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Molecular characterisation of the twisted wing endoparasitoid *Dipterophagus daci* (Strepsiptera) and its interactions with *Wolbachia* and multiple tephritid fruit fly host species

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Statement of authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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Sharon Chepkemoi Towett-Kirui
September 2021

Preface

This PhD thesis comprises five chapters; Chapter 1 provides the general introduction and overview of the fundamental aspects of this study, the general relevance and the knowledge gaps. It highlights the scope, the research questions, hypothesis and objectives of the thesis. Chapters 2, 3 and 4 are experimental chapters presented as independently publishable manuscripts. The experimental chapters present the background, methodology, findings and discussion of the key questions highlighted in Chapter 1. Chapter 5 is the general discussion of the key findings of the experimental chapters, it also places the findings of this thesis in the context of other studies. It also presents the limitations of this study and also gives key future directions. Chapter 2 has been published in *Environmental Microbiology* and is cited in the thesis as Towett-Kirui et al. (2021). Chapter 3 has been submitted to a peer-reviewed journal and Chapter 4 is in preparation for publication and will be submitted to a peer-reviewed journal in the near future.

I am the principal author of the thesis chapters. I conceptualised and designed the study, performed the experiments and data analysis with input and guidance from my supervisors, Assoc. Prof. Markus Riegler (principal supervisor) and Dr Jennifer L. Morrow (co-supervisor). The insect specimens used in this study were provided and identified by Dr Jane E. Royer and Shannon Close who are also co-authors of some thesis chapters.

This thesis was completed during the SARS-CoV-2 (COVID-19) pandemic. This resulted in no laboratory access for 3 months in a critical time period, and restricted access afterwards. This significantly affected parts of the data collection required for the thesis write-up. For instance, I was unable to acquire more field-collected fruit fly samples from Queensland for my fourth experimental chapter which would have involved an extensive survey of *Dipterophagus daci* and *Wolbachia* in Australian tephritid fruit flies. This is because a

planned trip to Queensland was not possible in March 2020 due to border closures. Furthermore, I had limited time to access the laboratory during and after the lockdown periods of 2020. Upon return to the lab, I used this opportunity to wrap-up the already started experiments for experimental chapters 1, 2 and 3. However, this also resulted in the omission of the fourth experimental chapter, despite the research efforts put in preliminary data collection. However, as part of this chapter, I was able to screen approximately 500 previously acquired fruit fly samples only for presence of *D. daci* with limited analysis of its genetic diversity. This data set will be completed and presented as a separate manuscript after thesis submission.

The thesis structure is highlighted below:

Chapter1: **General introduction**

Chapter 2: **Published in *Environmental Microbiology* as below:**

Towett-Kirui, S. Morrow, J. L. Close, S. Royer, J. E. & Riegler, M. (2021). Host-endoparasitoid-endosymbiont relationships: concealed Strepsiptera provide new twist to *Wolbachia* in Australian tephritid fruit flies. *Environmental Microbiology*. https://doi.org/https://doi.org/10.1111/1462-2920.15715.

Supporting information and supplementary figures and tables are provided in Appendix A at the end of the thesis.

Chapter 3: **Substantial rearrangements, -1 frameshift deletion and low diversity in the mitogenomes of *Wolbachia*-infected strepsipteran endoparasitoid when compared to its hosts.** Manuscript submitted to a peer-reviewed journal.

Supporting information and supplementary figures and tables are provided in Appendix B at the end of the thesis.

Chapter 4: **Bacterial communities are less diverse in the endoparasitoid *Dipterophagus daci* (Strepsiptera) than its fruit fly hosts and are dominated by *Wolbachia*.** Manuscript in preparation for submission to a peer-reviewed journal.

Supporting information and supplementary figures and tables are provided in Appendix C at the end of the thesis.

Chapter 5: **General discussion**

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Abbreviations

aa	amino acids
ANOVA	analysis of variance
atp	ATP synthase
AW-IPM	area-wide integrated pest management
CI	cytoplasmic incompatibility
cif	*cytoplasmic incompatibility factor*
cob	*cytochrome b*
cox1	*cytochrome oxidase 1*
Cq	quantification cycle
DsRed	red fluorescent protein from a *Discosoma* sp.
EGFP	enhanced green fluorescent protein
GSS	genetic sexing strains
IIT	incompatible insect technique
MAT	male annihilation technique
MLST	multi-locus sequence typing
MK	male killing
ML	maximum likelihood
mtDNA	mitochondrial DNA
nad	*NADH dehydrogenase*
NCBI	National Centre for Biotechnology Information
ORF	open-reading frame
PCGs	protein coding genes
PERMANOVA	permutational multivariate analysis of variance

PCR	polymerase chain reaction
QC	quality control
qPCR	quantitative PCR
RNA Pol II	*RNA polymerase II*
rRNA	*ribosomal RNA*
rrnL	large ribosomal subunit
rrnS	small ribosomal subunit
RSCU	relative synonymous codon usage
SIT	sterile insect technique
SNPs	single nucleotide polymorphisms
ST	sequence type
tRNA	transfer RNA
WGS	whole genome sequencing
wsp	*Wolbachia surface protein*

Abstract

Insects represent the most diverse and successful group of organisms. Therefore, they have diverse interactions with other organisms, including with gut bacteria, endosymbiotic bacteria and parasites such as parasitoids. Overall, these interactions affect the biology, ecology and evolution of the interacting partners. A substantial body of research work exists which has mainly focused on host-endosymbiont and host-parasite interactions; however, the combination of these in host-parasite-endosymbionts interactions is less explored. Australia hosts over 300 species of tephritid fruit flies, including the Queensland fruit fly, *Bactrocera tryoni*, Australia's most significant horticultural pest. Australian tephritid fruit fly species have previously been found to be infected by two strains of the common insect endosymbiont *Wolbachia*. *Wolbachia* are maternally inherited endosymbionts in about 50% of insect species and can affect host reproduction and fitness. The phylogenetic incongruence of *Wolbachia* and their hosts indicates that horizontal transfer between species can also happen. Previously reported *Wolbachia* infections in Australian tephritid fruit fly species were unusual because they were detected in only seven out of 24 tested species (29 %) at low prevalence and titres and were restricted only to individuals of tropical Australia. This PhD thesis investigated these *Wolbachia* infections further by performing whole genome sequencing (WGS) of the field-caught *Wolbachia*-positive flies. This revealed an unexpected presence of almost complete mitochondrial genomes (mitogenomes) of another insect, in addition, to the expected fruit fly mitogenome in the genomic libraries of fruit fly. The additional mitogenomes belonged to a twisted-wing endoparasitoid, *Dipterophagus daci* (Strepsiptera), suggesting a possible link between *Wolbachia* and the presence of *D. daci* in tephritid fruit flies. *Dipterophagus daci* is the only described strepsipteran endoparasitoid of Diptera (besides an undescribed species from platystomatid flies in Papua New Guinea) and

has previously been reported from 19 tephritid fruit fly species in Australia and the Solomon Islands.

This study therefore investigated the host-endoparasitoid-endosymbiont interaction between tephritid fruit fly species, *Dipterophagus daci* and *Wolbachia*. The introductory Chapter 1 describes the background information pertaining to the biology of tephritid fruit flies, *Wolbachia* and *D. daci*. It identifies the knowledge gaps and presents the scope of the study, research questions and aims of the thesis.

Chapter 2 details the analysis and findings of *D. daci* in the WGS data sets of the field-caught *Wolbachia*-positive flies. It presents the molecular diagnostics technique used to detect *D. daci* in *Wolbachia*-positive flies of seven fruit fly species that were not visibly parasitised (unstylopised). This then revealed that most *Wolbachia*-negative flies were *D. daci*-negative indicating a link between *Wolbachia* and *D. daci* in the tephritid fruit flies. To confirm this link, *D. daci* specimens were dissected from 23 fruit fly specimens that were visibly parasitised (stylopised), and screened for *Wolbachia* using PCR, and further evaluated by quantitative PCR. Interestingly, *Wolbachia* titres were higher in *D. daci* specimens relative to the fruit fly tissues, providing further evidence that *Wolbachia* infects *D. daci.* Therefore, it was concluded that concealed early stages of strepsipteran parasitisation had led to the incorrect previous assignment of *Wolbachia* infection to the seven tephritid species. Additionally, this chapter presented the first genetic characterisation of *D. daci*, its first phylogenetic placement within the strepsipteran family of Halictophagidae, and its relationship with *Wolbachia* and tephritids. It also provided three new host species records for *D. daci* (i.e. 22 recorded host species) and demonstrated the possibility of obtaining endoparasitoid, endosymbiont and host sequence data from the same individual libraries.

The genetic characterisation of *D. daci* was further explored in Chapter 3, which delved into the assembly and characterisation of the mitogenomes of *D. daci* and its fruit fly host species.

This work presented six mitogenomes of the *Wolbachia*-infected *D. daci* (and thereby the first sequenced mitogenomes of a halictophagid) and nine mitogenomes of fruit fly host species (*Bactrocera frauenfeldi*, *Bactrocera neohumeralis*, *Bactrocera tryoni* and *Zeugodacus strigifinis*). The *D. daci* mitogenomes were found to be highly rearranged relative to the ancestral pattern of insect mitogenomes, while this was not observed in the fruit fly host species' mitogenomes. Some of the gene rearrangements observed in *D. daci* had previously been observed in three other strepsipterans, *Xenos vesparum*, *Xenos moutoni* and *Mengenilla australiensis*, however, other rearrangements (including the -1 frameshift deletion of the *nad5* gene and rearrangement of ribosomal RNA genes) were unique to *D. daci*, suggesting that their mitogenomes experienced additional evolutionary steps. This could be linked to the more extreme level of parasitism seen in *D. daci* and Halictophagidae with an almost completely endoparasitic life cycle. Furthermore, intraspecific mitogenome diversity was very low in *D. daci*, while the fruit fly host species mitogenomes were polymorphic. This suggests that *Wolbachia* may have caused the loss of mitochondrial DNA diversity possibly by influencing *D. daci* reproduction or fitness.

Chapter 4 explored the bacterial communities associated with *D. daci* as the first characterised microbiome of a strepsipteran. The *D. daci* microbiome was not diverse and was dominated by very few Proteobacteria species while other bacterial taxa had low relative abundance. This dominance of Proteobacteria was due to the high relative abundance of *Wolbachia* (Alphaproteobacteria). Surprisingly, *Wolbachia* dominance was not observed in the fruit fly hosts that either had early stages of parasitisation or were not parasitised by *D. daci*. Further findings from this study showed that the *D. daci* microbiome was very distinct from all fruit fly microbiomes. Moreover, there was a distinct clustering of microbiomes of *Zeugodacus strigifinis* from *B. tryoni*, *Bactrocera neohumeralis*, *Bactrocera frauenfeldi* and *Bactrocera bryoniae*. *Zeugodacus strigifinis* is a minor flower pest of *Cucurbitaceae* while

the four *Bactrocera* species are important fruit pests, suggesting that host plant/diet may be a driver for this microbiome differentiation. The impact of parasitisation by early stages of *D. daci* on fruit fly microbiome was also investigated and this showed a change in relative abundance of bacterial communities. In addition, parasitisation by *Wolbachia*-positive *D. daci* also revealed a change in relative abundance of bacterial communities in parasitised fly samples. These findings also reveal that concealed parasitisation by *D. daci* could obstruct microbiome studies of field caught fruit flies.

Chapter 5 highlights and discusses the key findings of this thesis and suggests future research directions. Overall, this study investigated and resolved the enigma of *Wolbachia* infections previously detected in tephritid fruit flies and presented evidence that the presence of *Wolbachia* in tephritid fruit flies was due to concealed early stages of parasitisation by *D. daci*. This reveals that the detection of *Wolbachia* in host taxa could be due to concealed parasitisation by strepsipterans or other endoparasitoids, and this could lead to incorrect assignment of *Wolbachia* to a wrong host. Additionally, it suggests that Australian tephritid fruit flies may not be naturally infected by *Wolbachia* therefore making them amenable for control using *Wolbachia*-based incompatible insect technique in the future. Furthermore, this study presents six *D. daci* mitogenomes and nine fruit fly mitogenomes which will be a useful source for future studies, in particular of the biology and ecology of the unique strepsipteran *D. daci*, and its impact on fruit fly population dynamics. This work also presents the first microbiome of a strepsipteran, which is a valuable contribution to the parasite microbiome studies.

Chapter 1

General introduction

1.1 Insect interactions

Insects are the most significant group of organisms both in abundance and species diversity (Stork et al. 2015). Their success may be attributed to their small size, exoskeleton and diverse life cycles which make them adaptable to many ecological niches. The ability for insects to occupy diverse ecological niches provides a great avenue for them to form interactions with diverse organisms (Burnett, 1960; Dheilly et al. 2019; Dheilly et al. 2015; Eleftherianos et al. 2010; Zchori-Fein & Bourtzis, 2012). These interactions vary across insect species, life-stage as well as habitat (Audsley et al. 2018; Burnett, 1960; Cavichiolli de Oliveira & Cônsoli, 2020) and could include interactions such as host-symbiont or host-parasite interactions involving just two (or more) species; or more complex, multi-trophic interactions such as host-parasite-endosymbiont interactions (Burnett, 1960; Frago et al. 2012; Gupta & Nair, 2020)

1.1.1 Host-symbiont interactions

Insects have formed numerous close and long-term interactions with microorganisms (e.g. bacteria and fungi) living within them, also known as symbioses (Buchner, 1965; Salje, 2021). These symbiotic interactions play crucial roles in both the insect host's and the symbiont's biology, ecology and evolution. For example, an evolutionary outcome of close symbiotic interactions could lead to rapid evolution or genome loss/reduction in symbiont genomes as demonstrated for many symbiotic bacteria (Moran et al. 2003). Another example, ants and their bacterial symbiont *Blochmannia* (Gammaproteobacteria) have been observed to undergo cospeciation characterised by phylogenetic congruence between host and symbiont, and rapid evolutionary rates of the symbiont (Degnan et al. 2004). Reduced genome sizes are a characteristic of endosymbionts, for instance the psyllid endosymbiont *Carsonella ruddii*

(Gammaproteobacteria) has a genome size of ~160 kb (Nakabachi et al. 2006); the genome of *Nasuia deltocephalinicola* (Betaproteobacteria) in the leafhopper *Macrosteles quadrilineatus* is 112 kb and is currently the smallest bacterial genome known (Bennett & Moran, 2013). Symbiotic bacteria are diverse and their host effects can range from beneficial to harmful (Overstreet & Lotz, 2016). Beneficial bacteria can take part in vital host processes such as immunity, host development, reproduction and protection against natural enemies (Hooper et al. 2012; Oliver et al. 2014).

Symbiotic bacteria can either be obligate or facultative symbionts, and may also be endosymbiotic if they live within host cells (Baumann, 2005; Kucuk, 2020). Obligate symbionts (sometimes referred to as primary symbionts) are usually vertically transmitted from mother to offspring and are essential for their host's survival and reproduction (Baumann, 2005; Moran et al. 2008). Obligate endosymbionts provide essential nutrients that are inadequate in the host's diet; for instance the aphid symbiont, *Buchnera aphidicola* (Gammaproteobacteria), provides essential amino acids absent in its host's diet (Douglas, 1998). Similarly, the tsetse fly symbiont, *Wigglesworthia glossinidia* (Gammaproteobacteria) supplements its host with nutrients, however, it has also been shown to increase its host's susceptibility to trypanosome infection (Pais et al. 2008). Obligate endosymbionts often live within special host cells (bacteriocytes) and/or tissues (bacteriome). In contrast to obligate symbionts, facultative symbionts (sometimes referred to as secondary symbionts) are not essential for host growth and development but can provide protection against natural enemies. For instance, the facultative symbionts of aphids, *Hamiltonella defensa, Regiella insecticola* and *Serratia symbiotica* (all Gammaproteobacteria), can protect their hosts against parasitoid eggs and larvae (Ferrari et al. 2004; Oliver et al. 2003; Oliver et al. 2014). Facultative symbionts such as *Wolbachia, Rickettsia* (both Alphaproteobacteria), *Cardinium* (Sphingobacteria) and *Spiroplasma* (Mollicutes) can also live within insect cells but may not

always be restricted to particular host cells or host tissues. They are primarily transmitted vertically, however, occurrence of similar or identical facultative endosymbionts in phylogenetically diverse hosts, as well as their high prevalence suggest that they can be transmitted horizontally from one species to another (Chiel et al. 2009; Oliver et al. 2010). Several of these endosymbionts have been found to have numerous fitness benefits to their hosts (Moran et al. 2008; Teixeira et al. 2008; Xie et al. 2014), however, some can also manipulate host reproduction (Engelstädter & Hurst, 2009; Gherna et al. 1991; Montenegro et al. 2005; Renvoisé et al. 2011; Werren et al. 2008; Werren, 1997).

1.1.2 Host-parasitoid interactions

Besides interactions with endosymbionts, arthropods also interact with parasitoids. Parasitoids can either be ecto- or endoparasitoids (i.e. development outside or within host individuals) and are characterised by having one or more life stages developing on or in an insect host and killing them in the course of their lifecycle (Eggleton & Belshaw, 1992; Eggleton & Gaston, 1990; Gang & Hallem, 2016; Kathirithamby, 1991, 2009; Silveira et al. 2019; Tseng & Myers, 2014). Parasitoids are diverse and the interactions with their hosts are also varied. Due to their host-linked lifecycle parasitoids have evolved mechanisms to survive the host immunity, develop successfully and also allow successful development of their offspring (Kathirithamby et al. 2003; Volkoff et al. 2020). These mechanisms could have an impact on host fitness, ecology, evolution and behaviour (Libersat et al. 2018). Insect parasitoids mostly belong to the two insect orders Hymenoptera and Diptera, and can develop in one or more species as well as one or more host life stages (Silveira et al. 2019). For instance, the wasp *Nasonia vitripennis* is an ectoparasitoid of dipteran pupae (Rivers et al.

2002) while the wasp *Fopius arisanus* is an endoparasitoid of tephritid larval stages (Carmichael et al. 2005; Rocha et al. 2016).

1.1.3 Host-parasitoid-endosymbiont interactions

Host-parasitoid-endosymbiont interactions in arthropods are complex. These tripartite interactions can be viewed from several perspectives: the role of the endosymbiont on the host-parasite association; the role of the parasite on host-endosymbiont interaction; and/or the role of the parasite-endosymbiont interaction on the host. Studies of the relationship between *Drosophila simulans*, its *Wolbachia* endosymbiont and the parasitoid wasp *Leptopilina heterotoma* revealed that *Wolbachia* can have a negative impact on the immunity of both the parasitoid wasp and its fruit fly host (Fytrou et al. 2006). The incidence of particular endosymbionts in parasitoid and parasite species as well as their hosts suggests that parasitoids and parasites could play a role in horizontal transmission of endosymbionts from one host species to another (Cook & Butcher, 1999). For instance, ectoparasitic mites can transmit *Spiroplasma* between *Drosophila* species (Jaenike et al. 2007). Similarly, *Wolbachia* can be transmitted between individuals of the whitefly *Bemisia tabaci* by the parasitoid *Eretmocerus furuhashii* (Ahmed et al. 2015). Additionally, the strepsipteran endoparasitoid *Elenchus japonicus* has been found to carry the same *Wolbachia* as its two rice planthoppers hosts, *Laodelphax striatellus* and *Sogatella furcifera,* and due to this finding it was suggested that *E. japonicus* transmitted *Wolbachia* between the two hosts (Noda et al. 2001). However another scenario for this outcome was also raised: that *E. japonicus* shares the same *Wolbachia* strains with the two planthopper hosts due to their close association; and that due to their unique biology and that Strepsiptera effectively castrate their host, the likelihood of *E. japonicus* transmitting *Wolbachia* to its host species was also

questionable (Hughes et al. 2004). Rickettsia-like microorganisms have previously been detected in five strepsipteran species including *E. japonicas* (Kathirithamby, 1998), suggesting that Strepsiptera could be actual hosts of *Wolbachia*. A similarly intriguing interaction was found in another endosymbiont-parasitoid-host system where *Wolbachia* first detected in *Ixodes ricinus* ticks was later actually attributed to the presence of a concealed hymenopteran endoparasitoid *Ixodiphagus hookeri* (Plantard et al. 2012). Similar findings have also been reported in tick populations from the Netherlands (Tijsse-Klasen et al. 2011) which supported the hypothesis that the presence of *Wolbachia* in *I. ricinus* could be due to parasitisation with *I. hookeri*. Other than *Wolbachia*, the endosymbiont *Arsenophonus nasoniae* (Gammaproteobacteria) detected in *I. ricinus* ticks was also detected in the parasitoid wasp *I. hookeri* which was then found to likely be the true host of this bacterium (Bohacsova et al. 2016).

1.2 Tephritid fruit flies

The family Tephritidae contains approximately 5,000 species, and about 250 of these are considered pests (White & Elson-Harris, 1992). Most of these pests belong to the genera *Anastrepha, Bactrocera, Ceratitis, Dacus, Rhagoletis* and *Zeugodacus* (White & Elson-Harris, 1992). Tephritid fruit flies can be categorised into two groups based on their biological and ecological characteristics, (i) the univoltine group is characterised by an obligate diapause and occupies temperate climates while (ii) the multivoltine group lacks obligate diapause and can be found in tropical and subtropical climates (Bateman, 1972).

Australia has a wide range of climatic conditions and hosts over 300 species of tephritid fruit flies (Drew, 1989). Most Australian tephritid fruit flies are native and are not considered as pests, however, some native and introduced species cause significant economic damage

(Drew, 1989; Hancock et al. 2000). These include native *Bactrocera tryoni* (Froggatt), *Bactrocera neohumeralis* (Hardy), *Zeugodacus cucumis* (French), *Bactrocera musae* (Tryon), *Bactrocera jarvisi* (Tryon), *Bactrocera aquilonis* (likely the same species as *B. tryoni*), *Bactrocera bryoniae* and *Bactrocera kraussii*, and accidentally introduced/invasive *Ceratitis capitata* and *Bactrocera frauenfeldi* (Plant Health Australia (PHA), 2018; Drew, 1989). The Queensland fruit fly, *B. tryoni* was declared a horticultural pest in the early 20th century and is overall the most significant (Drew, 1989; Hancock et al. 2000). Additionally, the Mediterranean fruit fly (*C. capitata*), accidentally introduced to Australia in the 1890s is an equally serious pest now established in parts of Western Australia and is also a threat to horticultural industries in eastern Australia (Dominiak & Daniels, 2012; White & Elson-Harris, 1992) while *B. tryoni* and other damaging pest species do not occur in Western Australia, and are absent from South Australia and Tasmania (Dominiak & Daniels, 2012; Holz et al. 2010). However, incursions of *B. tryoni* and *C. capitata* into fruit fly free areas occur, presenting serious threats to horticultural industries requiring eradication efforts (Popa-Báez et al. 2021).

1.2.1 Queensland fruit fly, *Bactrocera tryoni*

Queensland fruit fly (*B. tryoni*) is recognised as the economically most damaging fruit fly species in Australia (Figure 1. 1). It originates from tropical and sub-tropical coastal Queensland and northern regions of New South Wales and is now widespread across eastern Australia, parts of the Northern Territory, northern Western Australia and several Pacific islands (Gilchrist & Ling, 2006; Popa-Báez et al. 2021). The original hosts of *B. tryoni* are native rainforest plant species, however, *B. tryoni* is polyphagous and can infest many

cultivated fruits and vegetables (Hancock et al, 2000; White and Elson-Harris, 1994). It does not exhibit an obligate diapause and its geographic range expansion is facilitated by favourable climatic conditions, agricultural practices, availability of the host fruit and transport of adult individuals or infested fruit (Meats, 1981; Meats & Edgerton, 2008; Yonow & Sutherst, 1998).

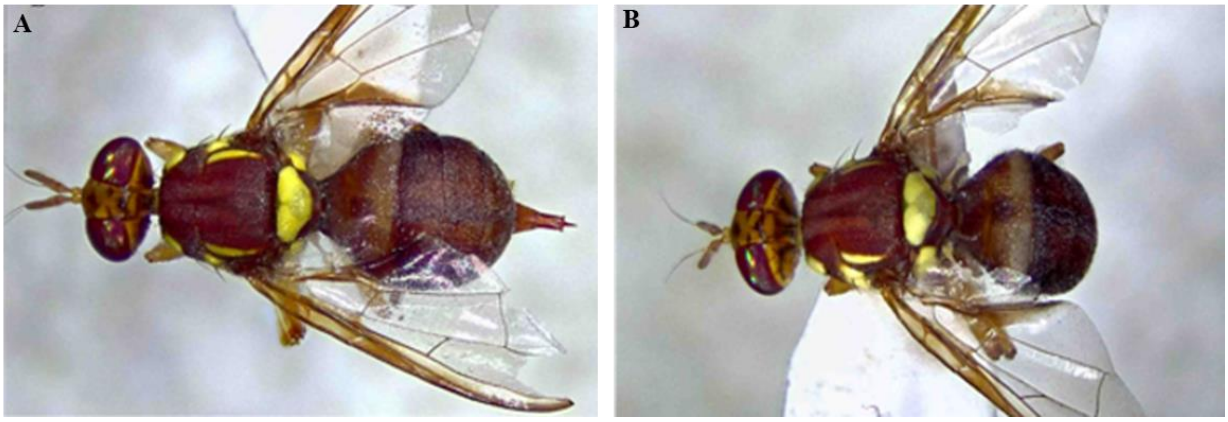

Figure 1. 1: *Bactrocera tryoni* morphology. (A) Female *B. tryoni* with distinct ovipositor. (B) Male *B. tryoni*

Damage of fruit occurs after the female fruit fly oviposits into the ripening or mature host fruit together with the deposition of bacteria that are important for larval development (Behar et al. 2008; Ben-Yosef et al. 2015). The eggs hatch a few days after oviposition and the larvae start feeding on the pulp causing premature ripening and rotting of the fruit. The infested fruit then falls to the ground, and, at the third instar, larvae leave the fruit to burrow into the soil where they pupate. Depending on temperature, the adult flies emerge after ten or more days. Adults then have to undergo adult maturation and mating before the start of a new generation (Clarke et al. 2011; Fletcher, 1987). Fruit fly outbreaks cause a severe decline in fruit and vegetable production resulting in a great economic impact on Australia's horticultural sector, in addition, infestation or presence of flies in a production area can mean loss of domestic and international market access and strict regulations imposed by importing states and countries (Plant Health Australia (PHA), 2008).

1.2.2 *Bactrocera tryoni* complex

The Queensland fruit fly (*B. tryoni*) belongs to a species complex with *B. neohumeralis* (Hardy), *B. aquilonis* (May) and *B. melas* (Perkins & May) (Drew, 1989). *Bactrocera tryoni* has the widest geographic distribution, with *B. neohumeralis* and *B. melas* nested within. Many studies have explored the relationship between *B. tryoni* and *B. neohumeralis*; the two species can be distinguished by their mating times whereby *B. tryoni* mates in the evening or near dusk while *B. neohumeralis* mates in broad daylight around midday (Smith, 1979). Another distinguishing trait is the colour of the humeral calli (shoulder pads): *B. tryoni* is characterised by bright yellow humeral calli while *B. neohumeralis* has brown humeral calli (Drew, 1989; Smith, 1979). However, intermediate colouration patterns of the humeral calli have been observed at different frequencies, thus, using the colour of humeral calli has been deemed a non-conclusive feature for differentiating the two species (Birch, 1961). Additionally, despite their distinct phenotypic and behavioural features, *B. tryoni* and *B. neohumeralis* readily hybridise in the laboratory, are genetically similar and share some polymorphic variation which further suggests the possibility of hybridisation between the two species in the field (Morrow et al. 2000).

The third sibling species, *B. aquilonis*, is restricted to northern parts of the Northern Territory and Western Australia (Drew, 1989; Popa-Báez et al. 2021). *Bactrocera aquilonis* is presumed to be a junior form of *B. tryoni* since they are genetically and morphologically similar, nonetheless, the two species have distinct host fruit preferences (May, 1963). *Bactrocera aquilonis* mates at dusk and laboratory experiments demonstrated that it can breed with *B. tryoni* and produce viable offspring (Drew & Lambert, 1986). The spread of *B. tryoni* into *B. aquilonis* territories has facilitated hybridisation between the two species resulting in bidirectional gene flow as well as eastward spread into some Melanesian

countries (Cameron et al. 2010; Popa-Báez et al. 2021). The other sibling species is *Bactrocera melas*, it is considered to occur in southern Queensland and is considered to be a melanic form of *B. tryoni*, however this requires taxonomic testing (Clarke et al. 2011).

1.3 Fruit fly management

1.3.1 *Bactrocera tryoni* management

The use of insecticides (including of organophosphates such as fenthion and dimethoate) have been key components of integrated pest management of *B. tryoni* and other fruit fly pest species, however, this method has received criticism due to its negative effects on the environment, humans and non-target species, and the possibility of the flies developing resistance, therefore, some of these insecticides have been banned and other environment-friendly management methods have been implemented. These include orchard sanitation (i.e removal and destruction of infested and fallen fruit), the male annihilation technique (MAT) which uses cue-lure to attract and kill males, and use of protein baits that attract and kill both male and females (Clarke et al. 2011; Dominiak & Ekman, 2013).

Under certain circumstances, the sterile insect technique (SIT) is a very effective control method, and the incompatible insect technique (IIT) relies on a similar concept and is very promising. The use of SIT is more effective in small populations and very costly in large target populations, additionally, effective control using SIT has been achieved when used as part of an area-wide integrated pest management (AW-IPM) program as a "mopping up tool" after chemical control (Dyck et al. 2005). Moreover, for tephritid fruit flies, IIT technique has only been tested in the laboratory and has not been applied to fruit fly control in the field (Zabalou et al. 2004). It is, however being successfully applied in mosquito control in several countries (Marris, 2017), including Australia (Ritchie et al. 2018)

1.3.2 Sterile insect technique (SIT)

Sterile insect technique (SIT) involves mass rearing and release of a large number of sterile insects in an affected area (Figure 1. 2). Sterilisation is usually achieved by irradiation. In the ideal situation it also involves male-only strains, as the release of females (albeit sterile) may distract the sterile males from seeking wild mates while sterile released female fruit flies can still oviposit and potentially cause fruit rot through introduction of bacteria without the development of any larvae (Dyck et al. 2005; Knipling, 1955). Similarly, for mosquitoes, the release of sterile females can still result in these females acquiring and transmitting disease by biting hosts. In the field, the sterilised males compete for mates with field males, and in such crosses between field females and the sterilised males, the fertilised eggs are not viable resulting in a reduced offspring population (Knipling, 1955). SIT is species-specific and environmentally friendly since the sterile males are non-replicating thus not permanently established in the wild.

The first successful implementation of SIT was in the area-wide integrated pest management (AW-IPM) of the screwworm *Cochliomyia hominivorax*, a potentially lethal parasite of livestock in USA and Mexico (Enkerlin, 2005) and this paved the way for other success stories. For instance, SIT has been useful in the control of economically important tephritid fruit flies, such as *C. capitata* , *Bactrocera dorsalis, B. tryoni*, *Bactrocera curcubitae*, *Anastrepha obliqua* and *Anastrepha ludens* (Enkerlin, 2005; Hendrichs et al. 2002; Yosiaki et al. 2003).

SIT application against *B. tryoni* was first trialed in New South Wales from 1962 to 1965 (Meats, 1996) and has since been further developed. SIT was applied in the eradication of *B. tryoni* in Western Australia (Perth) in 1995; this approach involved an integrated approach using male attractant, cue-lure, and protein baits to attract both females and males, followed

by the release of sterilised males (McPheron & Steck, 1996). Australia has also seen recent success in the containment of occasional outbreaks of the Queensland fruit fly in the former fruit fly exclusion zone (FFEZ) that included southern NSW, Victoria, and South Australia (Dominiak & Daniels, 2012; Dyck et al. 2005). The fruit fly exclusion zone has since been dissolved and currently only South Australia maintains its fruit fly free status (Dominiak & Mapson, 2017). Furthermore, SIT was used in the suppression of *C. capitata* in Mexico and USA (Enkerlin, 2005) and is now being applied in the control of *C. capitata* when occasional small outbreaks (based on a few flies) are recorded in otherwise fruit fly free South Australia (Enkerlin, 2005; Hendrichs et al. 1983; Hendrichs et al. 2002).

SIT efficiency relies on the ability of the sterile males to adequately court females and mate, inseminate and prevent them from remating with fertile males. However, studies on sterilised *C. capitata* males have shown that irradiation has an effect on the pre- and post-copulatory behaviour of fruit flies rendering them sexually incompetent (Pérez-Staples et al. 2013). These effects can lead to mating failure, especially in scenarios where the species form leks to mate and the females have mating preferences (Dyck et al. 2005).

1.3.3 Incompatible insect technique (IIT)

Incompatible insect technique (IIT) is a recommended alternative method (Figure 1. 2). It exploits cytoplasmic incompatibility (CI) to reduce pest invasion in a way analogous to the sterile insect technique, as it also relies on mass rearing, sex separation and mating sterility (here caused by *Wolbachia* infection) before release to the affected environment Initial field experiments of IIT were done to control *Culex pipiens* mosquitoes (Laven, 1967) and was followed by research and field trials for applications in the control of other mosquito species

as well as other insects like the almond moth *Ephestia cautella* (Brower, 1980), European cherry fruit fly, *Rhagoletis cerasi* (Blumel & Russ, 1989) and, in the laboratory, *C. capitata* (Zabalou et al. 2004). IIT has successfully been applied in mosquito control in several countries (Marris, 2017), including Australia, for the control of *Aedes aegypti* (Ritchie et al. 2018). However, against tephritid fruit flies, IIT has only been tested in the laboratory and has not been applied for fruit fly control (Zabalou et al. 2004).

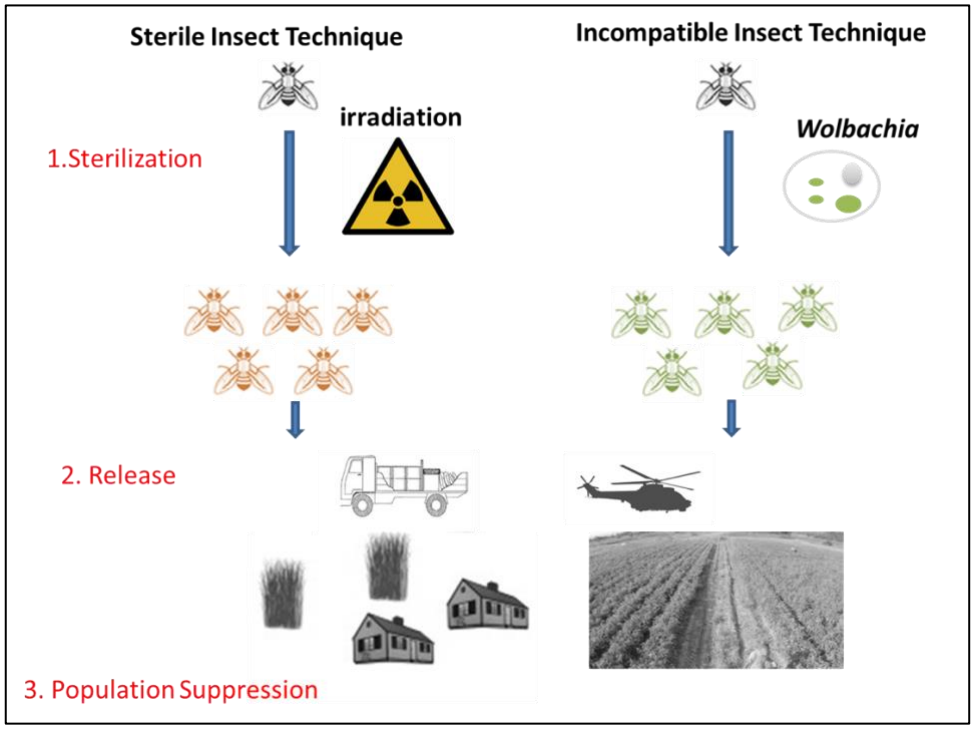

Figure 1. 2: Sterile Insect Technique (SIT) and Incompatible Insect Technique (IIT); male flies are sterilised by either irradiation or *Wolbachia* infection then released to the target environment for pest population suppression.

1.3.3.1 Genetic sexing strains

The success of SIT in AW-IPM of fruit flies is increased by male-only release as the release of sterile females distracts the sterile males from seeking wild mates. In SIT-based control of vector insects like mosquitoes, female release can lead to disease transmission by biting, while female fruit flies might sting and cause damage to fruits. The male-only release can be

achieved by either removing the females from the population before release or eliminating them at their immature stages. This requires a good sex separation technique which has been achieved in some insect species by using genetic sexing strains (GSS).

The use of GSS in SIT was proposed for the sheep blowfly, *Lucilia cuprina* where GSS were produced using pupal colour mutations (Whitten, 1969). In *C. capitata* GSS has been achieved by using the *temperature-sensitive lethal, tsl* and *white pupae, wp mutations* (Robinson, 2002) while in the silkworm, *Bombyx mori* where males produce more silk, the genetic sexing strains were produced using W-autosome translocations (cocoon and egg colour mutation*)* (Nagaraja et al. 2005). GSS has been developed for about 19 insect species and only in *C. capitata* and *Anopheles albimanus* has it been applied in SIT (Dyck et al. 2005). However, many important pests and disease vectors lack a good sex separation technique.

So far, *B. tryoni* SIT program has used mixed-sex releases due to the lack of a genetic sexing strain. However, attempts to develop GSS for *B. tryoni* have been made, for instance using translocation of autosomal mutations to the Y chromosome (Meats et al. 2002). Several translocations were recovered in this study, unfortunately, the translocation stocks showed inexplicable temperature-dependent lethality and were therefore not suitable for male-only release (Meats et al. 2002). Raphael et al. (2011) demonstrated a successful heritable germline transformation of *B. tryoni* using the *piggyBac* transposon vector which produced stable transgenic lines expressing fluorescent proteins EGFP or DsRed (Raphael et al. 2014). Furthermore, the sex-determining genes *transformer* (*tra*) and *transformer* (*tra-2*) previously recorded in *C. capitata* (Pane et al. 2002) have also been explored for *B. tryoni* GSS (Morrow et al. 2014; Raphael et al. 2014). Additionally, the CRISPR/Cas genome editing tool has been

used to produce *B. tryoni* with white eyes by targeting the *white* gene, demonstrating a promising approach for CRISPR/Cas to generate a GSS of *B. tryoni* (Choo et al. 2017).

1.4 *Wolbachia*

Wolbachia are gram-negative intracellular bacteria of the class Alphaproteobacteria. They are vertically transmitted from mother to offspring and can also be transmitted horizontally across species (Heath et al. 1999). *Wolbachia* infects a wide range of insects and other arthropod species (40-60 %), crustaceans and nematodes (Hilgenboecker et al. 2008; Werren, 1995; Rousset et al. 1992; Werren, 1997). *Wolbachia* was first described in *C. pipiens* mosquitoes, as a *Rickettsia*-like microorganism (Hertig & Wolbach, 1924), and later named *Wolbachia pipientis* (Hertig, 1936). *Wolbachia* is currently viewed as a single species with 17 supergroups (A-F, H-Q and S) (Kaur et al. 2021; Augustinos et al. 2011). However, there are ongoing debates as to whether *Wolbachia* is a single or multiple species (Lindsey et al. 2016), , and this remains unclear due to several factors, for instance gene recombination between strains of different *Wolbachia* supergroups (Baldo et al. 2005). *Wolbachia* can manipulate host reproduction in order to enhance its spread through host population. These reproductive manipulations include cytoplasmic incompatibility, feminization, male-killing, and parthenogenesis (Figure 2) (Stouthamer et al. 1999; Weeks et al. 2007; Werren et al. 2008).

1.4.1 Cytoplasmic incompatibility

Cytoplasmic incompatibility (CI) observed in insects, terrestrial isopods and arachnids prevents viable reproduction between uninfected females and *Wolbachia*-infected males (unidirectional CI), or when infected females mate with males infected with an incompatible *Wolbachia* strain (bi-directional CI) (Figure 1. 3) (Kageyama et al. 2012; Stouthamer et al. 1999; Werren et al. 2008). Infected females have a reproductive advantage as they are able to

mate with both uninfected males and males infected with the same or compatible *Wolbachia* strain hence increasing infection frequency in subsequent generations. Additionally, since *Wolbachia* transmission is cytoplasmic, and is linked to the maternal lineage (as males are a "dead end") *Wolbachia* can be selected to confer additional fitness advantages to the infected females so as to increase its frequency in the population (Weeks et al. 2007; Werren, 1997)

The molecular mechanisms of CI have remained unknown until recently. Studies have identified *cif* genes located in the eukaryotic association module of prophage WO as the genetic determinants of CI in *Wolbachia* (Bordenstein & Bordenstein, 2016; Lepage et al. 2017). The *cif* genes are encoded in a two-gene operon (Beckmann & Fallon, 2013; Lindsey et al. 2018). These genes (annotated as WD0631 and WD0632 in *w*Mel genome) are now named cytoplasmic incompatibility factors, *cifA*, and *cifB* and are responsible for *Wolbachia*-induced sperm modification resulting in CI, while expression of the *cifA* gene maternally rescues CI (Shropshire et al. 2018). In *Drosophila*, an expression of both genes in the same male shows reduction in hatch rates when crossed with uninfected females (Lepage et al. 2017). Additionally, Beckmann et al. (2017) showed that orthologues of *cifA* and *cifB* from *Wolbachia* of *C. pipiens* (called *CidA* and *CidB*) induce CI. The CI-inducing deubiquitylating enzyme (DUB), *CidB*, cleaves ubiquitin from substrates and is encoded in a two-gene operon, while *CidA* encodes a protein that binds *CidB*. Thus, both of these studies, in two different systems, show that coexpression of a pair of genes is sufficient to induce and rescue CI (Beckmann et al. 2017; Lepage et al. 2017). These findings have allowed for the investigation and detection of similar CI genes in other *Wolbachia* strains (Bing et al. 2020; Lindsey et al. 2018; Morrow et al. 2020). Interestingly, another endosymbiotic bacterium, *Cardinium*, can also induce CI, however it evolved this capacity independently and its molecular CI mechanism is still unknown (Penz et al. 2012).

1.4.2 Thelytokous parthenogenesis

Thelytokous parthenogenesis (PI) induced by *Wolbachia* and other bacteria such as *Cardinium* has been reported in insects and arachnids and it occurs when virgin females carrying *Wolbachia* produce infected female-only offspring, and removal of *Wolbachia* by antibiotics results in male-only production (Figure 1. 3, Stouthamer et al. 1990; Zchori-Fein et al. 2001). In some hymenopterans, thelytokous parthenogenesis has been attributed to gamete duplication caused by the failure in chromosome segregation during metaphase resulting in diploidization of the nucleus (Pannebakker et al. 2004; Stouthamer & Kazmer, 1994). However the case is different in a haplodiploid mite of the genus *Bryobia*; their *Wolbachia*-induced parthenogenesis results in heterozygous progeny identical to the mother (Rabeling & Kronauer, 2013). PI biases the sex ratio towards female-biased sex ratios and this then supports the endosymbiont's colonisation of the host population by producing more infected females (Zchori-Fein et al. 2001).

1.4.3 Feminisation

Some strains of *Wolbachia* cause feminisation of genetic males (Figure 1. 3), a phenomenon first described in isopods but has since been observed in a few species of Lepidoptera and one species of Hemiptera (Hiroki et al. 2002; Kageyama et al. 2012; Negri et al. 2006; Vandekerckhove, 2003; Werren et al. 2008). Feminisation in isopods has been attributed to over-proliferation of *Wolbachia* in the androgenic glands leading to hypertrophy and inhibited function during male development (Cordaux et al. 2011). Studies have shown that feminisation in insects is caused by the effect of *Wolbachia* on the sex-determination pathways (Narita et al. 2007). Additionally, an interaction between Z chromosome inheritance and feminisation has been demonstrated in *Wolbachia* infected *Eurema* butterflies

suggesting a possibility of *Wolbachia* causing a disruption of chromosome inheritance by meiotic drive (MD) against Z bearing gametes in feminised Z0 females (Kern et al. 2015). Further analysis of this system confirmed that *Wolbachia* is responsible for the disruption of the Z chromosome inheritance (either by MD or elimination of the maternal Z) and feminisation (Kageyama et al. 2017).

1.4.4 Male-Killing (MK)

Wolbachia has also been reported to cause male-killing (MK), where sons of infected mothers suffer from embryonic mortality (Figure 1. 3) (Fialho & Stevens, 2000; Jiggins et al. 2001; Zeh et al. 2005). While not clearly understood, male-killing has been attributed to interference in the sex determination pathway in a way analogous to feminization (Kageyama & Traut, 2004). MK can occur early during embryonic development or in late larval stages or early pupal stage (Kageyama et al. 2007). Early MK is seen as a way of eliminating male competition in broods and hence more resources remain for the female progeny (Ma et al. 2014). Besides *Wolbachia*, other microorganisms can cause late MK in their hosts and can invade host populations both vertically and horizontally (Kageyama et al. 2007; Nakanishi et al. 2008).

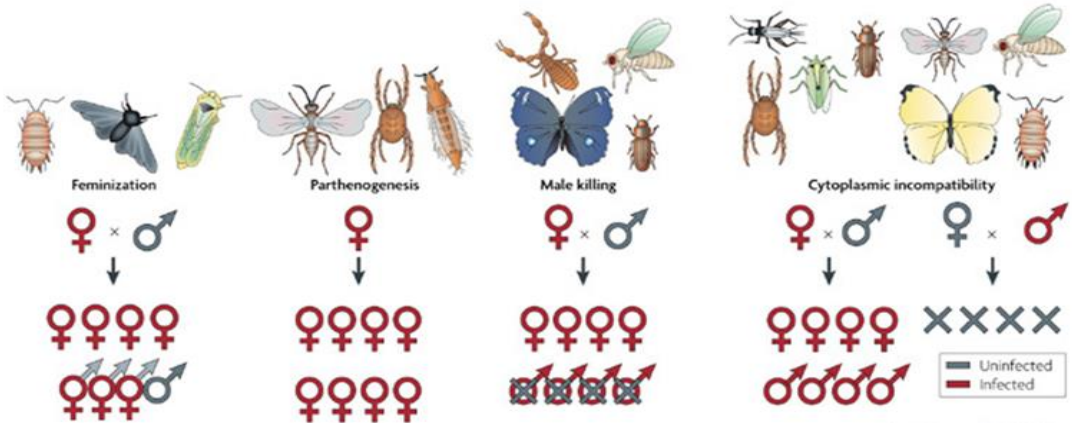

Figure 1. 3: Effects of *Wolbachia* on hosts. Figure from Werren et al. (2008), illustrating cytoplasmic incompatibility (CI), feminisation, thelytokous parthenogenesis and male-killing.

1.5 *Wolbachia* in Australian tephritid fruit flies

Two *Wolbachia* strains with complete multi-locus sequence typing (MLST) profiles (Baldo et al. 2006) were reported in seven out of 24 (29 %) Australian tephritid species (Morrow et al. 2015). These *Wolbachia* infections occurred at low prevalence (i.e detected in only 2-7 % of individuals per species), were restricted to northern Queensland and these *Wolbachia* strains were not linked to any particular mitochondrial haplotypes as often seen for *Wolbachia* infections that either manipulate host reproduction or provide host benefits (Morrow et al. 2014; Morrow et al. 2015). For these reasons, *Wolbachia* strains in the Australian tephritid fruit flies may not be maternally transmitted and may lack the capacity to manipulate host reproduction. Similarly, the detection of identical *Wolbachia* strains in several tephritid species may be a manifestation of horizontal *Wolbachia* transmission due to parasites, parasitoids and predators, hybridization and ecological interactions between species that share the same ecological niche (Werren, 1995; Raychoudhury et al. 2009; Rigaud & Juchault, 1995), but without proliferation in the fruit fly hosts. *Bactrocera tryoni* and *B. neohumeralis* are sibling species that can hybridise and produce viable offspring (Morrow et

al. 2000; Wang et al. 2003) therefore, detection of identical *Wolbachia* in these two species supported hybridisation as a possible route of horizontal transmission of *Wolbachia* between these two (but this mode of horizontal transmission would not support *Wolbachia* infections seen in the other species). Most fruit fly species are polyphagous in nature and hence share host plants which can also provide a suitable avenue for horizontal transmission of *Wolbachia*. Additionally, fruit fly host species can share common parasitoids, for instance, *Fopius arisanus* is a common parasitoid of most fruit fly host species (Carmichael et al. 2005) and the detection of identical *Wolbachia* strains in *Bactrocera frauenfeldi* and *F. arisanus* could suggest that the *Wolbachia* could be from *F. arisanus.*

1.6 Strepsiptera

The order Strepsiptera, also known as the twisted wing insects or twisted wing parasitoids are a small group of holometabolous insects that parasitise other insects (Kathirithamby, 2018). Strepsiptera have approximately 630 species belonging to ten extant families (Bahiaxenidae, Mengenillidae, Corioxenidae, Myrmecolacidae, Lychnocolacidae, Stylopidae, Xenidae, Bohartillidae, Elenchidae and Halictophagidae), plus another five extinct families (Kathirithamby, 2018; McMahon et al. 2009). Strepsiptera has two suborders, Mengenillidia, which comprises the family Mengenillidae, and Stylopidia which comprises eight extant families (Kathirithamby, 1989, 2005; McMahon et al. 2009). Strepsiptera are cosmopolitan and they parasitise 35 insect families and seven insect orders including Blattodea, Diptera, Hemiptera, Hymenoptera, Orthoptera, Mantodea and Zygentoma, (Kathirithamby, 1989). Strepsiptera are an unusual group characterised by a unique lifestyle and extreme sexual dimorphism (Kathirithamby, 1991, 2009). The males are short lived, free living and have the external morphological features of insects (Kathirithamby, 1991, 2009). However, the

females are neotenic in nature, lacking the external morphological features of an insect and are fully endoparasitic, except in Mengenillidae which have late instar larvae that leave the host to pupate on the outside of the host (Kathirithamby, 1991, 2009).

1.6.1 Strepsiptera life cycle

Strepsiptera first instar larvae (also called planidia) are produced viviparously in large numbers (up to ~750,000 per female) by the endoparasitic neotenic female (Kathirithamby, 1989). The larvae emerge through the brood canal located in the cephalothorax and are small in size (length of 0.08-0.30 mm) (Kathirithamby, 1989). The host-seeking larvae enter their hosts generally via the host abdominal cuticle, however, entry through any part of the host has been observed in other strepsipteran species (Kathirithamby, 2001; Maeta et al. 2012). For instance, *Eoxenos laboulbei* has been observed to enter its silverfish (Zygentoma) host via the coxa, while *Stichotrema dallatorreanum* enters its host via the tarsi (Kathirithamby, 2001). Upon entry into the host, the first instar larvae undergo hypermetamorphosis into 2nd and 3rd instar larval stages; at this stage the males can be distinguished from the females by the presence of prolegs (Kathirithamby, 1991). At the 4th larval instar, the females develop a cephalothorax while the males form a cephalotheca (Kathirithamby, 1989, 1991). At the end of the pupal stage, the Stylopidia males emerge by breaking the cap of the cephalotheca while the females remain endoparasitic with only the cephalothorax extruded in the host (Figure 1. 4). The parasitised hosts are referred to as stylopised. In Mengenillidae, both males and females emerge from their puparium (Kathirithamby, 1991). Strepsiptera adult males are short-lived (3-6 hours) and they search for a mate upon emergence (Kathirithamby, 2009). The males are attracted to pheromones produced by the female (Hughes et al. 2004). In Stylopidia (except Corioxenidae), the male fertilises the female through the brood opening in

the cephalothorax, whereas the free living Mengenillidae females are fertilised by traumatic insemination (Beani et al. 2005; Kathirithamby, 1989, 1991, 2009).

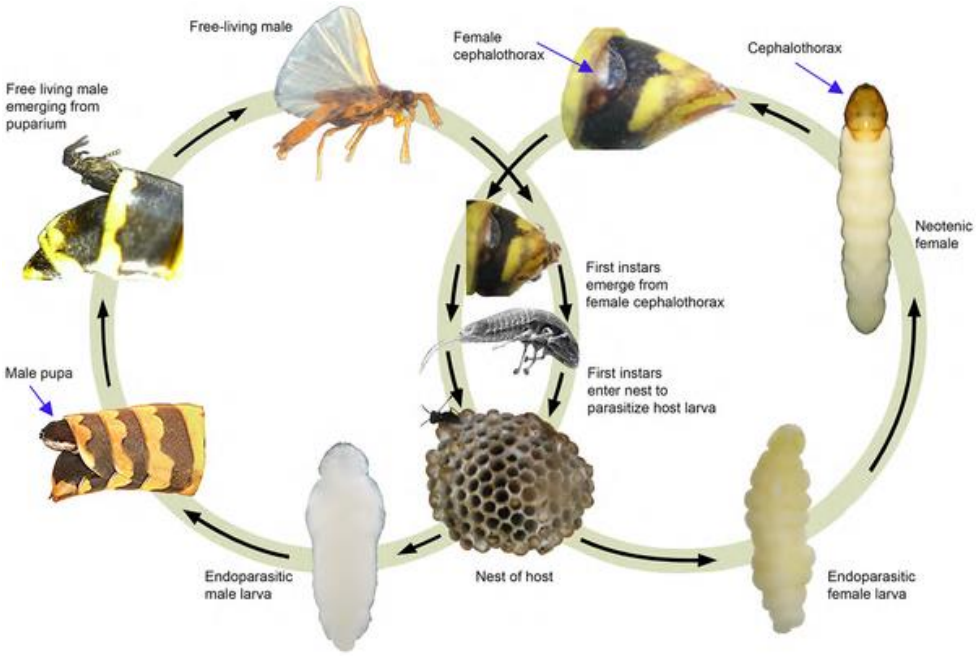

Figure 1. 4: Figure illustrating the lifecycle of the strepsipteran *Xenos vseparum* (Xenidae) within its paper wasp host, *Polistes domula*. (from Erezyilmaz et al. 2014)

1.6.2 Host-parasitoid association

Parasitisation of new hosts by Strepsiptera can occur in places frequented by hosts as the planidia are usually released in the same habitats as the hosts (Kathirithamby, 2009). However, in other hosts such as bees and wasps, planidia disperse to host nests, usually carried along phoretically (Kathirithamby, 2009). Phoresy has been observed in hymenopterans parasitised by *Pseudoxenos iwatai* Esaki (Maeta et al. 2012), as well as in the thread-waisted wasps, *Ammophila sp.* parasitised by *Paraxenos lugubris* (Kathirithamby et al. 2012). Similarly, *Stylops pacificus* parasitising *Andrena complexa* can be carried along with nectar into the honey crop of bees, then regurgitated onto the pollen cells in the nest (Kathirithamby, 2009).

Unlike other endoparasitoids strepsipterans do not kill their host as part of their development process, however, they castrate their hosts and the hosts eventually die as a direct/indirect effect of parasitisation (Cappa et al. 2014). Interestingly, strepsipterans keep their hosts alive until the strepsipteran males mature and emerge, and after the first instar larvae are produced by the neotenic females (Kathirithamby, 2009). Additionally, strepsipterans can allow their host larvae and pupa to undergo metamorphosis to adult stage, this has been attributed to the endoparasitic larvae lag phase that lasts until the host's pupal stage (Hughes & Kathirithamby, 2005; Kathirithamby, 2018). The lag phase involves reduced development in Strepsiptera which reduces the negative effects on the host during its early developmental stages (Hughes & Kathirithamby, 2005; Kathirithamby, 2018).

1.6.3 Strepsipteran parasitisation and host immunity

Stylopidia parasitised hosts (stylopised) are characterised by the presence of male cephalothecae and female cephalothorax extrusions (Kathirithamby, 1991). These extrusions occur commonly in adult hosts, however, in planthoppers and leafhoppers they occur in host nymphs (Kathirithamby, 1991). The stylopised hosts usually have one extrusion, however, superparasitisation with more than one extrusion with any combination of sexes has been observed (Drew & Allwood, 1985; Kathirithamby, 1991; Nakase & Kato, 2011; Vannini et al. 2008). Strepsipteran parasitisation may not always be visible especially when a host is parasitised by immature stages, unless molecular detection methods are applied.

Strepsipteran parasitisation has a substantial impact on host fitness (Hughes et al. 2004; Kathirithamby, 1989, 1998; Solulu et al. 1998). Other than the direct effects caused by castration, the physical deformities caused by the presence of Strepsiptera in the host can

inhibit mating as well as reproduction in general. Similarly, the exclusive dependence of Strepsiptera on the host for nutrients and secretion of waste could have an indirect effect on host fitness (Kathirithamby, 1998; Thomas et al. 2005). Additional morphological effects have been observed in hymenopterans and hemipterans. For instance, facial markings, anal fimbria and pollen-collecting apparatus in *Andrena* (mining bee) have been observed to change and resemble the opposite sex (Kathirithamby, 1989), whereas Delphacidae (planthoppers) have reduced/lost both the internal and external sexual organs (Kathirithamby, 1989). The European paper wasp *Polistes dominulus* when parasitised by *X. vesparum* has been observed to leave the colony and aggregate outside the nest, and this behavioural effect on the host has been interpreted as an adaptive strategy for Strepsiptera to complete its lifecycle (Hughes et al. 2004), by providing an avenue for mating.

Strepsiptera are obligate endoparasitoids meaning that they have to encounter the host immune system. The mechanism that strepsipterans use to avoid the host immune system and to continue developing without any effects has remained unknown until recently. A study of *Stichotrema dallatorreanum* revealed that the first instar larvae use host tissues to evade the host immune system: the first instar larva detaches the host epidermal layer from the endocuticle and wraps itself in it. It then moves into the host hemocoel and molts into the second instar inside the epidermal bag, and derives nourishment and secretion of waste through the host hemolymph which is in direct contact with the basal lamina (Kathirithamby, 2009; Kathirithamby et al. 2003).

1.6.4 Strepsipteran phylogeny and host specificity

Other than extreme sexual dimorphism, strepsipterans also exhibit unusual genetic characteristics for instance, they have one of the smallest insect genomes. Studies using flow cytometry revealed the genome sizes of *Caenocholax fenyesi*, and *Xenos vesparum* and *Xenos vesparum* to be 108 Mb, 130 Mb and 133 Mb, respectively (Johnston et al. 2004). Similarly, strepsipteran mitogenomes have been found to have short protein-coding genes as well as several mitogenome rearrangements (Carapelli et al. 2006; McMahon et al. 2011). Translocation of the tRNA gene $trnS_1$ has occurred in the mitogenomes of both *Mengenilla australiensis* and *X. vesparum,* while translocation of the tRNA genes *trnF* and $trnS_2$ has also occurred in *X. vesparum* (Carapelli et al. 2006; McMahon et al. 2011). Conversely, strepsipterans have one of the largest nuclear *18S* ribosomal RNA genes, attributed to large insertions in the gene (Gillespie et al. 2005). Additionally, phylogenetic analysis of Strepsiptera has shown that it has undergone rapid sequence evolution (McMahon et al. 2011).

Phylogenetic placement of Strepsiptera in the insect phylogeny had proved to be a challenge with Strepsiptera being placed in different phylogenetic positions over time. Earlier studies had hypothesised that Strepsiptera is either a member of Hymenoptera, Diptera or Coleoptera (Longhorn et al. 2010; Wheeler et al. 2001; Whiting et al. 1997). However, based on genomic data, primarily the *Mengenilla moldrzyki* genome, and the use of morphological analyses, Strepsiptera has more recently been found to be a sister group to the Coleoptera (McKenna & Farrell, 2010; Niehuis et al. 2013). Strepsiptera phylogenetic analyses have revealed a monophyletic grouping of all strepsipteran taxa (Figure 1. 5) (Kathirithamby, 2009; McMahon et al. 2011). Mengenillidia is a sister group to Stylopidia and is represented by *Mengenillidae*, and its members parasitise species of Zygentoma (Kathirithamby, 2009, 2018;

McMahon et al. 2011). The suborder Stylopidia consists of the family Corioxenidae and the infra order Stylopiformia which comprises the remaining Stylopidia families (Kathirithamby, 2018). Corioxenidae differs from Stylopiformia in that, females are inseminated through the cephalothorax, or an opening pierced by the male, while in Stylopiformia insemination occurs though the brood canal opening. Members of Corioxenidae parasitise Hemiptera (Kathirithamby, 2018). The infra order Stylopiformia consists of the families; Myrmecolacidae, Lychnocolacidae, Stylopidae, Xenidae, Bohartillidae, Elenchidae and Halictophagidae (Kathirithamby, 2018).

The family Myrmecolacidae consists of three extant genera including *Myrmecolax, Caenocolax* and *Stichotrema* (Kathirithamby, 2018; McMahon et al. 2011). Myrmecolacidae exhibit heterotrophic heteronomy, an extraordinary characteristic where males and females parasitise different hosts (either species or sex), in this case the males and females of Myrmecolacidae parasitise host species from different orders (Kathirithamby, 1998, 2009; Kathirithamby & Hamilton, 1992). Males of Myrmecolacidae parasitise Hymenoptera (ants) while females parasitise Orthoptera (crickets and grasshoppers) and Mantodea (mantids) (Kathirithamby, 2009, 2018). The family Lychnocolacidae consists of only one genus, *Lychnocolax* and its host is unknown (Kathirithamby, 2018). The family Stylopidae is the largest and its members parasitise Hymenoptera (bees and wasps). Stylopidae consists of nine genera: *Stylops* (the largest genus), *Crawfordia, Eurystylops, Halictoxenos, Hylecthrus, Jantarostylops, Kinzelbachus, Melittostylops* and *Rozenia* (Kathirithamby, 2018). The family Xenidae consists of four genera including *Xenos, Pseudoxenos, Paraxenos,* and *Paragioxenos* and they parasitise Hymenoptera (Vespidae and Sphecidae). The family Bohartillidae consists of only one genus *Bohartilla* and its host is unknown. Elenchidae family consist of five genera *Elenchus, Elencholax, Colacina, Deinelenchus* and

Protelenchus. The members of Elenchidae parasitise Hemiptera (Cicadellidae, Dictyopharidae, Eurybrachidae, Flatidae, Delphacidae) (Kathirithamby, 2018).

The family Halictophagidae is a polyphyletic group consisting of six subfamilies: Callipharixenidae, Dipterophaginae, Halictophaginae, Tridactylophaginae, Blattodeaphaginae and Coriophaginae. Halictophagidae includes seven genera *Callipharixenos, Dipterophagus, Halictophagus, Tridactylophagus, Stenocranophilus* and *Coriophagus* (Kathirithamby, 2018). The members of Halictophagidae parasitise diverse host species, for instance *Callipharixenos* parasitise Hemiptera, *Dipterophagus* parasitise Diptera (Tephritidae: Dacini), *Halictophagus* parasitise Hemiptera, *Tridactylophagus* parasitise Orthoptera (Tridactylidae), *Stenocranophilus* parasitise Hemiptera (Delphacidae) and *Coriophagus* parasitise Hemiptera (Pentatomidae and Coreidae). *Dipterophagus daci*, the only member of the genus *Dipterophagus* has previously been placed into a separate family, Dipterophagidae (Drew & Allwood, 1985). However, based on morphological characteristics shared by *D. daci* and the members of the family Halictophagidae, Dipterophagidae was placed as a subfamily of Halictophagidae (Kathirithamby, 1989).

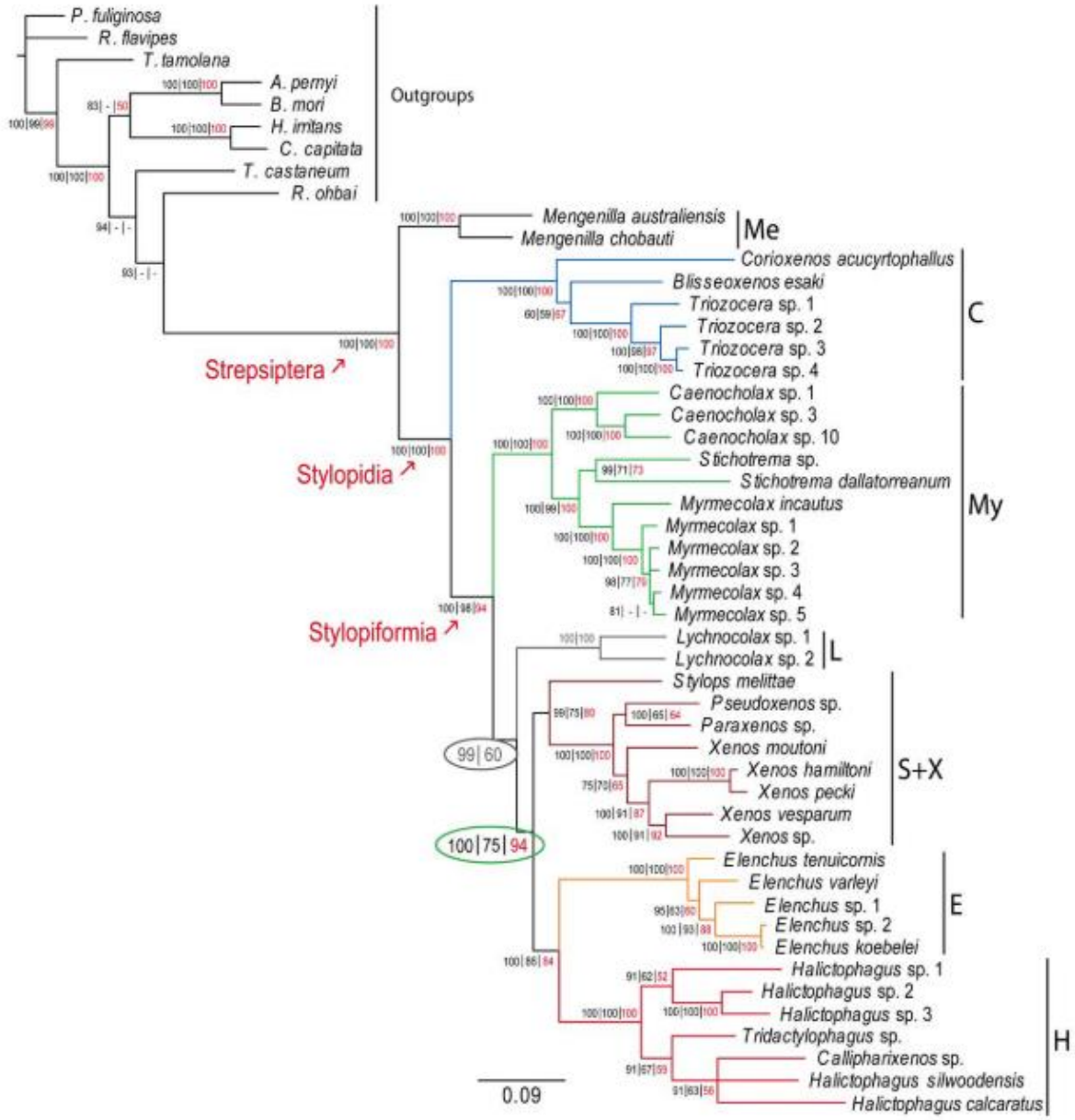

Figure 1. 5: Phylogenetic tree of Strepsiptera (from McMahon et al. 2011), showing eight strepsipteran families; Me= Mengenillidae, C= Corioxenidae, My =Myrmecolacidae, L = Lychnocolax, S+X= Stylopidae+Xenidae, E = Elenchidae, H= Halictophagidae. Note: the families of Bahiaxenidae and Bohartillidae are not presented in this phylogeny, possibly due to absence of specimen data to be included in the phylogenetic analysis.

1.6.5 *Dipterophagus daci*

Dipterophagus daci has so far been recorded as the only described strepsipteran parasite of Diptera, while there is another undescribed strepsipteran from platystomatid flies from Papua New Guinea (Drew & Allwood, 1985). The geographic distribution of *D. daci* includes Melville Island (Northern Territory), Cape York Peninsula, Townsville, Mt Glorious, Palmwoods, Redbay (all in Queensland) as well as some Torres Strait Islands (to the north of Queensland) and Solomon Islands (Allwood & Drew, 1996; Drew & Allwood, 1985). *Dipterophagus daci* parasitise 19 dacine hosts including *B. tryoni, B. neohumeralis, B. aquilonis, B. cacuminata, B. decurtans, B. mayi, B. peninsularis, B. tenuifascia, B. aeruginosa, B. abscondita, B. breviaculus, B. jarvisi, B. musae, B. perkinsi, B. umbrosa, B. tenuifascia, B. frauenfeldi, B. frogatti* and *Dacus bellulus* (Allwood & Drew, 1996; Drew & Allwood, 1985). Allwood & Drew (1996) observed that *D. daci* parasitisation in *B. aquilonis* and *B. tenuifascia* was dependent on the availability of the host and rainfall. However, low levels of parasitisation were observed when fly populations were at high peak and vice versa, suggesting that *D. daci* could not induce high levels of parasitisation despite high host numbers (Allwood & Drew, 1996). For instance, in a site the parasitisaton levels were observed to peak from 2.6 % during peak season of *B. aquilonis* to 7 % when fly populations were in low abundance (Allwood & Drew, 1996). The *D. daci* parasitised flies did not show any apparent external effects on their size, color and testes, except for the extrusions (Allwood & Drew, 1996). However, *D. daci* was observed to exhibit gregarious parasitism with more than one *D. daci* male or female extrusion (Allwood & Drew, 1996; Drew & Allwood, 1985), as well as large number of first instar larvae (over 3,000) released by individual females (Allwood & Drew, 1996).

1.7 Research scope and aims

The research detailed in this thesis focuses on a multipartite interaction involving three key players: tephritid fruit flies, their endoparasitoid *D. daci*, and its *Wolbachia* (Figure 1. 6). Furthermore, it also looks at the bacterial communities in both flies and *D. daci*. Fruit flies are devastating to the Australian horticultural industry and their management relies on the use of biosecurity measures, orchard hygiene, monitoring, insecticides and lures as well as other techniques such as SIT and potentially IIT. This research focuses on the biology of fruit flies and explores its interaction with *D. daci* endoparasites and *Wolbachia*, as this is pertinent to the understanding of fundamental aspects related to fruit fly biology that can be applied to improve the current control strategies for the Australian tephritid fruit flies.

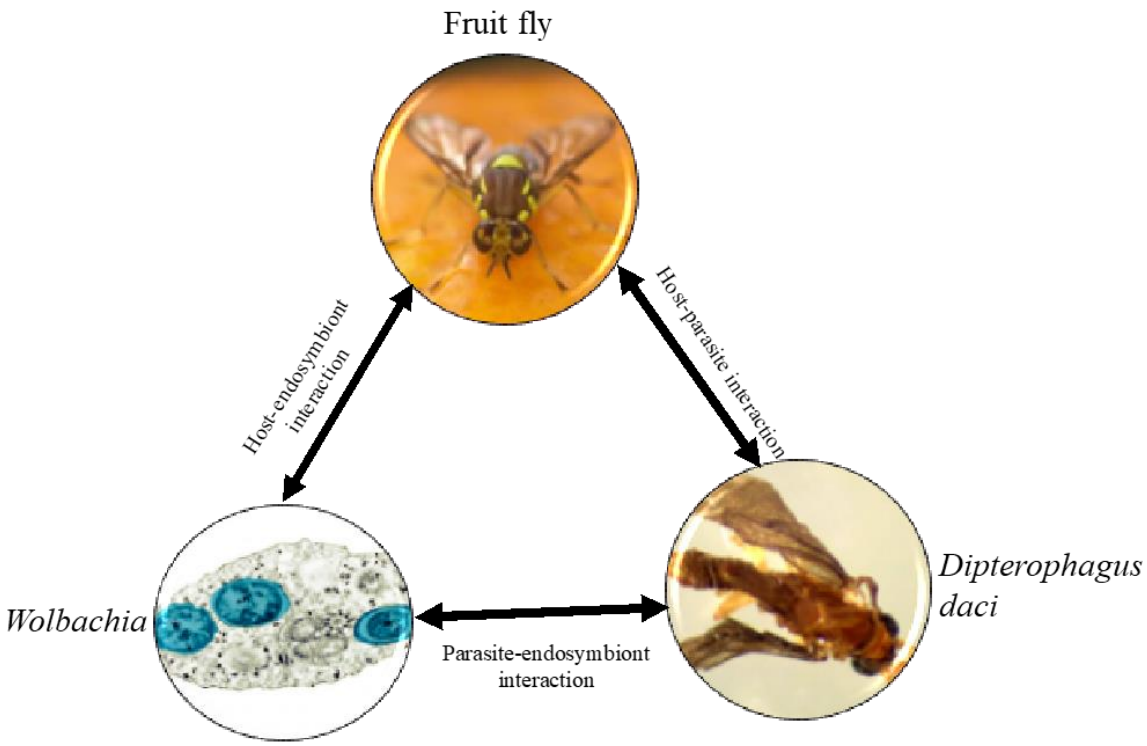

Figure 1. 6: The interactions of *Wolbachia, D. daci* and the fruit fly host. Images show *Wolbachia* (adapted from https://www.worldmosquitoprogram.org/), *Bactrocera tryoni* and *Dipterophagus daci.*

The key aims of this research were:

1. **To determine whether the presence of *Wolbachia* in Australian tephritid fruit fly species is correlated to concealed parasitisation of Australian tephritid fruit flies by *D. daci*.**
2. **To determine whether the highly rearranged and less diverse *D. daci* mitogenome relative to their fruit fly hosts, is correlated to its extreme parasitic lifestyle and *Wolbachia* infection.**
3. **To explore the less diverse and *Wolbachia* dominated *D. daci* microbiome and compare it with the microbiome of its tephritid fruit fly hosts.**

The ability for *Wolbachia* to manipulate its host's reproduction has resulted in the development of a new control strategy, IIT. However, many different strains of *Wolbachia* exist, and in addition to reproductive manipulation, they can also have other fitness effects on the host. Therefore, a potential *Wolbachia* candidate strain as well as its effects on the target host must first be explored. *Wolbachia* infections have previously been reported in Australian tephritid fruit flies (Morrow et al. 2014; Morrow et al. 2015). These infections were enigmatic since they occurred at very low prevalence and incidence, they were shared by multiple fruit fly species and were restricted to northern Queensland. Additionally, these infections did not have a clear link to a specific mitochondrial haplotype suggesting that they were neither vertically transmitted, nor did they induce CI.

Therefore, Chapter 2 of this study applied whole genome sequencing (WGS) on *Wolbachia*-infected tephritid fruit flies to further study the *Wolbachia* infections and explain the enigmatic *Wolbachia* infections at the genomic level. The WGS revealed the presence of the expected fruit fly mitogenome and notably, the mitogenome of another insect, the twisted wing parasitoid, *D. daci* (Allwood & Drew, 1996; Drew & Allwood, 1985), suggesting that the *Wolbachia*-infected fruit flies could also be parasitised by *D. daci*.

Dipterophagus daci has previously been reported in several Australian tephritid fruit fly species (Allwood & Drew, 1996; Drew & Allwood, 1985). However, its interaction with tephritid fruit fly hosts has not been explored. Chapter 2 further applies PCR, qPCR and Sanger sequencing on fruit fly samples previously tested for *Wolbachia*, that had no external signs of parasitisation, and dissected fruit fly samples that had visible signs of parasitisation (stylopisation). It was expected that *Wolbachia* would correlate with the presence of *D. daci* in fruit flies.

The aim of Chapter 3 was to sequence the mitogenomes of *D. daci* and those of its fruit fly hosts. Strepsipterans are endoparasitoids of insects and they have intimate relationship with their hosts (Kathirithamby, 2009), therefore we expected that *D. daci* mitogenomes will contain gene rearrangements similar to other available strepsipteran mitogenomes owing to their parasitic lifestyle. Chapters 2 and 3 delve into the relationship between *D. daci* and the Australian tephritid fruit flies. This is fundamental for understanding fruit fly biology and may further improve the development of fruit fly control strategies.

The interaction between fruit flies and organisms living within them, such as symbiotic bacteria or endoparasitoids, can have an overall effect on their fitness. The aim of Chapter 4 was to explore the microbiome of *D. daci* and compare it with the microbiome of its fruit fly hosts. This was done by performing high throughput bacterial 16S rRNA gene amplicon sequencing on the MiSeq platform. We hypothesized that the microbiome of *D. daci* is distinct from that of its fruit fly hosts. However, due to their close association, we expected that *D. daci* would share some bacterial taxa with the fruit fly host. Further, Chapter 4 aimed to investigate whether *D. daci* parasitisation may have an impact on the fruit fly microbiome.

Chapter 2

Host-endoparasitoid-endosymbiont relationships: concealed Strepsiptera provide new twist to *Wolbachia* in Australian tephritid fruit flies

Towett-Kirui, S. Morrow, J. L. Close, S. Royer, J. E. & Riegler, M. (2021). Host-endoparasitoid-endosymbiont relationships: concealed Strepsiptera provide new twist to *Wolbachia* in Australian tephritid fruit flies. *Environmental Microbiology*. https://doi.org/https://doi.org/10.1111/1462-2920.15715.

2.1 Abstract

Wolbachia are widespread endosymbionts that affect arthropod reproduction and fitness. Mostly maternally inherited, *Wolbachia* are occasionally transferred horizontally. Previously, two *Wolbachia* strains were reported at low prevalence and titres across seven Australian tephritid species, possibly indicative of frequent horizontal transfer. Here, we performed whole-genome sequence of field-caught *Wolbachia*-positive flies. Unexpectedly, we found complete mitogenomes of an endoparasitic strepsipteran, *Dipterophagus daci*, suggesting that *Wolbachia* in the flies are possibly linked to concealed parasitisation. We performed the first genetic characterisation and detected *D. daci* in *Wolbachia*-positive flies not visibly parasitised, but approximately 80% of *Wolbachia*-negative flies were *D. daci*-negative, presumably reflecting polymorphism for the *Wolbachia* infections in *D. daci*. We dissected *D. daci* from stylopised flies and confirmed that *Wolbachia* infects *D. daci*, but also found *Wolbachia* in stylopised fly tissues, likely somatic, horizontally transferred, non-heritable infections. Furthermore, no *Wolbachia cif* and *wmk* genes were detected, and very low mitogenomic variation in *D. daci* across sampled locations. Therefore, *Wolbachia* may influence host fitness without reproductive manipulation. Our study of 13 tephritid species highlights that concealed early stages of strepsipteran parasitisation led to the previous incorrect assignment of *Wolbachia* co-infections to tephritid species, obscuring ecological studies of this common endosymbiont and its horizontal transmission by parasitoids.

2.2 Introduction

Wolbachia are maternally inherited endosymbiotic Alphaproteobacteria widespread in arthropods (Hilgenboecker et al. 2008; Werren & Windsor, 2000; Weinert et al. 2015). *Wolbachia* can manipulate host reproduction or increase host fitness to persist despite imperfect maternal transmission (Hoffmann & Turelli 1997; Kriesner et al. 2013). The most common example of reproductive parasitism is cytoplasmic incompatibility (CI), which results in embryonic lethality when an infected male fertilises an uninfected female or a female bearing an incompatible *Wolbachia* strain, while presence of the same or a compatible *Wolbachia* strain in the embryo results in rescue of CI (Doremus & Hunter 2020; Werren et al. 2008; Werren, 1997). The modification and rescue functions of CI have recently been demonstrated to be encoded by pairs of *cytoplasmic incompatibility factor* (*cif*) genes within prophage regions of *Wolbachia* genomes (Beckmann et al. 2017; Bordenstein & Bordenstein, 2016; Chen et al. 2020; Lepage et al. 2017; Lindsey et al. 2018; Shropshire et al. 2020; Shropshire et al. 2018). Other reproductive manipulations are male-killing, thelytokous parthenogenesis and feminisation (Doremus & Hunter, 2020; Werren et al. 2008; Werren 1997). A candidate gene for male killing, *Wolbachia*-phage *WO-mediated killing* (*wmk*), has recently been identified as well (Perlmutter et al. 2019). Besides establishment in somatic host tissues (Cheng et al. 2000; Dobson et al. 1999; Ijichi et al. 2002), *Wolbachia* colonisation of the host germline is required to ensure successful maternal inheritance; induction of reproductive manipulations and/or host fitness benefits then increase infection frequencies in populations (Stouthamer et al. 1993; Werren et al. 2008). However, occasional horizontal transmission of *Wolbachia* from one host species to another occurs and has led to its wide distribution across different host lineages (Ahmed et al. 2015; Raychoudhury et al. 2009; O'Neill et al. 1992).

Several studies, however, have reported puzzling *Wolbachia* host associations. For instance, some insect species consistently deliver *Wolbachia*-positive diagnostic results only for a small subset of field-collected individuals, with sequence analyses indicating the presence of one or multiple *Wolbachia* strains in a host species (Chrostek & Gerth, 2019; Gichuhi et al. 2019; Hughes et al. 2011; Kittayapong et al. 2003; Sintupachee et al. 2006; Sun et al. 2007), or of the same strains across several host species (Jamnongluk et al. 2002; Morrow et al. 2014). Furthermore, these low prevalence *Wolbachia* positives may be regionally restricted and occur only in some populations of a species (Morrow et al. 2015). It remains an enigma how such consistently detected *Wolbachia* infections (thereafter referred to as enigmatic infections) can be maintained across host populations (even of different species) at low prevalence and titre. A possible explanation could be ongoing horizontal acquisition of *Wolbachia* by individuals of uninfected host populations through ecological interactions with individuals of a *Wolbachia*-infected species. This may occur naturally via direct ecological interaction of infected and uninfected hosts through the use of the same resources within a habitat (Chrostek et al. 2017; Kittayapong et al. 2003; Schuler et al. 2013; Sintupachee et al. 2006), or through common predators, parasites and parasitoids (Brown & Lloyd, 2015; Heath et al. 1999; Hoy & Jeyaprakash 2005; Kittayapong et al. 2003; Noda et al. 2001; Vavre et al. 1999; Le Clec'h et al. 2013). However, direct experimental evidence to demonstrate the early stages of *Wolbachia* establishment after horizontal transmission in field populations remains scarce (Ahmed et al. 2015; Chrostek et al. 2017; Ross et al. 2020).

An alternative explanation for the detection of enigmatic *Wolbachia* strains could be infections of a concealed host (e.g. a endoparasite) within a tested individual. This could lead to the inadvertent assignment of a *Wolbachia* infection to a wrong host and inflate the number of *Wolbachia*-host associations, while also obscuring the study of horizontal *Wolbachia* transmission. For instance, *Wolbachia* infections in *Ixodes ricinus* ticks have

previously been attributed to concealed parasitisation with an endoparasitoid wasp, *Ixodiphagus hookeri* (Plantard et al. 2012; Tijsse-Klasen et al. 2011). Similarly, the presence of another symbiont, *Arsenophonus nasoniae,* in *I. ricinus* has also been associated with *I. hookeri* parasitisation (Bohacsova et al. 2016).

Several invertebrate taxa which parasitise insects can be *Wolbachia* infected; these include the very distinctive, yet relatively little-studied order of twisted-wing insects (Strepsiptera) which consists entirely of endoparasitoid species (around 630 described species) with a macrynobiont strategy, i.e. continued development of the host insect, often to the adult stage, with host mortality occurring after completion of the strepsipteran life cycle (Kathirithamby 2009, 2018). For most strepsipteran families, all life stages are endoparasitic except for the mobile first instar larvae (planidia) which parasitise new hosts after release by the endoparasitic neotenic female, and the short-lived winged males that emerge to mate with neotenic females (Kathirithamby, 1991, 2009). Strepsipterans are known to parasitise a wide range of insect orders, and have several life history traits not shared with other parasitoid taxa, for example, they can parasitise adult hosts, affect their reproductive system and render them infertile (Kathirithamby, 1989b, 2009; Cappa et al. 2014), or can also extend host life span (Beani et al. 2021). Strepsipterans have very intimate associations with their host, for instance *Stichotrema dallatorreanum* is covered by host epidermal tissue, presumably to avoid the host immune system and it utilises host hemolymph for nourishment and secretion of materials (Kathirithamby et al. 2003). Previously, using electron microscopy, *Rickettsia*-like microorganisms were reported in the developing germ cells, embryos and larvae of strepsipteran species such as *Elenchus japonicus* and *Elenchus tenuicornis* that parasitise planthoppers, in *Xenos moutoni* that parasitises hornets, and in *Xenos vesparum* that parasitises paper wasps (Kathirithamby, 1998). However, so far, *Wolbachia* was only detected by PCR and sequence analysis in *E. japonicus* (Noda et al. 2001).

Australia hosts over 300 tephritid fruit fly species, with several species of *Bactrocera* (Tephritidae: Dacini), and in particular, Queensland fruit fly, *Bactrocera tryoni*, as the economically most significant (Dominiak & Daniels 2012; Drew, 1989; Hancock et al. 2000; Meats, 1981). *Wolbachia* gene sequences have previously been detected in male individuals of nine out of 24 tested Australian tephritid fruit fly species (Morrow et al. 2014, 2015). Only male individuals were analysed in these previous studies because sampling of adults in the field relied on the use of male lure traps. Multilocus sequence typing (MLST) revealed the presence of the complete MLST profiles of two different supergroup A *Wolbachia* strains with distinct MLST gene sequences, characterised as sequence types (ST) ST-285 and ST-289, that occurred in all positive individuals of seven Dacini species (*B. tryoni, Bactrocera neohumeralis, Bactrocera decurtans, Bactrocera frauenfeldi, Bactrocera bryoniae, Dacus axanus* and *Zeugodacus strigifinis*) as co-infections, and in a few individuals, as single infections (Morrow et al. 2014, Morrow et al. 2015). The remaining two species, *Bactrocera peninsularis* and *Bactrocera perkinsi*, did not have complete MLST sets and contained MLST alleles and pseudogenes not related to the two strains, possibly *Wolbachia* genome fragments that had been acquired by these two host species' genomes by lateral gene transfer (Morrow et al. 2014, Morrow et al. 2015). Across the seven species with the complete MLST profiles, the two *Wolbachia* strains co-occurred at a very low prevalence, with low but variable titres and were restricted to host populations of tropical Queensland. It is unknown whether the two *Wolbachia* strains have any effects on the fruit flies but mitochondrial haplotype analysis did not reveal a linkage of *Wolbachia* and any particular mitochondrial haplotypes (Morrow et al. 2014, 2015). Therefore, it remained unclear what maintains these two *Wolbachia* strains across these seven host species: either the previously tested adult flies had acquired *Wolbachia* from a common source, they had escaped parasitisation by a *Wolbachia* infected parasitoid in the larval stage, or the adult flies still harboured a

Wolbachia-infected parasite not seen during dissection of the field-caught flies before DNA extraction.

Our study applied a whole-genome sequencing (WGS) approach on DNA extracts of male flies previously found positive for one or both of the two *Wolbachia* strains to test whether these samples contained traces of concealed parasites that could be the potential hosts of *Wolbachia* in this system. Previous work demonstrated that opiine parasitoids of tephritids can carry *Wolbachia* (Morrow et al. 2014; Schuler et al. 2016). Therefore, we expected it was unlikely that adult fruit flies that had escaped parasitisation at an early developmental stage would still carry traces of an opiine parasitoid, but they could carry its *Wolbachia*. Furthermore, a strepsipteran endoparasitoid, *Dipterophagus daci,* has previously been described from adult individuals of *B. tryoni* and 18 other Dacini species across northern Australia and the Solomon Islands (Allwood & Drew, 1996; Drew & Allwood, 1985). *Dipterophagus daci* was originally assigned to a new and monotypic family, Dipterophagidae (Allwood & Drew, 1996; Drew & Allwood, 1985). However, this was questioned and an assignment to the Halictophagidae was proposed (Kathirithamby, 1989; Kathirithamby, 2018), and, therefore, the phylogenetic placement requires further evaluation with DNA sequence data. Here, by analysing WGS libraries of field-collected *Wolbachia*-positive Australian tephritid fruit flies without any obvious external signs of parasitisation (stylopisation) we unexpectedly detected the mitogenomes of a strepsipteran which we then confirmed as belonging to *D. daci*. We then developed a diagnostic tool to detect concealed early stages of *D. daci* in fruit fly individuals, and we demonstrated a link between *D. daci* and the two *Wolbachia* strains previously detected and characterised in the seven tephritid species. Finally, we applied qPCR in order to locate and quantify the two *Wolbachia* strains in *D. daci* and across dissected tissues of stylopised fruit flies.

2.3 Methods

Insect samples

Male tephritid fruit flies were collected from Queensland, Australia (Figure 2. 1, Table 2. 1) using male lure traps with insecticides as part of fruit fly monitoring programs (Royer & Hancock, 2012) in the years 1998, 2001, 2012 and 2013 (Morrow et al. 2014, 2015). The traps were emptied every 14 days and flies morphologically identified and kept as a dry sample for a few months. Flies were then transferred into pure ethanol and stored at -20 ºC for subsequent processing. Genomic DNA previously extracted using GenElute Mammalian Genomic DNA miniprep kit (Sigma) following previously described protocols (Morrow et al. 2014, 2015) was used in this study. The genomic DNA comprised extracts from abdomens of 64 individuals of seven tephritid species used in previous *Wolbachia* surveys with PCR primers for the *Wolbachia surface protein* (*wsp*) and *16S rRNA* genes, followed with further strain characterisation (ST-285 and ST-289) by cloning of *wsp* and MLST gene sequences and strain titre comparisons (Morrow et al. 2014, 2015). Here, an equal number of *Wolbachia*-positive and negative samples were used (Table 2. 1). These samples did not appear parasitised when the abdomens used for DNA extractions were removed under a dissection stereo microscope. At a later stage, another 23 male flies of eight species (Appendix A; Table A. 1) that had clear signs of parasitisation (stylopisation) were obtained from Cooktown, Cairns, Townsville, Airlie Beach and Mackay in 2019. The male and female strepsipteran extrusions of the stylopised flies were morphologically examined and confirmed as *D. daci*. The stylopised flies were then processed for dissection of fly and *D. daci* tissues for *Wolbachia* localisation and titre analyses. The DNA extracted from *D. daci* individuals was used for DNA barcoding of *D. daci*.

Table 2. 1: Tephritid fruit flies were screened with *wsp*-specific primers for the two *Wolbachia* strains ST-285 (*w*Ddac1) and ST-289 (*w*Ddac2), *16S rRNA* gene primers, *Dipterophagus daci*-specific *cox1* primers and with general insect *cox1* primers Pat and Dick used as a positive control of template quality (this primer pair only amplifies fruit fly and not *D. daci cox1*). All tephritid individuals (ordered by species and locality, from north to south) were male and provided fruit fly *cox1* amplicons. Positive and negative PCR results are denoted by y (yes) and n (no). Grey shading helps to identify linkages between *Wolbachia* and *D. daci*, and indicates PCR positives for *Wolbachia* and *D. daci*; no shading PCR-negative for *Wolbachia* and *D. daci*. Empty cells in the row of *D. daci cox1* denote samples not sequenced. All *D. daci cox1* sequences were identical to the *cox1* sequence of the mitochondrial genome of *D. daci* obtained from *Bactrocera frauenfeldi* 485.

Species	Sample ID No.	Collection Locality	Collection Year	*Wolbachia* ST-285	*Wolbachia* ST-289	*Wolbachia 16S* rRNA gene	*D. daci cox1*	*D. daci cox1* sequence	Fruit fly *cox1*
Bactrocera bryoniae	536	Lockhart	2013	y	y	y	y	y	y
Bactrocera bryoniae	535	Lockhart	2013	n	n	n	n		y
Bactrocera bryoniae	545	Cairns	2013	y	y	y	y	y	y
Bactrocera bryoniae	544	Cairns	2013	n	n	n	n		y
Bactrocera decurtans	85	Seisia	1998	y	y	y	y	y	y
Bactrocera decurtans	84	Seisia	1998	n	n	n	n		y
Bactrocera frauenfeldi	3	Lockhart	2019	y	y	y	y	y	y
Bactrocera frauenfeldi	5	Lockhart	2019	n	n	n	n		y
Bactrocera frauenfeldi	485	Cairns	2013	y	y	y	y	y	y
Bactrocera frauenfeldi	492	Cairns	2013	y	y	y	y	y	y
Bactrocera frauenfeldi	490	Cairns	2013	y	n	y	y	y	y
Bactrocera frauenfeldi	486	Cairns	2013	n	n	n	y	y	y
Bactrocera frauenfeldi	133	Cairns	2001	n	n	n	n		y
Bactrocera frauenfeldi	491	Cairns	2013	n	n	n	n		y
Bactrocera frauenfeldi	489	Cairns	2013	n	n	n	n		y
Bactrocera neohumeralis	243	Cairns	2012	y	n	y	y	y	y
Bactrocera neohumeralis	248	Cairns	2012	y	y	y	n		y
Bactrocera neohumeralis	34	Cairns	1998	n	n	n	n		y
Bactrocera neohumeralis	107	Cairns	1998	n	n	n	n		y
Bactrocera neohumeralis	246	Cairns	2012	n	n	n	n		y

Bactrocera neohumeralis	245	Cairns	2012	n	n	n	n		y
Bactrocera neohumeralis	171	Townsville	2019	y	y	y	y	y	y
Bactrocera neohumeralis	244	Townsville	2012	n	y	y	n		y
Bactrocera neohumeralis	168	Townsville	2019	n	n	n	n		y
Bactrocera neohumeralis	238	Mourilyan Harbour	2012	y	y	y	y	y	y
Bactrocera neohumeralis	135	Mourilyan Harbour	2019	y	y	y	y	y	y
Bactrocera neohumeralis	240	Mourilyan Harbour	2012	y	n	y	y	y	y
Bactrocera neohumeralis	239	Mourilyan Harbour	2012	n	n	n	n		y
Bactrocera neohumeralis	132	Mourilyan Harbour	2019	n	n	n	n		y
Bactrocera neohumeralis	342	Mackay	2012	y	y	y	y	y	y
Bactrocera neohumeralis	345	Mackay	2012	y	y	y	y	y	y
Bactrocera neohumeralis	343	Mackay	2012	n	n	n	n		y
Bactrocera neohumeralis	344	Mackay	2012	n	n	n	n		y
Bactrocera neohumeralis	347	Mackay	2012	n	n	n	n		y
Bactrocera neohumeralis	355	Gladstone	2012	y	y	y	y	y	y
Bactrocera neohumeralis	357	Gladstone	2012	n	n	n	n		y
Bactrocera neohumeralis	229	Gladstone	2012	n	n	n	n		y
Bactrocera tryoni	194	Cairns	2019	y	y	y	y	y	y
Bactrocera tryoni	275	Cairns	2001	y	n	y	y	y	y
Bactrocera tryoni	276	Cairns	2001	y	n	y	y	y	y
Bactrocera tryoni	277	Cairns	2001	n	n	n	n		y
Bactrocera tryoni	468	Cairns	2013	n	n	n	n		y
Bactrocera tryoni	190	Cairns	2019	n	n	n	n		y
Bactrocera tryoni	267	Townsville	2019	n	n	y	y		y
Bactrocera tryoni	265	Townsville	2019	n	n	n	n		y
Bactrocera tryoni	210	Mackay	2019	y	y	y	y	y	y
Bactrocera tryoni	439	Mackay	2013	y	n	y	y	y	y
Bactrocera tryoni	443	Mackay	2013	y	n	y	y	y	y
Bactrocera tryoni	441	Mackay	2013	n	n	n	n		y
Bactrocera tryoni	445	Mackay	2013	n	n	n	n		y
Bactrocera tryoni	207	Mackay	2019	n	n	n	n		y
Bactrocera tryoni	310	Gladstone	2019	n	n	y	n		y

Bactrocera tryoni	309	Gladstone	2019	n	n	n	n		y
Dacus axanus	87	Thursday Is	1998	n	n	n	n		y
Dacus axanus	86	Thursday Is	1998	n	n	n	n		y
Dacus axanus	88	Weipa	1998	y	y	y	y	y	y
Zeugodacus strigifinis	81	Bamaga	1998	y	y	y	y	y	y
Zeugodacus strigifinis	269	Bamaga	1998	y	y	y	y	y	y
Zeugodacus strigifinis	82	Bamaga	1998	n	n	n	n		y
Zeugodacus strigifinis	271	Bamaga	1998	n	n	n	n		y
Zeugodacus strigifinis	503	Cairns	2013	y	y	y	y	y	y
Zeugodacus strigifinis	504	Cairns	2013	y	y	y	y	y	y
Zeugodacus strigifinis	502	Cairns	2013	n	n	n	y	y	y
Zeugodacus strigifinis	505	Cairns	2013	n	n	n	y	y	y

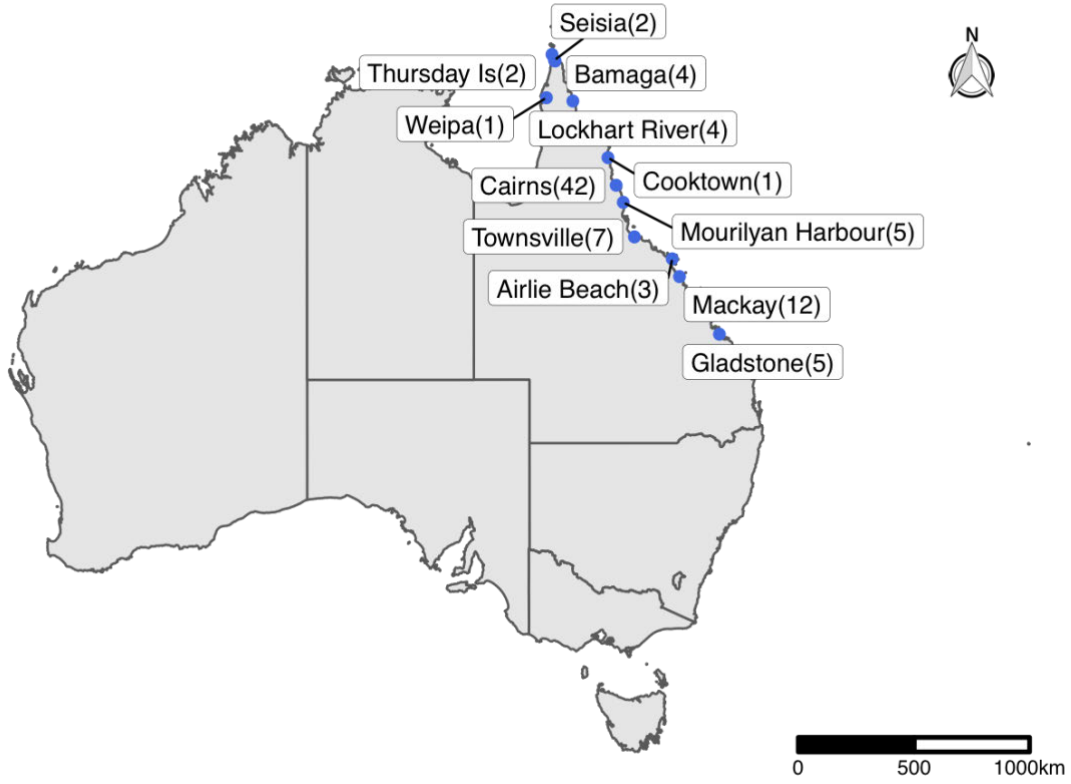

Figure 2. 1: Fruit fly collection sites. Map of Australia with the collection sites of male adult fruit flies (collected with male lure traps) that were tested for *Dipterophagus daci* and *Wolbachia*. Blue dots denote the collection localities, and the numbers in parentheses denote the number of fruit fly individuals collected from the localities and used for detection of *Wolbachia* and concealed and extruding *D. daci*. Flies were collected across central and northern Queensland, and, therefore, in an area that covers a large part of the known distribution of *D. daci* in Queensland, the Top End of the Northern Territory and the Solomon Islands (Allwood & Drew 1996).

Library construction and whole-genome sequencing (WGS)

Fourteen genomic DNA extracts previously characterised by Morrow et al. (2014, 2015) were used for WGS; these included genomic extracts of 13 specimens that were also PCR screened for the two *Wolbachia* strains ST-285 and ST-289 and *D. daci*, and a DNA extract of *B. peninsularis* which contained pseudogenes as described previously (Table 2. 2, Morrow et al. 2014, 2015). Prior to WGS, we performed multiple displacement amplifications using the

REPLI-g mini kit (Qiagen), followed by clean-up using the QIAamp DNA mini kit as per manufacturer's instruction and as previously described (Morrow et al. 2020; Morrow & Riegler 2021). The samples were then used for library construction using the TruSeq PCR-free library kit followed by sequencing on the Illumina HiSeq 2500 at Macrogen, Seoul Korea.

Read quality control and de novo assembly of mitogenomes

Sequence quality control (QC), trimming, *de novo* assembly and downstream processing were performed in CLC genomics workbench v12.0.3 (Morrow et al. 2020; Morrow & Riegler 2021). Raw reads were trimmed by removing the TruSeq adapter sequences and low sequence quality was maintained at 0.05. A maximum of two ambiguous nucleotides was allowed and sequences with fewer than 15 nucleotides and more than 1,000 nucleotides were removed. Trimmed reads were *de novo* assembled into contigs following CLC default parameters, with word size set at 24 and bubble size at 50.

To determine whether the WGS libraries contained eukaryotic host genomic data, we probed the 14 libraries for fruit fly mitogenomes. First, the *Ceratitis capitata* mitogenome (GenBank accession AJ242872.1) was queried against the contig list of the sample that returned the largest number of sequence reads, *B. frauenfeldi* 485. The contig with the best hit was extracted and verified using BLASTn search of the NCBI nucleotide database (downloaded February 2019) in CLC. *Bactrocera frauenfeldi* 485 trimmed reads were then mapped against the identified *B. frauenfeldi* draft mitogenome. The complete *B. frauenfeldi* draft mitogenome was then used as the reference genome to map and assemble the fly mitogenomes of the remaining 13 libraries. However, an additional complete mitogenome belonging to another insect that mapped only at 70% similarity to the *B. frauenfeldi* draft mitogenome was found

in six libraries. This other mitogenome was identified by BLASTn search as belonging to a strepsipteran. To determine its mitogenome coverage, the mitogenome of a strepsipteran, *Mengenilla australiensis* (GenBank accession GU188852.1), was queried against the *B. frauenfeldi* 485 contig list and contigs with the best hit were verified as previously stated. These contigs were extracted, assembled and used to determine the strepsipteran mitogenome coverage in the rest of the libraries (Table 2. 2). To further verify the presence of a strepsipteran, the presence of strepsipteran nuclear *18S rRNA* genes was queried using this gene of *M. australiensis* (GenBank accession JN082886.1) against the *B. frauenfeldi* 485 contig list, the best contigs extracted, joined and verified by read mapping and used to extract the strepsipteran *18S rRNA* genes from the remaining libraries. Previous phylogenetic analyses of the two *Wolbachia* strains ST-285 and ST-289 detected in Australian tephritids placed both strains into the *Wolbachia* supergroup A (Morrow et al. 2014, 2015). Therefore, the genome of the *Wolbachia* strain *w*Mel (Genbank accession CP042444.1) of *Drosophila melanogaster* was used as a reference to determine the presence of *Wolbachia* by querying the contig lists of each of the 14 libraries. The coverage of the *Wolbachia* strains was assessed by mapping the reads to the *w*Mel genome at 90% similarity and 90% length. The presence of *wsp* and two MLST genes of the two *Wolbachia* strains was determined by read mapping to *wsp*, *coxA* and *fbpA* reference sequences (Morrow et al. 2014, 2015). Furthermore, the presence or absence of *cif* and *wmk* genes in the libraries was determined by read mapping at the same stringency to sequences representing Type I (*w*Mel), Type III (*w*No), Type I and Type IV (*w*Pip) and Type V (*w*Stri) *cif* genes (Morrow et al. 2020) and the *wmk* gene (WD0626_of *w*Mel). The estimated average coverage for all the mappings were calculated using the formula: coverage = (read count * read length) / total genome size.

PCR screening for *D. daci*

Using the extracted strepsipteran mitogenome, *D. daci*-specific cytochrome c oxidase subunit I (*cox1*) primers (Appendix A; Table A. 2) were designed with Primer 3 v2.3.7 (Untergasser et al. 2012). This primer set was used for PCR screening of the 64 fruit fly DNA extracts. The PCR cycling conditions involved an initial denaturation for 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 56 °C and 30 s at 72 °C, then a final elongation step of 10 min at 72 °C. The MyTaq™ Mix (Bioline) PCR reagents were used following the manufacturer's instructions. The extracts were also tested for DNA quality using the mitochondrial *cox1* primers Pat and Dick (Simon et al. 1994) which amplified fruit fly *cox1* but not *D. daci cox1*. PCR products were electrophoresed in a 1% agarose gel stained using SYBR safe and visualised using UV-light transillumination. Positive and negative (non-template) controls were included in each PCR run and positive amplifications were confirmed by direct Sanger sequencing. Prior to sequencing, the PCR amplicons were purified using a mix of exonuclease I (New England Biolabs, Ipswich, MA, USA) and shrimp alkaline phosphatase (Promega) at 37°C for 30 min, then 95°C for 5 min (Morrow et al. 2014).

Wolbachia titre and localisation by qPCR

The 23 stylopised flies were obtained from the same fruit fly field monitoring programs as previously described, involving male lure traps. Stylopised flies were first stored dry until identification, and then stored in absolute ethanol at -20 ºC. Prior to dissection the samples were surface sterilised using 4 % bleach, followed by rinsing with 0.02 % triton-X and rinsed with Milli-Q water (Morrow et al. 2015). Dissections were performed on a clean glass slide under a stereo microscope using fine forceps for isolating different fly tissues and *D. daci*.

This involved dissection of fly head, thorax and abdomen, and then dissection of individual *D. daci* neotenic females with fully developed cephalothorax [fused head, pro- and mesothorax (Kathirithamby, 1989b)], individual male pupae and two individual adult males from the fly abdomen. The forceps were sterilised between dissections of different tissues by dipping them in 4% bleach then 0.02% Triton-X for a minute each, followed by three thorough washes in Milli-Q water. The isolation of *D. daci* females was difficult considering their neotenic nature and the fact that the abdominal segments were completely embedded in the host (Hughes et al. 2004; Kathirithamby, 1991). Therefore, dissections of *D. daci* females involved isolation of tissues around the female cephalothorax in the host abdomen and cannot be considered 'clean' dissections due to the high likelihood of including some fruit fly tissue. In contrast, dissections of *D. daci* pupa and adult male were easier and can be considered 'clean' dissections since the strepsipteran male puparium exists as a discrete chamber within the host abdomen.

The dissected tissues were placed into individual 1.5 mL microcentrifuge tubes for DNA extraction. DNA was extracted using GenElute Mammalian Genomic DNA miniprep kit (Sigma) following the manufacturer's instruction, and DNA elution in 100 µL of elution buffer. Standard PCR was used to detect *D. daci* using specific *cox1* primers, *Wolbachia* using *wsp* primers specific for ST-285 and ST-289 *Wolbachia* strains (Appendix A; Table A. 2, Morrow et al. 2014) and fruit fly DNA using the *cox1* primers Pat and Dick (Simon et al. 1994) (Appendix A; Table A. 2) which only amplified fruit fly but not *D. daci cox1*. Quantitative PCR (qPCR) was then used to determine *Wolbachia* localisation and titre in the dissected fly tissues and *D. daci* samples. For this, previously developed qPCR primer sets specifically targeting the *wsp* genes of the two *Wolbachia* strains (ST-285 and ST-289) and the *scarlet* gene of dacine fruit flies were used (Morrow et al. 2014; Zhao et al. 1998) (Appendix A; Table A. 2). Furthermore, qPCR primer pairs targeting a region of the *D. daci*

single copy *RNA polymerase II* gene were designed from sequences obtained from the WGS dataset (Appendix A; Table A. 2). qPCR was performed in duplicates using the SensiFAST SYBR® No-ROX kit as per manufacturer's instructions, quantification cycle (Cq) values for the *Wolbachia* and host genes were calculated from average of the duplicate Cq values and *wsp* Cq values were normalised against host gene Cq values as individual data points using 2-ΔCq (Schmittgen & Livak, 2008). In order to confirm the diagnostic reliability, we also included *Wolbachia*-negative and *D. daci*-negative samples in qPCR runs.

To determine if *Wolbachia* titres differed between the dissected *D. daci* and fruit fly tissues, we used an analysis of variance (ANOVA), followed by Tukey's HSD test to identify tissue samples that were significantly different from each other in their *Wolbachia* titres. The analyses were performed in R version 3.6.3 (R core Team, 2020, https://www.R-project.org/).

Species confirmation and first phylogenetic analysis of *D. daci*

SNPs were identified by aligning the 13 PCG sequences of the six strepsipteran mitogenomes (Bfra485, Bn171, Bn342, Btry194, Btry210, Zst503) and (Bn240 with low coverage that did not allow assembly of the mitogenome but allowed for SNP calling) and aligning the four *D. daci 18S rRNA* gene sequences (Bfra485, Bn342, Btry194, Btry210) obtained from the WGS libraries of this study. Similarly, the *cox1* gene sequences obtained by PCR and Sanger sequencing from *D. daci* individuals were aligned with the strepsipteran mitogenomes of the WGS libraries and analysed for SNPs. Multiple sequence alignments utilised CLUSTAL-W algorithm in Geneious v10.0.9 and Geneious variant/SNP finder tool was employed to determine the presence of SNPs and other differences applying the default settings and variant frequency (percentage relative frequency of the variant) displayed (Kearse et al. 2012)

In order to analyse the phylogenetic placement of *D. daci*, reference sequences for other

Strepsiptera were downloaded from GenBank (Appendix A; Table A. 3), and the corresponding sequences extracted from the WGS libraries. The *D. daci* mitochondrial genes *cox1*, NADH dehydrogenase subunit I (*nad1*) and the large subunit ribosomal RNA (*16S rRNA*) genes, as well as the nuclear small subunit ribosomal RNA (*18S rRNA*) gene obtained from the WGS were identical, therefore we used sequences obtained from *B. frauenfeldi* 485 WGS library for analysis. Multiple sequence alignment of *cox1* and *nad1* genes were performed using translated alignments while nucleotide alignments were performed for *16S rRNA* and *18S rRNA* genes in MEGA-7 (Kumar et al. 2016). The final alignment was used in phylogenetic analysis. Bayesian phylogenetic analysis was performed using BEAST v2.6.0 (Drummond & Rambaut, 2007a) applying the bModelTest package. A chain length of 10 million was applied with sampling every 1000 trees. BEAST output and burn-in were visualised in TRACER v1.4.7 (Drummond & Rambaut, 2007c) and the first 10% of the sampled trees were discarded as a burn-in cut off using TREEANNOTATOR. FigTree v1.4.4 was used for tree visualisation and drawing (Drummond & Rambaut, 2007b). In addition, a maximum likelihood phylogenetic analysis was performed for confirmation using MEGA applying the GTR model at 100 bootstraps, which was determined using find the best DNA model in MEGA-7 (Kumar et al. 2016).

2.4 Results

Evidence of *D. daci* genomic sequences in fruit fly WGS libraries

WGS analyses of 14 *Wolbachia*-positive fruit fly genomic extracts were performed and this revealed the presence of fruit fly mitochondrial and *Wolbachia* genomes. Mapping coverage for the fruit fly mitogenomes ranged from <1 to 5,427-fold. In six libraries we also found

nearly complete mitogenomes belonging to a different insect, while the remaining eight libraries had some to no traces of these additional mitogenomes (Table 2. 2). According to a BLASTn search, these mitogenomes belonged to a strepsipteran species which we confirmed as *D. daci* by sequencing the *cox1* gene of adult *D. daci* specimens obtained from our study. The libraries that had good coverage (>18-fold) for the *D. daci* mitogenome included *B. frauenfeldi* 485, *B. tryoni* 194 and 210, *B. neohumeralis* 171 and 342, and *Z. strigifinis* 503. The complete *D. daci* draft mitogenome was also extracted from *B. neohumeralis* 240, albeit at low coverage (<5-fold). The low and uneven coverage (<1-fold) in *B. bryoniae* 536, *B. neohumeralis* 135, *B. tryoni* 275 and *B. neohumeralis* 244 libraries precluded the assembly of additional complete mitogenomes from these libraries. The library of *B. peninsularis* was the only WGS library with no coverage for the *D. daci* mitogenome. The *18S rRNA* gene sequences of *D. daci* were extracted from four libraries (*B. frauenfeldi* 485, *B. tryoni* 194 and 210 and *B. neohumeralis* 342) using available reference sequences from other strepsipteran species while the remaining libraries had relatively low to no coverage of this gene (Table 2. 2). The mapping coverage of *Wolbachia* by using *w*Mel as a related supergroup A reference genome for the two strains ST-285 and ST-289 ranged from <1 to 37-fold. The four libraries that had good coverage for *Wolbachia* genomes included *B. frauenfeldi* 485, *B. tryoni* 194 and 210, and *B. neohumeralis* 135; the rest contained *Wolbachia* reads, however at very low coverage (<1-fold) (Table 2. 2). The high *Wolbachia* genome coverage (11.3-fold) without *D. daci* mtDNA in *B. neohumeralis* 135 may indicate a transient *Wolbachia* infection, possibly as a result of horizontal acquisition of *Wolbachia*. Furthermore, the presence of *wsp* and MLST gene sequences (*coxA* and *fbpA*) of ST-285 and ST-289 confirmed the presence of these two *Wolbachia* strains in our WGS libraries. *Wolbachia* genome reads were also analysed as to whether they contained *cif* and *wmk* genes, but no hits were detected by mapping reads to *cif* genes of *w*Mel (Type I), *w*No (Type III), *w*Pip (Type I and Type IV) and

*w*Stri (Type V) and the *wmk* gene (WD0626 of *w*Mel). Therefore, it is unlikely that the two *Wolbachia* strains can cause CI or male killing.

Sequences obtained in this study were deposited in GenBank: the *Dipterophagus daci* mitogenome extracted from *Bactrocera frauenfeldi* 485 GenBank accession number MW233588, *D. daci 18S rRNA* gene extracted from *B. frauenfeldi* 485 accession number MW241536 and *D. daci* RNA polymerase II gene extracted from *B. frauenfeldi* 485 accession number MW241535. Raw reads (for Bfra485, Bn171, Bn342, Bt194, Bt210 and Zst503) were submitted to NCBI Sequence Read Archive under the BioProject accession number PRJNA682518.

Linkage between *Wolbachia* and the presence of *D. daci* in fruit flies

The presence of nearly complete *D. daci* mitogenomes in the *Wolbachia*-positive fruit fly WGS libraries suggested a link between *Wolbachia* and the presence of *D. daci.* We therefore screened 64 genomic DNA extracts for both *Wolbachia* strains and *D. daci* (Figure 2. 1, Table 2. 1). Out of the 28 samples positive for at least one of the two *Wolbachia* strains using *wsp* and *16S rRNA* gene primers, 26 were positive for *D. daci* while two *B. neohumeralis* samples positive for either one or both *Wolbachia* strains were negative for *D. daci*. Out of two *B. tryoni* samples that were only positive for the *Wolbachia 16S rRNA* gene (but not for *wsp*), one was positive for *D. daci*. Out of the 34 samples that were negative for *Wolbachia*, 31 were negative for *D. daci* and the remaining three individuals, one *B. frauenfeldi* and two *Z. strigifinis* were *D. daci*-positive. Therefore, with the exception of six individuals, a linkage of *D. daci* and *Wolbachia* was confirmed. The absence of *Wolbachia* in the three *D. daci*-positive flies could indicate that the flies were parasitised by *D. daci* not infected by *Wolbachia* (possibly due to imperfect maternal transmission), and the three *Wolbachia*-positive flies in absence of *D. daci* could indicate a diagnostic resolution issue for very early stages of *D. daci*, or, alternatively, low levels of horizontal transmission of *Wolbachia* from *D. daci* to the fruit fly hosts for which we found further evidence (see below).

Table 2. 2: Sequence reads obtained from 14 tephritid fruit fly WGS libraries (ordered by species and locality) and the mapping coverage of the fly mitogenome, the *Wolbachia* genome (*w*Mel was used as reference genome*)*, the *Dipterophagus daci* mitogenome and the *D. daci 18S rRNA* gene. The presence and absence of *Wolbachia* and *Dipterophagus daci* by read mapping is denoted by (yes) and n (no), respectively; y* denotes low read quality.

Species	Sample ID	Collection locality	*Wolbachia* presence	*D. daci* presence	Number of reads after QC	Fly mt genome mapped reads (coverage)	*w*Mel mapping (coverage)	*D. daci* mt genome mapped reads (coverage)	*D. daci 18s rRNA* gene mapped reads (coverage)
Bactrocera bryoniae	536	Lockhart River	y	y	61,877,652	427,844 (2698)	3,526 (0.28)	2 (0.01)	164 (11.5)
Bactrocera decurtans	85	Seisia	y*	y*	70,030,306	152 (0.95)	10,858 (0.86)	74 (0.49)	2 (0.1)
Bactrocera frauenfeldi	485	Cairns	y	y	109,057,960	11,544 (72)	474,952 (37.46)	15,938 (104.5)	236 (16.6)
Bactrocera neohumeralis	244	Cairns	n	y	60,128,324	496,912 (3101)	2 (0)	52 (0.34)	16 (1.1)
Bactrocera neohumeralis	171	Townsville	y	y	77,482,368	62060 (387)	2,038 (0.16)	4,094 (26.8)	4 (0.3)
Bactrocera neohumeralis	240	Mourilyan Harbour	n	y	72,186,748	36,332 (228)	80 (0.006)	320 (2.10)	18 (1.3)
Bactrocera neohumeralis	135	Mourilyan Harbour	y	y*	68,308,764	86,7084 (5427)	143,684 (11.33)	6 (0.04)	6 (0.4)
Bactrocera neohumeralis	342	Mackay	y	y	67,282,474	47,976 (300)	1,334 (0.11)	4,086 (26.7)	11,388 (799)
Bactrocera peninsularis	197	Seisia	n	n	75,579,632	204,904 (1285)	112 (0.01)	0	8 (0.6)
Bactrocera tryoni	194	Cairns	y	y	79,574,356	530,836 (3312)	38,598 (3.04)	8,104 (53.2)	1750 (122.8)
Bactrocera tryoni	275	Cairns	n	n	68,023,992	15,178 (94.7)	126 (0.01)	6 (0.04)	0
Bactrocera tryoni	210	Mackay	y	y	63,859,882	18,486 (115.4)	58,058 (4.58)	6,892 (45.2)	3926 (275.5)
Dacus axanus	88	Weipa	y*	y*	95,439,898	2 (0.01)	430 (0.03)	70 (0.46)	6 (0.4)
Zeugodacus strigifinis	503	Cairns	y	y	65,468,734	579,200 (3646)	3,486 (0.27)	11,210 (73.5)	6 (0.4)

Wolbachia localisation and quantification

To determine the titre and tissue localisation of the *Wolbachia* strains ST-285 and ST-289, an additional 23 male individuals of eight tephritid species (*B. abscondita, B. aeruginosa, B. breviaculeus, B. frauenfeldi, B. neohumeralis, B. mayi, B. pallida* and *B. tryoni*) that were visibly parasitised (stylopised) by *D. daci* were selected. Most of these flies were superparasitised with two to six adult *D. daci*, as both females and males were observed to extrude from the ventral side of the fruit fly abdomen (Figure 2. 2). The stylopised fruit fly males were also observed to have the aedeagus unusually positioned and elongated (Appendix A; Figure A. 1). This unusual position was not observed in non-stylopised males (Appendix A; Figure A. 1). Dissections were carried out on stylopised flies that were categorised into three types of parasitisation: (1) parasitisation with *D. daci* male/s only, with the male/s emerged and exited (extrusion empty); (2) single parasitisation by either a *D. daci* male pupa or a neotenic female only, (3) superparasitisation with more than one *D. daci* individual (Appendix A; Figure A. 1). In each of two stylopised flies, one extrusion contained a fully developed adult male whereas all other extrusions were either male pupae or the cephalothoraxes of neotenic females.

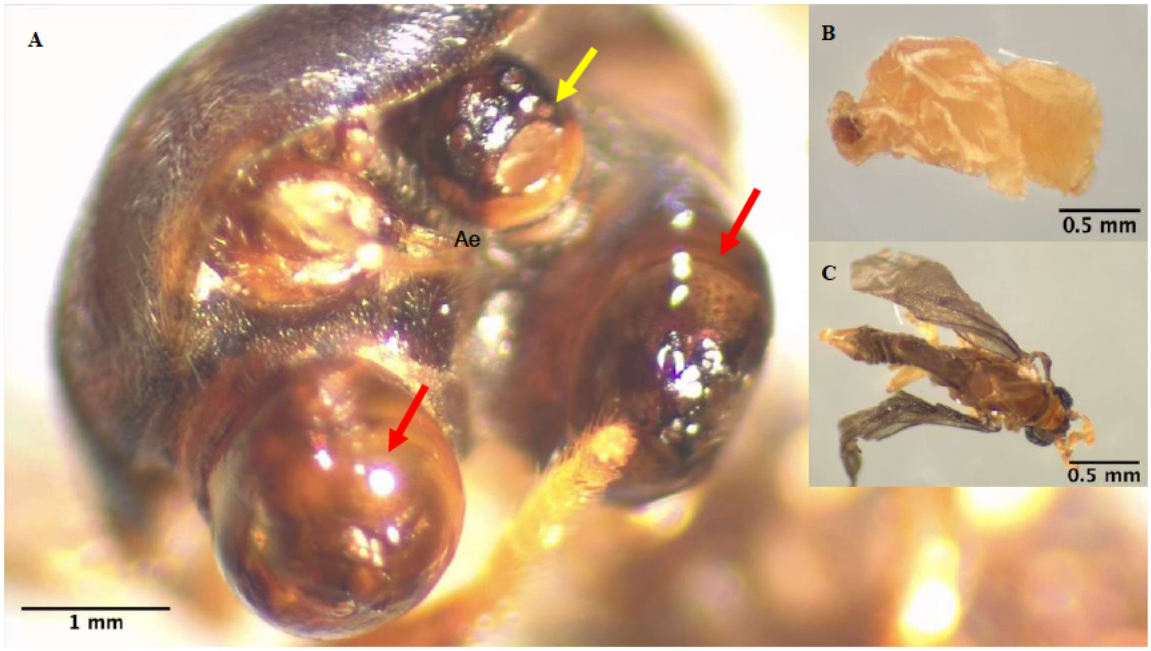

Figure 2. 2: Stylopised *Bactrocera tryoni* abdomen, *Dipterophagus daci* male pupa and adult male. (A) Posterior view of the abdomen of a male *B. tryoni* stylopised by *D. daci*. Red arrows indicate *D. daci* male extrusions, yellow arrow indicates a neotenic *D. daci* female cephalothorax on the ventral side of the fruit fly abdomen; unusually positioned and elongated fruit fly aedeagus (Ae) is visible; (B) *D. daci* male pupa dissected from an extrusion; (C) *D. daci* adult male (not yet emerged) dissected from an extrusion.

DNA was extracted from the dissected parts of stylopised flies: fruit fly heads, thoraces and abdomens after removal of extrusions; *D. daci* neotenic female cephalothoraces, male pupae and adult males. Then, the extracts were screened for the presence of *D. daci* DNA by using standard PCR. All the fly heads and thoraces were negative for *D. daci.* However, the fly abdomens had traces of *D. daci* (Appendix A; Table A. 1). *Dipterophagus daci* male pupae and adult males were all negative for fly DNA, however, *D. daci* females were positive for fly DNA (Appendix A; Table A. 1).

Quantitative PCR (qPCR) was then performed to determine the presence and the relative titres of ST-285 and ST-289 compared to host genes across the three tephritid body regions and *D. daci* individuals. The two *Wolbachia* strains were detected in all stylopised flies (except Bt_CN14.3 which contained only the ST-285 strain), albeit at variable titres (Appendix A; Figure A. 2). The titres of the strains ST-285 and ST-289 were different across

the dissected *D. daci* individuals and fruit fly host tissues ($F=6.45_{(5,190)}$, $p < 0.001$ and $F=18.16_{(5,192)}$, $p = < 0.001$; Figure 2. 3, Table 2. 3). Consistently, the *Wolbachia* titres were highest in *D. daci* individuals, albeit similar to titres in fruit fly abdomens, but significantly higher than in fly thoraces and heads (Figure 2. 3, Table 2. 3). Interestingly, *Wolbachia* was detected at high titres in the stylopised fly tissues from which *D. daci* male had emerged and left, including Bt_CN14.3 in which only ST-285 was detected (Appendix A; Figure A. 2). The ratios of the titres of ST-289 to ST-285 in co-infected individuals was very variable and ranged from 97.8 to 0.004 (Appendix A; Figure A. 2; Appendix A; Table A. 1), and this indicates that both strains are independent entities.

Table 2. 3: ANOVA of the *Wolbachia* titre and localisation in dissected *Dipterophagus daci* and fruit fly host tissues from 23 stylopised flies. ANOVA was performed for each *Wolbachia* strain (ST-285 and ST-289) independently. The Cq difference denotes the difference between the mean Cq values of the two categories of samples. Significantly different values are denoted by asterisks (ANOVA, P< 0.05 '*', 0.01 '**', 0.001 '***'). Comparisons that are significantly different are presented in bold.

	ST-285		**ST-289**	
	Cq Difference	**ANOVA**	**Cq Difference**	**ANOVA**
***D. daci* adult female - *D. daci* adult male**	9.128391	0.7689	9.5414384	0.0335*
***D. daci* adult female - *D. daci* male pupa**	5.170155	0.675	5.1452194	0.0164*
***D. daci* adult female - fly abdomen**	11.021712	0.0092**	9.3912346	0***
***D. daci* adult female - fly head**	14.074508	0.0003***	11.9251756	0***
***D. daci* adult female - fly thorax**	15.424312	0***	11.5751737	0***
D. daci adult male - *D. daci* male pupa	-3.958236	0.992	-4.3962191	0.7161
D. daci adult male - fly abdomen	1.893321	0.9997	-0.1502038	1
D. daci adult male - fly head	4.946117	0.9759	2.3837371	0.9703
D. daci adult male - fly thorax	6.29592	0.933	2.0337353	0.9853
***D. daci* male pupa - fly abdomen**	5.851557	0.3695	4.2460153	0.0226*
***D. daci* male pupa - fly head**	8.904353	0.0375*	6.7799562	0***
***D. daci* male pupa - fly thorax**	10.254157	0.0094**	6.4299543	0.0001***
Fly abdomen - fly head	3.052796	0.8593	2.5339409	0.3029
Fly abdomen - fly thorax	4.402599	0.5603	2.1839391	0.4736
Fly head - fly thorax	1.349804	0.9958	-0.3500018	0.9997

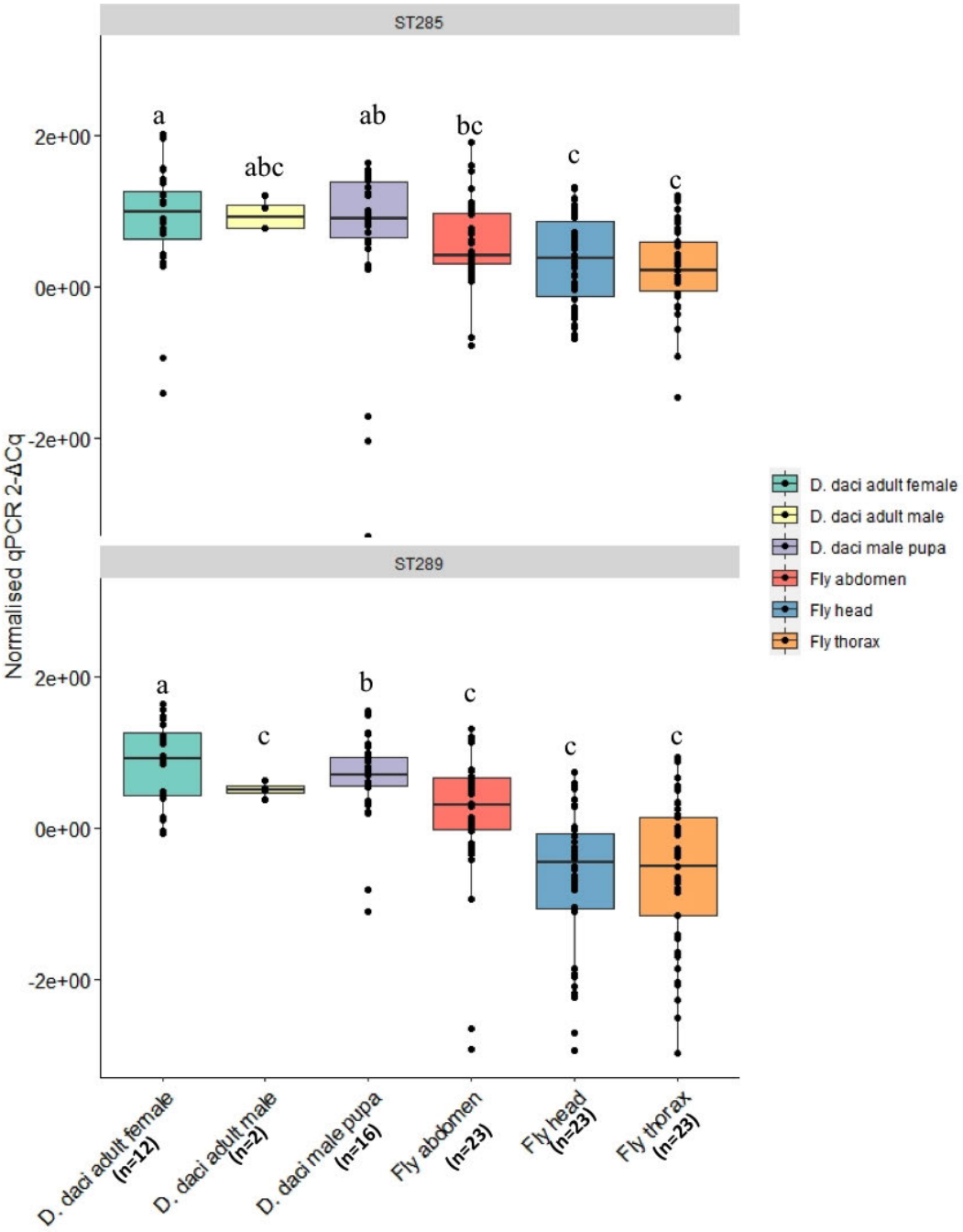

Figure 2. 3: Relative titre and localisation of the two *Wolbachia* strains ST-285 and ST-289 in *Dipterophagus daci* and dissected fruit fly tissues using qPCR, presented as Cq values normalised against *D. daci* and fruit fly host genes, and graphed on a logarithmic scale. Samples were obtained by dissection of 23 tephritid fruit flies parasitised by *D. daci*. Letters (a-c) denote a significant difference in means (Tukey's HSD test). Median values are shown as the middle line in each boxplot, and the interquartile range is represented by the boxed areas. Box and whiskers indicate the maximum and minimum values except the outliers. The dots represent the individual data points and n the number of samples.

Genetic diversity and phylogenetic placement of *D. daci*

To determine whether the strepsipteran mitogenomes obtained by WGS belonged to *D. daci*, we compared their *cox1* genes with PCR amplicon sequences obtained from adult *D. daci* DNA extracts. All *cox1* gene sequences were without any single nucleotide polymorphisms (SNPs), confirming that the mitogenomes belonged to *D. daci.* Next, we examined the mitogenomes and nuclear *18S rRNA* gene sequences of *D. daci* obtained by WGS for SNPs and other differences. Comparison of 13 protein coding genes (PCGs) of the six *D. daci* mitogenomes and one partial mitogenome with low coverage that did not allow assembly of the mitogenome revealed ten polymorphic sites (Appendix A; Table A. 4) and very few variable sites were found in the AT-rich region. In contrast, the nuclear *18S rRNA* gene (1,425 bp) of *D. daci* obtained from four WGS libraries (Bfra485, Bn342, Btry194, Btry210) contained 19 polymorphic sites (18 SNPs and one indel) (Appendix A; Table A. 4). Because of the low diversity, we used the mitochondrial and *18S rRNA* gene sequences of *D. daci* from the *B. frauenfeldi* 485 WGS library for the phylogenetic analysis of *D. daci* together with reference sequences of other species obtained from GenBank. Phylogenetic analysis using Bayesian inference (Figure 2. 4) and maximum likelihood (Appendix A; Figure A. 3) placed *D. daci* in the Halictophagidae and the topology of both trees was the same. Furthermore, the analysis showed monophyletic clusters in most genera except for *Halictophagus* which was a polyphyletic grouping with *Callipharixenos* and *Tridactylophagus* (Figure 2. 4; Appendix A; Figure A. 3).

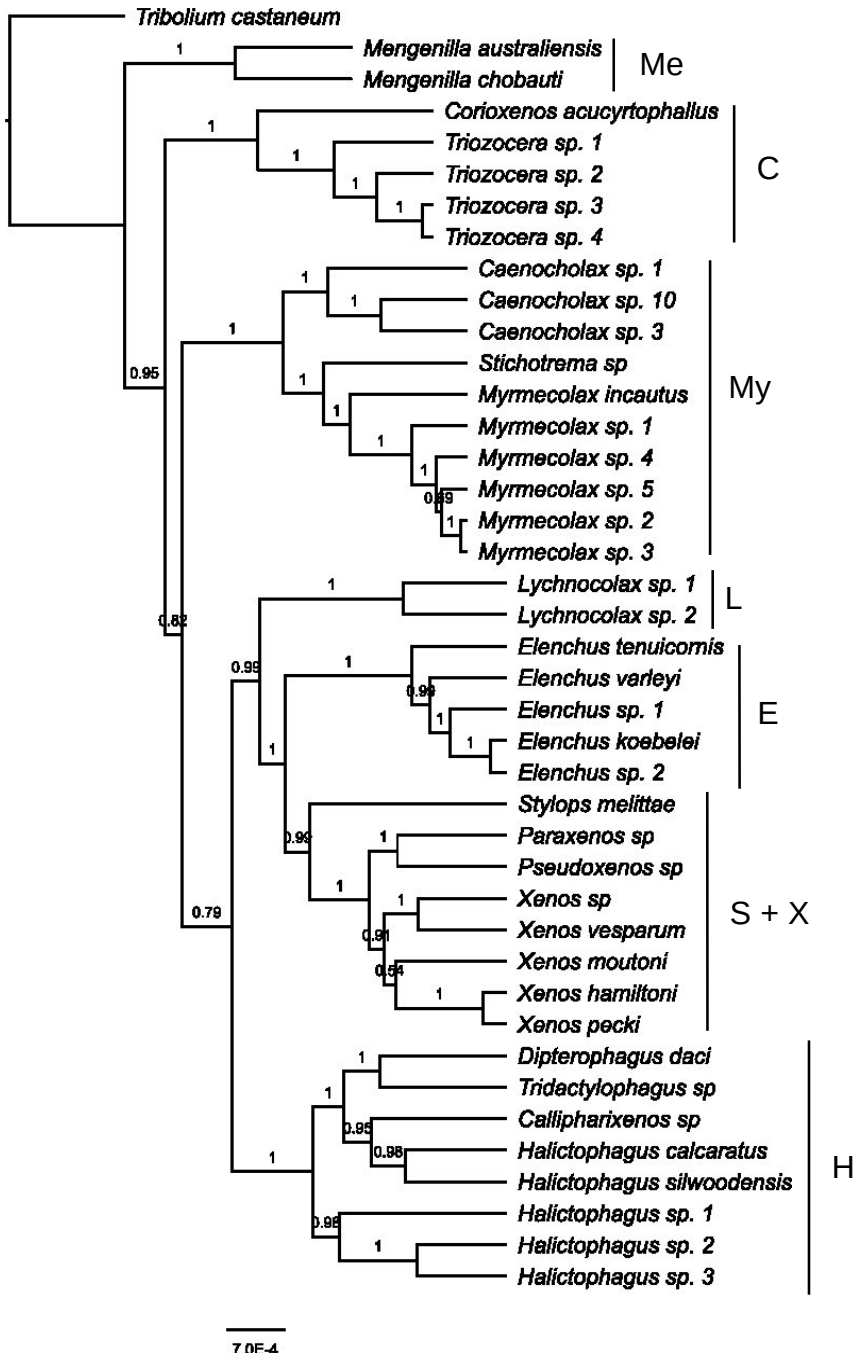

Figure 2. 4: Bayesian inference phylogenetic tree based on concatenated mitochondrial *cox1*, *nad1*, *16S rRNA* and nuclear *18S rRNA* gene alignments. The tree includes representative species of eight strepsipteran families as indicated by the vertical bars on the right: Mengenillidae (Me); Corioxenidae (C); Myrmecolacidae (My); Lychnocolacidae (L); Stylopidae (S); Xenidae (X); Elenchinidae (E); Halictophagidae (H). The reference sequences were obtained from NCBI GenBank. Branch labels are posterior probability values, and the scale bar shows the number of substitutions per site. The tree was rooted with *Tribolium castaneum* as an outgroup.

2.5 Discussion

We demonstrated that the detection of the two *Wolbachia* strains ST-285 and ST-289 in field-collected individuals of seven Australian tephritid fruit fly species collected using male lure traps was due to the presence of concealed *Wolbachia*-infected *D. daci* endoparasitoids. We disentangled this complex interaction of host-endoparasitoid-endosymbiont by using WGS analyses that uncovered the presence of *D. daci* genomic sequences in DNA extracts of *Wolbachia*-positive individuals of Australian tephritid species. Screening of additional male fruit fly individuals with *D. daci* and *Wolbachia* diagnostic markers verified a link between the endoparasitoid and the two *Wolbachia* strains in the majority of the tested individuals. Furthermore, localisation and quantification of *Wolbachia* across dissected *D. daci* individuals and fruit fly host tissues of stylopised flies confirmed the presence of the two *Wolbachia* strains ST-285 and ST-289 in *D. daci*, suggesting that the previously detected *Wolbachia* strains were linked to *D. daci* parasitism. This was further supported by the very low mitogenome variation across a large region of the known geographic distribution of *D. daci* – an outcome usually observed in species in which *Wolbachia* manipulates host reproduction or causes host fitness effects (Turelli et al. 1992; Hurst & Jiggins, 2005; Morrow & Riegler, 2021). Given that no *cif* and *wmk* gene orthologues were found in the *Wolbachia* reads of the WGS libraries we can exclude reproductive manipulations and conclude that the *Wolbachia* strains may have beneficial effects on *D. daci*. Furthermore, the presence of the MLST genes confirmed the presence of the two *Wolbachia* strains of *D. daci*, ST-285 (*w*Ddac1) and ST-289 (*w*Ddac1) in our libraries. However, future characterisation of their genomes should shed more light on their diversity and function.

Interestingly, in stylopised flies, we did not detect *D. daci* DNA in the fly heads and thoraces, yet these two body regions were *Wolbachia*-positive at low titre which is possibly indicative of somatic *Wolbachia* infections acquired by flies as a consequence of parasitisation and stylopisation by *Wolbachia*-infected *D. daci*. We also observed highly variable ratios between the titres of the two *Wolbachia* strains suggesting that the two *Wolbachia* strains are independent. Furthermore, we detected three out of a total of 88 tested flies of 13 tephritid fruit fly species (tested by WGS, standard PCR, tissue dissection and/or qPCR) that were *Wolbachia*-positive without presence of *D. daci*. This detection of *Wolbachia* DNA could be a consequence of failed parasitisation, involvement of very few or very early stages of *D. daci* in parasitisation that may be difficult to detect, and/or a consequence of horizontal transmission of *Wolbachia* from *D. daci*. We also detected three flies that were positive for *D. daci* but negative for *Wolbachia*, suggesting that the two *Wolbachia* strains are not fixed in *D. daci* populations, and their maternal transmission may be imperfect in *D. daci*, as seen in other *Wolbachia*-host interactions (Hoffmann & Turelli, 1997). Our study established *D. daci* as an attractive strepsipteran host species for further investigation of the two *Wolbachia* strains, their potential fitness effects for *D. daci* and its host species as well as the role of endoparasitoids in the horizontal transmission of *Wolbachia*.

Concealed strepsipterans can lead to incorrect host assignment of endosymbionts

Attack of host insects occurs by first instar strepsipteran larvae (or planidia), and parasitisation is only externally visible after the fourth larval instar when adult males and females extrude through the host cuticle (Kathirithamby, 1989b, 2009; Noda et al. 2001). Extrusion of *D. daci* only becomes apparent in adult host flies (Drew & Allwood, 1985). Therefore, there is a lack of external signs of parasitisation in hosts throughout much of

strepsipteran larval development. Strepsiptera are cosmopolitan and are known to parasitise a wide range of insect orders (Kathirithamby, 1989b, 2005, 2009). Thus, field-collected specimens used in *Wolbachia* surveys could be parasitised by *Wolbachia*-infected strepsipterans, potentially leading to incorrect *Wolbachia* host assignments as previously demonstrated for ticks parasitised by parasitoid wasps (Plantard et al. 2012; Tijsse-Klasen et al. 2011). However, in contrast to many other endoparasitoid taxa (such as parasitoid wasps and flies), strespsipteran endoparasitoids can also be found in adult hosts. Therefore, if *Wolbachia* surveys focus on adults (as many do) the issue of concealed parasitisation by strepsipterans may be more likely encountered (and therefore its lack of detection more serious) than concealed parasitisation by other endoparasitoid taxa.

The previous detection of *Rickettsia*-like microorganisms in four strepsipteran species by electron microscopy (Kathirithamby, 1998), including in *E. japonicus* by PCR (Noda et al. 2001) suggested that *Wolbachia* may be common in Strepsiptera. Furthermore, the *Wolbachia* strains detected in *E. japonicus* were identical with *Wolbachia* strains detected in individuals of the two rice planthoppers *Laodelphax striatellus* and *Sogatella furcifera* that are hosts of this species (Noda et al. 2001) or of closely related strepsipterans (Hughes et al. 2004). While it has been suggested that the strepsipterans mediated the horizontal transmission of *Wolbachia* between the planthopper species (Noda et al. 2001), these findings could also have been due to the detection of concealed *Wolbachia*-infected strepsipteran larval stages within the planthoppers. Another study detected similar *Wolbachia* strains at a very low prevalence in several hopper species, including *L. striatellus* and *S. furcifera* (Kittayapong et al. 2003) but this study did not test for strepsipterans. *Wolbachia* at low prevalence, low titres and without clear geographic patterns were also reported in the sugarcane planthoppers *Perkinsiella saccharicida* and *Perkinsiella vitiensis* (Hughes et al. 2011). Interestingly, *Perkinsiella* species are also known hosts of strepsipterans

(Kathirithamby, 2018; Osborn, 1969). Furthermore, *Wolbachia* at very low prevalence, with inconsistent results, and with closely related strains were detected in several tephritids (also belonging to Dacini) in Asia and Africa (Asimakis et al. 2019; Gichuhi et al. 2019; Jamnongluk et al. 2002; Sun et al. 2007). Therefore, further testing will be required to determine whether any previously reported enigmatic *Wolbachia* detection is due to the presence of concealed *Wolbachia*-infected strepsipterans (and other endoparasitoids). The diagnostic tools and approach that we have developed for Strepsiptera may be useful for such research.

Revisiting the contribution of endoparasitoids to horizontal transmission of *Wolbachia*

Horizontal transmission of endosymbionts from one host species to another can occur (Chrostek et al. 2017), however, the mechanisms are still poorly understood. Host-parasitoid interactions are regarded as a route of horizontal transfer of endosymbionts between species (Duron et al. 2010; Vavre et al. 1999; West et al. 1998). The detection of *Wolbachia* in three fruit fly individuals that were negative for *D. daci* has several possible explanations. This could be due to failed detection of *D. daci* (due to involvement of few or very early stages of endoparasitoids). It could also indicate possible horizontal transmission of *Wolbachia* from *D. daci* to the fruit flies, e.g. after failed parasitisation of tephritid fruit flies or environmental exposure to *Wolbachia* infected *D. daci*. We also detected *Wolbachia* in fly heads and thoraces of stylopised flies, while these body regions were negative for *D. daci* DNA, implying that *Wolbachia* detected in these tissues were somatic infections because they could not directly be linked to the presence of *D. daci* endoparasitoids in these same tissues of parasitised flies. Horizontal transfer of *Wolbachia* between strepsipteran endoparasitoids and their hosts is probable owing to their life-long intimate relationship, but any such detected

Wolbachia may not be inherited if it is not established in the germline tissue of the fruit flies (Morrow et al. 2015). Future research is required to investigate the role of *D. daci* in horizontal transmission of *Wolbachia*, e.g. using fluorescence in situ hybridization (FISH) for further resolution of the *Wolbachia* localisation across different tissues.

Phylogenetic placement of *D. daci*

Our phylogenetic analysis placed *D. daci* as a sister lineage to *Tridactylophagus* in the family Halictophagidae, and, thereby, confirmed the polyphyletic nature of *Halictophagus* (McMahon et al. 2011). The phylogenetic placement of *D. daci* in Halictophagidae is in agreement with earlier morphological analyses (Kathirithamby, 1989b) and argues against the previous proposal of the family of Dipterophagidae (Allwood & Drew, 1996; Drew & Allwood, 1985). Further research is required on the relationship of *D. daci* as member of the proposed subfamily Dipterophaginae within the Halictophagidae, the most species-rich family of Strepsiptera (Kathirithamby 2018), and its relationship to other, yet undescribed strepsipteran species from platystomatid flies from Papua New Guinea (Drew & Allwood, 1985; Riek, 1970). These other species and *D. daci* are the only strepsipterans known to parasitise Diptera.

Dipterophagus daci and dacine fruit flies

The first molecular characterisation of *D. daci* from seven fruit fly species collected across a very large area of its known distribution revealed very little genetic diversity supporting the idea that one strepsipteran species parasitised these seven fruit fly species (Towett-Kirui et al. 2021) . The very high *Wolbachia* prevalence in *D. daci*, the low genetic diversity across the

PCGs of *D. daci* mitogenomes and moderate diversity in the nuclear *18S rRNA* gene suggest that *Wolbachia* may have caused a reduction of mitochondrial diversity, possibly due to selective traits caused by the *Wolbachia* strains. Given that no *cif* and *wmk* gene orthologues were found in the WGS libraries, it is likely that *Wolbachia* does not manipulate host reproduction but rather confers a host fitness benefit to this host species. In contrast to interactions with *D. daci*, previous research did not demonstrate any links between *Wolbachia* and fruit fly mitochondrial haplotypes (Morrow et al. 2015). Future research will need to elucidate the fitness effects that the two strains *w*Ddac1 and *w*Ddac2 have on *D. daci.*

Dipterophagus daci has previously been recorded from 19 Dacini species (Allwood & Drew, 1996; Drew & Allwood, 1985). In the current study we detected *D. daci* in 13 fruit fly species. Ten of these species had previously been recorded as hosts (*B. abscondita, B. aeroginosa, B. breviaculeus, B. bryoniae, B. decurtans, B. frauenfeldi, B. mayi, B. neohumeralis, B. tryoni, D. axanus*), and for a further three species we have provided the first host record: we detected concealed *D. daci* in *B. bryoniae* and *Z. strigifinis*, and in stylopised *B. pallida*. We observed fruit flies with up to six *D. daci* male pupae and neotenic females, similar to previous reports (Drew & Allwood, 1985; Kathirithamby, 1989a; Nakase & Kato, 2011). Therefore, superparasitism by Strepsiptera is common and could be explained by the higher probability of a host species encountering multiple individuals of a strepsipteran species (Kathirithamby, 2018).

All strepsipteran species (except *D. daci*) parasitise host nymphs or larvae (sometimes eggs) and the host can go through metamorphosis to the adult stage (Hughes et al. 2003; Kathirithamby. 2009; Maeta et al. 2012). However, *D. daci* is assumed to parasitise the adult stage of the host by the planidial larvae entering the soft-bodied teneral adult stage (Drew & Allwood, 1985). This unusual behaviour could be an adaptation to the high mortality of the fruit fly larvae (Kathirithamby, 2018). However, it is still unclear how teneral fruit flies that

do not yet mate come in contact with planidia. Planidial attack of tenerals could occur at protein food source that flies, in particular immature flies, search out (Reynolds et al. 2014; Clarke, 2019). Alternatively, while fruit fly larvae develop within fruit, fully developed larvae leave the fruit to pupate in the soil (Clarke, 2019) and could perhaps still be targeted by planidia. The new diagnostic *D. daci* markers will help to investigate this in the future. Strepsiptera can affect host reproduction (Beani et al. 2011; Cappa et al. 2014; Kathirithamby, 1989b; Kirkpatrick, 1937). Previous examinations of testes of stylopised fruit flies did not reveal any apparent differences between stylopised and unstylopised flies and therefore it was not clear whether *D. daci* castrates males (Drew & Allwood, 1985). We found, however, that stylopised males had their aedeagus (Drew, 1969) unusually elongated possibly rendering them incapable of mating. In contrast, the non-stylopised males did not display this, suggesting that this unusual morphological difference is due to *D. daci* parasitisation. Finally, while our study demonstrates that the detection of *Wolbachia* in tephritid fruit flies is due to *D. daci* parasitisation, it is also likely that low levels of horizontal transmission of *Wolbachia* to fruit flies occurs. Therefore, future research should investigate what prevents the establishment of *Wolbachia* infections in Dacini fruit flies, as our study demonstrates that none of 24 Australian species tested so far have established heritable high-prevalence *Wolbachia* infections.

2.6 Conclusion

Several studies reported consistent detection of *Wolbachia* at low prevalence and titres in host species and populations, yet it is difficult to explain how such *Wolbachia* can be maintained by vertical transmission. Several of these enigmatic infections have been

attributed to the acquisition of *Wolbachia* from an infected host by horizontal transmission. The other possible reason that has not been explored as extensively is the detection of *Wolbachia* of a concealed host (e.g. endoparasites) within the studied species. We have provided clear evidence that the enigmatic *Wolbachia* infections previously reported in adult tephritid fruit flies are actually linked to a concealed strepsipteran endoparasitoid of these fruit flies. We have found highly similar strepsipteran mitogenomes in WGS libraries of *Wolbachia*-positive adult tephritid fruit flies. PCR assays of DNA extracts of flies with no external signs of parasitisation showed a clear link between *Wolbachia* and the presence of this strepsipteran, albeit *Strepsiptera* parasitisation is not always externally visible. Detection of *Wolbachia* at higher titres in *D. daci* dissected from stylopised flies showed that *D. daci* is indeed infected with *Wolbachia*, and that *D. daci* is the true host of the previously reported *Wolbachia* in tephritid fruit flies. Sequencing of *cox1* gene of *D. daci* individuals revealed that the strepsiptera mt genomes extracted from the WGS libraries belonged to this species. Findings from our study provide a new basis for future studies of the interaction between *Wolbachia*, *D. daci* and the fruit fly host. This future research should include more detailed genomic studies of the mitochondrial and *Wolbachia* genomes of *D. daci*, and the mitogenomes of the fruit fly hosts, as well as extensive field studies to understand the interaction between *D. daci* and fruit flies, in particular the parasitisation process, parasitisation frequencies, fitness effects and potential for horizontal transmission in host fruit flies. Furthermore, the bacterial communities in *D. daci* and fruit flies should be investigated further to better understand the interplay between *Wolbachia*, *D. daci* and the fruit fly host.

Chapter 3

Substantial rearrangements, -1 frameshift deletion and low diversity in the mitogenomes of *Wolbachia*-infected strepsipteran endoparasitoid when compared to its hosts

3.1 Abstract

Insect mitogenome organisation is highly conserved, yet, some insects, in particular with parasitic life cycles, have rearranged mitogenomes. Furthermore, intraspecific mitochondrial diversity can be reduced due to the presence of maternally inherited bacterial endosymbionts like *Wolbachia* which affect host fitness. We have sequenced mitogenomes of the *Wolbachia*-infected endoparasitoid *Dipterophagus daci* (Strepsiptera: Halictophagidae) and four of its 22 known tephritid fruit fly host species using total genomic extracts of parasitised flies collected across >700 km. This first-sequenced halictophagid mitogenome revealed extensive rearrangements relative to the four fly mitogenomes which exhibited the ancestral insect mitogenome pattern. Compared to the only four other strepsipteran mitogenomes, the *D. daci* mitogenome had additional transpositions of one rRNA and two tRNA genes, and a single nucleotide frameshift deletion in *nad5* requiring -1 translational frameshifting, or resulting in a large protein truncation. *Dipterophagus daci* displays an almost completely endoparasitic life cycle when compared to more basal Strepsiptera with free-living adults. Our results support the hypothesis that the transition to extreme endoparasitism evolved together with increased levels of mitogenome changes. Furthermore, intraspecific mitogenome diversity was substantially smaller in *D. daci* than the parasitised flies suggesting that *Wolbachia* reduced mitogenome diversity because of a role in the strepsipteran's fitness.

3.2 Introduction

Insect mitogenomes are double-stranded DNA molecules with a length of 15-18 kb. They are generally circular chromosomes consisting of 37 genes including 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes and two ribosomal RNA (rRNA) genes, and one AT-rich region, also known as the control region (Boore, 1999; Wolstenholme, 1992). Mitochondrial genes and mitogenomes have widely been used for DNA barcoding, and in phylogenetic and phylogeographic analyses across many insect taxa because of their conserved function yet relatively high mutation rates, maternal inheritance and very low levels of recombination (Barr et al. 2005; Cameron, 2014; Hebert et al. 2003). However, some studies focussing on individual species have revealed that some insect species have very low mitogenome diversity, and this has generally been attributed to bottleneck effects, also known as founder effects (Dobelmann et al. 2019; Yeun et al. 2019). Reduced mitogenome diversity can also be caused by maternally inherited bacterial endosymbionts such as *Wolbachia* that can invade host populations by either manipulating host reproduction or increasing host fitness in other ways (Kaur et al. 2021), resulting in the selective sweep and the hitchhiking of co-inherited mitogenome variants (Hurst & Jiggins, 2005; Morrow & Riegler, 2021; Turelli et al. 1992).

Comparative mitogenome analyses across multiple phylogenetically diverse insect taxa have revealed in some insect lineages unusual genome characteristics such as gene duplications, changes of gene order, indels and differences in codon usage, nucleotide content and secondary structures of tRNA genes (Beckenbach et al. 2005; Dowton et al. 2009; Mindell et al. 1998; Shao et al. 2001). The rearrangement of gene order can include transposition, inversion and inverse transposition of mitochondrial genes, and can be used to infer

phylogenetic relationships across different taxonomic levels (McMahon et al. 2009; Negrisolo et al. 2011; Shao et al. 2001). It has been hypothesised that mitogenome rearrangements may occur because of recombination, but recombination in animal mitogenomes is generally rare (Tsaousis et al. 2005). The more likely process may be tandem duplication of a set of genes followed by the random loss of a part of the duplication, also known as tandem duplication random loss (TDRL) events (Fujita et al. 2007; San Mauro et al. 2006; Zardoya, 2020). Mitochondrial gene duplications have been observed in the scorpion fly, *Microchorista philpotti* (Beckenbach, 2011) and in other invertebrates, such as *Leptotrombidium* chigger mites (Shao et al. 2006) and the parasitic nematode, *Camallanus cotti* (Zou et al. 2017). However, such duplications may not persist for long before they result in pseudogenisation and loss of duplicated genes, and are not frequently seen in lineages with rearranged mitogenomes (Fujita et al. 2007; San Mauro et al. 2006; Zardoya, 2020). Mitogenome fragmentation has also been observed in several arthropod taxa and other organisms, and can lead to mitogenomes consisting of several small circular chromosomes (Burger et al. 2003; Fu et al. 2020; Shao et al. 2012).

Mitogenome rearrangements have occurred in several insect and other arthropod lineages with parasitic life cycles, for example, some hymenopteran endoparasitoid taxa (Dowton & Austin, 1999). Mitogenome rearrangements have also been found in ectoparasites, such as the wallaby louse, *Heterodoxus macropus*, and the small pigeon louse, *Campanulotes bidentatus compar* (Covacin et al. 2006; Shao et al. 2001). Similarly, mitogenome fragmentation has been found in parasitic human lice, *Pediculus humanus, Pediculus capitis* and *Pthirus pubis* (Shao et al. 2012), the macaque louse, *Pedicinus obtusus* and the colobus louse, *Pedicinus badii* (Fu et al. 2020).

Strepsiptera is a small insect order, with approximately 630 described species (Kathirithamby, 2018). They are thought to have small genomes; using flow cytometry, the genome sizes of *Caenocholax fenyesi* and *Xenos vesparum* were estimated at 108 Mb and 130 Mb (Johnston et al. 2004). Their small genome size may be attributed to their endoparasitic life cycle, unusual morphological characteristics and unique features (Gillespie et al. 2005; Kathirithamby, 2009; McMahon et al. 2009). Strepsiptera display extreme sexual dimorphism. Adult females of most strepsipteran species are neotenic, with fused head and thorax, lacking typical characteristics of adult insects like wings, antennae, mouth and legs, and are permanently endoparasitic, except for the free-living adult females of the Mengenillidae (Kathirithamby, 1989; Pohl et al. 2012). In contrast, adult strepsipteran males undergo complete metamorphosis, and are free-living and winged (Kathirithamby, 1989b). Strepsiptera comprises two suborders, the basal Mengenillidia, with one family (Mengenillidae) and the more diverged Stylopidia with eight families, including the Xenidae and the Halictophagidae (Kathirithamby, 2018; Pohl & Beutel, 2005). Strepsiptera are endoparasitoids of a wide range of hosts across seven insect orders: Blattodea, Hemiptera, Hymenoptera, Diptera, Mantodea, Orthoptera and Zygentoma (Kathirithamby, 1989b, 2009). Host attack occurs by the free-living first instar larvae (planidia). After three more larval instars within their hosts, in Stylopidia the neotenic females and male pupae extrude through the host's cuticle; adult males then emerge from the pupae in the extrusions while the neotenic females remain fully endoparasitic. In contrast, females and males of Mengenillidae undergo pupation outside the host (Kathirithamby, 2009).

Until recently, the phylogenetic placement of Strepsiptera and its species has proved to be a challenge due to their morphological peculiarities and the scarcity of molecular data (McMahon et al. 2011; Pohl & Beutel, 2005; Wiegmann et al. 2009). The sequencing of the

mitogenomes of two species each of *Mengenilla* (Mengenillidae) and *Xenos* (Xenidae) has provided substantial progress (Carapelli et al. 2006; McMahon et al. 2009; Niehuis et al. 2012; Zhang et al. 2021). Comparative mitogenomics of *Mengenilla australiensis* and *Xenos vesparum* revealed more changes from the ancestral holometabolan mitogenome in *X. vesparum*. These additional changes arose with the transition from Mengenillidae which still leave the host for pupation and have free-living adult females and males, to Stylopidia with only free-living males and, therefore, a more extreme endoparasitic strepsipteran life cycle (McMahon et al. 2009). Nevertheless, molecular data of the largest and possibly most diverged strepsipteran family Halictophagidae is crucial for a more comprehensive understanding of strepsipteran evolution, and in particular, their interactions with hosts and the transition to the more extreme endoparasitic life cycle of Stylopidia, with males that pupate inside the host and neotenic females that are fully endoparasitic.

Dipterophagus daci is the only described strepsipteran parasitising Diptera (except for undescribed strepsipteran species from Papua New Guinean platystomatid flies) and has been recorded in 22 dacine fruit fly species (Tephritidae: Dacini) in Australia and the Solomon Islands (Allwood & Drew, 1996; Drew & Allwood, 1985; Towett-Kirui et al. 2021). Recent molecular analyses of whole genome sequencing (WGS) libraries of field-collected adult tephritid fruit flies from Australia detected genomic sequences of *D. daci*, including its entire mitogenome, indicative of concealed parasitisation of the sequenced flies (Towett-Kirui et al. 2021). Phylogenetic analyses of the *D. daci* mitochondrial *cox1*, *nad1*, *16S rRNA* and nuclear *18S rRNA* genes revealed that it belongs to the family Halictophagidae (Towett-Kirui et al. 2021), confirming earlier morphological analyses which placed it into the halictophagid subfamily Dipterophaginae (Kathirithamby, 1989b, 2018). The WGS analyses also revealed a link between *D. daci* and two *Wolbachia* strain sequence types, ST-285 and ST-289,

previously detected in these tephritid fruit fly samples (Morrow et al. 2015; Morrow et al. 2014), demonstrating that *D. daci* is the true host of these two strains, *w*Ddac1 and *w*Ddac2 (Towett-Kirui et al. 2021). Furthermore, no *Wolbachia* genes known to cause host reproductive manipulations were found, and there was a low diversity in the mitochondrial PCGs of *D. daci* when compared with its nuclear 18S rRNA gene sequences, suggesting *Wolbachia* influences host fitness (Towett-Kirui et al. 2021). However, it has not been analysed whether the extent of intraspecific mitogenome diversity differs between *D. daci* and its fruit fly host species, yet this may provide further evidence that *D. daci* is the actual host of *Wolbachia* rather than the fruit flies.

The host species of *D. daci* include several dacine fruit fly species that are destructive pests of fruits and vegetables, for example *Bactrocera tryoni* (Queensland fruit fly, Australia's most significant horticultural pest), its sibling species *Bactrocera neohumeralis*, and *Bactrocera frauenfeldi* (White & Elson-Harris, 1992) as well as many other species that are not major pests such as *Zeugodacus strigifinis* which develops in flowers of Cucurbitaceae (Doorenweerd et al. 2018; Vargas et al. 2015). Several Dacini mitogenomes have previously been sequenced, including of *B. tryoni* (Drosopoulou et al. 2021), however, the mitogenomes of *B. frauenfeldi*, *B. neohumeralis* and *Z. strigifinis* have not yet been sequenced and characterised.

In this study, we obtained six mitogenome variants of *D. daci* and nine mitogenome variants of four of its 22 tephritid host species, *B. frauenfeldi*, *B. neohumeralis*, *B. tryoni* and *Z. strigifinis* by WGS of DNA libraries obtained from parasitised individual hosts. We then compared the arrangement, nucleotide composition and codon usage of these mitogenomes together with the previously sequenced mitogenomes of four other strepsipterans, species of

closely related insect orders, and the host fruit flies. We expected that the *D. daci* mitogenome arrangement would differ from the ancestral insect and the fruit fly mitogenomes. We anticipated that *D. daci* mitogenomes contain more rearrangements compared to the mitogenomes of the more basal *Mengenilla* but share some differences with the mitogenomes of the more derived *Xenos.* Furthermore, we compared the intraspecific mitogenome diversity between *D. daci* and the fruit flies from which the *D. daci* mitogenomes were obtained. Due to the *Wolbachia* association, we expected that intraspecific mitogenome diversity would be less in *D. daci* than the fruit fly species.

3.3 Methods

Insect specimens

This study analysed WGS libraries of nine males of four tephritid fruit fly species: *B. frauenfeldi*, *B. neohumeralis*, *B. tryoni* and *Z. strigifinis*; representing field populations across a region from Mackay to Cairns (>700 km distance) in Queensland, Australia (Table 3. 1). These specimens formed part of a previous survey of *Wolbachia* in 24 Australian tephritid fruit fly species and were collected using traps with male attractants as previously described (Morrow et al. 2015; Morrow et al. 2014). DNA was extracted from fly abdomens and tested for *Wolbachia* using *Wolbachia surface protein* (*wsp*) and 16S rRNA gene primers; furthermore, two strains of *Wolbachia*-positive flies were characterised using multi-locus sequence typing (MLST) as ST-285 and ST-289 (Morrow et al. 2015; Morrow et al. 2014) (Table 3. 1), with later assignment of these strains to their actual host *D. daci* as *w*Ddac1 and *w*Ddac2 (Towett-Kirui et al. 2021). DNA extracts of 14 *Wolbachia*-positive flies were

selected, amplified by multiple displacement, and submitted for library construction and WGS as previously described (Towett-Kirui et al. 2021). Nine of these 14 WGS libraries produced sufficient mitogenome coverage and were used for analyses in the current study (Table 3. 1). The remaining five WGS libraries were of low quality and excluded from the analyses.

Sequencing and genome assembly

WGS was performed using the Illumina Hiseq2500 platform, and sequence quality control and *de novo* assembly done in CLC Genomics Workbench as previously described (Towett-Kirui et al. 2021). Sequence identification and extraction was achieved by querying the reference genomes against the WGS library contig lists. First, BLASTn using the *M. australiensis* partial mitogenome (GenBank GU188852) was performed to extract the *D. daci* mitogenome from the contig list of *Bactrocera frauenfeldi* Bfra485 (Table 3. 1). Contigs with the best hit were concatenated and manually gap-filled by iterative mapping of the trimmed reads at 90% similarity and 60-80% read length. The final *D. daci* draft mitogenome consensus sequence of this library was verified by mapping reads at 99% similarity. The final *D. daci* mitogenome extracted from the Bfra485 contig list was then used as a reference for the identification and extraction of *D. daci* mitogenomes from the other five libraries (Table 3. 1). Similarly, BLASTn using the *Ceratitis capitata* mitogenome (GenBank AJ242872) was performed to identify and extract the fruit fly mitogenomes from the libraries, and the contigs with the best hit in each library were then assembled by iterative mapping as described earlier. The extracted *D. daci* and fruit fly mitogenomes were manually aligned and inspected in Geneious v10.0.9 (Kearse et al. 2012).

PCR amplification and Sanger sequencing of *nad5*

The *D. daci* mitogenome assembly revealed an unusual deletion of one nucleotide in *nad5*. This genomic dataset was obtained from WGS libraries which underwent multiple displacement amplification using REPLI-g mini kit (Qiagen) prior to library preparation (Towett-Kirui et al. 2021). To verify that this mutation was not due to a rare amplification error, PCR primers were designed to specifically amplify *nad5* of *D. daci* to confirm the WGS results, using Primer-BLAST (NCBI); Dd_nad5F: 5' GAAACTGGAGTTGGAGCAGC 3' and Dd_nad5R: 5' ATAGCGTGTGATAAGTTAAATCGTT 3' with an expected amplicon size of 396 bp. MyTaq™ Mix (Bioline) PCR reagents were used according to the manufacturer's instructions. PCR cycling conditions began with an initial denaturation for 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, then a final elongation step of 7 min at 72 °C. Five additional *D. daci* samples (Appendix B; Table B. 1) were PCR amplified and visualised by capillary electrophoresis on a QIAxcel system using a QIAxcel DNA screening kit (Qiagen). Prior to sequencing, PCR amplicons were treated with ExoSAP [exonuclease I (New England Biolabs, Ipswich, MA, USA) and shrimp alkaline phosphatase (Promega)] and incubated at 37 °C for 30 min, then 95°C for 5 min. Sanger sequencing was performed using BigDye Terminator v3.1 kit (Applied Biosystems) and run on an Applied Biosystems 3500 Genetic Analyser.

Mitogenome annotation and analysis

The assembled *D. daci* and fruit fly mitogenomes were identified using MITOS2 with "RefSeq 63 Metazoa" provided by MITOS2 and the invertebrate genetic code (Bernt et al. 2013) , followed by manual verification of the coding regions and comparison with published mitochondrial sequences in Geneious v10.0.9 and NCBI BLASTn. The tRNA genes predicted by MITOS2 were confirmed using tRNAscan-SE (Lowe & Eddy, 1996), and ARWEN (Laslett & Canbäck, 2008). The circular mitogenomes were visualised in Geneious v10.0.9. Comparative analyses of the composition skewness of the mitogenomes were calculated using the formulae: AT skew= [A-T]/[A+T] and GC skew=[G-C]/[G+C]. Comparative analysis of the mitogenomes codon usage was computed in MEGA7 (Kumar et al. 2016).

Comparative mitogenomics

Comparative analyses were performed using the six *D. daci* and nine fruit fly mitogenomes from this study, the mitogenomes of four other strepsipterans [*M. australiensis* (GU188852.1), *M. moldryzki* (JQ398619.1), *X. vesparum* (DQ364229.1) and *X. moutoni* (MW222190)] and a representative member of other orders closely related to Strepsiptera including Coleoptera [*Tribolium castaneum* (AJ3124132)], Neuroptera [*D. pantherinus* (MK3012461)], Megaloptera [*Neochauliodes fraternus* (NC_0252821)], Raphidioptera [*Mongoloraphidia harmandi* (NC_0132511)] and Diptera [*B. tryoni* (NC_014611)].

Intraspecific mitogenome diversity analyses

To determine the intraspecific genetic diversity across the *D. daci* mitogenome variants, we performed multiple sequence alignments of the six *D. daci* mitogenome variants. Additionally, to compare the intraspecific genetic diversity in *D. daci* and the fruit fly host species, we performed individual multiple sequence alignments of 13 PCGs of the six *D. daci*, five *B. neohumeralis* and three *B. tryoni* mitogenome variants (including *B. tryoni* NC_014611 obtained from GenBank). The multiple sequence alignments and DNA diversity analyses were performed in Geneious.

3.4 Results

Genome sequencing

Whole genome sequencing was performed on genomic extracts of nine individuals of four tephritid fruit fly species that were parasitised with *Wolbachia*-infected *D. daci* and were collected across a range of >700 km (Table 3. 1). Of these, six sequence libraries produced a good coverage (≥26.7-fold) of *D. daci* mitogenomes and three other sequence libraries contained *D. daci* mitogenomic sequences but not of sufficient coverage to assemble mitogenomes (Table 3. 1). However, all nine sequence libraries included mitogenomes of the four fruit fly species. The *D. daci* and fruit fly mitogenomes were first extracted from the contig list of Bfra485 which had the highest read number. Its *D. daci* mitogenome comprised two contigs of approximately 12 kb and 3.2 kb while the fly mitogenome comprised one contig of approximately 15.9 kb. Then, iterative mapping using Bfra485 reads resulted in an

almost complete *D. daci* mitogenome with a minimum estimated length of 16,255 bp and a complete circular *B. frauenfeldi* mitogenome of 15,935 bp (Figure 3. 1, Appendix B; Table B. 2). These two mitogenomes were used for the extraction of the *D. daci* and fruit fly mitogenomes from the other sequence libraries. *Dipterophagus daci* mitogenomes were successfully assembled from six libraries: Bfra485, Bn171, Bn342, Bt194, Bt210 and Zst503; the fruit fly mitogenomes were successfully assembled from nine libraries: Bfra485, Bn135, Bn171, Bn 240, Bn244, Bn342, Bt194, Bt210 and Zst503 (Table 3. 1). The size of the mitogenomes ranged from 16,243 to 16,255 bp for *D. daci*, and from 15,858 to 15,935 bp for the fruit flies (Appendix B; Table B. 2).

The sequences obtained in this study were deposited in GenBank: the *Dipterophagus daci* mitogenome extracted from *Bactrocera frauenfeldi* 485 was deposited under accession number MW233588. Fruit fly mitogenomes from this study were deposited under accession numbers MZ520731- MZ520739. Raw reads (for Bfra485, Bn171, Bn342, Bt194, Bt210 and Zst503) were submitted to NCBI Sequence Read Archive under the BioProject accession number PRJNA682518.

Table 3. 1*:* Summary of nine fruit fly WGS libraries obtained from individuals of four tephritid fruit fly species parasitised by *Dipterophagus daci*, collection localities, *Wolbachia* infection status (+ or -) with *w*Ddac1 (ST-285) and *w*Ddac2 (ST-289), number of reads after QC and coverage for the *D. daci* and fruit flies mitogenomes. Mitogenomes with high coverage are presented in bold, with coverage number in parentheses.

Tephritid species	Sample ID	Collection locality	*w*Ddac1 (ST-285)	*w*Ddac2 (ST-289)	Number of reads after QC	*D. daci* mitogenome mapped reads (coverage)	Fly mitogenome mapped reads (coverage)
Bactrocera frauenfeldi	485	Cairns	y	y	109,057,960	**15,938 (104.5)**	**11,544 (72)**
Bactrocera neohumeralis	135	Mourilyan Harbour	y	y	68,308,764	6 (0.04)	**86,7084 (5,427)**
Bactrocera neohumeralis	171	Townsville	y	y	77,482,368	**4,094 (26.8)**	**62060 (387)**
Bactrocera neohumeralis	240	Mourilyan Harbour	y	n	72,186,748	320 (2.1)	**36,332 (228)**
Bactrocera neohumeralis	244	Cairns	n	y	60,128,324	52 (0.3)	**496,912 (3101)**
Bactrocera neohumeralis	342	Mackay	y	y	67,282,474	**4,086 (26.7)**	**47,976 (300)**
Bactrocera tryoni	194	Cairns	y	y	79,574,356	**8,104 (53.2)**	**530,836 (3,312)**
Bactrocera tryoni	210	Mackay	y	y	63,859,882	**6,892 (45.2)**	**18,486 (115.4)**
Zeugodacus strigifinis	503	Cairns	y	y	65,468,734	**11,210 (73.5)**	**579,200 (3,646)**

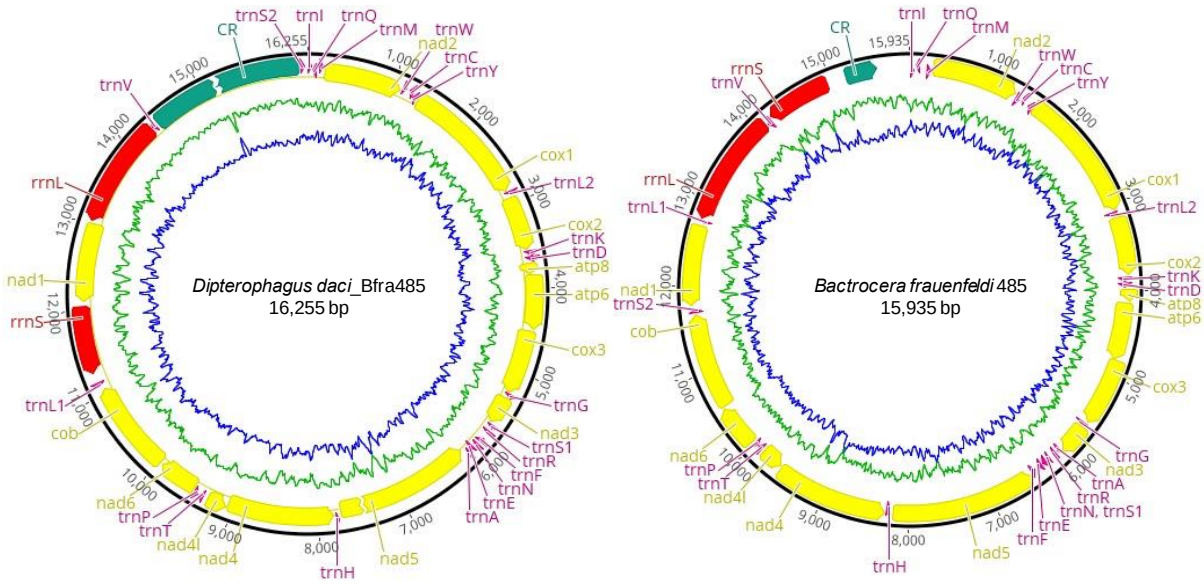

Figure 3. 1: Structure of the mitogenomes of *Dipterophagus daci* and *Bactrocera frauenfeldi* obtained from a whole genome sequencing library of the genomic extract of the parasitised specimen *B. frauenfeldi* Bfra485. PCGs are denoted in yellow, rRNA genes in red, tRNA genes in purple and control region in green. The AT content (blue) and GC content (green) were plotted as the deviation from the average AT and GC content of the overall sequence using sliding window analysis. The mitogenome of *D. daci* has not been closed and contains one unusual nucleotide deletion in the *nad5* gene.

Mitogenome structure

The six *D. daci* and nine fruit fly mitogenomes each contained 13 PCGs, two rRNA genes and 22 tRNA genes (Appendix B; Table B. 2). In the *D. daci* and fruit fly mitogenomes, nine PCGs (*nad2, cox1, cox2, atp8, atp6, cox3, nad3, nad6* and *cob*) and 14 tRNA genes (*trnI, trnM, trnW, trnL$_2$, trnK, trnD, trnG, trnS$_1$, trnR, trnN, trnE, trnA, trnT* and *trnS$_2$*) were located on the major strand (leading strand) while 4 PCGs (*nad5, nad4, nad4L* and *nad1*), eight tRNA genes (*trnQ, trnC, trnY, trnF, trnH, trnP, trnL$_1$* and *trnV*) and both rRNA genes (*rrnL* and *rrnS*) were located on the minor strand (lagging strand) (Figure 3. 1, Appendix B; Table B. 2). The fruit fly mitogenomes had an AT-rich region, located between *rrnS* and *trnI* and with an average length of 594 bp in fruit flies, while in *D. daci* the AT-rich region was located between *trnV* and *trnS$_2$*. Furthermore, the *D. daci* mitogenomes contained an unresolved sequence assembly gap between *trnV* and *trnS$_2$* resulting in variable lengths (Appendix B; Table B. 2).

Mitogenome base composition

The nucleotide composition of *D. daci* mitogenomes was AT-biased (approximately 84%) and this was similar to the mitogenomes of the other strepsipterans. The fruit fly mitogenomes were less AT-biased (approximately 72%) (Figure 3. 2, Appendix B; Table B. 3) and their AT contents were similar except for *B. frauenfeldi* 485 and *Z. strigifinis* 503, which had AT contents of 74.1% and 73.4% respectively (Figure 3. 2, Appendix B; Table B. 3). Comparative mitogenome analyses of *D. daci* and their fruit fly hosts revealed a clear bias in nucleotide composition with positive AT-skews and negative GC-skews (Appendix B;

Figure B. 1, Appendix B; Table B. 3). This was also noted for *X. vesparum* while *M. australiensis* and *Mengenilla moldryzki* had a negative AT skew (Appendix B; Figure B. 1, Appendix B; Table B. 3). All the insect taxa had a negative GC skew (Appendix B; Figure B. 1, Appendix B; Table B. 3).

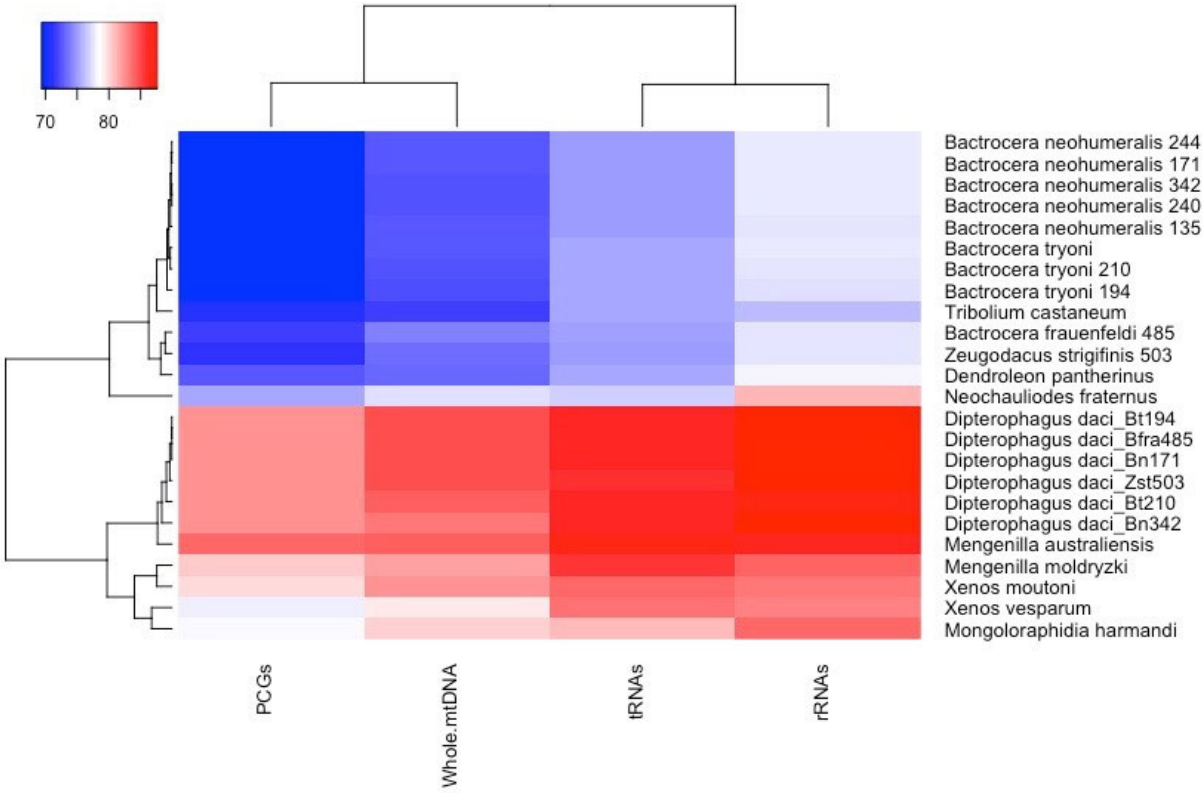

Figure 3. 2: Comparative analysis of AT content of mitogenomes of *Dipterophagus daci*, its host fruit fly species and other reference species.

Mitochondrial protein coding genes

The total length of the 13 PCGs of the *D. daci* mitogenomes was on average 10,696 bp and was relatively shorter than the total length of the PCGs of the fruit fly mitogenomes with an average length of 11,187 bp (Appendix B; Table B. 3). The start codons ATT, ATA and ATG were used in both *D. daci* and fruit fly PCGs, except the fruit fly *atp8* gene which started with GTG and *cox1* started with CAA (Appendix B; Table B. 3). In *D. daci* PCGs *nad1*, *nad2*, *nad3* and *nad4L* started with ATA, *cox2*, *atp8*, *nad5* and *nad6* with ATT, and *atp6*, *cox3*, *nad4* and *cob* with ATG (Appendix B; Table B. 3). Furthermore, the *D. daci* PCGs *nad2*, *atp8*, *nad6*, *cox3*, *nad4L* and *nad1* ended with TAA, while it is assumed that the remaining PCGs that ended with T and TA are completed by adding 3' A residues in the mRNA (Appendix B; Table B. 3).

The fruit fly PCGs *nad2*, *nad3*, *nad5* and *nad6* started with ATT, *cox2*, *atp6*, *cox3*, *nad4*, *nad4L* and *cob* with ATG, *atp8* with GTG, and *nad1* with ATA and *cox1* started with TCG (Table S2). Seven fruit fly PCGs stopped with TAA, while *nad3* and *nad4* stopped with TAG; *nad5*, *cob* and *nad1* that ended with T and TA are presumably completed by adding 3' A residues in the mRNA (Appendix B; Table B. 3). Comparative analyses of the relative synonymous codon usage (RSCU) revealed that across *D. daci*, the fruit fly and the other insect species, codons ending with A or T prevailed. Amino acids Ala, Gly, Leu, Pro, Arg, Ser, Thr and Val were commonly used, and Leu had the highest RSCU in all insect species (Appendix B; Table B. 4).

Surprisingly, the *nad5* gene contained an unusual deletion of one nucleotide (nucleotide position 291) in all six *D. daci* mitogenomes which introduced an in-frame stop codon (TAA) at amino acid position 98 (Figure 3. 3); the remainder of *nad5* further downstream, however,

still constituted an open reading frame but started from a different position. The unexpected finding of a single nucleotide -1 frameshift deletion was further verified by Sanger sequencing of the *nad5* region of *D. daci* from five samples in addition to those used for WGS; these samples did not undergo multiple displacement amplification using REPLI-g mini kit which was used for the WGS samples prior to library preparation (Morrow et al. 2020; Morrow & Riegler, 2021, Appendix B; Table B. 1). All *nad5* gene Sanger sequences were identical to the assembled mitogenomes and confirmed this nucleotide deletion. Subsequently, the domain architecture of *nad5* gene was checked using CDART (NCBI) (Geer et al. 2002). This revealed that, similar to other *nad5* genes, the second part of *D. daci nad5* downstream of the deletion contained the proton-conducting transporter domain starting at amino acid position ~ 100 in most full-length *nad5* genes (Figure 3. 3), suggesting that this larger fragment of *nad5* of *D. daci* could still encode for a functional yet truncated protein.

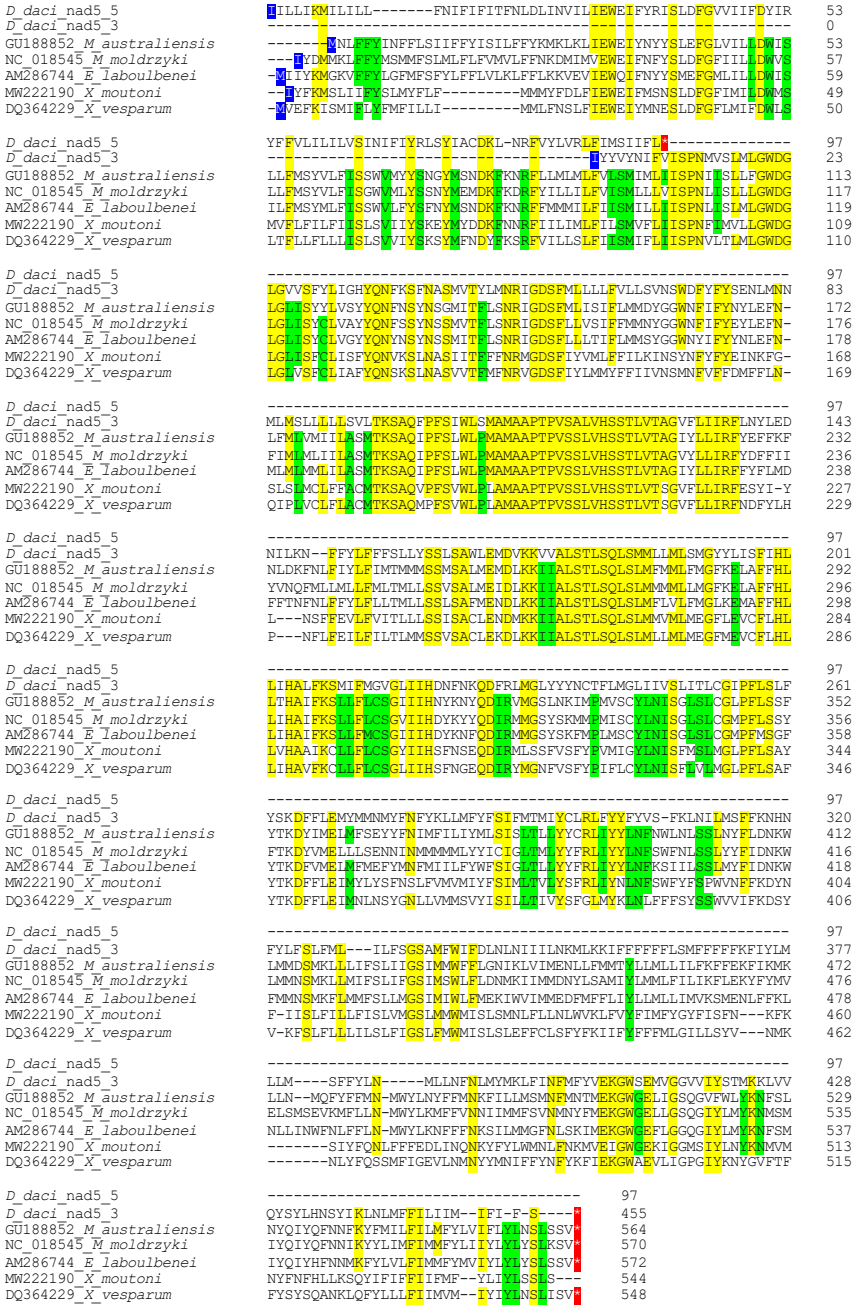

Figure 3. 3: Amino acid (aa) alignment of the nad5 gene of *Dipterophagus daci* (Bfra485) and five strepsipteran species, *Mengenilla australiensis*, *Mengenilla moldryzki*, *Eoxenos laboulbenei* (Mengenillidae), *Xenos vesparum* and *Xenos moutoni* (Xenidae), listed with their GenBank accession numbers. The red-highlighted star indicates stop codons, including a stop codon at position 98 in *D. daci*, with a new start codon (highlighted in blue) upstream of the mutation. Positions with >0.5 conserved aa across sequences are highlighted in yellow when *D. daci* displays the conserved aa, or green when *D. daci* is different; the 5' sequence of *D. daci* reads from an alternative open reading frame starting position than the 3' sequence due to the deletion that inserts a stop codon.

Mitochondrial tRNA and rRNA genes

The *D. daci* and fruit fly mitogenomes contained 22 tRNA genes (Figure 3. 1, Appendix B; Table B. 2). Their average total length was 1,424 bp in *D. daci* and 1,468 bp in fruit fly mitogenomes (Appendix B; Table B. 3). Both 16S rRNA and 12S rRNA genes (*rrnL* and *rrnS* respectively), had a total length of 2,074 bp in the *D. daci* mitogenomes, while both combined ranged from 2,081 to 2,110 bp in the fruit fly mitogenomes (Appendix B; Table B. 3). Across the six *D. daci* mitogenomes, MITOS2 could only identify one part (688 bp 3' section adjacent to the *nad1* gene) of the 16S rRNA gene because the 5' section flanked by *trnV* was highly diverged, but was confirmed by sequence alignment with 16S rRNA genes of the reference strepsipteran mitogenomes obtained from GenBank and by BLASTn. In fruit fly mitogenomes the 16S rRNA gene was flanked by $trnL_1$ and *trnV* and the 12S rRNA gene was flanked by *trnV* and the AT-rich region (Figure 3. 1, Appendix B; Table B. 2).

Mitochondrial gene arrangement

Significant gene rearrangements were observed in the *D. daci* mitogenomes relative to the ancestral insect mitogenome, while the gene arrangement of the fruit fly mitogenomes were identical to the ancestral insect mitogenome (Figure 3. 4A, Figure 3. 4B). Gene rearrangements in the *D. daci* mitogenomes were observed in two regions: the first region involved the transposition of *trnA*, $trnS_1$ and *trnF*; and the second region involved the transposition of $trnS_2$, $trnL_1$ and *rrnS* (Figure 3. 4A), resulting in a different rRNA gene order when compared to all other mitogenomes.

The *D. daci* mitogenome arrangement was also compared with the mitogenomes of the four other strepsipteran species, one representative species each of four closely related insect

orders (Coleoptera, Megaloptera, Neuroptera, Rhaphidioptera), *B. frauenfeldi* 485 and a reference *B. tryoni* (GenBank accession NC014611) (Figure 3. 4B). Generally, most genes in the *D. daci* mitogenome had a conserved gene arrangement position (Figure 3. 4B). However, comparisons revealed that *D. daci* contained more mitogenome rearrangements (6 transpositions) compared to *Xenos moutoni, X. vesparum, M. moldryzki* and *M. australiensis* that contained 4, 3, 2 and 1 transpositions, respectively (Figure 3. 5). The transposition of *trnS*$_1$ observed *in D. daci* was also observed in the four strepsipteran species, and the transposition of *trnA* and *trnL*$_1$ was also found in *X. moutoni* and *X. vesparum* (Figure 3. 5). The transposition of *trnF*, *trnS*$_2$ and *rrnS* were unique to *D. daci*, while the transposition of *trnM* (from I-Q-M in ancestral arrangement to M-I-Q) was unique to *X. moutoni* and not seen in *D. daci* (Figure 3. 5). Mitogenomes of the fruit flies as well as the three representative species of Coleoptera, Megaloptera and Rhaphidioptera were arranged according to the ancestral insect mitogenome pattern while *Dendroleon pantherinus* (Neuroptera) exhibited a C-W-Y (W-C-Y in ancestral) gene arrangement (Figure 3. 4B).

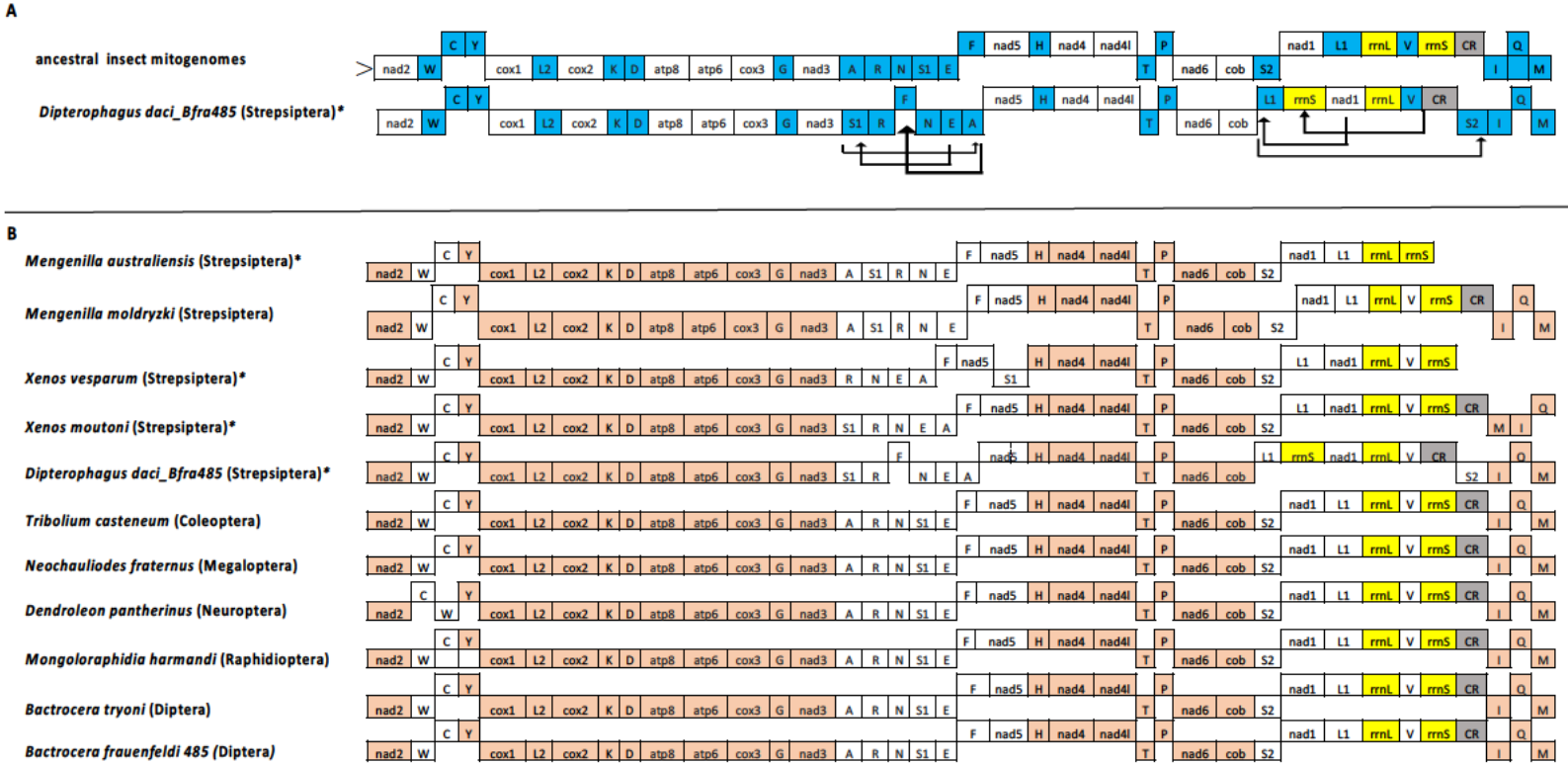

Figure 3. 4: Organisation and rearrangement of the *Dipterophagus daci* mitogenome (A) compared to the ancestral holometabolan pattern (Boore, 1999); tRNA genes are blue, rRNA genes are yellow, protein coding genes are white and the control region is grey. The major (leading) strand is denoted by > and arrows denote gene translocations; (B) compared to ten other insect species (including four strepsipteran species and the host species *Bactrocera frauenfeldi* and *Bactrocera tryoni*); brown shading and unshaded denote the conserved and different gene arrangements respectively in *D. daci* and the other species; grey denotes the control region. Mitogenome representation is not drawn to scale; * indicates species for which only incomplete mitogenomes are available.

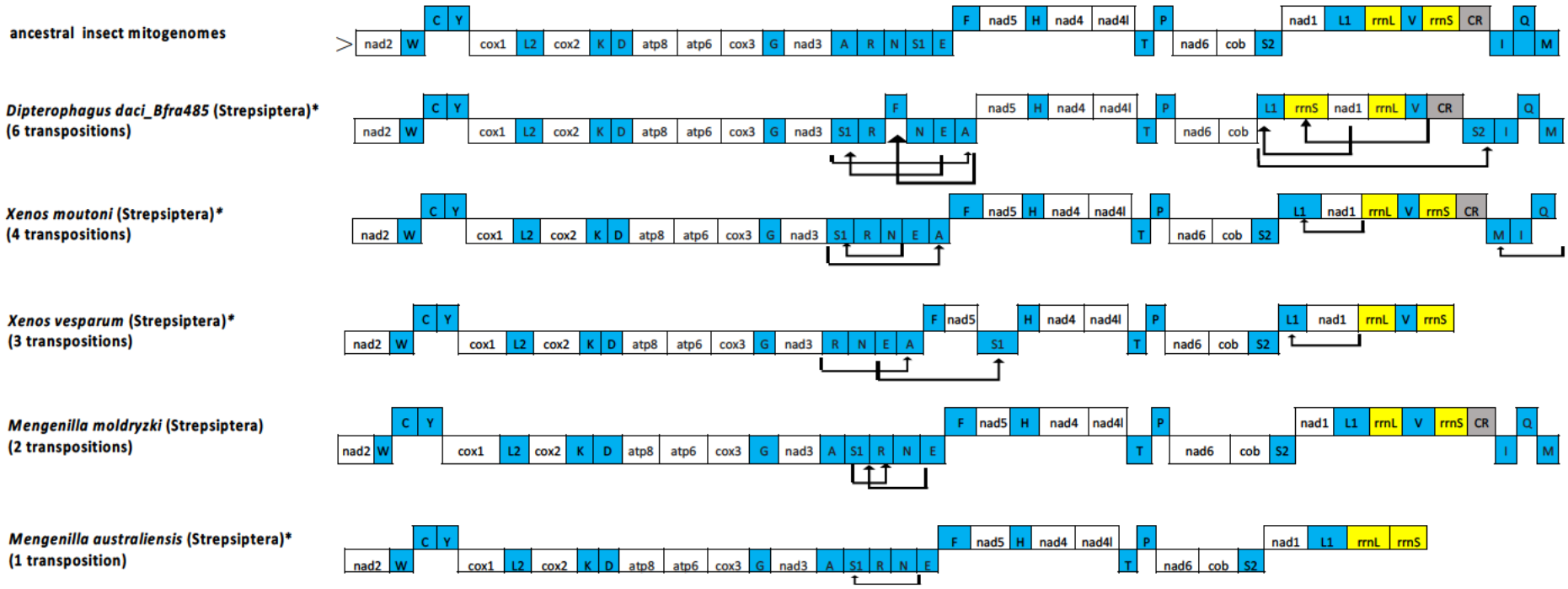

Figure 3. 5: Mitogenome organisation and rearrangement illustrating gene translocations and number of transpositions in *Dipterophagus daci* and four strepsipteran species relative to the ancestral pattern in insect mitogenomes (Boore, 1999; McMahon et al. 2009); tRNA genes are blue, rRNA genes are yellow, protein coding genes are white and the control region is grey. The major (leading) strand is denoted by >, arrows denote gene translocations and * indicate species for which only incomplete mitogenomes are available. Mitogenome representation is not drawn to scale.

Intraspecific mitogenome variation

We performed multiple sequence alignments to investigate the intraspecific diversity across the six *D. daci* mitogenome variants. We identified a total of ten single nucleotide polymorphisms (SNPs) occurring in four mitochondrial PCGs, including *cox1, nad5, nad4 and cob* (Table 3. 2) and a total of 34 SNPs occurring in the *D. daci* mitogenome variants (Appendix B; Table B. 5). To contrast intraspecific mitogenome variation, we investigated the diversity of the 13 PCGs of the six *D. daci*, five *B. neohumeralis* and two *B. tryoni* mitogenome variants obtained in this study and the reference *B. tryoni* mitogenome variant. Despite the relatively low mitogenome sample number, intraspecific nucleotide diversities were substantially lower in the PCGs of the *D. daci* mitogenome variants than in the PCGs of the fruit fly mitogenome variants (Table 3. 3). In contrast to the ten SNPs in the mitochondrial PCGs of *D. daci*, the mitochondrial PCGs of *B. neohumeralis* and *B. tryoni* had 298 and 133 SNPs, respectively, showing that the PCGs of the *B. neohumeralis* and *B. tryoni* mitogenomes were 33.1x and 14.7x more diverse than the *D. daci* mitogenome (Table 3. 3).

Table 3. 2: Mitogenome protein coding gene diversity of *Dipterophagus daci* mitogenomes, showing the collection locality, *Wolbachia infection* status (+ or -) with *w*Ddac1 (ST-285) and *w*Ddac2 (ST-289) and the single nucleotide polymorphism (SNP) position in the mitogenome. The * denotes the assembled reference mitogenome of *D. daci* from *Bactrocera frauenfeldi* Bfra485 (MW233588) and ^ denotes library with low coverage that did not allow assembly of the mitogenome. Empty cells indicate that the position has the same nucleotide as the assembled reference genome.

Collection locality	***w*Ddac1**	***w*Ddac2**	**Gene**	***cox1***		***nad5***				***nad4***		***cob***	
			SNP position in the mitogenome	**1,762**	**2,546**	**6,408**	**6,607**	**6,912**	**7,306**	**7,869**	**8,640**	**10,276**	**11,033**
Cairns	**y**	**y**	***Dipterophagus daci*_Bfra485***	**C**	**A**	**G**	**G**	**T**	**C**	**T**	**G**	**A**	**A**
Townsville	y	y	*Dipterophagus daci*_Bn171	T				C		C	A	C	G
Mourilyan Harbour	y	n	*Dipterophagus daci*_Bn240^						G	C	A	C	
Mackay	y	y	*Dipterophagus daci*_Bn342				A						
Cairns	y	y	*Dipterophagus daci*_Bt194			A							
Mackay	y	y	*Dipterophagus daci*_Bt210		G								
Cairns	y	y	*Dipterophagus daci*_Zst503			A							

Table 3. 3: Nucleotide diversity of the mitochondrial PCGs of *Dipterophagus daci* (n=6), *Bactrocera neohumeralis* (n=5) and *Bactrocera tryoni* (n=3), showing the number of single nucleotide polymorphisms (SNPs). The 5' part of the *D. daci nad5* gene with the stop codon is listed separately as nad5_5'.

	***Dipterophagus daci* (n=6) PCGs**		***Bactrocera neohumeralis* (n=5) PCGs**		***Bactrocera tryoni* (n=3) PCGs**	
Gene	**Total number of sites**	**SNPs**	**Total number of sites**	**SNPs**	**Total number of sites**	**SNPs**
atp6	642	0	678	16	678	8
atp8	150	0	162	5	162	1
cob	1,111	2	1,135	23	1,135	13
cox1	1,507	2	1,535	42	1,535	12
cox2	652	0	690	18	690	8
cox3	768	0	789	17	789	9
nad1	942	0	940	24	940	9
nad2	927	0	1,023	22	1,023	10
nad3	343	0	354	10	354	5
nad4	1,263	2	1,341	45	1,342	22
nad4L	264	0	291	7	297	1
nad5	1,350	4	1,720	55	1,720	26
nad5_5'	291	0	na	na	na	na
nad6	486	0	525	14	525	9
Total PCG	10,696	10	11,183	298	11,190	133

3.5 Discussion

We have analysed the mitogenome of *D. daci* as the first sequenced mitogenome of Halictophagidae, the largest strepsipteran family, together with the mitogenomes of four of its 22 tephritid fruit fly host species, *B. frauenfeldi*, *B. neohumeralis*, *B. tryoni* and *Z. strigifinis*. We obtained these sequences from fly individuals with concealed *D. daci* parasitisation. Mitogenome analyses revealed extensive mitogenome rearrangements in *D. daci* relative to the ancestral holometaboloan mitogenome arrangement and the fruit fly mitogenomes. Furthermore, in comparison to the other strepsipteran mitogenomes, *D. daci* has with six gene transpositions the most re-arranged strepsipteran mitogenome characterised so far. While it

shared some of the mitogenome rearrangements with other Strepsiptera, *D. daci* contained additional and unique mitogenome differences. This included a single nucleotide -1 frameshift deletion in the coding region of the *nad5* gene possibly requiring -1 translational frameshifting (Beckenbach et al. 2005; Mindell et al. 1998), other unknown compensation mechanisms, or, alternatively, leads to a significant truncation of the gene product. Another unusual feature was a different order of the rRNA genes because of the transposition of the *rrnS* gene. Our findings also revealed that *D. daci* mitogenomes have shorter PCGs which is typical for strepsipterans (Carapelli et al. 2006; McMahon et al. 2009). Despite the low sample number but whole-mitogenomic representation and similar sampling effort for *D. daci* and fruit fly species across a geographic range of >700 km, covering a large part of known *D. daci* distribution (Allwood & Drew, 1996), we observed substantially (15-33x) lower genetic diversity in the *D. daci* mitochondrial PCGs relative to their host fruit fly species, suggesting that *Wolbachia* may be the cause for the loss of mitogenome diversity in *D. daci*.

Mitogenome rearrangement and gene truncation in *Dipterophagus daci*

Insect mitogenomes have a fairly conserved gene order, however, gene rearrangements occur in several insect taxa (Chen et al. 2018). In the current study, we found extensive gene rearrangements in *D. daci* mitogenomes relative to the ancestral holometabolan pattern. Mitochondrial gene rearrangements are usually characterised by either transposition, inversion or inverse transposition (Dowton et al. 2002), and more frequently involve tRNA genes than PCGs and rRNA genes (Boore et al. 1995). In *D. daci*, rearrangements involved six transpositions of genes (five tRNA genes and one rRNA gene). These were more mitogenomic transpositions in *D. daci* than in any other strepsipterans further suggesting that *D. daci* is a more derived species of Strepsiptera. The transpositions of *trnF*, *trnS*$_2$ and *rrnS*

were unique to *D. daci*, however, the transpositions of *trnA* and *trnL₁* were also observed in *X. moutoni* and *X. vesparum*, while the transposition of *trnS₁* was common to the five strepsipteran species.

We also found that *nad5* of *D. daci* had one nucleotide -1 frameshift deletion that resulted in the introduction of a stop codon at amino acid position 98. However, the downstream part of the gene still had an open reading frame but starting with another nucleotide position. This could be indicative that *D. daci* possibly experiences -1 translational frameshifting, similar to the translational editing mechanism proposed to overcome the issues of single nucleotide insertion and deletions found in PCGs of some mitogenomes (Andreu-Sánchez et al. 2021). Previously, single nucleotide insertions have been observed in *cob* of ants (Beckenbach et al. 2005) and *nad3* of some bird and turtle species (Mindell et al. 1998). It is noteworthy that our finding is, to our knowledge, the first example of -1 frameshift deletion found in an invertebrate mitogenome. So far single nucleotide deletions in mitochondrial PCGs have only been found in a few turtle species (Andreu-Sánchez et al. 2021), and, overall, -1 frameshifts appear to be rarer than +1 insertions (Singh, 2013). Alternatively, the single nucleotide deletion in *nad5* of *D. daci* could result in the expression of a truncated but still functional *nad5* gene product because it still contained the proton-conducting transporter domain similar to *nad5* genes in other species (Chase et al. 2018), however, this scenario may be less likely because it would constitute a substantial truncation. Yet another scenario could be compensation of the frame shift mutation by an unknown mechanism other than translational frameshifting, via the *D. daci* nuclear genome, *Wolbachia* or the fruit fly mitochondrial or nuclear genomes. There are several examples of intracellular endosymbionts with degraded gene functions that are compensated by other endosymbionts (Monnin et al. 2020) or their hosts (Mao et al. 2018).

Mitogenome evolution and parasitism

It has previously been hypothesised that mitogenome rearrangements arose with the evolution of parasitic life cycles. This is because a transition to a parasitic life cycle in a lineage may come in hand with a relaxation of selective constraints acting on mitogenomes and their functions (McMahon et al. 2011). Based on our findings we can now add single nucleotide frameshift mutations that may also arise in lineages that have evolved parasitic life cycles. There is evidence for the association between mitogenome changes and evolution of parasitic life cycles, because mitogenomes of parasitic lineages of Hymenoptera are highly rearranged when compared to the conserved mitogenome arrangement patterns in the more basal lineages of Hymenoptera which are not parasitic (Dowton & Austin, 1999). Mitogenome rearrangements were also reported for the two egg parasitoids, *Trichogramma japonicum* and *Trichogramma ostriniae* (Chen et al. 2018) as well as a parasitoid of *Drosophila* larvae, *Leptopilina boulardi* (Oliveira et al. 2016). Similarly, rearrangements have been observed in three parasitoid wasp species of the genus *Psyttalia* which parasitise *Bactrocera oleae* (Powell et al. 2020). Furthermore, the numbers of mitogenome rearrangements in Strepsiptera correlated with the transition from moderate to extreme levels of parasitism. More gene rearrangements were observed in the mitogenomes of the more derived Stylopidia species *D. daci*, *X. moutoni* and *X. vesparum* compared to the more basal Mengenillidia species *M. australiensis* and *M. moldryzki*. The largest number of differences when compared to the ancestral insect mitogenome arrangement were observed in *D. daci*, and the single nucleotide frameshift deletion in *nad5* and the transposition of *rrnS* were unique, and possibly associated with the more extreme endoparasitism displayed by *D. daci* and its different host utilisation (i.e. Diptera). Rearrangements involving ribosomal RNA genes have been found in other insects, such as thrips (Kumar et al. 2019). It is unclear how the *nad5* nucleotide deletion

could have occurred, but its effect may not be as severe in an endoparasitic insect (Kathirithamby, 2009). Flight muscles rely heavily on mitochondrial function (Iwamoto, 2011; Sacktor, 1961), and an insect with limited flight function may be able to cope with a less efficient mitochondrial function.

Dipterophagus daci mitogenome characteristics

The overall length of the *D. daci* and fruit fly mitogenomes were within the expected length of 15-18 kb (Cameron, 2014). Both *D. daci* and the fruit fly mitogenomes contained the 37 genes and the AT-rich region usually found in animal mitogenomes (Boore, 1999; Wolstenholme, 1992). The conserved location for AT-rich region is between *rrnS* and *trnI*, however in the *D. daci* mitogenome the AT-rich region was located between *trnV* and *trnS*$_2$, which is similar to its position in a gnat bug, *Stenopirates* sp. (Li et al. 2012), while it is located in the conserved location in *M. moldrzyki* (Niehuis et al. 2012) and *X. moutoni*; however, incomplete information is available for *X. moutoni* (Zhang et al. 2021). The *D. daci* mitogenome assembly contained a gap in this region and hence the full length of the AT-rich region could not be estimated. Attempts to close the mitogenome by iterative mapping with short reads proved impossible. This region could be either too long and repetitive to be closed with bioinformatics approaches, or have secondary folding structures resulting in sequencing difficulties, as also found for *M. australiensis*, *X. moutoni* and *X. vesparum* (Carapelli et al. 2006; McMahon et al. 2009; Zhang et al. 2021).

Our study revealed that the mitochondrial PCGs of *D. daci* are shorter relative to the PCGs of their host fruit flies, and this could be associated with the evolution of the strepsipteran life cycle, as also suggested for *M. australiensis*, *X. moutoni* and *X. vesparum* (Carapelli et al.

2006; McMahon et al. 2009; Zhang et al. 2021). Similar to other parasitic insects (Cameron, 2014; Chen et al. 2018), the nucleotide composition of the *D. daci* mitogenomes were more AT-biased compared to fruit fly mitogenomes. The high AT bias observed in *D. daci* is similar to the other Strepsiptera (Carapelli et al. 2006; McMahon et al. 2009; Zhang et al. 2021). Furthermore, the *D. daci* mitogenome had a positive AT skew and a negative GC skew indicating that its genes contain more A than T and more C than G, as also reported in other insects (Wei et al. 2010).

Low mtDNA diversity in *Dipterophagus daci*

Low intraspecific mitogenome diversity is generally attributed to founder events (Kinziger et al. 2011; Wessel et al. 2013), or can be due to *Wolbachia* endosymbionts which manipulate host reproduction or have other host fitness effects (Turelli et al. 1992). Maternal coinheritance of mitogenomes and *Wolbachia* may facilitate *Wolbachia*-driven selective sweeps of the infected mitochondrial haplotype resulting in low mitochondrial genetic diversity (Hurst & Jiggins, 2005; Morrow & Riegler, 2021; Schuler et al. 2016; Turelli et al. 1992). In comparison to *B. neohumeralis* and *B. tryoni*, *D. daci* mitogenomes had only ten SNPs in PCGs and were 15-33× less diverse. Previously, it has been demonstrated that *D. daci* hosts two *Wolbachia* strains, *w*Ddac1 and *w*Ddac2; these two strains lack genes required for host reproductive manipulations, and therefore may have beneficial effects on host fitness (Towett-Kirui et al. 2021). Our extensive mitogenome analysis of *D. daci* together with the previous analysis of its nuclear *18S rRNA* gene extracted from the WGS libraries provides strong evidence that the low diversity observed in the *D. daci* mitogenome could be due to a past *Wolbachia* invasion with hitchhiking mitogenome types. It is unknown, however, whether both strains invaded this host at once, or in two separate waves. Further

characterisation of *D. daci* nuclear DNA diversity and the *D. daci*-*Wolbachia* relationship across a larger sample population will be required to ascertain beneficial *Wolbachia* host effects and mitogenome diversity patterns in this species.

3.6 Conclusions

Despite advancement in sequencing techniques, most host-parasite studies involving insect hosts and parasitoids have relied on direct sequencing of the parasitoid genomic DNA, however, this is more difficult in studies involving parasitoids which are almost entirely endoparasitic like Strepsiptera. The successful assembly of mitogenomes of *D. daci* and their fruit fly host species from individual libraries in the current study, demonstrate the possibility of obtaining both endoparasitoid and host sequences from the same library. We observed extensive gene rearrangements in *D. daci*, similar to observations in other Strepsiptera as well as other parasitic insects suggesting that this could be due to its transition to parasitism. Additionally, a single nucleotide -1 frameshift deletion in the coding region of the *nad5* gene of *D. daci* and rearrangement of the ribosomal RNA genes uniquely found in *D. daci* suggest mutation events that may be linked to their life history traits of extreme endoparasitism. However, further studies that focus on the relationship between hosts and parasites should investigate the functional consequences of these gene rearrangements and the deletion in *nad5*. The current study has provided molecular data that provides insights into *D. daci* evolution. *Wolbachia* has likely cause the low mtDNA diversity observed in *D. daci*. However, experiments involving a larger population dataset will need to be used to investigate whether *Wolbachia* has reduced the mtDNA diversity in *D. daci* populations, and whether this is due to a mechanism of reproductive manipulation or conferral of beneficial fitness effects to the host.

Chapter 4

Bacterial communities are less diverse in the endoparasitoid *Dipterophagus daci* (Strepsiptera) than in its fruit fly hosts and are dominated by *Wolbachia*

4.1 Abstract

Insect-microbe interactions play a vital role in insect biology and ecology. Several factors influence the composition and structure of bacterial communities within a host, including host-parasite interactions which involve microbiomes of both host and parasite. Studies have explored the microbiomes of free-living insects, however, the microbiomes of endoparasitic insects are less explored. Due to their high specialisation, it is expected that endoparasitoids have a simple and distinct microbiome relative to that of hosts. We compared the microbiomes of the strepsipteran endoparasitoid *Dipterophagus daci* and of seven of its tephritid fruit fly host species using 16S rRNA gene amplicon sequencing of dissected endoparasitoids and host flies with and without concealed early stages of parasitisation. The *D. daci* microbiome was distinct and less diverse than the microbiomes of its fruit fly hosts. It was dominated by Proteobacteria (> 96%), attributed to the dominance of *Wolbachia*, with other minor components. *Wolbachia* dominance was not observed in fruit fly hosts with and without parasitisation, with variability in the relative abundance of some bacteria also depending on the *Wolbachia* infection status of *D. daci*. Our study presents the first report of a strepsipteran microbiome. It reveals the effects of early stages of *D. daci* parasitisation on the fruit fly host microbiome that could interfere with microbiome studies of field collected fruit fly specimens. Furthermore, our study confirms that *D. daci* is the true host of *Wolbachia* previously detected in fruit flies which had suffered from concealed parasitisation.

4.2 Introduction

Insects have associations with diverse microbial communities. Some of these microbes play vital roles in aspects of host biology, for instance, nutrition, development and reproduction, or they can influence fitness and immunity, and provide protection against pathogens, parasitoids and toxins (Akman Gündüz & Douglas, 2009; Cheng et al. 2017; Coon et al. 2015; Eleftherianos et al. 2013; Oliver et al. 2008; Gill et al. 2010). Symbiotic microbes can reside within the digestive tract, in particular the gut lumen (Behar et al. 2008; Ben-Yosef et al. 2008), on the surface of the insect host (ectosymbionts) or within host cells and tissues (endosymbionts) (Aharon et al. 2013; Eleftherianos et al. 2013; Saridaki & Bourtzis, 2010; Zchori-Fein & Bourtzis, 2012). To the host, endosymbiotic bacteria can either be obligate or facultative. Obligate endosymbionts that are essential for host development can have long-term and evolutionary relationships with the host, are often maternally inherited, take part in vital host processes and provide essential nutrients to their host, in particular when feeding on diets that are limited in these nutrients (Douglas, 2009; Oliver et al. 2010; Buchner, 1965). For instance, the aphid endosymbiont, *Buchnera aphidicola* synthesises essential amino acids for its host (Akman Gündüz & Douglas, 2009). Facultative endosymbionts are not essential for host development but can promote host fitness and ecological adaptation (Feldhaar et al. 2011). They are highly diverse and their relationship with the host can be anywhere from beneficial to harmful (Brumin et al. 2011; Chen et al. 2000; Shaw et al. 2016; Zug & Hammerstein, 2015). Therefore, in order to understand the biology and dynamics of insects the study of the relationships with their microbial communities is of vital importance.
Of particular interest is *Wolbachia*, a maternally inherited facultative endosymbiont that occurs in over 50% of insects and other arthropod species where it can influence host biology and manipulate host reproduction to enhance its maternal transmission (Bandi et al.1998;

Doremus & Hunter, 2020; Hilgenboecker, 2008; Hurst et al. 1999; Stouthamer et al. 1999; Weinert et al. 2015; Werren et al. 2008). Cytoplasmic incompatibility (CI) is the most common reproductive manipulation caused by *Wolbachia*, and results in embryonic mortality when infected males mate with uninfected females or with females infected with a different *Wolbachia* strain (Doremus & Hunter, 2020; Werren, 1997). Other forms of reproductive manipulations of *Wolbachia* include male-killing, parthenogenesis and feminisation (Hurst et al. 1999; Werren, 1997). Some *Wolbachia* strains can also confer protection to their hosts against parasites, viruses and other pathogens (Bian et al. 2010; Hughes et al. 2011; Stevanovic et al. 2015; Teixeira et al. 2008). Additionally, *Wolbachia* strains can provide benefits as nutritional mutualists that synthesize vitamins deficient in hosts diet; for instance *Wolbachia* provides B vitamins to the bedbug, *Cimex lectularius* (Hosokawa et al. 2010; Ju et al. 2020). Furthermore, *Wolbachia* can influence the host's microbial diversity (composition) and relative abundance (structure) (Audisio et al. 2015; Audsley et al. 2018; Dittmer & Bouchon, 2018; Duan et al. 2020). For instance, *Wolbachia* can alter the relative abundance of microbial communities in adult mosquitoes (Audsley et al. 2018) as well as in the parasitoid wasp, *Nasonia vitripennis* (Duan et al. 2020). Conversely, other bacteria in host bacterial communities can influence *Wolbachia*, for example, *Asaia* can impede the establishment and stable transmission of *Wolbachia* after artificial introduction in mosquitoes (Hughes et al. 2014).

Studies of host-microbe interactions have been extensively performed on free-living stages of insects, and less on species that develop parasitically on or within free-living insects (such as endoparasitoids) (Dheilly et al. 2019), and never for any species of the endoparasitic insect order of Strepsiptera (Hammer & Moran, 2019), due to its extraordinary lifecycle. Strepsiptera is a small insect order that consists of 630 known species that are entirely obligate endoparasitoids (Kathirithamby, 1989b, 1991). With eight extant families,

Strepsiptera are diverse and parasitise hosts belonging to the seven insect orders Blattodea, Diptera, Hemiptera, Hymenoptera, Mantodea, Orthoptera and Zygentoma (Kathirithamby, 1998). Adult strepsipterans display extreme sexual dimorphism with adult males that have the external morphological features of a free-living adult insect while the females are neotenic and fully endoparasitic within their host, except for Mengenillidae females which are also free-living (Kathirithamby, 1989b, 1991, 2009). Parasitisation of the host occurs via the free-living first instar larvae (planidia) which enter the host (Kathirithamby, 2009, 2018). Once, inside the host, the first instar larvae undergo hypermetamorphosis (i.e change in morphology and behaviour between different instars) to the 4th larval instar. In Mengenillidae (suborder Mengenillidia) the 4th larval instars of both sexes leave and pupate on the outside of their hosts, while in all other families (all contained within the suborder Stylopidia) both males and females complete their larval development inside the host and the males extrude as cephalotheca (i.e extruded and externally visible anterior region of the male pupa) while females extrude as cephalothorax (i.e fused head, thorax and anterior abdominal segments) (Kathirithamby, 2009, 2018). Adult males emerge from the cephalotheca within the host while females remain neotenic and their cephalothorax extrudes through the host cuticle. Therefore, parasitisation with early stages of Strepsiptera may remain unknown unless detected by PCR (Towett-Kirui et al. 2021).

Other than host-microbe interactions, host-parasite interactions in insects can be modulated by microbes associated with either the host or the parasite (Dheilly et al. 2019; Dheilly et al. 2015). Host microbes can protect their hosts against parasites, for instance the aphid symbiont *Hamiltonella defensa* protects its hosts against the parasitoid wasp *Lysiphlebus fabarum* (Cayetano & Vorburger, 2015; Oliver et al. 2003). The host microbiome can also aid in the establishment of parasites in their insect hosts, as shown in the interaction between the cestode *Hymenolepis diminuta* and its intermediate host, the grain beetle, *Tenebrio molitor*

(Fredensborg et al. 2020). Endoparasitoids like Strepsiptera have an intimate relationship with the host and depend on the host for nourishment (Kathirithamby et al. 2003). Therefore, endoparasitoids can compete with the host's microbiota for resources. Host associated microbes can influence host immunity. Altering the bacterial communities of *Drosophila melanogaster* by antibiotic treatment influenced its resistance to *Asobara tabida* parasitoids by reducing the encapsulation rate of the parasitoid egg (Chaplinska et al. 2016). Similarly, parasitisation of the larvae of the two moth species, *Diatraea saccharalis* and *Spodoptera frugiperda* by the parasitoid wasp *Cotesia flavipes* changed the composition and structure of the larvae's bacterial communities (Cavichiolli de Oliveira & Cônsoli, 2020). Similarly, parasitism of *Spodoptera frugiperda* larvae with parasitoids *Cotesia marginiventris* changed the larvae's bacterial community composition (Wang et al., 2021), suggesting that the bacteria play a substantial role in host-parasite interactions.

While most animal species have diverse associations with their microbial communities, some animal species may have few/no microbial associations (Hammer et al, 2019). For example, parasites/parasitoids can be associated with simple microbiomes due to their unique lifestyle and exclusive dependence on their hosts for resources. For example, the parasitic plant *Orobanche hederae*, a root holoparasite of *Hedera*, exhibited a reduced microbiome compared to its host (Fitzpatrick & Schneider, 2020). In Strepsiptera, the neotenic females reproduce viviparously and obtain nutrients exclusively from the host hemolymph including for the production of their offspring (Kathirithamby et al. 2003). Strepsipteran larvae have a gut, and nutrient uptake from the host hemolymph occurs in the midgut, however, after extrusion of the females, the gut is degenerate and filled with host hemolymph (Giusti et al. 2007; Kathirithamby, 2009). Nutrient uptake in neotenic females may vary depending on the host (Giusti et al. 2007; Kathirithamby, 2009), and nutrient uptake from the host hemolymph occurs via a particular tissue, the *apron* (Kathirithamby, 2000).Therefore, the exclusive

dependence on the host by Strepsiptera could also predict that their microbiome has a low diversity and/or is less diverse than the microbiome of the host. This expectation is also in line with the general observation that the exclusive association between parasites or parasitoids with their hosts has led to the evolution of reduced morphological and genomic characteristics (Gillespie et al. 2005; Johnston et al. 2004; Kathirithamby, 1989, 2009; McMahon et al. 2009; Sun et al. 2018; Sundberg & Pulkkinen, 2015). Similarly, close host-endosymbiont associations can result in reduced genomic characteristics exhibited also by endosymbionts, for instance, the psyllid endosymbiont *Carsonella ruddii* which has a genome size of ~160kb (Nakabachi et al. 2006), and leafhopper endosymbiont, *Nasuia deltocephalinicola* which has a genome size of 112kb (Bennett & Moran, 2013), substantially smaller than the genomes of free-living bacteria.

Our study focused on *Dipterophagus daci*, a strepsipteran endoparasitoid of tephritid fruit flies (Drew & Allwood, 1985). To date, *D. daci* is the only described strepsipteran endoparasitoid of Diptera (besides another undescribed strepsipteran endoparasitoid of platystomatid flies) and has been reported from 22 species of the tephritid subfamily of Dacini (Allwood & Drew, 1996; Drew & Allwood, 1985; Towett-Kirui et al. 2021). A recent study revealed that the presence of two *Wolbachia* strains in seven Australian tephritid species (Morrow et al. 2014; Morrow et al. 2015) was due to concealed early parasitisation stages of *D. daci*, and that *D. daci* is the true host of *Wolbachia* (Towett-Kirui et al. 2021). The two *Wolbachia* strains were characterised using the *Wolbachia surface protein* (*wsp*) gene and five MLST loci (Morrow et al. 2014).

Tephritid fly pests are diverse, and include some species that can infest a wide range of fruit and vegetable crops (White & Elson-Harris, 1992). Across tephritid diversity, specialisations have occurred in which parts of a plant are infested by taxa, for instance fruit, flower, seed, stem or leaves (Bragard et al. 2020; Vargas et al. 2015). Furthermore, tephritids have diverse

bacterial communities that can vary in diversity and structure depending on host species, host phylogeny, host diet, rearing environment and life stage (Colman et al. 2012; Deutscher et al. 2018; Kolasa et al. 2019; Morrow et al. 2015; Woruba et al. 2019). For example, the bacterial communities of the island fly, *Dirioxa pornia* were distinct from the ones of *Bactrocera* species due to the different life histories (Morrow et al. 2015). Furthermore, diverse bacterial compositions were observed between different *Bactrocera* species (Morrow et al. 2015) suggesting that several factors play a role in shaping the microbiome of tephritid fruit flies. Our study aimed to explore the diversity and structure of bacterial communities in *D. daci.* We hypothesised that due to the endoparasitic life history, the microbial communities in *D. daci* are not very diverse, yet distinct from the communities of its fruit fly hosts. Furthermore, due to the detection of *Wolbachia* in this species at high prevalence (i.e. 60-100% of individuals are infected, we expected that *Wolbachia* would dominate the microbiome of *D. daci* as this has been seen in other host species of *Wolbachia* (Audsley et al. 2018; Chen et al. 2016; Duan et al. 2020). We also tested whether early stages of *D. daci* parasitisation had an effect on the fruit fly microbiome, and whether there was any interaction with the *Wolbachia* of *D. daci*. To address these questions, we performed 16S ribosomal RNA gene amplicon sequencing of (i) *D. daci* male pupae, (ii) fruit flies parasitised with early stages of *Wolbachia*-positive *D. daci*, (iii) fruit flies parasitised with early stages of *D. daci* without detectable *Wolbachia*, and (iv) unparasitised fruit flies. We expected that the *D. daci* microbiome is distinct from the microbiome of the fruit fly host species. We also expected to observe variability in the bacterial community structure across the seven fruit fly host species which were included in this study. However, we did not expect to see *Wolbachia* dominance in fruit flies parasitised by early stages of *D. daci* because *D. daci* and not fruit fly is the true host of *Wolbachia* previously detected in fruit flies.

4.3 Methods

Fruit fly collection and DNA extraction

This study sequenced and analysed the bacterial 16S rRNA gene diversity of total genomic DNA extracts of 84 adult male fruit flies and 17 *D. daci* male pupae obtained from previous studies, i.e. total of 101 samples (Figure 4. 1) (Morrow et al. 2014, 2015; Towett-Kirui et al. 2021). Only male flies (and no female flies) were available for this study as they were collected in the field using male attractant traps. The 84 adult male fruit flies comprised individuals of seven species including *Bactrocera tryoni* (32), *Bactrocera neohumeralis* (22), *Bactrocera frauenfeldi* (11), *Bactrocera decurtans* (2), *Bactrocera bryoniae* (4), *Dacus axanus* (2) and *Zeugodacus strigifinis* (11), collected from Queensland in 1998, 2001, 2012, 2013 and 2019, and stored in ethanol at -20 ºC prior to DNA extraction. The *D. daci* male pupae were dissected from visibly parasitised (stylopised) male fruit flies collected from Queensland in 2019 (Towett-Kirui et al. 2021). This involved the removal of the pupae from the cephalotheca of fruit fly host abdomens (Figure 4. 1A, 4. 1B). The other male flies were not visibly (concealed) parasitised or unparasitised (Figure 4. 1C, Towett-Kirui et al. 2021). Prior to DNA extraction, the male fruit fly specimens and *D. daci* male pupae were surface treated with 4 % sodium hypochlorite to remove any external microorganisms, then washed with 0.2 % Triton-X and rinsed thoroughly using Milli-Q water (Morrow et al. 2015).

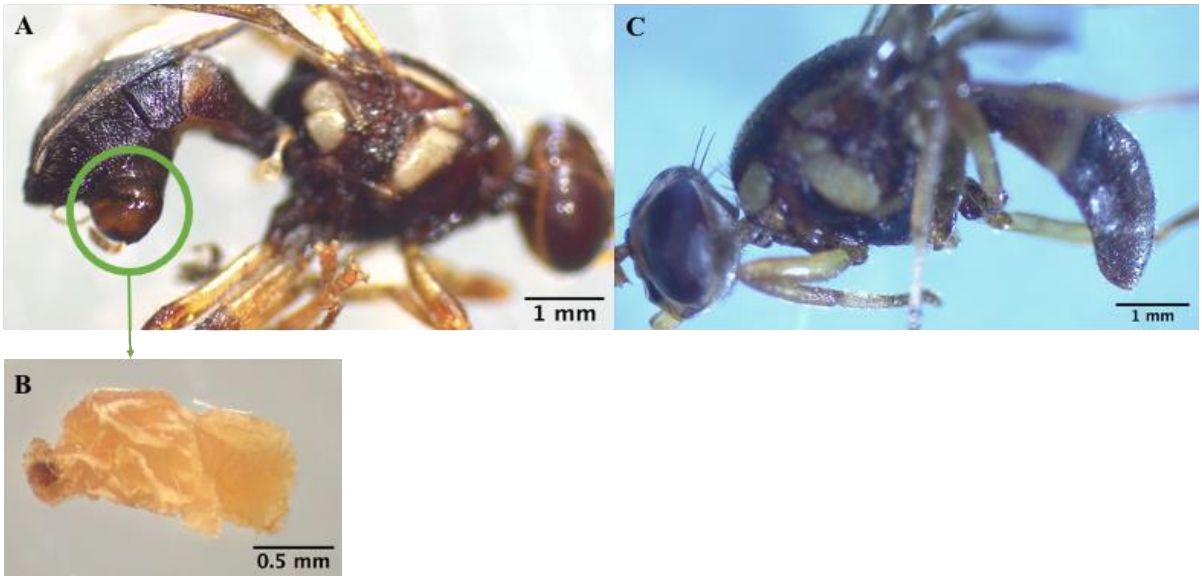

Figure 4. 1: Field-caught male tephritid fruit flies collected using male lure traps. (A) Stylopised male fruit fly (*Bactrocera neohumeralis*), (B) *Dipterophagus daci* male pupa dissected from a stylopised male fruit fly, (C) non-stylopised male fruit fly (*Bactrocera bryoniae*).

Total genomic DNA was extracted from individual fruit fly male abdomens and individual whole *D. daci* pupae using GenElute™ DNA Miniprep Kit (Sigma-Aldrich) as per manufacturer's instruction. The DNA quality was determined using Nanodrop and gel electrophoresis, and then stored at -20 ºC for subsequent experiments. The fruit fly and *D. daci* DNA extracts were screened by PCR using specific primers for the *Wolbachia surface protein* (*wsp*) and 16S rRNA genes (Morrow et al. 2014, 2015) and the *D. daci cytochrome c oxidase I* (*cox1*) gene (Towett-Kirui et al. 2021). Based on the PCR results the samples were categorised into four sample groups (i) *D. daci* male pupae (Dd) which were all positive for *Wolbachia* (ii) fruit flies parasitised by *Wolbachia*-positive *D. daci* (FliesDdW), (iii) fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd) and (iv) fruit flies without detectable *D. daci* DNA (unparasitised fruit flies; Flies) (Table 4. 1).

Bacterial 16S rRNA gene amplicon sequencing

The DNA extracts were submitted for 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform at the Western Sydney University Next Generation Sequencing Facility. Primers 341F (5' CCTACGGGNGGCWGCAG) and 805R (5' GACTACHVGGGTATCTAATCC) were used to amplify the V3-V4 region of the bacterial 16S rRNA gene with a total read length of 2 x 301 bp. Mock communities provided by the Next Generation Sequencing Facility was included.

Sequence analysis

After sequencing, the sequences were pre-processed, quality filtered and analysed using Quantitative Insight into Microbial Ecology (QIIME 2, v. 2019.7). Raw demultiplexed Illumina fastq sequence (Phred33 applied for QC) and mapping files were imported into QIIME 2 for downstream processes. First the manifest file was created by concatenating the forward and the reverse sequences. The DADA2 pipeline was used for denoising, quality filtering, dereplication and chimera removal (Callahan et al. 2016). Quality analysis was performed by trimming the primers and truncating the reads by using the commands (--p-trim-left-f 17 --p-trim-left-r 21 --p-trunc-len-f 290 --p-trunc-len-r 210). A naive Bayes classifier was trained using the Greengenes 99% OTUs at the V3-V4 region of the 16S rRNA gene. Amplicon sequence variants (ASVs) from DADA2 were used for taxonomic classification at a 99 % similarity threshold using QIIME 2 q2-feature-classifier plugin (Bokulich et al. 2018) and sample taxonomic composition and structure was visualised using QIIME 2 bar plot and plotted in R version 3.6.3 (R core Team, 2020, https://www.R-project.org/).

The core-metrics-phylogenetic pipeline was used to construct the phylogenetic tree. A rarefaction curve was used to assess adequate sampling of the microbial communities. Based on the rarefaction curve, the overall alpha and beta diversity analyses were performed at a sampling depth of 6,020. We estimated the alpha diversity between the four groups using Shannon's diversity index and Pielou's evenness. Beta diversity was assessed using weighted unifrac distance (phylogenetic relationships and relative abundance) and Bray-Curtis distance (relative abundance) to determine the microbial community variation in the four sample groups (Dd, FliesDdW, FliesDd and Flies) with pairwise comparisons (PERMANOVA) using qiime diversity beta-group-significance in QIIME 2 (v. 2019.7). Beta diversity results were also visualised using principal coordinates analysis (PCoA) plotted in R.

To determine whether *D. daci* parasitisation had an impact on the microbiome data obtained from the abdomen of parasitised male fruit flies, we assessed the differential relative abundance of bacterial taxa in fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd; n = 19) by comparing it to the unparasitised fruit flies (Flies; n = 34). Similarly, we aimed to determine whether parasitisation by concealed *Wolbachia*-positive *D. daci* (FliesDdW; n = 30) had an impact on the host fruit fly microbiome. We used the original taxonomic assignments of ASVs (at 99% identity) with the *Wolbachia* reads excluded to eliminate any biases. OTU datasets generated in QIIME and summarised at genus level were imported into Phyloseq. Genera with low variation across the samples were filtered out. The differential relative abundance was then performed in EDGER (Robinson et al. 2009).

4.4 Results

Sequence read analysis

The 101 sequenced 16S rRNA gene amplicon libraries (Table 4. 1) included 17 *D. daci* male pupae (Dd), 30 fruit flies parasitised by *Wolbachia*-positive *D. daci* (FliesDdW), 19 fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd) and 35 unparasitised fruit flies (Flies). After QC and filtering, we obtained a total of 2,274,402 sequence reads, with a mean sequence read number of 22,519 per sample (between 42 and 120,845 sequence reads per sample). After normalising the sequence read number at a sampling depth of 6,020 to minimise biases, we excluded one fruit fly specimen that contained less than 6,020 sequences (one *D. axanus* with 42 sequence reads) from the subsequent analysis (Table 4. 1).

Table 4. 1: Overview of the *Dipterophagus daci* and fruit fly specimens examined in this study. Table includes species identity, collection locality, collection year, sample group (*D. daci* male pupae (Dd), fruit flies parasitised by *Wolbachia*-positive *D. daci* (FliesDdW), fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd) and unparasitised fruit flies (Flies), and the number of reads for individual sample after filtering. The list is organised according to sample group and then fruit fly species**.**

Species	Sample ID	Collection locality	Collection year	Sample group	No of reads
Bactrocera bryoniae	Bbry544	Cairns	2013	Flies	14414
Bactrocera bryoniae	Bbry535	Lockhart River	2013	Flies	16769
Bactrocera decurtans	Bdec84	Seisia	1998	Flies	16741
Bactrocera frauenfeldi	Bfra61	Cairns	2013	Flies	6120
Bactrocera frauenfeldi	Bfra63	Cairns	2013	Flies	9082
Bactrocera frauenfeldi	Bfra135	Cairns	2001	Flies	36936
Bactrocera frauenfeldi	Bfra491	Cairns	2013	Flies	13205
Bactrocera frauenfeldi	Bfra5	Cape York - Lockhart	2019	Flies	11378
Bactrocera neohumeralis	Bn468	Brisbane	2019	Flies	12687
Bactrocera neohumeralis	Bn245	Cairns	2012	Flies	8273
Bactrocera neohumeralis	Bn242	Cairns	2019	Flies	16091
Bactrocera neohumeralis	Bn107	Cairns	2019	Flies	12639
Bactrocera neohumeralis	Bn246	Cairns	2012	Flies	7551
Bactrocera neohumeralis	Bn356	Gladstone	2012	Flies	11549
Bactrocera neohumeralis	Bn343	Mackay	2012	Flies	7079
Bactrocera neohumeralis	Bn344	Mackay	2012	Flies	11931
Bactrocera neohumeralis	Bn132	Mourilyan Harbour	2019	Flies	74895
Bactrocera neohumeralis	Bn239	Mourilyan Harbour	2012	Flies	10276
Bactrocera neohumeralis	Bn168	Townsville	2019	Flies	9689
Bactrocera tryoni	Bt229	Brisbane	2019	Flies	16447

Bactrocera tryoni	Bt431	Brisbane	2019	Flies	7546
Bactrocera tryoni	Bt376	Bundaberg	2019	Flies	22219
Bactrocera tryoni	Bt377	Bundaberg	2019	Flies	18389
Bactrocera tryoni	Bt277	Cairns	2001	Flies	7624
Bactrocera tryoni	Bt207	Mackay	2019	Flies	6679
Bactrocera tryoni	Bt444	Mackay	2013	Flies	10940
Bactrocera tryoni	Bt438	Mackay	2013	Flies	60986
Bactrocera tryoni	Bt265	Townsville	2019	Flies	120845
Bactrocera tryoni	Bt262	Townsville	2019	Flies	14329
Dacus axanus	Dax87	Thursday Island	1998	Flies	42
Zeugodacus strigifinis	Bst82	Bamaga	1998	Flies	24083
Zeugodacus strigifinis	Bst271	Bamaga	1998	Flies	21703
Zeugodacus strigifinis	Bst506	Cairns	2013	Flies	23846
Zeugodacus strigifinis	Bst507	Cairns	2013	Flies	30737
Zeugodacus strigifinis	Bst508	Cairns	2013	Flies	28086
Bactrocera frauenfeldi	Bfra486	Cairns	2012	FliesDd	8851
Bactrocera frauenfeldi	Bfra35	Cairns	2019	FliesDd	9877
Bactrocera neohumeralis	Bn517	Brisbane	2019	FliesDd	15354
Bactrocera neohumeralis	Bn790	Cairns	2019	FliesDd	24069
Bactrocera neohumeralis	Bn833	Cairns	2019	FliesDd	22592
Bactrocera tryoni	Bt213	Brisbane	2019	FliesDd	14736
Bactrocera tryoni	Bt215	Brisbane	2019	FliesDd	17304
Bactrocera tryoni	Bt216	Brisbane	2019	FliesDd	27508
Bactrocera tryoni	Bt372	Bundaberg	2019	FliesDd	16441
Bactrocera tryoni	Bt373	Bundaberg	2019	FliesDd	11721
Bactrocera tryoni	Bt374	Bundaberg	2019	FliesDd	15404
Bactrocera tryoni	Bt196	Cairns	2019	FliesDd	9512
Bactrocera tryoni	Bt187	Cairns	2019	FliesDd	7505
Bactrocera tryoni	Bt195	Cairns	2019	FliesDd	17911
Bactrocera tryoni	Bt197	Cairns	2019	FliesDd	10803
Bactrocera tryoni	Bt198	Mackay	2019	FliesDd	9373
Bactrocera tryoni	Bt199	Mackay	2019	FliesDd	8677
Zeugodacus strigifinis	Bst502	Cairns	2013	FliesDd	27178
Zeugodacus strigifinis	Bst505	Cairns	2013	FliesDd	19800
Bactrocera bryoniae	Bbry545	Cairns	2013	FliesDdW	19165
Bactrocera bryoniae	Bbry536	Lockhart River	2013	FliesDdW	13046
Bactrocera decurtans	Bdec85	Seisia	1998	FliesDdW	21897
Bactrocera frauenfeldi	Bfra492	Cairns	2013	FliesDdW	7978
Bactrocera frauenfeldi	Bfra485	Cairns	2012	FliesDdW	8913
Bactrocera frauenfeldi	Bfra490	Cairns	2013	FliesDdW	65333
Bactrocera frauenfeldi	Bfra3	Cape York - Lockhart	2019	FliesDdW	7460
Bactrocera neohumeralis	Bn243	Cairns	2012	FliesDdW	90253
Bactrocera neohumeralis	Bn355	Gladstone	2012	FliesDdW	12952
Bactrocera neohumeralis	Bn342	Mackay	2012	FliesDdW	9161
Bactrocera neohumeralis	Bn345	Mackay	2012	FliesDdW	11618
Bactrocera neohumeralis	Bn240	Mourilyan Harbour	2012	FliesDdW	10002
Bactrocera neohumeralis	Bn135	Mourilyan Harbour	2019	FliesDdW	10427
Bactrocera neohumeralis	Bn238	Mourilyan Harbour	2012	FliesDdW	11112
Bactrocera neohumeralis	Bn171	Townsville	2019	FliesDdW	11308
Bactrocera tryoni	Bt225	Brisbane	2019	FliesDdW	16393
Bactrocera tryoni	Bt214	Brisbane	2019	FliesDdW	18544
Bactrocera tryoni	Bt194	Cairns	2019	FliesDdW	8645
Bactrocera tryoni	Bt276	Cairns	2001	FliesDdW	10852
Bactrocera tryoni	Bt275	Cairns	2001	FliesDdW	8582
Bactrocera tryoni	Bt202	Mackay	2019	FliesDdW	9124
Bactrocera tryoni	Bt210	Mackay	2019	FliesDdW	8430
Bactrocera tryoni	Bt439	Mackay	2013	FliesDdW	14307

Bactrocera tryoni	Bt443	Mackay	2013	FliesDdW	10549
Bactrocera tryoni	Bt267	Townsville	2019	FliesDdW	10742
Dacus axanus	Dax88	Thursday Island	1998	FliesDdW	21580
Zeugodacus strigifinis	Bst81	Bamaga	1998	FliesDdW	24438
Zeugodacus strigifinis	Bst269	Bamaga	1998	FliesDdW	26351
Zeugodacus strigifinis	Bst503	Cairns	2013	FliesDdW	21186
Zeugodacus strigifinis	Bst504	Cairns	2013	FliesDdW	29379
Dipterophagus daci male pupa	Dd55	Airlie Beach	2019/2020	Dd	16745
Dipterophagus daci male pupa	Dd62	Airlie Beach	2019/2020	Dd	54222
Dipterophagus daci male pupa	Dd69	Airlie Beach	2019/2020	Dd	41185
Dipterophagus daci male pupa	Dd10	Cairns	2019/2020	Dd	36923
Dipterophagus daci male pupa	Dd41	Cairns	2019/2020	Dd	48956
Dipterophagus daci male pupa	Dd57	Cairns	2019/2020	Dd	27659
Dipterophagus daci male pupa	Dd91	Cairns	2019/2020	Dd	46539
Dipterophagus daci male pupa	Dd108	Cairns	2019/2020	Dd	57708
Dipterophagus daci male pupa	Dd110	Cairns	2019/2020	Dd	48687
Dipterophagus daci male pupa	Dd111	Cairns	2019/2020	Dd	48349
Dipterophagus daci male pupa	Dd45	Cairns	2019/2020	Dd	41325
Dipterophagus daci male pupa	Dd101	Cairns	2019/2020	Dd	47497
Dipterophagus daci male pupa	Dd11	Cairns	2019/2020	Dd	47479
Dipterophagus daci male pupa	Dd75	Cooktown	2019/2020	Dd	41656
Dipterophagus daci male pupa	Dd1	Townsville	2019/2020	Dd	41820
Dipterophagus daci male pupa	Dd64	Townsville	2019/2020	Dd	42561
Dipterophagus daci male pupa	Dd22	Townsville	2019/2020	Dd	18942

Microbiome of *D. daci*

In *D. daci* pupae, the phylum Proteobacteria was dominant, accounting for 96.2 % of the total bacterial community. Other phyla identified included Firmicutes (2.1 %) and Bacteroidetes (0.8 %), and other phyla with relative abundance of <1 % accounted for 0.7 %. Analysis at the class level showed that the class Alphaproteobacteria was relatively abundant. The classes Gammaproteobacteria, Deltaproteobacteria, Bacilli, Flavobacteria, Bacteroidia and other classes with relative abundance of <1 %, were also identified (Figure 4. 2A; Appendix C; Table C. 1). The bacterium with the highest relative abundance in *D. daci* pupae was *Wolbachia* accounting for 78.7 %. *Wolbachia* was present in all 17 *D. daci* pupae, however, at variable relative abundance (from 26.8 to 98 %). Other genera that were relatively abundant included *Serratia* (5.6 %), *Trabulsiella* (2.4 %), *Enterobacter* (1.6 %), one unknown Pasteurellales (2.4 %), one unknown Enterobacteriaceae (1.4 %) and *Lactococcus* (1.09 %) (Figure 4. 2B, Appendix C; Table C. 2). The relative abundance of *Serratia* increased with decreasing relative abundance of *Wolbachia* and vice versa (Figure 4. 2B). Alpha diversity analysis revealed low Shannon and Pielou evenness indices in *D. daci* (Figure 4. 3A, 4.3 B). Beta diversity analysis of bacterial communities using weighted unifrac and Bray-Curtis PCoAs showed that *D. daci* bacterial communities clustered separately from those of the host fruit flies (Figure 4. 3C, 4. 3D, Table 4. 2).

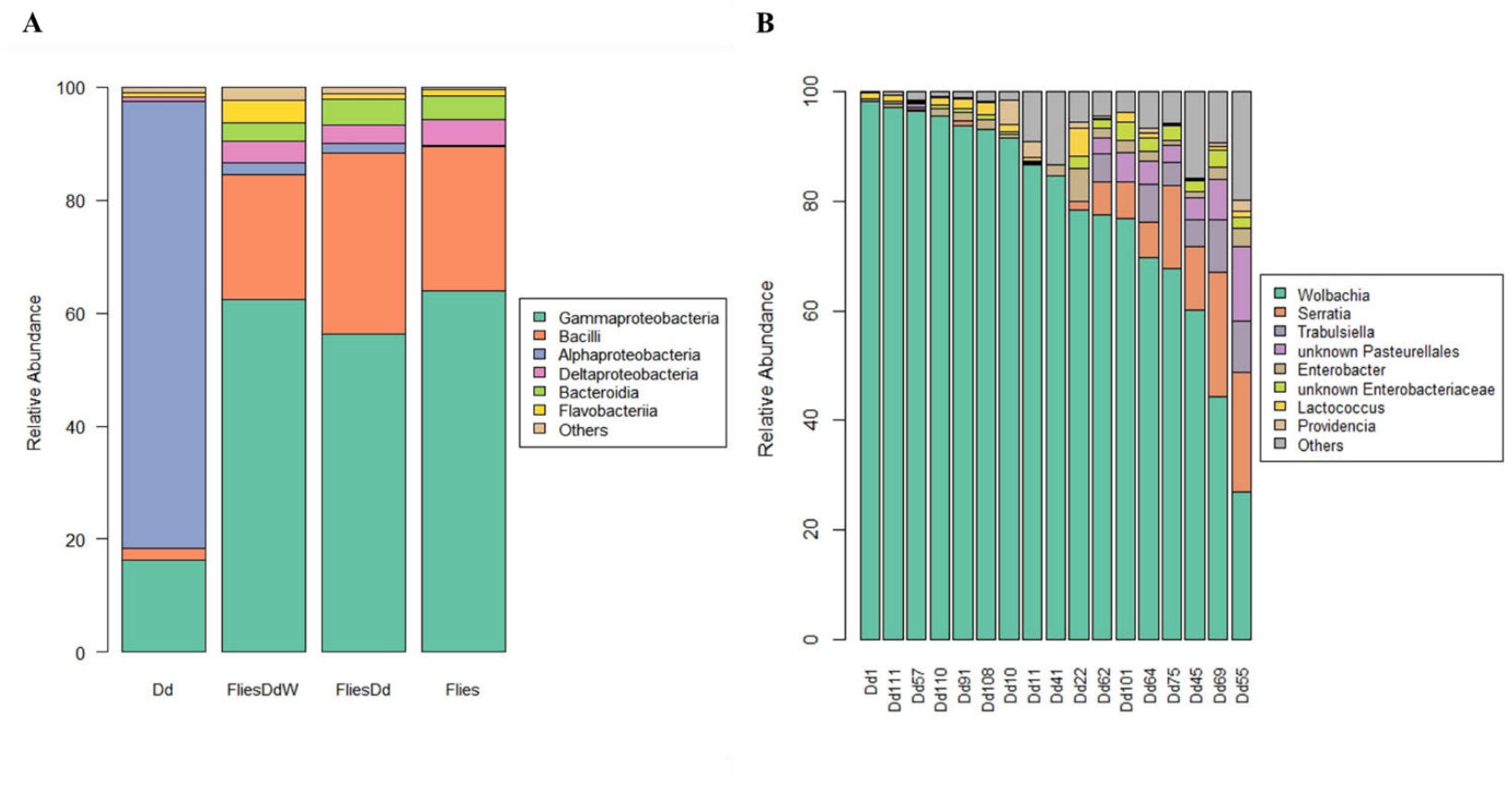

Figure 4. 2: Relative abundance of bacterial taxa in *Dipterophagus daci*. (A) Barplot of the relative abundance of bacterial classes in the four categories of samples (*D. daci* male pupae (Dd), fruit flies parasitised by *Wolbachia*-positive *D. daci* (FliesDdW), fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd) and unparasitised fruit flies (Flies), (B) Barplot of the relative abundance of bacterial genera in the 17 *D. daci* pupae samples. The highest available classification was used for taxa with no genus assigned**.**

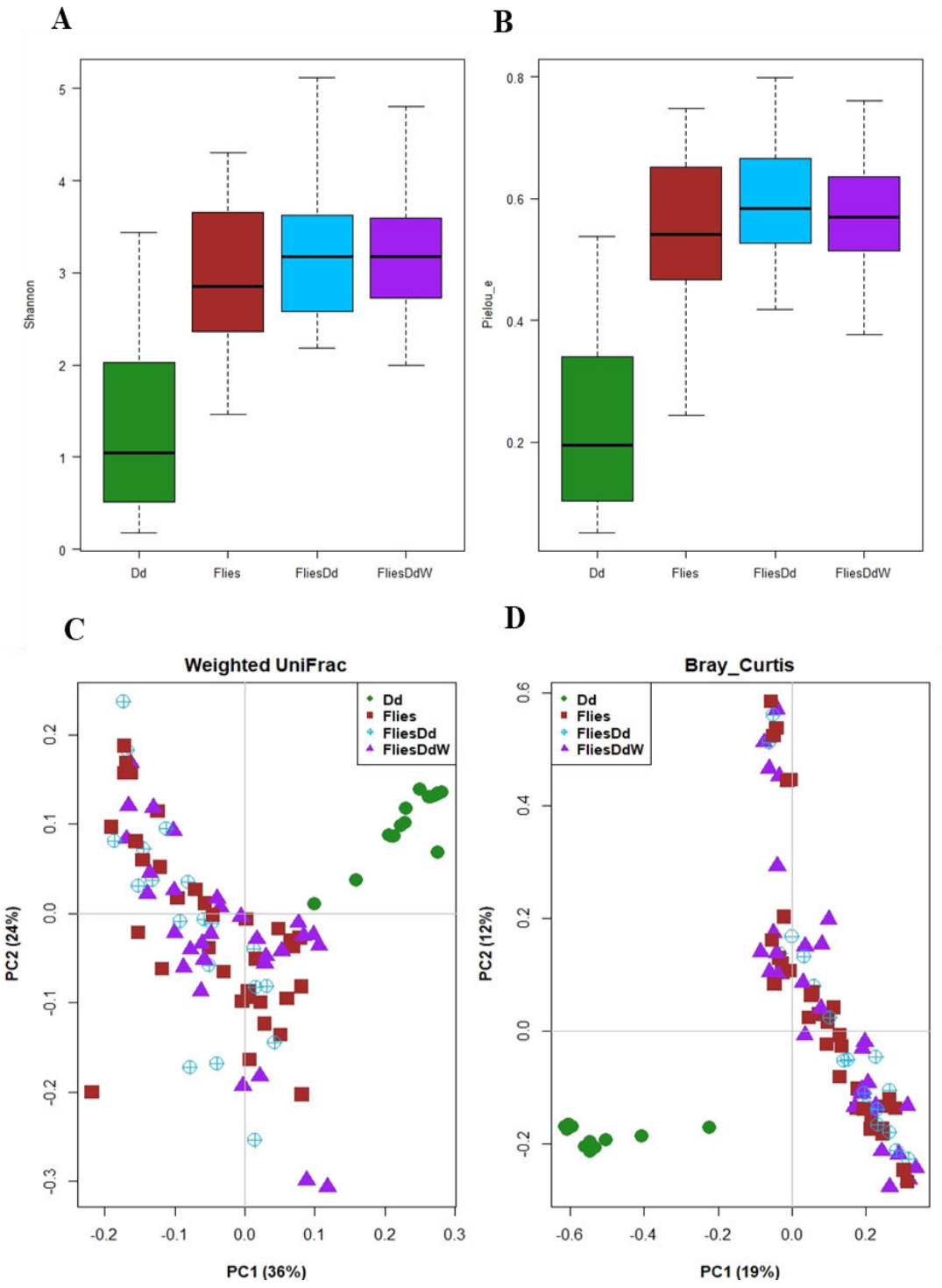

Figure 4. 3: Alpha and Beta diversity analysis of *Dipterophagus daci* male pupae (Dd), unparasitised fruit flies (Flies), fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd), fruit flies parasitised by *Wolbachia*-positive *Dipterophagus daci* (FliesDdW). (A) Shannon diversity (B) Pielou evenness diversity (C) Weighted Unifrac and (D) Bray-Curtis PcoA plots visualise the clustering and similarity of the bacterial communities of *D. daci* and the fruit fly sample groups**.**

Table 4. 2: Beta diversity metrics using PERMANOVA performed on the four sample groups; *D. daci* male pupae (Dd), fruit flies parasitised by *Wolbachia*-positive *D. daci* (FliesDdW), fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd) and unparasitised fruit flies (Flies). Comparisons that are significantly different are shown in bold.

PERMANOVA			Weighted_Unifrac		Bray-Curtis	
	Sample size	Permutations	pseudo-F	p-value	pseudo-F	p-value
Flies-Dd	51	999	41.014	**0.001**	18.898	**0.001**
FliesDd-Dd	36	999	47.850	**0.001**	22.044	**0.001**
FliesDdW-Dd	47	999	39.167	**0.001**	19.437	**0.001**
Flies- FliesDd	53	999	0.688	0.598	0.904	0.577
Flies- FliesDdW	64	999	0.708	0.615	1.058	0.341
FliesDd - FliesDdW	49	999	1.329	0.236	1.103	0.307

Comparison of bacterial communities between fruit fly host species

Multivariate analysis using Bray-Curtis PCoA revealed a distinct separation between the bacterial communities of *Z. strigifinis* and the four *Bactrocera* species; *B. tryoni, B. neohumeralis, B. frauenfeldi* and *B. bryoniae* (*B. decurtans* and *D. axanus* were not included, due to their low replication) (Figure 4. 4B), however the distinct clustering of *Z. strigifinis* was not observed in the weighted unifrac analysis (Figure 4. 4A). The alpha diversity analysis of the five fruit fly species revealed that the Shannon diversity indices ranged from (2.7 to 3.3) and Pielou evenness diversity indices ranged from (0.5-0.6), indicating bacterial diversity and evenness in the fruit fly hosts (Appendix C; Figure C. 1). Therefore, the fruit fly bacterial communities and abundance of *Z. strigifinis, B. tryoni, B. neohumeralis, B. frauenfeldi* and *B. bryoniae* were investigated to determine the difference.

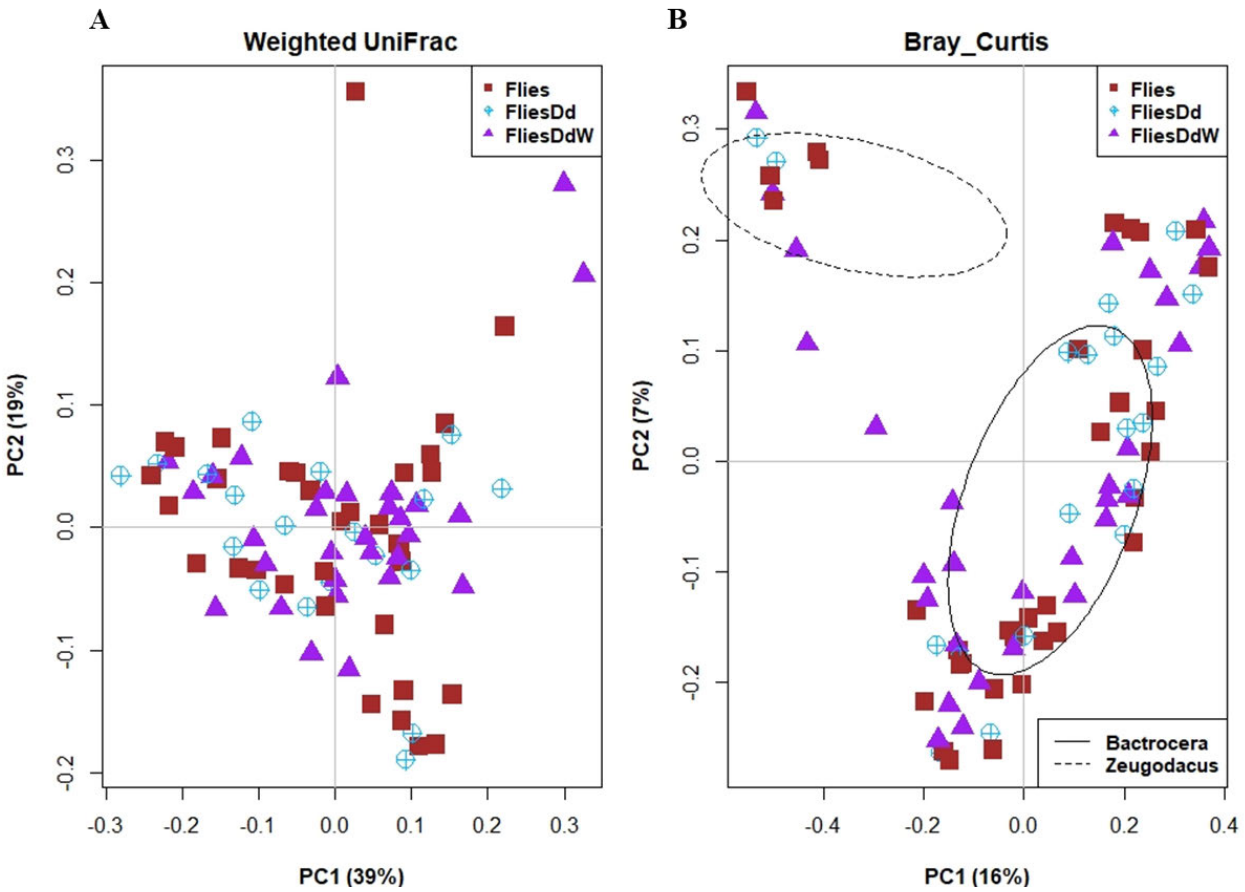

Figure 4. 4: Beta diversity analyses of fruit fly samples groups. (A) Weighted Unifrac and (B) Bray-Curtis PcoA plots visualise the clustering and similarity of the fruit fly sample groups. The ellipses show the clustering of the *Bactrocera* and *Zeugodacus* samples**.**

Barplot of the fruit fly bacterial communities revealed variability in the relative abundance of the bacterial communities. The most striking difference was the low relative abundance of the one unknown Pasteurellales bacterium in *Z. strigifinis* (1 %) compared to the other fruit fly species that had relative abundances ranging from 13 to 35 % (Figure 4. 5). Interestingly, bacteria from the genus *Acinetobacter* were relatively more abundant in *Z. strigifinis* (21 %) compared to *B. tryoni* (1.4 %), *B. neohumeralis* (0.01 %), *B. frauenfeldi* (8.9 %) and *B. bryoniae* (0.2 %) (Figure 4. 5, Appendix C; Table C. 3). PERMANOVA analyses based on Bray-Curtis results showed substantial differences not only for *Z. strigifinis* but also *B. frauenfeldi* and *B. bryoniae* (Table 4. 3). *Bactrocera frauenfeldi* exhibited low relative abundance of *Vagococcus* (7.3%) and high relative abundance of one unknown bacterium of

Enterobacteriaceae (15.1%) compared to the other fruit fly species, while *B. bryoniae* had relatively high abundance of *Vagococcus* (41.3%) compared to the other *Bactrocera* species. There was no significant difference between the bacterial communities of *B. tryoni* and *B. neohumeralis* (Table 4. 3).

Table 4. 3: Summary of PERMANOVA results assessing differences between the host fruit fly species (*Bactrocera bryoniae, Bactrocera frauenfeldi, Bactrocera neohumeralis, Bactrocera. tryoni, Zeugodacus strigifinis*). Comparisons that are significantly different are shown in bold

Bray-Curtis PERMANOVA	Sample size	Permutations	pseudo-F	p-value
B. bryoniae-B. frauenfeldi	15	999	3.025889	**0.002**
B. bryoniae-B. neohumeralis	26	999	1.548076	0.068
B. bryoniae-Z. strigifinis	15	999	15.05819	**0.001**
B. bryoniae-B. tryoni	36	999	1.778257	**0.017**
B. frauenfeldi-B. neohumeralis	33	999	2.430796	**0.002**
B. frauenfeldi-Z. strigifinis	22	999	7.418192	**0.001**
B. frauenfeldi-B. tryoni	43	999	1.850674	**0.004**
B. neohumeralis-Z. strigifinis	33	999	11.84403	**0.001**
B. neohumeralis-B. tryoni	54	999	1.323608	0.113
Z. strigifinis-B. tryoni	43	999	10.1723	**0.001**

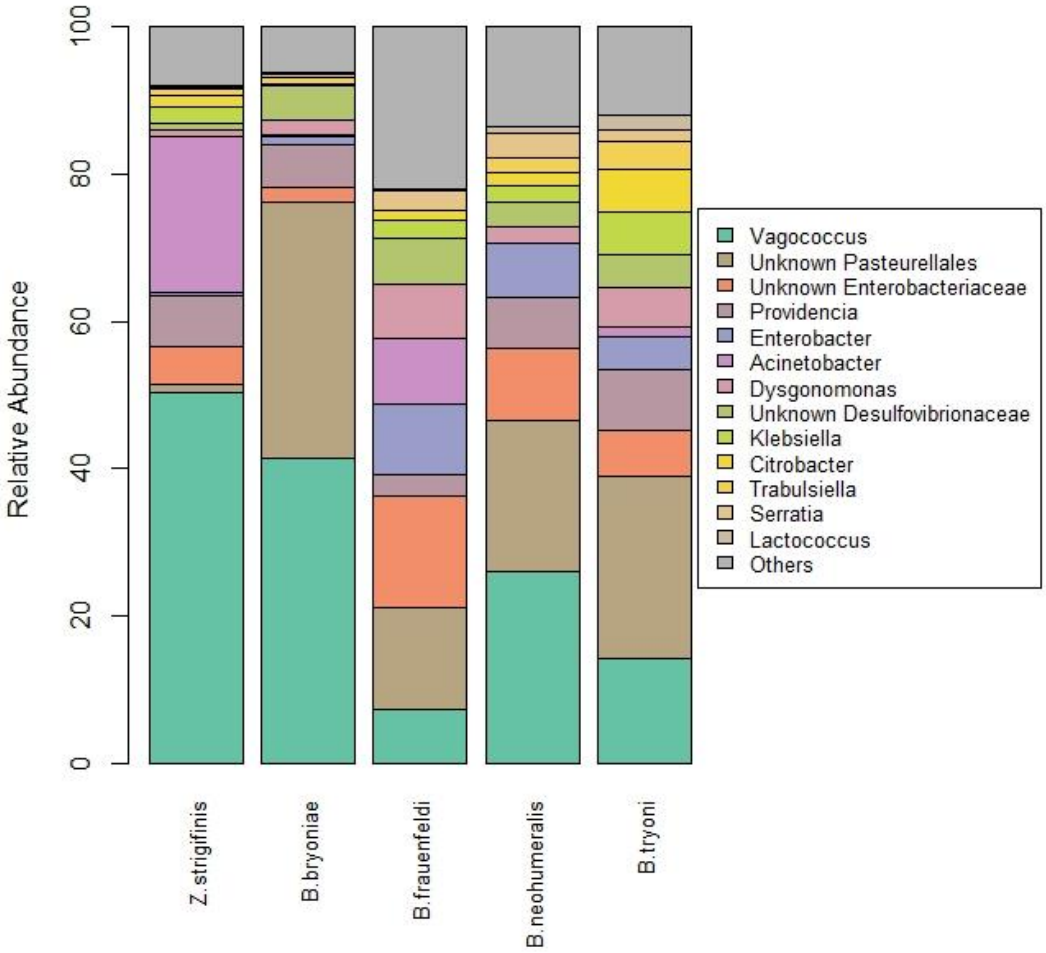

Figure 4. 5: Barplot of the most common bacterial genera in the host fruit flies *Zeugodacus strigifinis*, *Bactrocera bryoniae*, *Bactrocera frauenfeldi*, *Bactrocera neohumeralis,* and *Bactrocera tryoni*. Analysis performed on fruit fly hosts from all samples groups including, unparasitised fruit flies (Flies), fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd), fruit flies parasitised by *Wolbachia*-positive *Dipterophagus daci* (FliesDdW**).**

Influence of *D. daci* parasitisation on fruit fly microbiome

A comparison of the fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd) and unparasitised flies (Flies) was performed to determine the impact of *D. daci* parasitisation on the overall diversity of bacteria in fruit fly abdomen. *Wolbachia* was detected in relatively low abundance in both FliesDd (0.002 %) and Flies (0.002 %) sample groups when compared with the very high abundance in *D. daci* pupae. Therefore, sequences used in this analysis of fruit fly abdomens were corrected for *Wolbachia* and normalised to sequencing depth of 1,000, based on the minimum number of reads after excluding *Wolbachia*. The OTU datasets used were retrieved from QIIME and summarised to genus level. However, *Wolbachia* abundance was so low in fruit fly abdomens and may not have impacted the analysis. However, this comparison revealed an influence of *D. daci* on the relative abundance of Proteobacteria and Firmicutes in the abdomens of host fruit flies parasitised with early stages of *D. daci* (Figure 4. 6). The relative abundance of nine bacterial genera including *Proteus,* one unknown Enterobacteriaceae species, *Klebsiella,* one unknown Acetobacteriacea species, *Ochrobactrum, Morganella, Providencia,* three unknown Pasteurellales species and *Enterococcus* were increased in FliesDd, while three bacterial genera (*Enterobacter, Citrobacter* and one unknown Halomodacea species) decreased in *D. daci* unparasitised fly abdomens (Figure 4. 6).

Similarly, we compared the relative abundance of bacterial taxa between fly abdomens parasitised by *D. daci* with (FliesDdW) and without detectable *Wolbachia* (FliesDd). *Wolbachia* was also detected in relatively low abundance in FliesDdW (0.81%). We found that the relative abundance of 11 genera comprising *Proteus, Providencia, Dysgonomonas, Morganella,* one unknown Acetobacteriaceae species, two unknown Pasteurellales species, *Vagococcus, Serratia,* one unknown Enterobacteriaceae species, *Staphylococcus and*

Enterobacter were slightly increased in fly abdomens parasitised by *D. daci* without detectable *Wolbachia*, while *Klebsiella*, *Trabulsiella*, *Myroides* and *Citrobacter* were slightly decreased (Figure 4. 7).

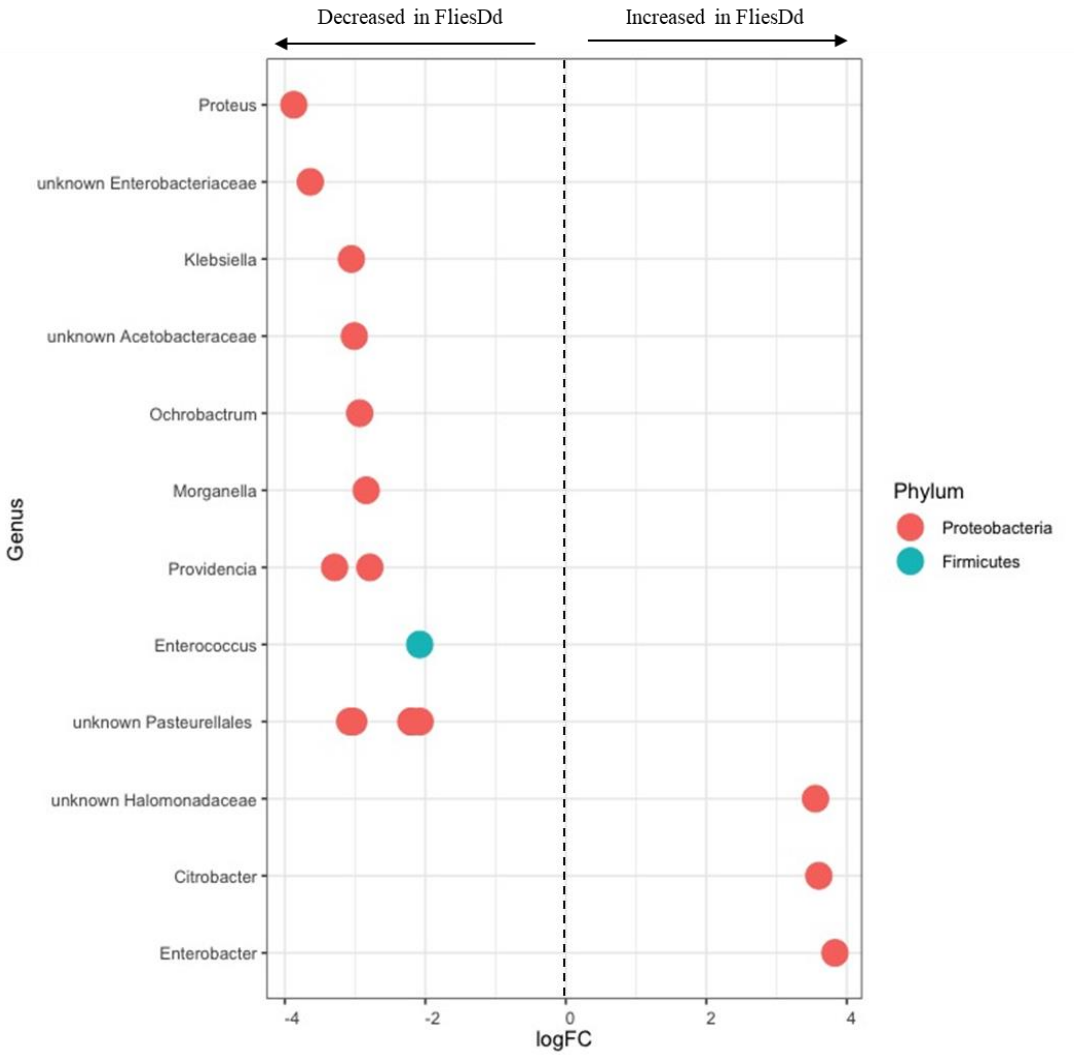

Figure 4. 6: Scatter plot of the bacterial taxa with differential relative abundance in fruit flies parasitised by *Dipterophagus daci* without detectable *Wolbachia* (FliesDd) compared to unparasitised fruit (Flies). A negative value denotes higher abundance in unparasitised flies and the taxa with significantly different relative abundances are coloured by phylum**.**

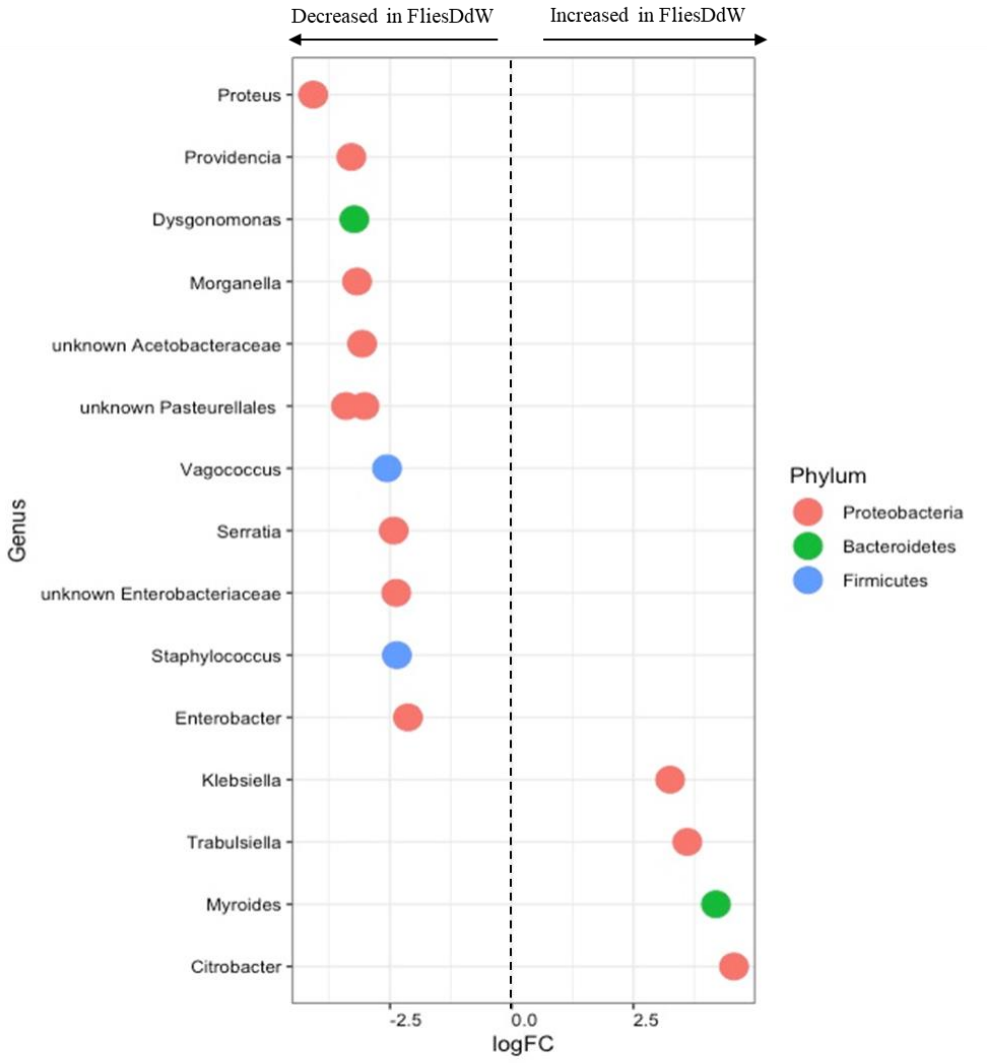

Figure 4. 7: Scatter plot of the bacterial taxa with differential relative abundance in fruit flies parasitised by *Wolbachia*-positive *D. daci* (FliesDdW) compared to fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd). A negative value denotes higher abundance in fruit flies parasitised by *D. daci* without detectable *Wolbachia* and the taxa with significantly different relative abundances are coloured by phylum.

4.5 Discussion

Host-parasite interaction studies in insects have revealed that individual microbe species associated with either the host or the parasite can play a role in shaping these interactions (Audsley et al. 2018; Fredensborg et al. 2020). In our study, we used 16S rDNA gene amplicon sequencing to explore the microbiomes involved in such an interaction, between the strepsipteran endoparasitoid *D. daci* and seven of its fruit fly host species. For this we conducted the first characterisation of the bacterial communities associated with a strepsipteran. We found that *D. daci* bacterial communities were dominated by *Wolbachia*. However, *Wolbachia* dominance was not observed in fruit flies parasitised by *Wolbachia*-positive *D. daci*, supporting previous findings of *D. daci* as the true host of *Wolbachia* (Towett-Kirui et al. 2021). For *D. daci* we found that the relative abundance of *Serratia* was reduced with increased relative abundance of *Wolbachia* and vice versa, possibly suggesting a competitive interaction between the two bacterial taxa (but this requires further quantification of the bacteria). Additionally, our study demonstrated that the *D. daci* microbiome is less diverse and distinct compared to the fruit fly host microbiomes. Comparison of bacterial taxa in abdomens of fruit flies parasitised by early stages of *D. daci* and abdomens of unparasitised flies revealed variability in relative abundance of bacterial taxa. Interestingly, comparing the bacterial communities of the fruit fly species, we found that the microbiome of *Z. strigifinis* was distinct from the microbiomes of the *Bactrocera* species. Furthermore, comparison of the bacterial communities of the fruit fly species revealed significant variation in relative abundance of bacterial communities.

Dipterophagus daci has a simple microbiome

The most abundant bacterial phylum in *D. daci* was Proteobacteria comprising 96.2 % of the total bacterial community, followed by Firmicutes and Bacteroidetes at relatively low abundance. A high relative abundance of Proteobacteria and Firmicutes had previously been detected in fruit flies (Deutscher et al. 2019; Morrow et al. 2015), as well as in other insect species (Colman et al. 2012; Yun et al. 2014). However, the abundance of *Proteobacteria* in *D. daci* was relatively higher compared to other insect species. We identified six relatively abundant bacterial classes including Alphaproteobacteria, Gammaproteobacteria, Bacilli, Deltaproteobacteria, Bacteroidia and Flavobacteria. However, the Alphaproteobacteria were by far the most dominant in *D. daci* samples owing to the relatively high abundance of *Wolbachia.* This indicates low bacterial diversity and uneven structure in *D. daci*, which is perhaps due to its parasitic lifestyle. *Dipterophagus daci*, similar to the other Stylopidia strepsipterans, have an almost fully endoparasitic life cycle in their host and depend exclusively on the host for nourishment and secretion (Kathirithamby, 1991, 2009; Kathirithamby et al. 2003). This exclusive dependence on the host is expected to reduce *D. daci* microbiome diversity. Low bacterial diversity and few bacterial co-associations have also been observed in the root holoparasite *Orobanche hederae* relative to its host *Hendera spp* (Fitzpatrick & Schneider, 2020). But as a consequence, this could also increase dependence of endoparasitoids/parasites on the very few bacteria that are present.

Wolbachia dominates *D. daci* microbiome

Wolbachia is a commonly occurring maternally inherited endosymbiont of insects that can manipulate host reproduction to increase its spread in host population (Hilgenboecker et al. 2008; Hurst et al. 1999; Stouthamer et al.1999; Werren et al. 2008). However, besides this trait of reproductive parasitism in many *Wolbachia* strains, some *Wolbachia* strains are also more mutualistic and provide fitness benefits which can also lead to high prevalence in host populations (Fry et al. 2004). *Wolbachia* dominance of host microbiomes has been found in several insect species (Diouf et al. 2018; Duan et al. 2020; Gottlieb et al. 2008; Novakova et al. 2017) and it is likely to apply to most insect hosts that have established and stably inherited *Wolbachia* infections (Audsley et al. 2018; Chen et al. 2016; Duan et al. 2020). The dominance of *Wolbachi*a in *D. daci* microbiome (but not in the host fruit fly microbiomes) in the current study supports earlier findings that demonstrated that the two *Wolbachia* strains previously detected in fruit flies (Morrow et al. 2014; Morrow et al. 2015) were actually associated with the fruit flies' endoparasitoid *D. daci*, and were detected because tested flies carried concealed early stages of *Wolbachia*-positive *D. daci* (Towett-Kirui et al.2021). Additionally, alpha diversity analysis revealed low Shannon diversity and Pielou's evenness values in *D. daci* bacterial communities (a consequence of the *Wolbachia* dominance) while this was not observed in fruit fly abdomens parasitised with *Wolbachia*-positive *D. daci*. Host-symbiont interactions can play key roles in host biology, fitness and function, as seen in the associations of aphids with *B. aphidicola* (Akman Gündüz & Douglas, 2009) and tsetse flies with *Wigglesworthia glossinidia* (Akman et al. 2002). There could be two reasons for *Wolbachia*'s dominance in the *D. daci* microbiome: *Wolbachia* either manipulates its reproduction, or it plays an essential role in *D. daci* nutrition or development. Both of these explanations are also supported by the earlier finding that the mitochondrial genome of *D.*

daci is depauperate of haplotype diversity across large parts of its geographic distribution. Most strepsipteran life stages are fully endoparasitic except for the free-living first instar larvae and adult males, and are therefore fully dependent on the host for nourishment (Kathirithamby, 2000). The host of parasitoids/parasites may not always provide all the essential nutrition and therefore endoparasitoids may form nutritional mutualisms with microbes like *Wolbachia. Wolbachia* has been observed as a nutritional mutualist in the bedbug, *Cimex lectularius*, providing B vitamins deficient in their diet (Hosokawa et al. 2010). Similarly, *Wolbachia* has been associated with synthesis of biotin and riboflavin to increase host fitness in the small brown planthopper *Laodelphax striatellus* and the brown planthopper *Nilaparvata lugens* (Ju et al. 2020). Furthermore, throughout its entire development, *D. daci* is exposed to the fruit flies' immune system and viruses. It has recently been demonstrated that tephritid fruit flies (including Australian tephritids) have a high incidence and prevalence of RNA viruses (Sharpe et al. 2021), suggesting that *Wolbachia* could play a role in protecting *D. daci* against the RNA viruses. *Wolbachia* protection against viruses has been observed in several insect species such as *Drosophila* (Stevanovic et al. 2015; Teixeira et al. 2008) and mosquitoes (Bian et al. 2010; Pimentel et al. 2021).

Bacterial communities in *D. daci* have a distinct composition and structure

The diversity and structure of bacterial communities in hosts can be attributed to several factors such as species identity and phylogeny, morphology and anatomy, life cycles and histories, diet, ecological and environmental factors (Morrow et al. 2015; Reese & Dunn, 2018; Yun et al. 2014). The weighted unifrac and Bray-Curtis beta diversity analyses revealed that the bacterial community of *D. daci* was distinct from that of the abdomens of its

fruit fly host species. This may be due to its phylogenetic history, which is diverse from the host fruit flies as well as host life cycle and diet.

Many insect-symbiont interaction studies focus on the interactions of bacterial symbionts with their hosts (Gündüz & Douglas, 2009; Brumin et al. 2011; Chen et al. 2000; Dale & Moran, 2006; Shaw et al. 2016; Zug & Hammerstein, 2015). However, the interaction between bacterial symbionts within a host could also have an effect on the host and its interactions with other insects (Audisio et al. 2015; Audsley et al. 2018; Dittmer & Bouchon, 2018; Duan et al. 2020; Hughes et al. 2014; Kondo et al. 2005; Mouton et al. 2004; Oliver et al. 2006). For instance, reduced *Wolbachia* densities have been observed in adzuki bean beetles, *Callosobruchus chinensis* individuals infected with multiple *Wolbachia* strains compared to individuals infected with just one strain (Kondo et al. 2005). Similarly, *Asaia* in *Anopheles* mosquitoes has been shown to impede *Wolbachia* transmission (Hughes et al. 2014). Such effects of bacterial taxa on each other could be attributed to competition for resources such as food and space (Dittmer et al. 2014; Osborne et al. 2012) or due to the priming of host immunity by one bacterial taxon to prevent overproliferation of another bacterial taxon (Hughes et al. 2011; Lee et al. 2013). Alternatively, *Wolbachia* has also been shown to protect its *Drosophila* host against harmful pathogens (Hedges et al. 2008; Osborne et al. 2012). However, another study did not observe any antibacterial protection in *Wolbachia* infected *Drosophila* (Wong et al. 2011). We observed that *Wolbachia* in *D. daci* led to reduced relative abundance of *Serratia*, suggesting a competitive interaction between the two taxa or induction of host immunity by *Wolbachia* against *Serratia*. *Wolbachia* alteration of host bacterial community structure has also been observed in adult *Aedes* mosquitoes (Audsley et al. 2018), the terrestrial isopod *A. vulgare* (Dittmer & Bouchon, 2018) and the parasitoid wasp *N. vitripennis* (Duan et al. 2020).

Variable bacterial communities in host fruit fly species

Tephritid fruit fly species exhibit diverse degrees of host plant specialisation and host plant utilisation, and this has been demonstrated to have an impact on their microbiome (Bragard et al. 2020; Doorenweerd et al. 2018; Morrow et al. 2015; Vargas et al. 2015). The Bray-Curtis analyses revealed that bacterial communities in *Z. strigifinis* abdomens were distinct from the ones in abdomens of *Bactrocera* species, possibly suggesting a fly genus effect or a host plant effect. However, a fly genus effect could not be confirmed in our study due to the availability of only one *Zeugodacus* species, and microbiomes of more *Zeugodacus* would need to be studied. The weighted unifrac analysis however did not show any distinct clustering, indicating that the variation between the bacterial communities of *Z. strigifinis* and the *Bactrocera* species was due to presence of distinct but phylogenetically close bacterial taxa. These findings revealed that the one unknown Pasteurellales and *Acinetobacter* bacterial taxa were the drivers of this difference. The fruit fly species *Z. strigifinis* is a flower pest of *Cucurbitaceae* plants while *B. tryoni, B. neohumeralis, B. frauenfeldi* and *B. bryoniae* are fruit pests (Doorenweerd et al. 2018; Vargas et al. 2015). This implies that the host plant, as well as the infested part of the host plant may contribute to the observed differences. In addition to the variable microbiome observed in *Z. strigifinis*, PERMANOVA revealed that *B. frauenfeldi* and *B. bryoniae* microbiomes also exhibited variability in the bacterial structure compared to *B. tryoni* and *B. neohumeralis*, while no difference was observed between bacterial communities of *B. tryoni* and *B. neohumeralis*. *Bactrocera tryoni* and *B. neohumeralis* are sibling species with close genetic similarity (Morrow et al. 2000; Yeap et al. 2020), therefore, this may explain the similarity of their microbiomes (Morrow et al. 2015).

Furthermore, it needs to be stated that in our study we compared bacterial communities characterised from genomic extracts of fruit fly abdomens, whereas previous studies have used genomic extracts of entire individuals, dissected guts as well other fly tissues (Deutscher et al. 2019; Morrow et al. 2015; Woruba et al. 2019). Given that a significant component of the bacterial communities in flies is located in the crop of the foregut it is possible that our sampling strategy has not well presented some of the bacterial diversity seen in other fruit fly microbiome studies – but in saying so the characterisation of the fruit fly microbiome diversity was not the primary aim of this study, but rather the interactions with the endoparasitoid *D. daci*.

Variable fruit fly microbiome due to *Dipterophagus daci* parasitisation

Microbes associated with the host, parasites and parasitoids can influence their interaction (Cavichiolli de Oliveira & Cônsoli, 2020; Dheilly et al. 2015; Fredensborg et al. 2020; Koch & Schmid-Hempel, 2011). For example, in a host-parasite/parasitoids relationships, the host microbiome can change to favour the establishment of a parasite/parasitoids; for instance, reduced establishment of the cestode, *H. diminuta* was observed in antibiotically treated grain beetle, *Tenebrio molitor* compared with the untreated group, suggesting that the host microbiome influenced the establishment of *H. diminuta* (Fredensborg et al. 2020). Additionally, a parasite/parasitoid can manipulate the host microbiota to its benefit (Dheilly et al. 2015). Conversely, the host microbiome can change to protect its host against parasite invasion, for instance, the symbiotic bacteria of bumble bees and honeybees can protect their hosts against the virulent trypanosomatid *Crithidia bombi* (Koch & Schmid-Hempel, 2011). In this study, we observed a significant decrease in the relative abundance of nine bacterial genera in the abdomens of fruit flies parasitised by concealed early stages of *D. daci*, while

three bacterial genera were increased, suggesting that *D. daci* parasitisation could influence the relative abundance of bacterial taxa of the host fruit fly microbiome. Alternatively, the changes in relative abundance of bacterial taxa observed could simply be due to the presence of *D. daci* in the fruit fly abdomen without an actual change in the gut microbiome of the fruit fly host, since our study did not dissect guts of parasitised flies. Irrespective of this, concealed parasitisation with early stages of *D. daci* could lead to misinterpretation of bacterial communities in field collected specimens. For instance, studies have showed that parasitisation can led to the assignment of endosymbionts' infections to wrong hosts (Bohacsova et al. 2016; Plantard et al. 2012; Towett-Kirui et al. 2021). The relative abundance of *Wolbachi*a in fruit flies parasitised by *Wolbachia*-positive *D. daci* was low compared to the *Wolbachia* abundance in *D. daci* tissues, confirming that *D. daci* is the true *Wolbachia* host. Despite the low *Wolbachia* relative abundance, we still found a slight increase in the relative abundance of 11 bacterial genera in fruit fly abdomens parasitised by *D. daci* without detectable *Wolbachia* while the relative abundance of four bacterial genera in fruit fly abdomens were slightly increased. This suggests that parasitisation of field collected host fruit flies by *Wolbachia* infected or uninfected *D. daci* could also influence the interpretation of their microbiome.

4.6 Conclusion

Our study is the first report of a strepsipteran microbiome. We demonstrate that bacterial communities of *D. daci* are dominated by Proteobacteria. This dominance is attributed to the high relative abundance of *Wolbachia.* We showed that *D. daci* microbiome is small and distinct from the microbiome of its host fruit fly species, which could be attributed to the difference in host life cycles and histories as well as host phylogeny. We observed variability in the relative abundance of bacteria in fruit fly species, irrespective of parasitisation by *D. daci*, suggesting that host diet and possibly phylogeny also play a role in shaping bacterial communities. In addition, this study greatly extends knowledge about host-endoparasitoid-microbe interactions, an area yet to be explored. We show that *D. daci* parasitisation could impact the relative abundance of bacteria in microbial communities of host fruit fly species as well as highlight that concealed parasitisation of host fruit flies by *D. daci* could influence fruit fly microbiome studies. Additionally, our study shows that *Wolbachia* infection status of *D. daci* could lead to variability in host fruit fly microbiome.

Chapter 5

General discussion

5.1 Overview

Insects have diverse interactions with other organisms, and these interactions can involve individuals of two or more species. This PhD thesis focused on the host-parasite-endosymbiont relationship of tephritid fruit fly species, their *Dipterophagus daci* endoparasitoid and its two *Wolbachia* strains (ST-285 and ST-289) which previously had been assigned to the fruit flies as hosts. WGS of field-collected *Wolbachia*-positive fruit flies revealed the presence of almost complete mitogenomes of *D. daci* in addition to the fruit fly mitogenomes in the WGS libraries, suggesting a link between *Wolbachia* and the presence of *D. daci*. A PCR diagnostic tool developed based on the WGS data confirmed this link by detecting *D. daci* in the majority of *Wolbachia*-infected fruit flies. Localisation and quantification of the two *Wolbachia* strains in dissected *D. daci* and fruit fly tissues of stylopised flies showed their presence at high titres in the dissected *D. daci* tissues relative to the fruit fly tissues. These findings demonstrate that the presence of the two *Wolbachia* strains previously detected in field-collected Australian fruit flies was due to concealed parasitisation by *Wolbachia*-infected *D. daci* parasitoids.

As part of this study six *D. daci* and nine fruit fly mitogenomes were assembled from the WGS data. The *D. daci* mitogenomes were highly rearranged, while the fruit fly mitogenomes had the conserved patterns of the ancestral insect mitogenome arrangement. Genome rearrangements observed in *D. daci* belonging to the family of Halictophagidae were common with those seen in other Strepsiptera, most similar to those of two species of Xenidae, and less similar to the more distantly related and more basal Mengenillidae. This suggests that genome rearrangements in Strepsiptera could be linked to evolution to parasitism as the more derived taxa have evolved more extreme characteristics of endoparasitic life cycles than the more basal lineage. In addition to the rearrangements seen

in the more basal Mengenillidae and also in Xenidae, the *D. daci nad5* gene contains a nucleotide deletion that could cause a truncation of its product, but could also be compensated by -1 translational frameshifting (Beckenbach et al. 2005; Mindell et al. 1998), and it also has a rearrangement of the order of ribosomal RNA genes. Furthermore, the *D. daci* mitogenomes displayed low diversity compared to the polymorphic fruit fly mitogenomes, suggesting a possible influence of *Wolbachia* on *D. daci* mitogenome diversity, and, therefore, on *D. daci* reproduction and fitness.

This thesis has for the first time presented the microbiome of a strepsipteran species. We found that the *D. daci* microbiome has low diversity, was simple and distinct from that of its hosts. Endoparasitoids such as *D. daci* have a highly specialised life cycle that could result in a less diverse microbiome. Microbiome of *D. daci* was dominated by Proteobacteria, which was due to a dominance of *Wolbachia*. *Wolbachia* dominance in *D. daci* suggest that it could play a vital role in *D. daci* biology and fitness. However, this dominance was not observed in the abdomens of host fruit flies parasitised by early stages of *Wolbachia*-infected *D. daci*, another piece of evidence that *D. daci* is the true host of *Wolbachia*. Some variability was observed in the fruit fly microbiomes, and in particular, the microbiome of *Z. strigifinis* was interestingly distinct from that of the analysed *Bactrocera* species, suggesting that its different specialisation in host plant species and host plant utilisation could influence its microbiome.

5.2 Key findings and limitations

Dipterophagus daci as the true host of *Wolbachia* previously detected in Australian tephritids

This study revealed that *D. daci* is the true host of the two *Wolbachia* strains previously reported in nine out of 24 Australian tephritid fruit flies (Morrow et al. 2014, 2015). Strepsiptera parasitise diverse insect orders and they enter the host as first larval instar and remain endoparasitic, develop to the fourth larval instar and, in Stylopidia, can only be visible once the adult male and female strepsipterans extrude through the host cuticle (Drew & Allwood, 1985; Kathirithamby, 1989b, 2009; Noda et al. 2001). Therefore, larval stages of Strepsiptera can remain concealed in the host unless detected by PCR. The presence of almost complete *D. daci* mitogenomes, in addition to the expected fruit fly mitogenome in the WGS libraries of *Wolbachia*-infected male fruit flies which were not visibly stylopised, suggested a link between *Wolbachia* and the presence of *D. daci* (Towett-Kirui et al., 2021).This confirmed that the presence of *Wolbachia* in tephritid fruit flies was due to *D. daci* parasitisation. *Wolbachia* quantification and localisation performed on dissected *D. daci* pupae and tissues of stylopised male fruit flies showed the presence of *Wolbachia* in high titres in the dissected *D. daci* pupae (Towett-Kirui et al., 2021). These findings confirmed the link between *D. daci* and *Wolbachia* and provided evidence that *D. daci* is indeed the true host of the *Wolbachia* detected in tephritid fruit flies.

The detection of *Wolbachia* in fruit flies with concealed *D. daci* parasitisation, demonstrate that *Wolbachia* infections can potentially be assigned to a wrong host if the presence of the endoparasitoids remains unknown. Similarly, studies in *Ixodes ricinus* ticks revealed that the presence of *Wolbachia* and *Arsenophonous nasoniae* endosymbionts was due to concealed

parasitisation with an endoparasitoid wasp, *Ixodiphagus hookeri* (Bohacsova et al. 2016; Plantard et al. 2012; Tijsse-Klasen et al. 2011). Additionally, *Rickettsia*-like microorganisms reported in the Strepsiptera species *E. japonicus, E. tenuicornis, X. moutoni* and *X. vesparum* by electron microscopy (Kathirithamby, 1998), and in *E. tenuicornis* also by PCR (Noda et al. 2001) suggest that *Wolbachia* infections in Strepsiptera is common.

Possible role of *D. daci* in horizontal transmission of *Wolbachia*

The PCR screening of 64 fruit fly specimens revealed that three fruit fly individuals were PCR positive for *Wolbachia* without the detection of *D. daci* (Towett-Kirui et al., 2021). While this may be due to parasitisation of the flies by undetectable early stages of *D. daci*, this could also be attributed to the detection of *Wolbachia* infections in fruit flies, possibly acquired horizontally from *D. daci* perhaps as a consequence of failed parasitisation. Additionally, the detection of *Wolbachia* in head and thorax tissues of stylopised flies while these tissues were negative for *D. daci* could also suggest a possibility of somatic infections by *Wolbachia* as a consequence of *D. daci* parasitisation of the fruit fly abdomen. While this could be interpreted as horizontal transmission it is unlikely that such horizontally acquired *Wolbachia* is transmitted to the next generation of the fruit flies (but also see (Hughes et al. 2004)). It has previously been hypothesised, and, in some examples, also demonstrated that parasitoids may constitute possible routes for horizontal transmission of *Wolbachia* between hosts due to their close associations with their hosts (Duron et al. 2010; Vavre et al. 1999; West et al. 1998). Due to the sample collection method used in the present study, fresh specimens were not available for *Wolbachia* localisation using fluorescence in situ hybridization (FISH), however, such approaches would be required for the further characterisation and localisation of any such horizontally acquired *Wolbachia* in fruit flies.

New record of *Dipterophagus daci* fruit fly host species

Strepsiptera comprise 630 described species and they parasitise diverse insect orders (Kathirithamby, 1989b, 2009; Osborn 1969). In Diptera, *Dipterophagus daci* has been detected in 19 Dacini species using morphological identification (Allwood & Drew, 1996; Drew & Allwood, 1985). The current study detected *D. daci* in 13 fruit fly species, 10 of which have previously been reported as hosts, and three new hosts species (Towett-Kirui et al., 2021). These findings clearly show that *D. daci* interacts with many Australian tephritid fruit fly species. Additionally, the molecular technique developed in the current study can be applied in the detection of concealed *D. daci* in fruit fly species, and possibly of other strepsipterans in other insect hosts.

Phylogenetic placement *Dipterophagus daci* in Halictophagidae

Besides the undescribed strepsipteran species that have been found to parasitise platystomatid flies in Papua New Guinea, *D. daci* is so far the only described strepsipteran endoparasitoid of Diptera (Drew & Allwood, 1985). Previous descriptions of *D. daci* were entirely based on morphological characterisation and placed *D. daci* in the newly formed family Dipterophagidae (Allwood & Drew, 1996; Drew & Allwood 1985). However, *D. daci* was later moved in the family Halictophagidae (subfamily Dipterophaginae) based on features that *D. daci* shared with other members of this family (Kathirithamby, 1989b). The family Dipterophagidae was again reinstated based on the combination of features unique to *D. daci* (Allwood & Drew, 1996) but this placement was still questioned (Kathirithamby, 2018). The present study presents the first molecular characterisation of *D. daci.* The phylogenetic analysis based on *cox1*, *nad1* and *16S rRNA* and the *18S rRNA* genes placed *D. daci* within

the Halictophagidae family, hence supporting the proposed morphological placement of *D. daci* in Halictophagidae (Towett-Kirui et al. 2021). Further molecular data however will also be required to demonstrate even the validity of the subfamily Halictophaginae. Our data make the validity of this subfamily somewhat questionable because of the position of *D. daci* amongst *Halictophagus* species in the phylogenetic tree which further supports earlier recommendations that the genus *Halictophagus* is polyphyletic and needs revision (McMahon et al. 2009). For this, the collection and analysis of new molecular data from the strepsipteran endoparasitoid of platystomatid flies from Papua New Guinea and other halictophagids will be very important.

Dipterophagus daci mitogenome has low mtDNA variation and is highly rearranged

The successful assembly of six *D. daci* mitogenomes and nine fruit fly mitogenomes in Chapter 3, demonstrated that it is possible to obtain both the endoparasitoid's and host's mitogenomes from WGS libraries of parasitised individuals. This is useful for the study of interactions of insects with endoparasitic lifestyles. Results show that *D. daci* mitogenomes contain all the genes expected in an animal mitochondrial genome, however, comparisons with the patterns of the ancestral insect mitogenome reveal a highly rearranged gene order (Chapter 3). Some of the gene rearrangements observed in *D. daci* were similar to four other strepsipteran mitogenomes, *M. australiensis*, *M moldryzki*, *X. vesparum* and *X. moutoni*, while some rearrangements were unique to *D. daci*. These gene rearrangements observed in *D. daci* could be attributed to tandem duplication-random loss events or recombination (Cameron, 2014; Jühling et al. 2012; Negrisolo et al. 2004; San Mauro et al. 2006). Besides these, some mutations and gene rearrangements were unique to *D. daci*. This includes a single nucleotide -1 frameshift deletion in the coding region of the *nad5* gene resulting in a

new in-frame stop codon which could result in a small peptide terminated by the new stop codon, and a larger, but truncated, gene product initiated with a new in-frame start codon (Chapter 3). However, this larger *nad5* gene product could still be functional since the truncated gene product still included the important proton-conducting transporter domain. Furthermore, perhaps *D. daci* is still able to develop and live with a less effective gene product of *nad5*. Alternatively, this mutation in the gene sequence could be compensated by a -1 translational frameshifting (Beckenbach et al. 2005; Mindell et al. 1998), or complemented by the *D. daci* nuclear genome or *Wolbachia*. Another unique feature of the *D. daci* mitogenome is the changed order of the two ribosomal RNA genes. Further research will need to investigate what the *nad5* mutation and ribosomal RNA gene order changes mean for the function of the *D. daci* mitogenome.

It was hypothesised that the extensive gene rearrangements and the nucleotide deletion in *nad5* gene observed in *D. daci* is due to evolution to more pronounced levels of parasitism in this species, because this species is more derived and has a more complete endoparasitic life cycle than the more basal Mengenillidae.

Another interesting finding of this study was the low mtDNA variation observed between the six *D. daci* mitogenomes. The current study explored the mtDNA diversity using the PCGs of the six *D. daci* mitogenomes and PCGs of the fruit fly host. Results revealed that the mitochondrial PCGs of *D. daci* had low/no polymorphism, while the mitochondrial PCGs of fruit flies were highly polymorphic (Chapter 3). Additionally, results in (Towett-Kirui et al. 2021) showed genetic diversity in the *D. daci* nuclear *18S rRNA* gene, suggesting that the lower diversity only occurred in the *D. daci* mitogenomes. One of the reasons for this low genetic variation could be due to frequent founder events and bottle necks caused by the life history of *D. daci* (Kinziger et al. 2011; Wessel et al. 2013). Alternatively, and more likely, the observed low genetic variation could be due to *Wolbachia*. *Wolbachia* are maternally

transmitted and can be co-transmitted with the host mtDNA and in the process facilitate *Wolbachia*-driven hitchhiking of mitochondria (a type of a selective sweep) resulting in the reduction/loss of mitochondrial genetic diversity (Hurst & Jiggins, 2005; Turelli et al. 1992).

Dipterophagus daci microbiome is simple, not diverse and dominated by *Wolbachia*

This study aimed to explore the microbiome of *D. daci* (Chapter 4). To achieve this, 16S rDNA gene amplicon sequencing of *D. daci* male pupae and its fruit fly host species was performed. Results showed the presence of six main bacterial classes in *D. daci*, however, Alphaproteobacteria was dominant, due to the relatively high abundance of *Wolbachia*. Additionally, the microbiome diversity of the *D. daci* samples was not diverse and less diverse than that of the fruit fly hosts. The low microbiome diversity was attributed to *Wolbachia* dominance which could have suppressed other bacteria however this will require further experiments that quantify the relative differences between different bacterial taxa. Importantly, the *Wolbachia* dominance observed in *D. daci* was not observed in the fruit flies parasitised by concealed *Wolbachia*-positive *D. daci.* This corroborates the findings of (Towett-Kirui et al. 2021) that demonstrated that *D. daci* is the true host of *Wolbachia* in this host-endoparasitoid-endosymbiont system.

Distinct *Dipterophagus daci* microbiome

Microbiome data analysis of fruit flies parasitised by early stages of *Wolbachia*-positive *D. daci*, fruit flies parasitised by early stages of *Wolbachia*-negative *D. daci* and fruit flies without *D. daci*, revealed that *D. daci* microbiome is distinct from that of its host fruit fly species. Based on previous studies, several factors can shape the host microbiome, for

instance, species identity, host phylogeny, host life cycle, host diet and ecology (Morrow et al. 2015; Reese & Dunn, 2018; Yun et al. 2014). The present study shows that factors such as host phylogenetic history and host life cycle could contribute to the distinct *D. daci* microbiome. *Dipterophagus daci* pupae are fully endoparasitic in their hosts and they depend exclusively on the host for nourishment. Therefore, this exclusive dependence on the host means that *D. daci* pupae have a restricted diet which could lead to the low microbiome diversity observed. Similarly, the distinct *D. daci* microbiome could also be attributed to large phylogenetic distance between *D. daci* and the host fruit fly species.

Variable microbiome in *Dipterophagus daci* parasitised host fruit fly microbiome

Interactions between hosts and parasites can be modulated by the microbes associated with either the host or the parasite (Cavichiolli de Oliveira & Cônsoli, 2020; Dheilly et al. 2015; Fredensborg et al. 2020; Koch & Schmid-Hempel, 2011). The 16S rRNA gene amplicon sequencing data demonstrated that early parasitisation resulted in a significant decrease in the relative abundance of bacteria from nine genera and an increase in bacteria from three genera in the host fruit fly abdomen microbiome. This could suggest that *D. daci* has influence over the growth and diversity of the microbiome of the fruit flies, however, the limitation of this study is that here, the guts of parasitised flies were not dissected, and instead entire parasitised abdomens were analysed. Therefore, the relative increase and decrease of bacterial genera in host fruit flies could also, and perhaps only, be due to the presence of *D. daci* in fruit fly abdomens. Irrespective of this, however, the study shows that microbiome studies of field-caught fruit flies that do not investigate the parasitisation status of the fly could be impacted by concealed *D. daci* parasitisation.

Variable microbial abundance in fruit flies

The current study found variability in the relative abundance (structure) but not composition (diversity) of microbial communities of *Z. strigifinis* from that of the *Bactrocera* fruit fly species (Chapter 4). This variability was caused by difference in abundance of one unknown Pasteurellales and *Acinetobacter* bacterial taxa. This variability could possibly be attributed to host plant and the infested host plant part because *Z. strigifinis* is pest of Cucurbitaceae flowers while the *Bactrocera* species are pests of fruits.

The microbiome variability observed within the *Bactrocera* species, but not between the two sibling species *B. tryoni* and *B. neohumeralis* suggests that host phylogeny influences microbiome diversity. Furthermore, this study used genomic extracts from fruit fly abdomens, therefore our findings may not be directly comparable to previous studies that used other fruit fly tissues such as dissected gut or entire flies (Deutscher et al. 2019; Morrow et al. 2015; Woruba et al. 2019).

5.3 Future research directions

There is a great scope in the study of the interactions between *Wolbachia* and *D. daci* in tephritid fruit flies. The samples used in the current study were stylopised flies collected using cue-lure and other lures (e.g. methyl eugenol) which only attract male flies. Therefore there are a couple of limitations. An effort should be made to investigate interactions in female flies as it is female flies which would have to transmit any horizontally acquired *Wolbachia* to a next generation of flies. Another limitation is the use of trap collected dead flies which are unsuitable for tissue localisation studies using molecular assays such as Fluorescence in situ hybridization (FISH) which require fresh specimens. FISH technique is

ideal in the visualisation of *Wolbachia* in *D. daci* and fruit fly tissues in situ. This could provide information on the horizontal transmission events of *Wolbachia* from *D. daci* to fruit flies. Additionally, live stylopised fruit flies can be collected and used to establish the laboratory cultures of *D. daci* parasitised flies. This will be useful for the study of *D. daci* life cycle, behavioural and fitness effects of *D. daci* parasitisation on the fruit fly hosts. The *D. daci* parasitised fly cultures can also be used to assess *Wolbachia* transmission and any phenotypic and fitness effects of *Wolbachia* on *D. daci*.

The present study has developed a molecular diagnostic technique that can be applied for an extensive screening of tephritid fruit fly samples to determine the prevalence of *D. daci* in populations of Australian tephritid fruit flies across sites and seasons. Similarly, there is need for an extensive screening of *D. daci* for *Wolbachia* in order to explore any underlying patterns of infections. The seasonal abundance and distribution of *D. daci* has previously been assessed using detection of stylopised flies collected from traps (Allwood & Drew, 1996), however, the developed molecular technique can be applied in order to detect flies that come to traps with concealed parasitisation and therefore also destined to die because of parasitisation. This means that so far, the parasitisation rate with *D. daci* has likely been underestimated and *D. daci* may have a stronger effect on fruit fly population dynamics than previously appreciated.

Results of *D. daci* mitogenome studies suggest that *D. daci* molecular architecture is unique. Therefore, there is need to perform further molecular characterisation of *D. daci*, for instance, exploring the nuclear genes of *D. daci*. This will be vital for further genetic characterisation of *D. daci*. The availability of *D. daci* DNA extracts provide an opportunity to sequence the whole genome of *D. daci*.

Furthermore, the current study found low mtDNA diversity in six *D. daci* mitogenomes. However, to further understand the relationship between *Wolbachia* and *D. daci* then further

research will be required to assess *D. daci* haplotype diversity in a large sample set of *Wolbachia*-positive and *Wolbachia*-negative *D. daci*.

Microbe interaction with their host is vital in understanding aspects of host biology. This study showed that *Wolbachia* is dominant in the *D. daci* microbiome, suggesting that *Wolbachia* could have a vital role in *D. daci*. Future studies should therefore investigate what the potential fitness benefits of both *Wolbachia* and other microbes in *D. daci* would be. This could include investigating the functional processes carried out by the specific microbes. Additionally, the characterisation of the microbial communities of *D. daci* from different climatic ranges can be performed to investigate the effects of host environment on *D. daci* microbiome. Additionally, the variability detected between *Z. strigifinis* and the *Bactrocera* fruit fly species suggest that there could be a host genus effect on the bacterial communities. Future studies could investigate this hypothesis by incorporating a good replicate of samples of different *Zeugodacus* and *Bactrocera* species.

5.4 Concluding remarks

This thesis has for the first time applied molecular techniques to detected *D. daci* in tephritid fruit flies. This study provides evidence that *D. daci* is the true host of *Wolbachia* previously found in tephritid fruit flies. It demonstrates that *Wolbachia* infections of a concealed strepsipteran host could lead to the assignment of detected *Wolbachia* to a wrong host. These findings show that caution should be taken in *Wolbachia* surveys of insect host communities and concealed parasitisation should be investigated. This study also presents for the first time the microbiome of a strepsipteran. It also explores the interaction between *D. daci* and its fruit fly hosts and highlights the impacts of parasitisation on the fruit flies. In general, the

findings from this study give insights on the biology of fruit fly host species and their interaction with *D. daci* and *Wolbachia*.

Finally, results from this study have provided molecular data that can be used to study the evolution of *D. daci* as well as additional information that can be used in phylogenetic studies of Strepsiptera.

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Appendices

Appendix A

Table A. 1: *Dipterophagus daci* and tephritid fruit fly tissues dissected from 23 stylopised male fruit flies collected from localities in central and northern Queensland and screened for *D. daci cox1* and fruit fly *cox1 gene* specific primers. Table also shows the qPCR mean quantification cycle (Cq) values for wsp normalised to the scarlet gene in fruit fly tissues and to the *RNA polymerase II* gene in *D. daci* samples for two *Wolbachia* strains (ST289 and ST285). Tephritid fruit flies had different types of parasitisation: (1) flies stylopised by *D. daci* male/s only, and the male/s has/have emerged and left (extrusion empty); (2) flies stylopised by one *D. daci*, either a female or male pupa; (3) flies stylopised by more than one *D. daci* individuals.

Species	**Sample ID**	**Collection Locality**	**Sample**	***D. daci cox1***	**Fly *cox1***	**Cq ST289**	**normalised mean ± sd 2^- (Cq Target/Cq Ref) ST289**		**Cq ST285**	**normalised mean ± sd 2^- (Cq Target/Cq Ref) ST285**		**Parasitisation type**
Bactrocera abscondita	Babsc_CN10	Cairns	Fly abdomen	y	y	21.41	0.54643643	± 0.05081321	19.33	2.30571883	± 0.05649934	1
			Fly head	n	y	28.82	0.30125811	± 0.1895289	24.92	4.14019422	± 1.35488488	1
			Fly thorax	n	y	27.725	0.32095771	± 0.1878102	22.765	9.18650443	± 1.83372983	1
Bactrocera breviaculeus	Bbrev_CN09	Cairns	Fly abdomen	y	y	22.875	0.11582351	± 0	18.415	2.5496724	± 0.07496931	1
			Fly head	n	y	27.705	0.00620706	± 0.0005772	22.325	0.2594108	± 0.03926392	1
			Fly thorax	n	y	28.975	0.00419267	± 0.00156492	21.415	0.76358796	± 0.03741069	1
Bactrocera breviaculeus	Bbrev_CN09.2	Cairns	Fly abdomen	y	y	26.925	5.29947405	± 0.67350894	24.645	26.6258477	± 10.180541	1
			Fly head	n	y	25.765	1.45960899	± 0.74469092	24.865	2.71698102	± 1.36297601	1
			Fly thorax	n	y	25.27	0.86478203	± 0.10569934	24.09	1.99301519	± 0.56843214	1
Bactrocera breviaculeus	Bbrev_CN14	Cairns	Fly abdomen	y	y	18.93	4.4102678	± 0.21607354	19.1	3.91852824	± 0.11521847	1
			Fly head	n	y	24.375	0.24839154	± 0.08702418	24.225	0.26971656	± 0.05383847	1
			Fly thorax	n	y	23.19	0.31100478	± 0.00152432	22.43	0.5267596	± 0.01290772	1
Bactrocera frauenfeldi	Bfra_CN4	Cairns	Fly abdomen	y	y	16.485	1.06074507	± 0.0155965	16.32	1.18992138	± 0.05829817	1
			Fly head	n	y	19.57	0.15442826	± 0.00075689	18.215	0.39562814	± 0.03099366	1
			Fly thorax	n	y	19.285	0.15395221	± 0.00603497	17.46	0.54541433	± 0.01870897	1
Bactrocera neohumeralis	Bn_AB1	Airlie Beach	Fly abdomen	y	y	21.54	4.23159035	± 0.74279885	20.27	10.141625	± 0.79449868	1
			Fly head	n	y	25.37	4.34901277	± 1.52368011	23.68	14.5765959	± 7.4369634	1
			Fly thorax	n	y	26.455	0.7307561	± 0.45107839	23.965	3.87874677	± 1.67482429	1
Bactrocera tryoni	Bt_CN14.3	Cairns	Fly abdomen	y	y	27.6	0.00038205	± 0.00019979	14.955	2.28359547	± 0.3013203	1
			Fly head	n	y	28.46	0.00030804	± 1.0567E-05	14.53	4.80726588	± 0.11779725	1
			Fly thorax	n	y	27.185	0.00075794	± 0.0004186	15.62	2.11408687	± 0.02072318	1

Bactrocera frauenfeldi	Bfra_CN2	Cairns	*D. daci* pupa	y	n	29.31	0.11542535	± 0.05289822	20.85	38.6744927	± 5.85369735	2
			Fly abdomen	y	y	29.31	0.00171055	± 0.00075378	22.47	0.18728542	± 0.02834716	2
			Fly head	n	y	25.9	0.0835119	± 0.00776578	23.39	0.47467391	± 0.00232651	2
			Fly thorax	n	y	29.31	0.30496514	± 0.33184377	24.285	7.57347366	± 5.85372962	2
Bactrocera neohumeralis	Bn_CN14	Cairns	*D. daci* male	y	n	18.5	3.21774083	± 0.17339792	17.64	5.83639683	± 0.08581454	2
			Fly abdomen	y	y	16.315	1.04246576	± 0	15.805	1.48484454	± 0.04365964	2
			Fly head	n	y	24.04	0.00938928	± 0.00191929	17.22	1.05133098	± 0.08236166	2
			Fly thorax	n	y	20.4	0.15004456	± 0.01468473	17.31	1.27606122	± 0.08749221	2
Bactrocera neohumeralis	Bn_CN16	Cairns	*D. daci* female	y	y	14.185	13.7489287	± 0.80818405	14.115	14.4785898	± 1.84008064	2
			Fly abdomen	y	y	15.125	3.25096103	± 0.15927529	15.605	2.33304953	± 0.18277197	2
			Fly head	n	y	18.325	0.48036966	± 0.03763234	17.37	0.92994455	± 0.02278736	2
			Fly thorax	n	y	18	0.51945763	± 0.00763776	16.835	1.16498542	± 0.03425466	2
Bactrocera neohumeralis	Bn_CN16.2	Cairns	*D. daci* pupa	y	n	16.04	8.0840485	± 0.11886253	15.645	10.6666	± 0.14769315	2
			Fly abdomen	y	y	16.04	1.34736304	± 0.02641358	15.645	1.77461067	± 0.14769315	2
			Fly head	n	y	23.41	0.01214772	± 0.00219097	19.26	0.21405421	± 0.01153497	2
			Fly thorax	n	y	22.015	0.02881677	± 0.00862451	18.735	0.27358	± 0.00268175	2
Bactrocera tryoni	Bt_CN14.2	Cairns	*D. daci* pupa	y	n	29.53	1.34677821	± 1.87959858	25.51	28.4428255	± 4.71800646	2
			Fly abdomen	y	y	22.71	0.40753191	± 0.04782813	21.065	1.27021964	± 0.01867647	2
			Fly head	n	y	22.67	0.33542346	± 0.07659216	21.835	0.59562463	± 0.11029757	2
			Fly thorax	n	y	26.435	5.91422645	± 0.63648862	24.975	0.07628634	± 0.06051903	2
Bactrocera tryoni	Bt_MA7	Mackay	*D. daci* female	y	y	17.69	28.769411	± 1.83185487	17.9	24.8901418	± 2.0714986	2
			Fly abdomen	y	y	20.22	17.3667586	± 3.88266025	20.68	12.4678312	± 0.2444182	2
			Fly head	n	y	22.78	4.42277762	± 1.2614299	20.79	17.4990232	± 4.49525046	2
			Fly thorax	n	y	23.795	1.75692492	± 0.52582645	22.69	3.70153987	± 0.34420678	2
Bactrocera tryoni	Bt_TV1.2	Townsville	*D. daci* pupa	y	n	19.785	12.5268224	± 0.92013522	19.265	18.0707927	± 3.08484212	2
			Fly abdomen	y	y	20.385	2.0994838	± 0.02058003	19.93	2.87788444	± 0.01410529	2
			Fly head	n	y	26.77	2.87121233	± 1.3284269	25.145	8.36993604	± 0.20509692	2
			Fly thorax	n	y	26.37	7.73417445	± 0.45462717	25.375	22.3708729	± 7.21704448	2
Bactrocera aeroginosa	Baer_CN11	Cairns	*D. daci* female	y	y	16.965	1.34393539	± 0.08557334	17.39	35.2685875	± 1.03702014	3
			D. daci female2	y	y	18.025	23.1843276	± 0.34088711	18.885	12.7820571	± 0.68880069	3
			Fly abdomen	y	y	18.09	0.56874316	± 0.07228144	13.895	10.3797637	± 0.45771972	3
			Fly head	n	y	23.7	0.01167879	± 0.00011448	13.58	13.0488357	± 1.65837352	3

Bactrocera breviaculeus	Bbrev_AB1	Airlie Beach	Fly thorax	n	y	22.23	0.02079264	± 0.0016289	12.71	15.2455035	± 0.44827126	3
			D. daci female	y	y	20.75	40.2608885	± 5.31242202	19.465	97.8308069	± 7.66410179	3
			D. daci pupa	y	n	23.33	5.39942368	± 0.47573764	22.76	8.01557173	± 0.70624373	3
			Fly abdomen	y	y	19.02	14.932755	± 1.82517936	17.795	60.8981541	± 29.2370638	3
			Fly head	n	y	24.08	0.49311392	± 0.08179619	22.105	1.92936429	± 0.17941189	3
Bactrocera breviaculeus	Bbrev_CN14.2	Cairns	Fly thorax	n	y	25.23	2.86927838	± 1.00525398	23.435	10.1409793	± 4.42384879	3
			D. daci female	y	y	18.36	0.87107344	± 0.04267676	17.195	1.95404512	± 0.12442128	3
			D. daci pupa	y	n	19.37	6.27682357	± 0.06152809	19.33	6.45375417	± 0.1265188	3
			Fly abdomen	y	y	17.39	1.12573422	± 0.05515343	16.49	2.10351984	± 0.18533896	3
			Fly head	n	y	26.65	0.00156001	± 0.00057516	15.475	3.48228591	± 0.03413484	3
			Fly thorax	n	y	23.92	0.00867601	± 0.00050999	16.805	1.20222064	± 0.0530147	3
Bactrocera mayi	Bma_AB1	Airlie Beach	*D. daci* pupa	y	n	24.465	5.67661798	± 2.74991849	23.18	13.4494665	± 4.89717002	3
			D. daci pupa2	y	n	21.66	4.29022238	± 1.14271302	20.88	7.23716423	± 0.24825142	3
			Fly abdomen	y	y	23.02	0.94292609	± 0.02310546	19.525	10.6386804	± 0.62535868	3
			Fly head	n	y	24.705	2.19495214	± 0.23622059	22.42	10.6849078	± 0.88925855	3
			Fly thorax	n	y	24.77	1.63215968	± 0.22329083	22.695	6.9605557	± 1.78806787	3
Bactrocera neohumeralis	Bn_CK1	Cooktown	*D. daci* pupa	y	n	19.21	33.4102051	± 2.61736789	19.33	30.9098101	± 5.1272221	3
			D. daci pupa2	y	n	23.07	17.6372588	± 0.69138551	22.42	27.6891201	± 1.62761084	3
			Fly abdomen	y	y	19.525	2.40444488	± 0.57197143	19.43	2.53206053	± 0.07445146	3
			Fly head	n	y	24.29	0.51943267	± 0.00254588	21.825	2.88142448	± 0.39419897	3
			Fly thorax	n	y	25.41	6.61270722	± 2.94322121	24.395	12.6907172	± 0.55962657	3
Bactrocera neohumeralis	Bn_CN2	Cairns	*D. daci* female	y	y	18.35	15.9608301	± 1.01628512	26.26	0.0755799	± 0.05133345	3
			D. daci male	y	n	23.06	3.28864048	± 1.28725431	21.01	13.2834069	± 3.22309496	3
			Fly abdomen	y	y	27.255	3.48218508	± 0.57761391	26.205	7.33705905	± 2.26449258	3
			Fly head	n	y	23.13	0.91737779	± 0.1566044	21.94	2.07982736	± 0.13243031	3
			Fly thorax	n	y	22.94	2.6703012	± 0.72385776	22.125	4.70747262	± 1.34262837	3
Bactrocera tryoni	Bt_TV1	Townsville	*D. daci* female	y	y	17.695	8.19768524	± 0.20087609	16.71	16.2268594	± 0.47712656	3
			D. daci pupa	y	n	20.59	3.57133577	± 0.22740017	20.51	3.77170436	± 0.09242185	3
			Fly abdomen	y	y	20.355	2.00753412	± 0.06886305	20.385	1.96829792	± 0.14457779	3
			Fly head	n	y	21.47	0.70955426	± 0.08327361	20.465	1.41933643	± 0.03477942	3
			Fly thorax	n	y	21.735	0.96329403	± 0.05191008	20.715	1.95216903	± 0.02870341	3
Bactrocera pallida	Bpal_CN02	Cairns	*D. daci* female	y	y	17.165	2.88175682	± 0.21167427	17.34	2.5530414	± 0.20000621	3

			D. daci female2	y	y	17.165	2.5501011	± 0.09996468	16.08	5.41937158	± 0.50394822	3
			D. daci female3	y	y	15.62	7.23855485	± 0.31920084	16.045	5.40460613	± 0.58164332	3
			D. daci pupa	y	n	17.84	3.59255107	± 0.01760807	25.915	0.0142666	± 0.00721791	3
			D. daci pupa2	y	n	19.36	1.59668307	± 0.02347658	18.185	3.63309722	± 0.63774142	3
			D. daci pupa3	y	n	19.35	3.98618499	± 0.01953737	18.78	5.97168691	± 1.13458902	3
			Fly abdomen	y	y	14.785	5.83611643	± 0.02860439	14.955	5.19860871	± 0.48341944	3
			Fly head	n	y	18.94	0.22539922	± 0.00883571	13.475	9.9736776	± 0.92745385	3
			Fly thorax	n	y	19.14	0.15129377	± 0.00518973	15.99	1.34322566	± 0.05923265	3
Bactrocera tryoni	Bt_CN14	Cairns	*D. daci* female	y	y	18.24	8.52805614	± 0.66809109	18.45	7.37583012	± 0.64987676	3
			D. daci pupa	y	n	21.235	9.48763252	± 0.51127043	21.37	8.66370978	± 1.01677686	3
			D. daci pupa2	y	n	20.23	4.61334379	± 1.17799988	20.81	0	± 0	3
			Fly abdomen	y	y	18.865	5.79663883	± 0.14204084	19.45	3.8645807	± 0.11363222	3
			Fly head	n	y	23.18	0.19031534	± 0.01211808	18.675	4.31701665	± 0.04231723	3
			Fly thorax	n	y	22.54	0.21201187	± 0.01660909	20.13	1.12516659	± 0.02205766	3

Table A. 2: PCR and qPCR primers used in this study.

Organism/locus	Primer name	Primer sequence (5'->3')	Reference
	Standard PCR		
D. daci cox1	Strep1189_F	GCAGGATGAACTWTMTARCCYCC	This study
	Strep1691_R	ATRTGRTGARCTCAAACRAKW	This study
Wolbachia wsp	81F	TGGTCCAATAAGTGATGAAGAAAC	Braig et al. (1998)
	691R	AAA AAT TAA ACG CTA CTC CA	Braig et al. (1998)
Wolbachia 16S rRNA gene	WspecF	CATACCTATTCGAAGGGATAG	Werren & Windsor (2000)
	WspecR	AGCTTCGAGTGAAACCAATTC	Werren & Windsor (2000)
Fruit fly *cox1*	Pat	TCCATTGCACTAATCTGCCATATTA	Simon et al. (1994)
	Dick	CCTACAGGAATTAAAATTTTTAGATGATTA	Simon et al. (1994)
	qPCR		
D. daci RNA Pol II	StrepRpol243	CTTGTGTGGGGCAGCAGAA	This study
	StrepRpol342	GATTCCGGTCCGTAGTCGTC	This study
Wolbachia wsp	wsp11_F	CTCCGGAAGTCAAACTTTATGCTGG	Morrow et al. (2014)
	wsp11_R	TGTCTTTGCCTGCAGCAGCATCTT	Morrow et al. (2014)
Wolbachia wsp	wsp661_F	GCTACGACGTAACTCCAGAAATCAAA	Morrow et al. (2014)
	wsp661_R	TTTACCTGCCGCACCAGCAGTTT	Morrow et al. (2014)
Fruit fly *scarlet*	scarBt_F	GCC ACATTCTTCGCCTTCAGCATA	Morrow et al. (2014)
	scarBt_R	TAATCGACGGGCACCAAATAAGCC	Morrow et al. (2014)

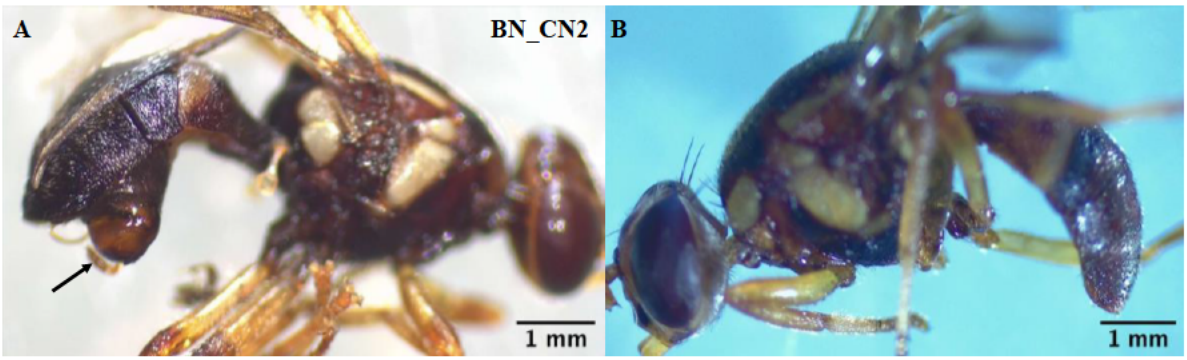

Figure A. 1: Field-caught male tephritid fruit flies collected using male lure traps, dried and then stored in ethanol; (A) stylopised (B) non-stylopised (*B. bryoniae*). Included is the specimen code, and arrow shows the elongated aedeagus in the stylopised fly.

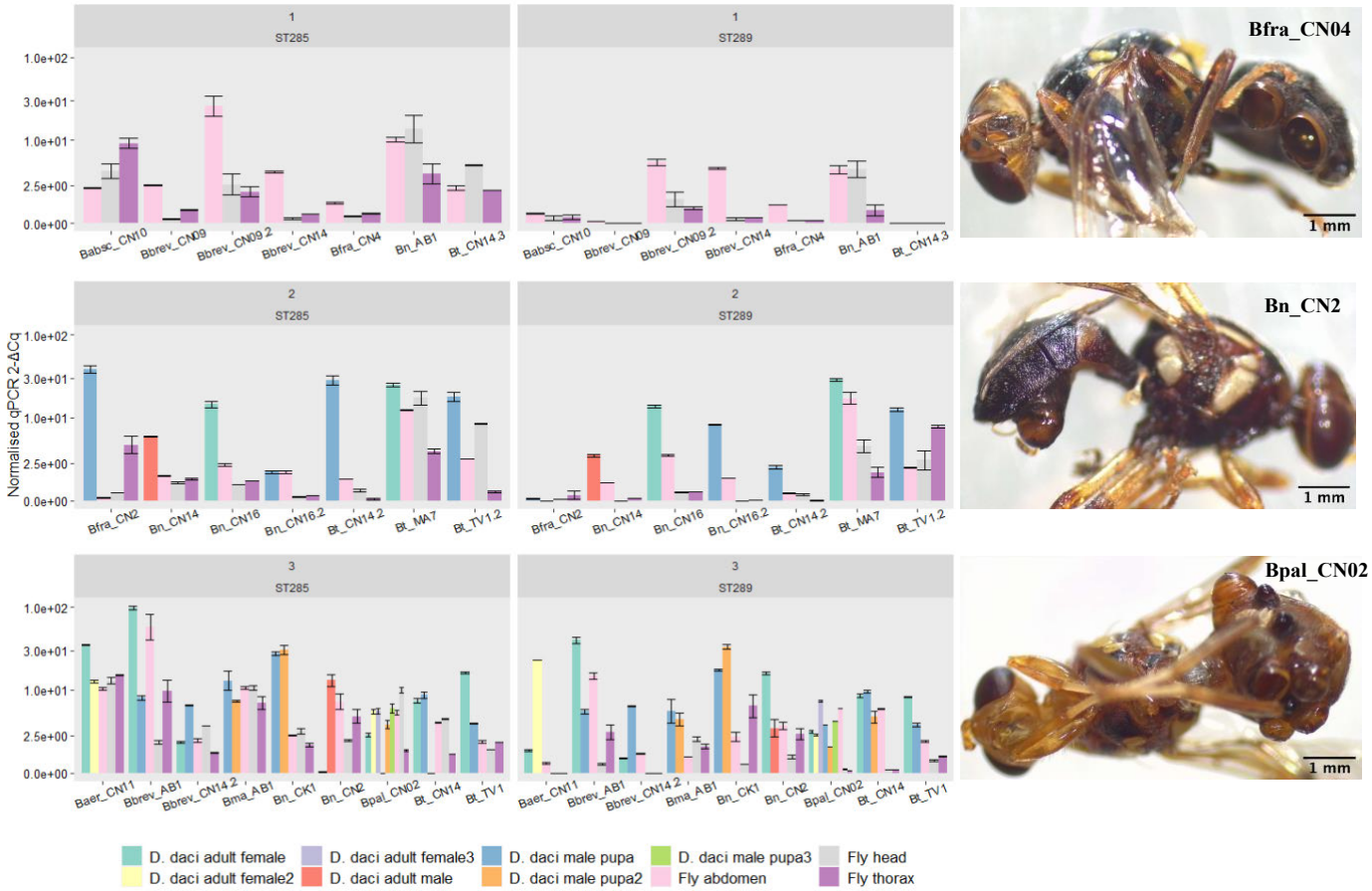

Figure A. 2: Relative titre and localisation of the two *Wolbachia* strains ST-289 and ST-285 in dissected insect tissues using qPCR. Dissected samples were obtained from tephritid flies with different types of parasitisation denoted by numbers in the barplot rows: (1) flies stylopised by *Dipterophagus daci* male/s only, and the male/s has/have emerged and left (extrusion empty); (2) flies stylopised by one *D. daci*, either a female or male pupa; (3) flies stylopised by more than one *D. daci* individuals. Fruit fly images with insect specimen codes illustrate the level of parasitisation. Numbers in inset legend were assigned randomly and refer to *D. daci* individuals dissected from each fly. The values are mean quantification cycle (Cq) values for *wsp* normalised to the scarlet gene in fruit fly tissues and to the *RNA polymerase II* gene in *D. daci* samples for the two *Wolbachia* strains**.**

Table A. 3: Taxon and sequence GenBank accession numbers for reference taxa used in the phylogenetic analyses.

TAXON	*cox1*	*nad1*	*16S rRNA*	*18S rRNA*
Outgroup				
Tribolium castaneum	AJ312413.2	AJ312413.2	AJ312413.2	HM156711.1
Mengenillidae				
Mengenilla australiensis	GU188852.1	GU188852.1	GU188852.1	JN082886.1
Mengenilla chobauti	JN082786.1	JN082825.1	JN082858.1	JN082887.1
Corioxenidae				
Corioxenos acucyrtophallus	JN082791.1	JN082830.1	JN082862.1	JN082893.1
Triozocera sp. 1	JN082787.1	JN082826.1	-	JN082888.1
Triozocera sp. 2	JN082788.1	JN082827.1	JN082859.1	JN082889.1
Triozocera sp. 3	JN082789.1	JN082828.1	JN082860.1	JN082890.1
Triozocera sp. 4	JN082790.1	JN082829.1	JN082861.1	JN082891.1
Myrmecolacidae				
Caenocholax sp. 1	JN082802.1	JN082838.1	-	JN082904.1
Caenocholax sp. 10	JN082804.1	JN082840.1	-	JN082906.1
Caenocholax sp. 3	JN082803.1	JN082839.1	JN082869.1	JN082905.1
Myrmecolax incautus	JN082796.1	JN082835.1		JN082898.1
Myrmecolax sp. 1	JN082797.1	-	JN082864.1	JN082899.1
Myrmecolax sp. 2	JN082798.1	-	JN082865.1	JN082900.1
Myrmecolax sp. 3	JN082799.1	-	-	JN082901.1
Myrmecolax sp. 4	JN082800.1	JN082836.1	JN082866.1	JN082902.1
Myrmecolax sp. 5	JN082801.1	JN082837.1	JN082867.1	JN082903.1
Stichotrema sp.	JN082795.1	JN082834.1	-	JN082897.1
Elenchidae				
Elenchus koebelei	JN082824.1	JN082857.1	JN082884.1	JN082922.1
Elenchus sp. 2	JN082823.1	JN082856.1	JN082885.1	-
Elenchus sp. 1	JN082822.1	JN082855.1	-	JN082920.1
Elenchus tenuicornis	JN082820.1	-	-	-
Elenchus varleyi	JN082821.1	JN082854.1	-	JN082921.1
Lychnocolax				
Lychnocolax sp. 1	JN082792.1	JN082831.1	-	JN082894.1
Lychnocolax sp. 2	JN082793.1	JN082832.1	JN082870.1	JN082895.1
Xenidae				
Paraxenos sp.	JN082810.1	JN082844.1	JN082876.1	JN082911.1
Pseudoxenos sp	JN082811.1	JN082845.1	JN082877.1	JN082912.1
Xenos hamiltoni	JN082807.1	-	JN082871.1	-
Xenos pecki	JN082808.1	JN082843.1	JN082874.1	JN082909.1
Xenos moutoni	JN082805.1	JN082841.1	JN082872.1	JN082907.1
Xenos sp.	JN082809.1	-	JN082875.1	JN082910.1
Xenos vesparum	JN082806.1	JN082842.1	JN082873.1	JN082908.1
Stylopidae				
Stylops melittae	JN082812.1	JN082846.1	JN082878.1	JN082913.1
Halictophagidae				
Halictophagus sp. 1	JN082815.1	JN082849.1	-	-
Halictophagus sp. 2	JN082816.1	JN082850.1	JN082883.1	JN082917.1
Halictophagus sp. 3	JN082817.1	JN082851.1	JN082881.1	JN082918.1
Tridactylophagus sp	JN082813.1	JN082847.1	JN082879.1	JN082914.1
Callipharixenos sp.	JN082819.1	JN082853.1	-	-
Halictophagus calcaratus	JN082814.1	JN082848.1	JN082880.1	JN082915.1
Halictophagus silwoodensis	JN082818.1	JN082852	JN082882.1	JN082916

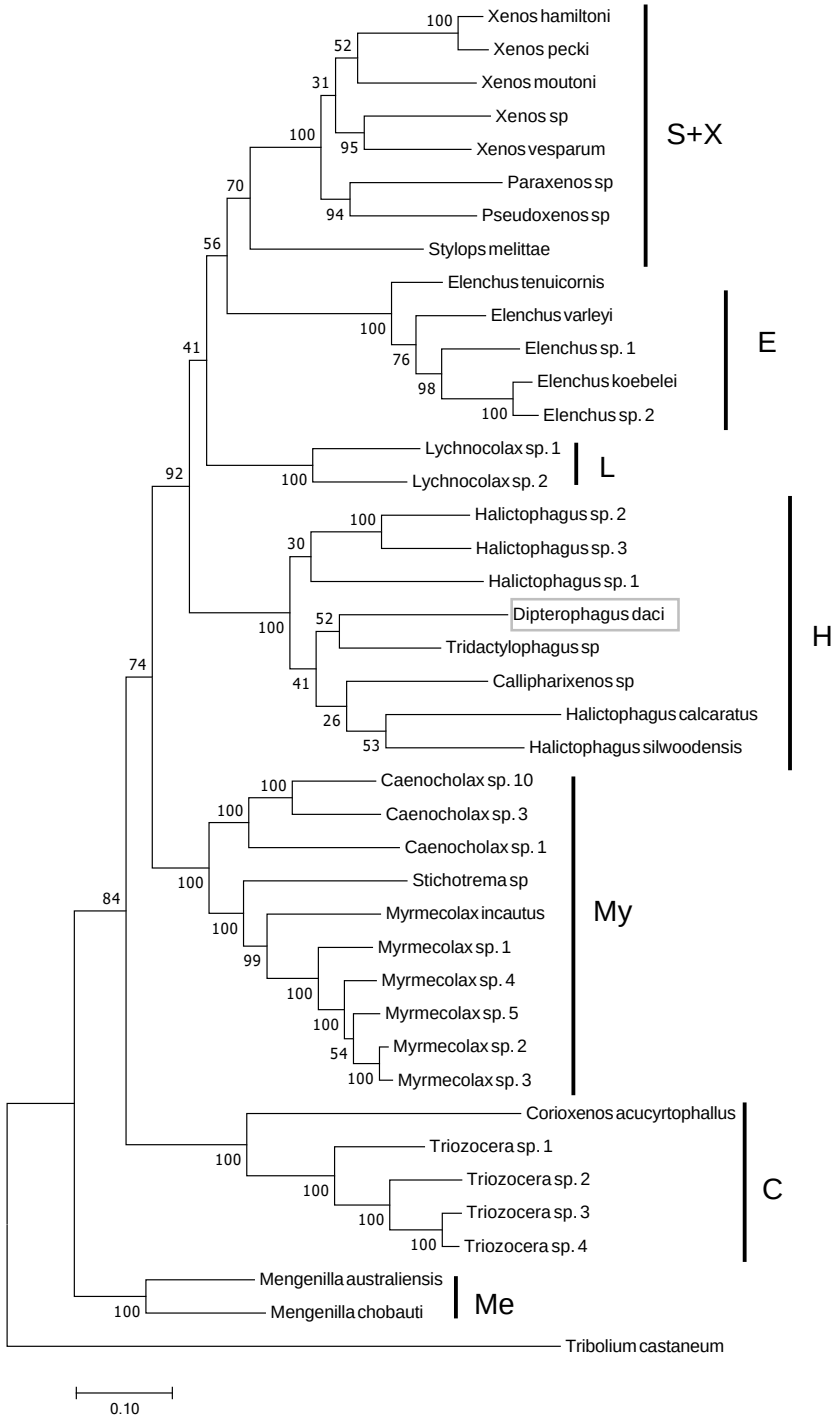

Figure A. 3: Maximum likelihood phylogenetic tree based on concatenated mitochondrial *cox1*, *nad1*, *16S rRNA* and nuclear *18S rRNA* gene alignments. The tree includes representative species of eight strepsipteran families as indicated by the vertical bars on the right: Mengenillidae (Me); Corioxenidae (C); Myrmecolacidae (My); Lychnocolacidae (L); Stylopidae (S); Xenidae (X); Elenchinidae (E); Halictophagidae (H). The reference sequences were obtained from NCBI GenBank. Branch labels indicate bootstrap values (100 replicates), and the scale bar shows the number of substitutions per site. The tree was rooted with *Tribolium castaneum* as an outgroup. The maximum likelihood tree topology is the same as the Bayesian tree (Figure 2. 4).

Table A. 4: Single nucleotide polymorphisms (SNPs) in the protein coding genes (PCGs) of six *Dipterophagus daci* mitogenomes (Bfra485, Bn171, Bn342, Btry194, Btry210, Zst503) and (Bn240 partial mitogenome with low coverage that did not allow assembly of the mitogenome, but allowed for SNP calling) and SNPs and indels in the nuclear *D. daci 18S rRNA* gene of four WGS libraries (Bfra 485, Bn 342, Btry194, Btry210).

PCGs					*18S rRNA* gene			
Mitogenome consensus position	Gene	Allele	Polymorphism type	Variant frequency	Gene consensus position	Allele	Polymorphism type	Variant frequency
1762	*cox1*	C->T	SNP	14.2%	108	T	Indel	75%
2546		A->G	SNP	14.2%	381	C->T	SNP	50%
6408	*nad5*	G->A	SNP	28.5%	522	A->G	SNP	50%
6607		G->A	SNP	14.2%	607	C->T	SNP	50%
6912		T->C	SNP	14.2%	658	C->T	SNP	75%
7306		C->G	SNP	14.2%	688	A->G	SNP	25%
7869	*nad4*	T->C	SNP	14.2%	691	C->T	SNP	50%
8640		G->A	SNP	14.2%	702	C->T	SNP	50%
10276	*cob*	A->C	SNP	14.2%	707	A->G	SNP	75%
11,033		A->G	SNP	14.2%	709	C->T	SNP	25%
					710	A->T	SNP	25%
					793	C->T	SNP	50%
					794	A->G	SNP	50%
					799	C->T	SNP	50%
					803	A->T	SNP	50%
					849	A->T	SNP	50%
					854	A->G	SNP	50%
					864	A->G	SNP	50%
					873	G->T	SNP	50%

Appendix B

Table B. 1: Summary of the *Dipterophagus daci* samples used for amplification and sequencing of *nad5* gene. Table shows the sample, sample ID, collection locality and collection year.

Sample	Sample ID	Collection locality	Collection year
Dipterophagus daci male	Dd45	Cairns	2019/2020
Dipterophagus daci male pupa	Dd10	Cairns	2019/2020
Dipterophagus daci male pupa	Dd57	Cairns	2019/2020
Dipterophagus daci male pupa	Dd55	Airlie Beach	2019/2020
Dipterophagus daci male pupa	Dd1	Townsville	2019/2020

Table B. 1: Annotation of a representative *Dipterophagus daci* mitogenome (*Dipterophagus daci*_Bfra485) and tephritid fruit fly mitogenomes. Table shows, genes, gene location, gene length, intergenic sequences and protein-coding genes' start and stop codons; + indicates genes coded on the major (leading) strand while - indicates genes encoded on the minor (lagging) strand. tRNA gene anticodons are shown in parentheses and positive values indicate intergenic nucleotides, negative values indicate overlaps and * indicates that TAA stop codon is presumably completed by addition of 3′A residues to mRNA.

***Dipterophagus daci*_Bfra485**					
Gene	**Location**	**Strand**	**Length**	**Intergenic sequence**	**Start/stop codon**
trnI(gat)	1-65	+	65	-3	
trnQ(ttg)	63-132	-	70	6	
trnM(cat)	139-202	+	64	0	
nad2	203-1129	+	927	-2	ATA/TAA
trnW(tca)	1128-1195	+	68	12	
trnC(gca)	1208-1274	-	67	0	
trnY(gta)	1274-1337	-	64	4	
cox1	1342-2848	+	1507	0	CAA/T*
trnL₂(taa)	2849-2910	+	62	9	
cox2	2920-3571	+	652	0	ATT/T*
trnK(ttt)	3572-3634	+	63	2	
trnD(gtc)	3637-3708	+	72	1	
atp8	3710-3859	+	150	-10	ATT/TAA
atp6	3850-4491	+	642	0	ATG/TAA
cox3	4491-5258	+	768	5	ATG/TAA
trnG(tcc)	5264-5323	+	60	-3	
nad3	5321-5663	+	343	0	ATA/T*
trnS₁(tct)	5664-5724	+	61	15	
trnR(tcg)	5740-5801	+	62	44	
trnF(gaa)	5846-5907	-	62	25	
trnN(gtt)	5933-5997	+	65	-3	
trnE(ttc)	5995-6059	+	65	5	
trnA(tgc)	6065-6132	+	68	-3	
nad5_3'	6130-7479	-	1350	-7	ATT/TAA
nad5_5'	7473-7763	-	291	-3	/TAA
trnH(gtg)	7761-7822	-	62	-2	
nad4	7821-9083	-	1263	0	ATG/T*
nad4L	9083-9346	-	264	11	ATA/TAA
trnT(tgt)	9358-9421	+	64	6	
trnP(tgg)	9428-9491	-	64	1	
nad6	9493-9978	+	486	4	ATT/TAA
cob	9983-11093	+	1111	10	ATG/T*
trnL₁(tag)	11104-11169	-	66	101	
rrnS	11271-12064	-	794	34	
nad1	12099-13040	-	942	-3	ATA/TAA
rrnL	13038-14317	-	1280	0	
trnV(tac)	14318-14380	-	63	0	
Control region	14399-16180	+	1781	gap	
trnS₂(tga)	16181-16247	+	67	0	

Table B. 2: continued

Bactrocera frauenfeldi 485					
Gene	**Location**	**Strand**	**Length**	**Intergenic sequence**	**Start/stop codon**
trnI(gat)	1-66	+	66	-3	
trnQ(ttg)	64-132	-	69	76	
trnM(cat)	209-277	+	69	0	
nad2	278-1300	+	1023	9	ATT/TAA
trnW(tca)	1310-1378	+	69	-8	
trnC(gca)	1371-1433	-	63	39	
trnY(gta)	1473-1539	-	67	-1	
cox1	1538-3072	+	1535	0	TCG/TA*
trnL$_2$(taa)	3073-3138	+	66	4	
cox2	3143-3832	+	690	4	ATG/TAA
trnK(ctt)	3837-3907	+	71	2	
trnD(gtc)	3910-3977	+	68	0	
atp8	3978-4139	+	162	-7	GTG/TAA
atp6	4133-4810	+	678	0	ATG/TAA
cox3	4810-5598	+	789	9	ATG/TAA
trnG(tcc)	5608-5672	+	65	0	
nad3	5673-6026	+	354	-2	ATT/TAG
trnA(tgc)	6025-6089	+	65	5	
trnR(tcg)	6095-6158	+	64	26	
trnN(gtt)	6185-6249	+	65	0	
trnS$_1$(gct)	6250-6317	+	68	0	
trnE(ttc)	6318-6384	+	67	18	
trnF(gaa)	6403-6467	-	65	0	
nad5	6468-8187	-	1720	15	ATT/T*
trnH(gtg)	8203-8268	-	66	0	
nad4	8269-9609	-	1341	-7	ATG/TAG
nad4L	9603-9893	-	291	8	ATG/TAA
trnT(tgt)	9902-9966	+	65	0	
trnP(tgg)	9967-10032	-	66	3	
nad6	10035-10559	+	525	0	ATT/TAA
cob	10559-11693	+	1135	0	ATG/T*
trnS$_2$(tga)	11694-11760	+	66	16	
nad1	11776-12715	-	940	10	ATA/T*
trnL$_1$(tag)	12726-12790	-	65	0	
rrnL	12791-14119	-	1329	0	
trnV(tac)	14120-14191	-	72	-1	
rrnS	14192-14983	-	792	0	
Control region	14984-15579	+	595	0	

Table B. 2: continued

Bactrocera neohumeralis 135					
Gene	**Location**	**Strand**	**Length**	**Intergenic sequence**	**Start/stop codon**
trnI(gat)	1-66	+	66	-3	
trnQ(ttg)	64-132	-	69	71	
trnM(cat)	204-272	+	69	0	
nad2	273-1295	+	1023	12	ATT/TAA
trnW(tca)	1308-1376	+	69	-8	
trnC(gca)	1369-1431	-	63	30	
trnY(gta)	1462-1528	-	67	-1	
cox1	1527-3061	+	1535	0	TCG/TA*
trnL$_2$(taa)	3062-3127	+	66	4	
cox2	3132-3821	+	690	4	ATG/TAA
trnK(ctt)	3826-3896	+	71	0	
trnD(gtc)	3897-3963	+	67	0	
atp8	3964-4125	+	162	-7	GTG/TAA
atp6	4119-4796	+	678	0	ATG/TAA
cox3	4796-5584	+	789	9	ATG/TAA
trnG(tcc)	5594-5658	+	65	0	
nad3	5659-6009	+	351	2	ATT/TAG
trnA(tgc)	6011-6075	+	65	7	
trnR(tcg)	6083-6146	+	64	34	
trnN(gtt)	6180-6244	+	65	0	
trnS$_1$(gct)	6245-6312	+	68	0	
trnE(ttc)	6313-6379	+	67	18	
trnF(gaa)	6398-6462	-	65	0	
nad5	6463-8182	-	1720	16	ATT/T*
trnH(gtg)	8198-8263	-	66	0	
nad4	8264-9604	-	1341	-7	ATG/TAG
nad4L	9598-9894	-	297	3	ATG/TAA
trnT(tgt)	9897-9961	+	65	0	
trnP(tgg)	9962-10027	-	66	3	
nad6	10030-10554	+	525	0	ATT/TAA
cob	10554-11688	+	1135	0	ATG/T*
trnS$_2$(tga)	11689-11755	+	66	16	
nad1	11771-12710	-	940	11	ATT/T*
trnL$_1$(tag)	12721-12785	-	65	0	
rrnL	12786-14082	-	1296	29	
trnV(tac)	14112-14183	-	72	0	
rrnS	14183-14972	-	790	0	
Control region	14973-15567	+	594	0	

Table B. 2: continued

Bactrocera neohumeralis 244					
Gene	**Location**	**Strand**	**Length**	**Intergenic sequence**	**Start/stop codon**
trnI(gat)	1-66	+	66	-3	
trnQ(ttg)	64-132	-	69	72	
trnM(cat)	205-273	+	69	0	
nad2	274-1296	+	1023	12	ATT/TAA
trnW(tca)	1309-1377	+	69	-8	
trnC(gca)	1370-1432	-	63	30	
trnY(gta)	1463-1529	-	67	-1	
cox1	1528-3062	+	1535	0	TCG/TA*
trnL$_2$(taa)	3063-3128	+	66	4	
cox2	3133-3822	+	690	4	ATG/TAA
trnK(ctt)	3827-3897	+	71	2	
trnD(gtc)	3900-3966	+	67	0	
atp8	3967-4128	+	162	-7	GTG/TAA
atp6	4122-4799	+	678	0	ATG/TAA
cox3	4799-5587	+	789	9	ATG/TAA
trnG(tcc)	5597-5661	+	65	0	
nad3	5662-6015	+	354	-2	ATT/TAG
trnA(tgc)	6014-6078	+	65	7	
trnR(tcg)	6086-6149	+	64	33	
trnN(gtt)	6183-6247	+	65	0	
trnS$_1$(gct)	6248-6315	+	68	0	
trnE(ttc)	6316-6382	+	67	18	
trnF(gaa)	6401-6465	-	65	0	
nad5	6466-8185	-	1719	16	ATT/T*
trnH(gtg)	8201-8266	-	66	0	
nad4	8267-9607	-	1341	-7	ATG/TAG
nad4L	9601-9897	-	297	3	ATG/TAA
trnT(tgt)	9900-9964	+	65	0	
trnP(tgg)	9965-10030	-	66	3	
nad6	10033-10557	+	480	0	ATT/TAA
cob	10557-11691	+	1137	0	ATG/T*
trnS$_2$(tga)	11692-11758	+	67	16	
nad1	11774-12713	-	940	11	ATT/T*
trnL$_1$(tag)	12724-12788	-	65	0	
rrnL	12789-14085	-	1296	29	
trnV(tac)	14115-14186	-	72	0	
rrnS	14186-14975	-	790	0	
Control region	14976-15570	+	594	0	

Table B. 2: continued

Bactrocera neohumeralis 171					
Gene	**Location**	**Strand**	**Length**	**Intergenic sequence**	**Start/stop codon**
trnI(gat)	1-66	+	66	-3	
trnQ(ttg)	64-132	-	69	72	
trnM(cat)	205-273	+	69	0	
nad2	274-1296	+	1023	12	ATT/TAA
trnW(tca)	1309-1377	+	69	-8	
trnC(gca)	1370-1432	-	63	30	
trnY(gta)	1463-1529	-	67	-1	
cox1	1528-3062	+	1535	0	TCG/TA*
trnL$_2$(taa)	3063-3128	+	66	4	
cox2	3133-3822	+	690	4	ATG/TAA
trnK(ctt)	3827-3897	+	71	2	
trnD(gtc)	3900-3966	+	67	0	
atp8	3967-4128	+	162	-7	GTG/TAA
atp6	4122-4799	+	678	0	ATG/TAA
cox3	4799-5587	+	789	9	ATG/TAA
trnG(tcc)	5597-5661	+	65	0	
nad3	5662-6015	+	354	-2	ATT/TAG
trnA(tgc)	6014-6078	+	65	7	
trnR(tcg)	6086-6149	+	64	33	
trnN(gtt)	6183-6247	+	65	0	
trnS$_1$(gct)	6248-6315	+	68	0	
trnE(ttc)	6316-6382	+	67	18	
trnF(gaa)	6401-6465	-	65	0	
nad5	6466-8185	-	1719	16	ATT/T*
trnH(gtg)	8201-8266	-	66	0	
nad4	8267-9607	-	1341	-7	ATG/TAG
nad4L	9601-9897	-	297	3	ATG/TAA
trnT(tgt)	9900-9964	+	65	0	
trnP(tgg)	9965-10030	-	66	3	
nad6	10033-10557	+	525	0	ATT/TAA
cob	10557-11691	+	1135	0	ATG/T*
trnS$_2$(tga)	11692-11758	+	67	16	
nad1	11774-12713	-	940	11	ATT/T*
trnL$_1$(tag)	12724-12788	-	65	0	
rrnL	12789-14085	-	1320	29	
trnV(tac)	14115-14186	-	72	0	
rrnS	14186-14975	-	790	0	
Control region	14976-15570	+	597	0	

Table B. 2: continued

Bactrocera neohumeralis 240					
Gene	**Location**	**Strand**	**Length**	**Intergenic sequence**	**Start/stop codon**
trnI(gat)	1-66	+	66	-3	
trnQ(ttg)	64-132	-	69	71	
trnM(cat)	204-272	+	69	0	
nad2	273-1295	+	1023	12	ATT/TAA
trnW(tca)	1308-1376	+	69	-8	
trnC(gca)	1369-1431	-	63	30	
trnY(gta)	1462-1528	-	67	-1	
cox1	1527-3061	+	1535	0	TCG/TA*
trnL$_2$(taa)	3062-3127	+	66	4	
cox2	3132-3821	+	690	4	ATG/TAA
trnK(ctt)	3826-3896	+	71	0	
trnD(gtc)	3897-3963	+	67	0	
atp8	3964-4125	+	162	-4	GTG/TAA
atp6	4122-4796	+	675	0	ATA/TAA
cox3	4796-5584	+	789	9	ATG/TAA
trnG(tcc)	5594-5658	+	65	0	
nad3	5659-6012	+	354	-2	ATT/TAG
trnA(tgc)	6011-6075	+	65	7	
trnR(tcg)	6083-6146	+	64	34	
trnN(gtt)	6180-6244	+	65	0	
trnS$_1$(gct)	6245-6312	+	68	0	
trnE(ttc)	6313-6379	+	67	18	
trnF(gaa)	6398-6462	-	65	0	
nad5	6463-8182	-	1719	16	ATT/T*
trnH(gtg)	8198-8263	-	66	0	
nad4	8264-9604	-	1341	-7	ATG/TAG
nad4L	9598-9894	-	297	3	ATG/TAA
trnT(tgt)	9897-9961	+	65	0	
trnP(tgg)	9962-10027	-	66	3	
nad6	10030-10554	+	525	0	ATT/TAA
cob	10554-11688	+	1135	0	ATG/T*
trnS$_2$(tga)	11689-11755	+	67	16	
nad1	11771-12710	-	940	11	ATT/T*
trnL$_1$(tag)	12721-12785	-	65	0	
rrnL	12786-14082	-	1320	29	
trnV(tac)	14112-14183	-	72	0	
rrnS	14183-14972	-	790	0	
Control region	14973-15566	+	594	0	

Table B. 2: continued

Bactrocera neohumeralis 342					
Gene	**Location**	**Strand**	**Length**	**Intergenic sequence**	**Start/stop codon**
trnI(gat)	1-66	+	66	-3	
trnQ(ttg)	64-132	-	69	71	
trnM(cat)	204-272	+	69	0	
nad2	273-1295	+	1023	12	ATT/TAA
trnW(tca)	1308-1376	+	69	-8	
trnC(gca)	1369-1431	-	63	30	
trnY(gta)	1462-1528	-	67	-1	
cox1	1527-3061	+	1535	0	TCG/TA*
trnL$_2$(taa)	3062-3127	+	66	4	
cox2	3132-3821	+	690	4	ATG/TAA
trnK(ctt)	3826-3896	+	71	0	
trnD(gtc)	3897-3963	+	67	0	
atp8	3964-4125	+	162	-4	GTG/TAA
atp6	4119-4796	+	678	0	ATA/TAA
cox3	4796-5584	+	789	9	ATG/TAA
trnG(tcc)	5594-5658	+	65	0	
nad3	5659-6012	+	354	-2	ATT/TAG
trnA(tgc)	6011-6075	+	65	7	
trnR(tcg)	6083-6146	+	64	34	
trnN(gtt)	6180-6244	+	65	0	
trnS$_1$(gct)	6245-6312	+	68	0	
trnE(ttc)	6313-6379	+	67	18	
trnF(gaa)	6398-6462	-	65	0	
nad5	6463-8182	-	1719	16	ATT/T*
trnH(gtg)	8198-8263	-	66	0	
nad4	8264-9604	-	1341	-7	ATG/TAG
nad4L	9598-9894	-	297	3	ATG/TAA
trnT(tgt)	9897-9961	+	65	0	
trnP(tgg)	9962-10027	-	66	3	
nad6	10030-10554	+	525	0	ATT/TAA
cob	10554-11688	+	1135	0	ATG/T*
trnS$_2$(tga)	11689-11755	+	66	16	
nad1	11771-12710	-	940	11	ATT/T*
trnL$_1$(tag)	12721-12785	-	65	0	
rrnL	12763-14082	-	1320	29	
trnV(tac)	14112-14183	-	72	0	
rrnS	14183-14972	-	790	0	
Control region	14973-15566	+	594	0	

Table B. 2: continued

Bactrocera tryoni 194					
Gene	**Location**	**Strand**	**Length**	**Intergenic sequence**	**Start/stop codon**
trnI(gat)	1-66	+	66	-3	
trnQ(ttg)	64-132	-	69	71	
trnM(cat)	204-272	+	69	0	
nad2	273-1295	+	1023	12	ATT/TAA
trnW(tca)	1308-1376	+	69	-8	
trnC(gca)	1369-1431	-	63	30	
trnY(gta)	1462-1528	-	67	-1	
cox1	1527-3061	+	1535	0	TCG/TA*
trnL$_2$(taa)	3062-3127	+	66	4	
cox2	3132-3821	+	690	4	ATG/TAA
trnK(ctt)	3826-3896	+	71	0	
trnD(gtc)	3899-3965	+	67	0	
atp8	3966-4127	+	162	-4	GTG/TAA
atp6	4121-4798	+	678	0	ATA/TAA
cox3	4798-5586	+	789	9	ATG/TAA
trnG(tcc)	5596-5660	+	65	0	
nad3	5661-6014	+	354	-2	ATT/TAG
trnA(tgc)	6013-6077	+	65	7	
trnR(tcg)	6085-6148	+	64	34	
trnN(gtt)	6182-6246	+	65	0	
trnS$_1$(gct)	6247-6314	+	68	0	
trnE(ttc)	6315-6381	+	67	18	
trnF(gaa)	6400-6464	-	65	0	
nad5	6465-8184	-	1719	16	ATT/T*
trnH(gtg)	8200-8265	-	66	0	
nad4	8266-9606	-	1341	-7	ATG/TAG
nad4L	9600-9896	-	297	3	ATG/TAA
trnT(tgt)	9899-9963	+	65	0	
trnP(tgg)	9964-10029	-	66	3	
nad6	10032-10556	+	525	0	ATT/TAA
cob	10556-11690	+	1135	0	ATG/T*
trnS$_2$(tga)	11691-11757	+	67	16	
nad1	11773-12712	-	940	11	ATT/T*
trnL$_1$(tag)	12723-12787	-	65	0	
rrnL	12765-14084	-	1320	29	
trnV(tac)	14114-14185	-	72	0	
rrnS	14185-14974	-	790	0	
Control region	14975-15569	+	594	0	

Table B. 2: continued

Bactrocera tryoni 210					
Gene	**Location**	**Strand**	**Length**	**Intergenic sequence**	**Start/stop codon**
trnI(gat)	1-66	+	66	-3	
trnQ(ttg)	64-132	-	69	71	
trnM(cat)	204-272	+	69	0	
nad2	273-1295	+	1023	12	ATT/TAA
trnW(tca)	1308-1376	+	69	-8	
trnC(gca)	1369-1431	-	63	30	
trnY(gta)	1462-1528	-	67	-1	
cox1	1527-3061	+	1535	0	TCG/TA*
trnL$_2$(taa)	3062-3127	+	66	4	
cox2	3132-3821	+	690	4	ATG/TAA
trnK(ctt)	3826-3896	+	71	0	
trnD(gtc)	3898-3964	+	67	0	
atp8	3965-4126	+	162	-4	GTG/TAA
atp6	4120-4797	+	678	0	ATA/TAA
cox3	4797-5585	+	789	9	ATG/TAA
trnG(tcc)	5595-5659	+	65	0	
nad3	5660-6013	+	354	-2	ATT/TAG
trnA(tgc)	6012-6076	+	65	7	
trnR(tcg)	6084-6147	+	64	34	
trnN(gtt)	6181-6245	+	65	0	
trnS$_1$(gct)	6246-6313	+	68	0	
trnE(ttc)	6314-6379	+	66	18	
trnF(gaa)	6398-6462	-	65	0	
nad5	6463-8182	-	1719	16	ATT/T*
trnH(gtg)	8198-8263	-	66	0	
nad4	8264-9604	-	1341	-7	ATG/TAG
nad4L	9598-9894	-	297	3	ATG/TAA
trnT(tgt)	9897-9961	+	65	0	
trnP(tgg)	9962-10027	-	66	3	
nad6	10030-10552	+	523	0	ATT/TAA
cob	10554-11688	+	1135	0	ATG/T*
trnS$_2$(tga)	11689-11755	+	67	16	
nad1	11691-12608	-	940	11	ATT/T*
trnL$_1$(tag)	12721-12785	-	65	0	
rrnL	12763-14082	-	1320	29	
trnV(tac)	14112-14183	-	72	0	
rrnS	14183-14972	-	790	0	
Control region	14973-15568	+	594	0	

Table B. 2: continued

Zeugodacus strigifinis 503					
Gene	**Location**	**Strand**	**Length**	**Intergenic sequence**	**Start/stop codon**
trnI(gat)	1-65	+	65	-3	
trnQ(ttg)	63-131	-	69	7	
trnM(cat)	139-207	+	69	0	
nad2	208-1230	+	1023	11	ATT/TAA
trnW(tca)	1241-1308	+	68	36	
trnC(gca)	1345-1407	-	63	0	
trnY(gta)	1408-1474	-	67	-1	
cox1	1473-3007	+	1535	0	TCG/TA*
trnL$_2$(taa)	3007-3072	+	66	5	
cox2	3078-3767	+	690	4	ATG/TAA
trnK(ctt)	3772-3842	+	71	-1	
trnD(gtc)	3842-3910	+	69	0	
atp8	3911-4072	+	162	-7	ATT/TAA
atp6	4066-4743	+	678	0	ATG/TAA
cox3	4743-5531	+	789	6	ATG/TAA
trnG(tcc)	5538-5602	+	65	0	
nad3	5603-5956	+	354	-2	ATT/TAG
trnA(tgc)	5955-6020	+	66	0	
trnR(tcg)	6021-6084	+	64	34	
trnN(gtt)	6119-6183	+	65	0	
trnS$_1$(gct)	6184-6251	+	68	0	
trnE(ttc)	6252-6319	+	68	18	
trnF(gaa)	6338-6402	-	65	0	
nad5	6403-8122	-	1720	16	ATT/T*
trnH(gtg)	8138-8203	-	66	0	
nad4	8203-9543	-	1341	-7	ATG/TAA
nad4L	9537-9833	-	297	2	ATG/TAA
trnT(tgt)	9836-9900	+	65	0	
trnP(tgg)	9901-9966	-	66	3	
nad6	9969-10493	+	525	0	ATT/TAA
cob	10493-11627	+	1135	0	ATG/T*
trnS$_2$(tga)	11628-11694	+	67	16	
nad1	11710-12649	-	940	11	ATT/T*
trnL$_1$(tag)	12659-12724		66	0	
rrnL	12725-14016	-	1292	37	
trnV(tac)	14053-14124	-	72	0	
rrnS	14124-14912	-	789	0	
Control region	14913-15502	+	589	0	

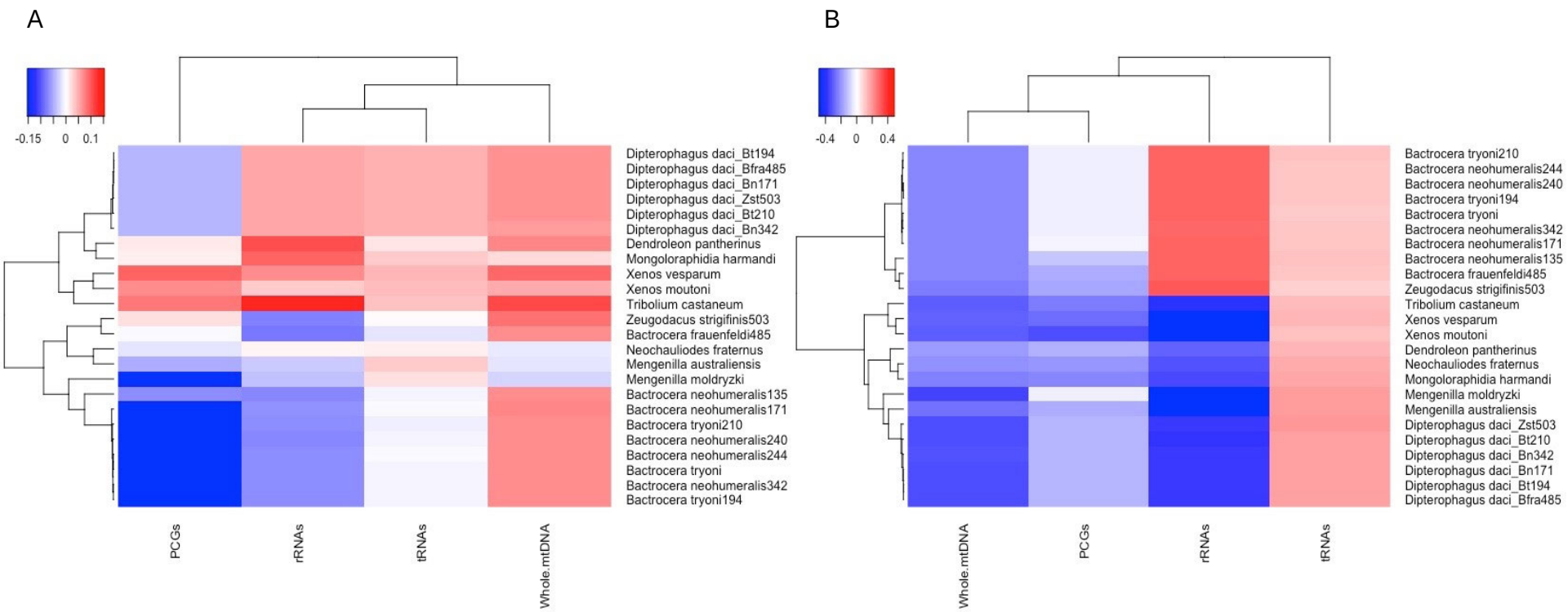

Figure B. 1: Comparative analysis of the mitogenomes of *Dipterophagus daci*, tephritid fruit fly species and other reference species (a) AT skew and (b) GC skew.

Table B. 3: Summary of the mitogenome comparisons between *Dipterophagus daci*, tephritid fruit fly species and reference species, showing genome size, A+T%, AT-skew and GC-skew. Values of the newly sequenced mitogenomes are listed in bold.

Species	Accession number	Order	Length				A+T %				Whole mtDNA AT-skew	Whole mtDNA GC-skew
			Whole mtDNA	PCGs	tRNAs	rRNAs	Whole mtDNA	PCGs	tRNAs	rRNAs		
***Dipterophagus daci*_Bfra485**	**MW233588**	**Strepsiptera**	**16,255**	**10,696**	**1,424**	**2,074**	**84.7**	**82.5**	**86.2**	**87.2**	**0.0649**	**-0.3333**
***Dipterophagus daci*_Bn171**			**16,248**	**10,696**	**1,424**	**2,074**	**84.4**	**82.5**	**85.9**	**87.2**	**0.0649**	**-0.3333**
***Dipterophagus daci*_Bn342**			**16,243**	**10,696**	**1,424**	**2,074**	**84.3**	**82.5**	**86.2**	**87.2**	**0.0588**	**-0.3289**
***Dipterophagus daci*_Bt194**			**16,247**	**10,696**	**1,424**	**2,074**	**84.7**	**82.5**	**86.2**	**87.2**	**0.0649**	**-0.3333**
***Dipterophagus daci*_Bt210**			**16,247**	**10,696**	**1,424**	**2,075**	**83.3**	**82.5**	**86.2**	**87.2**	**0.0652**	**-0.3333**
***Dipterophagus daci*_Zst503**			**16,248**	**10,696**	**1,424**	**2,074**	**84.7**	**82.5**	**86.2**	**87.2**	**0.0664**	**-0.3333**
Mengenilla australiensis	GU188852		13,421	10,736	1,046	1,632	84.3	83.8	86.7	85.9	-0.0154	-0.2692
Mengenilla moldryzki	JQ398619		15,363	11,052	1,260	1,963	81.9	80.5	85.6	84.2	-0.0256	-0.3516
Xenos moutoni	MW222190		16,717	10,663	1,379	1,969	82.5	79.8	83.8	83.4	0.052	-0.3028
Xenos vesparum	DQ364229		14,519	10,737	1,372	1,177	79.3	77.8	83.5	79.9	0.0921	-0.2913
***Bactrocera frauenfeldi* 485**	**MZ520731**	**Diptera**	**15,935**	**11,189**	**1,468**	**2,081**	**74.1**	**71.7**	**75.1**	**77.5**	**0.0688**	**-0.2231**
***Bactrocera neohumeralis* 135**	**MZ520732**		**15,924**	**11,185**	**1,467**	**2,087**	**72.5**	**69.6**	**75**	**77.5**	**0.0703**	**-0.2291**
***Bactrocera neohumeralis* 171**	**MZ520733**		**15,927**	**11,188**	**1,467**	**2,087**	**72.5**	**69.7**	**74.9**	**77.6**	**0.0731**	**-0.2291**
***Bactrocera neohumeralis* 240**	**MZ520734**		**15,922**	**11,188**	**1,467**	**2,087**	**72.4**	**69.7**	**74.9**	**77.6**	**0.0691**	**-0.2246**
***Bactrocera neohumeralis* 244**	**MZ520735**		**15,927**	**11,188**	**1,467**	**2,087**	**72.5**	**69.7**	**74.9**	**77.6**	**0.0703**	**-0.2291**
***Bactrocera neohumeralis* 342**	**MZ520736**		**15,923**	**11,188**	**1,467**	**2,087**	**72.4**	**69.7**	**74.9**	**77.6**	**0.0691**	**-0.2246**
***Bactrocera tryoni* 194**	**MZ520737**		**15,926**	**11,189**	**1,467**	**2,110**	**72.3**	**69.4**	**75.4**	**77.3**	**0.0691**	**-0.2246**
***Bactrocera tryoni* 210**	**MZ520738**		**15,925**	**11,186**	**1,467**	**2,110**	**72.4**	**69.6**	**75.3**	**77.5**	**0.0705**	**-0.2274**
Bactrocera tryoni	NC014611		15,925	11,187	1,467	2,115	72.5	69.6	75.3	77.5	0.0703	-0.2246
***Zeugodacus strigifinis* 503**	**MZ520739**		**15,858**	**11,189**	**1,469**	**2,081**	**73.4**	**71.1**	**74.9**	**77.5**	**0.0845**	**-0.2434**
Tribolium castaneum	AJ312413	Coleoptera	15881	11091	1369	2041	71.7	69.2	75.4	75.7	0.1102	-0.3074
Neochauliodes fraternus	NC_025282	Megaloptera	15768	11092	1372	2092	77.3	75.4	76.8	80.7	-0.0142	-0.207
Dendroleon pantherinus	MK301246	Neuroptera	15516	11158	1400	2087	73.2	72.2	75.4	77.7	0.071	-0.1908
Mongoloraphidia harmandi	NC_013251	Raphidoptera	16006	11100	1437	1602	80.3	78	81	80.7	0.0237	-0.2347

Table B. 4: Comparative analysis of the mitogenome relative synonymous codon usage (RSCU) of *Dipterophagus daci*, tephritid fruit fly species and reference species mitogenomes. Newly sequenced mitogenomes are listed in bold.

Species	Accession number	UUU(F)	UUC(F)	UUA(L)	UUG(L)	CUU(L)	CUC(L)	CUA(L)	CUG(L)	AUU(I)	AUC(I)	AUA(M)	AUG(M)	GUU(V)	GUC(V)
***Dipterophagus daci*_Bfra485**	**MW233588**	1.9	0.1	4.96	0.17	0.34	0.06	0.44	0.02	1.8	0.2	1.8	0.2	1.93	0.15
***Dipterophagus daci*_Bn171**		1.89	0.11	4.95	0.17	0.34	0.07	0.45	0.02	1.79	0.21	1.8	0.2	2.08	0.15
***Dipterophagus daci*_Bn342**		1.9	0.1	4.95	0.17	0.35	0.06	0.45	0.02	1.79	0.21	1.81	0.19	2.02	0.16
***Dipterophagus daci*_Bt194**		1.9	0.1	4.96	0.17	0.33	0.07	0.45	0.02	1.79	0.21	1.81	0.19	2.02	0.16
***Dipterophagus daci*_Bt210**		1.9	0.1	4.95	0.17	0.34	0.07	0.45	0.02	1.79	0.21	1.81	0.19	2.02	0.16
***Dipterophagus daci*_Zst503**		1.9	0.1	4.96	0.17	0.33	0.07	0.45	0.02	1.79	0.21	1.81	0.19	2.02	0.16
Mengenilla australiensis	GU188852	1.92	0.08	5.22	0.16	0.42	0.03	0.17	0	1.93	0.07	1.86	0.14	2.2	0.05
Mengenilla moldryzki	JQ398619	1.75	0.25	4.45	0.28	0.62	0.12	0.53	0.01	1.77	0.23	1.85	0.15	1.44	0.19
Xenos vesparum	DQ364229	1.71	0.29	3.9	0.38	0.58	0.12	0.95	0.07	1.71	0.29	1.77	0.23	1.85	0.08
Xenos moutoni	MW222190	1.55	0.45	3.19	0.56	0.92	0.39	0.78	0.16	1.55	0.45	1.7	0.3	1.21	0.42
***Bactrocera frauenfeldi*485**	**MZ520731**	1.55	0.45	3.76	0.52	0.64	0.08	0.89	0.1	1.8	0.2	1.68	0.32	1.71	0.19
***Bactrocera neohumeralis*135**	**MZ520732**	1.51	0.49	3.24	0.73	0.77	0.06	1.07	0.13	1.69	0.31	1.56	0.44	1.7	0.23
***Bactrocera neohumeralis*171**	**MZ520733**	1.53	0.47	3.26	0.72	0.76	0.07	1.04	0.15	1.68	0.32	1.58	0.42	1.68	0.23
***Bactrocera neohumeralis*240**	**MZ520734**	1.5	0.5	3.24	0.71	0.77	0.06	1.09	0.13	1.69	0.31	1.57	0.43	1.69	0.25
***Bactrocera neohumeralis*244**	**MZ520735**	1.5	0.5	3.31	0.7	0.75	0.08	0.98	0.17	1.69	0.31	1.56	0.44	1.7	0.22
***Bactrocera neohumeralis*342**	**MZ520736**	1.51	0.49	3.21	0.75	0.76	0.07	1.09	0.12	1.7	0.3	1.57	0.43	1.74	0.21
***Bactrocera tryoni*194**	**MZ520737**	1.51	0.49	3.26	0.73	0.76	0.07	1.06	0.12	1.71	0.29	1.55	0.45	1.69	0.25
***Bactrocera tryoni*210**	**MZ520738**	1.49	0.51	3.26	0.73	0.77	0.06	1.05	0.13	1.68	0.32	1.59	0.41	1.71	0.23
Bactrocera tryoni	NC0146111	1.52	0.48	3.21	0.75	0.78	0.06	1.06	0.14	1.7	0.3	1.59	0.41	1.69	0.25
***Zeugodacus strigifinis* 503**	**MZ520739**	1.32	0.68	2.31	0.73	0.95	0.45	1.24	0.32	1.45	0.55	1.57	0.43	1.44	0.56
Tribolium castaneum	AJ312413	1.4	0.6	2.57	0.84	0.99	0.37	1.11	0.13	1.6	0.4	1.58	0.42	1.73	0.22
Neochauliodes fraternus	NC_025282	1.84	0.16	4.79	0.14	0.68	0.08	0.28	0.02	1.88	0.12	1.82	0.18	2.06	0.18
Dendroleon pantherinus	MK301246	1.59	0.41	3.94	0.57	0.61	0.12	0.67	0.09	1.72	0.28	1.77	0.23	1.87	0.1
Mongoloraphidia harmandi	NC_013251	1.78	0.22	4.87	0.26	0.41	0.03	0.4	0.03	1.82	0.18	1.94	0.06	2.01	0.19

Table B. 4: continued

Species	Accession number	GUA(V)	GUG(V)	UCU(S)	UCC(S)	UCA(S)	UCG(S)	CCU(P)	CCC(P)	CCA(P)	CCG(P)	ACU(T)	ACC(T)	ACA(T)	ACG(T)
***Dipterophagus daci*_Bfra485**	**MW233588**	1.45	0.47	2.27	0.15	1.94	0.05	2	0.4	1.47	0.13	1.94	0.26	1.76	0.03
***Dipterophagus daci*_Bn171**		1.38	0.38	2.28	0.16	1.96	0.05	2	0.4	1.47	0.13	1.96	0.27	1.75	0.03
***Dipterophagus daci*_Bn342**		1.44	0.39	2.28	0.16	1.96	0.05	2	0.4	1.47	0.13	1.96	0.27	1.75	0.03
***Dipterophagus daci*_Bt194**		1.44	0.39	2.28	0.16	1.96	0.05	2	0.4	1.47	0.13	1.96	0.27	1.75	0.03
***Dipterophagus daci*_Bt210**		1.44	0.39	2.27	0.16	1.95	0.05	2	0.4	1.47	0.13	1.96	0.27	1.75	0.03
***Dipterophagus daci*_Zst503**		1.44	0.39	2.28	0.16	1.96	0.05	2	0.4	1.47	0.13	1.96	0.27	1.75	0.03
Mengenilla australiensis	GU188852	1.76	0	2.27	0.25	2.22	0.03	2.69	0.12	1.18	0	2.14	0.12	1.7	0.04
Mengenilla moldryzki	JQ398619	2.07	0.3	2.2	0.37	2.23	0.12	2.06	0.39	1.55	0	2.21	0.35	1.4	0.04
Xenos vesparum	DQ364229	1.62	0.45	1.63	0.37	2.01	0.07	1.81	0.87	1.25	0.08	1.32	0.57	2.08	0.03
Xenos moutoni	MW222190	1.74	0.63	1.72	1.24	1.81	0.29	1.66	1.06	1.15	0.13	1.53	0.96	1.27	0.24
***Bactrocera frauenfeldi*485**	**MZ520731**	1.84	0.26	2.56	0.44	2.15	0.17	2.39	0.47	1.05	0.09	1.64	0.4	1.92	0.04
***Bactrocera neohumeralis*135**	**MZ520732**	1.72	0.34	2.6	0.36	2.17	0.17	2.01	0.79	1.05	0.15	1.55	0.53	1.71	0.22
***Bactrocera neohumeralis*171**	**MZ520733**	1.77	0.32	2.63	0.34	2.19	0.17	2.03	0.79	1.12	0.06	1.57	0.53	1.67	0.24
***Bactrocera neohumeralis*240**	**MZ520734**	1.72	0.34	2.6	0.36	2.17	0.17	2.01	0.79	1.05	0.15	1.56	0.53	1.69	0.22
***Bactrocera neohumeralis*244**	**MZ520735**	1.72	0.36	2.62	0.34	2.16	0.19	2.09	0.74	1.06	0.12	1.57	0.53	1.67	0.24
***Bactrocera neohumeralis*342**	**MZ520736**	1.67	0.37	2.62	0.34	2.16	0.17	1.99	0.82	1.08	0.12	1.56	0.53	1.69	0.22
***Bactrocera tryoni*194**	**MZ520737**	1.7	0.36	2.61	0.36	2.18	0.17	1.99	0.82	1.08	0.12	1.56	0.53	1.69	0.22
***Bactrocera tryoni*210**	**MZ520738**	1.66	0.39	2.55	0.41	2.17	0.17	1.96	0.88	1.05	0.12	1.56	0.53	1.71	0.2
Bactrocera tryoni	NC0146111	1.72	0.34	2.64	0.34	2.16	0.17	1.97	0.82	1.09	0.12	1.52	0.57	1.73	0.18
***Zeugodacus strigifinis* 503**	**MZ520739**	1.72	0.28	1.55	0.88	1.82	0.46	1.18	1.11	1.34	0.36	1.21	1.05	1.39	0.35
Tribolium castaneum	AJ312413	1.65	0.4	2.1	0.51	2.23	0.38	1.58	0.69	1.49	0.24	1.45	0.7	1.69	0.15
Neochauliodes fraternus	NC_025282	1.63	0.14	2.61	0.36	2.07	0.07	2.58	0.34	1.02	0.06	1.86	0.48	1.66	0
Dendroleon pantherinus	MK301246	1.81	0.22	1.96	0.4	2.43	0.15	1.91	0.65	1.14	0.31	1.64	0.4	1.85	0.11
Mongoloraphidia harmandi	NC_013251	1.71	0.08	2.99	0.23	1.6	0.09	2.7	0.41	0.89	0	2.14	0.41	1.41	0.05

Table B. 4: continued

Species	Accession number	GCU(A)	GCC(A)	GCA(A)	GCG(A)	UAU(Y)	UAC(Y)	UAA(*)	UAG(*)	CAU(H)	CAC(H)	CAA(Q)	CAG(Q)	AAU(N)	AAC(N)
***Dipterophagus daci*_Bfra485**	**MW233588**	2.31	0.38	1.23	0.08	1.71	0.29	2	0	1.57	0.43	2	0	1.6	0.4
***Dipterophagus daci*_Bn171**		2.35	0.39	1.25	0	1.73	0.27	2	0	1.52	0.48	2	0	1.59	0.41
***Dipterophagus daci*_Bn342**		2.35	0.39	1.25	0	1.72	0.28	2	0	1.57	0.43	2	0	1.59	0.41
***Dipterophagus daci*_Bt194**		2.35	0.39	1.25	0	1.72	0.28	2	0	1.57	0.43	2	0	1.59	0.41
***Dipterophagus daci*_Bt210**		2.35	0.39	1.25	0	1.72	0.28	2	0	1.57	0.43	2	0	1.59	0.41
***Dipterophagus daci*_Zst503**		2.35	0.39	1.25	0	1.72	0.28	2	0	1.57	0.43	2	0	1.59	0.41
Mengenilla australiensis	GU188852	2.4	0.4	1.2	0	1.93	0.07	1.82	0.18	1.9	0.1	2	0	1.94	0.06
Mengenilla moldryzki	JQ398619	2.31	0.38	1.31	0	1.74	0.26	1.38	0.62	1.62	0.38	1.81	0.19	1.79	0.21
Xenos vesparum	DQ364229	2.16	0.74	1.11	0	1.74	0.26	1.85	0.15	1.61	0.39	1.65	0.35	1.71	0.29
Xenos moutoni	MW222190	1.71	1.03	1.14	0.11	1.65	0.35	1.78	0.22	1.42	0.58	1.51	0.49	1.66	0.34
***Bactrocera frauenfeldi*485**	**MZ520731**	2.12	0.41	1.4	0.06	1.58	0.42	1.23	0.77	1.25	0.75	1.73	0.27	1.68	0.32
***Bactrocera neohumeralis*135**	**MZ520732**	1.95	0.72	1.19	0.14	1.48	0.52	1.23	0.77	0.99	1.01	1.63	0.37	1.51	0.49
***Bactrocera neohumeralis*171**	**MZ520733**	1.92	0.74	1.18	0.16	1.52	0.48	1.23	0.77	1.05	0.95	1.68	0.32	1.48	0.52
***Bactrocera neohumeralis*240**	**MZ520734**	1.91	0.74	1.21	0.14	1.47	0.53	1.23	0.77	1.01	0.99	1.65	0.35	1.51	0.49
***Bactrocera neohumeralis*244**	**MZ520735**	1.9	0.76	1.18	0.16	1.51	0.49	1.23	0.77	1.04	0.96	1.65	0.35	1.49	0.51
***Bactrocera neohumeralis*342**	**MZ520736**	1.88	0.72	1.28	0.12	1.46	0.54	1.23	0.77	0.99	1.01	1.65	0.35	1.51	0.49
***Bactrocera tryoni*194**	**MZ520737**	1.9	0.74	1.22	0.14	1.49	0.51	1.23	0.77	0.99	1.01	1.65	0.35	1.51	0.49
***Bactrocera tryoni*210**	**MZ520738**	1.89	0.74	1.19	0.18	1.47	0.53	1.23	0.77	0.99	1.01	1.65	0.35	1.49	0.51
Bactrocera tryoni	NC0146111	1.92	0.71	1.24	0.12	1.49	0.51	1.23	0.77	0.99	1.01	1.63	0.37	1.51	0.49
***Zeugodacus strigifinis* 503**	**MZ520739**	1.13	1.46	1.3	0.11	1.44	0.56	1.49	0.51	1.4	0.6	1.3	0.7	1.47	0.53
Tribolium castaneum	AJ312413	1.42	0.93	1.5	0.15	1.38	0.62	1.54	0.46	1.05	0.95	1.51	0.49	1.31	0.69
Neochauliodes fraternus	NC_025282	2.3	0.46	1.15	0.09	1.66	0.34	1.8	0.2	1.76	0.24	1.78	0.22	1.84	0.16
Dendroleon pantherinus	MK301246	2.14	0.34	1.41	0.11	1.39	0.61	1.67	0.33	1.42	0.58	1.74	0.26	1.63	0.37
Mongoloraphidia harmandi	NC_013251	2.25	0.41	1.27	0.06	1.69	0.31	1.85	0.15	1.63	0.37	1.87	0.13	1.73	0.27

Table B. 4: continued

Species	Accession number	AAA(K)	AAG(K)	GAU(D)	GAC(D)	GAA(E)	GAG(E)	UGU(C)	UGC(C)	UGA(W)	UGG(W)	CGU(R)	CGC(R)	CGA(R)	CGG(R)
***Dipterophagus daci*_Bfra485**	**MW233588**	1.86	0.14	1.64	0.36	1.83	0.17	1.63	0.37	1.9	0.1	1.33	0.12	2.42	0.12
***Dipterophagus daci*_Bn171**		1.88	0.12	1.61	0.39	1.83	0.17	1.62	0.38	1.92	0.08	1.25	0.12	2.5	0.12
***Dipterophagus daci*_Bn342**		1.88	0.12	1.61	0.39	1.83	0.17	1.62	0.38	1.92	0.08	1.25	0.12	2.5	0.12
***Dipterophagus daci*_Bt194**		1.88	0.12	1.61	0.39	1.83	0.17	1.62	0.38	1.92	0.08	1.25	0.12	2.5	0.12
***Dipterophagus daci*_Bt210**		1.88	0.12	1.61	0.39	1.83	0.17	1.62	0.38	1.92	0.08	1.25	0.12	2.5	0.12
***Dipterophagus daci*_Zst503**		1.88	0.12	1.61	0.39	1.83	0.17	1.62	0.38	1.92	0.08	1.25	0.12	2.5	0.12
Mengenilla australiensis	GU188852	1.97	0.03	1.63	0.37	1.82	0.18	1.92	0.08	1.94	0.06	1.33	0.21	2.36	0.1
Mengenilla moldryzki	JQ398619	1.77	0.23	1.83	0.17	1.57	0.43	1.83	0.17	1.86	0.14	1.54	0.1	1.74	0.62
Xenos vesparum	DQ364229	1.72	0.28	1.57	0.43	1.4	0.6	1.76	0.24	1.89	0.11	1.38	0.5	1.63	0.5
Xenos moutoni	MW222190	1.77	0.23	1.62	0.38	1.62	0.38	1.4	0.6	1.54	0.46	1.33	0.19	1.52	0.95
***Bactrocera frauenfeldi*485**	**MZ520731**	1.3	0.7	1.51	0.49	1.87	0.13	1.95	0.05	1.7	0.3	1.17	0.28	2.14	0.41
***Bactrocera neohumeralis*135**	**MZ520732**	1.31	0.69	1.26	0.74	1.81	0.19	1.72	0.28	1.66	0.34	0.98	0.21	2.32	0.49
***Bactrocera neohumeralis*171**	**MZ520733**	1.26	0.74	1.34	0.66	1.73	0.27	1.81	0.19	1.72	0.28	1.05	0.14	2.25	0.56
***Bactrocera neohumeralis*240**	**MZ520734**	1.31	0.69	1.29	0.71	1.79	0.21	1.72	0.28	1.62	0.38	0.98	0.21	2.32	0.49
***Bactrocera neohumeralis*244**	**MZ520735**	1.29	0.71	1.23	0.77	1.79	0.21	1.67	0.33	1.7	0.3	1.05	0.14	2.32	0.49
***Bactrocera neohumeralis*342**	**MZ520736**	1.33	0.67	1.3	0.7	1.76	0.24	1.71	0.29	1.66	0.34	0.98	0.21	2.39	0.42
***Bactrocera tryoni*194**	**MZ520737**	1.31	0.69	1.26	0.74	1.79	0.21	1.77	0.23	1.66	0.34	0.98	0.21	2.32	0.49
***Bactrocera tryoni*210**	**MZ520738**	1.31	0.69	1.26	0.74	1.79	0.21	1.81	0.19	1.66	0.34	0.98	0.21	2.39	0.42
Bactrocera tryoni	NC0146111	1.36	0.64	1.32	0.68	1.73	0.27	1.77	0.23	1.68	0.32	0.98	0.21	2.46	0.35
***Zeugodacus strigifinis* 503**	**MZ520739**	1.56	0.44	1.29	0.71	1.24	0.76	1.29	0.71	1.43	0.57	0.55	1.03	1.45	0.97
Tribolium castaneum	AJ312413	1.33	0.67	1.38	0.63	1.6	0.4	1.48	0.52	1.84	0.16	1.14	0.21	2.29	0.36
Neochauliodes fraternus	NC_025282	1.57	0.43	1.69	0.31	1.83	0.17	1.88	0.12	1.84	0.16	1.53	0.22	2.04	0.22
Dendroleon pantherinus	MK301246	1.58	0.42	1.65	0.35	1.59	0.41	1.83	0.17	1.64	0.36	1.61	0.07	2.11	0.21
Mongoloraphidia harmandi	NC_013251	1.79	0.21	1.78	0.22	1.84	0.16	1.82	0.18	1.91	0.09	1.28	0.16	2.4	0.16

Table B. 4: continued

Species	Accession number	AGU(S)	AGC(S)	AGA(S)	AGG(S)	GGU(G)	GGC(G)	GGA(G)	GGG(G)
***Dipterophagus daci*_Bfra485**	**MW233588**	0.8	0.15	2.27	0.36	1.09	0.27	1.86	0.78
***Dipterophagus daci*_Bn171**		0.79	0.16	2.25	0.34	1.1	0.27	1.88	0.75
***Dipterophagus daci*_Bn342**		0.79	0.16	2.25	0.34	1.1	0.27	1.88	0.75
***Dipterophagus daci*_Bt194**		0.79	0.16	2.25	0.34	1.1	0.27	1.88	0.75
***Dipterophagus daci*_Bt210**		0.82	0.16	2.24	0.34	1.1	0.27	1.88	0.75
***Dipterophagus daci*_Zst503**		0.79	0.16	2.25	0.34	1.1	0.27	1.88	0.75
Mengenilla australiensis	GU188852	0.77	0.03	2.17	0.25	1.09	0.09	2.54	0.28
Mengenilla moldryzki	JQ398619	0.63	0.19	2.04	0.23	1.04	0.26	1.88	0.81
Xenos vesparum	DQ364229	0.73	0.26	2.36	0.57	1.13	0.44	1.83	0.6
Xenos moutoni	MW222190	0.53	0.57	1.29	0.55	1.28	0.64	1.28	0.8
***Bactrocera frauenfeldi*485**	**MZ520731**	1.29	0.15	1.24	0	1.01	0.12	2.09	0.78
***Bactrocera neohumeralis*135**	**MZ520732**	1.13	0.31	1.25	0	0.82	0.14	2.04	1
***Bactrocera neohumeralis*171**	**MZ520733**	1.04	0.39	1.25	0	0.88	0.12	1.96	1.04
***Bactrocera neohumeralis*240**	**MZ520734**	1.11	0.34	1.25	0	0.82	0.14	1.99	1.05
***Bactrocera neohumeralis*244**	**MZ520735**	1.03	0.38	1.27	0	0.87	0.14	2.07	0.92
***Bactrocera neohumeralis*342**	**MZ520736**	1.08	0.36	1.27	0	0.84	0.14	1.92	1.1
***Bactrocera tryoni*194**	**MZ520737**	1.14	0.29	1.26	0	0.82	0.14	1.97	1.07
***Bactrocera tryoni*210**	**MZ520738**	1.06	0.41	1.23	0	0.82	0.16	1.9	1.12
Bactrocera tryoni	NC0146111	1.06	0.38	1.25	0	0.84	0.12	1.92	1.12
***Zeugodacus strigifinis* 503**	**MZ520739**	0.95	0.73	1	0.62	0.91	0.73	1.39	0.97
Tribolium castaneum	AJ312413	0.55	0.09	1.88	0.27	0.68	0.31	2.23	0.77
Neochauliodes fraternus	NC_025282	0.97	0.21	1.69	0.02	1.38	0.09	1.93	0.6
Dendroleon pantherinus	MK301246	1.22	0.3	1.52	0.02	1.4	0.19	1.4	1.02
Mongoloraphidia harmandi	NC_013251	0.88	0.02	2.18	0	0.91	0.08	2.58	0.43

Table B. 5: Mitogenome diversity of *Dipterophagus daci* mitogenomes, showing the collection locality, *Wolbachia* infection status (+ or -) with *w*Ddac1 (ST-285) and *w*Ddac2 (ST-289), single nucleotide polymorphism (SNP) position in the mitogenome. The * denotes the assembled reference genome (MW233588), ^ denotes library with low coverage that did not allow assembly of the mitogenome and — denotes a nucleotide deletion and the # denotes the non-coding region.

Collection locality	***w*Ddac 1**	***w*Ddac 2**	**Gene**	***cox1***		**#**	***nad5***				***nad4***		***cob***		**#**				***rrnS***	
			Nucleotide position in the mitogenome	**1,762**	**2,546**	**3,738**	**6,408**	**6,607**	**6,912**	**7,306**	**7,869**	**8,640**	**10,276**	**11,033**	**11,207**	**11,208**	**11,209**	**11,209**	**11,837**	**11,983**
Cairns	**y**	**y**	***Dipterophagus daci*_Bfra485 ***	**C**	**A**	**C**	**G**	**G**	**T**	**C**	**T**	**G**	**A**	**A**	**A**	**A**	**T**	**A**	**G**	**—**
Townsville	y	y	*Dipterophagus daci*_Bn171	T					C		C	A	C	G					A	A
Mourilyan Harbour	y	n	*Dipterophagus daci*_Bn240^			T				G	C	A	C		—	—	—	—	A	A
Mackay	y	y	*Dipterophagus daci*_Bn342					A							—	—	—	—	A	A
Cairns	y	y	*Dipterophagus daci*_Bt194				A													
Mackay	y	y	*Dipterophagus daci*_Bt210		G										—	—	—	—	A	A
Cairns	y	y	*Dipterophagus daci*_Zst503				A													

Table B. 5: continued

Collection locality	*w*Ddac 1	*w*Ddac 2	Gene	*rrnL*	control region														
			Nucleotide position in the mitogenome	13,064	14,967	15,000	15,074	15,075	15,078	15,089	15,135	15,564	15,667	15,668	15,752	15,764	15,043	15,044	15,045	16,070
Cairns	**y**	**y**	***Dipterophagus daci*_Bfra485***	**A**	**G**	**C**	**G**	**T**	**A**	**T**	**G**	**G**	**—**	**—**	**T**	**C**	**T**	**A**	—	**A**
Townsville	y	y	*Dipterophagus daci*_Bn171		T					C	A	A				T				
Mourilyan Harbour	y	n	*Dipterophagus daci*_Bn240^	G																
Mackay	y	y	*Dipterophagus daci*_Bn342			A	T	A	T						A		A	—		
Cairns	y	y	*Dipterophagus daci*_Bt194																	
Mackay	y	y	*Dipterophagus daci*_Bt210										T	A			A		A	
Cairns	y	y	*Dipterophagus daci*_Zst503														A		A	A

Appendix C

Table C. 1*:* Relatively abundant bacterial classes in the four categories of samples (*D. daci* male pupae (Dd), fruit flies parasitised by *Wolbachia*-positive *D. daci* (FliesDdW), fruit flies parasitised by *D. daci* without detectable *Wolbachia* (FliesDd) and unparasitised fruit flies (Flies).

	Flies	FliesDd	FliesDdW	Dd
Alphaproteobacteria	0.19	1.81	2.24	79.23
Bacilli	25.60	32.03	22.06	2.13
Bacteroidia	4.17	4.52	3.29	0.14
Deltaproteobacteria	4.50	3.24	3.71	0.61
Flavobacteriia	1.17	1.06	4.01	0.66
Gammaproteobacteria	63.92	56.24	62.40	16.21
Others	0.44	1.10	2.29	1.03

Table C. 2: Relatively abundant bacterial genera in the 17 *D. daci* pupae samples. The highest available classification was used for taxa with not genus assigned.

Bacterial genera	**Dd1**	**Dd10**	**Dd41**	**Dd55**	**Dd57**	**Dd62**	**Dd64**	**Dd69**	**Dd75**	**Dd91**	**Dd108**	**Dd110**	**Dd111**	**Dd45**	**Dd101**	**Dd11**	**Dd22**
Wolbachia	98.32	91.54	84.71	26.85	96.59	77.56	69.70	44.32	67.68	93.91	93.15	95.60	97.16	60.23	76.87	86.70	78.36
Serratia	0.00	0.00	0.03	21.92	0.22	6.08	6.59	22.82	15.23	0.81	0.06	0.09	0.00	11.50	6.78	0.12	1.59
Trabulsiella	0.00	0.00	0.00	9.39	0.43	5.17	6.91	9.51	4.30	0.00	0.00	0.00	0.00	5.01	0.00	0.03	0.00
unknown Pasteurellales	0.00	0.00	0.00	13.54	0.52	2.79	4.27	7.46	2.96	0.05	0.00	0.00	0.08	3.93	5.24	0.03	0.00
Enterobacter	0.32	0.72	1.94	3.33	0.23	1.89	1.60	2.17	0.89	1.49	1.68	1.21	0.61	1.22	2.16	0.24	6.09
unknown Enterobacteriaceae	0.19	0.55	0.11	2.06	0.34	1.47	2.64	3.19	2.72	0.75	0.98	0.83	0.51	2.01	3.47	0.18	2.30
Lactococcus	0.94	1.18	0.01	1.11	0.08	0.22	0.74	0.46	0.33	1.69	2.12	1.30	0.94	0.18	1.75	0.63	4.97
Providencia	0.04	4.46	0.00	1.99	0.00	0.49	0.91	0.78	0.18	0.35	0.33	0.16	0.02	0.08	0.08	2.94	1.12
Others	0.19	1.54	13.21	19.82	1.58	4.32	6.65	9.30	5.71	0.96	1.69	0.79	0.69	15.84	3.64	9.11	5.58

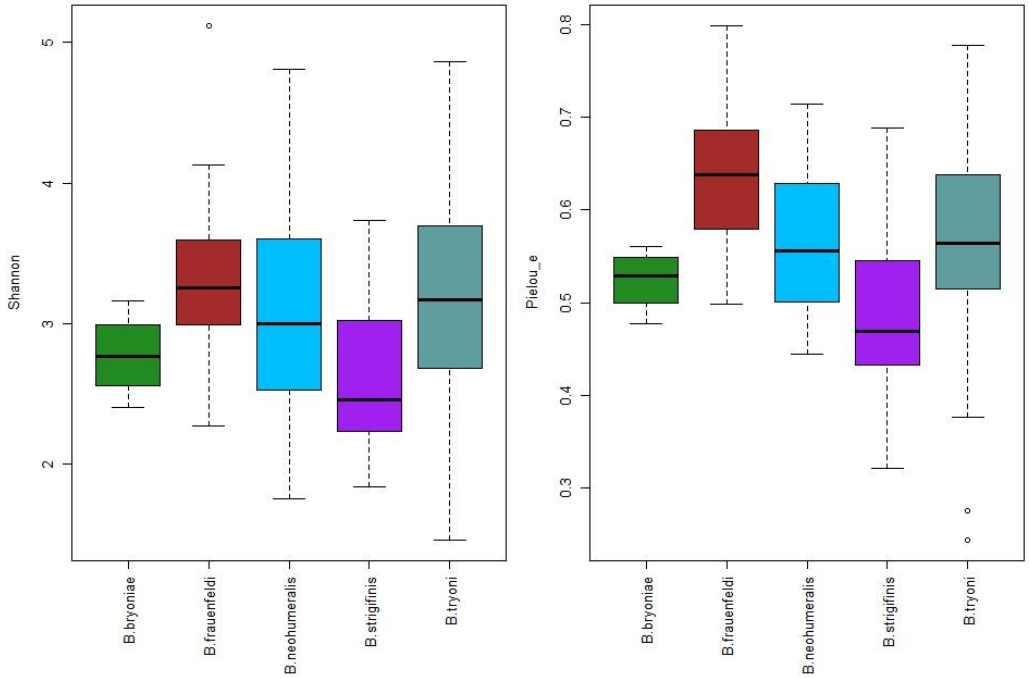

Figure C. 1: Alpha diversity indices of fruit fly host species (*Bactrocera bryoniae, Bactrocera frauenfeldi, Bactrocera neohumeralis, Bactrocera. tryoni, Zeugodacus strigifinis*). (A) Shannon diversity (B) Pielou evenness diversity.

Table C. 3: Relatively abundant bacterial genera in the host fruit flies *Zeugodacus strigifinis*, *Bactrocera bryoniae*, *Bactrocera frauenfeldi*, *Bactrocera neohumeralis*, and *Bactrocera tryoni*.

Bacterial genera	***Z. strigifinis***	***B. bryoniae***	***B. frauenfeldi***	***B. neohumeralis***	***B. tryoni***
Vagococcus	50.32	41.31	7.37	26.00	14.15
Unknown Pasteurellales	1.07	34.84	13.70	20.61	24.85
Unknown Enterobacteriaceae	5.15	2.05	15.11	9.74	6.27
Providencia	6.86	5.87	3.02	7.02	8.18
Enterobacter	0.63	1.12	9.58	7.17	4.43
Acinetobacter	21.14	0.25	8.92	0.02	1.43
Dysgonomonas	0.87	1.88	7.43	2.35	5.37
Unknown Desulfovibrionaceae	0.95	4.83	6.15	3.39	4.48
Klebsiella	2.19	0.03	2.38	2.20	5.75
Citrobacter	1.42	0.04	1.37	1.72	5.85
Trabulsiella	1.04	1.00	0.13	2.10	3.59
Serratia	0.24	0.48	2.63	3.27	1.66
Lactococcus	0.11	0.03	0.20	0.82	2.00
Others	8.01	6.27	22.01	13.59	12.00