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Characterisation of entomopathogenic nematodes and their symbiotic bacteria across eastern Australia and their potential in controlling Queensland fruit fly

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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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Sitaram Aryal

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Preface

This dissertation consists of five chapters that deal with the diversity of entomopathogenic nematodes in eastern Australia, and their potential as biological control agent against Queensland fruit fly (*Bactrocera tryoni*). Chapter 1 includes the general introduction and a review of the literature identifying knowledge gaps, followed by the scope, aims and hypotheses of the thesis. This is followed by three experimental research chapters (Chapter 2, 3 and 4). All three research chapters build on each other. Chapter 2 presents the results of a comprehensive survey of entomopathogenic nematodes (EPNs) from Australian soils, the isolation of EPNs and the molecular identification of each EPN isolate and its bacterial symbiont. Chapter 3 reports the outcome of about laboratory assays to test the pathogenicity of identified and successfully cultured EPNs in the Queensland fruit fly, *Bactrocera tryoni*. Chapter 4 assessed the effects of temperature on the persistence of particularly virulent EPNs identified in chapter 3 to evaluate their potential use as biological control agents of *B. tryoni*. The final part of thesis (Chapter 5) synthesizes the findings of Chapters 2-4 and highlights their importance in conjunction with other related research. This chapter also contains the future directions based on the findings and observations of the thesis.

Chapter 2, 3 and 4 were written in manuscript format for submission to international peer-reviewed journals. Therefore, their introduction and method sections might have some repetitive information. Chapter 2 has been published with the journal *BioControl*. Chapter 3 has been submitted for publication (currently under review with the journal Biological Control) and chapter 4 will be submitted to peer-reviewed journals after thesis submission.

This thesis is a compilation of my work with guidance from my supervisors: Assoc. Prof Markus Riegler (WSU) and Assoc Prof Uffe N Nielsen (WSU). The study was conceptualized jointly by me and my supervisors. I designed the experiments for each chapter with guidance of my supervisors. Soil sampling was done by me and Assoc. Prof Markus Riegler. I have undertaken all the laboratory assays and data analyses with suggestions and feedback from Assoc. Prof Markus Riegler, Assoc Prof Uffe N Nielsen and Dr. Nannette Hope Sumaya. I am the primary author of all thesis chapters with guidance and feedback from my supervisory panel. Each chapter is self-contained with the tables and figures and all references are listed at the end of the thesis.

Finally, this PhD work was completed during the COVID-19 pandemic and, therefore, some laboratory research was affected, and one chapter had to be dropped because of delays that the COVID-19 pandemic has caused with regard to the planned NGS amplicon sequencing of nematode communities (including of EPNs as part of these communities, together with plant parasitic and free-living nematodes) across the Australian ecosystems sampled for this PhD thesis. The DNA-based analyses of whole nematode communities using DNA meta barcoding approaches will be completed after the submission of the PhD thesis.

Supplementary information is presented in appendices as specified below, and details about each chapter contributions are detailed below:

Chapter 1: *General Introduction*

Chapter 2: *Isolation and molecular characterization of five entomopathogenic nematode species and their bacterial symbionts from eastern Australia.*

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This study was conceptualized by Sitaram Aryal, Markus Riegler and Uffe N Nielsen. Soil sampling was done by Sitaram Aryal and Markus Riegler with help from Stefano De Faveri. Data collection and molecular analysis were done by Sitaram Aryal with guidance and suggestions from Markus Riegler, Uffe Nielsen and Nannette Hope Sumaya. Craig Wilson (Ecogrow Environment Pty Ltd) provided guidance on the study design, supply of commercial nematode strains and the structure of the manuscript. Supplemental figures and tables are placed under Appendix A.

Chapter 3: *Virulence, penetration rate and reproductive potential of entomopathogenic nematodes from eastern Australia in Queensland fruit fly, Bactrocera tryoni.*

**Under review with the journal *Biological Control*.

The study was conceptualized by Sitaram Aryal, Markus Riegler and Uffe N Nielsen. Laboratory assays were conducted by Sitaram Aryal. Data collection and analysis were done by Sitaram Aryal with guidance from Markus Riegler and Uffe N Nielsen. Nannette Hope Sumaya helped with the experimental design, data analysis and structure of the manuscript. Craig Wilson provided guidance on the study design and the structure of the manuscript.

Chapter 4: *Persistence and effect of temperature on virulence of Australian entomopathogenic nematodes against Queensland fruit fly, Bactrocera tryoni.*

This study was conceptualized by Sitaram Aryal, Markus Riegler and Uffe N Nielsen. Laboratory assays were conducted by Sitaram Aryal. Data collection and analysis were done by Sitaram Aryal with guidance from Markus Riegler and Uffe N Nielsen. Nannette Hope Sumaya helped with the experimental design, data analysis and structure of the manuscript. Craig Wilson provided guidance on the study design and the structure of the manuscript.

Chapter 5: *Thesis Discussion*

Table of Contents

List of Tables

sandy loam; SOM: soil organic matter). Strain baited with *B. tryoni* is labelled with BT, all others were baited with *T. molitor*. Asterisks (*) denote commercial strains from Ecogrow. Strains with no available DNA sequences are labelled with NA. All localities mentioned are in Australia.

Supplementary Table S3: BLAST results showing closest relatives with GenBank accession numbers and percentage identity of EPN and its associated bacterium from this study. Strains with no available DNA sequences are labelled with NA. Species are listed in alphabetical order.

Supplementary Table S4: Isolation of EPN strains across New South Wales (NSW) and Queensland (QLD), Australia. Asterisk (*) indicates that nematode species infecting bait cadavers (as confirmed by dissection) could not be cultured and identified.

Supplementary Table S5: List of EPN species with their associated bacteria as previously reported and the ones found in this study. Species are listed in alphabetical order.

Supplementary Table S6: List of bacterial species with their associated nematodes as previously reported from Australia. Species in the table are listed in alphabetical order. All regions mentioned are in Australia.

List of Figures

Figure 3.1: *Bactrocera tryoni* packed with EPNs: a) larva infected with Hi.HRN; b) larva infected by Sf.ECCS; c) pupa infected with Hi.HRN; d) pupae infected with Hb.HIE; e-f): larvae that pupated after infection with Hz.NAR1 and Sf.Y13, respectively.

Figure 3.2: LD_{50} of new EPN strains (*Steinernema* grey, *Heterorhabditis* black) and commercial strains (white) tested against *Bactrocera tryoni* larvae after one week of exposure to IJs at 25 °C. Lower LD_{50} values resemble higher virulence. Error bars indicate SD across five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Tukey's HSD test ($p < 0.05$).

Fig. 3.3: LD_{50} of new EPN strains (*Steinernema* grey, *Heterorhabditis* black) and commercial strains (white) tested against *Bactrocera tryoni* pupae after one week of exposure to IJs at 25 °C. Lower LD_{50} values resemble higher virulence. Error bars indicate SD across five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Tukey's HSD test ($p < 0.05$). The blank space indicates no infection occurred.

Figure 3.4: Penetration percentage of IJs in *Bactrocera tryoni* larvae after individuals were inoculated with 100 IJs of each new EPN strain (*Steinernema* grey, *Heterorhabditis* black) and commercial EPN strain (white), assessed after five days at 25 °C. Error bars indicate SD of five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Dunn's test for multiple comparisons.

Figure 3.5: Reproduction potential (average number of IJs produced) of newly isolated EPN strains (*Steinernema* grey, *Heterorhabditis* black) and commercial EPN strains (white). Individual larvae were exposed to IJs for five days and the IJs leaving the larval cadaver was recorded for three weeks. Error bars indicate SD of five replicates. Different letters next to the error bars indicate that means are significantly different as per Tukey's HSD test ($p < 0.05$).

Figure 4.1: LD_{50} of EPN isolates tested against *Bactrocera tryoni* larvae after two weeks of incubation at 15 °C (green), 25 °C (orange) and 30 °C (red). Error bars indicate SD across five replicates. Different letters on top of the error bars indicate that means are significantly different from each other as per Tukey's HSD test ($p < 0.05$). Lower LD_{50} values resemble higher virulence.

Figure 4.2: LD_{50} of EPN isolates tested against *Bactrocera tryoni* larvae after 7, 14 and 21 days of incubation at 25 °C. Error bars indicate SD across five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Tukey's HSD test ($p < 0.05$). Lower LD_{50} values resemble higher virulence.

Figure 4.3: LD_{50} of EPN isolates tested against *Bactrocera tryoni* pupae after 7 and 14 days of incubation at 25 °C. Nematodes incubated for 21 days at 25 °C did not infect pupae (and are therefore not presented). Error bars indicate SD across five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Tukey's HSD test ($p < 0.05$). Lower LD_{50} values resemble higher virulence.

Figure 4.4: Survival (%) of EPN isolates at 15 °C (green), 25 °C (blue) and 30 °C (red). Error bars indicate SD across five replicates.

Supplementary Fig. S1: Variation in *Tenebrio molitor* cadaver color after infection with different EPN isolates. **a**. *Heterorhabditis zealandica* Hz.NAR1, with greenish coloration; **b**. *Heterorhabditis zealandica* Hz.NAR2, with red coloration; **c**. *Heterorhabditis marelatus* Hm. GOS2, with light red coloration; **d**. *Heterorhabditis indica* Hi.LMBt, with orange to brick-red coloration; **e**. *Heterorhabditis bacteriophora* Hb.HIE, with red coloration and ; **f**. *Steinernema feltia* Sf.ECCS, with light brown coloration. The average size of *T. molitor* larvae was 27.73 ± 2.76 mm.

Supplementary Fig. S2: Maximum Likelihood tree of the EPN isolates from eastern Australia (triangles) and other known *Heterorhabditis* spp. based on the analysis of ITS1 (406bp – 1057 bp), with *Oscheius tipulae* as an outgroup; bootstrap values at the nodes (1,000 replicates); GenBank accession numbers in parentheses; scale bar represents number of substitutions per site.

Supplementary Fig. S3: Maximum Likelihood tree of the EPN isolates from eastern Australia (triangles) and other known *Steinernema* spp. based on the analysis of ITS1 (434bp – 1067 bp), with *Oscheius tipulae* as an outgroup; bootstrap values at the nodes (1,000 replicates); GenBank accession numbers in parentheses; scale bar represents number of substitutions per site.

Supplementary Fig. S4: Maximum Likelihood tree of *Pseudomonas protegens* of two S*teinernema feltiae* isolates (triangles) and other *Pseudomonas* spp. based on the analysis of 16S rRNA gene (743 bp to1531 bp), with *Photorhabdus luminescens* as an outgroup; bootstrap values at the nodes (1,000 replicates); GenBank accession numbers in parentheses; scale bar represents number of substitutions per site.

Supplementary Fig. S5: Maximum Likelihood tree of *Delftia acidovorans* of a *Steinernema feltiae* isolate (triangle) and other *Delftia* spp. based on the analysis of 16S rRNA gene (784 bp to 1534 bp), with *Proteus mirabilis* as an outgroup; bootstrap values at the nodes (1,000 replicates); GenBank accession numbers in parentheses; scale bar represents number of substitutions per site.

Supplementary Fig. S6: Life stages of *Bactrocera tryoni* used in this study. a) Late instar larvae (L3) and early-stage pupae and b) pupae (> 3 days old).

Abbreviations

16S rRNA	16S ribosomal RNA gene
ANOVA	analysis of variance
ARC	Australian Research Council
BCA	biocontrol agent
BLAST	basic local alignment search tool
Bt	*Bactrocera tryoni*
χ^2	chi-square
DNA	deoxyribonucleic acid
EG	Ecogrow Environment Pty Ltd
EPN	entomopathogenic nematodes
FLN	free living nematode
Hb	*Heterorhabditis bacteriophora*
Hi	*Heterorhabditis indica*
Hm	*Heterorhabditis marelatus*
HSD	honestly significant difference
Hz	*Heterorhabditis zealandica*
IJ	infective juvenile
ITS	internal transcribed spacer
ITTC	Industrial Transformation Training Centre
KCl	potassium chloride
L	litre

L3	third instar larva
LD_{50}	lethal dose
MAT	male annihilation technique
mL	millilitre
ML	maximum likelihood
MQ	Milli-Q
NaHCO3	sodium bicarbonate
NaOCl	sodium hypochlorite
NCBI	National Center for Biotechnology Information
NSW	New South Wales
PCR	polymerase chain reaction
PPN	plant parasitic nematode
Ps	*Pseudomonas*
QFF	Queensland fruit fly
RH	relative humidity
rRNA	ribosomal ribonucleic acid
Sf	*Steinernema feltiae*
SIT	sterile insect technique

Thesis Abstract

Tephritid fruit flies (Tephritidae) include several destructive pest species that attack fruit and fruiting vegetables across many climatic regions of the world. The Queensland fruit fly, *Bactrocera tryoni*, is Australia's most significant horticultural pest. It lays eggs in the fruit of over 40 plant families, including many important fruit and vegetable crops, making it one of the most polyphagous and most economically important species among all tephritids. So far, one of the most effective control measures is the use of synthetic insecticides. However, many insecticides have been abolished because of their environmental and human health risks and their impact on non-target organisms. Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae and their associated bacteria *Photorhabdus* and *Xenorhabdus*, found in soils throughout the world, are important biocontrol agents of insect pests with soil-inhabiting stages. They have been commercially used as a safe alternative to chemical pesticides. However, the potential of EPNs to control *B. tryoni* is largely unknown.

This PhD thesis comprises five chapters. Chapter 1 reviews the biology and ecology of the Queensland fruit fly, EPNs and their associated symbiotic bacteria. It highlights the potential use of the EPNs as a biological control agent against *B. tryoni*. The factors affecting EPN efficacy against different insect pests, including fruit flies in laboratory and field conditions is explained. This chapter also outlines the scope, aims and hypotheses of my research program.

Chapter 2 comprises a survey and the identification of EPNs and their associated bacteria isolated from soils across eastern Australia. A total of 198 soil samples were collected from citrus orchards, grasslands and forests across temperate, subtropical and tropical climates and baited with *Tenebrio molitor, Galleria mellonella* and *B. tryoni*. This led to the characterisation

of 36 EPN isolates and six of their bacterial symbionts. DNA sequence analysis showed that EPN isolates belonged to five species, specifically *Heterorhabditis bacteriophora*, *Heterorhabditis indica*, *Heterorhabditis marelatus*, *Heterorhabditis zealandica* and *Steinernema feltiae*. Analysis of the bacterial 16S rRNA gene revealed the presence of *Photorhabdus heterorhabditis* in *H. zealandica*; *Photorhabdus laumondii* in *H. bacteriophora* and *H. marelatus*; *Photorhabdus tasmaniensis* in *H. indica* and *H. bacteriophora*; and *Photorhabdus namnaonensis* in *H. zealandica*. The bacterial symbiont of *Steinernema*, *Xenorhabdus*, was not detected in the *S. feltiae* isolates, while the bacteria *Pseudomonas protegens* and *Delftia acidovorans* were identified instead. This study provides the first record of *H. marelatus* from Australia, and *H. indica* and *H. zealandica* from New South Wales. It was also first to report *P. heterorhabditis*, *P. laumondii*, *P. tasmaniensis*, *Ps. protegens* and *D. acidovorans* from EPNs from Australia.

Chapter 3 provides an overview of the virulence of the 32 newly isolated and 4 commercial EPN strains on larval and pupal stages of *B. tryoni*. I also assessed their penetration rate and reproduction potential in *B. tryoni* larvae. All EPN strains were virulent against larvae while 29 strains remarkably caused pupal mortality. All isolates were able to penetrate and reproduce inside *B. tryoni* larvae. Of the tested isolates, Hz.NAR1, Hi.HRN, Hi.ECCH and Hb.HIE were the most virulent isolates and Hz.NAR4 the least virulent isolate against larvae. Similarly, Sf.Y13 and Hi.HIE2 were most virulent, and Sf.YNG the least virulent isolates against *B. tryoni* pupae. The highest reproductive potential was recorded in Sf.ECCS and Hz.NAR2, and the lowest in SC.EG. Finally, Hm.CB, Hi.ECCH, Hi.QF6 and Hz.NAR1 had the highest, and Sf.GG2 recorded lowest penetration rate in *B. tryoni* larvae.

Chapter 4 provides the result of the effects of temperature on survival and persistence of 17 EPN isolates against larvae and pupae of *B. tryoni* under laboratory conditions. I assessed the persistence after 7, 14 and 21 days of EPN inoculation. Furthermore, the effect of temperature (15 °C, 25 °C, and 30 °C) on EPN survival and virulence was tested. All isolates were virulent against larval stages after 7, 14 and 21 days of incubation, while 15 isolates caused pupal mortality after 7 days, two isolates after 14 days and no mortality was observed after 21 days of incubation. All isolates infected and killed larvae at all three temperatures, but the virulence was higher at lower temperatures. Similarly, the EPN survival rate of all isolates was higher at lower temperatures. EPNs were able to survive 18 weeks at 15 °C and 25 °C, while at 30 °C no EPN survived beyond 9 weeks. *Steinernema* showed a higher survival rate than *Heterorhabditis.*

Overall, my research expands substantially the previously available information about EPN diversity in Australia and provides the first comprehensive genetic characterization of this Australian diversity. Furthermore, the EPN isolates such as Hi.HRN2, Hz.NAR1, Hi.ECCH, Hi.LMI2, Hi.QF6, Hb.HIE and Sf.ECCS showed traits, such as high virulence and long persistence, that make them potential candidates for the biological control of fruit flies. The survival and virulence of these isolates, even at higher temperature suggest that they could be a good biological control agent of fruit flies in Australian conditions. However, it is necessary to further check their efficacy in the field before development of applications at a commercial scale.

Chapter 1: General Introduction

1.1. Overview

Several fruit fly species, including of the genus *Bactrocera* are potentially destructive pests of commercial fruits and fruiting vegetables. Queensland fruit fly, *Bactrocera tryoni* (Tephritidae), causes significant loss of horticultural products and is limiting domestic and international market access (Sutherst et al., 2000). It develops in maturing fruit of many plant species and pupates in the soil (Bateman, 1972). Several chemical insecticides for fruit fly control have recently been abolished, and there are few alternative control strategies. Natural enemies of insect pests may provide a solution.

Entomopathogenic nematodes (EPNs) from the families of Steinernematidae and Heterorhabditidae together with their associated bacterial symbionts *Xenorhabdus* and *Photorhabdus* can be used as a potential biological control agent (BCA) targeting *B. tryoni* when they leave the fruit to pupate in the soil. However, the diversity of EPNs and their interactions with symbiotic bacteria and tephritid fruit flies in Australia has not been thoroughly investigated. Australia is a continent with diverse climates and soils which could host yet unknown EPNs which might be more virulent and effective against *B. tryoni* than commercially available EPNs. Collecting locally occurring EPNs may uncover isolates with a higher efficacy due to their better adaptation to local climate and population regulators.

EPNs have been proven effective against several insect pests worldwide under laboratory and field conditions (Ehlers, 2001; Pilz et al., 2014; Toledo et al., 2005), but their effectiveness against *B. tryoni* is mostly unknown. Laboratory trials by Langford et al. (2014) showed the possibility of using three commercially available EPN strains to control *B. tryoni*. However, the EPN efficacy against *B. tryoni* in the field or natural conditions is yet to be tested. The keys to

success with EPNs are to understand their life cycles and functions, matching the correct nematode species with the pest species and applying them during appropriate environmental conditions. EPNs need insect hosts to complete their life cycle. Therefore, EPNs will only persist if there is an ongoing presence of appropriate hosts. Finally, it is important to know the virulence of EPNs against *B. tryoni*, and the persistence of these EPNs, to use them as a BCA in the field.

The overarching hypothesis of my thesis was that Australia harbored a diverse EPN and bacterial community and some of these EPNs together with their bacterial symbiont could be potential biocontrol agents of *B. tryoni*. I setup a series of studies to address the following questions i) how diverse and abundant are the naturally occurring EPN communities in different habitats and climatic regions; ii) do Australian EPNs show host specificity during the baiting process and are isolates captured from *B. tryoni* are likely to provide better control of this pest; iii) are naturally occurring EPN isolates from Australian soils infective and reproductive in *B. tryoni*; and iv) can the EPN isolates survive and remain virulent at field relevant temperatures? This thesis addresses gaps in current knowledge about EPN diversity and abundance in Australia and highlights the potential of Australian native EPN isolates to control *B. tryoni*.

1.2. Literature Review

1.2.1. Queensland fruit fly (*Bactrocera tryoni*)

1.2.1.1. Pest status and importance

Several species of the family of tephritid fruit flies (Diptera: Tephritidae) are destructive pests of commercial fruits and fruiting vegetables, with a wide distribution across the globe (Christenson and Richard, 1960). Key pest genera include *Anastrepha*, *Bactrocera*, *Ceratitis*, *Dacus*, *Rhagoletis* and *Zeugodacus* (Clarke, 2019; White and Elson-Harris, 1992). *Bactrocera* is a

tephritid fly genus with at least 440 species distributed primarily in tropical Asia, the south Pacific, and Australia (White and Elson-Harris, 1992). Most *Bactrocera* species occur in tropical and subtropical regions, and many are not a threat to agriculture. However, approximately 70 species are pests that attack many economically important fruits and fruiting vegetables, causing significant crop losses (Hancock et al., 2000). The Queensland fruit fly, *Bactrocera tryoni* (Froggatt), is Australia's worst horticultural pest insect that can develop in around 240 host plant species, making it one of the most economically important species among all tephritids (Clarke et al., 2011; Hancock et al., 2000). It can cause significant damage in a wide range of fruits and fruiting vegetables and has significant economic impacts in terms of production loss and limiting market access, as even very small levels of infestation (which barely damage produce) can result in complete cancellation of export contracts as importers want to prevent the introduction of any infested produce which could result in the establishment of this highly invasive pest elsewhere. *Bactrocera tryoni* originates from rainforest environments of Queensland and northern New South Wales, but is now widespread in eastern Australia, with the climate's suitability for development declining in the south (Clarke et al., 2011; Sutherst and Yonow, 1998). Currently the species is distributed across most of Queensland and New South Wales, and parts of Victoria and the Northern Territory. Climate is most favourable for *B. tryoni* along the northern and eastern coastal regions of the continent, extending south to central New South Wales (Sutherst and Yonow, 1998). The species has high invasion potential, and has invaded several Pacific Islands (Dominiak and Daniels, 2012; Drew et al., 1978), and regularly experiences outbreaks in South Australia, Northern Territory and Western Australia which require eradication efforts (Cameron et al., 2010; Dominiak and Daniels, 2012; White and Elson-Harris, 1992). Fruit fly has major implications for the sustainable production and market access of Australia's multi-billion

dollar horticultural industry causing direct and indirect annual losses of over $100 million (Clarke, 2019).

1.2.1.2. Life cycle of *B. tryoni*

The life cycle of *B. tryoni* starts with the oviposition of eggs in maturing fruit. The larval stages feed and develop inside the fruit causing direct fruit damage and induce bacterial decay and premature fruit drop (Sutherst et al., 2000). Fully developed larvae (L3) enter the soil soon after they leave the fruit and pupate within one or two days (Bateman, 1972) (Figure 1.1).

Figure 1.1: Life cycle of *Bactrocera tryoni* with different stages above and belowground.

After maturation *B. tryoni* mates at dusk (Pike and Meats, 2002) and adult females require intake of protein before they can produce and lay their eggs into fruit throughout the day. In the absence of sufficient fruits, mature adults disperse to find fruit suitable for oviposition (Bateman and Sonleitner, 1967). Although most of the temperate species of fruit flies overwinter as obligately diapausing pupae, Australian tephritids, including *B. tryoni*, have no pupal diapause and mostly survive as adults with low numbers in winter (Bateman, 1972), albeit females may experience a reproductive diapause during the winter (Fletcher, 1979; O'Loughlin et al., 1984). This multivoltine species produces up to six overlapping generations in a year with population peaks in late summer and early autumn after which populations rapidly decline (Bateman and Sonleitner, 1967; Bot, 1965).

1.2.1.3. Factors affecting fruit fly abundance

Temperature, moisture, availability of hosts and natural enemies are some of the factors influencing the distribution of fruit fly species such as *B. tryoni* (Bateman, 1972; Sutherst and Yonow, 1998). Light quality affects mating behaviour as well as fecundity of *B. tryoni* (Barton-Browne, 1956, 1957). Flies in constant darkness feed little and do not mate, whereas illuminance with 240 lm/sq.ft resulted in increased feeding and egg production (Barton-Browne, 1956). The minimum temperature required for development and mating of *B. tryoni* determines the southern extent of its distribution in cooler climates (O'Loughlin et al., 1984; Sutherst and Yonow, 1998). Reproduction can occur where daily maximum temperatures exceed 20 °C (Meats and Fay, 2000). Even a slight increase in the average temperature (0.5 °C) is expected to result in the southerly spread of *B. tryoni*, primarily as a result of longer summer and autumn seasons, increased development and, consequently, an increase in the number of generations per year (Clarke et al., 2011). However, temperatures over 38 °C for a week or more result in mortality of

all stages (Meats, 1984). Water limitation is considered another key factor restricting the distribution and abundance of *B. tryoni* because of its susceptibility to desiccation in several life stages (Dominiak et al., 2006; Sutherst and Yonow, 1998). Hulthen and Clarke (2006) showed nearly complete pupal mortality in soils with no soil moisture; however, increasing soil moisture to only 10% resulted in nearly 100% pupal survival. Another important factor for *B. tryoni* distribution and abundance is availability and distribution of host plants.

1.2.1.4. Control measures

Several management practices have been applied to bring the population of fruit fly below the damaging threshold in fruit fly endemic areas, or local eradication of outbreaks in areas where fruit fly usually does not occur. Some of them include combination and/or sole use of orchard hygiene and field sanitation (Piñero et al., 2009), protein bait sprays (Piñero et al., 2009), cover sprays (Dominiak and Ekman, 2013), male annihilation technique (MAT) relying on male attractants/lures (Reynolds et al., 2016), attract-and-kill systems (Chuang and Hou, 2008), sterile insect technique (SIT) (Barnes et al., 2015; Reynolds et al., 2014), female based attractants (Siderhurst and Jang, 2010) and parasitoids (Chuang and Hou, 2008; Spinner et al., 2011). Morever, commercial growers together with local communities are practicing area-wide management (AWM) programs as an additional fruit fly management approach which does not just focus on the production areas, but the areas surrounding orchards such as home gardens, public spaces and natural environments (Lloyd et al., 2010). While it is not widely studied, there are indications that natural enemies can have a large impact on *B. tryoni* populations. For example, many soil dwelling insects like ants were found responsible for causing at least 10% mortality in soil-inhabiting stages of a population of *B. tryoni* (Bateman, 1972). Therefore,

farmers need to follow management practices that encourages natural enemies in orchards. This includes practices that use less synthetic chemicals.

So far there has been an overreliance on chemical control, which has led to environmental and human health issues and therefore bans of many chemicals. Malathion has been the most frequently used insecticide against fruit flies in the past (Steiner et al., 1961). Yee and Alston (2012) suggested zeta-cypermethrin as an effective control measure against fruit flies. More targeted neonicotinoid insecticides have been applied in attract-and-kill systems against fruit flies exhibiting longer persistence in the field than other conventional toxicants (Chuang and Hou, 2008). However, efficient organophosphate and neonicotinoids are now banned in many countries because of their hazardous non-target effects (Prokopy et al., 2000; Van der Sluijs et al., 2013). This leads to the urgency towards identifying alternative control options against fruit flies that are safe for humans, beneficial insects (pollinators and natural enemies) and the environment. Therefore, it is necessary to explore robust biological control methods such as encouraging natural enemies already in the agro-ecosystem, introducing specific natural enemies from the area of origin of the pest (also known as classical biological control) (Messing and Wright, 2006) or releasing commercially available natural enemies like entomopathogenic nematodes (EPNs) in an inoculative or inundative way (Ehlers, 2001). Finding effective control practices against fruit fly pests is a challenging task as eggs and most of the larval stages occur inside the fruit (Sutherst et al., 2000). However, late instar larvae leave the fruit for pre-pupal and pupal development in the soil and therefore can be targeted. Also, adult flies emerge from the pupae in the soil and have to crawl to the surface, are mobile and need to feed on diverse food sources (including protein sources) and can be targeted besides larvae and pupae. Once infected

by EPNs, the adults will not die immediately but still have some time to actively fly and move around which can therefore increase dispersal of EPNs.

1.2.2. Entomopathogenic nematodes

Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae associated with the symbiotic bacteria *Xenorhabdus* and *Photorhabdus*, respectively, are used as biological control agents to control a range of insect pests in a variety of crops. The phylogeny of EPNs (De Ley and Blaxter, 2002) and their symbiotic bacteria (Boemare et al., 1993; Fischer-Le Saux et al., 1999b; Thomas and Poinar, 1979) is presented in Figure 1.2 and Figure 1.3.

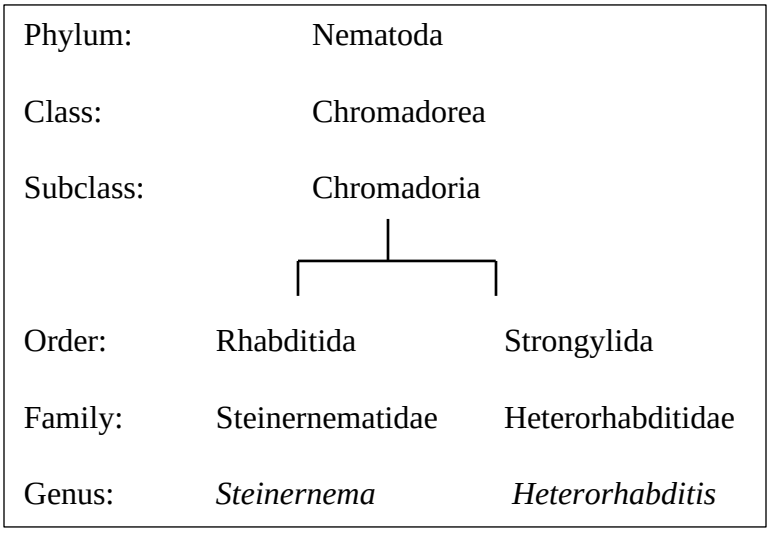

Figure 1.2: Taxonomic positions of entomopathogenic nematodes.

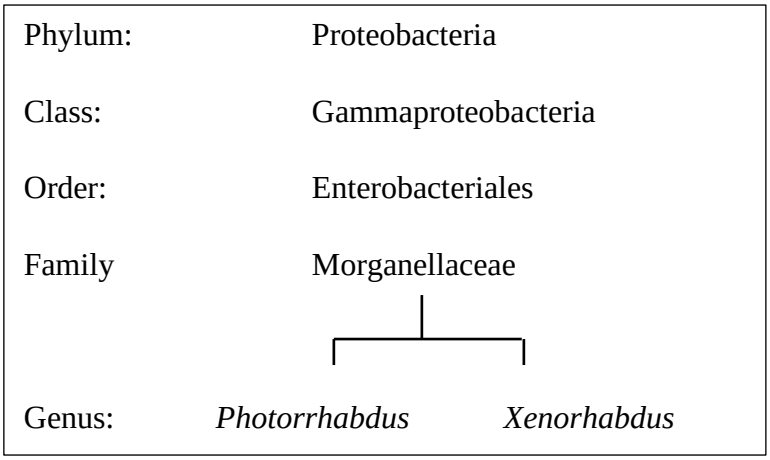

Figure 1.3: Taxonomic positions of bacteria

These nematodes together with their symbionts cause death of insect hosts due to the combined action of the nematode and their symbiotic bacteria (Grewal et al., 2005; Shapiro-Ilan et al., 2009). EPNs have a wide range of insect hosts of the orders Blattodea, Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Orthoptera, Siphonaptera and Thysanoptera (Dlamini et al., 2019; Ehlers, 2001; Georgis et al., 2006; Glazer, 1992; James et al., 2018; Peters, 1996; Shapiro-Ilan and Mizell, 2012). Some examples of economically important insect pests that were successfully targeted using EPNs in the field include *Anastrepha ludens* (Toledo et al., 2005), *Choristoneura occidentalis* (Kaya and Reardon, 1982), *Cydia pomonella* (Odendaal et al., 2016), *Diabrotica virgifera virgifera* (Pilz et al., 2014), *Hyphantria cunea* (Yamanaka et al., 1986), *Phyllocnistis citrella* (Beattie et al., 1995), *Planococcus citri* (Van Niekerk and Malan, 2015), *Plutella xylostella* (Nyasani et al., 2008), *Ostrinia nubilalis* (Ben-Yakir et al., 1998), *Scolytus scolytus* (Finney and Walker, 1979) and *Synathedon pictipes* (Shapiro-Ilan et al., 2016). In general, EPNs are good alternatives to synthetic chemicals as they are safe to humans and the environment (Ehlers, 2003). However, EPNs applied to control certain pests might also attack non-pest insects including predatory insects in soil that may be beneficial. As most of the known EPNs were isolated with a susceptible bait like the wax moth *Galleria mellonella*, the natural hosts of more than 50% of EPNs is unknown (Akhurst and Smith, 2002). Furthermore, there is limited knowledge of EPN effects on non-target organisms in field conditions and very few studies related to EPNs impact on non-target organisms have been carried out. Some examples of reported effects of EPNs on non-target insects are listed in Table 1.1.

Table 1.1: Examples of reported effects of entomopathogenic nematodes on non-target invertebrates, adapted from (Akhurst and Smith, 2002).

Non-target organism	Nematode species	Effect	Reference
Parasitoid insects			
Apanteles militaris	*S. carpocapsae, H. bacteriophora*	Indirect (host death) in lab tests	(Kaya, 1978)
Compsilura concinnata	*S. carpocapsae*	Indirect (host death) in lab tests	(Kaya, 1984)
Apanteles ultor	*S. feltiae, H. bacteriophora*	Indirect (host death) in lab tests	(Triggiani, 1985)
Cephalcia arvensis	*S. feltiae*	Reduced emergence in field	(Battisti, 1994)
Predatory insects			
Harmonia axyridis	*S. carpocapsae*	Some beetles were temporarily paralysed and others were killed in Petri dish assays	(Lemire et al., 1996)
Harpalus sp., *Pterostaticus* sp., *Cicindela* sp., *Tetracha* sp., *Philonthus* sp., *Labidura riparia*	*S. carpocapsae* *H. bacteriophora*	Immature stages but not adults killed in lab test; no effect on populations in field	(Georgis et al., 1991)
Bembidion proerans, Pterostichus cupreus	*S. carpocapsae*	Adults but not larvae killed in lab test; small reduction in field populations	(Ropek and Jaworska, 1994)

Coleomegilla maculata, Olla v-nigrum, Harmonia axyridis, Coccinella septempunctata	*H. bacteriophora, S. carpocapsae*	Mortality of the moth *Agrotis ipsilon* was higher than in all lady beetles. *H. axyridis* and *C. septempunctata* were less susceptible than *C. maculata and O. v-nigrum.* Lower host suitability in *H. axyridis* compared to other beetles.	(Shapiro-Ilan and Cottrell, 2005)
Adalia bipunctata, Chrysoperla carnea	*S. feltiae, S. carpocapsae, H. bacteriophora*	Higher dose exhibits a pronounced effect on the larvae of both predators.	(Rojht et al., 2009)
Other invertebrates			
Onychiurus	*S. carpocapsae*	Reductions in field population	(Poinar Jr, 1989)
Scutigerella immaculata	*S. carpocapsae*	Killed in lab tests	(Poinar Jr, 1989)
Armadillidium vulgare, Porcellio scaber	*S. carpocapsae, H. bacteriophora, S. glaseri*	Killed by *S. carpocapsae* and *H. bacteriophora* in lab test but not by *S. glaseri*	(Poinar Jr, 1989)
Porcellio scaber, Blaniulus guttulattus	*S. carpocapsae, S. feltiae*	Killed in lab tests. *S. feltiae* also killed 50% in glasshouse tests.	(Jaworska, 1991)
Various species of Arachnida	*H. bacteriophora*	Killed in lab tests	(Poinar Jr, 1989)
Amblyomma variegatum, Boophilus microplus, B. annulatus	*Steinernema and Heterorhabditis spp.*	*B. annulatus* was killed by 17 species of nematodes in lab tests but B. *microplus* and *A. variegatum* were	(Mauleon et al., 1993)

		not killed by any nematode species	
Ixodes scapularis	*13 species of Steinernema and Heterorhabditis*	Only engorged females were killed in lab test	(Hill, 1998)
Aporrectodea sp.	*S. carpocapsae*	Intact earthworms were not infected	(Capinera et al., 1982)
Allolobophora caliginosa	*S. scapterisci*	Only dead or injured earthworms were infected. However, nematodes developed on the bacteria associated with earthworm segments	(Nguyen and Smart Jr, 1991)
Aporrectodea caliginosa	*Steinernema sp.*	No effect on earthworm cocoons in lab test	(Nuutinen et al., 1991)
Aporrectodea turgida, Aporrectodea trapezoids, Lumbricus terrestris, and *Eisenia* sp.	*S. carpocapsae, S. glaseri*	No effect in lab tests	(Potter et al., 1994)

1.2.2.1. Life cycle of EPNs

The key to success with the application of EPNs in biological control is to understand their life cycles and functions in order to match the most effective nematode species with the target pest species. This requires a robust understanding of the life cycle of the insect host, its susceptibility and, furthermore, the right environmental conditions for the application, efficacy and persistence of EPNs. EPNs spend most of their life cycle feeding and reproducing in the host's cadaver. The Dauer juveniles, also known as infective juveniles (IJs), are the only free-living stages and are means of transmission from host to host. They live in the soil until they find a new host and enter the host through the insect's natural openings such as anus, mouth, spiracles and, in some cases, through the cuticle (Dowds and Peters, 2002; Koppenhöfer and Fuzy, 2007). The EPNs reach the insects hemolymph through its digestive tract or trachea, and then release their symbiotic bacteria in the hemolymph. Thereafter, the bacteria proliferate, secrete proteins and secondary metabolites that are toxic to the insect hosts resulting in the host's death within 24 to 48 hours. This specific condition is suitable for nematode development and reproduction (Poinar, 1990). As soon as the hosts' nutrients are depleted, the nematodes enter the Dauer stage in which bacterial retention occurs in a vesicle (*Steinernema*), or the anterior and middle part of the intestine (*Heterorhabditis*). Then they leave the host cadaver, search for new hosts, resume feeding and exit from the developmentally arrested Dauer stage due to signals released from the insect hosts or its symbiotic bacteria (Strauch and Ehlers, 1998) and develop into a fourth stage juvenile (J4). After recovery and development into J4, *Steinernema* spp. individuals develop either as males or females, whereas IJs of *Heterorhabdits* spp. always develop into hermaphrodites. In hermaphroditic females, unfertilized eggs produced in the ovary moves to the uterus and are fertilized by sperm in receptaculum seminis (the area between ovary and uterus)

and enter the uterus where embryo development occurs (Johnigk and Ehlers, 1999). Thus, heterorhabditids consist of self-fertile hermaphrodites during the first generation, while subsequent generations produce males, females and hermaphrodites (Dix et al., 1992). On the other hand, steinernematids consist of males and females in all generations and thus reproduce by amphimixis (Poinar, 1990). Both heterorhabditids and steinernematids complete two or more generations inside the host depending upon food availability. At the time of food depletion and increased nematode population density, the pre-Dauer juveniles (J2d) are colonized by the symbiotic bacteria and completely develop into IJs (Ciche et al., 2008). Depending on the body size of the host, a few to hundreds of thousands of IJs emerge from the host cadaver and look for a new host (Griffin, 2012).

Before implementing EPNs as biological control agents, a good knowledge of interactions between EPNs and their symbiotic bacteria is needed (Ciche et al., 2006). Many studies have reported association of bacteria other than their usual symbionts from EPNs (Gouge and Snyder, 2006; Razia et al., 2011). The actual relationship of these other bacteria with EPNs and their usual symbionts, and their role during EPN infection are unknown. However, *Pseudomonas protegens* and *Pseudomonas chlororaphis* from *Steinernema carpocapsae* have been found to be pathogenic against *Galleria mellonella* (Ogier et al., 2020). Therefore, more research needs to be done to understand the complex EPN-bacteria relationships. Although a number of EPN species of the genera *Heterorhabditis* and *Steinernema* and their symbiotic bacteria has been identified across the globe, little is known about their traits like insect host specificity, infectivity, virulence, reproductive ability, and response to of these species of different environmental stress factors.

1.2.2.2. The EPN-bacteria symbiosis and its importance in biological control

The interaction of EPNs and their associated bacterial symbionts are required during the process of infection. The symbiotic bacteria of EPNs have never been found outside nematodes or insect hosts, implying that they would not survive without their host nematodes. However symbiotic bacteria can be cultured in the laboratory on oily agar plates (16 g of nutrient broth, 5 g of yeast extract, 5 g of commercially available vegetable oil, 15 mL of NaPO4 buffer (pH 7.0), and 15 g of Bacto Agar [Difco] in a final volume of 1 L) (Volgyi et al., 1998). The nematodes facilitate the dispersal of the bacteria from one insect host to another, provide shelter from environmental stressors and soil antagonists (Orozco et al., 2013). The bacteria are carried within the entire intestine of *Heterorhabditis* (Ciche and Ensign, 2003) or inside an intestinal vesicle in *Steinernema* (Bird and Akhurst, 1983) .

The bacteria use nematodes as a vector to enter the insect hemocoel (Brillard et al., 2002) and also to persist outside the insect host (Ciche et al., 2006; Daborn et al., 2002). The insect hemocoel could be seen as a sterile environment for bacterial development, where they take up nutrients, replicate and kill insects and provide nutrients for nematode growth, development and reproduction (Ciche et al., 2006; Waterfield et al., 2009). The bacteria proliferate in the hemolymph before the insect dies and must therefore be able to survive attacks by the insect immune response (Li et al., 2014). EPNs employ bacteria to overcome the cellular and humoral defences of insect hosts, to protect the insect cadaver against saprophytic microorganisms, scavenging insects and bacterivorous nematodes, and as a substrate for growth and reproduction (Ciche et al., 2006; Murfin et al., 2012). After penetration of the insect haemocoel, encapsulation of EPNs has been reported in many insect orders including Lepidoptera, Diptera and Coleoptera. Nematodes may resist encapsulation in insects by evasion, tolerance or suppression (Dowds and

Peters, 2002). The bacteria can persist for many weeks within IJs until becoming pathogenic when released within the insect hemocoel (Ciche et al., 2006). Once nutrient availability is depleted, IJs reassociate with the bacterial symbionts and exit the insect cadaver in search of a new host (Orozco et al., 2013). The bacterial symbiont has two alternate life cycle phases, the pathogenic and the symbiotic phase, which show differential expression of genes associated with both virulence and symbiosis (Daborn et al., 2002).

Nematode and its bacterial symbiont both are needed during the infection process. Gerritsen et al. (1998) injected *P. luminescens* in larvae of wax moth G. *mellonella* and crane fly *Tipula oleraceae* and found that the bacteria are able to kill the *G. mellonella* larvae but were not pathogenic to *T. oleraceae*, while the combination of nematode and bacterium killed *T. oleraceae* also. This might be because the nematode was needed to evade a defense mechanism of *T. oleraceae* against the bacteria. Another study showed that in the absence of *P. luminescens*, *H. bacteriophora* is unable to reproduce or cannot develop beyond J1 offspring, and the main significance of this symbiosis is the nutritional dependence of the nematodes on their bacterial associates (Han and Ehlers, 2001). An exception for the strict obligate symbioses of EPNs with bacteria is *S. scapterisci* that can reproduce without its described bacterial symbiont, *Xenorhabdus innexi* (Nguyen and Smart Jr, 1990) and is preferentially activated upon exposure to cricket tissue (Lu et al., 2017b). Besides, Lu et al. (2017a) showed that *S. carpocapsae* can releases venom proteins and therefore have a more active role during infection than just relying upon their symbiotic bacteria.

1.2.2.3. Symbiont host specificity and bacterial host switching

Many studies have investigated host specificity of bacterial symbionts associated with EPNs and found a high degree of specificity between bacteria and nematodes (Emelianoff et al., 2008;

Gaudriault et al., 2006). Gaudriault et al. (2006) observed highly specific association between EPNs and their bacterial strains such as for *H. bacteriophora* (*P. luminescens* subsp. *laumondii*) and *H. megidis* (*P. temperata* subsp. *temperata*). However, studies have demonstrated that bacterial strains can be experimentally switched. For this, bacteria that are extracted from infected insect and cultured in oily agar plates (Volgyi et al., 1998) are grown together with aposymbiotic EPNs (without bacteria), where the EPNs acquire the non-cognate symbiont (McMullen et al., 2017). Association with bacteria other than their original symbionts result in decreased EPN fitness, infectivity, reproductive potential and virulence (Gerritsen and Smits, 1993; McMullen et al., 2017). This also negatively affects the bacterial transmission to the next generation, thus resulting in lower virulence of progeny (McMullen et al., 2017). Similarly, when cultured on the bacterial symbionts of *H. megidis*, no or only a few *H. bacteriophora* infective juveniles retained these symbiotic bacteria, and the IJs without symbiotic bacteria were not pathogenic to insects (Gerritsen et al., 1998). Furthermore, *H. bacteriophora* failed to reproduce on the bacterial symbionts of *H. indica*, and *H. indica* did also not reproduce on the bacterial symbiont isolated from another *Heterorhabditis* species (Q6) (Han and Ehlers, 1998). This can be attributed to bacterial specificity and preference for the nematode's development and reproduction. On the other hand, *S. scapterisci* was able to use and retain symbionts of other Steinernematids, but IJ recovery was delayed indicating a high degree of specificity between the nematode and its symbiont for optimal IJ recovery (Grewal et al., 1997). Furthermore, the pathogenicity, development and reproduction of *S. scapterisci* IJs seems significantly higher in the presence of *Xenorhabdus* strain UY61 than with other bacterial strains (Bonifassi et al., 1999). Akhurst et al. (1996) showed that the *Photorhabdus* strains HbT and D1 isolated from Australian *H. bacteriophora* and *H. indica* populations, respectively, are only compatible with

their hosts. Primary-specific genes are involved in the bacteria-nematode symbiotic interaction and the symbiotic relationships involve complex molecular interactions that require coordinated gene expression within, and transspecies signalling between the bacteria and the host (Forst and Clarke, 2002). Both *Xenorhabdus* and *Photorhabdus* produce bacteriocins, which are proteins that have antimicrobial activity against closely related strains or species of bacteria (Adams et al., 2006; Boemare et al., 1997). Overall, one can therefore conclude that the nematode-bacteria associations are highly species-specific. These associations as well as bacterial preference have a profound influence on the development and reproduction of the nematodes and the degree of infectivity caused to the insect hosts. Association of bacterial symbiont with different EPN species is presented in Table 1.2.

Table 1.2: *Photorhabdus* and *Xenorhabdus* association with different EPN species

Symbiotic bacteria	Associated nematode species	References
X. beddingii	*Steinernema* sp., *S. longicaudum*	(Akhurst and Boemare, 1988; Lee and Stock, 2010)
X. bovienii	*S. affine, S. anatoliense, S. costaricense, S. feltiae, S. intermedium, S. jollieti, S. kraussei, S. litorale, S. nguyeni, S. oregonense, S. puntauvense, S. sichaunense, S. weiseri, S. silvaticum*	(Akhurst, 1983; Akhurst and Boemare, 1988; Boemare and Akhurst, 1988; Boemare et al., 1993; Dreyer et al., 2017; Kazimierczak et al., 2016; Kuwata et al., 2006; Lee and Stock, 2010; Malan et al., 2016; Tailliez et al., 2006)
X. budapestensis	*S. bicornutum, S. ceratophorum*	(Lengyel et al., 2005; Tailliez et al., 2006)
X. cabanillasii	*S. riobrave*	(Tailliez et al., 2006)
X. doucetiae	*S. diaprepesi*	(Tailliez et al., 2006)
X. eapokensis	*S. eapokense*	(Kämpfer et al., 2017)
X. ehlersii	*S. serratum, S. longicaudum*	(Lengyel et al., 2005; Tailliez et al., 2006)
X. griffiniae	*S. hermaphroditum (*Previously referred to as *S. dharanai*) *S. litchi, S. khoisanae*	(Dreyer et al., 2018; Tailliez et al., 2006)
X. hominickii	*S. karii, S. monticolum*	(Fischer-Le Saux et al., 1998; Tailliez et al., 2006)
X. indica	*S. thermophilum, S. yirgalemense, S. abbasi*	(Ferreira et al., 2016; Somvanshi et al., 2006; Tailliez et al., 2006)
X. innexi	*S. scapterisci*	(Lengyel et al., 2005)
X. ishibashii	*S. aciari*	(Kuwata et al., 2013)
X. japonicus	*S. kushidai*	(Nishimura et al., 1994)

X. khoisanae	*S. khoisanae, S. jeffreyense, S. sacchari, S. beitlechemie*	(Ferreira et al., 2013b)
X. koppenhoeferi	*S. scarabaei*	(Tailliez et al., 2006)
X. kozodoii	*S. arenarium, S. apuliae, S. boemarei*	(Boemare et al., 1993; Lee and Stock, 2010; Tailliez et al., 2006)
X. magdalenensis	*S. australe*	(Tailliez et al., 2012)
X. mauleonii	*Steinernema sp.*	(Tailliez et al., 2006)
X. miraniensis	Nematode from the family Steinernematidae, isolated from Australia	(Boemare et al., 1993)
X. nematophila	*S. carpocapsae* (previously referred to as *S. anatoliense*) *S. websteri*	(Lee and Stock, 2010; Park et al., 1999)
X. poinarii	*S. glaseri, S. cubanum*	(Akhurst, 1983; Fischer-Le Saux et al., 1999a)
X. romanii	*S. puertoricense*	(Tailliez et al., 2006)
X. stockiae	*S. siamkayai*	(Tailliez et al., 2006)
X. szentirmaii	*S. rarum, S. costaricense*	(Lee and Stock, 2010; Lengyel et al., 2005)
X. thuongxuanensis	*S. sangi*	(Kämpfer et al., 2017)
X. vietnamensis	*S. sangi*	(Tailliez et al., 2010)
P. asymbiotica	*H. indica (one single strain only)*	(Akhurst et al., 2004; Fischer-Le Saux et al., 1999b; Machado et al., 2018)
P. australis		(Akhurst et al., 2004)
P. luminescens	*H. indica, H. bacteriophora*	(Boemare et al., 1993; Fischer-Le Saux et al., 1999b; Machado et al., 2018)

P. akhurstii	*H. indica, H. bacteriophora*	(Akhurst et al., 2004; Fischer-Le Saux et al., 1999b; Machado et al., 2018)
P. laumondii subsp. *laumondii*	*H. bacteriophora, H. safricana*	(Fischer-Le Saux et al., 1999b; Geldenhuys et al., 2016; Machado et al., 2018; Malan et al., 2008)
P. hainanensis	*Heterorhabditis sp.*	(Machado et al., 2018; Tailliez et al., 2010)
P. carribeanensis	*H. bacteriophora*	(Machado et al., 2018; Tailliez et al., 2010)
P. noenieputensis	*H.* noenieputensis	(Ferreira et al., 2013a; Machado et al., 2018; Malan et al., 2014)
P. kayaii	*H. bacteriophora*	(Hazir et al., 2004; Machado et al., 2018)
P. bodei		(Machado et al., 2018)
P. kleinii	*H. bacteriophora; H. georgiana*	(An and Grewal, 2011; Machado et al., 2018; Maneesakorn et al., 2011)
P. thracensis	*H. bacteriophora*	(Hazir et al., 2004; Machado et al., 2018; Tailliez et al., 2010)
P. khaini	*H. bacteriophora, H. megidis, H. Helioditis*	(Machado et al., 2018; Maneesakorn et al., 2011; Tailliez et al., 2010)
P. tasmaniensis	*H. zealandica, H. marelatus*	(Machado et al., 2018; Maneesakorn et al., 2011; Tailliez et al., 2010)
P. temperata	*H. downesi, H. bacteriophora, H. megidis, H. zealandica*	(Fischer-Le Saux et al., 1999b; Machado et al., 2018; Maneesakorn et al., 2011)

P. cinerea	*H. downesi, H. megidis*	(Machado et al., 2018; Maneesakorn et al., 2011)
P. stackebrandtii	*H. bacteriophora, H. georgiana, H. megidis*	(An and Grewal, 2010; Machado et al., 2018; Maneesakorn et al., 2011)
P. namnaonensis	*H. baujardi*	(Glaeser et al., 2017; Machado et al., 2018)
P. heterorhabditis	*H. zealandica*	(Ferreira et al., 2014; Machado et al., 2018)

1.2.2.4. EPN-bacterium complex and virulence factors

Nematodes develop into IJs that become colonized by symbiotic bacteria (Ciche et al., 2006). These symbiotic bacteria are a good source of anti-insecticidal, antimicrobial and other bioactive molecules (Murfin et al., 2012). In the intestine, the complex of the EPN and its bacteria faces insect humoral and cellular defence mechanisms (Ciche et al., 2006) and to cope with such insect defences, several virulence factors including bacterial toxins (Bowen et al., 1998; Dowling and Waterfield, 2007), insecticidal proteins and hydrolytic enzymes (e.g. proteases, lipases, and chitinases) (Bode, 2009; Brillard et al., 2002) are released by the bacteria in the insect hemocoel which eventually kill the insect host. The toxin complexes are large multi-subunit insecticidal toxins of high molecular weight produced both by gram-negative and gram-positive bacteria and placed on their outer surface (Dowling and Waterfield, 2007). Wang and Gaugler (1999) extracted *Steinernema glaseri* surface coat proteins and found that surface coat protein 3a can suppress the insect host immune system which then protects nematode species from encapsulation and hemocyte lysis and its symbiotic bacteria from phagocytosis. Münch et al. (2008) found that Plu4122 is a putative novel *Photorhabdus* toxin, which contains proteins that bundle actin filaments, thereby affecting cell division, adhesion and motility. Furthermore, the same author found that plu1463 encodes a tail-fibre protein that is induced in *P. luminescens* upon entering the insect. A dominant virulence factor like the makes caterpillar floppy toxin is however found to be critical for pathogenesis causing a rapid destruction of the insect gut epithelium, resulting in the caterpillars losing all body turgor and becoming floppy (Daborn et al., 2002; Waterfield et al., 2003). Moreover, antibiotics that inhibit the growth of competing microorganisms in the insect cadaver, toxic proteins (e.g. hemolysins), cell surface proteins that mediate bacterial attachment, cell surface carbohydrates and proteins that protect bacteria are

also produced during the infection process. These enzymes and proteins help EPN and bacteria to establish within the hosts, protect against host defence mechanisms and helps in killing and bioconversion of the insect host (Brillard et al., 2002; Ciche et al., 2006; Derzelle et al., 2004; Waterfield et al., 2009). Although, many virulence factors are involved in the infectivity of EPNs and their associated bacteria, it is not clear which factors are important for EPN symbionts.

1.2.3. Factors affecting EPN efficacy under field conditions

Production costs and limited regional availability of EPNs are two key factors limiting the widespread adoption of entomopathogenic nematodes in biological control programs. Morever, host seeking ability and ability to persist in soil after application plays an important role in the effectiveness of any EPNs. For their development, EPNs require insect hosts. Without the presence of hosts EPNs have limited ability to survive in the soil, yet insect hosts (in particular target pests) might not be always available. Therefore, the success of EPN hugely depends upon the ability of EPNs to survive and remain infective against target pests in the soil until they find the host. It has also been reported that EPNs survive longer in the field than under laboratory conditions (Pilz et al., 2014; Susurluk and Ehlers, 2008). The long persistence in the field might be due to offspring production in other suitable insect hosts. Although the presence of alternate hosts can support maintenance of EPN populations in the soil during the time without the target hosts, this field of research is yet to be further explored.

Several biotic and abiotic factors, EPN traits and insect host traits affect EPN efficacy (Labaude and Griffin, 2018). Presence of competitors, scavengers and predators can adversely affect EPN abundances in the soil (Mertz et al., 2015; Ulug et al., 2014). Most importantly, the insect cadaver killed by EPNs can be attacked by scavengers or omnivores which could result in death

of nematodes and the bacterial symbionts that are developing inside the cadaver (Kaya, 2002). Other biotic factors including susceptibility of insect hosts and life stages to be targeted (Bedding et al., 1993), EPN species/ isolates, and the associated bacteria (Grewal et al., 1997) can impact the virulence of EPNs and therefore the abundancy. Abiotic factors like soil type, pH, moisture and temperature affect the survival and abundance of EPNs (Koppenhöfer and Fuzy, 2007; Kung et al., 1990a, b; Rohde et al., 2010). Furthermore, different EPNs react differently to UV light (Shapiro-Ilan et al., 2015) and desiccation (Mukuka et al., 2010a; Shapiro-Ilan et al., 2014), which have a detrimental effect on the density or abundance of EPNs in soil. Soil moisture plays an important role in EPN efficacy and is the main factor affecting survival and movement of nematodes (Baimey et al., 2015; Pilz et al., 2014). Furthermore, EPN populations are also affected by habitat, vegetation and land use type (Bal et al., 2017; Canhilal et al., 2017; Hoy et al., 2008). Therefore, it is necessary to conduct laboratory and field tests to check EPN compatibility with different factors and their efficacy against target insect hosts before application at a larger scale.

1.2.4. Fruit fly control with EPNs

Several laboratory studies have been conducted to test the effectiveness of different species of EPNs against fruit flies, and mostly focused on larval stages (Langford et al., 2014; Sirjani et al., 2009; Stark and Lacey, 1999; Toledo et al., 2005). Adults of *B. zonata*, *Dacus ciliates*, *R. indifferens* were found susceptible to EPNs (Kamali et al., 2013; Mahmoud et al., 2016; Yee and Lacey, 2003). Furthermore, some of the previous studies reported pupal infection (Barbosa-Negrisoli et al., 2009; Godjo et al., 2018b; Heve et al., 2017) while others reported no pupal infection (Abbas et al., 2016; Karagoz et al., 2009a; Langford et al., 2014; Yee and Lacey, 2003) with EPNs against several species of fruit flies. Sirjani et al. (2009) found the potential of *S.*

feltiae to suppress the populations of mature larvae of *Bactrocera oleae* in soil as well as in dropped infested olive fruits. Although EPNs like *S. feltiae* (Langford et al., 2014; Mahmoud et al., 2016), *H. bacteriophora* (Abbas et al., 2016; Barbosa-Negrisoli et al., 2009; Langford et al., 2014) and *S. carpocapsae* (Rohde et al., 2010; Toledo et al., 2009) were found to be virulent against several tephritid pests, research on EPN-based *B. tryoni* biocontrol has not yet been explored under field conditions. These findings demonstrated that EPNs have potential to control fruit flies. However, it is necessary to improve the efficacy of EPNs and further test their virulence in the fields to select isolates that can be used as an effective biological control agent against fruit flies.

1.3. EPNs in Australia

The interest and research into the biology, ecology and applications of EPNs has grown remarkably over the past few decades. The search for EPNs has led to the isolation of numerous new isolates and strains of EPNs. Despite this increase in surveys to discover native EPN species, their efficacy and success entail a comprehensive knowledge and understanding of the biology, ecology and distribution across variable environments and biogeographic regions. For instance, obtaining environmental parameters associated with the prevalence of EPN during surveys could unravel crucial factors that drive the distribution of these nematode species. There is also a need to more clearly define specific relationship between EPNs and the symbiotic bacteria, as little is known about bacterial diversity associated with EPNs from Australia. The symbiotic bacteria play a crucial role for insect host infection and nematode growth and development. Their dual nature as a pathogen and mutualist can be an excellent study system to elucidate bacteria-EPN-insect host interactions. EPN-bacteria interactions, thus, cannot be ignored. It is also essential to know and understand the biology and specificity of associated

bacteria for effective biological control using EPNs. Furthermore, knowledge of the role of non-related symbionts on EPN efficacy is another interesting field of research.

Australia is a continent with diverse climates, ecosystems, soil ecology, and land use systems. This diversity is expected to provide a large diversity of microorganisms including those of EPNs that might be more virulent and effective against insect pests. Knowledge on the diversity and distribution of EPNs will serve as a basis to improve the efficiency of biological control programs. Although EPNs such as *H. bacteriophora, H. indica, H. zealandica, S. feltiae, S. carpocapsae, S. longicaudam* have been previously recorded from Australia (Akhurst and Bedding, 1986; Fischer-Le Saux et al., 1998; Hominick, 2002; Poinar, 1975; Poinar, 1990; Sagun et al., 2015; Stack et al., 2000)(), very little is known about their potential for the control of *B. tryoni*. Furthermore, previous research presented promising use of EPNs against fruit flies (Godjo et al., 2018b; Langford et al., 2014). However, in order to be an effective biocontrol agent of insect pests, EPNs need to be adapted to the local environmental conditions (Bedding, 1990; Stock et al., 2008). Therefore, the isolation and characterization of indigenous or native EPN populations is an important research task to obtain candidate EPNs with better adaptation to local conditions and higher efficacy against local pests. As Australian soils have not yet been fully explored for their EPN diversity, this is the rationale for the primary scope to explore new EPN species and/or isolates, including first occurrence records for EPN species and strains. A more detailed survey to look for EPNs and their symbiotic bacteria needs to be carried out to identify nematode-bacteria combination that can be potential candidates for biological control of *B. tryoni* and other tephritids.

1.4. Research scope and aims

This PhD includes a detailed survey and identification of EPNs and their related bacteria from eastern Australia to assess their biological diversity in these regions. This also comprises assays to test their virulence, persistence and the effect of temperature on survival and virulence of EPNs against *B. tryoni* to identify species with commercial potential. With this fundamental information, it is hoped that better management strategies are obtained for the significant pest *B. tryoni*. In general, the target was to acquire EPN isolates with high virulence, fast host-seeking behaviour and long persistence.

The specific aims of this thesis were to:

1. determine the diversity and abundance of naturally occurring EPNs in orchards, forests and grasslands from eastern Australia;
2. isolate and characterize the bacterial symbionts associated with these EPNs;
3. assess the host preferences of EPNs during the baiting process;
4. investigate the virulence, penetration rate and reproductive fitness of isolated EPN strains against larvae and pupae of *B. tryoni*;
5. test the persistence and effect of temperature on EPN survival and virulence against *B. tryoni*.

The specific hypotheses of this thesis were:

1. EPN species abundance varies among habitats such as forests, orchards, and grasslands. The forests and grasslands soil are less disturbed and therefore suitable for EPN to survive and persist for longer due to higher nutrient and mineral availability along with favourable

soil texture, temperature and moisture conditions. Hence, higher EPN abundances are likely observed in forests and grasslands (Chapter 2).

2. Symbiotic bacteria vary with EPN species across regions. It is hypothesised that there is a likely substantial diversity of EPN-associated bacteria in Australian soils that are yet to be described. It is expected that several species of bacteria are associated with more than one nematode species, and with any newly isolated EPNs, new bacterial isolates or subspecies may also be found (Chapter 2).
3. Insect hosts belonging to different orders and with varying susceptibility such as *G. mellonella*, *T. molitor* and *B. tryoni* can be used to bait different EPN isolates and/or species. As the life stage development (i.e., late instar larva to pupa) of *B. tryoni* in the soil is around 1-2 days, only nematodes with better host finding ability and higher penetration rate can locate and successfully enter them. Hence, *B. tryoni* can provide unique isolate(s) (Chapter 2).
4. EPN species and/or isolates differ in survival, virulence and reproductive capacity against a specific insect pest. It is assumed that some of the newly recovered and identified species and/or isolates may have high potential as biological control agents of pests. There can be a higher reproductive potential in the EPN virulent species. Nematodes baited with *B. tryoni* could be more virulent against fruit fly than the nematodes baited with other insects. It was assumed that a higher infectivity with higher reproduction potential is observed with EPNs baited with *B. tryoni* due to initial selection pressure and early host-EPN interactions (Chapter 3).
5. Temperature plays a vital role in EPN survival and influences the infectivity and virulence of EPNs. It was expected that EPN survival was reduced with increasing

temperatures. Considering that EPN isolates were obtained from harsh Australian sub-tropical and warm temperate climates, their infectivity and virulence could be higher at higher temperatures. It was hypothesized that nematode isolates will persist longer than the commercial strains at higher temperature (Chapter 4).

Chapter 2: Isolation and molecular characterization of five entomopathogenic nematode species and their bacterial symbionts from eastern Australia

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2.1. Abstract

Entomopathogenic nematodes (EPNs) are used in biological control of pest insects but their potential may be limited by strain availability from different bioregions and effectiveness against specific pests. Here, we isolated and characterized EPNs and their symbiotic bacteria from Australia where their diversity is scarcely known. We collected 198 soil samples from citrus orchards, grasslands and forests across temperate, subtropical and tropical eastern Australia. EPNs were isolated by baiting with mealworm, greater wax moth and Queensland fruit fly, the Australia's most significant horticultural pest. We obtained 36 isolates which, according to DNA sequence analyses, represented five species, *Heterorhabditis bacteriophora*, *Heterorhabditis indica*, *Heterorhabditis marelatus*, *Heterorhabditis zealandica* and *Steinernema feltiae*, including the first report of *H. marelatus* from Australia, and *H. indica* and *H. zealandica* from New South Wales. Thirty-five isolates were baited with mealworm, one with fruit fly, and none with wax moth. *Heterorhabditis marelatus* was recovered from forests, *H. bacteriophora* from citrus orchards, *S. feltiae* from citrus orchards and grasslands, *H. indica* and *H. zealandica* from all three habitats. According to bacterial DNA analyses, *Photorhabdus heterorhabditis* occurred in *H. zealandica* and a reference strain of *H. bacteriophora*; *Photorhabdus laumondii*[1] in *H. bacteriophora* and *H. marelatus*; *Photorhabdus tasmaniensis* in *H. indica* and *H. bacteriophora*; and *Photorhabdus namnaonensis* in *H. zealandica*. Unexpectedly, *Pseudomonas protegens* and *Delftia acidovorans* were found in *S. feltiae* while its expected symbiont *Xenorhabdus* was undetected, possibly due to our approach. The newly isolated EPNs should be tested as biological control agents against pest insects.

[1] *P. laumondii* was also detected in *H. indica*.

Keywords: *Bactrocera tryoni*, *Tenebrio molitor*, 16S rRNA gene, D2-D3 region, ITS1, phylogenetic analysis

2.2. Introduction

Entomopathogenic nematodes (EPNs) are ubiquitous in terrestrial ecosystems and have been recorded from all continents except Antarctica (Hominick, 2002). Continental scale variation in climate and environmental variables are likely to affect local EPN communities and thus assessing EPN assemblages from different climatic regions and habitats with varying land use and management is important. EPNs serve as important population regulators of insect species with soil-dwelling stages, and have been applied as biological control agents for a variety of economically important pest insects (Griffin et al., 2005). Despite a current surge in surveys to discover native EPN species for biological control (Godjo et al., 2018a), their efficacy and success in pest management entail a comprehensive understanding of their biology, ecology, diversity, distribution and adaptation to local environmental conditions.

EPNs encompass *Steinernema* (Steinernematidae) and *Heterorhabditis* (Heterorhabditidae). As obligate parasites of insects they feed and reproduce within the host's cadaver, then leave as infective juveniles (IJ) and remain in the soil until they find a new host (Grewal et al., 2005). EPNs are associated with symbiotic bacteria, *Xenorhabdus* (*Steinernema*) and *Photorhabdus* (*Heterorhabditis*), which have never been found freely in soil. The symbionts are essential for host infection. Conversely they require the EPNs to enter the insect hemocoel (Brillard et al., 2002) and persist outside the insect host (Ciche et al., 2006). The EPNs reach the insect hemolymph through the natural openings such as anus, mouth and spiracles, and, in some cases, through the cuticle (Dowds and Peters, 2002). They then release the bacteria that proliferate in the insect hemolymph resulting in sepsis and insect death within 24 to 48 hours, creating suitable conditions for EPN development and reproduction (Poinar, 1990). As soon as the nutrients in the cadaver are depleted, the EPNs enter the Dauer stage in which bacterial retention occurs in a

vesicle (*Steinernema*) or the anterior and middle part of the intestine (*Heterorhabditis*) before they leave as IJs. While *Xenorhabdus* is always found in *Steinernema*, and *Photorhabdus* always in *Heterorhabditis*, the bacterial species can vary with EPN species. Furthermore, several bacterial species have been found in more than just one EPN species (Maneesakorn et al., 2011), and some EPNs appear to have other bacteria such as *Alcaligens*, *Pseudomonas*, *Delftia* and *Ochrobactrum* (Ogier et al., 2020). However, their role and specificity remain unclear.

The diversity and abundance of EPNs may vary across biogeographic and climatic regions, and only a few tolerant and unique EPNs may be found in more extreme environments. EPNs are also affected by edaphic variables including soil texture, soil organic matter (SOM), moisture, temperature, pH as well as biotic factors like host availability, predators, pathogens and vegetation (Hoy et al., 2008; Susurluk and Ehlers, 2008). However, the distribution of EPN species remains poorly studied.

EPNs have been recovered from different habitats across biogeographic and climatic regions, with isolation success rates ranging from around 2% to over 45% (Hominick, 2002). Globally, ~ 100 *Steinernema* species (Cimen et al., 2016; Liu et al., 1998), 26 *Xenorhabdus* species (Lengyel et al., 2005), > 25 *Heterorhabditis* species (Akhurst and Bedding, 1986; Malan et al., 2014) and 25 *Photorhabdus* species (Machado et al., 2021; Machado et al., 2018) have been described. However, the EPN diversity in Australia is with only six species (*H. bacteriophora, H. indica*, *H. zealandica S. carpocapsae*, *S. feltiae, S. longicaudam*) scarcely known (see Supplementary Table S1), and the bacterial symbiont diversity nearly unknown. Most efforts to isolate EPNs from soils use larvae of the greater wax moth (*Galleria mellonella*) and, to a lesser degree, mealworm (*Tenebrio molitor*) as baits (Bedding and Akhurst, 1975; James et al., 2018). For most EPNs, *G. mellonella* larvae are better hosts than *T. molitor* larvae because of their softer cuticle

and a larger body size (Boff et al., 2000). Although less susceptible, *T. molitor* might increase chances of obtaining more diverse EPN species that are possibly also more effective against beetle pests (Koppenhöfer and Fuzy, 2003). Using the target pest as a bait might also increase the chances of obtaining EPN strains that are particularly virulent against that pest. Queensland fruit fly (*Bactrocera tryoni*) is Australia's most significant horticultural pest, and occurs throughout tropical, subtropical and temperate regions of eastern Australia (Dominiak and Mapson, 2017). While it oviposits eggs into ripening fruit, mature third instar larvae (L3) leave the fruit, pupate in the soil and remain in the soil until adult emergence (Hulthen and Clarke, 2006). Therefore, this pest is naturally exposed to EPNs in the soil.

Our aim was to investigate the diversity and distribution of Australian EPNs and their symbiotic bacteria throughout the range of *B. tryoni*, including the recovery of EPNs by using different bait species, as this may provide more diversity and higher infectivity due to better adaptation to particular insect hosts such as *B. tryoni*. Ecological studies have demonstrated that EPNs are sensitive to climatic and edaphic properties. Hence, we isolated and characterized the EPN and bacterial symbiont diversity in natural and managed ecosystems across tropical, subtropical and temperate Australia, focusing on species that may be tested as potential biological control agents against *B. tryoni* in the future.

2.3. Material and methods

2.3.1. Soil sampling

We collected 198 soil samples from orchards, grasslands and forests across tropical, subtropical and temperate regions of eastern Australia (Figure 2.1; Supplementary Table S2) following a random sampling method (Orozco et al., 2014). At each location we identified three sampling

sites with a minimum distance of 100 m. Five subsampling sites, all within a 3 x 3 m^2 area plot were identified and a composite soil sample of 3 kg was obtained at a depth of 0-30 cm (De Brida et al., 2017). The soil was placed in zip lock plastic bags, stored at 15 °C and processed within five days of collection. Most samples were collected in October-November 2018 (late spring) and March 2019 (early autumn) when *B. tryoni* populations peak (Fletcher, 1974).

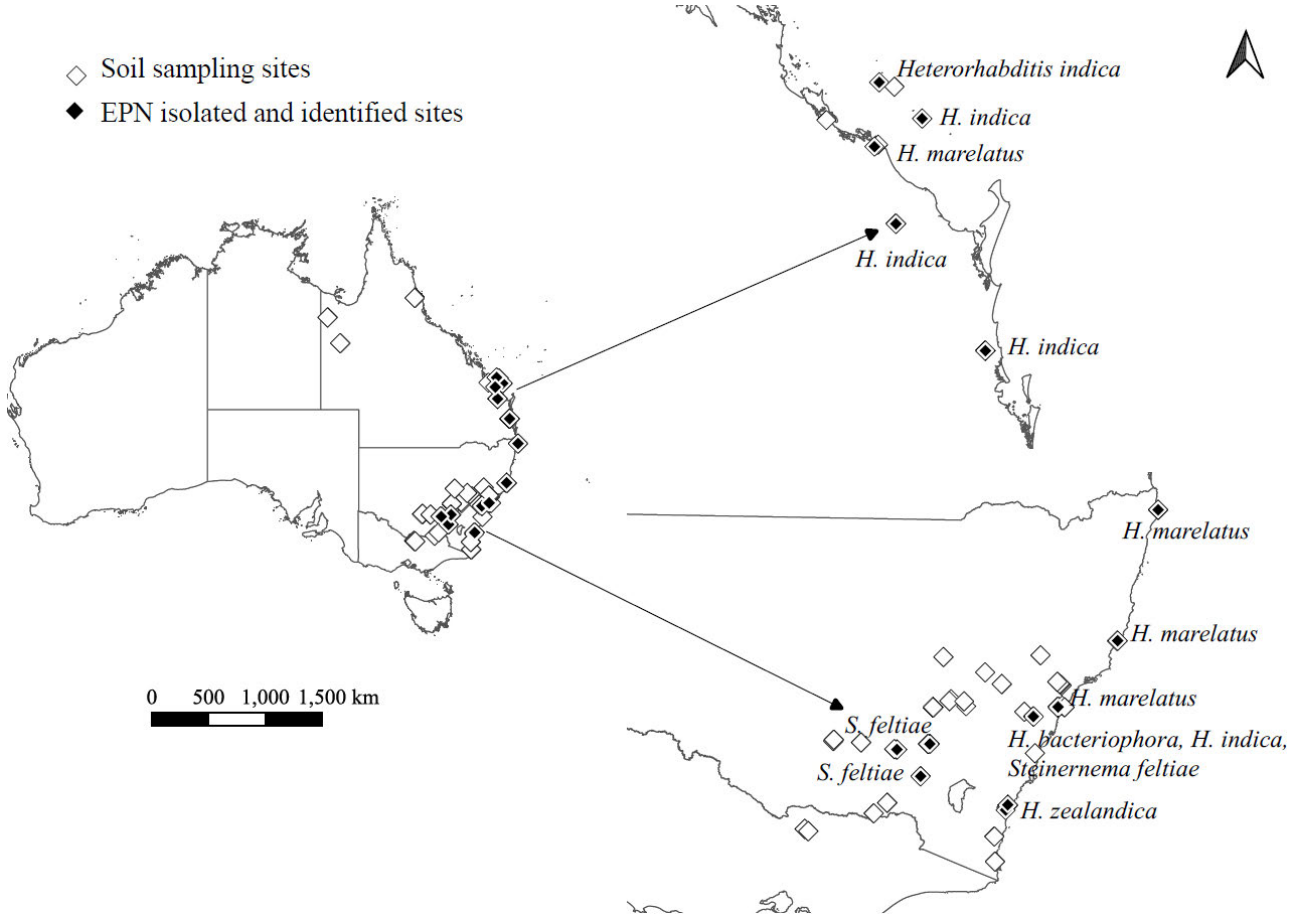

Figure 2.1: Sampling sites (empty diamonds) in tropical, subtropical and temperate regions of eastern Australia from where EPNs were successfully extracted and identified (filled diamonds)[2].

[2] *Heterorhabditis indica* was the only EPN found on the two islands.

Table 2.1: Number of soil samples, EPN positive soil samples and identified EPN strains from different climatic regions and habitats.

Region	Soil samples	EPN positive	Success rate (%)	Samples (EPN positive samples) per habitat			Identified EPN strains
				Orchard	Grassland	Forest	
Tropical	18	7	39	6 (4)	6 (1)	6 (2)	0
Sub-tropical	40	15	37	8 (4)	10 (2)	16 (9)	10
Temperate	140	76	54	37 (18)	54 (22)	55 (36)	26
Total	**198**	**98**	**-**	**51 (26)**	**70 (25)**	**77 (47)**	**36**

2.3.2. Nematode isolation and storage

Late instar larvae of *T. molitor*, *G. mellonella* and *B. tryoni* were used as a bait EPNs from the soil. *Tenebrio molitor* larvae were purchased from a BioSupplies (Sydney, NSW, Australia) and kept at 25 °C and 70 % RH on wheat bran with some carrot slices on top. Individuals of *G. mellonella* were collected from the Western Sydney University apiary and reared at 37 °C and 70% RH on media modified from Kaya and Stock (1997), i.e. 37.5% wheat bran, 23.4% honey, 11.7% bees wax, 21.4% glycerol, 0.5% nipagin[3] and 5.5% yeast extract. Late instar larave of *G. mellonella* and *T. molitor* were used for baiting. Adult flies of *B. tryoni* were maintained in cages in a glasshouse chamber at 25 °C and 70% RH, and eggs obtained for larval rearing in 120ml cups filled with larval diet as previously described (Langford et al., 2014). The cups were then

[3] Chemical name for nipagin is methylparaben. Nipagin is commonly used as an antifungal preservative in insect diets (including of waxmoth and fruit flies), does not have antibacterial function and should therefore not impact bacterial symbionts.

transferred to a plastic container (355 mm × 235 mm × 120 mm) with a fine sterile sand layer. After 9 days the mature L3 started to leave the cups. L3 which had recently jumped (<4 h) and were still moving were selected for baiting.

Each soil sample was divided into nine plastic containers (120 mm × 170 mm × 35 mm), each with 300 g of soil (three containers per insect species), and 20 larvae were added to each container. The containers were then kept at 25 °C (Canhilal et al., 2017). After three days, the larvae were checked for infection by observing larval color changes. The infected cadavers were individually transferred to a modified White trap (Kaya and Stock, 1997) to harvest IJs (see Supplementary Method S1). New bait individuals were inoculated with 1 mL Ringer's solution with 1000 harvested IJs. This process was repeated for three generations to obtain pure cultures (Hoy et al., 2008). For each of these three generations, one infected larva was placed onto a new White trap and nematodes were collected, washed and stored in Ringer's solution at 15 °C for use within two months. Furthermore, we dissected bait cadavers which did not yield IJs using White traps to check whether they were infected by EPNs.

2.3.3. Soil properties

Soil moisture was calculated as the difference between wet and dry soil weight (oven dried at 105 °C for 48 hours) proportional to the original wet weight. Dry soil was burned to ash in a muffle furnace at 360 °C for 4 hours and then SOM was calculated as the difference between dry and ashed soil weight, proportional to the dry weight (Hoy et al., 2008). For soil pH, 5 g fresh soil was sieved (2 mm), thoroughly mixed with 25 mL Milli-Q (MQ) water in a 50 mL centrifuge tube and left for two hours. Soil pH was measured using a SevenCompact S220 pH

meter (Mettler-Toledo). Soil texture was calculated based on hydrometer readings, obtained following the protocols described in Supplementary Method S2.

2.3.4. Molecular characterization of EPNs and associated bacteria

IJs were collected after three generations of subsampling with White traps and surface sterilized by soaking in 2% NaOCl for five minutes. The IJs were transferred to 5 mL MQ water, individually picked and transferred to fresh MQ water. Then, 5-10 live IJs were transferred into 200 µL MQ water on a sterile glass slide and crushed with a sterile pipette tip. 40 µL were used for DNA extraction using the GenElute extraction kit (Sigma) following the manufacturer's protocol. DNA was eluted with 50 µL of elution solution. The ribosomal internal transcribed spacer 1 (ITS1) and the D2-D3 segment of the 28S rRNA gene of the EPNs, and the 16S rRNA gene of the bacteria were PCR amplified using the primers TW81/AB28, D2A/D3B and 27F/1492R (Supplementary Method S3). Amplicons were visualized on agarose gels and prepared for sequencing (Macrogen Korea) using the ExoSAP method (Supplementary Method S4). The sequences were inspected by eye, submitted to BLASTn and aligned with ClustalW using the default parameters. Phylogenetic trees were calculated in Mega X using the Maximum Likelihood (ML) method with the Tamura-Nei model (Kumar et al., 2018; Saitou and Nei, 1987).

2.4. Results

2.4.1. Isolation and identification of the entomopathogenic nematodes

Out of the 198 soil samples collected across eastern Australia (Figure 2.1), 95 (48%)[4] samples were EPN positive (Table 2.1). Overall, 51, 70 and 77 soil samples were collected from citrus orchards, grasslands and forests, and 26 (51%), 25 (36 %) and 47 (61%) were EPN positive. The EPN infected bait larvae were sorted according to cadaver color (Supplementary Fig. S1). A total of 36 EPN isolates were cultured and molecularly characterized, with BLAST similarities of 96.5-100% for D2-D3 and 92.5-99.87% for ITS1 (Supplementary Table S3). The phylogenetic trees of D2-D3 (Figs. 2.2, 2.3) and ITS1 (Supplementary Fig. S2-S3) had similar topologies and strong bootstrap support. There was one unresolved D2-D3 clade with *H. bacteriophora*, *H. georgiana*, Hb.HIE1, Hb.HIE2, and Hb.ECOGROW because of very few variable nucleotide sites. However, based on ITS1, Hb.HIE1, Hb.HIE2, and Hb.ECOGROW clustered with *H. bacteriophora*, and were distinct from *H. georgiana* and *H. beicherriana*. Overall, the EPNs belonged to *H. bacteriophora*, *H. indica*, *H. marelatus*, *H. zealandica* and *S. feltiae* (Supplementary Table S2-S3). *Heterorhabditis* was recovered from 29 samples (14.6 %) while *Steinernema* was isolated from 7 samples (3.5%). *H. indica* (14 isolates) was the most frequently encountered species followed by *H. zealandica* (seven isolates), *S. feltiae* (seven isolates), *H. marelatus* (six isolates) and *H. bacteriophora* (two isolates). *Heterorhabditis* was mostly isolated from the subtropical and temperate regions while *Steinernema* was recorded only from the temperate region. While 39% of tropical samples produced EPN-infected insects as verified by dissection, we were unsuccessful in isolating EPNs from them (Supplementary Table

[4] 98 (49%) samples were EPN positive.

S2). The EPNs were isolated from soil with pH 3.4-7.6, soil moisture 6- 21.6%, and SOM 2.5-88 % (Supplementary Table S2). Most EPN isolates (85% of *Heterorhabditis* and 50% of *Steinernema*) were obtained from sandy soil, while less were obtained from loamy sand and sandy loam[5]. One isolate was baited with *B. tryoni* larvae (Hi.LMBT) while the remaining isolates with *T. molitor* larvae. Out of the 28 soil samples that caused *G. mellonella* infections no EPNs were recovered, 27 of these samples were from temperate region (Supplementary Table S4).

[5] Number of positive samples/number of samples baited for different soil types:
Sand- 22/35; Sandy loam- 42/87; Loamy sand- 15/40; Sandy clay loam- 15/28; Sandy clay- 0/5; Clay- 1/3

Figure 2.2: Maximum likelihood tree of EPN isolates from eastern Australia (triangles) and other *Heterorhabditis* spp. based on the analysis of the D2-D3 segment (482–1018 bp), with *Oscheius tipulae* as an outgroup. Bootstrap values are indicated at the nodes (1000 replicates). GenBank accession numbers are given in parentheses. Scale bar represents number of substitutions per site.

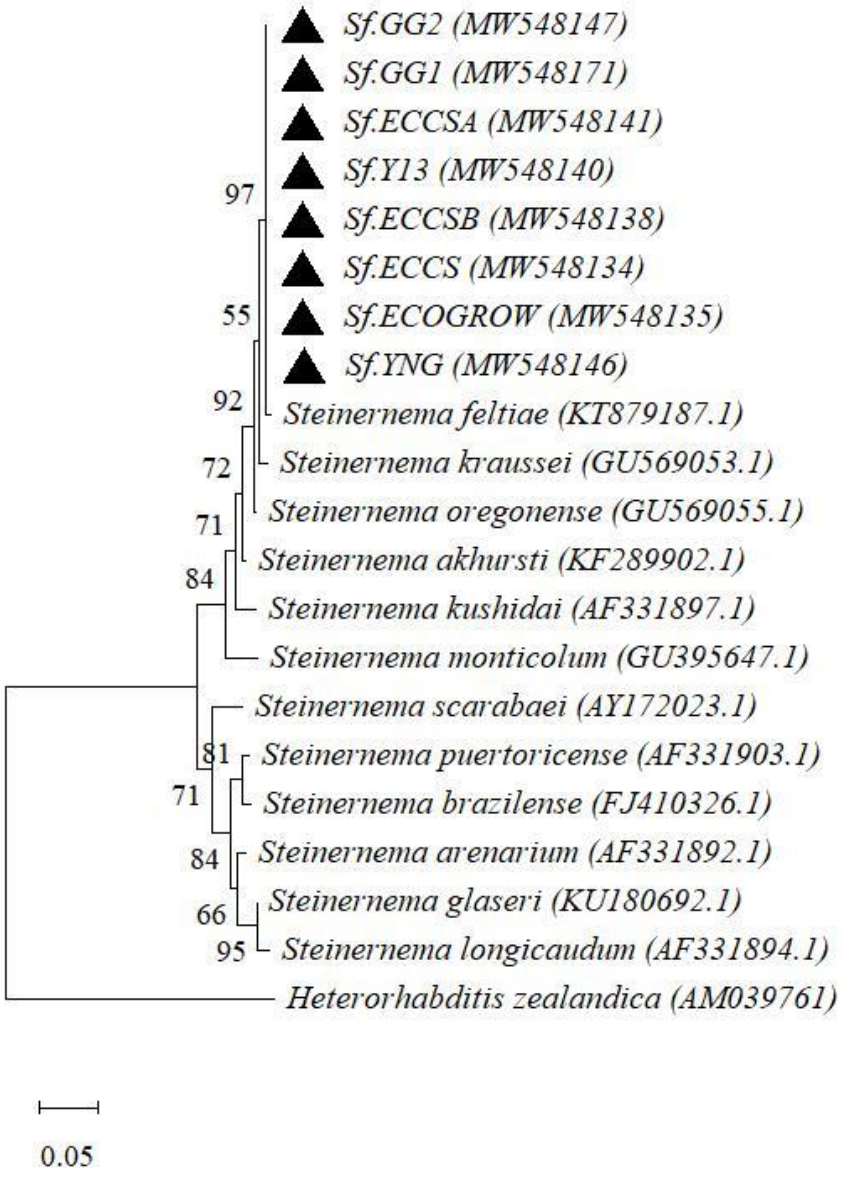

Figure 2.3: Maximum likelihood tree of EPN isolates from eastern Australia (triangles) and other *Steinernema* spp. based on the analysis of the D2-D3 segment (520–3493 bp), with *Heterorhabditis zealandica* as an outgroup. Bootstrap values are indicated at the nodes (1000 replicates). GenBank accession numbers are given in parentheses. Scale bar represents number of substitutions per site.

2.4.3. Identification of bacteria

We obtained 16S rRNA gene sequences from 12 EPN isolates, representing six bacterial species (Supplementary Table S2-S3). According to BLASTn and phylogenetic analyses they were 91.6-99.47 % similar to known bacteria (Supplementary Table S3), including bacterial symbionts of EPNs (Figure 2.4, Supplementary Fig. S4-S5). We identified *Photorhabdus heterorhabditis* from Hb. ECOGROW and Hz.NAR4; *Photorhabdus namnaonensis* from Hz. NAR2; *Photorhabdus tasmaniensis* from Hb.HIE2, Hi.ECCSA and Hi.QFSC6; *Photorhabdus laumondii* from Hb.HIE, Hi.LMBT and Hm.CB. Interestingly, *H. zealandica* infected cadavers showed either green or red coloration. The 16S rRNA gene analysis indicated that green coloration was linked with *P. heterorhabditis* and the red coloration with *P. namnaonensis*. Furthermore, two strains of *Pseudomonas protegens* and a strain of *Delftia acidovorans* were isolated and identified from *S. feltiae* isolates Sf.GG1, Sf.YNG and Sf.Y13.

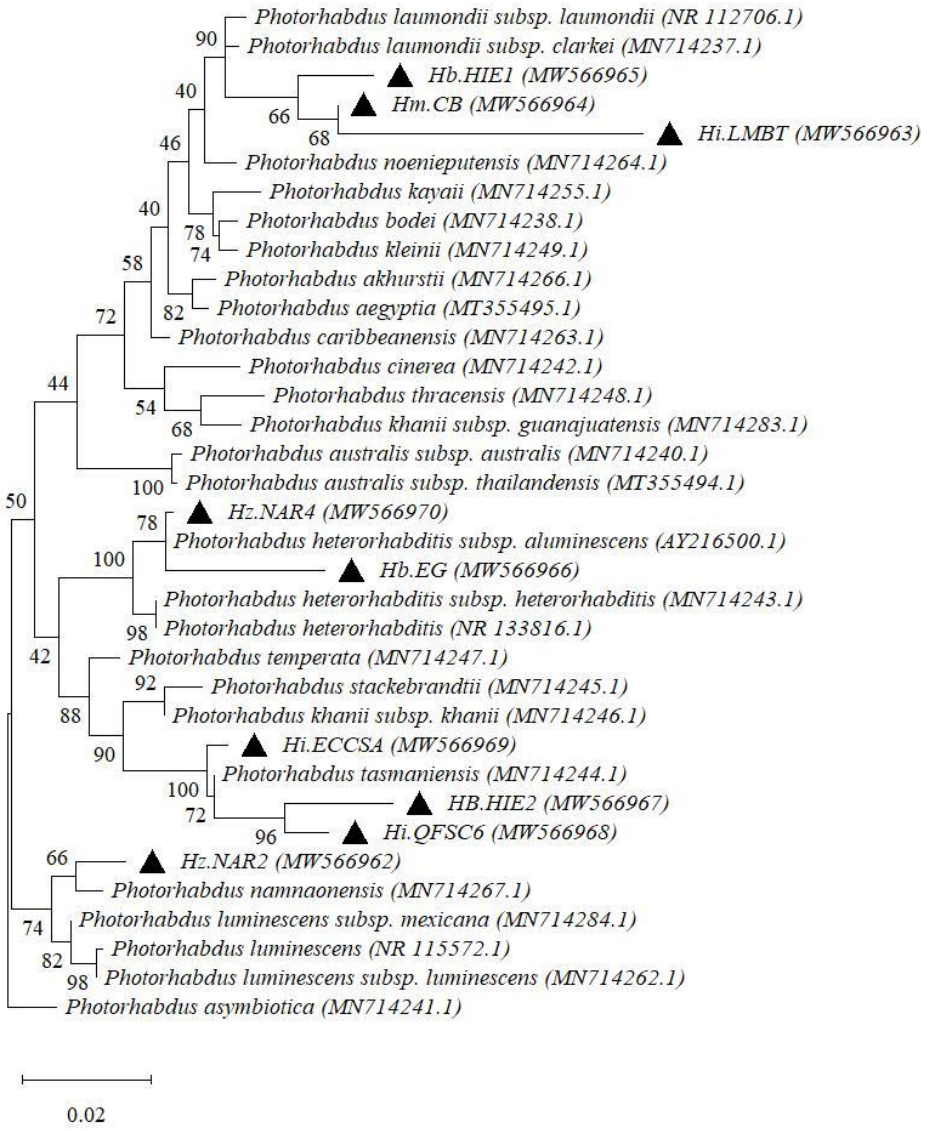

Figure 2.4. Maximum likelihood tree of *Photorhabdus* spp. of new Australian EPN isolates (triangles) and other *Photorhabdus* spp. based on the analysis of 16S rRNA gene (616–1538 bp), with *Photorhabdus asymbiotica* as an outgroup. Bootstrap values are indicated at the nodes (1000 replicates). GenBank accession numbers are given in parentheses. Scale bar represents number of substitutions per site.

2.5. Discussion

We isolated and cultured 36 EPNs from three habitat types across three climate regions of eastern Australia. According to ITS1 and D2-D3 sequences they were *H. bacteriophora*, *H. indica*, *H. marelatus*, *H. zealandica* and *S. feltiae*. For the first time *H. marelatus* was reported from Australia, and *H. indica* and *H. zealandica* from NSW. The 16S rRNA gene analyses revealed six bacterial species: *P. heterorhabditis*, *P. laumondii*, *P. namnaonensis*, *P. tasmaniensis*, *D. acidovorans* and *Ps. protegens*.

Distribution and recovery of the EPNs

At a global scale, *S. feltiae* is the most commonly recorded EPN, followed by *H. bacteriophora* and *H. indica* (Hominick, 2002). Besides these, *H. zealandica* has been recovered from a few countries including Australia (Malan et al., 2006; Poinar, 1990), while *H. marelatus* has previously only been reported from the USA (Liu and Berry, 1996; Stock et al., 1999). Previous surveys of EPNs in Australia reported *H. bacteriophora*, *H. indica*, *H. zealandica*, *S. carpocapsae*, *S. feltiae* and *S. longicaudum* (Supplementary Table S1). In our study, *H. indica* was the most frequently recovered EPN followed by S. *feltiae* and *H. zealandica*. *Heterorhabditis bacteriophora*, *H. zealandica* and *S. feltiae* were recorded only from sites in the temperate region. *Heterorhabditis marelatus* was only found in three rainforest habitats and this may suggest a habitat preference. Previous record of this species were from sandy soils in the USA which is in agreement with five of the six *H. marelatus* isolates from our study. Recovery of *H. marelatus* from rainforest soil may also suggest that more diverse and rarer EPN species may be present in more species-diverse and less disturbed habitats. Soil texture is another important factor. We did not recover EPNs from clay soil. Many previous studies showed

negative correlation of EPN occurrence with soil clay content, and higher EPN abundance in sandy soils (Campos-Herrera et al., 2008; Koppenhöfer and Fuzy, 2006). We were successful in isolating EPNs from soils with pH values between 3.40 to 7.6. The optimum pH range for EPNs without affecting survival and infectivity is between 4 to 8, yet optimal pH levels can be species-specific (Stuart et al., 2015). For example, higher pH reduces steinernematid survival and infectivity (Kung et al., 1990a). Soil temperature, moisture and texture can also influence the diversity, distribution, survival and infectivity of EPNs (Abate et al., 2017; Kanga et al., 2012).

Surprisingly, we were not able to isolate EPNs form infected *T. molitor* obtained from 39% tropical soil samples. Yet, several EPN species have previously been recorded from tropical regions (Fischer-Le Saux et al., 1998; Poinar, 1975). This could possibly have been due to the presence of soil bacteria or other soil factors contaminating the EPN-infected bait insects leading to intoxication, poor nutrition and/or death of EPNs in the bait. Recently, Upadhyay and Mohan (2021) found that the presence of non-symbiotic bacteria in *G. mellonella* affects EPN development and, thus, reducing the number of progeny. This could also reduce the recovery of EPNs. The low recovery of *Steinernema*[6] in our study could be due the particular *G. mellonella* population used, or also because of potential presence of contaminating bacteria in *G. melonella* or the soil. Recently, another study did not detect *Steinernema* using *G. mellonella*, yet it reported the isolation of *Heterorhabditis* (Kour et al., 2020). It may be important to not rely on *G. mellonella* in order to bait diverse EPNs. A future metagenomics soil analysis to characterize the EPN diversity and distribution in Australian soils will be independent from EPN recovery success and, therefore, more informative.

[6] *Steinernema* spp. reproduce by amphimixis and therefore invasion by only one sex will not produce progenies, which might be the possible reason for lower extraction rate compared to occurrence of EPNs.

Diversity of bacterial associations

We found six bacterial species in EPN isolates, including several bacteria that had previously not been recorded from Australia (Supplementary Tables S5-S6). The bacterial symbionts of EPNs have always been considered highly host-specific (monoxenous), and, conversely, each EPN strain having only one symbiotic bacterial strain (McMullen et al., 2017). However, in *S. feltiae* we were not able to isolate *Xenorhabdus* despite this being its commonly described symbiont (Boemare et al., 1993; Tailliez et al., 2006). This does not preclude that *Xenorhabdus* is associated with this EPN, as our approach may have favored the detection of other bacteria instead of *Xenorhabdus*. Bacteria other than *Photorhabdus* and *Xenorhabdus* have previously also been reported from other EPNs. Bonifassi et al. (1999) suggested that these bacteria may be contaminants. In contrast, Ogier et al. (2020) showed that bacteria other than *X. nematophila* are sustainably associated with *S. carpocapsae*, and two of the species identified, *P. protegens* and *P. chlororaphis*, killed insects as efficiently as the cognate *Xenorhabdus* symbiont, suggesting that they may belong to the *Steinernema* pathobiome. It has long been hypothesized that there is coevolution between EPNs and associated bacteria, and that EPNs might require their cognate symbionts to be effective in biological control (Maneesakorn et al., 2011). We have found more complexity with some bacteria being shared across different EPNs. For instance, *P. tasmaniensis* was found in *H. bacteriophora* and *H. indica*, and *P. heterorhabditis* in *H. bacteriophora* and *H. zealandica* (Supplementary Table S5). The occurrence of non-cognate bacteria and more than one bacterial species within different isolates of the same EPN species needs further investigation regarding EPN efficacy in biological control. Furthermore, the bacteria of many EPNs have not yet been characterized, or their phylogenetic relationships based on 16S rRNA

gene sequence data remain unresolved, requiring the application of multi-gene and genomic approaches.

Isolation of locally adapted EPNs

In many parts of Australia, soils experience extreme conditions. High temperatures and prolonged dry periods could result in reduced EPN diversity and abundance as these conditions reduce EPN survival and mobility. However, harsher conditions may select EPNs that are better adapted to these conditions. Isolating and selecting EPNs with higher heat and desiccation tolerance will be advantageous, as susceptibility to heat and desiccation are major constraints for commercial use (Strauch et al., 2004). Solomon and Glazer (1999) found that a *S. feltiae* isolate collected from an Israeli desert region showed the highest desiccation tolerance, while a German isolate showed the lowest desiccation tolerance. Therefore, the EPN isolates collected from Australian soils that experience extreme conditions might be more tolerant to heat and dessication, and this should be investigated further. These native isolates may also be more suitable to control Australian pests than introduced EPNs. Furthermore, introduced EPNs can alter the soil nematode communities (Ishibashi and Kondo, 1986). Therefore, information about EPN biogeography and diversity may also be important for decisions about import and release applications for EPN isolates (Abate et al., 2017; Bedding et al., 1996).

We attempted to isolate EPNs using larvae of a target pest, *B. tryoni*. However, the infection and extraction success were low. It is possible that the small fruit fly larvae which pupate soon after entering the soil are less efficient baits to detect EPNs at low abundance than the larger and more persistent larvae of *T. molitor*. However, EPN isolates baited with a target pest might be more virulent and effective against that pest due to initial natural selection process (Koppenhöfer and Fuzy, 2003). Therefore, exposure of EPN isolates to target pests early on in EPN product

development may improve EPN virulence for target pests. Next, the isolates from this study will be tested against *B. tryoni* under laboratory and field conditions and compared with commercial EPNs that have previously been tested against this pest (Langford et al., 2014).

Chapter 3: Virulence, penetration rate and reproductive potential of entomopathogenic nematodes from eastern Australia in Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae)

3.1. Abstract

Queensland fruit fly *(Bactrocera tryoni*) is Australia's most significant horticultural pest, yet limited options are available for its biological control. We assessed the virulence, penetration rate and reproductive potential of 32 newly isolated Australian entomopathogenic nematode (EPN) strains of *Heterorhabditis bacteriophora*, *Heterorhabditis indica*, *Heterorhabditis marelatus*, *Heterorhabditis zealandica* and *Steinernema feltiae* in late larval and pupal stages of *B. tryoni* under laboratory conditions and compared their performance with four commercially available strains of *H. bacteriophora*, *H. zealandica*, *S. feltiae* and *S. carpocapsae*. All EPN strains caused larval mortality, and remarkably, 29 also pupal mortality. The mean LD_{50} value in *B. tryoni* ranged from 25 to 114 infective juveniles (IJs) per larva, and from 114 to 360 per pupa; therefore, a 3.2 to 4.6x higher dose was required to kill pupae. The penetration rates of IJs in *B. tryoni* larvae varied from 2 to 6.8%, and the number of IJs recovered varied between 2,100 to 5,683. Our findings highlight the potential of Australian native EPN strains for controlling *B. tryoni*. Overall, based on the three tested traits, one *H. zealandica* strain (Hz.NAR1), three *H.indica* strains (Hi.ECCH, Hi.HRN and Hi.HIE2), one *H. bacteriophora* strain (Hb.HIE), one *H. marelatus* strain (Hm.ENCBF2) and one *S. feltiae* strain (Sf.Y13) were the most promising candidates as potential biological control agents against *B. tryoni*. Further laboratory testing is required to assess their persistence under a broader range of environmental conditions before their biological control efficacy can be trialled under field conditions.

3.2. Introduction

Several fruit fly species of the family Tephritidae are major pests due to the damage they cause in fruits and fruiting vegetables; furthermore, they are major biosecurity threats because of their invasion potential, and therefore, they also constitute key barriers for trade and market access for horticultural produce (Clarke, 2019; White and Elson-Harris, 1992). The Queensland fruit fly, *Bactrocera tryoni* (Froggatt), Australia's most damaging horticultural pest can infest fruits of over 40 plant families, including many important crop species (Clarke et al., 2011; Dominiak and Daniels, 2012; Hancock et al., 2000; White and Elson-Harris, 1992). Like other tephritid pests, females of *B. tryoni* oviposit their eggs through the fruit's pericarp into ripening fruit and by doing so also inoculate the oviposition site with bacteria which are important for larval development (Clarke et al., 2011). Then, larval feeding and bacterial decay cause substantial damage to fruit. Eventually, the fully developed third instar larvae (L3) enter a pre-pupal larval stage that can last from 1-2 days (Clarke, 2019). They stop feeding, leave the fruit, drop to the soil and disperse for a few minutes to up to two hours after which they burrow into the top layer of the soil for pupation (Hulthen and Clarke, 2006). In contrast to the relatively short pre-pupal stage in the soil, the pupal stage lasts about 11 days at 26 ºC (FAO/IAEA/USDA, 2019).

During the transition from the infested fruit to the soil, the larvae and pupae of tephritid fruit flies are exposed to natural enemies, including soil-borne entomopathogens. In order to have an impact on fruit fly populations, entomopathogens will need to be able to quickly locate or encounter and infect the larvae entering the soil as the time window of this stage in soil is short. Furthermore, while the pupal stage is exposed to entomopathogenic nematodes (EPN) over longer time periods, the pupa may be more protected from entomopathogen infections because it

is enclosed in a puparium formed from the cuticle of the L3 stage (Chapman, 1998). However, adult flies emerging from puparia and crawling to the soil surface can also be infected (Garriga et al., 2020), and targeting adults may be beneficial given their potential to disperse after infection which may also increase the dispersal of EPNs.

Several management strategies with or without synthetic chemicals exist for *B. tryoni* and are integrated to either reduce fruit fly abundance below a damage threshold or to eradicate outbreaks. Control measures can be applied at the orchard scale, but also beyond, as part of area-wide management (Lloyd et al., 2010). Besides biological control, control measures without insecticides include removal of unmanaged host plants, orchard hygiene, frequent monitoring (Piñero et al., 2009) and the sterile insect technique (Reynolds et al., 2014). Insecticides mostly target adult flies, and are used in cover sprays (Dominiak and Ekman, 2013), attract and kill methods, such as protein bait sprays (Balagawi et al., 2014), the male annihilation technique which relies on male on lures combined with insecticides (Reynolds et al., 2016), and in combination with other fruit fly attractants, such as fruit extract (Siderhurst and Jang, 2010). However, several effective organophosphates and neonicotinoids are now banned in many countries because of their hazardous non-target effects on pollinators, other beneficial animals, humans and the environment more broadly (Prokopy et al., 2000; Van der Sluijs et al., 2013). Hence, there is a need for alternative control options for fruit fly, including biological control agents (BCA) that are safe for humans, beneficial insects and the environment. BCAs can be used in introduction programs of natural enemies from the area of origin of the pest, or the augmentation of natural enemies that are locally present by releasing them or enhancing them. A number of BCAs have been tested against *B. tryoni* such as parasitoids (Spinner et al., 2011; Zamek et al., 2012), fungi

(Carswell et al., 1998; Gava et al., 2020) and EPNs (Langford et al., 2014), but their use has not yet been extensively researched and implemented at larger scales.

The portfolio of natural enemies includes entomopathogenic nematodes (EPNs). EPNs, together with their bacterial symbionts (*Photorhabdus* in *Heterorhabditidae*, and *Xenorhabdus* in *Steinernematidae*), reside in the soil and are lethal to important insect pests, in particular insects with developmental stages in the soil. In general, EPNs are good alternatives to synthetic chemicals as they are safe to humans and the environment (Ehlers, 2003). Previously, EPNs have been tested for their efficacy against several tephritids fruit flies with promising results (Table 3.1).

Table 3.1: Laboratory tests performed against tephritids in genera *Anastrepha*, *Bactrocera*, *Ceratitis*, *Dacus* and *Rhagoletis* with different EPN species.

Target pest	EPN species and strain	Tested life stage	Result	References
A. fraterculus	*Heterorhabditis* spp., *Steinernema* spp.	Larvae	Larval infection	(Foelkel et al., 2017)
A. fraterculus	*H. bacteriophora, S. feltiae* (RS76), *S. glaseri* (RS38), *S. riobrave, S. rarum*	Pre-pupae, pupae	Pupal infection	(Barbosa-Negrisoli et al., 2009)
A. ludens, A. serpentine	*H. bacteriophora*	Larvae	Larval infection	(Toledo et al., 2005; Toledo et al., 2006a; Toledo et al., 2006b; Toledo et al., 2014)
A. oblique	*S. carpocapsae*	Larvae	Larval infection	(Toledo et al., 2009)
A. suspense	*Steinernema* sp., *Heterorhabditi* ssp.	Larvae, pupae	Larval and pupal infection	(Heve et al., 2017)
B. dorsalis	*H. taysearae, H. indica, Steinernema* sp.	Larvae, pupae	Larval and pupal infection	(Godjo et al., 2018b)
B. oleae	*S. feltiae*	Larva and pupa	Larval and pupal infection	(Torrini et al., 2020a)
B. oleae	*S. feltiae, S. carpocapsae, S. riobrave, S. glaseri, H.*	Larvae	Larval infection	(Sirjani et al., 2009)

	bacteriophora, H. marelatus			
B. tryoni	*S. feltiae, S. carpocapsae, H. bacteriophora*	Larvae, pupae	No pupal infection	(Langford et al., 2014)
B. zonata, C. capitata	*S. riobrave, H. bacteriophora*	Larvae, pupae	Larval and pupal infection	(Soliman et al., 2014)
B. zonata	*S. feltiae*	Larvae, pupae and adults	Larval, pupal and adult infection	(Mahmoud et al., 2016)
C. capitata	*S. feltiae, S. riobrave, S. carpocapsae, S. glaseri, H. bacteriophora*	Larvae	Larval infection	(Gazit et al., 2000)
C. capitata	*S. carpocapsae, S. feltiae, S. weiseri, H. bacteriophora*	Larvae, pupae	No pupal infection	(Karagoz et al., 2009a)
C. capitata	*S. carpocapsae, Heterorhabditis* sp. RSC01	Larvae	Larval infection	(Rohde et al., 2010)
C. capitata, B. zonata	*S. carpocapsae; H. bacteriophora* (HP88 strain)	Pupae, adults	No pupal infection	(Abbas et al., 2016)
C. capitata, C. rosa	*H. bacteriophora, H. zealandica, S. khoisanae*	Larvae, pupae, adults	No pupal infection	(Malan and Manrakhan, 2009)
D. ciliatus	*H. bacteriophora and S.carpocapsae*	Larvae, pupae, adults	Very low pupal infection;	(Kamali et al., 2013)

			larvae and adults infected	
R. cerasi	*S. feltiae, S. carpocapsae, H. bacteriophora, H. marelatus*	Larvae	Larval infection	(Kepenekci et al., 2015)
R. indifferens	*S. feltiae, S. carpocapsae S. riobravis, H. marelatus, H. bacteriophora*	Larvae	Larval infection	(Stark and Lacey, 1999)
R. indifferens	*S. feltiae, S. carpocapsae, S. intermedium*	Larvae, Pupae, adults	No pupal infection	(Yee and Lacey, 2003)

Several surveys have been carried out to search for EPN species and strains across the world (Hominick, 2002; Hominick et al., 1996; Torrini et al., 2020b; Yoshida et al., 1998). However, in depth follow-up experiments are needed to characterise the pathogenicity and virulence of newly isolated EPNs in target insect pest species, including across different stages, in order to identify the most suitable strain for biological control. EPN traits like virulence, persistence in soil, heat and desiccation tolerance, and host seeking ability all vary between nematode species and strains (Bedding et al., 1983; Levy et al., 2020). Furthermore, EPN virulence also depends on the pathogenicity of their bacterial symbionts (Gerritsen et al., 1998; McMullen et al., 2017). Hence, laboratory assays are necessary to assess the infectivity of EPNs, as the first important step to test their suitability as BCA in field applications (Bedding, 1990). The EPN dose effect, the required exposure time and effectiveness in causing mortality in target pests and the effect of soil

and environmental conditions on survival, virulence and persistence of EPNs are key parameters to test the usefulness of EPNs in biological control programs. Improvements in the infectivity and persistence of EPNs may allow lower application rates (Bedding et al., 1993), which can reduce the cost of EPN-based products and thereby increase their efficiency. Furthermore, it is important to assess for any EPN the susceptibility of targeted insect pest species and the targeted developmental stages before they can be developed as a potential BCA (Bedding et al., 1993).

Larvae of the greater wax moth, *Galleria mellonella*, are commonly used for baiting EPNs from soil samples (Bedding and Akhurst, 1975; Canhilal et al., 2017; De Brida et al., 2017). Larvae of Mealworms, *Tenebrio molitor*, are also used (Griffin et al., 2000), albeit less frequently. Larvae of *T. molitor* are less susceptible to EPNs than *G. mellonella* larvae, and, therefore, EPNs baited with *T. molitor* may be more effective against beetles and other harder to infect insects (Koppenhoeffer and Fuzy, 2003). In order to be an effective BCA, of any insect pest, EPNs need to also be adapted to the local environmental conditions and the target pest (Bedding, 1990; Stock et al., 2008). Baiting EPNs from the biogeographic regions of target pest, and using target insect pests as a bait to isolate EPNs might further increase the chance of obtaining EPNs that are more virulent against that pest (Koppenhöfer and Fuzy, 2003).

In this study, we focused on Australian native EPN strains that have recently been baited with *T. molitor* and *B. tryoni* larvae from soil samples collected across eastern Australia (Chapter 2). The aim of this study was to assess the potential of these recently collected Australian EPN strains as BCAs of larval and pupal stages of *B. tryoni* under laboratory conditions, and compared them with commercially available EPN strains which have been isolated from Australian soil using *G. mellonella* over 30 years ago (Akhurst and Bedding, 1986). Virulent strains could be candidate EPNs for the development of a biological control program of *B. tryoni* at a commercial scale, or

valuable additions to existing control measures. We hypothesized that some of the newly isolated strains are more virulent and have a higher reproductive potential in *B. tryoni* larvae and pupae than commercially available strains in *B. tryoni* larvae and pupae, because of their more recent collection with *T. molitor* and *B. tryoni* as baits, as opposed to the commercially available strains that were isolated using *G. mellonella* over 30 years ago. Furthermore, the more virulent strains could be candidates for further development as BCAs against *B. tryoni.*

3.3. Material and methods

3.3.1. Nematode collection and storage

Thirty-two EPN strains collected from Eastern Australia in a previous study (Chapter 2) and four commercially available strains obtained from Ecogrow Environment Pty Ltd (NSW, Australia) were used in this study (Table 3.2). Infective Dauer juveniles (IJs) were stored in Ringer's solution (9.0 g NaCl, 0.42 g KCl, 0.37 g $CaCl_2 \times 2H_2O$, and 0.2 g $NaHCO_3$ dissolved in 1 L of distilled water) at 15 °C and used for laboratory assays within a week.

Table 3.2: EPN strains used in this study: 32 strains were isolated from soils in New South Wales (NSW) and Queensland (QLD) (Chapter 2), and four strains were obtained from Ecogrow, Australia (*). All new strains were baited with larvae of *Tenebrio molitor* except Hi.LMBT which was baited with *Bactrocera tryoni* larvae.

EPN species	Isolates	Locality
H. bacteriophora	Hb. ECOGROW *	-
H. bacteriophora	Hb.HIE1	Richmond, NSW
H. bacteriophora	Hb.HIE2	Richmond, NSW
H. indica	Hi.ECC1H	Richmond, NSW
H. indica	Hi.ECCSA	Richmond, NSW
H. indica	Hi.HIE1	Richmond, NSW
H. indica	Hi.HIE2	Richmond, NSW
H. indica	Hi.HIE3	Richmond, NSW
H. indica	Hi.HRN	Heron Island, QLD
H. indica	Hi.HRN2	Heron Island, QLD
H. indica	Hi.LMBT	Lady Musgrave Island, QLD
H. indica	Hi.LMI1	Lady Musgrave Island, QLD
H. indica	Hi.LMI2	Lady Musgrave Island, QLD
H. indica	Hi.QF6	Palmwoods, QLD

H. indica	Hi.QFSC6	Palmwoods, QLD
H. indica	Hi.QGL	Duingal, QLD
H. indica	Hi.QGLB	Duingal, QLD
H. marelatus	Hm.CB	Byron Bay, NSW
H. marelatus	Hm.CBF1A	Coralville, NSW
H. marelatus	Hm.ENCBF2	Eurimbula, QLD
H. marelatus	Hm.GOS1	Somersby, NSW
H. marelatus	Hm.GOS2	Somersby, NSW
H. marelatus	Hm.GOS3	Somersby, NSW
H. zealandica	Hz.BB1	Batemans Bay, NSW
H. zealandica	Hz.BB2	Batemans Bay, NSW
H. zealandica	Hz.BB3	Broulee, NSW
H. zealandica	Hz.ECOGROW *	-
H. zealandica	Hz.NAR1	Narara, NSW
H. zealandica	Hz.NAR2	Narara, NSW
H. zealandica	Hz.NAR3	Narara, NSW
H. zealandica	Hz.NAR4	Narara, NSW
S. feltiae	Sf.ECCS	Richmond, NSW
S. feltiae	Sf.ECCSA	Richmond, NSW
S. feltiae	Sf.ECCSB	Richmond, NSW
S. feltiae	Sf.ECOGROW*	-
S. feltiae	Sf.GG1	Gundagai, NSW
S. feltiae	Sf.GG2	Gundagai, NSW

S. feltiae	Sf.Y13	Temora, NSW
S. feltiae	Sf.YNG	Temora, NSW

3.3.2. Insect cultures

Tenebrio molitor larvae were purchased from a commercial supplier of live insects (BioSupplies, Sydney, NSW) and reared on wheat bran at 25 °C and 70 % RH with the addition of some carrot slices on top of the bran to keep it moist. A laboratory population of *B. tryoni* established from flies collected in Bathurst, NSW, in summer 2009 was used and cultured in a glasshouse chamber at 25 °C and 70 % RH (Langford et al., 2014). Eggs were collected from adult flies kept in cages (30 cm each side) using 120 mL cups filled with larval diet (Meats et al., 2004). The cups filled with larval diet were covered with parafilm perforated with needles and exposed to fruit flies in the cages for 2 hours to allow oviposition into the larval diet. The cups with eggs were then transferred to a box with a ventilated lid on the top and a fine layer of sterile sand on the bottom. The larvae and pupae were collected from this sand layer (Supplementary Fig. S6). Late instar larvae which had recently jumped (<4 hours) from the larval diet and were still moving were selected. For the pupal experiments, dark brown pupae (approximately 3 days old) were chosen for all laboratory assays. The average weight of larvae and pupae used were 14.87 mg (± 1.16) and 9.18 mg (± 1.90), respectively.

3.3.3. Virulence assay against *B. tryoni* larvae and pupae

A sand plate assay was performed for each strain to measure their virulence as larval and pupal mortality of B. tryoni using different IJ concentrations of EPN strains (Glazer, 1992; Ricci et al.,

1996). Sand was autoclaved, oven dried and maintained at 10 % moisture. Petri dishes (150 mm diameter) were filled with 250 g of sand. For each EPN strain, fresh IJs were harvested from five White traps (White, 1927) from different time period, each with one EPN-infected *T. molitor* larva. All strains (including the commercially available strains) were treated in the same way and propagated in *T. molitor* larvae. EPNs collected from five different White traps were used as replicates for each EPN isolate. Each sand plate was inoculated with 1 mL of Ringer's solution with 0 (control), 50, 100, 200, 500 and 1000 IJs per mL. Immediately after EPN inoculation, 20 *B. tryoni* larvae or pupae were added on top of the sand at the centre of the plate. The plates were incubated at 25 °C in the dark and the insect mortality due to EPN infection was recorded after seven days. All cadavers were dissected to confirm the presence of nematodes.

3.3.4. Host penetration with *B. tryoni* larvae

The number of IJs penetrating third instar larvae of *B. tryoni* was assessed in a 24 well plate. Individual larvae were added to each empty well and, then, half-filled with sand with 10% mositure. Each well received 100 IJs in 50 μL of Ringer's solution on top of the sand. Five replicates were tested for each strain. After five days, the infected cadaver was dissected to record the number of hermaphrodites for *Heterorhabditis* spp. and first-generation females and males for *Steinernema* spp., and nematode penetration was calculated as the percentage of added IJs which developed into hermaphrodites or first-generation females and males.

3.3.5. Reproduction potential with *B. tryoni* larvae

The reproductive potential measured as the number of IJs produced by each strain (Nimkingrat et al., 2013) was assessed in pre-pupal larvae of *B. tryoni.* Individual larvae were added to each well of an empty 24 well plate, then half-filled with 10% moist sand and inoculated with 100 IJs

in 50 µL of Ringer's solution. Five replicates were performed for each strain. After five days, the infected cadavers were placed on White traps, with one individual per trap. The total number of IJs produced by each White trap were counted for three weeks to ensure all IJs had emerged. The cadavers were then dissected for any IJs that were not trapped via White traps.

3.3.6. Data analysis

The dose of IJs required to kill 50 % of the insects (LD_{50}) for each replicate was calculated and compared between different strains (Finney, 1952). The percentage of dead insects (mortality) due to EPN infection were incorporated in the following formula: mortality = a (1-ln(-bx)) +c, whereby a equals the total number of insects tested, corrected by the control mortality; b the slope; c the control mortality; x the dose used per insect. The LD_{50} values were calculated by fitting a saturation curve to the mortality data through minimization of the chi-square (χ^2) for the comparison of the theoretical distribution and the observed distribution using Microsoft Excel application solver. The data for virulence (LD_{50}), penetration rate and reproduction potential were analysed for normality with the Shapiro-Wilkinson test ($p > 0.05$). For normally distributed data, one way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test for multiple comparisons were performed. For non-normal distributed data (penetration %), Kruskal-Wallis test with post hoc Dunn's test for multiple comparisons was used. All data analyses were performed in R version 4.0.2 (R Core Team, 2020).

3.4. Results

3.4.1. Virulence

All 36 EPN strains were able to infect *B. tryoni* larvae (Figs. 3.1 and 3.2) and 29 of these also infected pupae (Figs. 3.1 and 3.3). Across all strains, the mean LD_{50} value of *B. tryoni* ranged from 25.32 (± 4.22) to 113.88 (± 16.79) for larvae, and 114.24 (± 43.46) to 360.22 (± 104.36) for pupae. The insect mortality caused by the IJs of different EPN strains was significantly different for *B. tryoni* larvae ($F_{35,144} = 28.24$; $p \leq 0.0001$) and *B. tryoni* pupae ($F_{28,116} = 11.31$; $p \leq 0.0001$). Similarly, the mortality caused in larvae was significantly higher than in pupae ($F_{64,260} = 41.35$; $p \leq 0.0001$). The lowest and highest LD_{50} were found for Hz.NAR1 and Hz.NAR4 for *B. tryoni* larvae, and for Sf.Y13 and Sf.YNG for *B. tryoni* pupae. Seven out of 36 tested strains, Sf.ECCSA, Sf.ECCS, Hi.QGLB, Sc.EG, Hb.EG, Hz.NAR3, Hz.NAR4, were not able to infect *B. tryoni* pupae. The strain baited with *B. tryoni* (Hi.LMBt) and commercial strains showed no significant differences against both larvae ($F_{4,20} = 12.93$; $p \leq 0.0001$) and pupae ($F_{2,12} = 14.68$; $p \leq 0.0006$). The strains Hz. NAR1, Hb.HIE, Hi.ECCH and Hz.NAR1 were substantially more

virulent against larvae than the commercial strains Hb.EG and Hz.EG. On the other hand, Sf.Y13 and Hi.HIE2 were more virulent than the commercially available strains against pupae.

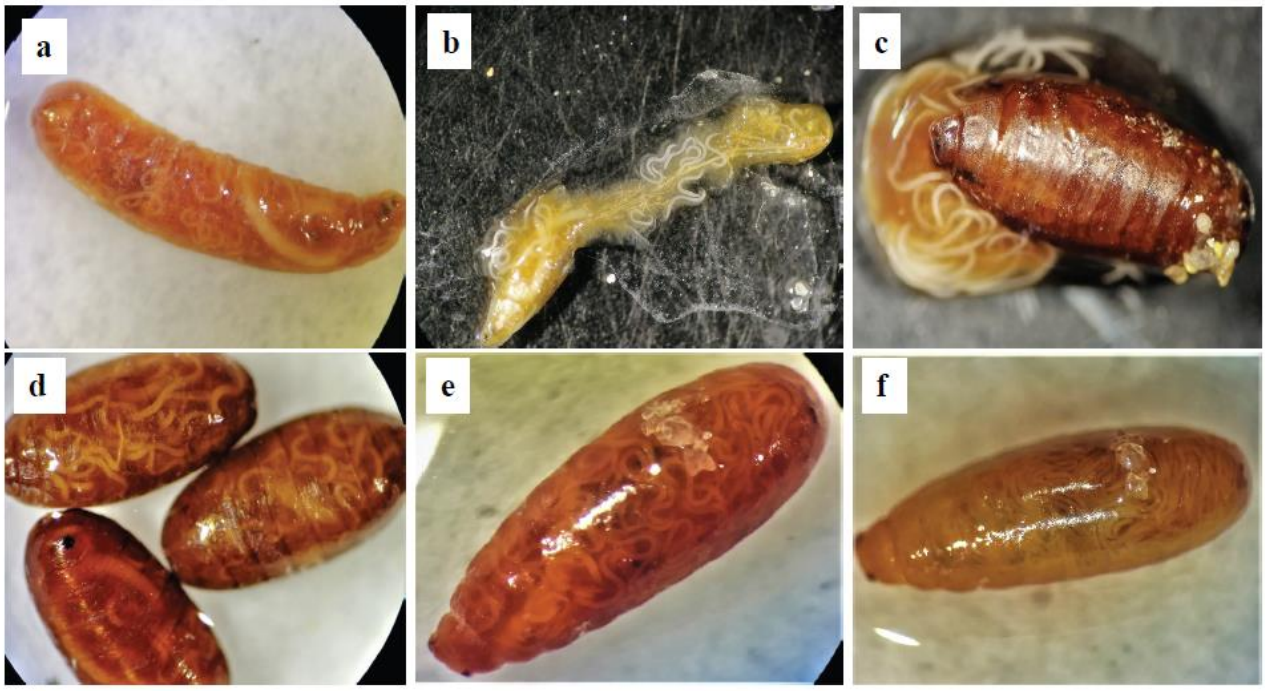

Fig. 3.1: *Bactrocera tryoni* packed with EPNs: a) larva infected with Hi.HRN; b) larva infected by Sf.ECCS; c) pupa infected with Hi.HRN; d) pupae infected with Hb.HIE; e-f): larvae that pupated after infection with Hz.NAR1 and Sf.Y13, respectively.

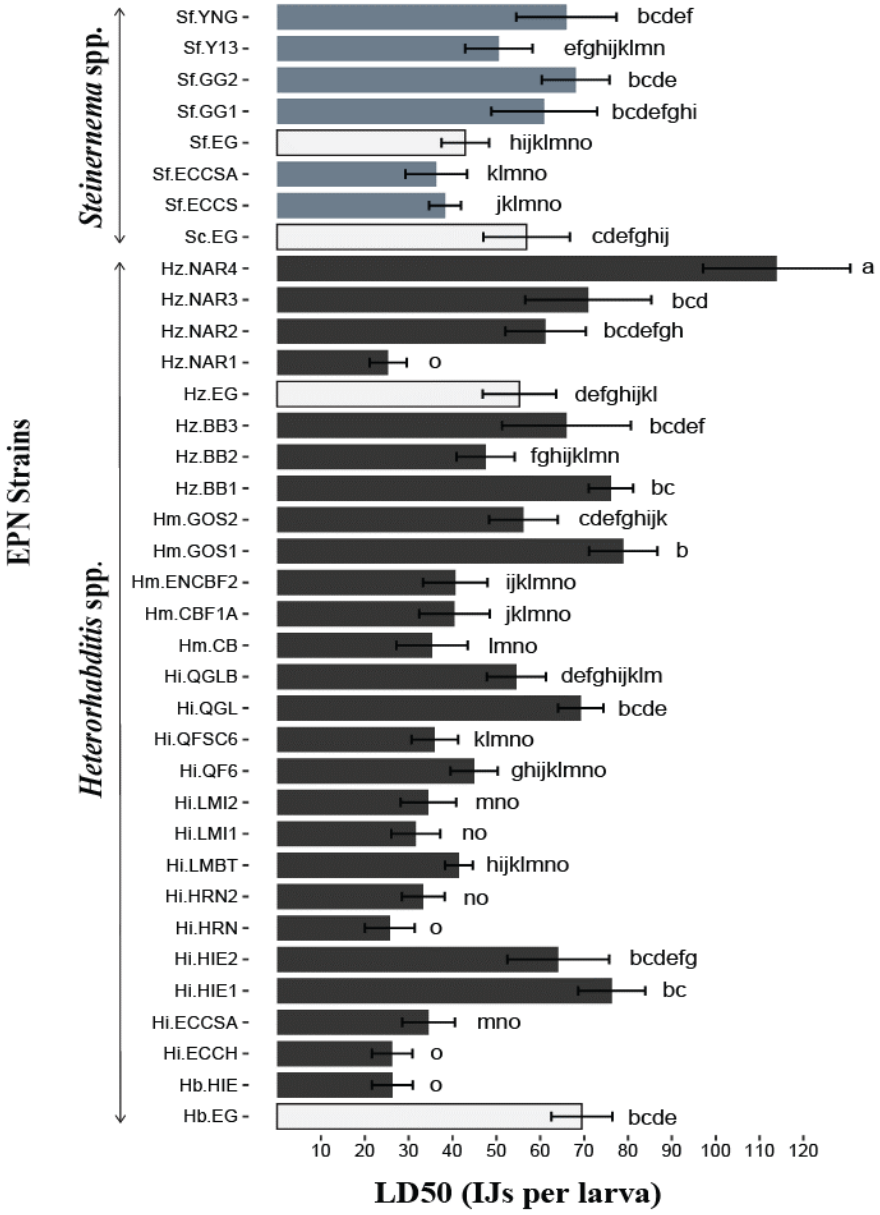

Fig. 3.2: LD_{50} of new EPN strains (*Steinernema* grey, *Heterorhabditis* black) and commercial strains (white) tested against *Bactrocera tryoni* larvae after one week of exposure to IJs at 25 °C. Lower LD_{50} values resemble higher virulence. Error bars indicate SD across five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Tukey's HSD test ($p < 0.05$).

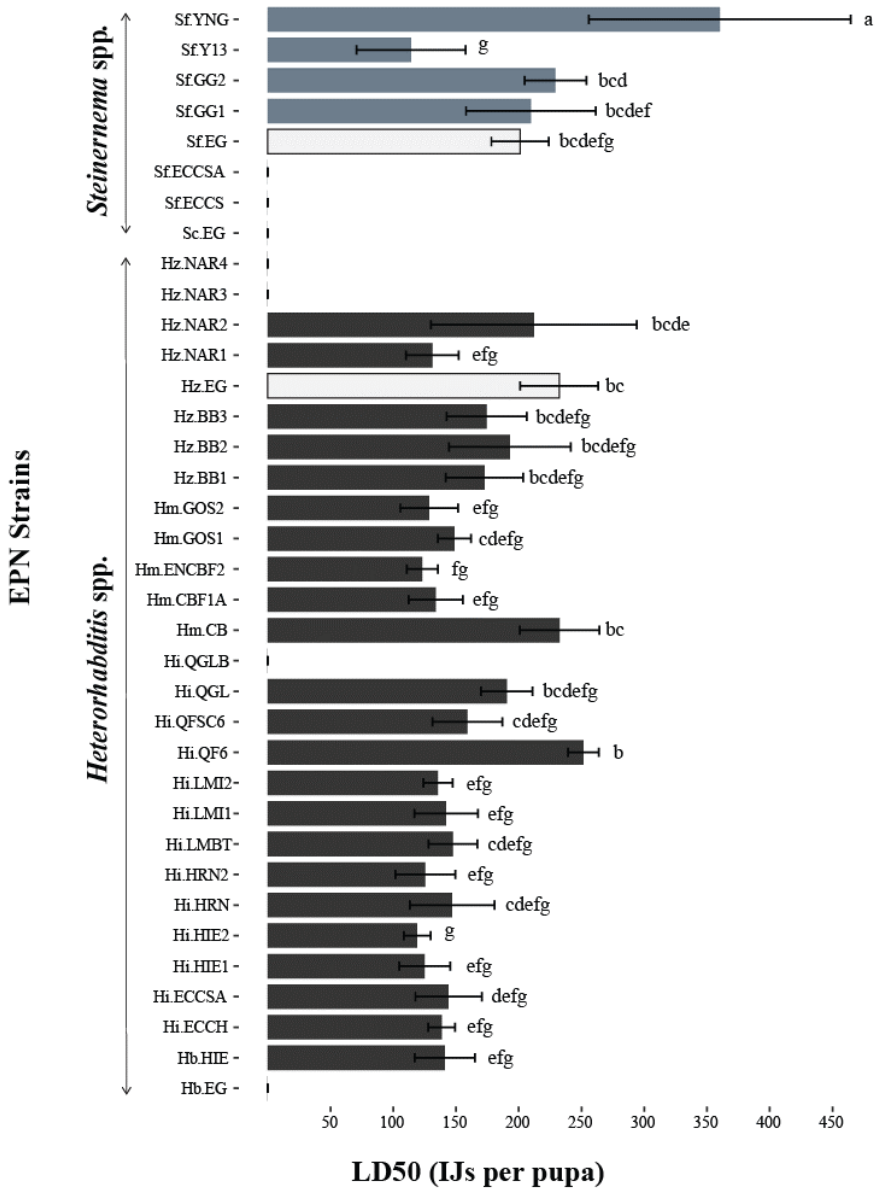

Fig. 3.3: **LD_{50} of new EPN strains (*Steinernema* grey, *Heterorhabditis* black) and commercial strains (white) tested against *Bactrocera tryoni* pupae after one week of exposure to IJs at 25 °C. Lower LD_{50} values resemble higher virulence. Error bars indicate SD across five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Tukey's HSD test ($p < 0.05$). The blank space indicates no infection occurred.**

3.4.2. Host penetration

Among the 36 tested strains, the penetration rate in *B. tryoni* larvae differed significantly (Kruskal-Wallis, χ^2 (35) = 125.08, $p < 0.0001$) and ranged from 2 % (± 0.71) (Sf.GG2) to 6.80 % (± 0.84) (Hm.CB) (Figure 3.4). No significant differences were found among strains of *Steinernema* spp., among strains of *H. bacteriophora*, or among strains of *H. marelatus*. Overall, *H. indica* strains Hi.ECCH, Hi.QF6, *H. marelatus* strains Hm.ENCBF2, Hm.CBF1A, Hm.CB, and *H. zealandica* strains Hz.NAR1, Hz.NAR2, Hz.NAR3 and Hz.NAR4 had significantly higher penetration rates than the commercial strains Sc.EG, Hz.EG and Hb.EG. The EPN strain baited with *B. tryoni larvae* (Hi.LMBt) displayed a higher penetration rate than the commercial strain Hb.EG.

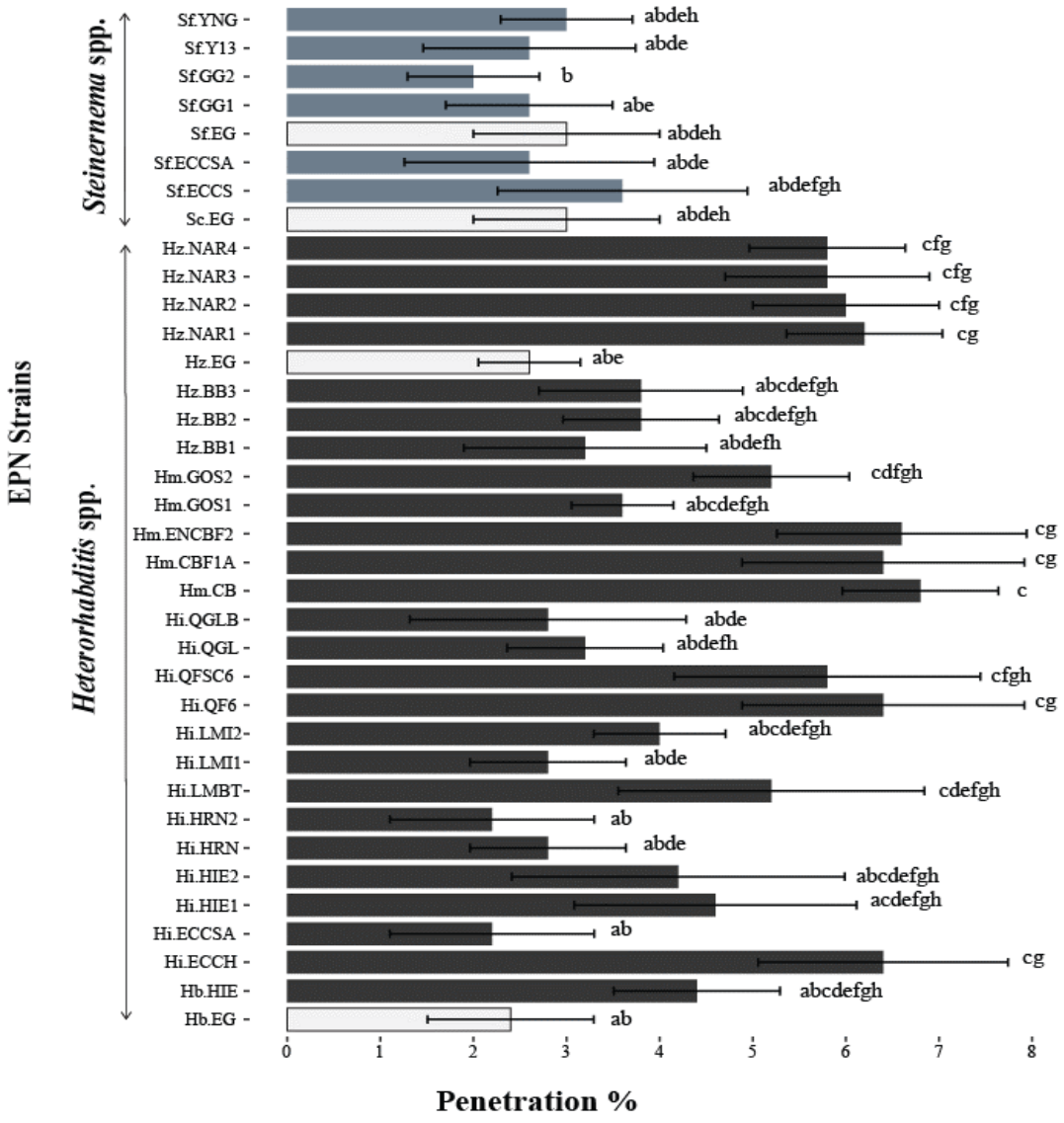

Fig 3.4: Penetration percentage of IJs in *Bactrocera tryoni* larvae after individuals were inoculated with 100 IJs of each new EPN strain (*Steinernema* grey, *Heterorhabditis* black) and commercial EPN strain (white), assessed after five days at 25 °C. Error bars indicate SD of five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Dunn's test for multiple comparisons.

3.4.3. Reproduction potential

The mean number of IJs recorded in infected *B. tryoni* larvae was between 2,100 (± 252.87) and 5,683.20 (± 231.43) (Figure 3.5). There were significant differences in the number of IJs produced in *B. tryoni* larvae ($F_{35,144} = 29.64$; $p \leq 0.0001$). Hz.NAR3, Sf.ECCS, Hz.NAR4, Hi.HIE2 and Hb.HIE had a higher mean IJs count than commercial strains. Furthermore, the EPN strain baited with *B. tryoni* larvae (Hi.LMBt) displayed a higher reproduction rate than the commercial strains Sf.EG and Sc.EG.

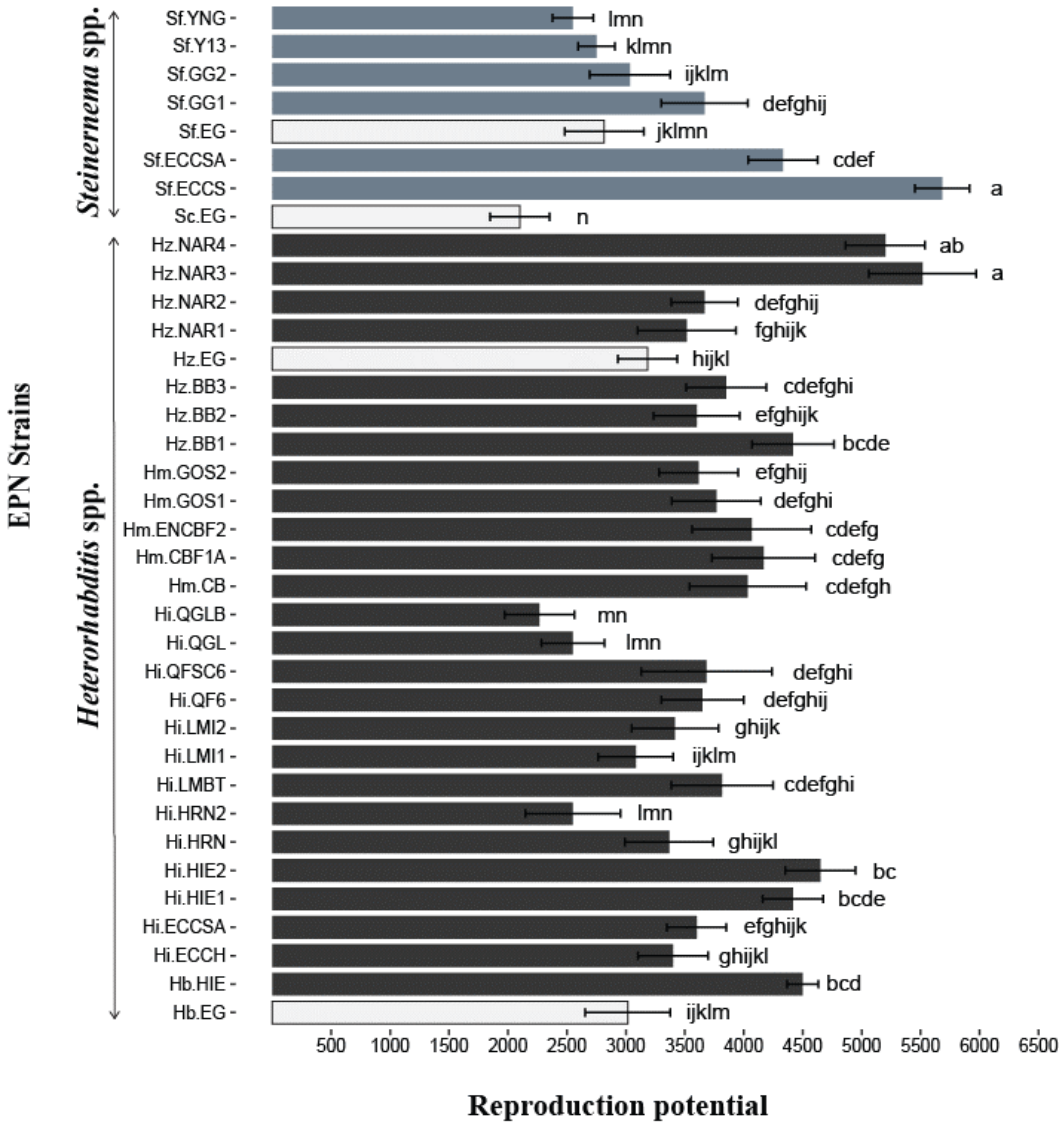

Fig. 3.5: Reproduction potential (average number of IJs produced) of new EPN strains (*Steinernema* grey, *Heterorhabditis* black) and commercial EPN strains (white). Individual larvae were exposed to IJs for five days and the IJs leaving the larval cadaver was recorded for three weeks. Error bars indicate SD of five replicates. Different letters next to the error bars indicate that means are significantly different as per Tukey's HSD test ($p < 0.05$).

3.5. Discussion

Our study demonstrates that both larval and pupal stages of *B. tryoni* are susceptible to EPNs, but the efficacy of EPN strains in causing larval and pupal mortality varied considerably. All 36 EPN strains penetrated and killed *B. tryoni* larvae and displayed high reproductive potential in them. Of these, 29 strains also caused pupal mortality. All but one of these EPN strains were baited with *T. molitor*, while one was baited with *B. tryoni* (Hi.LMBT). The virulent strains with promising reproduction potential in *B. tryoni* need further testing for virulence and persistence under a broader range of environmental conditions that better represent field conditions prior to field testing.

Virulence in larval and pupal stages of B. tryoni

For *B. tryoni* lavae we found that the *H. bacteriophora* strain Hb.HIE, *H. zealandica* strain Hz.NAR1, *H.indica* strains Hi.HRN and Hi.ECCH were more virulent than *H. marelatus* and *S. feltiae*. In contrast, Kepenekci et al. (2015) found for another tephritid fruit fly species, *Rhagoletis cerasi*, that *H. bacteriophora* was less virulent than *H. marelatus*, while *S. feltiae* was the most virulent species against larvae. Against *B. tryoni* pupae, two different *S. feltiae* strains showed the highest (Sf.Y13) and lowest virulence (Sf.YNG). *Steinernema feltiae* may be a good option for field application because of its intermediate ambush to cruise foraging behavior in which it waits for or even looks for hosts (Lewis, 2002). On the other hand, *Heterorhabditis* spp. may be a better option against the pupal stage due to their terminal tooth which they use to rupture the insect cuticle and penetrate the insect host (Bedding and Molyneux, 1982), including pupae. In contrast, *Steinernema* spp. do not have this terminal tooth. We saw variation in the

virulence, host penetration and reproduction potential in *B. tryoni* larvae and pupae between tested EPN strains. These traits are important criteria for the selection of highly performing BCAs against pests. Furthermore, it is important to undertake laboratory trials with a diverse pool of EPNs to characterise EPN performance against the target pest,and this will allow the selection of the better performing strains for further field testing. We note here that our laboratory experiments were carried out in 150 mm Petri dishes, which may not reveal behavioral differences between EPN strains. However, these behavioural differences might play a large role in their field efficacy as BCA and will need to be investigated further.

Significance of EPN infectivity against pupae

Our study is the first to report successful EPN infectivity of *B. tryoni* pupae, whereas the only other EPN study with *B. tryoni* did not detect pupal mortality caused by EPNs (Langford et al., 2014). We found *S. feltiae* (Sf.Y13) and *H. indica* (Hi.HIE2) were the most virulent strains against *B. tryoni pupae.* Similar to our result, Stark and Lacey (1999) found high pupal mortality with *S. feltiae* (70 %), *H. bacteriophora* (62.5 %), *H. marelatus* (55 %) against the early pupal stage of another tephritid pest, *Rhagoletis indifferens*, possibly because IJs entered the puparia and pupae either through spiracles and intersegmental membranes before pupal cuticle sclerotization. It remains unclear how *Steinernema* spp. can penetrate pupae, but they may be able to enter puparia through their spiracles.

Interestingly, our study observed pupal mortality caused by one commercially available strain, *S. feltiae*, but not for the three other commercial strains. Previously, Langford et al. (2014) tested the same commercial strains of *S. feltiae* and *H. bacteriophora* as well as *S. carpocapsae* against

B. tryoni but found no pupal mortality. Besides this, many previous studies which tested EPNs against other tephritids did not find pupal infectivity (Abbas et al., 2016; Karagoz et al., 2009a; Langford et al., 2014; Yee and Lacey, 2003), while some of them reported pupal infection (Barbosa-Negrisoli et al., 2009; Godjo et al., 2018b; Heve et al., 2017). Also, some larvae inoculated with EPNs still pupated and were found infected in the pupal stage. We assume that some larvae pupated after they were infected by EPNs; the exact time point of infection remains unclear.This is in agreement with other studies on different tephritid fruit fly species (Sirjani et al., 2009; Torrini et al., 2020a). We found a generally lower EPN virulence in pupae than larvae of *B. tryoni*. Lower pupal infection might be because of the closure of anal and oral apertures and the formation of a protective puparium (LeBeck et al., 1993) which made it difficult for IJs to penetrate. Moreover, the burrowing movement of larvae into the soil probably helps IJs in finding larvae; it may be more difficult for IJs to locate stationary pupae. Considering the short time that pre-pupal fruit fly larvae spend in burrowing into the soil before pupation, and the pupal stage as the longest life stage of *B. tryoni* in the soil, it is important to consider strains that are also highly effective against pupae.

Variation in penetration rate and number of IJs produced among EPN strains

Strains of all five tested EPN species displayed variable penetration and reproduction rates in *B. tryoni*, both among and within EPN species. Penetration and reproduction potential are very important traits of EPNs and need to be high for any strain to be considered for the development as a BCA. The ability of EPNs to penetrate and reproduce inside insect hosts increases EPN populations in the soil after field application, with higher chances of infecting more hosts over longer time periods and thereby providing better pest population control. There is no other report

of EPN penetration and reproduction rates in *B. tryoni* larvae. However, previous studies with *Ceratitis rosa* (Malan and Manrakhan, 2009) and *Bactrocera dorsalis* (Godjo et al., 2018b) showed that in these hosts, *Heterorhabditis* spp. have a higher reproduction potential than *Steinernema* spp.; in contrast, in our study targeting *B. tryoni*, both the highest and lowest reproduction rates were found for *S. feltiae* strains.

Conclusions

We found variability in the effectiveness of Australian-native EPNs to penetrate, infect and cause mortality of *B. tryoni* larvae and pupae. The EPN strains' virulence against larvae and pupae differed and, therefore, EPN efficacy varied with insect life stage. One aim of our study was to identify EPN strains that can penetrate and kill the pupal stage of *B. tryoni* which cover a longer time period in the soil than larvae but may be more challenging for EPNs to infect. Focussing on this target pest stage-specific aspect may lead to the selection of different EPN strains for further assessment of their persistence and performance under different environmental conditions. Furthermore, EPNs traits can be improved by cross breeding of strains which are superior in different traits to obtain strains with all desirable traits (Mukuka et al., 2010b). Finally, EPNs that are effective against different stages of *B. tryoni*, particularly pupae, are likely to be successful against other pest of economic significance with different life stages in the soil, in Australia and other parts of the world. In conclusion, based on the higher larval and pupal virulence together with medium to high penetration rate and reproduction potential, *H. indica* (Hi.HIE2, Hi.ECCH, Hi.HRN), *H. bacteriophora* (Hb.HIE), *H. marelatus* (Hm. ENCBF2), *H. zealandica* (Hz.NAR1) and *S. feltiae* (Sf.Y13) were the best performing isolates. While these EPN traits were assessed in the laboratory, their efficiency needs to be tested under

environmental conditions that are more similar to field conditions. Only then can EPN isolates be chosen as BCA candidates against a particular insect pest.

Chapter 4: Survival, persistence and effect of temperature on virulence of Australian entomopathogenic nematodes against Queensland fruit fly, *Bactrocera tryoni*

4.1. Abstract

Entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae, together with their symbiotic bacteria, have been used as biological control agents. Their efficacy, however, depends on their ability to persist and control target species at locally relevant environmental temperatures. Here, the survival, persistence and effect of temperatures on virulence of 17 isolates of five EPN species (*Heterorhabditis bacteriophora*, *Heterorhabditis indica*, *Heterorhabditis marelatus*, *Heterorhabditis zealandica*, and *Steinernema feltiae*) from Australian soils were assessed against larvae and pupae of Australia's most significant horticultural pest, Queensland fruit fly *(Bactrocera tryoni*) under laboratory conditions. After incubation at 25 °C, all isolates still infected and killed *B. tryoni* larvae after 7, 14 and 21 days, with mean LD_{50} values ranging from 24.72 (± 3.29) to 70.82 (± 10.6), 51.02 (± 4.57) to 117.24 (± 20.71) and 84.62 (± 12.45) to 144.02 (± 15.93), respectively. Interestingly, 15 isolates still infected *B. tryoni* pupae after 7 days with a mean LD_{50} value between 129.64 (± 16.5) and 209.28 (± 46.49), and 2 isolates after 14 incubation days with a mean LD_{50} value between 228.82 (± 27.8) to 209.42 (± 20.48), but no pupal mortality was seen after 21 days. After incubation at respective temperatures (15 °C, 25 °C, 30 °C) for two weeks, all isolates were capable to infect *B. tryoni* larvae at all temperatures; however, the mean LD_{50} value was generally lower at 15 °C than at 25 °C and 30 °C, ranging from 34.56 (± 4.6) to 62.44 (± 9.74), 48.26 (± 3.1) to 85.26 (± 4.86) and 82.16 (± 12.54) to 149.92 (± 19.79), respectively. EPN isolates survived longer at 15 °C and 25 °C when compared to 30 °C. Complete EPN mortality was observed after 9 weeks at 30 °C, and after 18 weeks at 15 °C and 25 °C, except for some survival in Sf.ECCS. Overall, isolates Hi.HRN2, Hz.NAR1, Hi.LMI2, Hi.QF6, Hb.HIE and Sf.ECCS are potential biological control agents for the control of fruit flies such as *B. tryoni*. Finally, isolates Hb.HIE, Hz.NAR1

and Sf.ECCS could be better in hot environments, while isolates Hi.LMI2, Hi.QF6 and Hi.HRN2 could be better in cooler climates.

4.2. Introduction

Fruit flies (Diptera: Tephritidae) are an important and widespread group of insect pests. These destructive pests cause substantial losses of fruit and fruiting vegetable crops and also limit market access of fresh fruits and fruiting vegetables because of biosecurity restrictions imposed by many countries. The Queensland fruit fly, *Bactrocera tryoni* (Frogatt), is among the most damaging tephritids and has a high invasion potential, being one of the most polyphagous and destructive horticultural pest species in Australia (Clarke et al., 2011). The adult female lays their eggs into fruit where the larvae develop. Finding reliable and effective control practices against fruit fly is a challenging task as a large fraction of the life cycle (oviposition and larval development) occurs inside the fruit. However, late instar larvae leave the fruit prior to the pre-pupal stage and pupation in the soil. Adult flies emerge from the pupae after ten or more days and have to crawl to the soil surface (Bateman, 1972). These stages outside the fruit can be targeted by soil-borne natural enemies and biological control agents.

EPNs of the families Heterorhabditae and Steinernematidae with their associated bacteria *Photorhabdus* and *Xenorhabdus* are well studied as effective biological control agents of diverse pest insects (Kaya and Gaugler, 1993; Tailliez et al., 2010). Unlike synthetic chemicals, EPNs are environment-friendly as they do not haveany detrimental side effects on humans, animals other than arthropods and the environment (Ehlers, 2003). However, their effects on non-target insects and other arthropods including other natural enemies of pest insects needs to be considered. Several studies showed that EPNs effects on non-target or beneficial pests are low (Georgis et al., 1991; Nuutinen et al., 1991; Potter et al., 1994) compared to their positive impact as biological control agent. The symbiotic relationship between EPNs and their bacteria provides the nematodes with a symbiotic partner that kills the insect hosts. EPNs have been proven

effective against several fruit fly pests including *B. tryoni* (Godjo et al., 2018b; Langford et al., 2014; Sirjani et al., 2009).

Persistence is described as the ability of EPNs to survive and remain infective in the soil before finding new insect hosts. EPNs have limited ability to survive in the soil without insect hosts, and, therefore, persistence remains a major constraint of EPN field applications (Strong, 2002). To successfully use EPNs as biological control agents, it is essential to evaluate the susceptibility of the target pest to a given EPN across its life cycle and under relevant environmental conditions to optimize the application efficacy and persistence of EPN isolates in the field. Improvements in the infectivity and persistence may allow lower application rates (Bedding et al., 1993), which can reduce the cost of EPN-based biocontrol agents and could provide farmers with an affordable and efficient control option against *B. tryoni*.

The survival and persistence of EPNs can be affected by several biotic and abiotic factors in the soil. The ability of EPN to tolerate environmental stress including heat and drought can impact their survival and virulence. High mortality of EPNs after field application remains the major constraint for EPN efficacy in the field, and can depend on temperature, moisture, soil type and pH (Griffin, 1993; Koppenhöfer and Fuzy, 2007; Kung et al., 1990a, b). The effect of temperature on EPN virulence against several fruit fly species has been studied (Aatif et al., 2020; Khoury et al., 2018; Langford et al., 2014; Shaurub et al., 2015). (Griffin, 1993) highlighted temperature as the major factor lowering the effectiveness of EPN applications in the field. However, considering varying effects of temperature were found for different EPN species, more research is needed in this field.

Survival and infectivity differ between nematode species (Georgis and Gaugler, 1991) and is influenced by lipid reserves (Hass et al., 2002), bacterial strain (Grewal et al., 1997) and bioassay

methods (Grewal et al., 1994). IJs movement and infectivity are directly affected by depletion of lipid reserves with higher lipid contents allowing EPN to survive longer (Fitters and Griffin, 2004; Patel et al., 1997). In addition, the susceptibility of the insect host and the developmental stages to be attacked by a given EPN isolate are also important factors to consider (Bedding et al., 1993). The infectivity levels can vary with EPN isolates, and studies have shown that not all EPNs are able to cause infection or mortality (Bohan and Hominick, 1997; Converse and Miller, 1999; Selvan et al., 1993a).

A challenge in the development of EPNs as biological control agent is that the activity of fruit flies can be characterized by considerable variation in the abundance and mobility of fruit fly individuals across the seasons. Furthermore, previous research has shown that larval and adult stages of fruit fly are more susceptible to EPN infection than pupae (Kamali et al., 2013; Karagoz et al., 2009a; Langford et al., 2014; Malan and Manrakhan, 2009; Yee and Lacey, 2003), yet fruit fly larvae and adults are unlikely to be exposed to EPNs for large part of their development outside of the soil, and the pupal stage is the longest developmental stage of fruit flies in the soil (Bateman, 1972). Therefore, in order to have an effect on fruit fly populations, EPNs must persist at least a period of time when no fruit fly individuals are in the soil, or be able to infect alternative hosts (Kurtz et al., 2007; Susurluk and Ehlers, 2008). They also need to infect quickly as the available time window of infection of larvae and adults during their short time in the soil is relatively short. Should one be able to select an EPN isolate with longer persistence, it might be possible to apply less frequently which in return may be more economic, likewise when using more virulent isolates which would allow application of lower concentrations.

We investigated the survival ability and virulence of nematode isolates at different temperatures together with persistence at room temperature to identify potential target candidates for the biological control of Queensland fruit fly. We aimed to test whether EPNs recently isolated from Australian soils may be better adapted to Australian conditions, and therefore, have better survivability, temperature response and/or persistence when compared with other EPNs from (1) other parts of the world (e.g., cooler climates) and/or (2) previously isolated from Australia but since been in long-term laboratory culture (i.e., the commercially available strains). We further aimed to identify EPN isolates that are superior in terms of virulence and persistence and are less affected by variation in temperature. We hypothesized that higher temperature has detrimental effect on EPN survival leading to lower virulence.

4.3. Materials and methods

4.3.1. Nematode collection and storage

Fifteen EPN isolates collected from eastern Australia from our previous study (Chapter 2) and two commercial isolates obtained from Ecogrow Environment Pty Ltd (NSW, Australia) were used in this study (Table 4.1). The nematodes were reared at 25 °C on *T. molitor* larvae and freshly emerged IJs were harvested from White traps and stored in Ringer's solution (9.0 g NaCl, 0.42 g KCl, 0.37 g $CaCl_2 \times 2H_2O$, and 0.2 g $NaHCO_3$ dissolved in 1 L of distilled water) at 15 °C before use.

Table 4.1: EPN isolates used in this study: Fifteen isolates were isolated from soils in New South Wales and Queensland (Chapter 2), and two isolates (*) were obtained from Ecogrow, Australia. All new isolates were baited with larvae of *Tenebrio molitor* except Hi.LMBT which was baited with *Bactrocera tryoni* larvae.

EPN isolates	EPN species	Locality
Hb.HIE1	*H. bacteriophora*	Richmond, NSW
Hi.ECC1H	*H. indica*	Richmond, NSW
Hi.HRN2	*H. indica*	Heron Island, QLD
Hi.LMBT	*H. indica*	Lady Musgrave Island, QLD
Hi.LMI2	*H. indica*	Lady Musgrave Island, QLD
Hi.QF6	*H. indica*	Palmwoods, QLD
Hi.QGL	*H. indica*	Duingal, QLD
Hm.ENCBF2	*H. marelatus*	Somersby, NSW
Hm.GOS1	*H. marelatus*	Somersby, NSW
Hz.ECOGROW*	*H. zealandica*	
Hz.BB1	*H. zealandica*	Batemans Bay, NSW
Hz.BB3	*H. zealandica*	Batemans Bay, NSW
Hz.NAR1	*H. zealandica*	Narara, NSW
Sf.ECOGROW*	*Steinernema feltiae*	
Sf.ECCS	*S. feltiae*	Richmond, NSW
Sf.GG1	*S. feltiae*	Gundagai, NSW
Sf.Y13	*S. feltiae*	Temora, NSW

4.3.2. Insect collection and rearing

A laboratory population of *B. tryoni* established from flies collected in Bathurst, New South Wales, in summer 2009 was used and cultured in a glasshouse chamber at 25 °C and 70 % RH (Langford et al., 2014). Eggs were collected from adult flies kept in cages (30 cm each side) using 120 mL cups filled with larval diet (Meats et al., 2004). The cups filled with larval diet were covered with parafilm perforated with needles and exposed to fruit flies in the cages for two hours to allow oviposition into the larval diet. The cups with eggs were then transferred to a container with a lid fitted with a mesh and a fine layer of sterile sand on the bottom, and the larvae and pupae were collected from this sand layer. Late instar larvae which had recently jumped (less than four hours ago) from the larval diet and were still moving were selected. Dark brown pupae (approximately three days old) were chosen for all laboratory assays.

4.3.3. Effect of temperature on EPN virulence

The effect of three temperature, 15 °C, 25 °C and 30 °C, on EPN virulence against *B. tryoni* larva was assessed using a sand plate assay. This temperature range falls within the average soil temperature in Australia, from Autumn to Spring, that is cooler by some degrees (°C) compared to air temperature. The sand was autoclaved, oven dried and maintained at 10 % moisture by weight. Petri dishes (150 mm) were filled with 250 g of sand. Five replicates were tested for each strain. Freshly harvested IJs from White traps were used for the assay. Nematodes were stored at 15 °C, 25 °C and 30 °C for two weeks in a culture flask before use. Nematode concentrations of 50, 100, 200, 500 and 1000 IJs were prepared in 1 mL of Ringer's solution and inoculated at the centre of each plate separately. The control received 1 mL of Ringer's solution. Immediately after EPN inoculation, 20 *B. tryoni* larvae were added to the centre of each plate on top of the

sand. The plates were incubated at their respective temperatures in the dark and the insect mortality due to EPN infection was recorded after seven days. The LD_{50} for each replicate was calculated.

4.3.4. EPN persistence

The ability of EPNs to infect and kill *B. tryoni* larvae and pupae after 7, 14 and 21 days of incubation at 25 ℃ was assessed using another sand plate assay. Petri dishes (150 mm) were filled with 250 g sterile sand with 10 % moisture. Then, EPN isolates used for survival estimation were chosen. Five replicates were tested for each strain. Nematode concentrations of 50, 100, 200, 500 and 1000 IJs in 1 mL of Ringer's solution were inoculated at the centre of the plates and incubated at 25 °C. After 7, 14 and 21 days of storage, 20 *B. tryoni* larvae or pupae were supplied to each plate and stored at 25 °C. Insect mortality due to EPN infection was recorded after 7 days. The LD_{50} for each replicate was calculated.

4.3.5. Effect of temperature on EPN survival ability

Survival ability of IJs of the 17 EPN isolates was estimated in a 24 well plate. For each isolate, 10,000 IJs in 2 ml Ringer's solution were placed into each well and stored at 15 °C, 25 °C and 30 °C. Five replicates were tested for each strain. The dead nematodes were counted every week for 18 weeks or till 100 % mortality was observed. The IJs were homogenously mixed before counting. For each well, 100 µl was counted, repeated three times and average mortality was used. The counted EPNs were placed back to their respective well and the volume was maintained at 2 ml after each count by adding additional Ringer's solution.

4.3.6. Statistical analysis

The dose required to kill 50 % of the insects (LD_{50}) for each replicate was calculated and compared between different isolates (Finney, 1952). The mortality (percentage of dead insects) due to EPN infection was calculated using the following formula:

mortality = a (1-ln(-bx)) +c

where **a** equals the total number of insects corrected by the control mortality; **b** is the slope; **c** is the control mortality; **x** is the dose used per insect. The LD_{50} values were calculated by fitting a saturation curve to the mortality data through minimization of the chi-square (χ^2) for the comparison of the theoretical distribution and the observed distribution using Microsoft Excel Solver. The data for the temperature effect on virulence and persistence (LD_{50}) were analysed for normality with the Shapiro-Wilkinson test ($p > 0.05$). For normally distributed data, one way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test for multiple comparisons were performed. For non-normal distributed data (survival %), Kruskal-Wallis test with post hoc Dunn's test for multiple comparisons were used. All data analyses were performed in R version 4.0.2 (R Core Team, 2020).

4.4. Results

4.4.1. Effect of temperature on EPN virulence

All 17 EPN isolates were able to infect *B. tryoni* larvae at 15 °C, 25 °C and 30 °C (Figure 4.1). Across the isolates, the mean LD_{50} value at 15 °C was lower than at 25 °C and 30 °C and ranged from 34.56 ± 4.6 to 62.44 ± 9.74, 48.26 ± 3.1 to 85.26 ± 4.86 and 82.16 ± 12.54 to 149.92 ± 19.79, respectively. The larval mortality caused by EPNs between different temperatures were

significantly different ($F_{19,235}$ = 50.56; df = 19, 235; $p \leq 0.0001$). Furthermore, the mean LD_{50} was significantly different between isolates at 15 °C ($F_{16,68}$ = 7.531; $p \leq 0.0001$), 25 °C ($F_{16,68}$ = 6.475; $p \leq 0.0001$) and 30 °C ($F_{16,68}$ = 4.156; $p \leq 0.0001$). The lowest and highest LD_{50} were found for Hi.LMI2 and Hm.GOS1 at 15 °C, for Hz.NAR1 and Hm.ENCBF2 at 25 °C, and for Hz.NAR1 and Hm.GOS1 at 30 °C. The commercial strain Hz.EG was more virulent than Hm.GOS1 at 15 °C and 30 °C, and ENCBF2 at 25 °C. In contrast, the commercial strain Sf.EG

was less virulent than Hi.QF6, Hi.HRN2 and Hi.LMI2 at 15 °C, and Hz.BB1, Hz.BB3, Hi.QF6 and Hz.NAR1 at 25 °C.

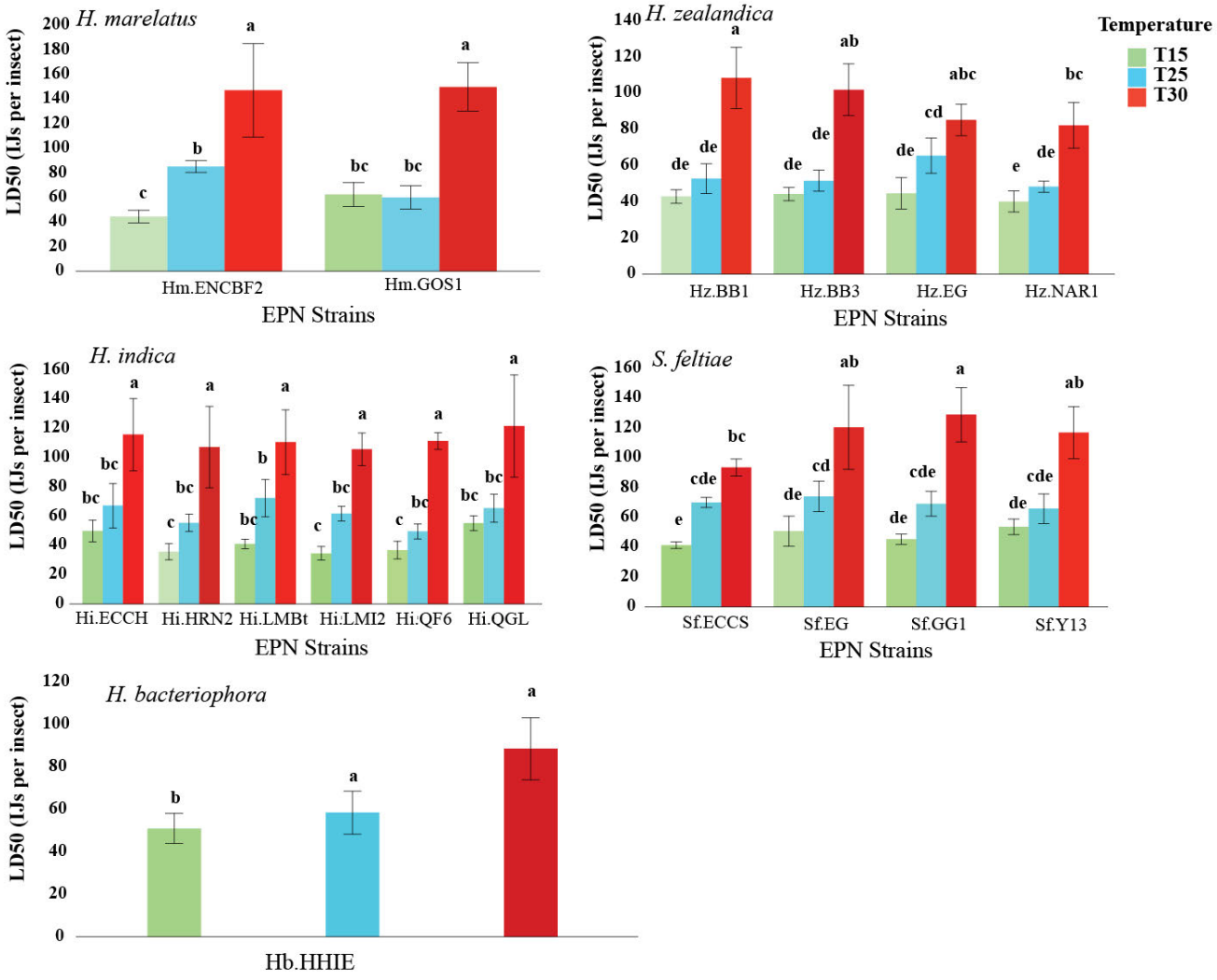

Figure 4.1: LD_{50} of EPN isolates tested against *Bactrocera tryoni* larvae after two weeks of incubation at 15 °C (green), 25 °C (blue) and 30 °C (red). Error bars indicate SD across five replicates. Different letters on top of the error bars indicate that means are significantly different from each other as per Tukey's HSD test ($p < 0.05$). Lower LD_{50} values resemble higher virulence.

4.4.2. EPN persistence

All EPN isolates were able to infect and kill *B. tryoni* larvae after 7, 14 and 21 days of incubation at 25 °C (Figure 4.2), while 15 and 2 isolates infected and killed *B. tryoni* pupae after 7 and 14 days of incubation, respectively (Figure 4.3). No pupae were infected by EPN isolates incubated for 21 days. The mean LD_{50} value against *B. tryoni* larvae after 7, 14 and 21 days of incubation ranged from 24.72 ± 3.29 to 70.82 ± 10.6, 51.02 ± 4.57 to 117.24 ± 20.71 and 84.62 ± 12.45 to 144.02 ± 15.93, respectively. There were significant differences in larval mortality between isolates incubated for 7 days ($F_{16,68} = 28.1$; $p \leq 0.0001$), 14 days ($F_{16,68} = 13.57$; $p \leq 0.0001$) and 21 days ($F_{16,68} = 8.44$; $p \leq 0.0001$). Overall, the larval mortality caused by EPNs incubated for different time periods were significantly different ($F_{20,234} = 67.4$; df = 19, 235; $p \leq 0.0001$). The pupal mortality caused by EPN isolates after 7 days of incubation ranged from 129.64 ± 16.5 to 209.28 ± 46.49. Two EPN isolates, Hz. NAR1 (LD_{50} 228.82 ± 27.8) and Hi.ECCH (LD_{50} 209.42 ± 20.48), killed pupae after 14 days of infection. The pupal mortality caused by EPN isolates were significantly different ($F_{15,69} = 5.493$; $p \leq 0.0001$). Hb. HIE was more virulent than both commercial isolates for all time periods.

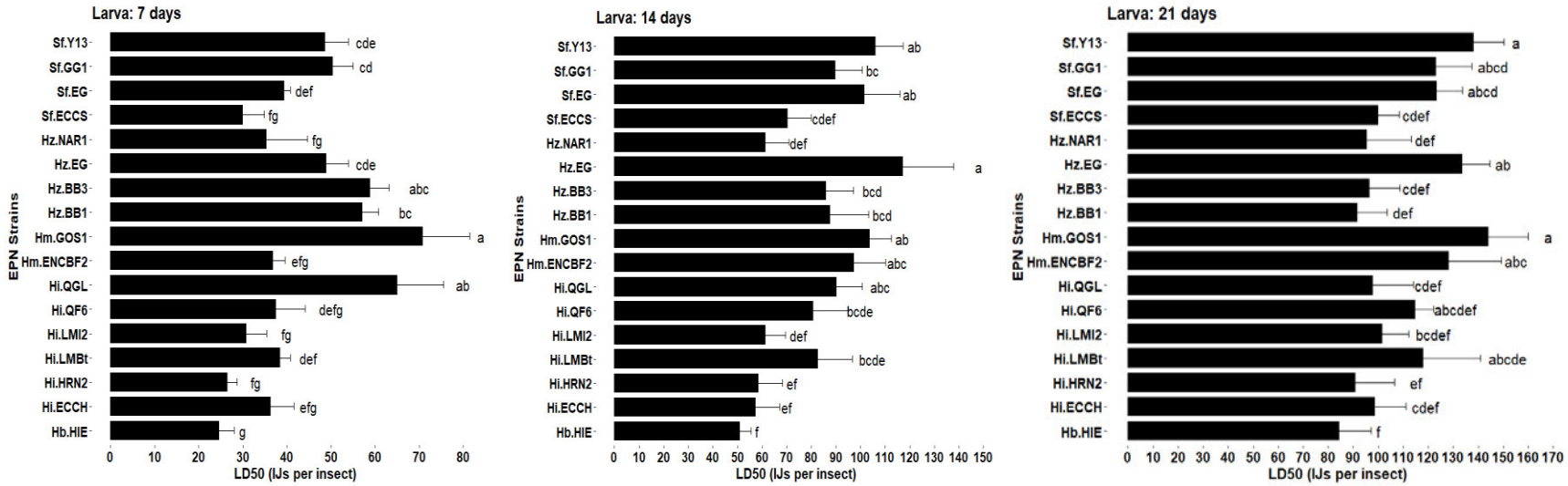

Figure 4.2: LD_{50} of EPN isolates tested against *Bactrocera tryoni* larvae after 7, 14 and 21 days of incubation at 25 °C. Error bars indicate SD across five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Tukey's HSD test ($p < 0.05$). Lower LD_{50} values resemble higher virulence.

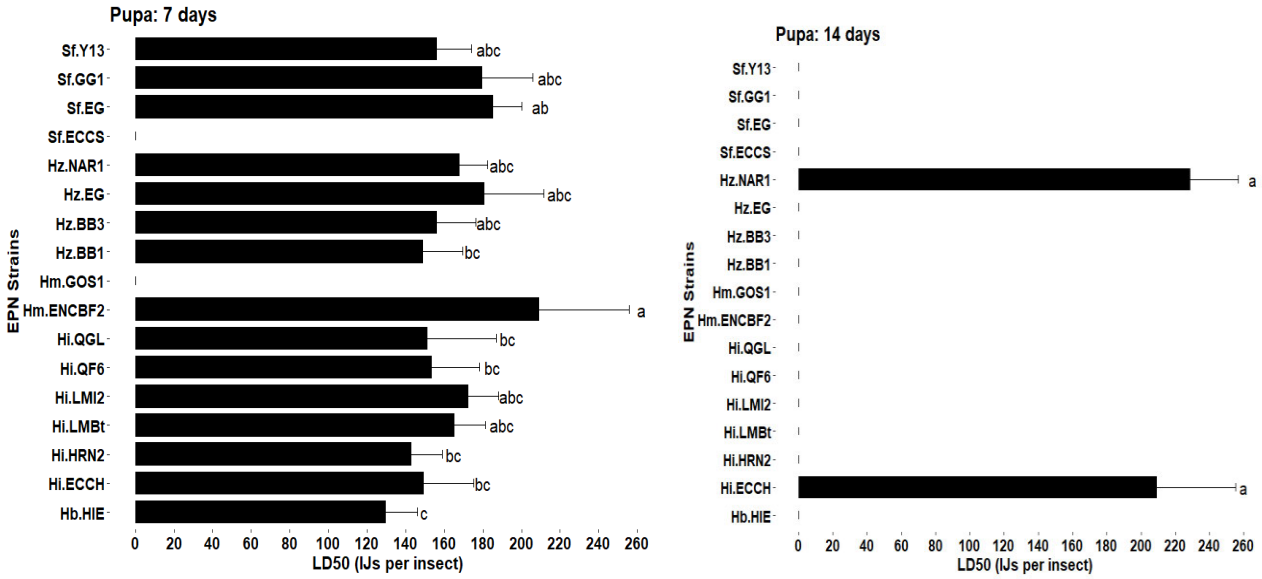

Figure 4.3: LD_{50} of EPN isolates tested against *Bactrocera tryoni* pupae after 7 and 14 days of incubation at 25 °C. Nematodes incubated for 21 days at 25 °C did not infect pupae (and are therefore not presented). Error bars indicate SD across five replicates. Different letters next to the error bars indicate that means are significantly different from each other as per Tukey's HSD test ($p < 0.05$). Lower LD_{50} values resemble higher virulence.

4.4.3. EPN survival

Temperature had a significant effect on the survival of tested EPN isolates (Figure 4.4). All isolates survived for at least 16 weeks at 15 and 25 °C, and 9 weeks at 30 °C. *Steinernema* spp. had the highest survival rate and were the only species to survive 18 weeks and more at 15 and 25 °C and 9 weeks at 30 °C. Sf.ECCS had the highest survival rate after 17 weeks at 15 °C (54.28 ± 5.26), 25 °C (56.6 ± 7.5) and after 8 weeks at 30 °C (11.78 ± 2.91). Similarly, Hi.ECCH had the highest survival rate among *Heterorhabditis* spp. after 17 weeks at 15 °C (8.28 ± 5.07) and 25 °C (14.2 ± 1.92) and Hz.BB1 (15.2 ± 4.64) at 30 °C after 8 weeks.

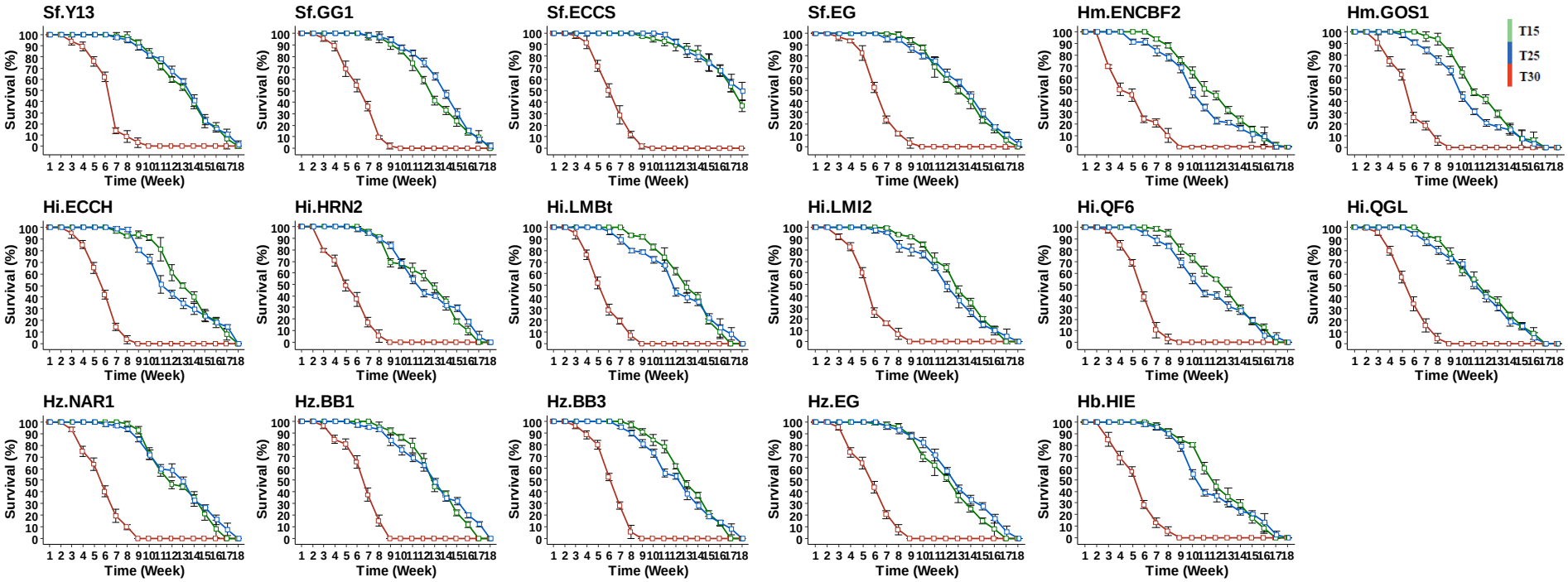

Figure 4.4: Survival (%) of EPN isolates at 15 °C (green), 25 °C (blue) and 30 °C (red). Error bars indicate SD across five replicates.

4.5. Discussion

All isolates survived and remained virulent at all temperatures and persisted for 3 weeks to still infect larvae and 2 weeks to still infect pupae at room temperature. We recorded higher nematode survival rates at lower temperatures. Nematodes survived for a maximum of 9 weeks at 30 °C and more than 16 weeks at 15 °C and 25 °C. At 15 °C, *H. indica* was most virulent followed by *H. zealandica*, at 25 °C, *H. zealandica* was most virulent followed by *H. indica*, and at 30 °C, *H. zealandica* was most virulent followed by *H. bacteriophora*. *Heterorhabditis marelatus* isolates were the least virulent at all temperatures. Although nematodes were originally collected from soils in warm-temperate and subtropical regions, all isolates (incubated for two weeks at the respective temperatures) were able to infect *B. tryoni* larvae at all three temperatures, but virulence was highest at 15 °C following reduction in virulence with increase in temperatures. In contrast many previous studies have found higher virulence at higher temperatures with EPNs (including *H. marelatus*) collected from tropical and sub-tropical climates with mild winters and hot summers (Kepenekci et al., 2015; Rohde et al., 2010). All isolates were able to infect *B. tryoni* larvae after 7, 14 and 21 days of incubation at 25 °C. We found higher infection rates after 7 days than after 14 and 21 days of incubation. Previous studies found that ageing of nematodes reduced the ability to locate and penetrate insect host, thereby reducing their infectivity and virulence (Alonso et al., 2018; Lee et al., 2016; Yoder et al., 2004).

The isolates from this study were collected from warm-temperate and subtropical climate regions, but we observed higher EPN survival and virulence at lower temperatures against *B. tryoni*. This implies that EPN isolates that originated from these warmer regions may also be able to persist in cooler climates. A similar result was obtained by Khoury et al. (2018) who recorded

higher mortality of *G. mellonella* due to *S.feltiae* and *H. bacteriophora* at 15 °C and 20 °C compared to higher temperatures of up to 35 °C. A factor that may contribute is lipid reserves which are EPNs sole energy source until they find a new host (Andaló et al., 2011; Patel et al., 1997). Higher temperatures might results in increased physiological activity leading to consumption of stored energy resulting in restriction of movement and the mortality of EPNs (Hass et al., 2002; Smits, 1996). Conversely, many studies reported increased EPN infectivity at higher temperatures (Hussaini et al., 2005; Rohde et al., 2010; Yul et al., 2002). Rohde et al. (2010) observed an increase in EPN-caused mortality of *C. capitata* with increases in temperature, with highest mortality seen at 31 °C and lowest mortality at 19 °C. Similarly, Kepenekci et al. (2015) found that temperature has a significant effect on EPN virulence, with higher virulence at higher temperatures. Previous reports suggested that higher temperature not only affects virulence, but also the progeny development. Hazir et al. (2001) recorded 100% mortality of *G. mellonella* at temperatures between 8 °C to 28 °C but no progenies were found at 28 °C, even with tropical *S. feltiae* isolates. Similar result was recorded by Lankin et al. (2020) who found absolute mortality of *Agrotis deprivata* at 28 °C, but no progeny was obtained.

Control of pests in the soil can be challenging, thus EPN isolates with ability to survive longer periods in the soil are better candidates as biological control agents. In this study EPNs were able to kill *B. tryoni* larvae after 3 weeks of incubation, while pupae were susceptible to some isolates stored for 2 weeks at 25 °C. Several studies have been carried out to find EPN isolates with better persistence in soil after application in the field. Blatt et al. (2021) showed that *S. feltiae* and *H. bacteriophora* survived and remained infective for 9 weeks in soil without irrigation. We found that the survival rate of *Steinernema* was higher than that of *Heterorhabditis* and this corroborates previous studies (Abate et al., 2019). The higher survival rate of *Steinernema* might

be the result of higher lipid reserves compared to *Heterorhabditis* (Selvan et al., 1993b). EPNs can persist for up to 150 days in field, with *H. bacteriophora* infectivity having higher persistence than *S. feltiae* (Şahin and Gözel, 2021). Furthermore, (Harvey and Griffin, 2016) recorded *S. carpocapsae* two years post application in soil and under bark of EPN treated tree stumps which was correlated with the number of insect host emerging from untreated tree stumps.

Native EPN isolates may easily adapt to the local environmental conditions and may be more virulent compared to introduced or commercial isolates (Abate et al., 2019). As the efficacy of EPNs is determined by several biotic and abiotic factors including EPN and target pest species, proper bioassays are important, in particular ones that consider the specific life cycle aspects of the target pest in the soil. EPN persistence can also be improved by managing the soil environment to be more suitable for EPN survival and dispersal; for example maintaining a neutral to acidic soil pH, adequate soil moisture and addition of soil amendments including mulching (Campos-Herrera et al., 2019). Besides, biotic factors such as soil bacteria, fungi, mites and collembolans should not be ignored as several are major antagonists that can cause EPN mortality post application (Karthik Raja et al., 2020). Moreover, to improve the survival and persistence ability of EPN post application, application of EPN-infected cadavers in place of aqueous suspension as indicated by Gulzar et al. (2020) may be useful. Besides, adults can play an important role for EPN persistence in the field as indicated by Garriga et al. (2020) who found that around 21% of nematode infected adults of *Drosophila suzukii* could fly therefore aiding for EPN dispersal. We suggest more detailed research on the significance and role of adults for dispersal and maintenance of EPN population in the field.

In conclusion, higher temperature showed a significant effect in survival and virulence of EPNs against *B. tryoni*. EPNs virulence and persistence was higher in larvae than in pupae. Overall, based on the tested traits, our findings demonstrated that *H. indica* (Hi.HRN2, Hi.LMI2, Hi.QF6), *H. zealandica* (Hz.NAR1), *H. bacteriophora* (Hb.HIE) and *S. feltiae* (Sf.ECCS) can be potential candidates for the control of *B. tryoni*. However, to test their potential as biological control agents their efficacy against *B. tryoni* will need to be evaluated under semi-field and field conditions.

Chapter 5: General discussion

5.1. Overview

Fruit flies are a biosecurity threat to horticulture worldwide. Australian horticultural industries are severely affected by Queensland fruit fly (*B. tryoni*). Conventionally, many measures to control *B. tryoni* rely on the use of synthetic chemicals, including several with negative effects on human health, pollinators and the environment. Development of resistance to chemicals by several target and non-target insect pests can be another problem with extensive use of chemicals. Therefore, sustainable farming will have to seek for alternatives that are less hazardous to nature than synthetic chemicals.

EPNs have previously shown to effectively control fruit flies including *B. tryoni* (Langford et al., 2014). Several species of *Heterorhabditis* and *Steinernema*, and their respective bacterial symbionts *Photorhabdus* and *Xenorhabdus*, have been recorded from around the world. But since Akhurst and Bedding (1986), no comprehensive survey of EPN diversity have been done in Australia. Therefore, Australian EPNs have not yet been fully explored. Several EPN isolates with a potential as biological control agent against *B. tryoni* can likely be isolated from Australia. Furthermore, the virulence of Australian native EPNs against *B. tryoni* or any other fruit fly pests in Australia has not yet been extensively tested, except for three commercially available Australian strains tested by Langford et al. (2014). Therefore, this study aimed to find potential EPN isolates that can effectively control *B. tryoni*.

This PhD research comprises two major work components: i) a comprehensive survey for EPN isolates and their associated bacterial symbionts from soil samples collected across eastern Australia and ii) detailed tests of their virulence and persistence against different stages of *B. tryoni*, as well as the evaluation of temperature effects. Four commercial isolates of Australian

EPNs were included in this study to compare their efficacy with the EPNs newly isolated from various ecosystem. This is the first comprehensive study to test the efficacy of Australian native entomopathogenic nematodes against *B. tryoni*, and comparably, also the largest effort to test EPN diversity as potential biocontrol agents against tephritid fruit flies.

5.2. Key findings and future directions

5.2.1. Extraction and identification of EPNs and their bacterial symbiont

My thesis is a comprehensive survey of EPNs, and their symbiotic bacteria, in eastern Australia, and is only the second effort of this kind in Australia since a first study was performed by (Akhurst and Bedding (1986). The present study revealed a large diversity of EPN species and their associated symbiotic bacteria from eastern Australia (Chapter 2). Previously reported EPNs were mostly extracted using *G. mellonella*, and some using *T. molitor* as a bait. On top of these universal EPN baits, the target pest *B. tryoni* was used as a more specific bait insect. However, EPN extraction with *B. tryoni* was not as successful an approach as was hoped for. The reason for this low success rate is not clear. It is possible that the larvae we used as bait were too advanced in their pre-pupal development and, therefore, more difficult to penetrate. Furthermore, the relatively small fruit fly larvae might be less efficient in attracting EPNs when compared to larger bait insects. However, *B. tryoni* baited isolate could be more virulent against fruit flies in the field. Moreover, the one isolate obtained with *B. tryoni* was not an unexpected species, nor was it behaving differently in the laboratory with regard to virulence and persistence. However, it will be interesting to see how this isolate performs against *B. tryoni* in the field.

The survey resulted in 36 new isolates of EPNs belonging to *H. bacteriophora*, *H. indica*, *H. marelatus*, *H. zealandica* and *S. feltiae*. Combined molecular and morphological approaches

might be ideal for EPN identification to species level, but I used molecular identification as a primary approach considering the highly conserved morphology with many common characteristics within the two genera of EPN species. In addition to the previously recorded Australian diversity of 6 EPN species, this study reported *H. marelatus* for the first time from Australia and *H. zealandica* and *H. indica* from NSW. Furthermore, *Photorhabdus heterorhabditis*, *P. laumondii*, *P. namnaonensis*, *P. tasmaniensis*, *Pseudomonas protegens* and *Delftia acidovorans* were also reported as EPN associated bacteria from Australia for the first time. Unexpectedly *Ps. protegens* and *D. acidovorans* were found associated with *S. feltiae* while its expected *X. bovienii* symbiont was not detected. Several other studies have also found EPNs with bacteria other than their usual symbiont (Gouge and Snyder, 2006; Ogier et al., 2020; Razia et al., 2011). Bonifassi et al. (1999) stated that the presence of these other bacteria might be because of contamination. However, we believe that these are not contaminant as these bacteria were detected in the nematodes after at least three generations of laboratory rearing, and the EPNs were surface sterilized before DNA extraction. On a similar note, Ogier et al. (2020) reported the association of a dozen of Proteobacteria species with laboratory reared *Steinernema*. The same author further confirmed the pathogenic role of two species of *Pseudomonas* and mentioned that they might be associated with parasitic life stage of *Steinernema*. Hence, the interactions of EPN with bacteria other than their known symbionts need further research to obtain a proper understanding of their role during the infection process.

In my PhD project 35 of the 36 isolates were obtained/baited with *T. molitor* larvae and one isolate with *B. tryoni* larvae. The latter indicated that there is potential to use the target pest as a bait for EPNs, albeit this was not highly successful. Although *G. mellonella* were infected with EPNs during the baiting process, no IJs were recovered throughout the process. Similar results

were also observed with infected *T. molitor* larvae from northern Queensland from which no live EPNs could be isolated. This failure of recovering EPNs from infected *G. mellonella* and *T. molitor* might have been due to the presence of contaminants during the baiting process leading to intoxication and/or lower nutrition, and, consequently, the death of EPNs. This may have prevented the EPNs to continue their life cycle inside the infected host cadaver resulting in no reproduction and formation of IJs. *Steinernema* reproduce by amphimixis that requires both male and female to produce progenies. Insect invasion by a single sex of steinernematids can kill the insect but cannot produce progenies and this might be the reason for lower extraction rate compared to EPN occurrence. Beside this, the higher baiting success of *Heterorhabditis* spp. with *T. molitor* and isolation of a single isolate with *B. tryoni* warrants the use of more than one insect bait species (including the target pest species) in the future surveys.

Soil texture, moisture, pH and EPN natural habitats affect EPN abundancy (Koppenhöfer and Fuzy, 2007; Kung et al., 1990a, b). In this study, no EPNs were recovered from clay soil while, 14 isolates including *H. marelatus* were recovered from forest soil. To date, *H. marelatus* has only been recorded from the USA, making it a relatively rare EPN species. Here, recovery of *H. marelatus* was only from forest soil suggesting that it has different habitat preferences, and, therefore, more natural and diverse ecosystems with undisturbed soil, and possibly with higher organic matter in the soil.

The diversity, abundance and ecology of EPNs in Australian soil is scarcely known. In this study 49% of the soil samples were found EPN positive. This is relatively high compared with previous reports of EPN isolation success rates found in other studies where they ranged between 2% and 45% (Hominick, 2002). The rainfall prior to soil sampling might have played a role in yielding more EPN-positive samples. Furthermore, as the main aim of this study was to find

EPNs to control *B. tryoni*, rather than their ecological interactions with other organisms in their ecosystems, we followed selective sampling regimes, avoiding areas with very dry soil. This might be one of the reasons for the higher EPN isolation success rate. Moreover, most of the EPNs isolated in this study were recovered from acidic soils with a high moisture content. It needs to be noted, however, that the EPN diversity recovered in this study might not represent the whole range of EPNs from eastern Australia. Therefore, additional surveys covering every representative type of Australian soils and climates are necessary to obtain an improved knowledge about EPN diversity and distribution in Australia.

5.2.2. EPN virulence, penetration rate and reproduction potential

EPNs can infect different life stages of *B. tryoni* in the soil, thereby reducing fruit fly abundance. In Chapter 3, EPNs newly isolated from soils across eastern Australia were characterised for their virulence, penetration rates and reproductive rates in *B. tryoni*. EPNs that are virulent and have higher penetration and reproduction rates in a target pest should be selected as a biological control agent against this pest. My PhD thesis is the second, yet more detailed, study to have tested these traits of Australian native EPN isolates against different stages of *B. tryoni*. An earlier study tested the virulence of three commercial EPN isolates against *B. tryoni* and recorded high levels of larval mortality, but no pupal infection by EPNs was observed (Langford et al., 2014). Pupal virulence is very important for EPNs to have higher efficacy in controlling field populations of *B. tryoni*, as the pupal stage in soil is considerably longer (around 11 days at 26 °C) than the very short period that the late instar larvae spend in the soil (few hours).

In this study, *B. tryoni* larvae were susceptible to all 36 EPN isolates, and 29 isolates caused pupal mortality. Many previous studies with fruit flies reported no pupal infection with EPNs (Langford et al., 2014; Yee and Lacey, 2003). Furthermore, all EPN isolates successfully

penetrated and reproduced inside *B. tryoni* larvae. From these assays, one *H. zealandica* isolate, three *H. indica* isolates, one *H. bacteriophora* isolate, one *H. marelatus* isolate and one *S. feltiae* isolate were identified as promising candidates as biological control agents against *B. tryoni*. The one isolate baited with *B. tryoni* displayed high rates of penetration, reproduction and virulence in *B. tryoni*. Interestingly, we observed some isolates of EPNs had higher penetration rate, reproductive potential and virulence, and others had lower virulence and other traits irrespective of whether they were *Heterorhabditis* spp. or *Steinernema* spp. Even within a species there was variation in traits including virulence. Furthermore, while these EPN traits were assessed in the laboratory, the better performing isolates need to be selected and tested under field conditions. Only then can EPN isolates be chosen as a candidate for biological control against a particular insect pest.

5.2.3. EPN persistence and temperature effect on survival and virulence of EPNs

Among the important EPN traits to be considered for the selection of a successful biocontrol agent, the ability to tolerate high temperatures and remain virulent in the ecosystem for a long time are the most important ones. EPNs are applied in large numbers in the field to suppress the target pest insect and for this they are expected to survive and persist for some time. EPNs persistence and efficacy can be affected by several factors including soil parameters such as pH, texture, moisture, temperature and organic matter content; further factors are exposure to UV light, the presence of predators and scavengers that can feed on infected pests and cadavers as well as soil microbes that can affect the development and life cycle of EPNs inside an infected pest individual. To ensure high efficacy of EPNs it is important to have sufficient mulch cover and/or cover crops during EPN application and establishment. This will help to prevent direct exposure of EPNs to UV light and also preserve moisture and organic matter content of the soil.

Furthermore, agricultural and horticultural fields may have been exposed to chemicals used in plant protection including fertilizers and soil amendments that reduce infectivity and survival of EPNs (Grewal, 1998; Patel and Wright, 1996; Rovesti and Deseö, 1991; Zimmerman and Cranshaw, 1990). Shapiro et al. (1996) found that *Steinernema* virulence is reduced by urea and fresh manure in laboratory tests against *G. mellonella*, while under field conditions virulence was reduced only by fresh manure application. Moreover, Bednarek and Gaugler (1997) reported that organic manure resulted in increased densities of native *S. feltiae* while NPK fertilizers negatively affected EPN populations. However, EPN susceptibility to chemical pesticides can differ with nematode species and isolates (Grewal, 2002) and therefore assays both in the laboratory and the field should be carried out to find isolates that are less susceptible to chemical pesticides and fertilizers.

Chapter 4 assessed the persistence and temperature effect on survival and virulence of EPNs against *B. tryoni* larvae and pupae under laboratory conditions. Considering EPN isolates in this study were collected from warm temperate and sub-tropical climates, it was expected that their survival and virulence increased with temperature. However, it was found that high temperature had a detrimental effect on survival and virulence of all EPN isolates. Furthermore, similar levels of EPN survival were observed at 15 °C and 25 °C but EPN survival was drastically reduced at 30 °C. Similarly, Khoury et al. (2018) observed decreased mortality of *G. mellonella* at higher temperature when testing EPN isolates from Mediterranean soils. On the other hand, Rohde et al. (2010) found that virulence of tropical and subtropical isolates against *C. capitata* increased with temperature. In general, *Heterorhabditis* spp. are adapted to warmer temperatures and *Steinernema* spp. to cooler temperatures (Hazir et al., 2001; Karagoz et al., 2009b). Interestingly survival of *Steinernema* was found to be higher than *Heterorhabditis* both at higher and lower

temperatures. Therefore, more detailed studies on the thermal niche of species of both genera of EPN are necessary.

This study has great importance for EPN survival, establishment and persistence, as nematode that can survive and remain virulent at higher temperature under laboratory conditions would possibly persist longer under field conditions a well. However, EPNs might encounter complex interactions with either predators or alternate hosts that could have negative or positive effects on EPN survival and persistence. Presence of natural enemies like invertebrate predators, nematophagous fungi, omnivores and scavengers might result in EPNs death both outside and inside insect cadavers (Kaya, 2002). On the other hand, Pilz et al. (2014) mentioned that the presence of alternate host in soil might allow nematodes to survive and complete their life cycle when the target pest is not available. Therefore, if EPNs can manage to survive in alternate hosts they can be ready to attack the target pest when it is present. However, EPNs may also have negative side effects when they attack beneficial insects in the soil (Rojht et al., 2009; Shapiro-Ilan and Cottrell, 2005). Yet, many previous studies have shown that EPNs effects on non-target insects and beneficial insects are very low (Hill, 1998; Potter et al., 1994).

Several interesting approaches have been proposed with the aim to increase EPN persistence in the field. One is the use of infected insect cadavers instead of aqueous application (Gulzar et al., 2020). This can be both advantageous and disadvantageous. The positive aspect is that application of infected cadavers creates an ideal environment for EPNs inside the cadaver and so IJs can possibly delay their emergence under adverse soil and climatic conditions improving their survival and persistence. Furthermore, the EPNs might gradually leave the cadaver, thus freshly emerged nematodes that are superior in terms of infectivity and virulence compared to older ones will be available across a longer time period. However, there might be a case where

IJs failed to emerge from the infected cadaver because of insufficient development or mortality. Moreover, scavengers or omnivores in the soil can feed on the applied infected cadavers resulting in death of developing nematodes inside the cadavers.

Another important research focus should be on the use of adult flies that have recently emerged from the puparium. A study by Garriga et al. (2020) showed that live infected adults can still be a good vector for the dispersal of EPNs until they succumb to the infection. Furthermore, should it be difficult to infect pupae, targeting the adults may be a better approach as previous research has shown that adults are more susceptible to EPNs than pupae (Kamali et al., 2013; Yee and Lacey, 2003). Malan and Manrakhan (2009) found that both *H. bacteriophora* and *H. zealandica* were virulent against adult *C. capitata* and *C. rosa*. Similarly, Abbas et al. (2016) reported 60% adult *B. zonata* were infected with *H. bacteriophora* after inoculation of the pupae. So, the next phase of study should investigate the effectiveness of Australian native EPNs between pupae and emerging infected adults to see their role in EPN dispersal and establishment in field environments.

5.3. Concluding remarks and recommendations for the future

This thesis is a comprehensive and detailed survey of EPNs and their associated bacterial symbionts isolated from soil across eastern Australia, revealing higher than previously recognized EPN diversity. However, isolation success with *B. tryoni* was not very high, and this warrants further research, using different stages of *B. tryoni* (larvae, pupae and adults) as a bait, with soils collected from throughout Australia, covering more regions and habitats. Laboratory assays showed promising results of EPN virulence and persistence against pupae of *B. tryoni* warranting further research to test the efficacy of the EPN isolates in semi-field and field

conditions. Assays to test the virulence of EPNs in particular against recently emerged and mature adults should be carried out, and it should also be checked if EPN infection has an impact on adult behavior and dispersal, as this may provide further insight into potential EPN dispersal by infected adult flies. EPNs can be effective against several insect pests including fruit fly species but might not be the only solution for all pest problems. Therefore, incorporation of EPNs in IPM programs is important for effective pest control. EPN application together with other means of fruit fly management like other biological control agents (such as parasitoids and entomopathogenic fungi) and SIT may be more effective than applying each control measure by itself. This integration of different measures needs further research, as some measures may be antagonistic and others synergistic. Besides this, more research to find isolates that are more specific to the target host need to be done. This can also include breeding programs, including the genetic hybridization and crossing of EPNs followed by selection for better traits to improve the performance of strains against particular pests and in particular environments so that they can tolerate harsh soil conditions, survive and persist for longer while still remaining virulent. It may also include experiments to switch bacterial isolates of EPN species to improve traits. As mentioned earlier, EPNs in Australia are not fully explored, and less explored than in other parts of the world. Therefore, additional studies that better understand the biogeography of EPNs in Australia but also across the world need to be done. It is necessary to understand how these nematode species interacts with each other and with several biotic and abiotic factors in natural and agricultural ecosystems. Finally, it is important to research and develop the upscaling of the application and production of better performing isolates at a commercial scale. In-vivo multiplication or IJs production using insects is laborious and therefore liquid culture or in-vitro

mass production is a common and successful technique for IJs production at a commercial scale (Ehlers, 2001).

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Appendices

Supplementary Table S1: List of EPNs previously characterized from Australia. Species are listed in alphabetical order. All regions mentioned are in Australia.

EPNs	Regions	References
H. bacteriophora	Brecon (Victoria); Darwin (Northern Territory)	(Akhurst and Bedding, 1986; Poinar, 1975; Sagun et al., 2015)
H. indica	Darwin (Northern Territory)	(Fischer-Le Saux et al., 1998; Stack et al., 2000)
H. zealandica	Tasmania; Queensland	(Poinar, 1990)
S. caropcapsae	New South Wales; Tasmania	(Poinar, 1990)
S. feltiae	Tasmania; Canberra (Australian Capital Territory); Victoria; New South Wales; Queensland	(Poinar, 1990)
S. longicaudum	Australia (but no state/territory or specific location provided)	(Hominick, 2002)

Supplementary Method S1

IJ isolation

A modified White trap (Kaya and Stock, 1997) was used to harvest IJs from infected bait cadavers. This consisted of a smaller Petri dish (35 mm) covered with a filter paper contained within a larger Petri dish (90 mm) filled with Ringer's solution (9.0 g NaCl, 0.42 g KCl, 0.37 g $CaCl_2 \times 2H_2O$, and 0.2 g $NaHCO_3$ dissolved in 1 l distilled water). The filter paper overlapped the smaller Petri dish so that it touched the surrounding Ringer's solution. Each dead insect was placed on top of the wet filter paper. After depletion of the food resources, the IJs migrated towards the Ringer's solution. After 14 days, the Ringer's solution with EPNs was transferred to a 15 ml centrifuge tube and the nematodes were allowed to settle on the bottom. The EPNs were then transferred to a cell culture flask (growth area 25 cm^2; total volume 50 ml) using a 1 ml pipette and 6 ml fresh Ringer's solution was added. Subsequently, new insect host individuals were inoculated with 1 ml of the collected IJs (1000 IJs per ml).

Supplementary Method S2

Soil texture analysis

Soil texture was calculated based on hydrometer readings. For this, 25 g oven-dried, 2 mm sieved soil was placed in a beaker with 25 ml5% Calgon solution and filled with 250 ml water. The soil solution was mixed rigorously for 5 minutes using a blender and transferred to a 500 ml measuring cylinder. Water was added to a volume of 500 ml. This solution was thoroughly mixed by inverting the cylinder several times and then the time was noted as time zero (T_0). After 5 minutes, a hydrometer reading (Rt_5) was recorded which represented the concentration of both silt and clay particles. Furthermore, the temperature of the solution was recorded (T °C).

The next reading was taken 90 minutes after T_0 which represented the clay particles (Rt_{90}). A blank solution with Calgon and water was made and topped up to 500 ml and another hydrometer reading was taken (R_0) to enable a correction to be made for Calgon. The texture was calculated following equations and the triangular diagram was used to find the textural class name (McDonald et al., 1998).

Summation % P1 at 5 minutes = $[Rt_5 - R_0 + (T - 19.5) \times 0.3] / (2 \times 25) \times 100$

Summation % P2 at 90 minutes = $[(Rt_{90}) - R_0 + (T - 19.5) \times 0.3] / (2 \times 25) \times 100$

Sand % = 100 - P1

Clay % = P2

Silt % = P1 - P2

Supplementary Method S3

PCR conditions

The PCR reaction mixture contained 4 µl of 5 × MyTaq buffer (Bioline), 0.4 µl of 20 mM forward and reverse primer, 0.1 µl (2 U/µl) MyTaq DNA polymerase, 1 µl DNA extract, and PCR H_2O up to a volume of 20 µl. Thermocycling conditions were initial denaturation at 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 90 s, and final extension at 72 °C for 5 minutes (D2-D3); an initial denaturation at 94 °C for 4 minutes, followed by 34 cycles of 94 °C for 1 minute, 55 °C for 90 s and 72 °C for 2 minutes, and final extension at 72 °C for 10 minutes (ITS1); and initial denaturation at 94 °C for 2 minutes, followed by 31 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 90 s, and final extension at 72° C for 5 minutes (16S rRNA gene).

Primers used in the study.

Gene locus	Primer name	Primer sequence (5' – 3')	Reference
ITS1 (EPN)	TW81	GTTTCCGTAGGTGAACCTGC	(Joyce et al., 1994)
	AB28	ATATGCTTAAGTTCAGCGGGT	
D2-D3 (EPN)	D2A	ACAAGTACCGTGAGGGAAAGTTG	(De Brida et al., 2017)
	D3B	TCGGAAGGAACCAGCTACTA	
16S rRNA (bacteria)	27F	AGAGTTTGATCCTGGCTCAG	(Frank et al., 2008)
	1492R	GGTTACCTTGTTACGACTT	

Supplementary Method S4

ExoSAP treatment of PCR products prior to sequencing

This was performed using the protocol described in Morrow et al. (2014): 2.5 µl of exonuclease (20 U/µl) and 25 µl of shrimp alkaline phosphatase (SAP) (1U/µl) were mixed with 172.5 µl of PCR water, 2 µl was then added to 12 to 18 µl PCR product and incubated at 37 °C for 30 minutes followed by 94 °C for 5 minutes.

Supplementary Table S2: EPN species (in alphabetical order) isolated from soil baited with larvae of *Tenebrio molitor* and *Bactrocera tryoni* with the geographical location, habitat type (C citrus; G grassland; F forest) and soil physical properties (Texture: lS loamy sand; S sand; sL sandy loam; SOM: soil organic matter). Strain baited with *B. tryoni* is labelled with BT, all others were baited with *T. molitor*. Asterisks (*) denote commercial strains from Ecogrow. Strains with no available DNA sequences are labelled with NA. All localities mentioned are in Australia.

EPN species	Strains	Bacteria	Coordinates	Locality	Habitat	Texture	SOM (%)	pH	Moisture (%)
H. bacteriophora	Hb. ECOGROW*	*Photorhabdus heterorhabditis*	-	-	-	-	-	-	-
H. bacteriophora	Hb.HIE1	*P. laumondii*	33.609965, 150.746438	Richmond NSW	C	lS	8.86	6.18	8.98
H. bacteriophora	Hb.HIE2	*P. tasmaniensis*	33.609965, 150.746438	Richmond NSW	C	lS	8.86	6.18	8.98
H. indica	Hi.ECC1H	*NA*	33.616071, 150.754905	Richmond NSW	C	S	12.75	5.83	9.90

H. indica	Hi.ECCSA	*P. tasmaniensis*	33.616071, 150.754905	Richmond NSW	C	S	12.75	5.83	9.90
H. indica	Hi.HIE1	NA	33.609965, 150.746438	Richmond NSW	C	lS	8.86	6.18	8.98
H. indica	Hi.HIE2	NA	33.610506, 150.745450	Richmond NSW	G	S	9.62	6.25	11.11
H. indica	Hi.HIE3	NA	33.610506, 150.745451	Richmond NSW	G	S	9.62	6.25	11.11
H. indica	Hi.HRN	NA	23.442316, 151.915879	Heron Island QLD	F	S	11.60	6.81	20.32
H. indica	Hi.HRN2	NA	23.442316, 151.915879	Heron Island QLD	F	S	12.20	6.91	21.64
H. indica	Hi.LMBT	*P. laumondii*	23.906647, 152.393471	Lady Musgrave Island QLD	F	S	76.84	3.41	18.30

H. indica	Hi.LMI1	NA	23.906647, 152.393470	Lady Musgrave Island QLD	F	S	87.96	3.40	15.87
H. indica	Hi.LMI2	NA	23.906647, 152.393471	Lady Musgrave Island QLD	F	S	76.84	3.41	18.30
H. indica	Hi.QF6	NA	26.703622, 152.949518	Palmwoods QLD	F	S	8.23	6.12	14.68
H. indica	Hi.QFSC6	*P. tasmaniensis*	26.703622, 152.949518	Palmwoods QLD	F	S	5.18	6.08	8.44
H. indica	Hi.QGL	NA	25.129154, 151.996016	Duingal QLD	G	S	4.33	7.35	8.67
H. indica	Hi.QGLB	NA	25.129154, 151.996016	Duingal QLD	G	lS	5.26	7.51	11.04
H. marelatus	Hm.CB	*P. laumondii*	28.671484, 153.612721	Byron Bay NSW	F	S	10.01	5.37	16.16

H. marelatus	Hm.CBF1A	NA	31.784866, 152.707818	Coralville NSW	F	sL	6.62	5.26	18.12
H. marelatus	Hm.ENCBF2	NA	24.198639, 151.806083	Eurimbula QLD	F	S	5.90	6.36	5.89
H. marelatus	Hm.GOS1	NA	33.374125, 151.32519	Somersby NSW	F	S	3.18	4.81	16.73
H. marelatus	Hm.GOS2	NA	33.374125, 151.32519	Somersby NSW	F	S	3.18	4.81	16.73
H. marelatus	Hm.GOS3	NA	33.374125, 151.32519	Somersby NSW	F	S	5.25	5.56	17.00
H. zealandica	Hz.BB1	NA	35.725000, 150.172000	Batemans Bay NSW	F	S	3.02	7.63	8.51
H. zealandica	Hz.BB2	NA	35.711306, 150.178111	Batemans Bay NSW	G	S	5.96	7.26	10.65
H. zealandica	Hz.BB3	NA	35.837972, 150.129306	Broulee NSW	G	sL	6.56	7.61	13.29

H. zealandica	Hz.ECOGROW*	NA	-	-	-	-	-	-	-
H. zealandica	Hz.NAR1	NA	33.392056, 151.332417	Narara NSW	C	S	2.82	5.03	10.86
H. zealandica	Hz.NAR2	*P. namnaonensis*	33.392056, 151.332417	Narara NSW	C	S	2.51	5.49	11.71
H. zealandica	Hz.NAR3	NA	33.392056, 151.332417	Narara NSW	C	S	3.06	6.51	13.56
H. zealandica	Hz.NAR4	*P. heterorhabditis*	33.392056, 151.332417	Narara NSW	C	S	5.20	6.10	13.13
S. feltiae	Sf.ECCS	NA	33.616071, 150.754905	Richmond NSW	C	S	12.75	5.83	9.90
S. feltiae	Sf.ECCSA	NA	33.616071, 150.754905	Richmond NSW	C	S	12.75	5.83	9.90
S. feltiae	Sf.ECCSB	NA	33.616071, 150.754905	Richmond NSW	C	S	12.75	5.83	9.90

S. feltiae	Sf.ECOGROW*	NA	-	-	-	-	-	-	-
S. feltiae	Sf.GG1	*Pseudomonas protegens*	35.072445, 148.102235	Gundagai NSW	G	sL	7.62	6.37	13.27
S. feltiae	Sf.GG2	NA	35.072445, 148.102235	Gundagai NSW	G	sL	9.77	7.06	13.68
S. feltiae	Sf.Y13	*Delftia acidovorans*	34.449722, 147.533528	Temora NSW	C	sL	9.78	5.88	18.24
S. feltiae	Sf.YNG	*Pseudomonas protegens*	34.449278, 147.533417	Temora NSW	C	lS	10.60	6.18	16.21

Supplementary Table S3: BLAST results showing closest relatives with GenBank accession numbers and percentage identity of EPN and its associated bacterium from this study. Strains with no available DNA sequences are labelled with NA. Species are listed in alphabetical order.

EPN isolates	ITS1		D2-D3		16S rRNA gene	
	Related EPN species (accession number)	Percent identity	Related EPN species (accession number)	Percent identity	Related bacterium species (accession number)	Percent identity
Hb.ECOGROW	*Heterorhabditis bacteriophora* (MK421486.1)	99.61	*Heterorhabditis bacteriophora* (MK421468.1)	99.82	*Photorhabdus heterorhabditis* (NR133816.1)	96.35
Hb.HIE1	*H. bacteriophora* (MK421504.1)	99.48	*H. bacteriophora* (MK421468.1)	99.82	*P. laumondii subsp. laumondii* (NR028870.1)	97.52
Hb.HIE2	*H. bacteriophora* (MG334257.1)	99.48	*H. bacteriophora* (MK421468.1)	99.82	*P. tasmaniensis* (NR11651.1)	97.09
Hi.ECC1H	*H. indica* (MH489028.1)	99.87	*H. indica* (MK421431.1)	100	NA	

Hi.ECCSA	*H. indica* (MH489028.1)	99.59	*H. indica* (MK421431.1)	99.65	*P. tasmaniensis* (NR11651.1)	99.47
Hi.HIE1	*H. indica* (MN922779.1)	99.53	*H. indica* (MK421431.1)	100	NA	
Hi.HIE2	*H. indica* (MH489028.1)	99.86	*H. indica* (MK421431.1)	100	NA	
Hi.HIE3	*H. indica*	NA	*H. indica* (MK421431.1)	100	NA	
Hi.HRN	*H. indica* (MN922779.1)	99.82	*H. indica* (MK421432.1)	97.54	NA	
Hi.HRN2	*H. indica* (MN028773.1)	99.73	*H. indica* (MK421432.1)	99.65	NA	
Hi.LMBT	*H. indica* (MN028773.1)	99.73	*H. indica* (MK421432.1)	99.83	*P. laumondii subsp. laumondii* (NR028870.1)	94.06
Hi.LMI1	*H. indica*		*H. indica* (MK421432.1)	98.44	NA	

Hi.LMI2	*H. indica* (MN922779.1)	99.68	*H. indica* (MK421432.1)	96.57	NA	
Hi.QF6	*H. indica* (MH489028.1)	99.73	*H. indica* (MK421431.1)	100	NA	
Hi.QFSC6	*H. indica* (MH489028.1)	99.59	*H. indica* (MK421431.1)	100	*P. tasmaniensis* (NR11651.1)	91.6
Hi.QGL	*H. indica* (MN922779.1)	99.55	*H. indica* (MK421431.1)	97.27	NA	
Hi.QGLB	*H. indica* (MH489028.1)	99.87	*H. indica* (MK421431.1)	99.82	NA	
Hm.CB	*H. marelatus* (EF043441.1)	99.86	*H. marelatus* (EU100412.1)	100	*P. laumondii subsp. laumondii* (NR028870.1)	92.35
Hm.CBF1A	*H. marelatus* (EF043441.1)	99.86	*H. marelatus* (EU100412.1)	100	NA	
Hm.ENCBF2	*H. marelatus* (EF043441.1)	99.86	*H. marelatus* (EU100412.1)	99.82	NA	

Hm.GOS1	*H. marelatus* (EF043441.1)	99.19	*H. marelatus* (EU100412.1)	98.25	NA	
Hm.GOS2	*H. marelatus* (EF043441.1)	99.31	*H. marelatus* (EU100412.1)	100	NA	
Hm.GOS3	*H. marelatus* (EF043441.1)	99.29	*H. marelatus* (EU100412.1)	100	NA	
Hz.ECOGROW	*H. zealandica* (MT476870.1)	99.38	*H. zealandica* (MH443381.1)	97.73	NA	
Hz.BB1	*H. zealandica* (EF530041.1)	97.96	*H. zealandica* (DQ145666.1)	100	NA	
Hz.BB2	*H. zealandica* (EF530041.1)	97.8	*H. zealandica* (DQ145666.1)	100	NA	
Hz.BB3	*H. zealandica* (AY321481.1)	99.58	*H. zealandica* (DQ145666.1)	99.82	NA	
Hz.NAR1	*H. zealandica* (EF530041.1)	97.64	*H. zealandica* (DQ145666.1)	100	NA	

Hz.NAR2	*H. zealandica* (MT476870.1)	99.87	*H. zealandica* (MH443381.1)	100	*P. luminescens* (NR115332.1)	98.57
Hz.NAR3	*H. zealandica* (EF530041.1)	95.44	*H. zealandica* (DQ145666.1)	99.26	NA	
Hz.NAR4	*H. zealandica* (MT476870.1)	98.94	*H. zealandica* (DQ145666.1)	99.45	*P. heterorhabditis* (NR133816.1)	98.66
Sf.ECOGROW	*Steinernema feltiae* (KM016354.1)	99.86	*Steinernema feltiae* (JF728852.1)	99.82	NA	
Sf.ECCS	*S. feltiae* (JF728858.1)	96.51	*S. feltiae* (JF728852.1)	100	NA	
Sf.ECCSA	*S. feltiae* (JF728858.1)	98.75	*S. feltiae* (JF728852.1)	99.82	NA	
Sf.ECCSB	*S. feltiae* (MK256355.1)	98.84	*S. feltiae* (JF728852.1)	100	NA	
Sf.GG1	*S. feltiae*	NA	*S. feltiae* (JF728852.1)	100	*Pseudomonas protegens* (NR114749.1)	97.98

Sf.GG2	*S. feltiae* (KM016346.1)	92.58	*S. feltiae* (JF728852.1)	100	NA	
Sf.Y13	*S. feltiae*	NA	*S. feltiae* (JF728852.1)	100	*Delftia acidovorans* (NR113708.1)	94.77
Sf.YNG	*S. feltiae*	NA	*S. feltiae* (JF728852.1)	99.81	*Ps. protegens* (NR114749.1)	96.42

Supplementary Table S4: Isolation of EPN strains across New South Wales (NSW) and Queensland (QLD), Australia. Asterisk (*) indicates that nematode species infecting bait cadavers (as confirmed by dissection) could not be cultured and identified.

	temperate NSW	**sub-tropical QLD**	**tropical QLD**
Number of sites examined	56	17	3
Number of samples	140	40	18
Number of EPN-positive samples	76	15	7
Number of EPN strains identified	26	10	0
Number of sites with nematodes detected			
H. bacteriophora	1	0	0
H. indica	4	4	0
H. marelatus	3	1	0
H. zealandica	4	0	0
S. feltiae	4	0	0
Not identified*	26	6	3
Number of samples with nematodes detected			
H. bacteriophora	2	0	0
H. indica	5	5	0
H. marelatus	5	1	0
H. zealandica	7	0	0
S. feltiae	7	0	0
Not identified*	50	9	7

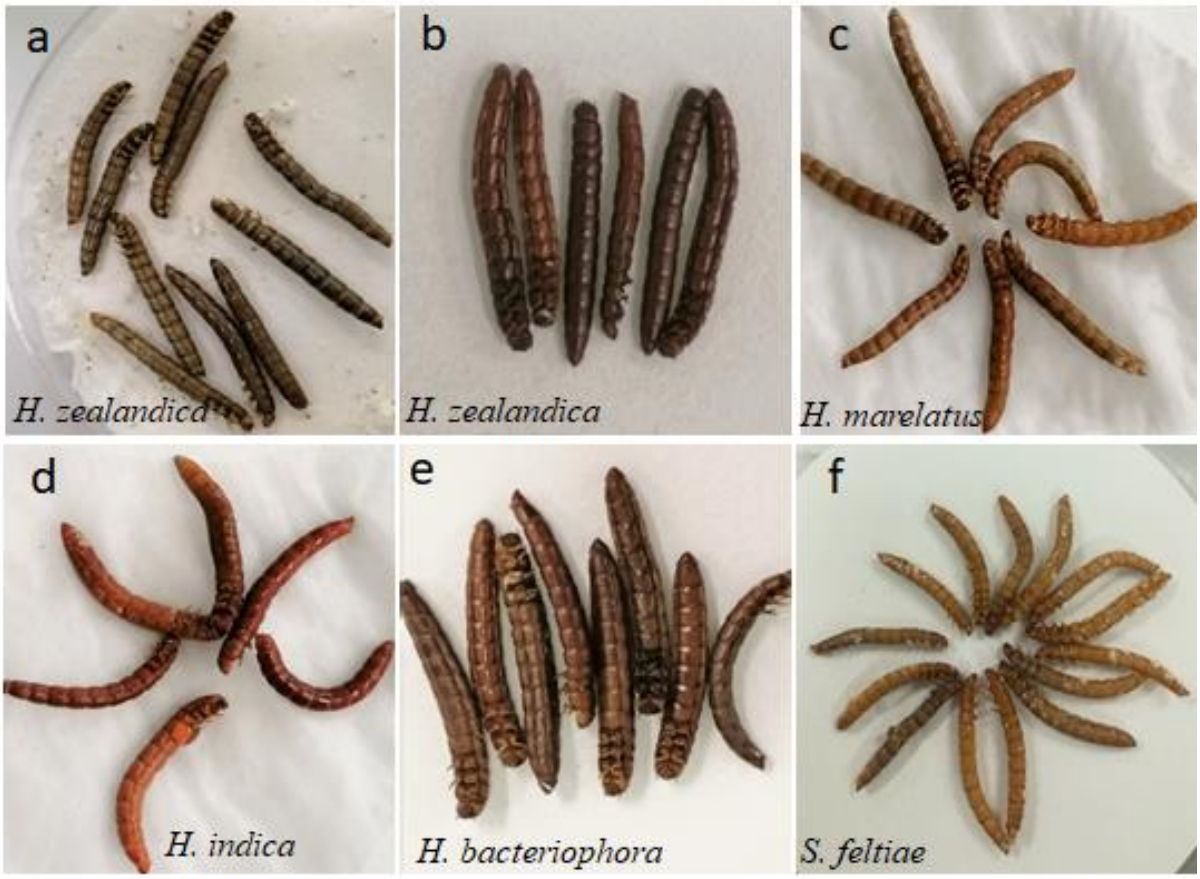
Supplementary Table S5: List of EPN species with their associated bacteria as previously reported and the ones found in this study. Species are listed in alphabetical order.

EPNs	Associated bacteria	References	Bacteria from this study
H. bacteriophora	*P. laumondii, P. laumondii* subsp. *laumondii, P. laumondii* subsp. *clarkei, P. thracensis, P. kayaii, P. carribbensis, P. temperata, P. luminescens, P. khaini, P. akhurstii, P. kleinii, P. stackebrandtii*	(An and Grewal, 2010, 2011; Boemare et al., 1993; Fischer-Le Saux et al., 1999b; Hazir et al., 2004; Machado et al., 2018; Maneesakorn et al., 2011; Tailliez et al., 2010; Thomas and Poinar, 1979)	*P. laumondii* Hb.HIE1, *P. tasmaniensis* Hb.HIE2, *P. heterorhabditis* Hb.ECOGROW
H. indica	*P. akhurstii, P. noenieputensis*	(Ferreira et al., 2013a; Fischer-Le Saux et al., 1999b; Machado et al., 2018)	*P. laumondii* Hi.LMBT, *P. tasmaniensis* Hi.QFSC6, *P. tasmaniensis* Hi.ECCSA
H. marelatus	*P. tasmaniensis*	(Machado et al., 2018; Tailliez et al., 2010)	*P. laumondii* Hm.CB

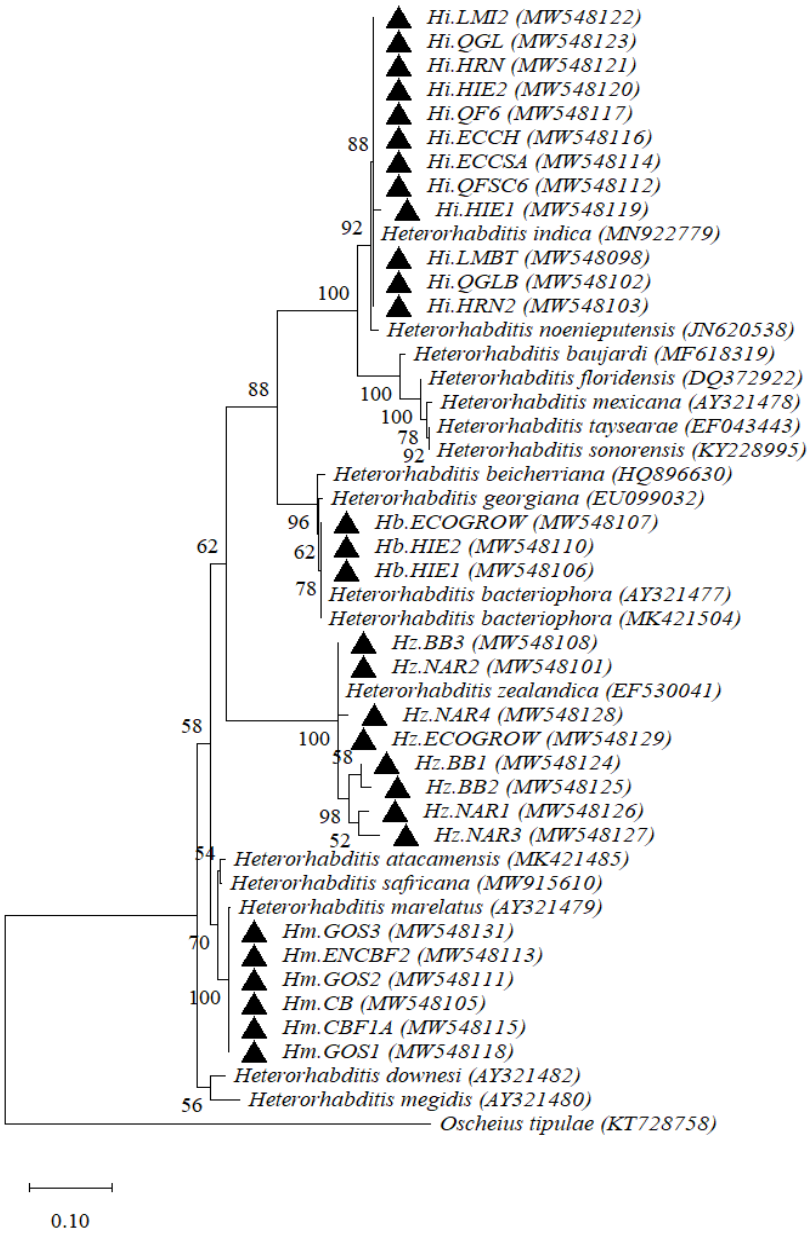
H. zealandica	*P. heterorhabditis, P. temperata*	(Ferreira et al., 2014; Fischer-Le Saux et al., 1999b)	*P. namnaonensis* Hz.NAR2, *P. heterorhabditis* Hz.NAR4
S. feltiae	*X. bovienii*	(Boemare et al., 1993; Tailliez et al., 2006)	*D. acidovorans* Sf.Y13, *Ps. protegens* Sf.GG1, *Ps. protegens* Sf.YNG

Supplementary Table S6: List of bacterial species with their associated nematodes as previously reported from Australia. Species in the table are listed in alphabetical order. All regions mentioned are in Australia.

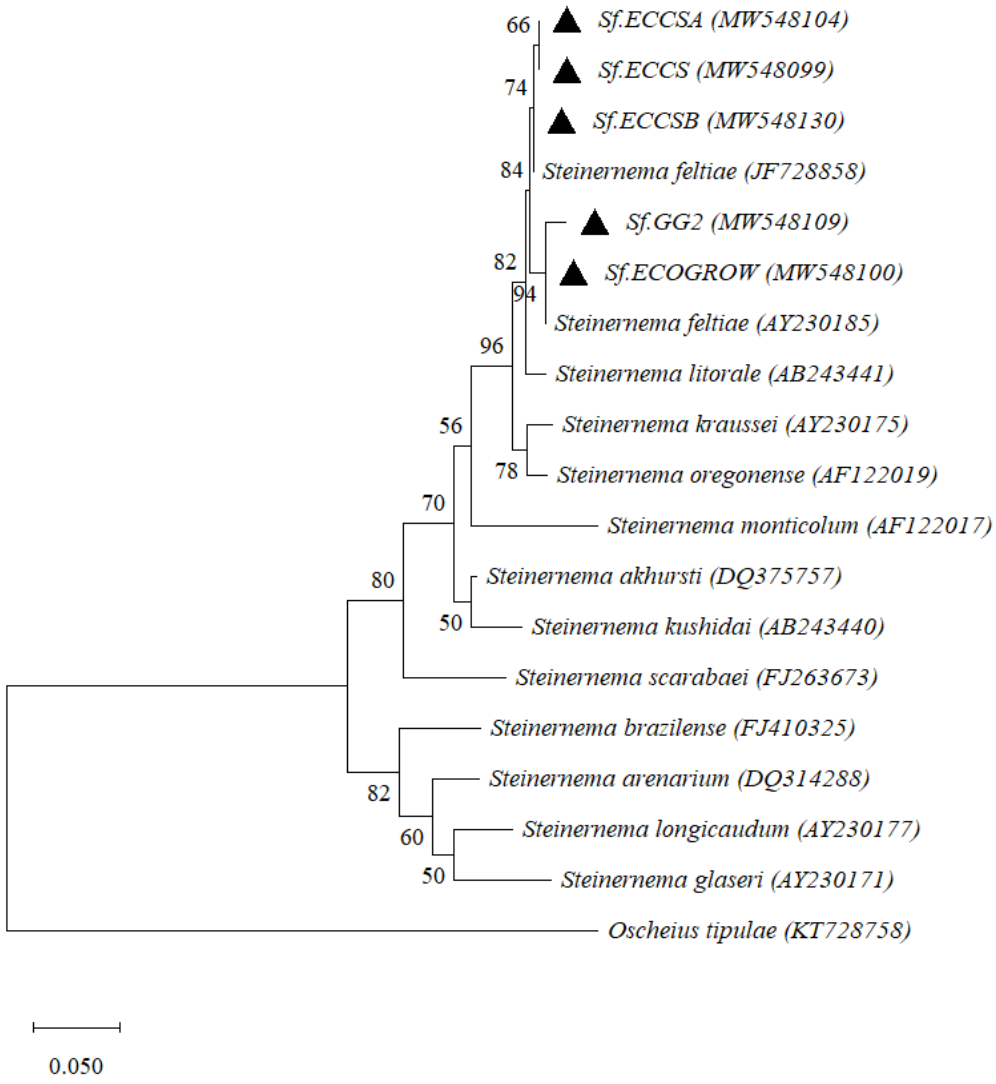
Bacteria	Strains	Associated EPNs	Regions	References
P. luminescens	Hb	*H. bacteriophora*	Victoria	(Fischer-Le Saux et al., 1998)
P. akhurstii	D1	*H. indica*	Northern Territory	(Fischer-Le Saux et al., 1998)
P. luminescens	Q614	*Heterorhabditis* sp.	Queensland	(Fischer-Le Saux et al., 1998)
X. beddingii	Q58	*Steinernema* sp.	Queensland	(Akhurst, 1986; Akhurst and Boemare, 1988; Boemare et al., 1993; Brunel et al., 1997; Fischer-Le Saux et al., 1998; Sicard et al., 2004; Tailliez et al., 2006)
X. bovienii	T228, N60	*S. feltiae*	Tasmania	(Akhurst, 1983; Akhurst and Boemare, 1988; Boemare et al., 1993; Brunel et al., 1997; Fischer-Le Saux et al., 1998; Tailliez et al., 2006)
X. miraniensis	Q1	*Steinernema* sp.	Queensland	(Boemare et al., 1993; Tailliez et al., 2006)



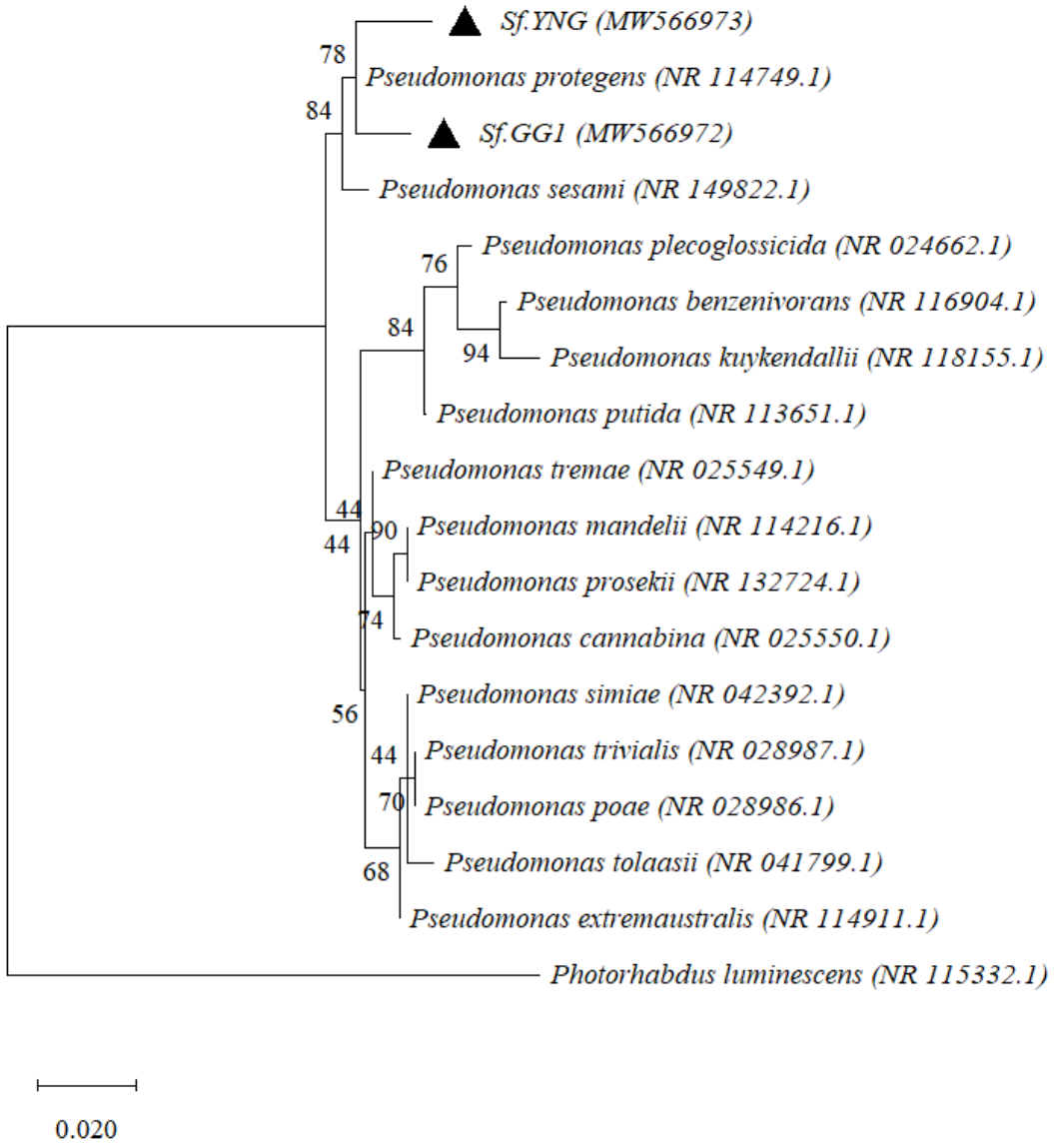
Supplementary Fig. S1: Variation in *Tenebrio molitor* cadaver color after infection with different EPN isolates. a. *Heterorhabditis zealandica* Hz.NAR1, with greenish coloration; b. *Heterorhabditis zealandica* Hz.NAR2, with red coloration; c. *Heterorhabditis marelatus* Hm. GOS2, with light red coloration; d. *Heterorhabditis indica* Hi.LMBt, with orange to brick-red coloration; e. *Heterorhabditis bacteriophora* Hb.HIE, with red coloration and ; f. *Steinernema feltia* Sf.ECCS, with light brown coloration. The average size of *T. molitor* larvae was 27.73 ± 2.76 mm.



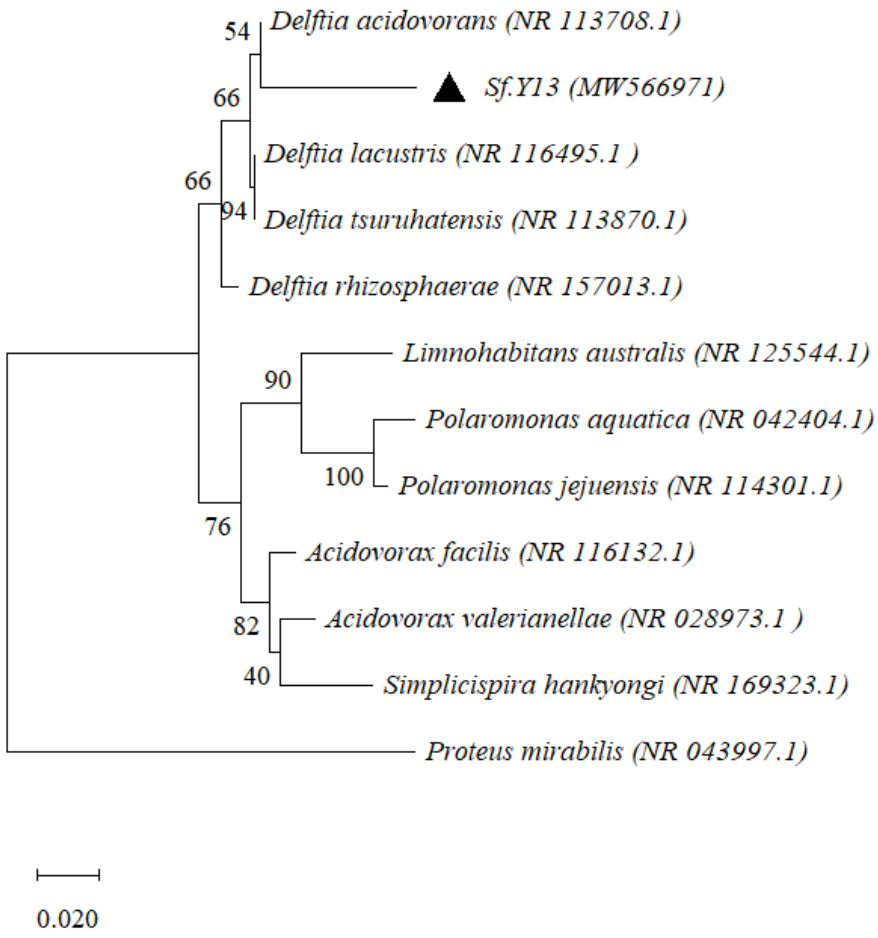
Supplementary Fig. S2: Maximum Likelihood tree of the EPN isolates from eastern Australia (triangles) and other known *Heterorhabditis* spp. based on the analysis of ITS1 (406bp – 1057 bp), with *Oscheius tipulae* as an outgroup; bootstrap values at the nodes (1,000 replicates); GenBank accession numbers in parentheses; scale bar represents number of substitutions per site.



Supplementary Fig. S3: Maximum Likelihood tree of the EPN isolates from eastern Australia (triangles) and other known *Steinernema* spp. based on the analysis of ITS1 (434bp – 1067 bp), with *Oscheius tipulae* as an outgroup; bootstrap values at the nodes (1,000 replicates); GenBank accession numbers in parentheses; scale bar represents number of substitutions per site.



Supplementary Fig. S4: Maximum Likelihood tree of *Pseudomonas protegens* of two S*teinernema feltiae* isolates (triangles) and other *Pseudomonas* spp. based on the analysis of 16S rRNA gene (743 bp to1531 bp), with *Photorhabdus luminescens* as an outgroup; bootstrap values at the nodes (1,000 replicates); GenBank accession numbers in parentheses; scale bar represents number of substitutions per site.



Supplementary Fig. S5: Maximum Likelihood tree of *Delftia acidovorans* of a *Steinernema feltiae* isolate (triangle) and other *Delftia* spp. based on the analysis of 16S rRNA gene (784 bp to 1534 bp), with *Proteus mirabilis* as an outgroup; bootstrap values at the nodes (1,000 replicates); GenBank accession numbers in parentheses; scale bar represents number of substitutions per site.

Supplementary Fig. S6: Life stages of *Bactrocera tryoni* used in this study. a) Late instar larvae (L3) and early-stage pupae and b) pupae (> 3 days old).