CSIRO PUBLISHING

Invertebrate Systematics, 2019, **33**, 618–627
https://doi.org/10.1071/IS18065

A genome-wide approach for uncovering evolutionary relationships of Australian *Bactrocera* species complexes (Diptera: Tephritidae)

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Abstract. Australia and Southeast Asia are hotspots of global diversity in the fruit-fly genus *Bactrocera*. Although a great diversity of species has been long recognised, evolutionary relationships are poorly understood, largely because previous sequencing techniques have provided insufficient historical signal for phylogenetic reconstruction. Poorly understood biogeographic history in *Bactrocera* has prevented a deeper understanding of migratory patterns in this economically important pest group. Using representatives from Australia and Malaysia, we tested the utility of a genome-reduction approach that generates thousands of single-nucleotide polymorphisms for phylogenetic reconstructions. This approach has high utility for species identification because of the ease of sample addition over time, and the species-level specificity able to be achieved with the markers. These data have provided a strongly supported phylogenetic tree congruent with topologies generated using more intensive sequencing approaches. In addition, our results do not support taxonomic assignments to species complex for a number of species, such as *B. endiandrae* in the dorsalis complex, yet find a close relationship between *B. pallida* and the dorsalis species. Our data have further validated non-monophyletic evolution of male response to primary attractants. We also showed at least two diversification events between Australia and Southeast Asia, indicating trans-regional dispersal in important pest species.

Received 10 August 2018, accepted 4 February 2019, published online 2 August 2019

Introduction

Fruit flies in the family Tephritidae are major global agricultural pests, attacking a wide variety of crops (Hancock *et al.* 2000; Abdalla *et al.* 2012). There are over 4000 species in the Tephritidae (White and Elson-Harris, 1992), with ~650 species in the *Bactrocera* genus (Vargas *et al.* 2015). Globally, ~73 *Bactrocera* species have economically important impacts on agricultural production (Vargas *et al.* 2015). Diversity within *Bactrocera* is centred in Southeast Asia, Australia and the South Pacific (Krosch *et al.* 2012). However, evolutionary relationships among *Bactrocera* species remain poorly understood, including the patterns of diversification within and between these regions. It is currently unknown whether major bioregions contain largely monophyletic groups of species, or whether species readily diversify across oceanic barriers.

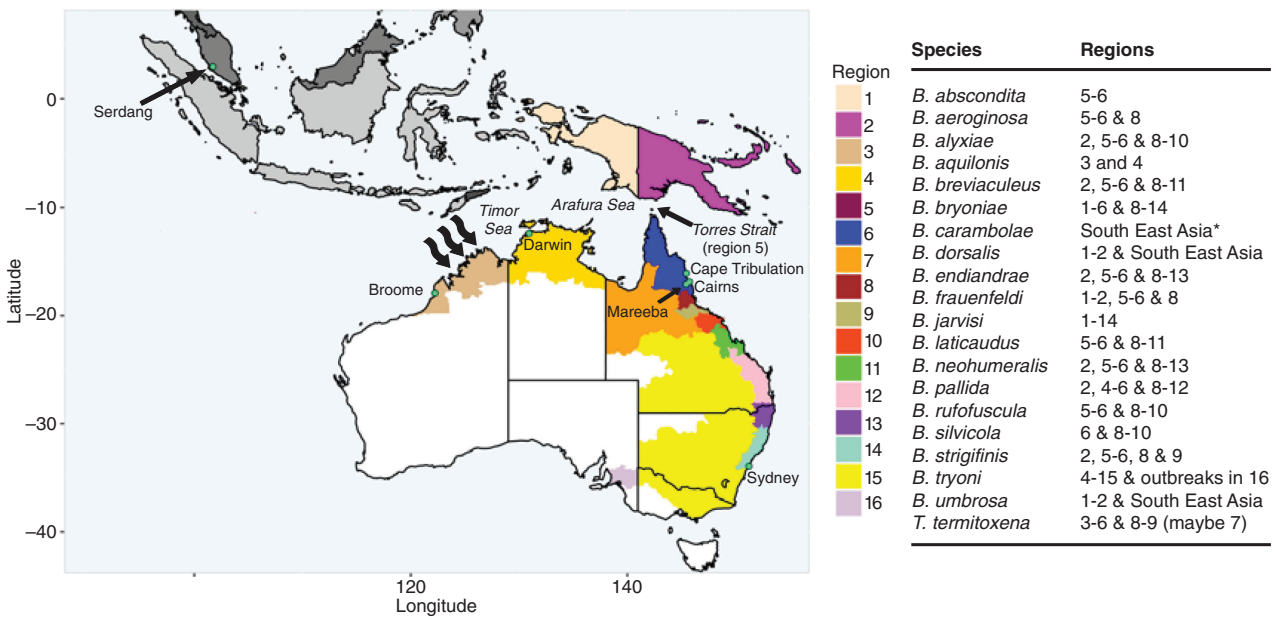
Australia has ~280 native species of *Bactrocera*, with the following seven species having a substantial economic impact: *B. tryoni*, *B. neohumeralis*, *B. aquilonis*, *B. cucumis*, *B. frauenfeldi*, *B. jarvisi* and *B. musae* (Vargas *et al.* 2015). Benefits to Australian

agriculture from the control of *Bactrocera* are estimated at AU $29–37 million per annum (Abdalla *et al.* 2012). The possible introduction of a new pest *Bactrocera* species has been categorised as 1 of 12 potential *megashocks* for Australian agriculture, being 'a significant, sudden, and potentially high impact event. . .based on what the biosecurity community identified as some of the most important threats we might face over the coming 20–30 years' (Simpson and Srinivansen 2014; p. 6). *Bactrocera dorsalis*, a Southeast Asian endemic, represents a particular threat to Australian agriculture. It is a major pest in Southeast Asia and is highly suited to the climate of Australia (Stephens *et al.* 2007). A recent incursion of the species was eradicated only at a significant cost (AU$33 million; Cantrell *et al.* 2002). Australia expends significant resources preventing, detecting and eradicating introduced *Bactrocera* species (Hafi *et al*. 2013).

Monitoring for invasive *Bactrocera* in Australia is primarily focused on the Torres Strait, a narrow gap of shallow water separating Australia from New Guinea (Abdalla *et al.* 2012). However, it is unclear whether the Torres Strait has been the sole route for migration between Australia and Southeast Asia, rather than across the Timor and Arafura Seas (Fig. 1). Directional movement into Australia across these seas may be possible in association with monsoonal storms, which blow north to south during the cyclonic activity of the wet season. These storms have the potential to act as engines of diversification, enabling isolated but rapid movements in one direction and, thereby, promoting invasion and vicariant speciation.

Improving our understanding of relationships within *Bactrocera* offers an opportunity to identify patterns of diversification, the timing of historical diversification, and climate conditions promoting trans-oceanic movements. Patterns and timing of diversification can be reconstructed using a phylogenetic framework (e.g. BioGeoBears; Matzke 2014), given sufficient sampling of taxa in the group of interest. Recent phylogenetic analyses of *Bactrocera* find support for diversification across oceanic barriers for Australian species (Krosch *et al.* 2012). For example, the close relationship between *B. dorsalis* from Malaysia and *B. pallida* from Australia (San Jose *et al.* 2018), or the relationship between *B. umbrosa* from Malaysia and *B. abscondita* from Australia (Dupuis *et al.* 2018) are only recently resolved. This only recent understanding of relationships within the Dacine flies is likely to be due to the relatively rapid and recent diversification of *Bactrocera* into thousands of species over the past 50–35 million years (Krosch *et al.* 2012), which provides a particularly challenging phylogenetic question. Rapid diversification requires significant amounts of genetic data to reconstruct evolutionary relationships, due to the high likelihood of incomplete lineage sorting (McVay and Carstens 2013).

A recent study using amplicon sequencing (Dupuis *et al.* 2018) was able to resolve inter- and intra-complex relationships in a global sampling of Dacine flies, with high levels of support. However, this method is not particularly tractable for the identification of individuals over time (e.g. samples collected during biosecurity operations). Sequencing approaches using single-nucleotide polymorphisms (SNPs) isolated using a double-digest restriction site ssociated DNA sequencing (RADseq; Melville *et al.* 2017) approach may provide a cost-effective opportunity for the identification of individual flies to species. SNP-based approaches to phylogenetic reconstruction have also been found to produce topologies congruent with phylogenies produced using ultra-conserved elements (Leaché and Oaks 2017). This suggests that SNP-based approaches may



Species	Regions
B. abscondita	5-6
B. aeroginosa	5-6 & 8
B. alyxiae	2, 5-6 & 8-10
B. aquilonis	3 and 4
B. breviaculeus	2, 5-6 & 8-11
B. bryoniae	1-6 & 8-14
B. carambolae	South East Asia*
B. dorsalis	1-2 & South East Asia
B. endiandrae	2, 5-6 & 8-13
B. frauenfeldi	1-2, 5-6 & 8
B. jarvisi	1-14
B. laticaudus	5-6 & 8-11
B. neohumeralis	2, 5-6 & 8-13
B. pallida	2, 4-6 & 8-12
B. rufofuscula	5-6 & 8-10
B. silvicola	6 & 8-10
B. strigifinis	2, 5-6, 8 & 9
B. tryoni	4-15 & outbreaks in 16
B. umbrosa	1-2 & South East Asia
T. termitoxena	3-6 & 8-9 (maybe 7)

Fig. 1. Geographic region of samples across Australia (white and Regions 3–16), Indonesia (light grey and Region 1), Timor Leste (dark grey), Papua New Guinea (Region 2) and Malaysia (medium grey). Sample locations are denoted by circles with location labels, oceanic barriers between Australia and other regions are in italics, and wavy arrows represent directionality of cyclonic winds during the monsoon season. Colours represent regions based on Drew and Hancock (1999), Hancock *et al*. (2000), Leblanc *et al*. (2015) and Royer and Hancock (2012) and the Atlas of Living Australia. *East to Sumbawa, not in the Philippines, unknown status in Sulawesi.

provide the phylogenetic information needed to identify trans-oceanic dispersal, at a relatively low cost and in a method allowing continuous addition of samples through time.

In the present study, we test the utility of a genome-reduction sequencing approach for phylogenetic reconstruction of Australian and Southeast Asian *Bactrocera*, and contrast our results to recent phylogenies constructed using more intensive sequencing methods (Dupuis *et al*. 2018 (739 amplicons totalling 151 511 bp); San Jose *et al.* 2018 (7 genes totalling 5303 bp)). We here present a phylogenetic analysis of representatives from different species complexes within the *Bactrocera* subgenus. In particular, we seek to test for diversification between Australia and Southeast Asia, *versus* monophyly within the Australian region. Our samples are from northern Australia, Sydney, and Serdang, Malaysia, and include five of the seven agriculturally important Australian pest species, and two Malaysian pest species.

Materials and methods

Sampling

Nineteen *Bactrocera* species, including 16 and three collected in Australia and Malaysia respectively, and one tephritid non-*Bactrocera* species from Australia (*Termitorioxa termitoxena* as an intended outgroup) were sampled from infested fruits, lure traps or laboratory lines (Fig. 1, Table 1, Table S1 and text available as Supplementary Material 1 to this paper). Species identification was performed on the basis of morphological characters described in Drew (1989), White and Elson-Harris (1992) and Drew and Romig (2013). Identification was performed by species experts (AKWH for Malaysian species and JC and SDF for Australian species). A more detailed description of the methodology is presented in Supplementary Material 1.

DNA sequencing and SNP selection

Whole flies were provided to Diversity Arrays Technology Ltd (DArT; Canberra, Australia) for DNA extraction, complexity reduction using endonucleases targeting low-copy DNA regions, polymerase chain reaction (PCR) amplification of fragments, and sequencing on an Illumina HiSeq2500 (DArTseq; detailed in Melville *et al.* 2017; Pazmiño *et al.* 2017). Genome reduction allows the detection of informative SNPs, resulting in ~70-bp DNA fragments after next-generation sequencing (Wenzl *et al.* 2004). In short, 150–200 ng of gDNA was digested using two methylation-sensitive restriction enzymes, namely, the frequent cutter *Pst*I (5′-CTGCA^G-3′) and the rare cutter *SphI* (5′-GCATG^C-3′). Fragments of <200 bp were ligated to a barcoded adaptor, and amplified using PCR. The PCR products were standardised and pooled for sequencing on an Illumina HiSeq2500.

Sequence reads were processed using a proprietary DArT analytical pipeline (detailed in Petroli *et al.* 2012; Melville *et al.* 2017; Pazmiño *et al.* 2017). The *B. tryoni* draft genome assembly (Gilchrist *et al.* 2014) was used as the reference genome for annotation of sequences. Each individual was identified as homozygous for the reference allele, homozygous for the alternate allele, or heterozygous for each SNP. DArTseq markers are relatively randomly dispersed across a genome but disproportionally represent coding regions (Petroli *et al.* 2012).

The SNP dataset (full dataset available at http:10.6084/m9.figshare.7886348) was further filtered in R v3.4 (R Core Team, 2017) using the dartR package (Gruber et al 2018). All successfully sequenced individuals were included in the genetic clustering analyses. For the phylogenetic analyses, only species with two successfully sequenced individuals were included. This is because, in preliminary analyses (Fig. S2.1), the placement of species that were represented by

Table 1. Taxonomic classification (based on Drew 1989) and site locations of *Bactrocera* species in the present study

Species	Subgenus	Complex	Location	Collection method	Primary male attractant
B. jarvisi	Bactrocera	–	Darwin/Mareeba	Protein bait trap or fruit	Zingerone
B. laticaudus	Bactrocera	–	Cape Tribulation	Male lure trap	Methyl eugenol
B. umbrosa	Bactrocera	–	Serdang, Malaysia	Male lure trap	Methyl eugenol
B. alyxiae	Bactrocera	alyxiae	Cape Tribulation	Male lure trap	Cuelure
B. aeroginosa	Bactrocera	bidentata	Cape Tribulation	Male lure trap	Cuelure
B. bryoniae	Bactrocera	bryoniae	Cape Tribulation	Male lure trap	Cuelure
B. carambolae	Bactrocera	dorsalis	Serdang, Malaysia	Male lure trap	Methyl eugenol
B. dorsalis	Bactrocera	dorsalis	Serdang, Malaysia	Laboratory line	Methyl eugenol
B. endiandrae	Bactrocera	dorsalis	Cape Tribulation	Male lure trap	Methyl eugenol
B. frauenfeldi	Bactrocera	frauenfeldi	Cairns	Fruit	Cuelure
B. pallida	Bactrocera	mayi	Cairns	Fruit	Methyl eugenol
B. abscondita	Bactrocera	silvicola	Cape Tribulation	Male lure trap	Cuelure
B. breviaculeus	Bactrocera	silvicola	Cape Tribulation	Male lure trap	Cuelure
B. rufofuscola	Bactrocera	silvicola	Cape Tribulation	Male lure trap	Cuelure
B. silvicola	Bactrocera	silvicola	Cape Tribulation	Male lure trap	Cuelure
B. aquilonis	Bactrocera	tryoni	Broome	Fruit	Cuelure
B. neohumeralis	Bactrocera	tryoni	Cape Tribulation	Male lure trap	Cuelure
B. tryoni	Bactrocera	tryoni	Sydney	Laboratory line	Cuelure
B. strigifinis	Sinodacus	–	Cape Tribulation	Male lure trap	Cuelure
Termitorioxa termitoxena	–	–	Darwin	Protein bait trap	None

a single individual depended on the minor allele frequency filter that was applied to SNP loci. The likely reason is that a minor allele frequency filter can remove all private alleles for species represented by one individual, but not for other species, which can still have private minor alleles (depending on the threshold value). Therefore, filters were run including all species for clustering approaches, and only included species with two individuals for phylogenetic analysis.

Downstream SNP quality control included removing loci missing in a set portion of individuals (call rate threshold: values are discussed below for each analysis). The SNPs were further filtered to those with a reproducibility of 1 (a measure of the precision of genotyping of a locus across technical replicates provided by Diversity Arrays), and by the minor allele frequency as discussed below for each analysis. A single SNP was then randomly selected from any DNA fragment with multiple SNPs.

Genetic distance and clustering analyses

For genetic distance and clustering analyses using all samples, including species where only one individual was successfully sequenced, the minor allele frequency was set to a minimum of two alleles out of 32 individuals ($2n = 64$), which allowed a homozygous SNP for a single-individual-represented species to be retained. Loci that sequenced for 85% of individuals were kept for analysis. Nei's genetic distance (Nei's *D*) and Fixation index (F_{st}) were calculated between all species in R, using the library StAMPP (Pembleton *et al.* 2013). The F_{st} value was calculated using 100 bootstrap replicates to generate confidence intervals and *P*-values. A Gower principal component analysis ordination using Euclidean distance was run on the filtered SNP data using the gl.pcoa function in the dartR package (Gruber *et al.* 2018). The same genetic distance and clustering analyses were conducted on the phylogenetic SNP alignments, to ensure that clustering was consistent across datasets (Supplementary Material 2).

Phylogenomics

For the SVDquartets and maximum likelihood analyses, species represented by a single individual were removed, and the minor allele frequency was set to a minimum of four alleles out of 28 individuals ($2n = 56$). These filters were determined by a series of preliminary phylogenetic analyses, in which it was observed that the placement of species represented by a single individual tended to be poorly supported, and was sensitive to the minor allele-frequency threshold (Supplementary Material S2.7). This retained SNPs homozygous, at minimum, for both individuals of a species, and primarily identified phylogenetically informative SNPs. Loci that sequenced for 85% of individuals were kept for analysis.

Coalescent species trees were constructed using SVDquartets (Chifman and Kubatko 2014, 2015). SVDquartets, as implemented in Paup* Version 4.0a (build 159; Swofford 2002), uses a coalescent model to infer topology between randomly sampled quartets of species, and then a quartet method to assemble the random-sampled quartets into a species tree. We sampled all possible quartets from the data matrix, used Quartet FM (QFM) quartet assembly for tree inference, plus non-parametric bootstrapping with 1000 replicates.

Maximum likelihood (ML) analyses were conducted with IQ-TREE v.1.5.3 (Nguyen *et al.* 2015), using 10 000 replicates of ultrafast bootstrapping (UFBoot; Minh *et al.* 2013) and 10 000 bootstrap replicates of the SH-like approximate likelihood-ratio test (SH-aLRT; Guindon *et al.* 2010). Substitution model selection used IQ-TREE's model selection procedure for SNP data (-m TEST+ASC; Kalyaanamoorthy *et al.* 2017), using the Bayesian information criteria, resulting in the TVMe+ASC+G4 model.

Bayesian estimation of the species tree was directly estimated from SNP data by using the SNAPP module (Bryant *et al.* 2012) in Beast v.2.4.6 (Bouckaert *et al.* 2014). For the SNAPP analyses, which are more computationally intensive than the ML or SVDquartets analyses, locus number was reduced by applying a stricter 5% missing loci filter. Two independent runs were conducted, with 10 million Markov-chain Monte Carlo (MCMC) generations, sampled every 1000 generations, and default model parameters. The first 10% of each run was removed as burn-in. Convergence of run parameters was assessed using Tracer v.1.6.0 (Rambaut *et al.* 2013), and convergence of separate runs was assessed using RWTY v. 1.102 (Warren *et al.* 2017). The Bayesian SNP alignments were also run in IQ-TREE using the same parameters (Fig. 2.6 available as Supplementary Material).

Because of the failure to amplify a significant number of DNA fragments for our selected outgroup species (discussed below), we used data on the presence/absence of the DNA fragment to assign an outgroup. These data were assigned on the basis of the ability of the selected restriction enzymes to cut a DNA fragment for a species, and were broadly analogous to restriction fragment length polymorphisms. This was then quantified solely on the basis of fragment presence. A phylogenetic tree was constructed using Euclidean distance neighbour joining with 1000 bootstrap replicates in Past3 (v.3.16; Hammer *et al.* 2001), and rooted using the non-bactroceran Dacine species *Termitorioxa termitoxena*.

Results

DNA sequencing and SNP selection

DNA was successfully extracted from all individuals. Amplification of fragments following restriction digest was successful for all individuals, except the single *T. termitoxena* and both *B. strigifinis* (*Zeugodacus stringifinis* in De Meyer *et al.* 2015) individuals. Both of these species did not successfully amplify sufficient homologous DNA fragments for inclusion, and are outside the *Bactrocera* subgroup (Table 1), suggesting restriction site evolution. In total, 22 714 SNPs with an average coverage of 46.7 reads per SNP were obtained using a DArTSeq medium-density assay and proprietary pipeline (Table S2.1). After filtering (Table S2.1) the SNP data for genetic clustering analyses, including species with only one sequenced individual, the alignment consisted of 32 individuals with 1222 SNPs. Filtering of the dataset for phylogenetic analysis was undertaken for the 14 species with two individuals. The filtering pipeline for the SVDquartets and ML analyses resulted in 1874 SNPs. These SNPs had an average read count of 46.7 per individual. The more stringent filtering pipeline for the Bayesian phylogenetic analysis resulted in 578 SNPs across 28 individuals.

Genetic distance and clustering analyses

Calculations of Nei's *D* (Table 2, Table S2.2) and F_{st} (Table S2.3) between all species identified *B. umbrosa* as the most distinct from all other species. Nei's *D* analyses (Table 2) found very low (0.02) differentiation between *B. tryoni*, *B. aquilonis*, *B. neohumeralis*, *B. breviaculeus* and *B. jarvisi*. Nei's *D* was also low (0.051) between *B. dorsalis* and *B. carambolae*, with the Australian species *B. pallida* as the next closest relative.

The first three axes of the principal component analysis (PCA) explained 37.6% of the variance (15.7%, 12.6%, 9.3% respectively) in the SNP data (Fig. 2) when all species were analysed. *Bactrocera laticaudus* was located closest to the overall mean on the first and second axes, suggestive of a mid-point root consistent with the phylogenetic results (below). The PCA plot of Axis 1 versus Axis 2, and Axis 1 versus Axis 3 showed the clustering of *B. tryoni*, *B. aquilonis* and *B. neohumeralis*. *Bactrocera jarvisi* and *B. breviaculeus*, as did *B. bryoniae*, *B. laticaudus*, *B. rufofuscula*, *B. abscondita*, *B. silvicola*, *B. endiandrae* and *B. frauenfeldi. Bactrocera dorsalis* and *B. carambolae* also clustered together. *Bactrocera alyxiae*, *B. aeroginosa*, *B. pallida* and *B. umbrosa* did not cluster closely with other species. The first three axes of the PCA of the SNP data for the SVDQuartets/ML phylogenetic analyses explained 44.8% of the variance, and recovered similar species clusters (Fig. S2.2). The increase in variance explained in this analysis is likely to be due to the reduction in 'noise' associated with the low minor allele frequency in the all-species analysis (Fig. S2.1).

Phylogenomics

Because of the failure of our intended outgroup species to sequence, phylogenetic trees are rooted with *B. laticaudus*, because this species was strongly supported as the most divergent in the DNA fragment presence/absence analysis in Past3 (Fig. S2.4). The SVDQuartets phylogenetic analysis found strong support for three major clades (Fig. 3*A*), although the relationships between these three clades formed a soft polytomy (bootstrap support (BS) = 65). Clade 1 consists of the Australian *B. tryoni*, *B. aquilonis*, *B. neohumeralis, B. jarvisi, B. breviaculeus* and *B. alyxiae* (BS = 100). Within this clade, the close relationship of *B. tryoni* and *B. aquilonis* was poorly supported (BS = 86). The sequential sister relationships to the *B. tryoni–B. aquilonis* clade of *B. neohumeralis*, *B. jarvisi*, *B. breviaculeus* and *B. alyxiae* respectively, were all strongly supported (BS > 98).

The second strongly supported clade (Clade 2; BS = 97.5) consists of the two Malaysian pest species, *B. dorsalis* and *B. carambolae*, plus the Australian species *B. pallida* and *B. bryoniae*. Strong support was found for the clustering of *B. dorsalis*, *B. carambolae* and *B. pallida* (BS = 100), although the sister relationship between *B. dorsalis* and *B. pallida* was only poorly supported (BS = 88). Clade 3 (BS = 93.6) consists of the Malaysian species *B. umbrosa* and the two Australian species *B. aeroginosa* and *B. endiandrae*. However, relationships among species within this clade are not strongly supported.

Table 2. Nei's genetic distance between all species pairs, organised by current species-complex designation

Complex	Species	*B. jarvisi*	*B. laticaudus*	*B. umbrosa*	*B. alyxiae*	*B. aeroginosa*	*B. bryoniae*	*B. carambolae*	*B. dorsalis*	*B. endiandrae*	*B. frauenfeldi*	*B. pallida*	*B. abscondita*	*B. breviaculeus*	*B. rufofuscola*	*B. silvicola*	*B. aquilonis*	*B. neohumeralis*
–	*B. laticaudus*	0.12																
–	*B. umbrosa*	0.22	0.21															
alyxiae	*B. alyxiae*	0.17	0.18	0.29														
bidentata	*B. aeroginosa*	0.17	0.16	0.23	0.24													
bryoniae	*B. bryoniae*	0.15	0.15	0.26	0.22	0.21												
dorsalis	*B. carambolae*	0.12	0.11	0.22	0.18	0.17	0.13											
dorsalis	*B. dorsalis*	0.11	0.11	0.21	0.18	0.16	0.13	0.05										
dorsalis	*B. endiandrae*	0.12	0.11	0.19	0.18	0.15	0.15	0.11	0.11									
frauenfeldi	*B. frauenfeldi*	0.15	0.14	0.21	0.19	0.17	0.18	0.14	0.13	0.13								
mayi	*B. pallida*	0.19	0.19	0.30	0.25	0.25	0.21	0.14	0.11	0.19	0.22							
silvicola	*B. abscondita*	0.14	0.14	0.22	0.21	0.18	0.18	0.13	0.13	0.12	0.16	0.22						
silvicola	*B. breviaculeus*	0.09	0.12	0.23	0.16	0.17	0.15	0.12	0.11	0.12	0.15	0.19	0.15					
silvicola	*B. rufofuscola*	0.12	0.04	0.21	0.18	0.15	0.15	0.10	0.10	0.10	0.13	0.18	0.13	0.11				
silvicola	*B. silvicola*	0.13	0.11	0.20	0.20	0.15	0.16	0.12	0.12	0.10	0.15	0.19	0.14	0.13	0.11			
tryoni	*B. aquilonis*	0.08	0.13	0.23	0.17	0.17	0.16	0.12	0.12	0.12	0.16	0.18	0.16	0.08	0.12	0.14		
tryoni	*B. neohumeralis*	0.07	0.12	0.22	0.16	0.16	0.15	0.11	0.11	0.11	0.15	0.19	0.15	0.08	0.11	0.13	0.02	
tryoni	*B. tryoni*	0.08	0.13	0.23	0.17	0.17	0.16	0.13	0.12	0.12	0.16	0.20	0.16	0.08	0.12	0.14	0.02	0.02

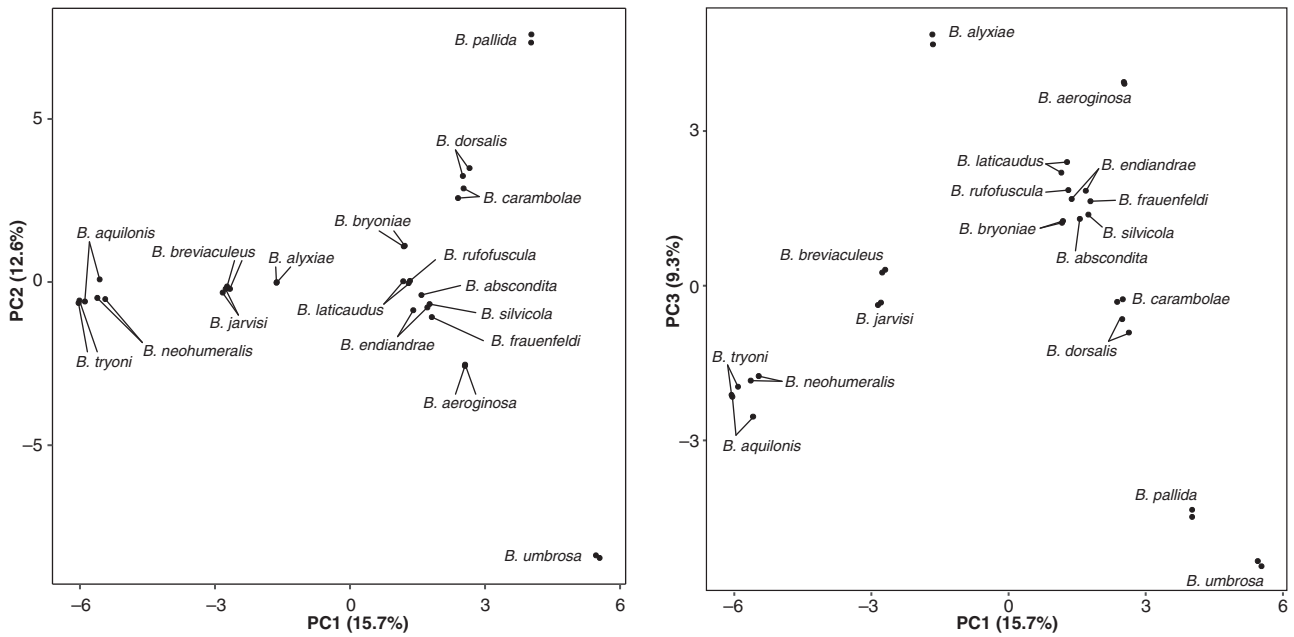


Fig. 2. Principal component analysis of single-nucleotide polymorphism (SNP) data for 18 *Bactrocera* species.

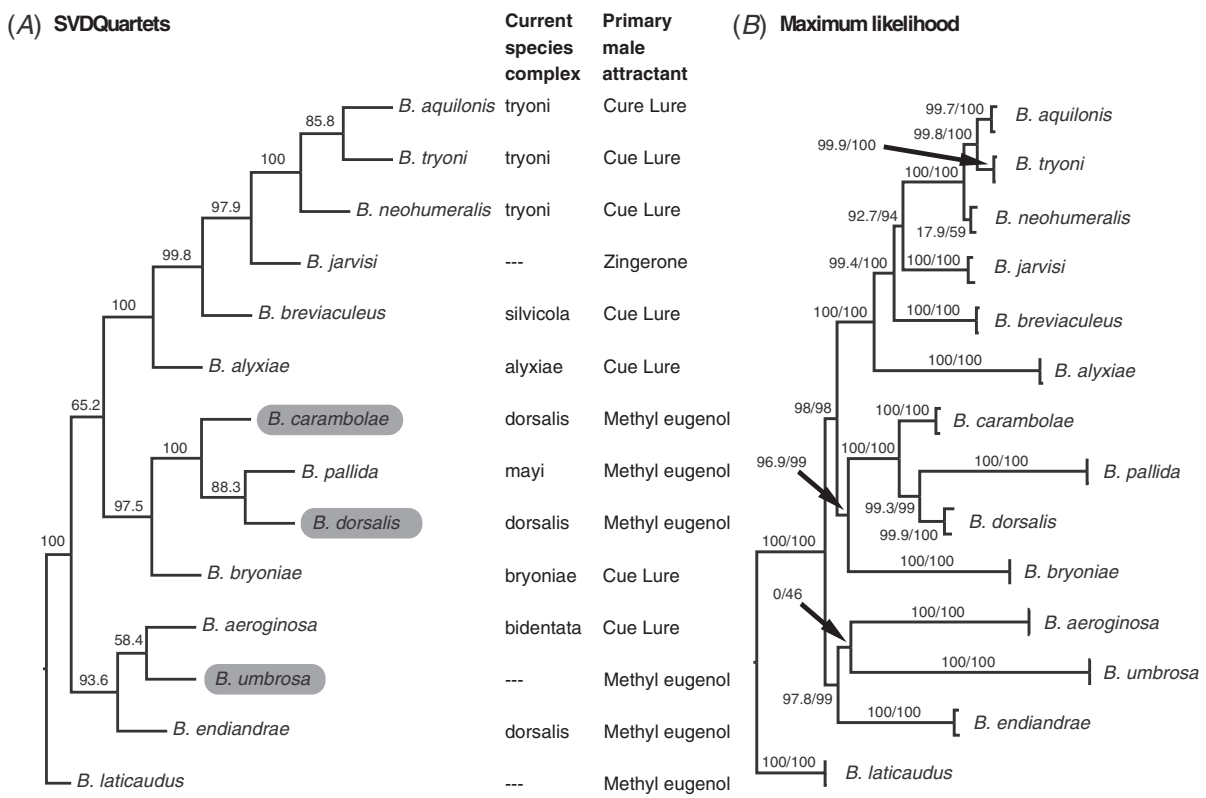


Fig. 3. (*A*) SVDQuartets (see text) and (*B*) maximum likelihood phylogenies of 14 *Bactrocera* species. In *A*, values on branches represent bootstrap support, and columns indicate currently assigned species complex and primary male attractant. Species highlighted in grey are from Malaysia, all other species are from Australia. In *A*, values on branches represent bootstrap support across 1000 replicates. In *B*, values on branches represent ultrafast bootstrap/SH-like approximate likelihood ratio test support across 10 000 replicates.

The ML phylogenetic analysis found the same species-level topology as the SVDQuartets analysis (Fig. 3*B*). Unlike the SVDQuartets analysis, the ML analysis strongly supported (UFBoot $\geq$ 0.95, SH-aLRT $\geq$ 0.80) the sister relationship between Clades 1 and 2 (as defined above), and the sister relationship between *B. dorsalis* and *B. pallida.* The ML analysis also found low support for the clustering of the two *B. neohumeralis* individuals, although they were still strongly supported as sister to the *B. tryoni–B. aquilonis* clade.

The SNAPP species tree analysis resolved only some portions of the phylogeny (Fig. S2.5), with generally low support throughout the tree. Low support values were due to the low number of SNPs included in the analysis, as computation time becomes intractable with greater SNP numbers. Differences between the SNAPP analysis and the SVDQuartets topology were always associated with poor posterior probabilities (PP < 0.80). The Bayesian analysis strongly supported the clustering of clade 1 (as defined above) species (excepting *B. alyxiae*) as per the SVDQuartets analysis (PP = 1), namely a clade consisting of *B. tryoni*, *B. aquilonis*, and *B. neohumeralis* (PP = 1) and another consisting of *B. dorsalis*, *B. carambolae*, and *B. pallida* (PP = 1). However, unlike the SVDQuartets and ML analyses, this analysis found strong support (PP = 1) for the sister relationship of *B. dorsalis* and *B. carambolae*, with *B. pallida* as the next closest relative.

Discussion

Recent phylogenetic analyses of *Bactrocera* relationships have finally resolved several relationships at the species-complex level (Dupuis *et al.* 2018; San Jose *et al.* 2018). In particular, Dupuis *et al*. (2018) was able to resolve the phylogeny by using an amplicon sequencing approach. While providing an excellent contrast to test the quality of our sequencing approach, the amplicon sequencing method utilised by Dupuis *et al*. (2018) is not suitable for the continuous addition of samples over time (e.g. for biosecurity assessments of individual flies), and is bioinformatically intensive. By contrast, our nuclear-genome-wide dataset is capable of continuous addition of flies over time, with relatively simple bioinformatic pipelines providing a fast resolution of individual clustering for biosecurity purposes.

Taxonomic implications

The three species traditionally classified in the dorsalis complex in our study (*B. dorsalis*, *B. carambolae* and *B. endiandrae*) showed a paraphyletic relationship in Dupuis *et al*. (2018), San Jose *et al*. (2018) and our phylogenies (Figs 2, 3, S2.5). *Bactrocera dorsalis* and *B. carambolae* form a well-supported clade that also included *B. pallida* from the mayi complex, whereas *B. endiandrae* is not a member of this clade (Fig. 3). Indeed, both these recent studies and our genetic distance analyses (Table 2) placed *B. endriandrae* closer to the two species in the silvicola complex (*B. rufofuscula* and *B. silvicola*) than to the dorsalis complex. The original assignment of *B. endiandrae* to the dorsalis complex was based on morphological resemblance (general characteristics and colouration) with *B. dorsalis* (Hardy 1951). Krosch *et al*. (2012), using 16S, *COII* mitochondrial and *white-eye* nuclear gene fragments, also placed *B. endiandrae* outside the dorsalis complex, although in a less basal position than in our results. The evidence from all the most recent studies, including ours, thus, does not support the original inclusion of *B. endiandrae* in the dorsalis complex.

The strong support for *B. laticaudus* as the outgroup for our set of species has not been found in other studies. Virgilio *et al*. (2015) was able to place *B. laticaudus* only within the subgenus with strong support. The seven-gene phylogeny of San Jose *et al*. (2018) found strong support for *B. laticaudus* as being closely related to the mayi complex, and moderate support for the tryoni complex as the outgroup. However, Dupuis *et al*. (2018) were not able to strongly resolve structure within the subgenus, and did not include *B. laticaudus* in their analysis. Our phylogenetic results suggest that this finding is consistent with a midpoint rooting of the phylogeny, and this orientation could change with addition of further samples.

The strong support for the placement of *B. pallida* with the dorsalis species complex is well supported by Dupuis *et al*. (2018), San Jose *et al*. (2018) and our study (Fig. 3). *Bactrocera pallida* has been considered part of the mayi complex, which also includes *B. mayi and B. tenuifascia* (Drew 1989). Prior to the formal description of the mayi complex (Drew 1989), *B. mayi* was assigned to the dorsalis complex (Hardy 1969), whereas *B. pallida* was unassigned to any complex. However, the placement of *B. mayi* in the dorsalis complex by Hardy (1969) has not been supported by any molecular study, and there is no for support a close evolutionary relationship of these complexes (Krosch *et al.* 2012; Dupuis *et al.* 2018; San Jose *et al.* 2018).

The three species of the tryoni complex (*B. tryoni*, *B. aquilonis* and *B. neohumeralis*) cluster together in a well-supported clade (Fig. 3). The sister group to this clade is *B. jarvisi*, which has been traditionally placed in the subgenus *Afrodacus* (Drew 1989), a finding that is congruent among the present study, the study of San Jose *et al*. (2018) and that of Dupuis *et al*. (2018). Although it has been recently revised to the *Bactrocera* subgenus (Copeland *et al.* 2004), *B. jarvisi* was unassigned to a species complex, and is attracted to a different lure than are the species of the tryoni complex (Table 1). Yet, interspecific crosses between *B. jarvisi* and *B. tryoni* are readily achievable (Cruickshank *et al.* 2001; Shearman *et al.* 2010), supporting a close relationship.

Bactrocera breviaculeus, a member of the silvicola complex, also clustered with the tryoni complex and *B. jarvisi* in all our analyses (Figs 2, and 3). This monophyletic placement is also consistent with results in Krosch *et al*. (2012). The assignment of *B. breviaculeus* into the silvicola complex was based on synapomorphy (Drew 1989), yet the PCA of our genome-wide data (Fig. 2) suggested that *B. breviaculeus* clusters with *B. jarvisi*, and not the silvicola complex (*B. silvicola* and *B. rufofuscola*). In conjunction with the clustering of *B. endiandrae* with *B. silvicola* (Fig. 2), our data suggest uncertainty about species assignments within the silvicola complex.

Consistent with earlier studies (Smith *et al.* 2003; Krosch *et al.* 2012), our analysis suggested that lure attraction is not a reliable feature for phylogenetic placement. Non-monophyly of this trait is observed within most of our clades. For example, *B. aeroginosa* males were responsive to Cuelure and *B. umbrosa*

and *B. endiandrae* males responded to methyl eugenol, but the three species were monophyletic on our phylogenetic tree. Even different species within a complex can be attracted to different attractants. Clarke *et al*. (2005) examined 75 species in the dorsalis complex, of which 35 responded to Cuelure and 26 to methyl eugenol, whereas lures for the 12 other species were unknown. Following a recent revision (Drew and Romig 2013), another 11 species were added to the dorsalis complex, with seven responding to Cuelure and four to methyl eugenol. It must be noted that, for that complex, the highly destructive methyl eugenol-responding flies (*B. philippinensis* and *B. papayae*) have been synonymised (Drew and Romig 2013), whereas *B. papayae* and *B. invadens* have been synonymised with *B. dorsalis* (Schutze *et al.* 2015). Although *B. papayae* and *B. invadens* were later withdrawn from the synonymy (Drew and Romig 2016), this withdrawal has been rejected because it was not subjected to a peer review (Schutze *et al.* 2017).

Biogeographic implications

Previous studies have suggested that the subgenus arose in Asia (Drew and Hancock 1999; Krosch *et al.* 2012), and have not found reciprocal monophyly in regions, suggesting repeated dispersal between regions (Krosch *et al.* 2012). Despite the inclusion of only three non-Australian species in our phylogeny (*B. dorsalis*, *B. carambolae* and *B. umbrosa*), our phylogeny also suggests that movements are required between Southeast Asia and Australia to explain the patterns of diversity (Figs 2, 3, S2.5). Both *B. dorsalis* and *B. carambolae* are non-Australian species, but they are closely related to the PNG–Australian *B. pallida*, requiring at least one trans-continental dispersal event in this clade. Similarly, the non-Australian *B. umbrosa* is related to several Australian species, requiring historical dispersal between the regions to explain this pattern of diversity. The routes for these invasions remain unclear and may have changed over time. Both *B. dorsalis* and *B. umbrosa* are present in New Guinea (Regions 1 and 2 in Fig. 1), providing support for dispersal across the Torres Strait, together with the recent (now eradicated) incursion of the papaya fruit fly, *B. papaya–B. dorsalis*, into northern Queensland, Australia (Cantrell *et al.* 2002; Meats *et al.* 2008).

In view of the small number of species that we have studied, there may have been many more successful dispersal events than those evidenced in our phylogenies. Given the recent dorsalis radiation suggested by Krosch *et al*. (2012), some of these events may have occurred significantly more recently than 6.2 million years ago (95% CI 8.4–4.3). The frequency of events implied by our findings confirms the high biosecurity threat to Australia posed by Asian species in the *Bactrocera* subgenus. In particular, our study lacked sampling of species from across the Indonesian archipelago and northern Australia (particularly the Kimberley), which limited our ability to test for additional wind-driven pathways across the Timor Sea (Fig. 1). A better understanding of both the phylogeny and biogeography of the subgenus across Southeast Asia and northern Australia would seem to be a high priority for the future *Bactrocera* research agenda.

Future research directions

Our analyses found strong support for most nodes in our SVDQuartets and ML analyses (Fig. 3). However, the relatively low number of SNPs post-quality filtering and the poor SNP coverage of non-*Bactrocera* subgenus species suggest that some changes in the sequencing strategy would be needed to extend the methodology to a wider set of subgenera. A low number of SNPs was successfully amplified for *B. strigifinis* (not shown), too few for the species to be included in analyses. This suggests that minor revision of the restriction enzymes used to reduce genome complexity is likely to improve representation across subgenera. Increasing the level of sequencing is also likely to increase sufficiently the number of sequence fragments amplified across all species, so as to allow for their inclusion in future analyses.

With these minor modifications, our sequencing approach provides several opportunities to support Australian biosecurity. Besides the development of invasion pathways research as discussed above, these data, with a combination of genetic clustering and phylogenetic analyses, may enable a fast and accurate identification of individual *Bactrocera* to species. In conjunction with the development of a reference genomic library, with sequenced individuals verified by taxonomic experts, rapid assignment of individuals to species is likely to be possible. This approach offers an opportunity to automate taxonomic assignments of individually detected biosecurity pests.

Conclusions

Our results have provided a strongly supported phylogenetic hypothesis for species in the *Bactrocera* subgenus, with a similar topology to more time- and bioinformatically intensive sequencing processes. Recovered relationships do not support several species complex assignments, and do not support the use of primary attractant type for taxonomic assignment of species to complex. Despite a low number of non-Australian samples, we recovered at least two dispersal events between Southeast Asia and Australia, suggesting a strong history of dispersal between the regions. Our results demonstrated the utility of genome reduction approaches to assessing evolutionary relationships in rapidly diversifying *Bactrocera*, a straightforward approach that may simplify taxonomic assessments in a significant biosecurity risk group.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of funding

Sequencing was funded by a Macquarie University Early Career Researcher Grant (to RAC) and a Western Sydney University Early Career Researcher Grant (to RAC).

Acknowledgements

Field collection was undertaken by the Macquarie–CSIRO collaboration as part of the SITPlus consortium funded by Horticulture Innovation Australia. We particularly thank Jane Royer for assistance with *Bactrocera* species identification. We thank Ian Lacey and Bill Woods from DAFWA for assisting with field collection in Broome and providing temporary rearing space in Perth, and Stuart Gilchrist, Deborah Shearman, Marianne Frommer and John Sved of UNSW for the Sydney *B. tryoni* line.

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Handling editor: Gonzalo Giribet