer primer) PCR fragment length for these species is 149 bp. Fixed SNP genotypes for distinguishing the two species are color highlighted in the alignment at their 3' positions on inner primers.

interest and morphological "intermediates" taken from the Queensland Department of Agriculture and Fisheries Market Access reference colonies (see Supplementary file 1 for details).

HTS reads were filtered using fastp (Chen *et al.*, 2018) to remove any residual adapter sequences and retain only those reads with a mean quality >20, >50 bp in length, and containing no ambiguous "N" bases. Filtered reads were then aligned to the more recent *B. tryoni* RefSeq genome assembly (NCBI BioProject Accession PRJNA695304) using BWA-MEM (Li, 2013). The nuclear gene region of interest was extracted from each aligned BAM file using bcftools (Danecek *et al.*, 2021) and filtered to keep bases with >5 reads and >100 bp of alignment. The two adjacent SNP sites for separation of *B. tryoni* and *B. neohumeralis* were identified and cross checked by eye for consistency. This confirmed the potential for this locus as the basis for diagnostic assay development.

Sanger sequenced specimens were extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) with modifications, as per Starkie *et al.* (2022) and sequenced using primers Outer-F and Outer-R (Table 1). Sequencing was carried out at the Australian Genome Research Facility, Brisbane; the Molecular Genetics Research Facility, QUT, Brisbane; and Macrogen Inc. (Seoul, South Korea). Voucher specimens are stored in ethanol at −20 °C at the Queensland Department of Agriculture and the New South Wales Department of Primary Industries.

Tetra-primer ARMS-PCR assay design and validation

To bind at the site of the adjacent SNPs, and to work alongside the outer primers under the same reaction conditions, two internal primers were designed by eye based on an in-house reference alignment of the sequenced specimens (Fig. 3). Primers were designed to be specific for the target locus, and include sufficient GC content to ensure that the annealing temperature would be compatible with PCR amplification (see Table 1), specifically, an internal forward primer "Inner-Btry-F" to pair with "Outer-R" targeting *B. tryoni* and an internal reverse primer "Inner-Bneo-R" to pair with "Outer-F", targeting *B. neohumeralis* (Table 1).

For the tetra-primer ARMS-PCR assay, each PCR reaction consisted of 12.5 μL OneTaq® Hot Start Quick-Load® 2X Master Mix with Standard Buffer (New England BioLabs, UK); 0.25 μL 10 μmol/L Outer-F primer; 0.25 μL 10 μmol/L Outer-R primer, 1.5 μL 10 μmol/L Inner-Btry-F primer; 0.75 μL 10 μmol/L Inner-Bneo-R primer; 1 μL 20 mg/mL bovine serum albumin (New England BioLabs, UK); 2 μL template DNA; and 6.75 μL H_2O to a total volume of 25 μL. Each reaction was run on a ProFlex PCR thermal cycler (Thermo Fisher Scientific), with an initial denaturation of 94 °C for 2 min; 35 cycles of denaturing at 94 °C for 30 s; annealing at 50 °C for 30 s; and extension at 68 °C for 30 s. This was followed by a final extension at 68 °C for 2 min. PCR products were visualized on a 1.5% agarose gel run for 40 min at 90 V.

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Table 2 Genotypes of *Bactrocera* and *Zeugodacus* species within the *pan* primer region established from Piper (2021) and newly generated Sanger sequence in this study. *Bactrocera tryoni* species complex members and additional species resolved in the clade are shaded in gray.

		SNP positions when aligned against reference genome			
Species	*n*	17154192−17154193	17154199	17154203	17154215−17154223
B. tryoni	312	AA	C	C	TGC—TT
B. neohumeralis	27	GG	C	C	TGC—TT
B. aquilonis	3	AA	C	C	TGC—TT
B. melas	3	AA	C	C	TGC—TT
B. curvipennis	2	GG	C	T	TGC—TT
B. erubescentis	4	GG	C	C	TGC—TT
B. mutabilis	1	GG	C	T	TGC—TT
B. alyxiae (May)	1	GG	C	C	TACATACTT
B. bancroftii	2	AG	C	C	TGC—TT
B. frauenfeldi	8	AA and GG	G and C	C	TGC—TT
B. kraussi (Hardy)	1	GG	C	C	TGC—TT
B. raiensis Drew and Hancock	1	GG	C	C	TGC—TT
B. visenda (Hardy)	1	AA	C	C	TGC—TT
Z. hochii (Zia)	1	AA	C	C	TGC—TT

Results

Diagnostic SNP region

A total of 477,934,035 sequence reads were generated from the GBS data, which were collapsed into 3,466,218 unique sequence tags with >3 supporting reads; 1,966,511 (56.7%) of these tags were successfully aligned to unique positions on the *B. tryoni* reference genome, while 145 477 (4.2%) aligned to multiple positions, and 1,354,230 (39.1%) could not be aligned based on the settings used here. After quality filtering, a total of 75 025 SNPs were identified within the dataset, but only five were found to have an allele frequency difference of 1 between the sequenced *B. tryoni* ($n = 118$) and *B. neohumeralis* ($n = 72$) specimens (i.e., diagnostic SNPs). Primers designed as specific for both species at each of the five loci provided amplicons that ranged between 94 and 291 bp (data not shown), but only one primer pair was selected for full species screening based on consistency of the SNPs and strength of amplification (see below). PCR amplification produced a 149 bp amplicon encompassing an adjacent 2 bp SNP region of AA for *B. tryoni* and GG for *B. neohumeralis*, matching positions 17,154,192−17,154,193 of an unplaced scaffold (Fig. 3) when aligned to the RefSeq genome assembly (NCBI BioProject Accession PRJNA695304) (note: the current assembly aligns in reverse). Annotations at this location on the reference genome identified the target for our new diagnostic assay as being within the *pangolin* (*pan*) gene.

Geographic validation, assay performance and diagnostic capability of the pan SNP region

Sequences of the *pan* region from existing HTS data (Piper, 2021) and new Sanger sequence data (GenBank accessions OQ694046−OQ694313), adding to that identified through the GBS, amounted to $n = 430$ for *B. tryoni* and $n = 99$ for *B. neohumeralis* specimens sampled across >40 geographic collection localities (see Fig. S2) to provide evidence that the two adjacent SNPs were fixed between *B. tryoni* and *B. neohumeralis* (Table 2) across their entire distributional ranges (>40 geographic collection localities; see Supplementary file 1). Intermediate samples included from colonies were accurately identified and results were concordant with Sanger sequencing results (see Fig. S2).

Genotypes of other nontarget species included in this bioinformatic assessment were identical to *B. tryoni* and *B. neohumeralis* sequences from this primer region (Table 2), indicating that this adjacent 2 bp SNP genotype is not specific to this species pair. The 2 bp adjacent SNPs at this site (positions 17154192−17154193 of reference genome) were identified to be either adjacent GG or AA

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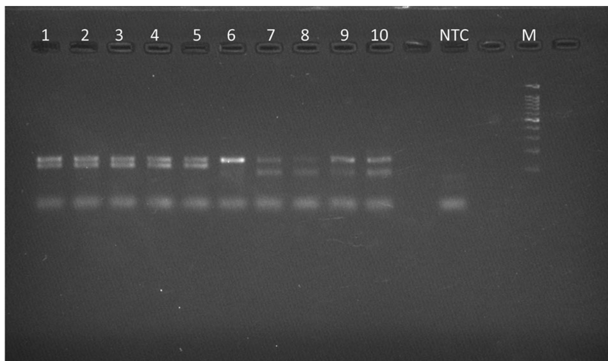

Fig. 4 Gel electrophoresis image of genotype primer performance in the *pan* tetra-primer diagnostic assay for examples of the target species *Bactrocera tryoni* and *Bactrocera neohumeralis*. Lanes 1−5: *Bactrocera neohumeralis*; 6−10: *Bactrocera tryoni*; NTC: no template control; and M: 100 bp DNA ladder (Biotium).

SNPs (as per *B. tryoni* and *B. neohumeralis*), except for *B. bancroftii* (Tryon), which was AG, although this variant may also occur in other species not considered here. Additional SNP mutations and small indels (<4 bp) were found at other positions in the *pan* sequence across other nontarget species, indicating further diagnostic potential (Table 2).

Species-specific tetra-primer ARMS-PCR diagnostic assay

Using all four primers in the tetra-primer ARMS-PCR diagnostic assay (named from hereon, the tetra-primer assay), the anticipated difference in double amplicons from the respective outer and inner primer pairings was consistently observed for *B. tryoni* and *B. neohumeralis*, indicating the *B. tryoni* internal primer (Inner-Btry-F) did not amplify *B. neohumeralis* (total tested = 10) and similarly the *B. neohumeralis* internal primer (Inner-Bneo-R) did not amplify *B. tryoni* (total tested = 10) (examples are illustrated in Fig. 4). Variation in band intensity was seen in PCR amplicons using these primers including some that show preferential amplification of one band over another (e.g., Fig. 4, lane 6). Performance of other species tested in the assay can be seen in Fig. S1, which shows multiple bands for some *B. dorsalis* complex members.

Discussion

Morphological intermediates of both *B. tryoni* and *B. neohumeralis* have long caused identification issues. Our study provides the first molecular diagnostic marker for differentiation of *B. tryoni* and *B. neohumeralis*, two adjacent SNPs in a small region of *pan* which are fixed across the distributional range of these species. We further validated a tetra-primer PCR-based assay to amplify this marker, enabling cost-effective, same-day diagnostics, which can be undertaken with little technical expertise. While only applicable to the *B. tryoni* clade, the assay will be a useful tool for the necessary quality control in *B. tryoni* research and SIT colonies, as well as for distinguishing ambiguous field-caught adults of *B. tryoni* and *B. neohumeralis*. Similarly, the inability to separate *B. tryoni* from the other two species in the taxonomic complex, *B. aquilonis* and *B. melas*, which share the same AA genotype, does not detract from useful application of the assay based on geographic separation of *B. aquilonis* (which occurs in the north-west of Australia) and the rarity and non-pest status of *B. melas*. Difficulty in diagnosing these three species (*B. tryoni/B. aquilonis/B. melas*) is consistent with their postulated recent evolutionary divergence and questionable species status, as has been debated elsewhere (Clarke *et al.*, 2011).

The *pan* region flanked by the outer primers of the tetra-primer assay suggests the potential for diagnosis of other species that resolve in the *B. tryoni* complex clade (Starkie *et al.*, 2022). Specifically, while *B. curvipennis* and *B. mutabilis* have identical GG SNP genotypes targeted by the Inner-Bneo-R primer, they can be distinguished from both *B. tryoni* and *B. neohumeralis* by mutations at other locations within our amplicon (i.e., T instead of C; Table 2). Sequence data comparison for *B. erubescentis* revealed this species also has a unique mutation alongside the GG genotype. Further development of this tetra-primer assay could look to target these additional SNPs for species that share fruit hosts with *B. tryoni* or *B. neohumeralis* to aid in diagnosis of larvae. To achieve this, additional primers would need to be developed, or the diagnostic laboratory could send the products for sequencing and potentially utilize additional SNPs and indels for diagnosis.

Beyond the *B. tryoni* clade, the diagnostic value of the assay is limited as most of our other test panel species (seven total) also exhibited the adjacent double AA or GG SNP genotype, except for *B. bancroftii* which was AG. Unfortunately, some other species were not able to be characterized due to the primers binding to additional nontarget regions (see Fig. S1). For example, gel visualization of *B. dorsalis* amplicons showed preferential primer binding for larger fragments over the smaller target regions of our assay. Conversely, all *B. cacuminata* (Hering) (a *B. dorsalis* complex member) specimens consistently amplified three bands of the same size.

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However, the inconsistency of other members of the *B. dorsalis* complex, such as *B. dorsalis*, *B. opiliae* (Drew & Hardy) and morphologically similar *B. musae* (Tryon) suggest that the fragment of *pan* amplified here will likely not be a viable region for *B. dorsalis* complex diagnostics.

In insects, *pan* is a component of the Wingless cell-to-cell signaling pathway that produces proteins critical for embryogenesis and morphogenesis (Bejsovec, 2018) and is responsible for regulating wing pattern development in *Drosophila* (Valenta *et al.*, 2012; Bejsovec, 2018). *Pangolin* encodes a transcription factor that is a known component of the Wingless pathway (Brunner *et al.*, 1997) and is essential for cell proliferation, wing margin specification, and wingless protein self-refinement (Schweizer *et al.*, 2003). In our study, almost all fruit fly *pan* sequences obtained exhibited the adjacent double SNP genotype of AA or GG, showing a deficit of AG genotypes, which is highly suggestive that this region has been under strong selection. Regardless of the functional significance of this gene in relation to its consistent and stable difference between the target taxa, this study has identified *pan* as a novel diagnostic locus able to discriminate very closely related tephritid species thus rendering it a valuable addition to the few nuclear coding loci used in species-level dacine diagnostics (Krosch *et al.*, 2019; Plant Health Australia, 2020), with potential for other insect cryptic species groups. This assumes heterozygous allele haplotypes are not prevalent to complicate amplification by production of both allele amplicons (Zhang & Hewitt, 2003), as may have been a problem here contributing to the nonspecific amplification observed for the *B. dorsalis* complex and shown elsewhere to be an issue for the use of other nuclear loci *period* and CAD (Boykin *et al.*, 2014). While we focused our assay on a 149 bp region, the complete *pan* gene (∼ 44 945 bp in *Drosophila*) (Gramates *et al.*, 2022) could be explored for other diagnostic regions toward which comparative genomic data in Genbank for other tephritids (Calla *et al.*, 2014; Geib *et al.*, 2014) and predicted *pan* sequence for *B. tryoni* (NCBI BioProject Accession PRJNA695304), *B. neohumeralis* (NCBI BioProject Accession PRJNA875056), and *B. latifrons* (Hendel) (NCBI BioProject Accession PRJNA281765) could be useful.

This diagnostic region and accompanying tetra-primer assay developed here will now provide confidence to those who maintain *B. tryoni* and *B. neohumeralis* colonies; encounter ambiguous adults in the field; or larvae in fruit, where diagnosis is difficult. Ambiguous samples encountered in reference or SIT colonies can be easily diagnosed to prevent the destruction of entire colonies, saving time and money. In addition, those who trap or rear adults in the field can be confident that reports of new incursions or distributions are accurate, which is critical in the early stages of pest detection and response.

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Disclosure

The authors declare that there are no financial competing interests (political, personal, religious, ideological, academic, intellectual, commercial, or any other), neither are there other competing interests in the production of this manuscript.

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Supporting Information

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

Fig. S1 Gel electrophoresis image of species beyond the *Bactrocera tryoni* taxonomic complex that were tested in this study using the tetra-primer assay.

Fig. S2 Sampling locations of *Bactrocera tryoni* and *Bactrocera neohumeralis* used for HTS, NGS, and Sanger sequencing in this study.

Supplementary file 1 Collection details, specimen codes, and sample analyses.

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