

**Barcoding alien invertebrates and biological control
agents in South Africa, with a focus on the natural enemies
of *Thaumatotibia leucotreta* (Meyrick)**

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Abstract

Alien invertebrate species and agricultural pests pose economic challenges through impeding crop production and threatening biodiversity in South Africa. Invertebrates are particularly successful invaders owing to their short generation times, high reproductive output, widespread occurrence across terrestrial, freshwater and marine environments, and considerable morphological diversity. The false codling moth, *Thaumatotibia leucotreta* (Meyrick), is a notable pest in South Africa, as it attacks important export crops such as citrus and stone fruit, greatly reducing returns on these exports, while threatening the country's access to international markets. While several wasp species have been recorded parasitising *T. leucotreta*, their distribution in South Africa is poorly documented, while only two species have been trialled as biological control agents against the pest.

The projected shifts in species distribution patterns due to globalisation and climate change underscore the need for comprehensive baseline genetic and distribution data of alien invertebrate species and biological control agents. Such data are important for mitigating and managing biological invasions. A useful genetic tool in this respect, is DNA barcoding, which uses a short genetic marker to aid in the delimitation of species. However, there is no recent database of barcoded alien invertebrates and biological control agents in South Africa. In addition, thermal physiological traits of invertebrates are useful data for modelling species distributions, especially under climate change. However, current baseline physiological data of important pests and their biological control agents remain limited, negatively impacting our understanding of their present and future distribution patterns. This study aimed to: 1) review the DNA barcoding status of alien invertebrates and biological control agents in South Africa; 2) sample *T. leucotreta* parasitoid wasps in organic and IPM orchards within the Western Cape; and 3) to determine the critical thermal limits of *Trichogrammatoidea cryptophlebiae* Nagaraja, an egg parasitoid used as a biological control agent against *T. leucotreta*, in the laboratory.

The review of alien invertebrate species in South Africa identified major gaps in DNA barcoding, particularly among arachnids and platyhelminths (flatworms). While 66.8% of all alien invertebrate species in the South African dataset were accessioned on BOLD (Barcoding of Life Datasystems), only 24.3% of these records represented specimens collected in South Africa. Furthermore, 44.6% of alien invertebrate species records on BOLD were not accompanied by images. This research has identified important gaps in the baseline knowledge of alien invertebrate species, pests, and biological control agents in South Africa. The paucity of South African specimens and image records on BOLD constrains the effectiveness of these records for identification. Despite conducting sampling under ideal conditions, no *T. leucotreta* parasitoids were sampled in the Western Cape. This absence may

be attributed to low parasitoid population densities or suboptimal sampling methodology. The lack of *T. leucotreta* parasitoids sampled in the Western Cape suggests that natural wasp populations may be insufficient for pest control, supporting the case for augmentative biological control whilst highlighting the need for more intensive sampling and trials of parasitoid wasps as biological control agents. Using thermal tolerance assays, the critical thermal minimum and maximum of *T. cryptophlebiae* were determined to be $8.15^{\circ}\text{C} \pm 0.58^{\circ}\text{C}$ and $44.50^{\circ}\text{C} \pm 0.41^{\circ}\text{C}$ (mean $\pm$ SE), respectively. The critical thermal minimum of *T. cryptophlebiae* determined in this study exceeds the wasp's lower developmental threshold, indicating that adult wasps are less cold-tolerant than other life stages. These thermal tolerance data provide valuable baseline information for predicting potential distribution shifts of *T. cryptophlebiae* under climate change scenarios. Overall, this study contributed to baseline data on alien invertebrate, pest and biological control agent species, including *T. leucotreta* and its parasitoids, in South Africa. These baseline data underscore the need for further research on the distribution and biology of these important species.

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Table of contents

Abstract.....	iii
Acknowledgements.....	v
Table of contents.....	vii
CHAPTER 1: General introduction.....	1
Background.....	1
Preserving insect biodiversity in agroecosystems.....	1
Biological control in agroecosystems.....	3
Integrated Pest Management.....	4
The false codling moth, <i>Thaumatotibia leucotreta</i>	5
Egg.....	6
Larva.....	6
Pupa.....	6
Adult.....	7
Management of false codling moth.....	8
Pest monitoring.....	8
Chemical control.....	8
Cultural control (orchard sanitation).....	9
Biological control.....	9
DNA barcoding.....	17
The Barcode of Life Data System (BOLD).....	18
Barcoding for biosecurity: aliens and biological control agents.....	19
Other genetic markers.....	19
Aims and objectives.....	21
References.....	21
CHAPTER 2: DNA barcoding of alien invertebrates and biological control agents in South Africa: a review.....	30
Introduction.....	30
Methods.....	33
Results.....	34
Discussion.....	38
Conclusion.....	41
References.....	43
CHAPTER 3: Parasitoid wasps of the false codling moth (<i>Thaumatotibia leucotreta</i>) in stone fruit and citrus orchards: sampling and barcoding.....	50
Introduction.....	50

Methods	52
Results	55
Discussion.....	60
References.....	62
CHAPTER 4: Critical thermal limits of <i>Trichogrammatoidea cryptophlebiae</i> Nagaraja (Hymenoptera: Trichogrammatidae).....	67
Introduction	67
Methods	68
Results	70
Discussion.....	71
References.....	74
CHAPTER 5: General discussion and conclusion	79
Supplementary material	86

CHAPTER 1

General introduction

Background

Preserving insect biodiversity in agroecosystems

Insects are crucial to the functioning of terrestrial ecosystems (Losey & Vaughan, 2006; Yang & Gratton, 2014; Wagner, 2020). They are fundamental components of trophic systems, since they fulfil the nutritional needs of many small mammals, birds and reptiles. In addition, insects also provide fundamental services to ecosystems such as nutrient cycling (Yang & Gratton, 2014). These services are important for the functioning of ecosystems and thus for maintaining biodiversity (Yang & Gratton, 2014). For humans, agroecosystems (agricultural land and all the biotic and abiotic components thereof) are the most important ecosystem type for which insects perform ecological services (Peden, 1998; Losey & Vaughan, 2006). Losey and Vaughan (2006) estimated the annual value of ecological services performed by wild insects in the United States of America to be about \$57 billion. Their study explicitly focused on wild species, not taking into account domesticated insects such as the honeybee *Apis mellifera* (Linnaeus) (Hymenoptera: Apidae) that are deliberately used to improve pollination rates, which would increase the economic value of insects significantly (Losey & Vaughan, 2006). The value that insects add to global agriculture warrants greater investment in the conservation of insects associated with agroecosystems.

Trends of global insect declines have been increasing (Simmons et al., 2019; Thomas, Jones & Hartley, 2019), especially declines in local insect populations, biomass, and species richness being recorded (Hallmann et al., 2017; Wagner, 2020; Wagner et al., 2021). However, there are also well-documented examples of local increases in populations of certain species (Schuch et al., 2012; Simmons et al., 2019; Thomas, Jones & Hartley, 2019). Nevertheless, local insect declines can be detrimental to insect biodiversity and ecosystem functioning (Losey & Vaughan, 2006; Raven & Wagner, 2021). A recent review of insect declines by Sánchez-Bayo and Wyckhuys (2019) reported substantial declines in North America and Europe and predicted extreme future global declines of insects, with a 40% decrease in species richness over the next few decades. Their article attracted significant media attention, with the sensationalist term “Insectageddon” being used in news headlines (Simmons et al., 2019; Thomas, Jones & Hartley, 2019). Although the article by Sánchez-Bayo and Wyckhuys (2019) drew attention to the important shifts in insect populations, it had some significant shortcomings. Firstly, their methodology was fundamentally flawed by only reviewing articles that studied declines (instead of population studies generally). Secondly,

the studies reviewed were overwhelmingly from North America and Europe, with far fewer studies from Africa, Asia, South America and Australia, although this could be attributed to a lack of studies in the underrepresented countries. Thirdly, the authors discussed drivers of insects declines, such as agricultural intensification, pesticide pollution, afforestation, climate change and invasive species, without explicitly testing these (Simmons et al., 2019; Thomas, Jones & Hartley, 2019). While the article by Sánchez-Bayo and Wyckhuys (2019) helped popularise the problem of insect declines, it was not a rigorous study. Furthermore, insect declines are much more heterogeneous than the authors claimed.

One of the putative drivers of local declines in insect populations is the increase and intensification of agricultural land use (Grubisic et al., 2018; Wagner, 2020; Raven & Wagner, 2021). Agroecosystems are often composed of large areas of land supporting a single crop (Wagner, 2020). These homogenous monocultures exclude much of the natural insect diversity, while attracting crop pests (Wagner, 2020; Raven & Wagner, 2021). Furthermore, agroecosystems are usually treated with pesticides, herbicides and other chemicals (Wagner, 2020; Rani et al., 2021). Pesticides are often not target-specific and may harm many insects and other non-target organisms in the treated agroecosystem (Raven & Wagner, 2021). Genetically modified (GM) crops may also threaten agroecosystem biodiversity (Zhang, Wohlueter & Zhang, 2016). Some GM crops are imbued with herbicide resistance, meaning that herbicide use is less reserved, which may be deleterious to beneficial plants and any beneficial invertebrates, such as natural enemies of crop pests (Zhang, Wohlueter & Zhang, 2016). Since the farming of GM crops was permitted since 1995 (Mabaya et al., 2015), the use of GM crops in South Africa may be reducing insect species richness (Raven & Wagner, 2021). Artificial light at night is another factor that can negatively impact entomofauna in agroecosystems by increasing the successfulness of predators by improving their vision at night (Grubisic et al., 2018). While many factors harm native insects in agroecosystems, insects in adjacent areas may also be affected by the loss of foraging areas or nesting sites (Ferreira et al., 2020). Although there is conflicting evidence for a global extinction-level threat to insect biodiversity, local insect declines do occur (Hallmann et al., 2017; Raven & Wagner, 2021; Wagner et al., 2021). Some of the potential drivers of declines, such as intensifying agriculture and pollution, seem highly probable (Wagner, 2020). However, more rigorous meta-analyses and more baseline species richness data from undersampled regions are needed to determine global trends of insect abundance or whether a global trend exists at all (Simmons et al., 2019; Thomas, Jones & Hartley, 2019).

Global climate change is a growing driver of biodiversity loss (Hallmann et al., 2017; Trisos, Merow & Pigot, 2020). However, climate change does not affect all ecosystems equally

(Bellard et al., 2012). For instance, climate refugia are small, climatically stable areas within a larger ecosystem that is more impacted by climate change than its refugia (Keppel et al., 2012). Agroecosystems may be antithetic to refugia in that they compound the adverse effects of climate change (Boxall et al., 2009; Wagner, 2020). Furthermore, changing climates may cause shifts in the ranges of agricultural pests or the species that naturally attack them (Raven & Wagner, 2021; Skendžić et al., 2021).

Biological control in agroecosystems

Monocrops are ideal for the establishment and spread of crop pests (Wagner, 2020; Raven & Wagner, 2021). Subsequently, pesticides are often applied to crops to kill pests (Raven & Wagner, 2021). However, pesticides can lead to unintended problems, including pesticide resistance in pests, the destruction of local populations of natural enemies of the pest, and excessive pesticide residues, associated with pesticide misuse (Wagner, 2020). Furthermore, the South African government has legislated the restriction or outright elimination of several insecticides since the late 1970s as part of its “National Bio-Economy Strategy” (Moore, 2021). Therefore, farmers, especially in South Africa, are increasingly favouring non-chemical control methods and management strategies that employ multiple control methods, including biological (Moore, 2021).

Biological control is the use of an organism to control the population of another organism (Hajek, 2004). Classical biological control involves using the natural enemies of an undesired species, or pest, to control its population (Hajek, 2004). Natural enemies are species that attack a pest in its native range (Hajek, 2004). Classical biological control is most often used for invasive species (Hajek, 2004). The invasive species’ native range is surveyed for its natural enemies, and these are collected and shipped to a quarantine laboratory (Hajek, 2004). Before a natural enemy is used as a biological control agent against a pest, biologists must first ensure that the enemy does not attack any species other than the pest, a step known as a host specificity test (Hajek, 2004). Once host specificity is determined, the natural enemy must be approved for mass rearing and release into target zones (Hajek, 2004). Finally, the biological control agent is released on pest population, and the agent’s performance is monitored (Hajek, 2004).

South Africa has a long history of biological control (Greathead, 2003; Hill, 2003). The first documented biological control effort in Africa was the introduction of *Rodolia cardinalis* (Mulsant) (Coleoptera: Coccinellidae) against the cottony cushion scale *Icerya purchasi* (Maskell) (Hemiptera: Monophlebidae) in South Africa in 1892 (Greathead, 2003). *Icerya purchasi* was a pest of citrus and ornamental plants (Greathead, 2003). In fact, many of the

early biological control efforts in Africa were against insect pests (Greathead, 2003). The first biological control of a weed in Africa was also in South Africa when *Dactylopius ceylonicus* (Green) (Hemiptera: Dactylopiidae) was used to control *Opuntia vulgaris* (Miller) (Cactaceae) in 1913 (Greathead, 2003). This biological control programme was very successful, and within decades there were biological control programmes for other invasive *Opuntia* species (Greathead, 2003).

Currently there are a total of 466 established alien invertebrate species in South Africa (Janion-Scheepers & Griffiths, 2020). Of these, 101 are invasive alien pests of crops and pastures (Prinsloo & Uys 2015), while 95 are biological control agents. All of these are insects except two arachnids, the lantana flower gall mite *Aceria lantanae* (Cook) (Janion-Scheepers & Griffiths, 2020) and the water hyacinth mite *Orthogalumna terebrantis* Wallwork. However, far fewer biological control agents have been used to control invasive invertebrates than plants, and information about such programmes is less readily available. In South Africa, biological control is one of the most important long-term strategies for managing agricultural pests, especially given that the alternative chemical control options can cause to ecosystems and human health (Moore, 2021; Rani et al., 2021; Raven & Wagner, 2021).

Integrated Pest Management

By the 1970s, there was a realisation of the disadvantages of reliance on a single control option like pesticides in Africa (Greathead, 2003). This, together with the long delay period between the release of biological control agents and the reduction of the pest population, motivated the need for an integration of multiple control methods into a unified control strategy that suited each pest (Greathead, 2003). Integrated pest management (IPM) is a pest control decision-making system that uses multiple distinct techniques in the most compatible way to control a pest population or eradicate it (Kogan, 1998; Steyn et al., 2019). Often, IPM implies the integration of classical biological control as the primary control method, but this is not always the case (Kogan, 1998).

There are many IPM systems targeting insect pests in Africa (Cherry et al., 2003; Greathead, 2003; Legg, Gerling & Neuenschwander, 2003; Neuenschwander, 2003). These include for the control of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Cherry et al., 2003), whiteflies in sub-Saharan Africa (Legg, Gerling & Neuenschwander, 2003), and cassava and mango mealybugs (Neuenschwander, 2003), among others (Greathead, 2003). An African example where classical biological control was not the primary component of an IPM system is the control of the desert locust, *Schistocerca gregaria* (Forskål) (Greathead, 2003). An outbreak of the locust in 1986-1988 coincided with the banning of dieldrin, an insecticide that

had been the cornerstone of locust control since the 1960s (Greathead, 2003). Subsequently, research began on the use of semiochemicals as biopesticides (Greathead, 2003). An entomophagous fungus that attacks the muscles in locusts was isolated and later used in the product Green Muscle™ for the control of *S. gregaria* in South Africa (Greathead, 2003). IPM has proven to be an invaluable strategy for the control of invasive alien species and agricultural pests in Africa (Greathead, 2003; Moore, 2021).

The false codling moth, *Thaumatotibia leucotreta*

The false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), is indigenous to Africa. It is thought to have originated in and near modern Ethiopia, although it is now present in more than 40 African countries and islands (Adom et al., 2021). *Thaumatotibia leucotreta* is also considered an indigenous pest in Israel (Kaspi, Kontsedalov & Ghanim, 2018; Adom et al., 2021). South Africa is the African country with the most comprehensive distribution analysis and research of this species (Adom et al., 2021). *Thaumatotibia leucotreta* occurs in a vast array of ecological zones, including desert and xeric shrubland, tropical and subtropical grasslands, savannas and moist broadleaf forest (Adom et al., 2021).

Thaumatotibia leucotreta is a pest of more than 70 plant species across 40 families globally (EPPO, 2019; Adom et al., 2021). In southern Africa, *T. leucotreta* is a pest of citrus, table grapes, peppers, pomegranates and stone fruit (Moore, 2021). *Thaumatotibia leucotreta* also attacks litchi, cotton, and maize to a lesser extent (EPPO, 2013). In South Africa, *T. leucotreta* is the most destructive pest of *Citrus* species, except for lemons, *Citrus limon* ((L.) Osbeck) (Sishuba, 2003; Moore, Kirkman & Hattingh, 2015; Malan et al., 2018). Although *T. leucotreta* larvae accept lemon fruit after hatching, larval development is rarely, if ever, completed; as such, *C. limon* is not regarded as a host for *T. leucotreta* (EPPO, 2013; Moore, Kirkman & Hattingh, 2015). *Thaumatotibia leucotreta* causes preharvest fruit drop and postharvest fruit decay, reducing the yield of orchards and the quality of the commercial product (Zimba et al., 2016; Moore, 2021). In addition, *Thaumatotibia leucotreta* has phytosanitary status in most markets to which fresh South African fruit is exported, including Europe, the United States of America and many countries in East Asia (Moore, 2021). Phytosanitary status is synonymous with quarantine status and implies a zero-tolerance for *T. leucotreta* in fresh produce (Moore, 2021). The detection of *T. leucotreta* in a single fruit can result in the disposal and destruction of entire citrus containers (Moore, 2021). The EU has placed strict trade regulations on countries where *T. leucotreta* is indigenous (Adom et al., 2021; Moore, 2021). If the fruit is to

be exported to the EU, the exporting countries must implement a management strategy condoned by the EU and EPPO (EPPO, 2013; Adom et al., 2021; Moore, 2021).

All life stages of *T. leucotreta* are associated with the host crop (Adom et al., 2021). Adult female *T. leucotreta* oviposit on the surface of the host fruit or on the flower bud in the case of *Rosa* spp. (Linnaeus) (Rosales: Rosaceae) (EPPO, 2019). An adult female can lay up to 800 eggs in its lifetime and may lay between three and eight eggs per fruit at the optimum temperature of 25°C (Adom et al., 2021). The complete life cycle ranges from 30 days (under optimum conditions) to 174 days and requires approximately 800 degree days (Adom et al., 2021).

Egg

Thaumatotibia leucotreta eggs (Figure 1A) are small, flat and slightly oval (width: ~0.60 mm, length: ~0.77 mm). Although the eggs are initially a translucent whitish colour, later becoming orange, they are often the same colour as the fruit surface and thus very difficult to detect by eye (EPPO, 2019). Eggs take 2-22 days to hatch, depending on ambient temperature and humidity (Adom et al., 2021).

Larva

After the larvae (Figure 1B) hatch, they immediately cut through the fruit rind and bore into the fruit (Adom et al., 2021). The larvae feed on the fruit pulp and seeds and are thus the life stage that causes damage to crops (EPPO, 2019; Adom et al., 2021). Other than eating the fruit pulp, larvae also leave unappealing frass inside the fruit and around their entrance hole (Figure 1C) (EPPO, 2019). Larval development time varies for different host fruit and is longer in poor quality fruit and at lower ambient temperatures (Adom et al., 2021). It is rare for more than one larva to develop per fruit because *T. leucotreta* larvae are cannibalistic (Adom et al., 2021). *Thaumatotibia leucotreta* has five larval instars (Adom et al., 2021). If the host fruit has not dropped by the time the larva has reached maturity, the larva will exit the fruit and use a silken thread to drop to the ground where it pupates (Adom et al., 2021).

Pupa

Before pupating, the larva enters a prepupal stage of 2-27 days, depending on ambient conditions, before moulting into a pupa (Figure 1D) (Adom et al., 2021). Pupation occurs in a silken cocoon hidden beneath the soil surface, under leaf litter or in bark crevices (EPPO, 2019). The pupal stage is the most sensitive in the *T. leucotreta* life cycle and can be influenced by temperature, relative humidity, soil cover, rainfall and soil pathogens (Adom et al., 2021).

Adult

Thaumatotibia leucotreta adults (Figures 1E&F) are most active at night, which is when females usually oviposit (Adom et al., 2021). Adults are dimorphic, with females (Figure 1E) being larger than males (Figure 1F) (Adom et al., 2021). Males have semicircular scent pockets on their hind wings and large genital tufts (EPPO, 2019; Adom et al., 2021). The scent pockets produce and store a scent used in courtship (Grant, 1978).

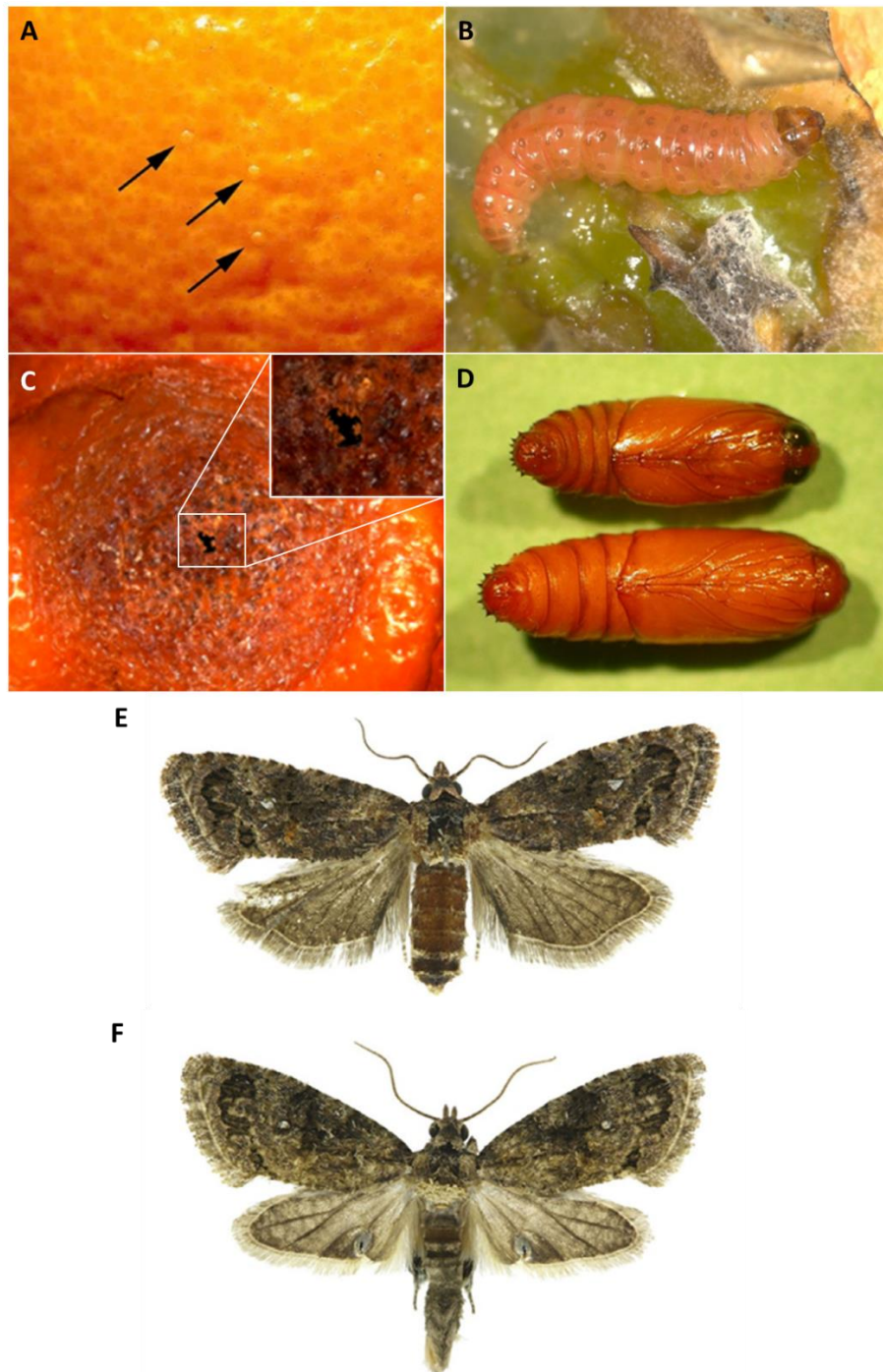


Figure 1. *Thaumatotibia leucotreta* life stages. A; eggs, B; larva, C; larval entry hole with frass protruding therefrom, D; male (top) and female pupae, E; adult female, F; adult male (EPPO, 2019).

Management of false codling moth

Pest monitoring

In the past, many fruit orchards used pheromone traps to track the prevalence of male *T. leucotreta* to determine whether interventions are necessary at a particular orchard (Moore, 2021). However, since the designation of *T. leucotreta* as a phytosanitary pest, pheromone traps are no longer used for this purpose because the domestic loss of fruit is outweighed by the economic loss of exported produce being rejected (Moore, 2021). Essentially, it is a given that intervention, now management, of *T. leucotreta* is required at any orchard (Moore, 2021). Pheromone traps are now used as a precision management tool for optimising the timing of treatments and determining whether the infestation of an orchard is low enough to qualify for export to markets where *T. leucotreta* is deemed a phytosanitary pest (Moore, 2021). Therefore, pest monitoring is crucial for controlling *T. leucotreta* and maintaining South Africa's important fruit export markets (Moore, 2021).

Chemical control

There are three insect growth regulator products for *T. leucotreta* control (Moore, 2019). Triflumuron (Alsystin®) and teflubenzuron (Nomolt®) are chitin synthesis inhibitors of the benzoyl urea group (Moore & Hattingh, 2016; Moore, 2019). They are both ovicides that disrupt the embryonic development of larvae in eggs (Moore, 2019). *Thaumatotibia leucotreta* has become resistant to triflumuron and teflubenzuron in areas where they have been applied frequently and erroneously (Moore, 2019). Methoxyfenozide (sold as Runner® and in several generics) is an ecdysone agonist that affects both eggs and larvae (Moore, 2019). Methoxyfenozide triggers an incomplete and lethal premature moult in the larvae (Moore, 2019). Field trials showed that the application of both triflumuron and teflubenzuron reduced fruit loss by up to 80% (Moore & Hattingh, 2016).

There are two registered pyrethroids for *T. leucotreta* control: cypermethrin and Meothrin (Moore, 2019). They are larvicides that kill *T. leucotreta* larvae by contact and are most effective when applied to fruit just before harvest (Moore, 2019). Cypermethrin and Meothrin reduced fruit drop by an average of 90% in field trials (Moore & Hattingh, 2016). Although effective, pyrethroids are potentially toxic to natural enemies of *T. leucotreta* (Moore, 2019). Thus, careful consideration of their necessity should be taken before application (Moore, 2019). Other chemicals available for *T. leucotreta* control include Spinetoram, Coragen® and Warlock®. In Spinetoram, the active ingredient in Delegate®, which targets the nicotinic acetylcholine receptor, causing death (Moore, 2019). Coragen® is an anthranilic diamide insecticide that inhibits muscle contractions, causing death by paralysis (Moore, 2019).

Emamectin benzoate (registered as Warlock®) functions by first being absorbed into the crop plant (Moore, 2019). Warlock has nerve and muscle activity, killing larvae within four days of ingestion (Moore, 2019).

Cultural control (orchard sanitation)

A poorly kept orchard with many injured and fallen fruit promotes *T. leucotreta* infestation (Moore, 2019; Adom et al., 2021). *Thaumatotibia leucotreta* larvae may emerge from infested fruit before or after they fall (Adom et al., 2021). Injured and fallen fruit may be more attractive to females seeking an oviposition site than healthy, unfallen fruit (Moore, 2019). Furthermore, fallen fruit can contribute to postharvest decay of the harvested fruit (Moore, 2019). Thorough and regular orchard sanitation can substantially decrease *T. leucotreta* infestation (Malan et al., 2018; Moore, 2019; Adom et al., 2021). Orchard sanitation is often referred to as the cornerstone of *T. leucotreta* control (Moore, 2019; Adom et al., 2021). Indeed, Moore and Kirkman (2008) found that weekly orchard sanitation between December and June reduced *T. leucotreta* infestation in a citrus orchard by up to 75%. Other research has also found sanitation to be very effective against *T. leucotreta* infestation of crops other than citrus, including eggplant and chillies (Adom et al., 2021).

Moore (2019) laid down sanitation directions for farmers to follow. Orchard sanitation should begin at the end of the fruiting season, immediately after harvesting is complete (Moore, 2019). All fruit, including fallen fruit and any left hanging on trees, should be removed. In the following fruiting season, orchard sanitation should commence before the fruit are marble-sized. Orchard sanitation cannot be conducted too frequently, but a week between sanitations is the recommended interval. Since insect activity and development are temperature-dependent, *T. leucotreta* infestation may be more severe in warmer months (Moore, 2019). Therefore, orchard sanitation should be conducted more frequently in warmer months (Moore, 2019). Although lemons are not true *T. leucotreta* hosts, larvae can still develop in overripe and small lemons (Moore, 2019). Therefore, all citrus orchards, including lemons, should be sanitised according to the above regime (Moore, 2019).

Biological control

Classical biological control often involves the use of exotic natural enemies to control an invasive pest (Hanner, Lima & Floyd, 2009). However, biological control agents have had unintended negative ecological impacts in the past (Hanner, Lima & Floyd, 2009). A well-known example is the cane toad in Australia (Shine, 2010). Cane toads were introduced into the country to control sugar cane pests but, because of their size, fecundity and polyphagous diet, have now invaded many regions of Australia and threaten native biodiversity (Shine,

2010). Given the potential ecological risk of biological control agents, accurate knowledge of species taxonomy, biology, and ecology is crucial for the success of a biological control programme (Hanner, Lima & Floyd, 2009). Biological control agents are often deployed by farmers who purchased the agents from commercial insectaries that began their culture from a small population of the agent (Hanner, Lima & Floyd, 2009). Ensuring that, at every stage, the correct agent species is being deployed is vital for successful biological control and native biodiversity protection (Hanner, Lima & Floyd, 2009). A recent review demonstrated that it is possible to control *T. leucotreta* using only biological control, eliminating the need for chemical insecticides (Moore, 2021). Although there are strict regulations for the amount of pesticide administered to a crop (quantified as maximum residue level; MRL), noncompliance can cause serious health risks to humans, while acceptable MRLs may already be damaging the environments around agroecosystems (Moore, 2021). The problems associated with chemical control emphasise the need for more effective use of biological control options for *T. leucotreta*, of which there are many (Moore, 2021).

Insects used for biological control of *T. leucotreta*

Ichneumonidae

Apophua (= *Glypta*) *leucotretae* (Wilkinson) (Figure 2) is the only ichneumonid wasp known to attack *T. leucotreta* larvae (Table 1) (Ulyett, 1939; Sishuba, 2003). *Apophua leucotretae* is an endoparasitoid, meaning its immature stages develop within the body cavity of its host. *Apophua leucotretae* heavily parasitises *T. leucotreta* in Zimbabwe and is thus a valuable natural enemy there (Ulyett, 1939; Sishuba, 2003). Until 2003, *A. leucotretae* had only been recorded in eastern Mpumalanga in South Africa (Sishuba, 2003). However, Sishuba (2003) reared *A. leucotretae* from *T. leucotreta* larvae collected in Citrusdal, Western Cape, in February 2002. This may suggest a range expansion of *A. leucotretae* and its potential to become a more important parasitoid of *T. leucotreta* in South Africa in the future.



Figure 2. *Apophua leucotretae* specimen from the Iziko South African Museum, Cape Town (van Noort, 2025).

Braconidae

Agathis bishopi (Nixon) is a koinobiont (meaning the host lives most of its life normally while the parasitoid develops within it) larval endoparasitoid of *T. leucotreta* (Zimba et al., 2016). It is mostly found in the Eastern Cape and has been recorded parasitising up to 34% of *T. leucotreta* larvae in fruit (Zimba et al., 2016). This indicates that *Agathis bishopi* is a strong candidate for biological control of *T. leucotreta* (Zimba et al., 2016; Moore, 2019). Citrus Research International (CRI) is currently investigating the use of *Agathis bishopi* as a biological control agent in other provinces in South Africa (S.D. Moore, pers. comm.).

Agathis bishopi has been investigated for its ability to detect *T. leucotreta* larvae in unopened citrus fruit (Zimba et al., 2015). This ability is part of the adult female's normal behaviour since female wasps are able to identify infested fruit to oviposit into the larva within (Zimba et al., 2015). Zimba et al. (2015) investigated the mechanism by which *Agathis bishopi* females locate infested fruit and found that females were strongly attracted to infested fruit over uninfested fruit, which was expected. Fruit volatiles were identified to be the putative olfactory cues the wasp uses to locate infested fruit. Female wasps were more attracted to infested fruit if they were previously exposed to fruit volatiles or had oviposition experience, suggesting some associative learning by *Agathis bishopi* (Zimba et al., 2015). However, given that the volatiles identified are present in many fruits, there is likely another olfactory cue (perhaps from frass left by larvae around their entry hole) that narrows down the female wasp's search, and this requires further investigation (Zimba et al., 2015).

Apanteles leucotretae and *Apanteles typhon* are both endoparasitoids of *T. leucotreta* (Sishuba, 2003). *Apanteles leucotretae* has also been recorded parasitising the codling moth

Cydia pomonella (Linnaeus) (Lepidoptera: Tortricidae) (Sishuba, 2003). Both species are known to occur only in Mpumalanga (Sishuba, 2003).

Chelonus curvimaculatus (Cameron) is an egg-larval parasitoid that attacks *T. leucotreta* and the potato tuber moth *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) (Sishuba, 2003). The adult wasp emerges around the same time as the unparasitised adult *T. leucotreta* (Sishuba, 2003). This species is found in potato fields throughout South Africa but has only been recorded on citrus in Mpumalanga (Sishuba, 2003).

Chalcididae

Nothing is known about the biology of *Oxycoryphe* (= *Haltichella*) *edax* (Waterson) except that it is a larval parasitoid of *T. leucotreta* (Waterston, 1916; Sishuba, 2003). This species has only been recorded in the Mpumalanga (Waterston, 1916; Sishuba, 2003).

Trichogrammatidae

The genus *Trichogrammatoidea* (Girault) was erected by Girault (1911) with the type specimen *Trichogrammatoidea nana*. Girault (1911) also described *Trichogrammatoidea lutea* for the first time. The most influential systematic work on this genus was done by Nagaraja (Nagarkatti & Nagaraja, 1977; Nagaraja, 1978, 1987; Nagaraja & Mohanraj, 2012), describing 10 new species and one subspecies in India (Nagaraja, 1978). Currently, there are about 25 known species of *Trichogrammatoidea* (Querino, Zucchi & Pinto, 2009). *Trichogrammatoidea* species are minute, with *Trichogrammatoidea cryptophlebiae* (Nagaraja) (Figures 3 & 4) being only ~0.5 mm long (Nagaraja, 1978; Prinsloo, 1984). Compared to their sister genus, *Trichogramma*, *Trichogrammatoidea* have much more simplified male genitalia (Nagaraja, 1978; Wahner, Addison & Timm, 2008). This has made species identification difficult, often requiring a specialist, of which there are few.

Trichogrammatoidea cryptophlebiae is a highly efficient egg parasitoid of *T. leucotreta* (Malan et al., 2018; Moore, 2021). It is native to South Africa, occurs naturally in citrus orchards there and can also be augmented from mass-reared cultures (Moore, 2021). Due to extraneous factors, such as pesticide spraying and the timing of parasitoid release, the performance of *T. cryptophlebiae* in orchards has been highly variable (Malan et al., 2018). However, recent research has shown that releasing 25 000 *T. cryptophlebiae* individuals per hectare per month for four months can reduce *T. leucotreta* infestation in an orchard by up to 60% (Moore, 2021). *Trichogrammatoidea cryptophlebiae* is highly sensitive to chemical pesticides (Moore, 2021). Therefore, cultivars should consider the health of their orchard's natural parasitoid populations when deciding on the use of pesticides, as chemical control of pests can reduce parasitoids

to negligible levels (Moore, 2021). Vital Bugs (Limpopo Province, South Africa) is the only insectary mass-rearing *T. cryptophlebiae* for biological control of *T. leucotreta*, making augmentation with *T. cryptophlebiae* spatially limited (Moore, 2021). Thus, conservation of the natural *T. cryptophlebiae* populations in an orchard is one of the most cost-effective means of controlling *T. leucotreta* (Moore, 2021).

Wahner et al. (2008) are the only authors to sequence DNA from South African *Trichogrammatoidea* species. They sequenced the ITS2 (internal transcribed spacer 2) genes from *T. cryptophlebiae* and *T. lutea*, as this gene region has become the popular choice for trichogrammatid species (Wahner, Addison & Timm, 2008). Using the ITS2 sequences, the authors constructed a phylogeny including *T. cryptophlebiae*, *T. lutea* and 22 randomly selected *Trichogramma* species. The phylogeny confirmed that *T. cryptophlebiae* and *T. lutea* were distinct species and the phylogeny assigned these species to a group separate from the *Trichogramma* species in 100% of the bootstrap replications. Thus, Wahner et al. (2008) show that the two *Trichogrammatoidea* species are distinct from each other and, as a group, from the 22 *Trichogramma* species they used in their study. Given the distinct difference in the ITS2 sequences of *T. cryptophlebiae* and *T. lutea*, the genetic differences between and within the morphologically distinct *nana* and *lutea* groups would be an interesting inquiry.

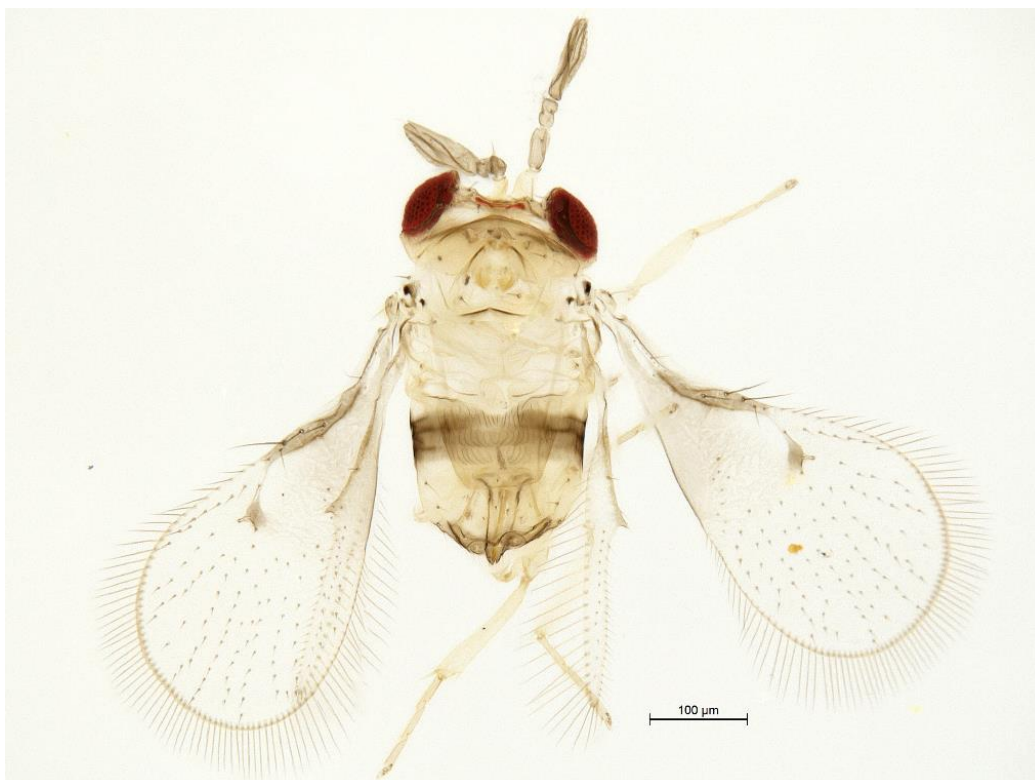


Figure 3. Slide-mounted adult female *T. cryptophlebiae* specimen from Iziko South African Museum (van Noort, 2025).



Figure 4. Live adult male *T. cryptophlebiae* (Moore & Hattingh, 2012).

Diptera

There are only two known dipteran parasitoids of *T. leucotreta* in southern Africa: *Silba virescens* Macquart (Lonchaeidae) and a species of the genus *Mintha* (Tachinidae) (Newton, 1998; Moore, 2002). One species of fly, *Actia cuthbertsoni* (Curran), was reported parasitising *T. leucotreta* in Uganda (Moore, 2002). There is a paucity of knowledge on these species (Newton, 1998).

Table 1. List of *Thaumatotibia leucotreta* parasitoids (Moore, 2002; Sishuba, 2003).

Order	Family	Species	Author	Host stage attacked	Crop	Country
Hymenoptera	Braconidae	<i>Agathis bishopi</i>	(Nixon, 1941)	larva/pupa	citrus	SA
		<i>Agathis leucotretae</i>	Latreille, 1804	larva	citrus	Zim
		<i>Apanteles leucotretae</i>	Ulyett, 1946	larva	citrus	SA & Zim
		<i>Apanteles typhon</i>	Nixon, 1965	larva	cotton	SA & Uga
		<i>Ascogaster</i> sp.	Wesmael, 1835	larva	cotton	Uga
		<i>Chelonus curvimaculatus</i>	Cameron, 1906	egg/larva	citrus	SA
		<i>Chelonus</i> sp.	Panzer, 1806	egg/larva	cotton	Nig
		<i>Phanerotoma curvicarinata</i>	Cameron, 1911	larva	citrus	SA
	Chalcididae	<i>Oxycoryphae edax</i>		larva	citrus	SA
	Elasmidae	<i>Elasmus johnstoni</i>	Ferrière, 1929	larva	cotton	Uga
	Ichneumonidae	<i>Apophua leucotretae</i>	Wilkinson, 1932	larva/pupa	citrus	Zim
	Trichogrammatidae	<i>Trichogrammatoidea cryptophlebiae</i>	Nagaraja, 1979	egg	citrus	SA
		<i>T. fulva</i>	Nagaraja, 1979			
		<i>Trichogramma</i> sp.	Riley, 1871	egg	cotton	Uga
Diptera	Tachinidae	<i>Actia cuthbertsoni</i>	Curran, 1933	larva	cotton	Uga
		<i>Mintha</i> sp.		larva/pupa	citrus	SA
	Lonchaeidae	<i>Silba virescens</i>	Macquart, 1851	larva/pupa	citrus	SA

Nig = Nigeria, SA = South Africa, Uga = Uganda, Zim = Zimbabwe

Granuloviruses

Moore (2002) developed the *Cryptophlebia leucotreta* granulovirus (CrleGV), a granulovirus that attacks *T. leucotreta* larvae. Initial field trials showed that CrleGV was highly effective, reducing *T. leucotreta* infestation in navel oranges by up to 60% for 39 days (Moore, 2002: 200). The virus enters larvae through ingestion, potentially even before active larval feeding begins (Moore, 2019). The larvae die, rupturing and releasing more of the virus back into the environment for uptake by other larvae (Moore, 2019).

CrleGV is now commercially available in three registered spray products: Cryptogran™, Cryptex and Gratham (Moore, 2019). They differ in their prescribed application regimes (Moore, 2019). The main advantage of using CrleGV-based products is that they are highly specific and will not harm beneficial insects and natural enemies of *T. leucotreta* (Moore, 2019). Furthermore, the sprays can be used in conjunction with chemical sprays (Moore, 2019). Thus, Cryptogran, Cryptex and Gratham are suitable for IPM programmes (Moore, 2019). It should be noted that CrleGV is UV-sensitive and should thus be applied in the late afternoon or evening (Moore, 2019).

Nucleopolyhedrovirus

Recently, the first nucleopolyhedrovirus in the Grapholitini tribe of Tortricidae was discovered, being that of the litchi moth, *Cryptophlebia peltastica* (Meyrick) (Moore, 2021). The *Cryptophlebia peltastica* nucleopolyhedrovirus (CrpeNPV) was found to be similarly virulent to *T. leucotreta* as its original host (Moore, 2021). Preliminary field trials have been performed with promising results (Moore, 2021). CrpeNPV has been evaluated for use in conjunction with CrleGV (Moore, 2021). Some mixtures showed greater insecticidal activity than either virus in isolation (Moore, 2021). Moore (2021) suggests that a CrpeNPV-based product will be on the market shortly. Its application and affordability will likely be similar to CrleGV-based products.

Entomopathogenic fungi

Beauveria bassiana is an entomopathogenic fungus (EPF) that affects the larvae of *T. leucotreta* (Moore, 2019). When the spores land on the larva, they germinate and secrete enzymes that weaken the larva's cuticle (Moore, 2019). With the larva's cuticle weakened, the fungus can invade the body of its host, growing throughout until death (Moore, 2019). There are two EPF products registered for control of *T. leucotreta*, namely Broadband and Eco-Bb (Moore, 2019). Both contain *B. bassiana*, are sprayed onto crops and differ only in prescribed application frequency (Moore, 2019). Like CrleGV, *B. bassiana* is UV-sensitive (Moore, 2019).

Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) are among the most recent categories of biological control agent for agricultural pests (Malan et al., 2018; Moore, 2021). Like EPFs and granuloviruses, EPNs are highly host-specific, making them ideal biological control agents (Malan et al., 2018). Nematodes from the genera *Heterorhabditis* and *Steinernema* are obligate parasites of insects (Malan et al., 2018). Species in these genera attack the soil-dwelling *T. leucotreta* pupa, a life stage that has previously been ignored (Moore, 2021). Cryptonem, containing *Heterorhabditis bacteriophora*, is the only EPN-based product currently registered for *T. leucotreta* control (Moore, 2021). Cryptonem is administered by spraying the product onto trees (Moore, 2021). Recently, a product containing *Steinernema feltiae* has been approved for registration (Moore, 2021). *Heterorhabditis bacteriophora* and *S. feltiae* performed well in trials, with larval infection frequently exceeding 80% and larval infestation of fruit being reduced by up to 88% (Moore, 2021).

While EPN-based products are proven to be very effective, naturally occurring EPNs, such as *Heterorhabditis zealandica*, can be valuable passive controls of *T. leucotreta* infestation (Moore, 2021). *Heterorhabditis zealandica* reduced *T. leucotreta* fruit infestation by 59% in orchards where the EPN was not suppressed by the nematicide Cadusafos (Moore, 2021).

The remarkable passive *T. leucotreta* control that *H. zealandica* offers underpins the importance of nematode conservation in orchards (Moore, 2021).

The biological control agents described above represent both established and potential tools for *T. leucotreta* management. However, realising their full potential requires overcoming taxonomic challenges, particularly for morphologically cryptic species and minute parasitoids, such as *Trichogrammatoidea*. Modern molecular approaches, particularly DNA barcoding, provide useful tools for addressing these identification challenges and advancing our understanding of the natural enemies of *T. leucotreta*.

DNA barcoding

The estimated number of eukaryotic species is about 8.7 million (± 1.7 million SE) (Mora et al., 2011). Of these, 86% of terrestrial species and 91% of marine species have yet to be described (Mora et al., 2011). It is estimated that 303 000 taxonomists would be required to work at a consistent rate for 1 200 years to fully describe these yet undescribed species (Mora et al., 2011). Furthermore, the presence of cryptic species means that the number of species on Earth may be underestimated. Indeed, discoveries of cryptic species are becoming more common, especially in insect taxa such as Lepidoptera and in small, morphologically conservative taxa such as *Trichogramma* (Hebert et al., 2003; Kaspi, Kontsedalov & Ghanim, 2018). While morphology can sometimes be used to delineate cryptic and closely related species, it is time-consuming and an expert taxonomist is required (Hebert et al., 2003; Mora et al., 2011).

Morphology has been the traditional diagnostic tool for taxonomic delimitation and description (Cook et al., 2010). However, morphological delimitation has four key limitations: (1) phenotypic plasticity and genetic variability of the morphological characters used to delineate species can lead to incorrect identification, (2) cryptic species may not be morphologically delineable, depending on their crypticness, (3) morphological keys often only identify a particular life stage or gender and (4) morphological keys (even the more robust interactive keys) can lead to misidentifications (Cook et al., 2010). Thus, DNA-based taxon discrimination is becoming more popular (Hebert et al., 2003). While whole-genome sequencing allows precise taxonomic review of taxa, it is costly and time-consuming (Ratnasingham & Hebert, 2007). A cost-effective and more readily available alternative for identifying specimens and delineating species is DNA barcoding (Ratnasingham & Hebert, 2007, 2013), although this is not always informative as a species diagnostic tool for all taxa (Goldstein & DeSalle, 2011; Zamani et al., 2022).

The Barcode of Life Data System (BOLD)

The Barcode of Life Data System (BOLD) is an online registry of DNA barcodes (Ratnasingham & Hebert, 2007). A DNA barcode is a roughly 658 base pair (bp) region of the cytochrome oxidase I (COI) gene that can identify species molecularly (Hebert et al., 2003). BOLD also associated specimens with an operational taxonomic unit (OTU) (Ratnasingham & Hebert, 2013). An OTU is a cluster of individuals grouped by genetic similarity and is a proxy for a species (Ratnasingham & Hebert, 2013). In many ways, an OTU functions as a species without formally being described as such (Ratnasingham & Hebert, 2013). In BOLD, OTUs are determined by the similarity of the DNA barcode gene of specimens (Ratnasingham & Hebert, 2013). Besides species name and DNA barcode sequence, BOLD records can contain other data, including voucher data, collection details and images (Figure 5).

BOLD SYSTEMS
DATABASES IDENTIFICATION TAXONOMY WORKBENCH RESOURCES LOGIN

Barcode Index Number Registry For BOLD: AAN0342
Back to: Last Page

Go to: Public records in this BIN

BIN DETAILS

BIN URI:	BOLD:AAN0342	Average Distance:	0.45% (p-dist)
DOI:	dx.doi.org/10.5883/BOLD:AAN0342	Maximum Distance:	1.64% (p-dist)
Member Count:	123 [125 Public]	Distance to Nearest Neighbor:	5.69% (p-dist)
Barcode Compliant Members:	1		
Founding Record:			

SPECIMEN IMAGES:

BIN COMPLIANT WITH METADATA REQUIREMENTS

GMKMY1430-15 {Trichogrammatoidea lutea}

License: Creative Commons Attribution NonCommercial ShareAlike (2016)

License Holder: CBG Photography Group, Centre for Biodiversity Genomics

Lateral

NEAREST NEIGHBOR (NN) DETAILS

Nearest BIN URI:	BOLD:AAJ8432	Average Distance:	0.71% (p-dist)
Member Count:	94	Maximum Distance:	3.4% (p-dist)
Nearest Member:	GBMNA19976-19	Distance Variance:	0.39% (p-dist)
Nearest Member Taxonomy:	Arthropoda, Insecta, Hymenoptera, Trichogrammatidae, Trichogramma, Trichogramma sp. CH11_Mart		

TAXONOMY

Phylum:	Arthropoda [125]	
Class:	Insecta [125]	
Order:	Hymenoptera [125]	

COLLECTION SITE:

Figure 5. A screenshot of the BIN page for *Trichogrammatoidea lutea* on the BOLD datasystems platform showing barcode information (www.boldsystems.org).

Barcoding for biosecurity: aliens and biological control agents

DNA barcoding has been identified as an important tool in biosecurity (Armstrong & Ball, 2005). For a simple species identification, DNA barcodes are sufficient, provided there are enough correctly-identified reference specimens on BOLD (Ratnasingham & Hebert, 2007). Species identification through DNA barcodes is efficient and more cost-effective compared to morphological identification (Armstrong & Ball, 2005). The materials required for sequencing a COI barcode are becoming less expensive and extraction can be performed by most scientists familiar with laboratory techniques (Hanner, Lima & Floyd, 2009). DNA extraction, amplification and sequencing services are also available commercially, meaning identification can easily be sub-contracted by biosecurity practitioners (e.g. national border officials). These advantages of DNA barcoding have resulted in it being used more widely for the early detection of invasive alien species (Armstrong & Ball, 2005; Hanner, Lima & Floyd, 2009; Comtet et al., 2015).

Correct species identification is foundational to biological control (Hajek, 2004; Miller, 2007). Both pests and biological control agents must be correctly identified in order to ensure host compatibility. Incorrect identifications account for many of the failures in biological control programmes (Miller, 2007). Despite the usefulness of DNA barcoding in biological control programmes, the lack of accessioned biological control agents on BOLD limit its use for this purpose (Garipey, Haye & Zhang, 2014).

Other genetic markers

Phylogenetics is crucial in the systematic review of taxa. While it is preferable to use multiple genes to construct a robust phylogeny (Castresana, 2007), a single gene can be useful for constructing a provisional phylogeny. Wahner et al. (2008) constructed a phylogeny of trichogrammatids, including *Trichogramma* and *Trichogrammatoidea*, from *ITS2* sequences. Their phylogeny assigned specimens to their traditional taxa and corroborated some assumptions about trichogrammatid taxonomy (Wahner, Addison & Timm, 2008). *COI* is well-suited to resolving phylogenetic relationships at the family to species level, while *ITS1* and *ITS2* are better suited for the genus to population-level (Hwang & Kim, 1999). Thus, DNA barcodes can be useful for constructing provisional phylogenies. In conjunction with other genes, DNA barcoding can be used for more robust phylogenies (Castresana, 2007).

Thermal physiology

Insects are ectotherms with a limited ability to regulate their body temperature (Chown & Nicolson, 2004a; Ali et al., 2019). As such, the distribution of insect species is partly

determined by their thermal tolerance, which differs between species (Chown & Nicolson, 2004b). Temperature affects several aspects of insect thermal physiology, including muscle function, fecundity and ontogenesis (Cui et al., 2018). Insect mobility is largely determined by temperature, resulting in limited motor function at very cold and very warm temperatures, which affects behaviour, including parasitism (Mawela, Kfir & Krüger, 2013). Temperature also affects fecundity and generation time, thus influencing population growth (Matthee, 2000; Tabebordbar et al., 2022). These aspects of thermal biology are particularly relevant to biological control using parasitoid wasps, where fecundity and motor function directly influence the control of the target pest.

There is evidence that periods of unusually high climatic temperatures incur generational changes in body size (Klockmann, Günter & Fischer, 2017; Ma, Ma & Pincebourde, 2021), though this may not be the case for all ectotherms (Di Santo & Lobel, 2017). Conversely, within a single generation, adult body size is inversely proportional to temperature, with exposure to higher temperatures during an insect's development resulting in smaller adults (Ma, Ma & Pincebourde, 2021). This is likely due to high temperatures shortening development time, thus producing less-developed, smaller adults (Chown & Nicolson, 2004c).

Body size affects the fitness in parasitoid wasps, with implications for their parasitism behaviour (Colinet, Boivin & Hance, 2007; Gao et al., 2016; del Pino et al., 2020). Larger females tend to live longer, parasitise more hosts, and produce more offspring (Gao et al., 2016; Beukeboom, 2018). In addition, females who mate with larger males also produce more offspring (Beukeboom, 2018). The size of female parasitoids also affects the fitness and body size of its offspring (Klockmann, Günter & Fischer, 2017). Given the role of temperature in limiting parasitoid performance, it is important to understand the thermal tolerance of parasitoids used for biological control.

A useful metric for assessing thermal tolerance in ectotherms is critical thermal limits (CTLs). These are the nonlethal low (critical thermal minimum; CT_{min}) and high (critical thermal maximum; CT_{max}) temperatures above and below which an animal loses motor function. CT_{min} is often defined as the temperature of loss of coordinated muscle function, while CT_{max} is the temperature of onset of muscle spasms (Terblanche et al., 2007). Unlike lethal temperatures, CTLs can help researchers predict behaviour relative to temperature, especially in the ability of an insect to move through its environment, seek out a mate and escape predation, among other abilities (Porter et al., 2019).

Trichogrammatoidea cryptophlebiae is minute, with adults being ~0.5 mm long (Nagaraja, 1978). Only one study has tested the effect of temperature on the development and reproduction of the wasp (Matthee, 2000). The lower development threshold (LDT) of *T. cryptophlebiae* was 7.65°C, while the mean generation time decreased with increasing temperature, being ~23 days at 15 °C and 7 days at 30 °C (Matthee, 2000). Since more mature life stages tend to tolerate higher and lower temperatures than larvae and pupae (Klockmann, Günter & Fischer, 2017), it is expected that *T. cryptophlebiae* adults will express CT_{min} below the LDT reported by Matthee (2000). This study aimed to determine the CT_{min} and CT_{max} of mass-reared *T. cryptophlebiae*.

Aims and objectives

Given the importance of DNA barcoding for biosecurity and cataloguing important species, this study aimed to revise the DNA barcoding of alien invertebrates in South Africa. I also used *COI* barcodes to catalogue *T. leucotreta* parasitoids in the Western Cape Province of South Africa. In addition, due to its importance as a commercial biological control agent of *T. leucotreta*, the thermal physiology of *T. cryptophlebiae* was investigated. Specifically, this study aimed to:

1. Review the DNA barcoding status of alien invertebrates and biological control agents in South Africa (Chapter 2);
2. Investigate the presence of *T. leucotreta* parasitoid wasps in organic and IPM orchards within the Western Cape and barcode all sampled parasitoids (Chapter 3); and
3. Determine the critical thermal limits of *T. cryptophlebiae* in the laboratory (Chapter 4).

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CHAPTER 2

DNA barcoding of alien invertebrates and biological control agents in South Africa: a review

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Introduction

The era of globalisation has seen an increase in the rate and range of human-induced spread of species due to an increase in international travel and trade (Lockwood et al., 2007a; Gariepy, Haye & Zhang., 2014). Invasion pathways, such as intercontinental shipping, international road networks and overseas flights, are ubiquitous and far-reaching, allowing species to spread into non-native regions (Lockwood et al., 2007a; Faulkner et al., 2016). Developing countries with growing trade markets are particularly at risk of new invasions (Faulkner et al., 2016). Points of entry, such as harbours and airports, are where alien species should be intercepted to limit their invasion.

Once established, alien species can disrupt communities and ecosystem functions in their invaded territories (van Wilgen, Zengeya & Richardson, 2022). In natural ecosystems, alien species disrupt native communities by competing with native species for resources or by preying upon them (Armstrong & Ball, 2005; Fortuna et al., 2022). As in many other countries globally, agroecosystems in South Africa are threatened by pests, many of them alien (Janion-Scheepers & Griffiths, 2020). Here, alien species can be direct pests by consuming crop plants or acting as vectors for plant diseases. This is concerning as South Africa has a highly valuable agricultural export market, totalling US\$13.2 billion in 2023 (SARS, 2023; Sihlobo, 2024). Although it is unlikely that the spread can be halted entirely, prevention strategies can minimise the future spread of alien species (Hill et al., 2020).

A basic prerequisite for managing an alien population is the correct species identification of a representative specimen (Pyšek et al., 2008; Hanner, Lima & Floyd, 2009). Species identification traditionally relies on morphology, often requiring an expert in a particular taxon, which is time-consuming, especially for very diverse taxa such as insects (Myburgh, Thabang Madisha & Coetzer, 2021). Yet, the detection and management of an alien species requires rapid species identification (Hanner, Lima & Floyd, 2009). Moreover, there is a global lack of

taxonomic expertise across many taxa (Coleman, 2015; Ge et al., 2021). Molecular techniques can provide a time-effective means to identify a specimen, though baseline genetic data is required for efficiency and accuracy (Hanner, Lima & Floyd, 2009).

DNA barcoding is a broad term for using short nucleic acid sequences for taxonomic identification (Ratnasingham & Hebert, 2007; 2013). In animals, the mitochondrial *COI* region is the standard barcode gene (Armstrong & Ball, 2005; Ratnasingham & Hebert, 2007). However, *COI* is not the most effective marker for all animal taxa, and other genetic markers have been used for certain taxa where *COI* is less effective in delineating species (Guo et al., 2022; Liu et al., 2023). Different genes are used as barcodes in other taxa, such as *rbcL* and *matK* in plants and ITS and the large subunit ribosomal rRNA in fungi (Schoch et al., 2012; Batley, 2015). A public database containing *COI* sequences from confirmed species is required to use the sequence data to identify a specimen. The Barcode of Life Data System (BOLD) is an open-access DNA barcode database with ~1.3 million specimen records, comprising 623,442 Barcode Index Numbers (BINs), the molecular operational taxonomic units (MOTUs) used to represent putative species in BOLD (BOLD, 2023). DNA barcoding is a baseline tool that can facilitate integrative taxonomic approaches to early detection of alien species (Armstrong & Ball, 2005; Hanner, Lima & Floyd, 2009; Madden et al., 2019). *Ad hoc* detection is achieved by individually sampling suspected alien specimens (e.g. Niemann et al., 2022). However, metabarcoding can also detect known alien species in taxonomically complex samples containing many specimens (Singh et al., 2021). The efficacy of metabarcoding taxonomically complex zooplankton samples was tested in South Africa, comparing the technique to traditional morphological sorting and identification. Singh et al. (2021) found that metabarcoding provided a higher taxonomic resolution of the samples than morphological analysis, identifying more species. However, the small number of DNA barcodes retrieved from BOLD limited the power of metabarcoding (Singh et al., 2021). Metabarcoding is increasingly being suggested as an approach for the detection and management of alien species in terrestrial, freshwater and marine environments (Comtet et al., 2015), including for insects (Piper et al., 2019). However, this method is only effective if there are sufficient baseline barcode data available for comparison.

DNA barcoding in South Africa began in earnest in 2011 when an International Barcode of Life Project (iBOL) node was established in South Africa (Table 1), indicating South Africa's commitment to the iBOL's goals for barcoding global biodiversity. That same year, the Urban DNA Barcode Project was launched in KwaZulu-Natal (da Silva & Willows-Munro, 2016). In 2016, the first review of DNA barcoding of animals in South Africa was published (da Silva & Willows-Munro, 2016). At the time, BOLD contained approximately 48,000 animal records

from South Africa, grouped into 10,526 BINs and representing 1,487 species (da Silva & Willows-Munro, 2016). Since there are over 65,000 known South African animal species (Hamer, 2013), only 2.3% of the country's species diversity was represented on BOLD at the time. Insects were underrepresented, with only 1% (37,105 records in 8,223 BINs, representing 513 species) of native insect species represented on BOLD (da Silva & Willows-Munro, 2016). The low barcoding rate was attributed to a lack of taxonomists, with only 28 vertebrate taxonomists and 23 insect taxonomists working in South Africa at the time (da Silva & Willows-Munro, 2016). However, due to focused funding initiatives such as the NRF-FBIP (National Research Foundation Foundational Biodiversity Information Programme) and other endeavours, a subsequent review of South Africa's contribution to BOLD found the representation of insects to have improved considerably, with over 56,000 records in 10,492 BINs representing 9,504 species (Myburgh, Thabang Madisha & Coetzer, 2021) – an 18-fold increase in insect representation. Estuarine macroinvertebrates have also received greater attention recently, with Fagg et al. (2021) barcoding 15 species associated with seagrass meadows, eight of which were not previously barcoded. Despite these important achievements, more focused barcoding needs to be done to increase the country's biosecurity since roughly half of all alien animal species in South Africa are insects (Picker & Griffiths, 2017).

Table 1. A brief history of DNA barcoding globally and in South Africa.

Date	Milestone	Reference
2003	Paper published showing that the <i>COI</i> region in eukaryotes can identify specimens with satisfactory accuracy and proposes this gene be adopted as the standard DNA barcode region for animals.	Hebert et al. (2003)
	Three meetings concerning DNA barcoding, sponsored by the Sloan Foundation, are held at the Banbury Centre in Cold Spring, USA.	DeSalle & Goldstein (2019)
2005	The African Centre for DNA Barcoding is founded in Johannesburg.	(Bezeng et al., 2017)
2010	The International Barcode of Life Project (iBOL) is launched.	da Silva and Willows-Munro (2016)
2011	An iBOL node is opened in South Africa to facilitate the country's commitment to the iBOL.	da Silva and Willows-Munro (2016)

2011	The Urban DNA Barcode Project, a part of the eThekweni Municipality-University of KwaZulu-Natal Joint Research Partnership, is launched to establish a biodiversity inventory for eThekweni.	da Silva and Willows-Munro (2016)
2016	Review published of DNA barcoding in South Africa. This article remains one of few on the topic and outlines the state of barcoding in South Africa at the time.	da Silva and Willows-Munro (2016)
2019	About 3 700 articles with “DNA barcoding” in their titles have been published.	DeSalle and Goldstein (2019)
2021	A review of the contributions of insect DNA barcodes to BOLD made by South Africa.	Myburgh et al. (2021)

The representation of South African invertebrate species on BOLD has substantially increased in the last decade (Myburgh et al., 2021; Stewart, Van Der Bank & Davies, 2024). Between 2014 and 2023, there has been a roughly 20-fold increase in the number of South African records accessioned on BOLD and a 30-fold increase in the number of South African species represented on the database (Table S1) (da Silva & Willows-Munro, 2016). Platyhelminths have had a 30-fold increase in the number of records accessioned on BOLD, while arachnids have had a 110-fold increase in the number of species represented on the database. This reflects the efforts of researchers and institutions to catalogue the country's biodiversity on the database.

To help facilitate these efforts, this study aimed to collate progress made in DNA barcoding of alien invertebrates in South Africa to highlight gaps which could inform future projects. The most recent summary of alien invertebrates focused on terrestrial species (Janion-Scheepers & Griffiths, 2020), while this study will include marine and freshwater species, as well as those on South Africa's sub-Antarctic island territories, the Prince Edward Islands.

Methods

Data collection

A dataset of terrestrial, marine and aquatic alien invertebrates in South Africa was initially compiled using published articles and reports, including Picker & Griffiths (2011), Picker & Griffiths (2017), Prinsloo & Uys (2015), Robinson, Peters & Brooker (2020), Skowno et al. (2019), Zachariades (2021), Janion-Scheepers et al. (2015) and Janion-Scheepers & Griffiths (2020). In cases where data were lacking from these sources or when a species' alien status

was uncertain, the Global Biodiversity Information Facility (GBIF), Barcode of Life Data System (BOLD) and the National Centre for Biotechnology Information's (NCBI) GenBank were searched, particularly for nucleotide sequences in the latter two. Species names were also added from the species list provided in 'The status of biological invasions and their management in South Africa in 2019' (Zengeya & Wilson, 2020).

An advanced literature search was performed on the Web of Science using the following search terms: (alien OR invasive OR non-native OR "biological control agent" OR "biocontrol agent" OR biocontrol) AND (invertebrate OR arthropod OR insect OR arachnid OR crustacea OR myriapod OR collembola OR mollusc OR platyhelminth OR annelid OR porifera OR sponge OR cnidaria OR echinoderm) AND ("South Africa"). Three relevant review articles were Dittrich-Schröder et al. (2020), Musundire et al. (2011) and Stokwe & Malan (2016). The final dataset contained South African alien species published up until July 2023.

Alien taxa were divided into either phyla or groups, as used by da Silva & Willows-Munro (2016). The latter was chosen to compare this study's results with previous literature on South African barcoding. The distribution of species in terrestrial, marine and freshwater realms was analysed using the list from Zengeya & Wilson (2020). Provincial distribution of species was determined from the collection locality from BOLD records.

Data analysis

The dataset was analysed using R v4.3.1 (R Core Team, 2023). The *ggplot* package was used to produce all the figures. The *rgbif* package was used to retrieve taxon names for each species, namely phylum, class, order, and family (Chamberlain, Oldoni & Waller, 2024). The *bold* package was used to mine DNA barcode metadata for each species from BOLD using species names only (Dubois & Chamberlain, 2023). These data were 1) the number of available records, 2) the number of associated BINs, 3) the number of available images and 4) the country where specimens were collected. These data were mined from BOLD on 16 December 2023. The R code used is available in the supplementary material.

Results

The final dataset comprised 1013 alien species, including 132 biological control agents, of which 96.9% were insects and 3.1% were arachnids. Of these species, 677 were represented on BOLD, including 56 biological control agents. Arthropods made up the majority of the barcoded alien species (461), followed by molluscs (61), annelids (46), nematodes (15) and cnidarians (11), followed by other taxa (Table S2). Within the arthropods, insects were the

largest taxon (Figure 1) and made up 49.7% (329) of the barcoded alien arthropod species in the dataset. Of the insect orders, the bugs (Hemiptera) accounted for the majority (40.4%) of barcoded species (Figure 2).

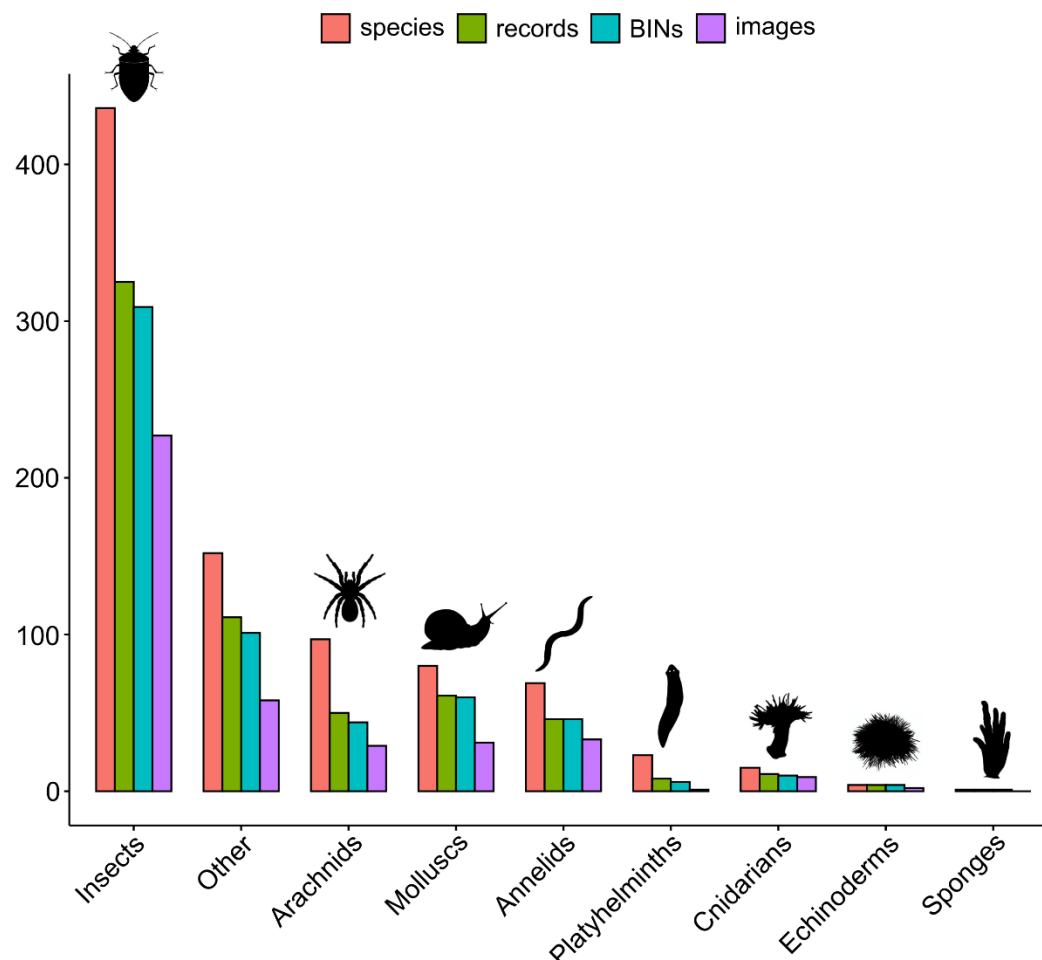


Figure 1. The number of alien species of different invertebrate groups in South Africa (red, from da Silva & Willows-Munro, 2016), the number of those species represented by records on BOLD (green), the number of species with associated BINs (blue) and the number of species with images available on BOLD (purple). 'Other' includes the phyla Nematoda, Chordata, Bryozoa, Brachiopoda, Ctenophora, Euglenozoa and Myxozoa, and the arthropod classes Malacostraca, Collembola, Diplopoda, Maxillopoda, Chilopoda, Copepoda, Branchiopoda, Pycnogonida and Thecostraca.

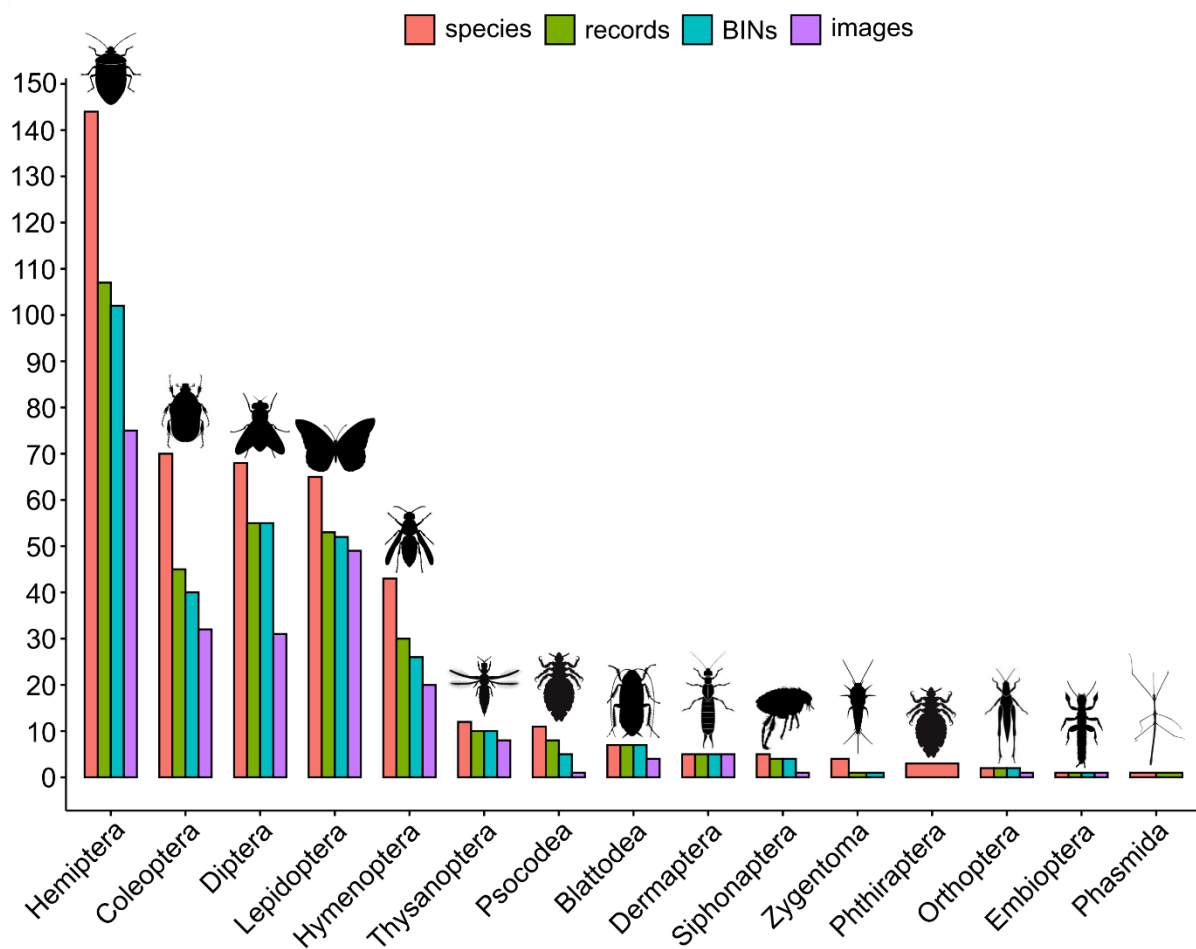


Figure 2. The number of alien insect species in South Africa by order and their representation on BOLD. The y-axis is a count of the number of species (red), species represented by records on BOLD (green), species represented in BINs (blue) and those represented with images on BOLD (purple) per order.

Most alien species in the dataset, including biological control agents, were terrestrial, with 855 species, while 103 were marine and 55 were from freshwater environments (Table 2). The Western Cape had the highest number of alien species (214), of which insects and molluscs were the most barcoded, at 72 and 32 species respectively (Figure 3, Table S3). The Prince Edward Islands had the least with 26 species, mostly insects, of which 16 were accessioned on BOLD. Gauteng had the highest number of barcoded alien insect species at 81, of which 74 were barcoded.

Table 2. Number of alien and biological control agent species from terrestrial, marine and freshwater environments.

Environment	Alien	Biological control agent	Total
Terrestrial	726	129	855
Marine	103	0	103
Freshwater	52	3	55
Total	881	132	1013

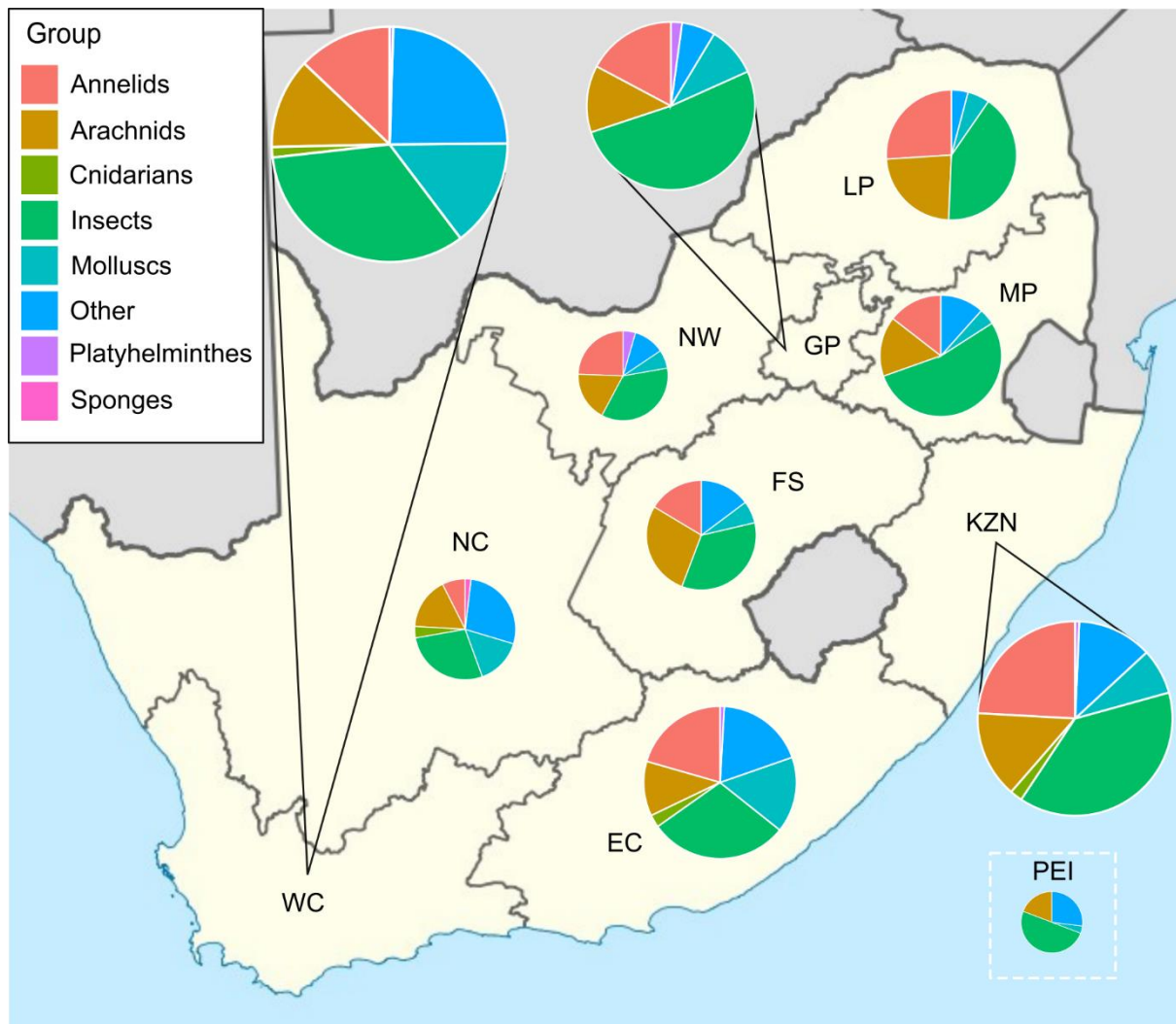


Figure 3. The distribution of barcoded alien invertebrates in South African provinces and island territories (EC = Eastern Cape, FS = Free State, GP = Gauteng, KZN = KwaZulu-Natal, LP = Limpopo, MP = Mpumalanga, NC = Northern Cape, NW = North West, PEI = Prince Edward Islands, WC = Western Cape). The groups used here reflect those da Silva & Willows-Munro (2016) used.

Of the alien species in the dataset, 66.8% are represented by records on BOLD (Figure 4). Of these records, 92.6% were associated with BINs (meaning these records are assigned to MOTUs), while 55.4% had specimen images (meaning record specimens can be compared

to other specimens morphologically). Of the biological control agents, 42.4% were represented by BOLD records. Of these, 86.6% were associated with BINs, while 59.7% had specimen images. The majority of all BOLD records were from specimens collected outside of South Africa, with 17.1% of alien records and 8.3% of biological control agent records barcoded from specimens collected locally.

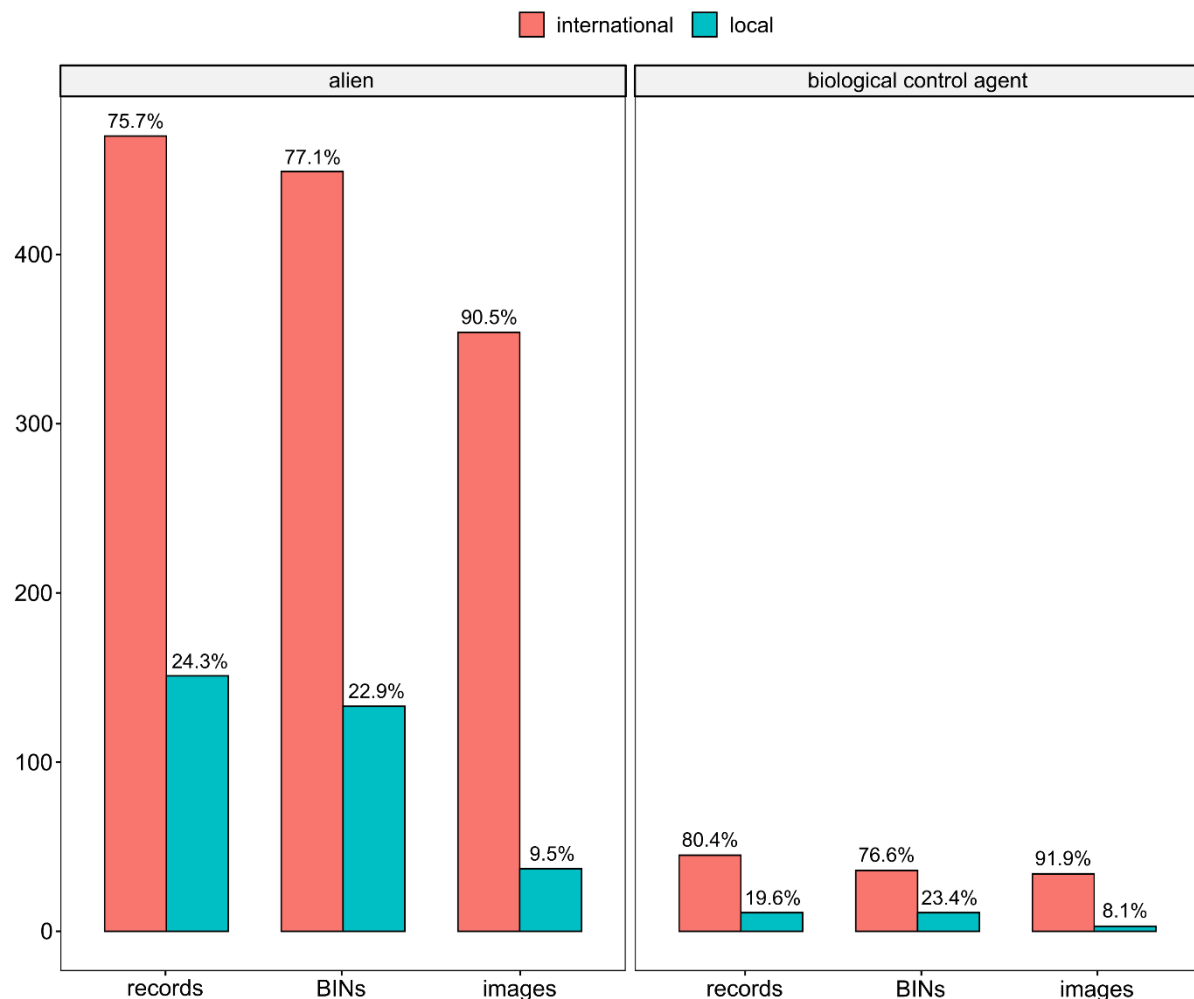


Figure 4. The number of barcoded invertebrate alien (left) and biological control agent (right) species in South Africa with records in BOLD, the number of species associated with BINs and the number of species with images on BOLD. The blue bars represent species for which a South African BOLD record is available, while the red bars represent species for which only international specimen records are available. The percentages indicate the portion of the total alien or biological control agent species for each bar.

Discussion

Alien invertebrate species are ubiquitous but poorly documented (Pyšek et al., 2020). The total number of alien invertebrates present in South Africa is substantial and comprises 62% of all

known alien species in the country (van Wilgen et al., 2020). Terrestrial invertebrates make up about one-third of the alien species in South Africa (van Wilgen et al., 2020), and insects globally far outnumber other alien taxa (Pyšek et al., 2020). However, invertebrates still receive disproportionately less attention than vertebrates from public and scientific communities (Pyšek et al., 2008; Di Marco et al., 2017; Troudet et al., 2017; Eisenhauer et al., 2019; Eisenhauer & Hines, 2021). This study illustrated that despite concerted efforts to barcode diverse taxa (da Silva & Willows-Munro, 2016; Myburgh et al., 2021), there remains a gap in the DNA barcoding of invertebrates generally and alien invertebrates and biological control agents specifically.

Alien invertebrates and barcoding

In this study, we found that the majority of barcoded alien invertebrate species in South Africa were arthropods. Insects are the largest barcoded arthropod group, most of which are hemipterans. Most of these were of the suborder Sternorrhyncha (aphids and scale insects), which are all sap-sucking insects (Janion-Scheepers & Griffiths, 2020). This feeding guild is particularly harmful to crop plants and native plant species (Janion-Scheepers & Griffiths, 2020; Huang et al., 2020). Furthermore, Sternorrhyncha are common vectors of plant diseases, which can spread rapidly in monocultures (Huang et al., 2020). The representation of Sternorrhyncha in South Africa's BOLD records is partly due to barcoding done by Sethusa (2014).

The most poorly represented invertebrate groups on BOLD were the arachnids and platyhelminths. Arachnids comprise economically important species such as ticks and mites, the latter including candidate biological control agents (Smith Meyer & Craemer, 1999; Klein, 2011). Since many are predatory, alien arachnids are a concern for local biodiversity. Alien tarantulas (Arachnida: Araneae) are a popular invertebrate pet in South Africa (Shivambu et al., 2020). The pet trade is an important pathway of alien invertebrate introduction in South Africa, but it is poorly studied except in the case of tarantulas (Nelufule et al., 2020; Shivambu et al., 2020). However, rather than being sold as pets, most alien invertebrates are co-introduced as parasites or commensals through the pet trade (Nelufule et al., 2020). This is also the case for platyhelminths, which comprise many parasitic species that have been co-introduced in South Africa along with their fish hosts (Smit, Malherbe & Hadfield, 2017). Indeed, nearly all the platyhelminths in the dataset have parasitic life histories (Table S2). Unfortunately, little is known about the distribution of platyhelminths in South Africa (Schockaert et al., 2008). The poorly understood distribution of alien arachnids and platyhelminths, coupled with the deficit of barcoding of these groups, is a major hurdle in the ability to identify and control future invasions in South Africa.

Most alien species in the dataset were terrestrial, while only about 10% were marine and 5% were from freshwater environments. Given that ~75% of the world's invertebrates are insects (Eisenhauer & Hines, 2021) and that insects were the largest group in this study's dataset, it is not surprising that most species were terrestrial. In other parts of the world such as Europe, however, the number of terrestrial (~600) and marine (514) alien invertebrates is roughly the same (Keller et al., 2011; European Environment Agency, 2023). In addition, marine biological invasions receive less attention globally than terrestrial invasions, suggesting a gap in the cataloguing of marine alien species in South Africa (Giakoumi & Pey, 2017).

The Western Cape had the highest number of barcoded alien invertebrates in this study. However, da Silva & Willows-Munro (2016) showed that Gauteng and KwaZulu-Natal superseded the Western Cape in the number of barcoded native and alien species, especially insects, which was confirmed by Stewart, Van Der Bank & Davies (2024) and attributed to intensive malaise trapping in the former provinces. The high number of barcoded alien invertebrates from the Western Cape may be explained by not only a greater abundance of alien species occurring in the province, but also a public interest in cataloguing the biodiversity in the Cape Floristic Region, which includes the famous fynbos biome (Ashwell et al., 2006), or more resources being allocated to barcoding aliens here than in other provinces. The North West Province was the least represented province on BOLD, followed by the Northern Cape and the Free State. The ranking of the Free State reflects the findings of Myburgh et al. (2021), who suggested that intense agriculture may be fragmenting ecosystems in the province, reducing the overall biodiversity and making certain species scarcer and more difficult to sample (Statistics South Africa, 2023). However, barcoding is important in provinces with high agricultural productivity, as it facilitates the cataloguing of alien species that may have negative impacts on crop yield and natural enemies that could be used to suppress pest populations. The provincial occurrence of 153 species recorded in South Africa is unknown from the literature and BOLD. This represents a significant knowledge gap of alien invasions in South Africa. To limit the spread of alien species, they should be intercepted at the earliest point in their invasion, and their distribution data should be made readily accessible to the public so that researchers can establish invasion paths.

Although many alien invertebrates have been accessioned on BOLD, few records were generated from South African specimens. Instead, most of the specimens were collected outside of South Africa. This creates a potential for misidentifications, as species native to South Africa may closely resemble alien species collected outside of the country. A small portion of all the alien species in this study had BOLD records which were not associated with

BINs. Records that are not associated with BINs represent a lack of sequencing of that species and, thus, limited reliability of the molecular confirmation of the specimen's species identity. Finally, only about half the BOLD records for the alien species had associated images, which prevents a BOLD record from being compared to a physical specimen or the identification being revised based on the morphology. The BOLD records accessioned by van Noort et al. (2022) include good examples of high-resolution images that will be beneficial for specimen identification and taxonomic revision. However, there are practical reasons that images are not always feasible, particularly when metabarcoding the contents of traps containing many specimens or when metabarcoding environmental DNA (eDNA) samples. In addition, many insect groups cannot be identified from images, especially if the resolution and quality of the image are low. The poor quality of BOLD records for alien invertebrates in South Africa limits the application of DNA barcoding for detection and monitoring and biosecurity programmes in the country.

Biological control agents and barcoding

Although many alien species have been accessioned on BOLD, just over half the biological control agents in South Africa have not. An important part of the selection process of a biological control agent is understanding its host specificity. Host specificity can be better understood if both the host and natural enemy are identified to species, a process aided by barcoding. Furthermore, isolated cases of biological control agents hybridising in the field after release have been recorded (Goldson, McNeill & Proffitt, 2003; Hopper et al., 2019; Viciriuc et al., 2021). Not all hybrids are equally effective against the alien species or pests they were introduced to control (Goldson, McNeill & Proffitt, 2003; Viciriuc et al., 2021). While COI barcodes cannot distinguish hybrids, other genetic markers, such as nuclear microsatellites (Abdul-Muneer, 2014), can enable hybrid identification and, thus, ideal agent selection and management. Therefore, sequencing DNA barcodes and other genetic markers of biological control agents enables hybrid identification and, thus, ideal agent selection and management.

Conclusion

Globalisation and climate change will continue to drive species range shifts in complex ways, which may include more rapid invasions of certain species, including pest species (Liebholt et al., 1995; Robinet & Roques, 2010; Pyšek et al., 2020; Fortuna et al., 2022). Invertebrates are poorly documented compared to vertebrates, yet they form a significant portion of alien species in South Africa and globally (Pyšek et al., 2020). Furthermore, insects, in particular, are damaging to local communities in complex ways (Fortuna et al., 2022) and are more likely to establish at long distances from their natural range in a changing climate (Robinet &

Roques, 2010). Identification of specimens using morphological taxonomy is slow which means that it is generally inappropriate for monitoring the spread of alien species, so barcoding will be crucial for monitoring and managing alien populations in the future. DNA barcoding enables rapid species cataloguing and identification, though it is limited in its efficacy by the low representation of species on public databases such as BOLD. While BOLD records are helpful, they are most useful once they have all their metadata available (e.g. place of collection, image and collection details) and when they are assigned to a BIN with many other records. Initiatives such as the Urban DNA Barcode Project are important recipients of funding to maximise barcoding. In addition, a concerted national sampling effort, including malaise trapping for insects (Stewart, Van Der Bank & Davies, 2024), could help generate important baseline data on invertebrate diversity. Metabarcoding of bulk trap samples offers a time- and cost-effective approach compared to traditional methods, making it an increasingly valuable tool for detection and monitoring programs, especially in biosecurity and trade contexts.

Advances in the use of environmental DNA (eDNA) have made it possible to detect the presence of alien species without a specimen (e.g. Larson et al., 2020). eDNA is helpful for elusive species or those inhabiting inaccessible to humans (Larson et al., 2020), for example detecting the spread of invasive silver carp inhabiting difficult-to-sample river systems in the Kruger National Park (Crookes et al., 2020). Given its simple sampling protocol, eDNA can aid in biosecurity monitoring through citizen science, an advantage for countries lacking biosecurity resources, such as South Africa (Larson et al., 2020).

South African biodiversity and agriculture are under threat from invasions. Alien invertebrates make up a significant portion of alien species in South Africa but, despite consistent increases in barcoding of this group in the country, these have not been proportionately barcoded. Alien arachnids and platyhelminths in the country are underrepresented on BOLD. This and underrepresentation of other taxa may be due to difficulties related to the amplification of barcode genes or the ineffectiveness of barcode genes for certain groups or species (Waugh 2007). Reasons for the deficit of alien invertebrate records in Gauteng and KwaZulu-Natal provinces should be addressed through additional investigations, as well as the general gap in occurrence data for alien invertebrate species in the country. Although there is an increased effort to barcode South Africa's alien invertebrate species, some BOLD records are incomplete or represent specimens collected outside South Africa.

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CHAPTER 3

Parasitoid wasps of false codling moth (*Thaumatotibia leucotreta*) in stone fruit and citrus orchards: sampling and barcoding

Introduction

Parasitoid wasps are widely used in the biological control of agricultural pests (Hajek, 2004a). As is the case with all biological control agents, parasitoid wasps can reduce the need for pesticides that decrease the biodiversity in and around agroecosystems (Rossbacher & Vorburger, 2020). Generally, parasitoid wasps demonstrate high host specificity (Quicke, 1997; Hajek, 2004a; Wang et al., 2019), and their population dynamics naturally track their host populations (Heimpel & Mills, 2017), making them self-regulating in agroecosystems. Once established in an orchard, they require fewer applications compared to conventional pesticides, making them more cost-effective (Bale, van Lenteren & Bigler, 2008). Taxonomically, parasitoid wasps historically comprised the infraorder Parasitica, although this grouping is now considered obsolete (Capinera, 2008; van Noort & Broad, 2024). Within Parasitica, the superfamilies Chalcidoidea and Ichneumonoidea are the largest parasitic groups (Quicke, 1997; van Noort & Broad, 2024).

In citrus and stone fruit orchards, parasitoid wasps are used in biological control to manage pest populations. Several species effectively control several economically important agricultural pests, reducing the need for chemical pesticides. For example, *Trichogramma* spp. (Hymenoptera, Chalcidoidea, Trichogrammatidae) parasitise lepidopteran pest eggs, and several species are used to control *Cydia pomonella* (Linnaeus, 1758) (Lepidoptera: Tortricidae) in stone fruit orchards (Sigsgaard et al., 2017). Parasitoid wasps that are used as biological control agents are bred in insectaries under controlled temperatures and humidities (Hajek, 2004b). Then, wasps are released in orchards before each harvest season, with the timing of release depending on the type of parasitoid (egg, larval and pupal) (Hajek, 2004a). The release of these wasps as biological control agents requires consideration of environmental factors that may influence their efficacy, such as temperature, among others (del Pino et al., 2020).

Thaumatotibia leucotreta (Meyrick) (Tortricidae, Olethreutinae) (false codling moth or FCM) is a pest of several fruit crops in South Africa, particularly stone fruit and citrus (EPPO, 2019; Moore, 2021). The larvae are polyphagous and burrow into fruit where they consume the flesh, causing early fruit drop, substantially reducing the quality of the harvest, and incurring high costs to farmers (Malan et al., 2018; Adom et al., 2021). Losses in the citrus industry alone

have been estimated at about R100 million annually (Stotter, 2009). The status of *T. leucotreta* as an invasive species in many of South Africa's key export markets further compounds its economic impact through phytosanitary restrictions (Moore, 2021). Entire consignments of fruit exports infested with the moth have been rejected at borders in the past (Stotter, 2009). Therefore, South African farmers spend substantial resources closely managing *T. leucotreta* populations in their orchards and disinfesting fruit after harvest (Moore, 2021).

Biological control is an effective method of managing *T. leucotreta* in fruit orchards (Moore, 2021). Currently, *Trichogrammatoidea cryptophlebiae* Nagaraja (Hymenoptera: Trichogrammatidae), an egg parasitoid, is the only commercially available wasp biological control agent for *T. leucotreta*. *Agathis bishopi* (Nixon) (Braconidae), a larval parasitoid of *T. leucotreta*, has been trialled as a biological control agent (Zimba et al., 2016) but has not been marketed commercially. While *A. bishopi* has been released in the field in several trials, a population has not successfully been established in the Western Cape (S.D. Moore, pers. comm.). Twelve other species in various families are reported to parasitise *T. leucotreta* (Table 1).

Despite the substantial economic impact of *T. leucotreta* and the documented existence of multiple natural enemies, research on these species and their potential as commercial biological control agents is scarce. The primary reference for *T. leucotreta* parasitoids continues to be Prinsloo's (1984) species checklist. Furthermore, little is known of the distribution of these species, with the most recent records being over 20 years old and only at the country level (Table 1). This highlights a gap in both the development of new biological control agents, and our understanding of conservation biological control of *T. leucotreta* that may or may not be occurring naturally in orchards.

This limited understanding of *T. leucotreta* parasitoid diversity and distribution challenges effective pest management. Climate change is expected to alter parasitoid distributions (Raven & Wagner, 2021; Skendžić et al., 2021), while varying environmental conditions such as temperature and humidity can significantly impact parasitoid efficacy across different regions (Colinet et al., 2015; del Pino et al., 2020). Additionally, parasitoid species likely exhibit differential resistance to pesticides (Stark, Banks & Acheampong, 2004; Theenoor, Ghosh & Venkatesan, 2024), a factor that remains largely under studied.

This study aimed to address these knowledge gaps through systematic sampling of *Thaumotibia leucotreta* parasitoids in Western Cape citrus and stone fruit orchards using modified pan traps. The identification also includes molecular analysis through *COI* barcoding

of specimens matching reference materials from museum collections, mass-reared *Trichogrammatoidea*, and specimens described by Prinsloo (1984).

Table 1. List of known *Thaumatotibia leucotreta* parasitoids (Moore, 2002; Sishuba, 2003). Nig = Nigeria, SA = South Africa, Uga = Uganda, Zim = Zimbabwe.

Family	Species	Host stage attacked	Country where present
Braconidae	<i>Agathis bishopi</i> (Nixon, 1941)	larva/pupa	SA
	<i>Apanteles leucotretae</i> Ulyet, 1946	larva	SA & Zim
	<i>Apanteles typhon</i> Nixon, 1965	larva	SA & Uga
	<i>Ascogaster</i> sp.	larva	Uga
	<i>Chelonus curvimaculatus</i> Cameron, 1906	egg/larva	SA
	<i>Chelonus</i> sp.	egg/larva	Nig
	<i>Phanerotoma curvicarinata</i> Cameron, 1911	larva	SA
Chalcididae	<i>Oxycoryphae edax</i> (Waterston, 1916)	larva	SA
Elasmidae	<i>Elasmus johnstoni</i> Ferrière, 1929	larva	Uga
Ichneumonidae	<i>Apophua leucotretae</i> (Wilkinson, 1931)	larva/pupa	Zim
Trichogrammatidae	<i>Trichogrammatoidea cryptophlebiae</i> Nagaraja, 1979	egg	SA
	<i>Trichogrammatoidea fulva</i> Nagaraja, 1978	egg	Unknown
	<i>Trichogramma</i> sp.	egg	Uga

Methods

Site selection and study area

Sampling took place between November 2023 and February 2024. To sample *T. leucotreta* parasitoids, study sites were selected from four stone fruit and citrus orchards — excluding lemon orchards — in the Western Cape (Table 2 & Figure 1). Selected orchards were either organic or were managed using integrated pest management (IPM). Trees in the selected sites were chosen for the absence of pesticide application for a minimum of one month prior to sampling. This was to ensure optimal conditions of collecting natural parasitoids of *T. leucotreta*. Farmers from several of the selected sites had previously noted the presence of *T. leucotreta* damage to fruit. Sites were sampled on warm days in summer. Days with a

forecasted low chance of rain were chosen to avoid rain limiting the number of flying insects, as well as flooding the traps. Each site, except Verdun Estates, was sampled twice over the sampling period.

Table 2. List of sampling sites with coordinates.

Name	Crop	Pest control method	Coordinates
Welgevallen	Plum	IPM	-33.947794, 18.871500
Somerset West	Plum	Organic	-34.088192, 18.866778
Tierhoek	Plum	Organic	-33.727975, 19.792114
Verdun Estates	Nectarine	IPM	-33.292539, 19.319828



Figure 1. Map of sampling sites in the Western Cape, South Africa.

Sampling protocol

Plastic pan traps were used to sample wasps in orchards. In an attempt to maximise the chances of sampling success, several colours were used (Saunders & Luck, 2013; Buffington et al., 2021; Dunlap, Casey & VanOverbeke, 2023). Following Dunlap, Casey & VanOverbeke (2023), yellow, blue, and white pan traps were deployed at each site, using propylene glycol as a trapping medium. *Thamatotibia leucotreta* eggs (XSIT, Citrusdal) were mounted on polystyrene floats and placed on the propylene glycol surface to attract egg parasitoids. These eggs are known as sentinel eggs (Laminou et al., 2020). At each site, 15 traps were set directly

on the ground beneath trees and 15 were suspended approximately one meter above the ground in trees. Sentinel eggs were placed in this way in order to increase the chance of capturing wasps flying at different elevations. All traps were collected within 24 hours of being set.

In addition to pan traps, active sampling was conducted by collecting fallen fruit that had visible larva entry holes. *Thaumatotibia leucotreta* larvae leave pin-sized holes on the fruit surface that is quickly surrounded by a dark patch as the larva feeds on the fruit flesh (Figure 2) (EPPO, 2019). The number of fruit collected were not counted.

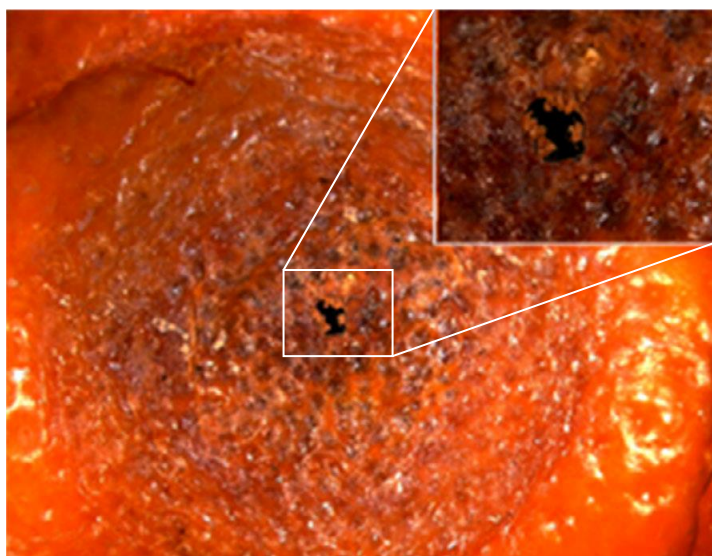


Figure 2. Larval entry hole on surface of citrus fruit (EPPO, 2019).

Sample processing and specimen identification

The contents of traps were transferred to ethanol within 24 hours of collection and stored at 5°C until processing. Fallen fruit were placed into a clear plastic container, covered securely with a mesh cloth (<1 mm mesh) and kept at 25°C, monitored daily for larval emergence. Emergent *T. leucotreta* larvae were removed from the container and placed in glass vials containing a feeding medium, covered with mesh cloth at the open end. The feeding medium was changed every two to three days until pupation, after which the pupae observed for larval parasitoid emergence.

Initial sorting focused on isolating wasp specimens from bycatch (all other taxa), with particular attention to documented *T. leucotreta* parasitoids. Reference specimens from the National Insect Collection (ARC, Roodeplaat, South Africa) and Iziko South African Museum (Cape Town, South Africa) were used to facilitate morphological identification of known *T. leucotreta*

parasitoid species. Only documented *T. leucotreta* wasps were identified and the remaining wasp species were not identified further. All specimens were stored in 96% ethanol.

DNA sequencing and PCR

DNA extraction was performed using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek). The standard procedure was adapted by including an overnight lysis step. PCR amplification targeted the *COI* barcode region using the universal DNA primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). The PCR cycle described by Kaspi, Kontsedalov & Ghanim (2018) for amplifying the *COI* region of *T. cryptophlebiae* was used: 94 °C for 3 min, followed by 33 cycles of 94 °C for 40 sec, 55 °C for 1 min and 72 °C for 1 min, with a final extension period at 72 °C for 5 min. Successfully amplified products were sequenced by the Central Analytical Facility (Stellenbosch University, South Africa) using Sanger sequencing. In addition to sampled specimens, insectary-reared specimens (Vital Bugs, Limpopo) of *T. cryptophlebiae* were sequenced.

Sequence analysis

Sequence editing and alignment were conducted using BioEdit software. Species identification was achieved through sequence comparison using the National Center for Biotechnology Information's (NCBI's) Basic Local Alignment Search Tool (BLAST) against reference databases. A maximum likelihood phylogenetic tree with 1000 bootstrap replications was produced using MEGA 11 (Tamura, Stecher & Kumar, 2021) and included sequences retrieved from GenBank (NCBI). As a proof of concept, and to confirm the identity of the commercial insectary specimen (sold as *T. cryptophlebiae*), these sequences were searched on BLAST and the results were used to choose published GenBank sequences with which to develop a phylogenetic tree (Figure 3). In addition, the BLAST identifications were recorded for the sequenced specimens.

Results

Sampling

In total, 691 wasps were sampled at the four study sites (Table 3). No *T. leucotreta* parasitoids were found during sampling. At all sites, yellow traps yielded the greatest numbers of wasps (Table 4).

Table 3. Number of specimens collected at sampling sites.

Site	Wasp	Bycatch
Somerset West Farm	478	2416
Welgevallen	67	868
Tierhoek	122	1251
Verdun Estates	24	477
Total	691	5012

Table 4. Number of wasps sampled using different trap colours.

Site	Trap colour		
	Blue	White	Yellow
Somerset West Farm	37	48	233
Welgevallen	21	23	23
Tierhoek	13	13	96
Verdun Estates	5	1	15

DNA sequences and analysis

While no *T. leucotreta* parasitoids were sampled, several sampled trichogrammatid wasps (B33T.1, B59G and Y57G), and one specimen from a commercial insectary (TC09), were sequenced successfully. DNA of several other insectary specimens were extracted and sequenced, but the resulting sequences were noisy and not fit for use in phylogenetic analysis. The BLAST results and tree confirmed the identity of the insectary trichogrammatid as *T. cryptophlebiae*. The sampled wasps clustered with several *Megaphragma* (Trichogrammatidae) species in the phylogenetic tree (Figure 3). The nucleotide sequences for the specimens sequenced and their BLAST identifications are given below.

TC09

Nucleotide sequence:

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TTGGTTTATCTATAAGTATAATTATTCGTTTAGAATTAGGAAATCCTGGTTCCTTAATTGGTAATGATCAAATT
TATAATTCAATTGTTACTAGACACGCTTTTTTAATAATTTTTTTTTTTGTAATACCTGTAATAATAGGGGGTTT
TGGAATTACTTAATTCCTTTAATATTAGGTAGTCCAGATATAGCTTTTCCACGAATAAATAATATAAGTTTTT
GGTTATTACCTCCTAGGTTATTATTATTATTATCTAGAATGTTTGTAGGAACTGGAACAGGTACAGGATGAACT
GTGTACCCTCCTTTATCTTCTAATTTATCTCATAGTGGCCCTTCAGTTGATTTATCAATTTTTCTTTACATAT
TGCAGGGGTTTCTTCAATTATAGGTTCTATTAATTTTATTTCTACTATTCTTAATATAAAAATTTATAAGTTTG
AGTTAATTTCTTTATTTTCTTGGGCTATATTATTAAGTCTATTTTATTATTATTATCATTACCTGTTT TAGCA
GGTGCATTACTATGTTATTATTTGATCGAAATTTAAATAC
```

BLAST results:

Species: *Trichogrammatoidea cryptophlebiae*
Sequence ID: MH102410.1
Query Cover: 100%
Percentage Identity: 99.82%
E Value: 0.0
Reference: Kaspi, Kontsedalov & Ghanim (2018)

B33T.1

Nucleotide sequence:

TGGGAGTATTARGATTATCTATAAGAATAATTATTCGTTTAGAGTTAGGAAATCCTGGGTCTTTAATTGGAAAT
GATCAAATTTATAATTCAATTGTTACAACCTCATGCTTTTACAATAATTTTTTTTTTTTGTATACCTGTAATAAT
AGGGGGATTTGGAAATTATTTAATTCCTTTAATATTAGGTTCTCCTGATATAGCTTTTCTCGAATAAAWAATA
TAARATTTTGATTATTACCTCCAAGAATTATTYTATTAATAAGAAGAATATTTATTGGGACTGGAACCTGGAAC
GGATGAACAGTTTATCCTCCTTTATCTTCTAATTTATCTCATGCAGGTCCTTCAGTAGATTTATCTATTTTTTC
TCTTCATATTGCTGGGGTTTCTTCAATTATAGGTTCTATTAATTTTATTACAACAATTATTAATATAAAAAATTT
TTAAAATTGAAATAGTTGTTTTATTTTCTTGAGCTATAATTTTAACAGCTATTTTATTATTATTATCATTACCA
GTATTAGCAGGAGCTATTACTATA

BLAST results:

Species: *Megaphragma polilovi*
Sequence ID: ON557484.1
Query Cover: 97%
Percentage Identity: 90.17%
E Value: 0.0
Reference: Polaszek, et al. (2022)

B59G

Nucleotide sequence:

TTTTTGGTATGTGAGCTGGAATTATAGGTTTATCATTAAGAATAATTATTCGTTTAGAATTAGGAAATCCTGGT
TCTTTAATTGGAAATGATCAAATTTATAATTCTGTAGTGACTACTCATGCTTTTATAATAATTTTTTTTTTTGT
TATACCTGTGATAATAGGTGGGTTTGGTAATTATTTAATTCCTTTAATATTAGGATCTCCTGATATAGCTTTTC
CTCGAATAAATAATATGAGATTTTGATTGTTACCCCAAGGATATTATTGTTAATTTCTAGAATATTTGTAGGA
AGGGGTACTGGTACTGGTTGAACAGTTTATCCTCCATTATCTTCTAATTTAGCTCATTCTGGACCTTCTGTAGA
TTTATCTATTTTTTCTTTACATATTGCTGGGGTTTCTTCGATTTTAGGATCTATTAATTTTATTACTACAATTT
ATAATATAAAAAATTTATAAGTTTGAATATATTACTTTATTTTGTGAGCTATATTATTAACAGCTATTTTATTG

TTATTGTCTTTACCAGTTTTAGCGGGTGCAATTCCTATATTATTGTTTGATCGTAATTTAAATACTTCTTTTTT
TGATCCAGCAGGAGGA

BLAST results:

Species: *Megaphragma polilovi*
Sequence ID: ON557484.1
Query Cover: 100%
Percentage Identity: 89.80%
E Value: 0.0
Reference: Polaszek, et al. (2022)

Y57G

Nucleotide sequence:

ATTATTGGATTATCTTTAAGTATAATTATTCGTTTGGAATTAGGAATTCCTGGTTCTTTAATTGGTAATGATCA
AATTTATAATTCTGTAGTTACTGCTCATGCTTTTATAATAATTTTTTTTTTTGTTATACCTGTAATAATAGGTG
GTTTTGGAAATTATTTAATTCCTTTAATATTAGGTGTTCTGATATAGCTTTTCCTCGTATAAATAATATAAGA
TTTTGATTACTTCCTCCTAGAATAATTTTATTAATTTCTAGAATATTTTATTGGTACTGGAAGTGGTACTGGAT
GAACAGTATATCCTCCTTTATCTTCAAATATTGCTCATGCGGGTCCTCAGTTGATTTATCAATTTTTCTTTA
CATATTGCTGGAGTTTCTTCAATTATAGGTTCAATTAATTTTATTACTACTATTATAAATATAAAGATTTTTAA
ATTTGAAATAGTTGTATTATTTGCTTGAGCAATAATTTTAACAGCAATTTTATTATTATTATCTTTACCTGTTT
TAGCTGGTGCAATTACTATATTATTATTTGATCGAAATTTAAATACTTCTTTTTTTGATCCTGCTGGAGGAGGA
GA

BLAST results:

Species: *Megaphragma noyesi*
Sequence ID: ON557518.1
Query Cover: 99%
Percentage Identity: 90.99%
E Value: 0.0
Reference: Polaszek, et al. (2022)

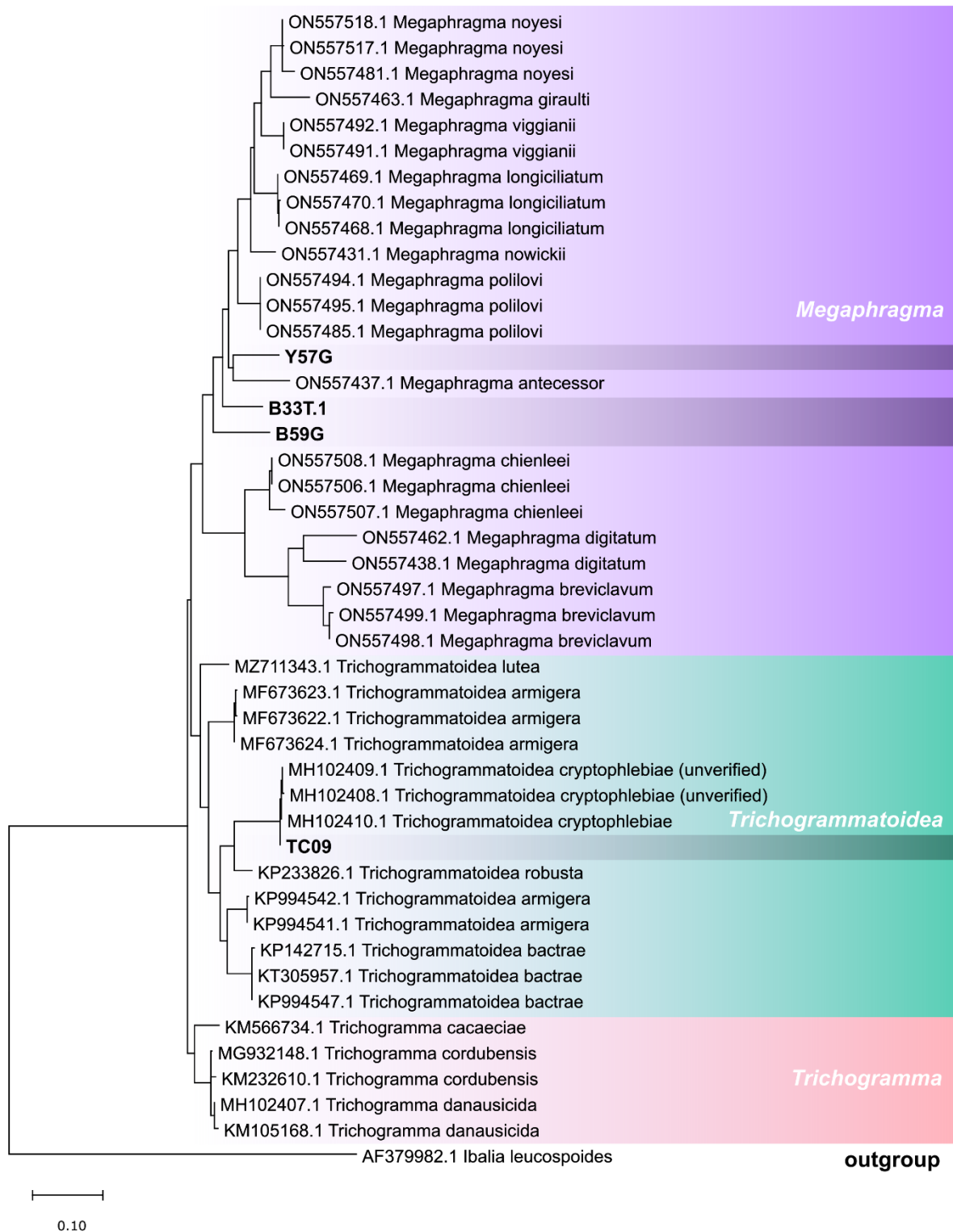


Figure 3. Maximum likelihood phylogenetic tree of published GenBank sequences of trichogrammatid wasps and four samples from this study (bold). Sequence IDs are provided for each of the GenBank specimens. Bootstrap values are given in Figure S1.

Discussion

Sampling of parasitoid wasps at four Western Cape fruit orchards yielded no specimens of known *T. leucotreta* parasitoids, despite sampling conditions that were favourable for parasitoid presence. The selection of organic and IPM-managed orchards (Kogan, 1998), combined with summer sampling and the use of sentinel eggs as attractants (Fatouros et al., 2008), should have provided the most favourable conditions to attract the natural enemies of the moth. The lack of parasitoids suggests that natural populations may be insufficient for effective passive biological control of *T. leucotreta*. This supports Moore's (2021) emphasis of the necessity of augmentative approaches in *T. leucotreta* management. In addition, Haran et al. (2019) showed that parasitoids of fruit flies were lower in the Western Cape than in other provinces, and this may be true for *T. leucotreta* parasitoids too.

The low numbers of parasitoids in the samples may reflect broader landscape-level effects of conventional pest management practices (Raven & Wagner, 2021). Extensive use of chemical pesticides in surrounding agricultural areas could suppress both host and parasitoid populations, creating ecological barriers to parasitoid establishment and persistence (Moore & Hattingh, 2012; Cruz et al., 2017; Pinheiro et al., 2020). This pattern has been observed in other agricultural systems where intensive pest management practices can inadvertently impact beneficial insect populations (Theenoor, Ghosh & Venkatesan, 2024).

Since this study only looked for known *T. leucotreta* parasitoids with descriptions or reference specimens, it is acknowledged that undocumented parasitoids could have been in the samples. This study would have benefited from the sequencing of all specimens sampled, but this was not possible as it would carry high costs. Metabarcoding could have been used to cost-effectively develop a dataset of COI barcodes for all wasps sampled (Piper et al., 2019). The focus on identification of parasitoids through morphological means without considering the aforementioned alternative options limited the value of this study and taxonomic expertise is needed for this group. Nevertheless, the lack of documented *T. leucotreta* parasitoids sampled in the Western Cape remains an important result.

While not known to parasitise *T. leucotreta*, *Megaphragma* species, such as those sampled in this study, are parasitoids of several species of thrips (Thysanoptera) (Loomans, 2003; Froud & Stevens, 2004; Polaszek et al., 2022; Lahey et al., 2025). *Megaphragma* species parasitise the eggs of thrips in the subfamilies Panchaetothripinae and Thripinae, and comprise nearly all known egg parasitoids of these groups (Loomans, 2003). *Megaphragma* species are cosmopolitan and have been collected in a wide range of habitats, including from crops

including tea, coffee, avocado and citrus (Loomans, 2003). Several species have been recorded on single host species, while *Megaphragma mymaripenne* Timberlake has been recorded attacking four species of thrip (Loomans, 2003; Froud & Stevens, 2004). While the parasitism of two *Megaphragma* species have been studied (Bernado & Viggiani, 2002), there is no evidence of any species being used in biological control. This may be due to the low number of progeny (usually one) per host egg, potentially making their population growth slow, limiting their efficacy as biological control agents (Lahey et al., 2025).

Recently, Polaszek et al. (2022) revised 32 *Megaphragma* species, including 6 new synonymies and the description of 22 new species using morphological and molecular techniques. This represents important new baseline data that could facilitate the investigation of these species as biological control agents. The specimen Y57G from this study is grouped in the phylogeny (Figure 3) with one of the species described in the recent revision of *Megaphragma*, namely *M. antecessor* (Polaszek et al., 2022). Since there have been no studies on the use of *M. antecessor* for the biological control of thrips in South Africa, this finding represents an opportunity for such work.

The sampling method used in this study may need to be improved for future sampling of *T. leucotreta* parasitoids. While pan traps are widely used for hymenopteran sampling (Buffington et al., 2021), the specific challenge of targeting *T. leucotreta* parasitoids may require more specialised methods. Future sampling efforts could be enhanced through the incorporation of chemical attractants that simulate cues used by foraging parasitoids. Specifically, the identification and utilisation of *T. leucotreta* oviposition-induced plant volatiles or plant defensive compounds released during larval boring could improve trap specificity and capture rates (Fatouros et al., 2008). Yellow pan traps were more successful than blue or white in trapping wasps. This supports the findings of Buffington et al. (2021), suggesting that yellow should be the primary colour used for pan trapping in future studies. However, the effectiveness of different colours may depend on several factors, including geographic location and taxon, and research on the colour preferences of parasitoid wasps is scarce in the literature (Buffington et al., 2021) and no such research on South African parasitoids has been done to date. Use of additional complimentary methods to pan trapping, such as Malaise trapping or sweeping will be necessary to assess the presence or absence of *T. leucotreta* in orchards. Different methods target different taxa, and *T. leucotreta* could potentially be more successfully sampled using these additional methods. Turnover of return on ichneumonid species between sampling methods is high with each method sampling a unique portion of the species assemblage present in a given locality (van Noort, 2004).

Besides sampling method, this study failed to first assess the presence and estimated population size of *T. leucotreta* at each site. This is a significant shortcoming of the study, as the host of the target parasitoids may have been absent at the sites. However, anecdotal evidence of *T. leucotreta* was given by landowners, although this cannot be used as definitive proof of the presence of the moth, nor does it indicate how abundant *T. leucotreta* was at each site. Future studies should begin by assessing *T. leucotreta* population sizes using pheromone traps. In addition, it is possible that other closely related moths serve as reservoirs for parasitoids of *T. leucotreta*, and this could be a possible avenue for sampling these parasitoids.

These results highlight the need for expanded sampling of *T. leucotreta* parasitoids. While the current study indicates the potentially low densities of parasitoids in the field, long-term monitoring across multiple seasons and a broader geographical range would provide a more comprehensive understanding of parasitoid population dynamics.

The results of this study have important implications for IPM strategies in South African fruit production. The apparent scarcity of natural parasitoid populations suggests that successful biological control of *T. leucotreta* will likely continue to depend on augmentative releases. This underscores the importance of maintaining and potentially expanding current mass-rearing programmes for *T. cryptophlebiae*, while also investigating the potential for commercialization of other parasitoid species, such as *A. bishopi*.

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CHAPTER 4

Critical thermal limits of *Trichogrammatoidea cryptophlebiae* Nagaraja (Hymenoptera: Trichogrammatidae)

Introduction

Insects are ectotherms with a limited ability to regulate their body temperature (Chown & Nicolson, 2004a; Ali et al., 2019). As such, the distribution of insect species is partly determined by their thermal tolerance, which differs from species to species (Chown & Nicolson, 2004b). In addition, insect mobility is largely determined by temperature, resulting in limited motor function at very cold and very warm temperatures, which affects behaviour, including parasitism (Mawela, Kfir & Krüger, 2013). Temperature also affects fecundity and generation time, thus influencing population growth (Matthee, 2000; Tabebordbar et al., 2022).

There is a strong correlation between temperature and body size in many ectothermic species (Di Santo & Lobel, 2017; Klockmann, Günter & Fischer, 2017; Ma, Ma & Pincebourde, 2021). There is evidence that periods of unusually high climatic temperatures incur generational changes in body size (Klockmann, Günter & Fischer, 2017; Ma, Ma & Pincebourde, 2021), though this may not be the case for all ectotherms (Di Santo & Lobel, 2017). In the tropical butterfly *Bicyclus anynana* (Butler), Klockmann et al. (2017) found that body size was positively correlated with upper lethal temperature (ULT₅₀) (i.e., the upper temperature at which 50% of all individuals in an experiment die) with larger life stages generally having higher thermal tolerance. Conversely, within a single generation, adult body size is inversely proportional to temperature, with exposure to higher temperatures during an insect's development resulting in smaller adults (Ma, Ma & Pincebourde, 2021). This is likely due to high temperatures shortening development time, thus producing less-developed, smaller adults (Chown & Nicolson, 2004c). This can impact the overall fitness of an adult, since smaller adults are generally weaker, making foraging and mating more challenging (Griffith, 2018).

Body size and temperature affects fitness in parasitoid wasps, with implications for their parasitism behaviour (Colinet, Boivin & Hance, 2007; Gao et al., 2016; del Pino et al., 2020). Larger females tend to live longer, parasitise more hosts, and produce more offspring (Gao et al., 2016; Beukeboom, 2018). In addition, females who mate with larger males also produce more offspring (Beukeboom, 2018). The size of female parasitoids also affects the fitness of its offspring (Klockmann, Günter & Fischer, 2017). *Trichogramma achaeae* Nagaraja and Nagarkatti (Hymenoptera: Trichogrammatidae) is a biological control agent for several lepidopteran pests, including *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) and

Chrysodeixis chalcites (Esper) (Lepidoptera: Noctuidae) (del Pino et al., 2020). In this species, development time and adult longevity was impacted by temperature, with adults being smallest at the highest temperature of 30 °C (del Pino et al., 2020). In addition, temperature had a significant effect on levels of parasitisation in adult females, with the number of parasitised eggs being highest at 20 °C and lowest at 15 °C (del Pino et al., 2020). As well as affecting the number of offspring, female body size may have a proportional relationship with the body size of offspring, with smaller females producing smaller female offspring. This would have negative knock-on effects for populations of parasitoids (Beukeboom, 2018; Wise & Jaeger, 2021), although it may not affect all species to the same degree (Gao et al., 2016).

A useful metric for assessing thermal tolerance in ectotherms is critical thermal limits (CTLs). These are the nonlethal low (critical thermal minimum; CT_{min}) and high (critical thermal maximum; CT_{max}) temperatures above and below which an animal loses motor function. CT_{min} is often defined as the temperature of loss of coordinated muscle function, while CT_{max} is the temperature of onset of muscle spasms (Terblanche et al., 2007). Unlike lethal temperatures, CTLs can help researchers predict behaviour relative to temperature, especially in the ability of an insect to move through its environment, seek out a mate and escape predation, among other abilities (Porter et al., 2019).

Trichogrammatoidea cryptophlebiae Nagaraja (Hymenoptera: Trichogrammatidae) is an egg parasitoid of the false codling moth *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae) (Moore, 2021). *Trichogrammatoidea cryptophlebiae* is minute, with adults being ~0.5 mm long (Nagaraja, 1978). While the effects of pesticides on the parasitism rates and fitness of *T. cryptophlebiae* have been investigated (Moore, 2021), only one study has tested the effect of temperature on the development and reproduction of the wasp (Matthee, 2000). The lower development threshold (LDT) of *T. cryptophlebiae* was 7.65°C, while the mean generation time decreased with increasing temperature, being ~23 days at 15 °C and 7 days at 30 °C (Matthee, 2000). Since more mature life stages tend to tolerate higher and lower temperatures than larvae and pupae (Klockmann, Günter & Fischer, 2017), it is expected that *T. cryptophlebiae* adults will express CT_{min} below the LDT reported by Matthee (2000). This study aimed to determine the CT_{min} and CT_{max} of mass-reared *T. cryptophlebiae*.

Methods

Insect culture and maintenance

Egg sheets containing parasitised, unhatched *T. leucotreta* eggs were provided by Vital Bugs (Tzaneen, South Africa). In the Vital Bugs insectary, unparasitised *T. leucotreta* eggs were

exposed to mated female wasps, which then parasitised the eggs. The parasitised eggs were then kept at 25 °C and shipped to the University of Cape Town in a coolbox with ice packs to reduce heat stress and prevent premature emergence. While cold storage during transport may have affected the results through cold-hardening the unhatched wasps, this was the only way of ensuring wasps did not hatch prematurely. A Thermochron iButton (DS1921, Semiconductor Corporation, Dallas/Maxim, TX, USA) was placed in the cooler box to measure the temperature during one transit. The mean temperature in over the three-day transit period was 15.58 °C (Figure S2). Upon receipt, individual egg sheets were placed in 15 mL plastic vials, each with a tube filled with 50% honey solution. The tube had a fabric wick to draw out the honey solution as it was consumed to avoid rapid evaporation and large droplets of honey that may have trapped and killed wasps. The vials were kept at 25 °C, 12:12 (L:D) until the assays were performed, checked daily for emergence. Assays were performed within one to three days of emergence.

Critical thermal limit assays

While lethal temperatures are a useful metric for predicting species distribution, and their assay is simple, CTLs are more indicative of the limits of physiology in that they indicate when an insect can no longer move. Therefore, CTLs describe the functional thermal range of an insect better than lethal temperatures. The CT_{min} and CT_{max} of *T. cryptophlebiae* were measured using a 50 mL plastic vial with a moistened Plaster-of-Paris substrate (to prevent desiccation) set in a custom-built double-jacketed aluminium thermal stage (Figure 1), connected to with silicone pipes to a programmable water bath (Model R2, Grant Instruments, Cambridge, UK). Temperature was monitored with a 40-gauge Type T (copper-constantan) thermocouple connected to a digital thermometer (CHY 507, Thermometer, Taiwan), and the chamber was sealed with a glass viewing window. The window was weighed down lightly with hot glue to keep it in place. To avoid observer bias, a single observer (TP) undertook all of the experimental work.

Given the small size of *T. cryptophlebiae*, special care had to be taken while handling it so as not to affect the fitness of individuals. Thus, a moistened 00-size paintbrush helped move 15 wasps into the chamber (Figure 1). Once all wasps were in the chamber, the wasps were given a resting period of 15 min to minimise the effects that handling them may have had on the results (Ali et al., 2019). Thereafter, the ramping programme was set to run. The rate of temperature increase and decrease was 0.1 °C·min⁻¹. This rate was chosen to resemble natural rates of temperature change that *T. cryptophlebiae* may experience in the field and to reduce the effects that rapid temperature change may have on thermal tolerance of the wasp (Terblanche et al., 2007; Allen et al., 2016; Scotta et al., 2021).

The wasps were monitored using a Leica microscope every $\sim 3^{\circ}\text{C}$ for the first 12°C and then every $\sim 1^{\circ}\text{C}$ until the end of the assay. This method was selected after preliminary trials and according to Chown et al. (2009). $\text{CT}_{\min}$ or $\text{CT}_{\max}$ for an individual was determined as the temperature at which individuals were unable to right themselves (Terblanche et al., 2007). If the wasps remained immobile, the final recorded temperature was taken as the thermal maximum or minimum. The experiment was terminated as soon as the last wasp stopped moving. The wasps were left for 24 hours at 25°C and checked for survival. Three replicates of 10 individuals were undertaken for each thermal limit, giving a total samples size of 45 individuals for $\text{CT}_{\min}$ and 45 for $\text{CT}_{\max}$.

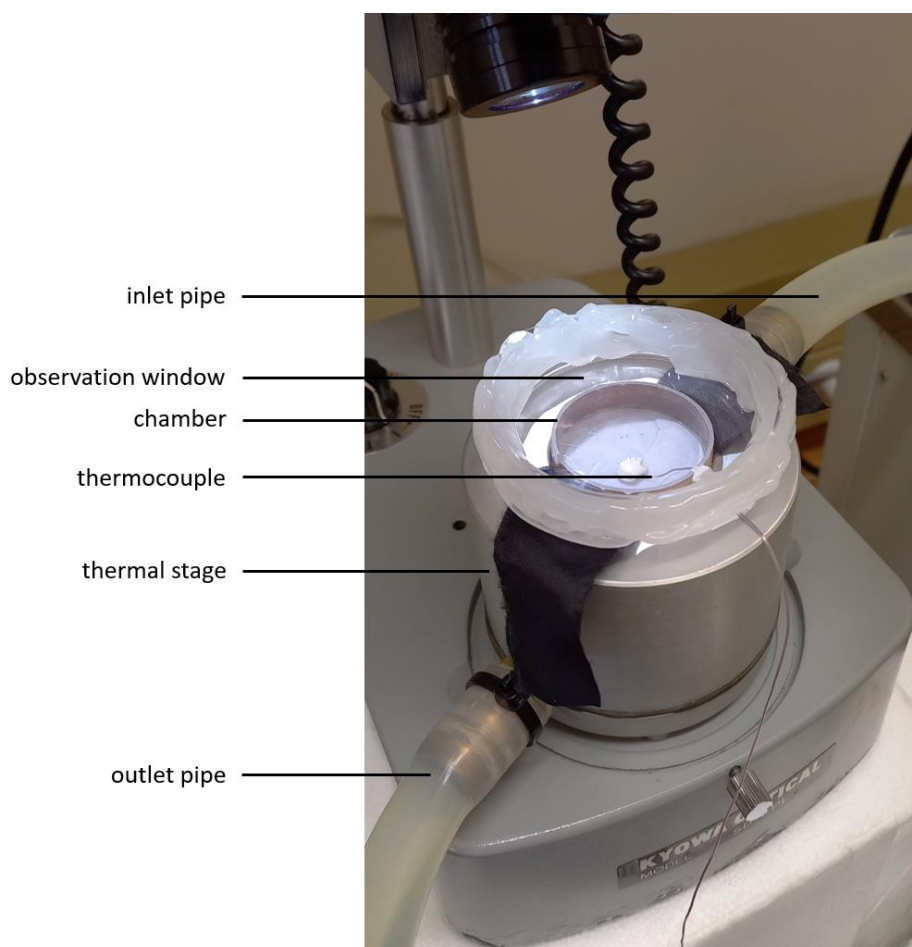


Figure 1. The thermal stage setup for CT assays of *T. cryptophlebiae*.

Results

The $\text{CT}_{\min}$ and $\text{CT}_{\max}$ were $8.15^{\circ}\text{C} \pm 0.58^{\circ}\text{C}$ and $44.50^{\circ}\text{C} \pm 0.41^{\circ}\text{C}$ (mean $\pm$ SE), respectively (Table 1 and Figure 2). The violin plots in Figure 2 show the distribution of individual data points (individual wasps) for each critical thermal limit. This difference is also shown by the

higher standard error for CT_{min} . Following each assay, none of the wasps survived after the 24-hour period.

Table 1. CT_{min} and CT_{max} at rates of -0.1 and 0.1 $^{\circ}C \cdot min^{-1}$, respectively, for *Trichogrammatoidea cryptophlebiae*.

Thermal limit	Mean ($^{\circ}C$)	Standard deviation	Standard error
CT_{min}	8.15	1.17	0.58
CT_{max}	44.50	0.82	0.41

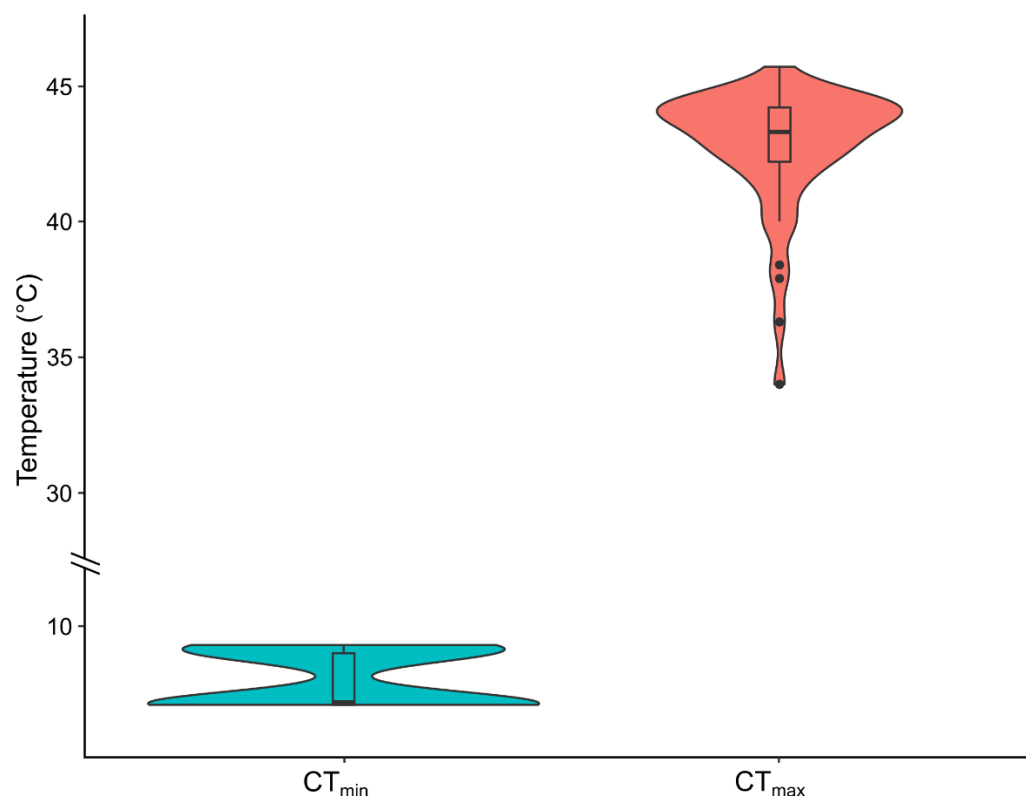


Figure 2. Violin plots of CT_{min} (blue) and CT_{max} (red) of *T. cryptophlebiae*. The coloured shapes represent the distribution of temperature values, with wider sections indicating clusters of similar values, while thinner sections represent lower densities of given values.

Discussion

This was the first study to determine the thermal limits of the parasitoid *T. cryptophlebiae*. The CT_{min} of 8.15 $^{\circ}C$ determined for adults in this study was higher than the 7.65 $^{\circ}C$ lower developmental threshold reported by Matthee (2000), which suggests that younger life stages of this species may exhibit greater cold tolerance than adults. This corroborates findings of other research, where eggs typically demonstrate the greatest cold tolerance, often being

tolerant to freezing (Sinclair, 1999; Papadopoulos et al., 2023). However, while immature stages may withstand colder temperatures, cold exposure during female larval development can severely reduce subsequent adult fecundity in parasitoid wasps (Hance et al., 2007). However, since the lower developmental threshold reported by Matthee (2000) was estimated using a regression of measured development rates, the actual threshold may be different. This underscores the need for comprehensive thermal tolerance studies across all life stages of *T. cryptophlebiae*.

Trichogrammatoidea cryptophlebiae is more thermally tolerant than other closely-related trichogrammatid wasps. Del Pino et al. (2020) reported the lower developmental threshold for *Trichogramma achaeae* as 10.5 °C. Not only is this higher than the threshold Matthee (2000) reported for *T. cryptophlebiae*, but if the margin between LDT and CT_{min} is similar in both species, then the CT_{min} of *T. achaeae* is likely higher than that of *T. cryptophlebiae*, making the latter more cold tolerant. In regards to heat tolerance, Negahban et al. (2021) estimated the CT_{max} of *Trichogramma brassicae* to be between 36 and 41 °C. Again, *T. cryptophlebiae* is more tolerant to heat stress than a related trichogrammatid wasp.

With predicted increase in temperature (van Wilgen et al., 2023), *Trichogrammatoidea cryptophlebiae* may not survive in all parts of the Western Cape in the future. Although maximum daily temperatures seldom exceeded 36.7 °C in the Western Cape between 1991 and 2020, minimum daily temperatures were recorded as low as 0.42 °C in winter in the province (World Bank Group, 2025). This minimum falls well below the developmental threshold and CT_{min} of *T. cryptophlebiae*, suggesting this species' movement will be hampered by these low temperatures if they continue to occur. However, projections indicate that temperatures may increase by 1.3 and 2 °C in coastal and inland regions of Southern Africa — including the Western Cape — by 2050 (van Wilgen et al., 2023). It is important to note that these recordings and projections are average values for the province, and while heatwaves may cause temperature anomalies in smaller regions within the Western Cape (Mbokodo et al., 2020), the microclimate will likely be different. In addition, behavioural responses of ectotherms in these microclimates should also be taken into account in future studies (Duffy et al., 2015). As such, it is likely that the performance of *T. cryptophlebiae* as a biological control agent will not be substantially affected by increasing temperatures on average. However, anomalously high and low temperatures in certain areas of the Western Cape may affect its performance and distribution.

This study acknowledges that the exposure to low temperatures during transport of the *T. cryptophlebiae* eggs may have affected the thermal tolerance of the emerged adults. In

particular, cold-hardening of the eggs in transport may have reduced the CT_{max} of the emerged adults (Gray, 2013; Porter et al., 2019). Conversely, the CT_{min} may have been higher were the wasps not exposed to a low temperature for an extended period during their development. Ideally, an experimental colony would have been established closer to the laboratory in which the experiment was conducted, which was not possible for logistic reasons. Future studies could be conducted closer to insectaries breeding *T. cryptophlebiae*, for example, Vital Bugs in Tzaneen, Limpopo Province in this case.

While critical thermal limits are more useful in indicating how behaviour, rather than survival, is affected by temperature, other metrics can explain how specific behaviour is affected by temperature. Upper and lower lethal temperatures (ULTs and LLTs) describe the thermal limits beyond which a species will die (Willot et al., 2022). Lethal temperatures are particularly important for modelling species abundance and distribution by indicating the absolute limits of a species thermal tolerance (Chown & Nicholson, 2004b; Nyamukondiwa et al., 2013). Assessing lethal temperatures is simple and comes at little cost (Nyamukondiwa et al., 2013). Supercooling points are also a useful metric for predicting how insect behaviour and distribution are limited by low temperatures (Sinclair, 1999). The relationship between temperature and parasitism has been studied for other trichogrammatids (Krechemer & Foerster, 2015; Abbes et al., 2020; Tabebordbar et al., 2022) and *Trichogrammatoidea lutea* (Mawela, Kfir & Krüger, 2013), but not in *T. cryptophlebiae*. Determining the effect of temperature on parasitism in *T. cryptophlebiae* would contribute to the understanding of the ideal conditions for biological control with the wasp.

The thermal tolerance data obtained for *T. cryptophlebiae* in this study can be used to model the distribution of the wasp. Ecological niche models (ENMs), a subset of species distribution models, can incorporate thermal tolerance data to project both current and future distributions of species (Elith & Leathwick, 2009; Ashraf, Chaudhry & Peterson, 2021). Such models prove particularly valuable for predicting distribution shifts under climate change scenarios and identifying optimal release sites for biological control programmes (Pérez-De La O et al., 2020).

Acclimation can greatly improve the thermal tolerance of biological control agents (Porter et al., 2019). Phenotypic plasticity has been shown to allow the thermal tolerance of several insect groups to change within a single lifespan (Colinet, Boivin & Hance, 2007; Gray, 2013). Both cold (Jørgensen et al., 2021) and warm (Gray, 2013; Janion-Scheepers et al., 2018) acclimation improve tolerance of cold and warm temperatures, respectively. The results of this study provide a baseline thermal tolerance for *T. cryptophlebiae* in CTLs, which can be used

for comparison by future studies that investigate the effects of acclimation on the wasp's thermal tolerance (Krechemer & Foerster, 2015). In addition, the experimental design in this study, while not necessarily novel, was optimised for very small wasps, which are difficult to handle and observe. The methodology of this study may benefit future studies on the thermal physiology of *T. cryptophlebiae*.

Understanding climate compatibility between biological control agents and their target environments improves the effectiveness of agents. Poor climate matching can result in lower rates of parasitism and can prevent agents from establishing in the target area (Griffith, 2018). The resources erroneously spent on failed releases of biological control agents could be better directed toward acclimating agents to the target climate or investigating alternative natural enemies (Muskett, Paterson & Coetzee, 2020). Currently, there exists a notable research gap regarding thermal tolerance studies of biological control agents, particularly in the context of climate matching (Griffith, 2018; Harms et al., 2020).

The results of this study contribute to addressing the gap in knowledge of an important parasitoid biological control agent by providing critical thermal limits for *T. cryptophlebiae*. These findings can inform release strategies and improve predictions of parasitism success across different thermal environments. Future research should focus on determining thermal tolerances across all life stages, assessing the relationship between temperature and parasitism rates, investigating thermal acclimation of this species, and modelling current and future distributions of *T. cryptophlebiae*.

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CHAPTER 5

General discussion and conclusion

Climate change and agricultural expansion are altering environments globally. Increasing temperatures are expected to change the distribution of fauna and flora, while globalisation and the growing demand for food grown in large monocultures are increasing the risk of biological invasions (Harvey et al., 2022). In addition, these climatic and land-use changes will likely change the distribution of agricultural pests as new environments become suited to their thermal physiology (Skendžić et al., 2021). Invertebrates are especially likely to spread into new environments due to their ectothermic nature (Chown & Nicolson, 2004) (although, this trait may also limit spread), rapid reproduction, short life cycles, and small size, which often allows them to bypass biosecurity measures.

Given these challenges, biological control will become increasingly important to manage pests and invasive species (Armstrong & Ball, 2005). Biological control is a cost-effective and organic alternative to chemical pesticides (Bale, van Lenteren & Bigler, 2008). Unlike pesticides, biological control agents have species-specific hosts, limiting the non-target effects on other, potentially beneficial, invertebrates in the agroecosystem or invaded environment (Hajek, 2004; Ndakidemi, Mtei & Ndakidemi, 2016). To improve the effectiveness of biological control, more research is needed to identify natural enemies of pests or invasive species using both morphological and molecular techniques (Armstrong & Ball, 2005; Chesters et al., 2012). DNA barcoding is a particularly well-suited tool for molecular description and identification, since it is cost-effective and barcodes can be referenced against a large and growing online database, namely the Barcoding of Life Data System (BOLD) (Ratnasingham & Hebert, 2007).

Despite the importance of molecular techniques for the rapid identification of alien invertebrates, our results show that there remains a large gap in the DNA barcoding of this group in South Africa. In particular, platyhelminths and arachnids were the least barcoded groups, and it is suggested that more resources should be allocated to their barcoding. Biological control agents were even less well barcoded than aliens, which is a concern since correct identifications are a foundation for approving and using agents. In addition, only about a quarter of BOLD records of alien invertebrates invasive to South Africa were collected in the country, and just over half of the alien invertebrate records on BOLD included specimen images. These factors limit the effectiveness of DNA barcodes of alien species and biological control agents, because of potential morphological misidentifications due to similar or cryptic species (Hebert et al., 2004; Park et al., 2011; Sikes, 2020), a challenge which could be

overcome by analysing high-resolution images. These results highlight the need for a greater DNA barcoding effort of alien invertebrates in South Africa.

While COI barcodes are valuable for species identification and can supplement morphological descriptions of new species, other genetic markers, such as microsatellite loci, are important in understanding how population genetics of invasive species and natural enemies change during their introduction (Hopper et al., 2019; Li et al., 2022). Populations of introduced invasive species and natural enemies usually differ from populations in their native ranges, because the former are usually founded by a small subset of the latter (Hopper et al., 2019). This can reduce genetic diversity in the introduced populations through founder events and bottlenecks (Hopper et al., 2019; Li et al., 2022). Conversely, different populations and even closely-related species of biological control agents can hybridise in the field, which can increase their genetic diversity (Szűcs et al., 2019; Li et al., 2022). There remains a gap in our understanding of population genetics in alien invertebrates, pests and biological control agents, which has implications for thermal physiology, as well as invasion biology.

Several natural enemies attack the important agricultural pest, *Thaumatotibia leucotreta* (Meyrick) (Tortricidae, Olethreutinae) (false codling moth or FCM) (Prinsloo, 1984; Moore, 2002). *Thaumatotibia leucotreta* larvae develop in citrus and stone fruit in South Africa, causing substantial annual losses in exports of these fruit (Moore, 2021). Despite this, many known natural enemies, mostly parasitoid wasps, which attack *T. leucotreta* have not been studied since their description and first recorded parasitisation. Two important parasitoids are *Trichogrammatoidea cryptophlebiae* Nagaraja, which is a commercial biological control agent of *T. leucotreta* (Moore, 2021), and *Agathis bishopi* (Nixon), which can be used to identify *T. leucotreta*-infested fruit in packing houses (Zimba et al., 2015). This study did not find any of these parasitoids in organic and IPM orchards in the Western Cape Province of South Africa, despite sampling under ideal conditions using pan trapping, an effective standard method for sampling wasps (Saunders & Luck, 2013; Buffington et al., 2021). It is unclear whether these results are due to a lack of *T. leucotreta* parasitoids in these orchards or the sampling method used, and alternative methods such as Malaise trapping is suggested for future sampling of these parasitoids (van Noort, 2004). This result suggests that more sampling and baseline life history studies are needed for natural enemies of *T. leucotreta* and underscores the importance of such studies for other important agricultural pests.

In addition to the need for increased DNA barcoding of natural enemies, more work is needed to understand the thermal physiology of biological control agents, as this affects their efficacy and suitability to future climates (Byrne, Currin & Hill, 2002; Ali et al., 2019). This study

contributed to the understanding of the thermal physiology of *T. cryptophlebiae* by determining its critical thermal limits. These results are a baseline for investigations into the plasticity of these limits. In addition, these limits are important for modelling the distribution of the wasp under current and future climates (Elith & Leathwick, 2009; Ashraf, Chaudhry & Peterson, 2021). Acclimation increases the thermal tolerance of some species of wasp, increasing their efficacy as parasitoids (Thomson, Robinson & Hoffmann, 2001; Jerbi-Elayed et al., 2021). Acclimating *T. cryptophlebiae* prior to release in citrus and stone fruit orchards could increase parasitism rates on *T. leucotreta*, improving the effectiveness of biological control using the wasp. This will be especially important in ensuring the continued efficacy of *T. cryptophlebiae* under a changing global climate (Hance et al., 2007; Harvey et al., 2022). It is also important to study other aspects of *T. cryptophlebiae*'s thermal physiology, particularly the relationship between temperature and parasitism rates and fecundity (Krechemer & Foerster, 2015; del Pino et al., 2020).

Genetically distinct populations incur phenotypic differences that have implications for thermal physiology (Martin-Roy et al., 2021; Rogers, Terblanche & Owen, 2021) and parasitism rates (Goldson, McNeill & Proffitt, 2003) in biological control agents. Rogers et al. (2021) showed that two distinct South African populations of the exotic weevil *Neochetina eichhorniae* Warner (Coleoptera: Curculionidae), a biological control agent of water hyacinth, *Eichhornia crassipes* (Martius) (Pontederiaceae), had different thermal tolerances. The CT_{min} differed by 1 °C between the two populations, while the super cooling point (the point at which an insect's body fluids spontaneously freeze) differed by ~3 °C (Rogers, Terblanche & Owen, 2021). In addition, using microsatellite loci, Hopper et al. (2019) showed that *N. eichhorniae* hybridises with a closely-related biological control agent, *Neochetina bruchi*, and results of other studies suggest that these hybrids may have different thermal tolerances to the parental species (Goldson, McNeill & Proffitt, 2003; Martin-Roy et al., 2021). While *T. leucotreta* and *T. cryptophlebiae* have not undergone changes due to invasion (being native to South Africa), genetically-distinct wild populations of each may have different thermal tolerances. This has implications for the future distribution of each species. Identifying genetically-distinct populations of *T. cryptophlebiae* may help researchers choose the population which is more effective in controlling *T. leucotreta*.

The results from this study highlight several important gaps in biological control research in South Africa. Increased sampling efforts are essential for both invasive species and their natural enemies, particularly potential biological control agents. Increased DNA barcoding efforts will facilitate the creation of thorough checklists for both groups. In addition, greater emphasis should be placed on barcoding biological control agents to ensure accurate

identifications and facilitate monitoring. Future research should focus on understanding the life histories, physiology, and potential hybridisation of biological control agents in the field, as this knowledge can enhance agent selection and improve overall efficacy of biological control.

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Supplementary material

Table S1. The representation of South African invertebrates on BOLD in 2014 and 2023.

Group	Number of known species in SA ^b	2014 ^a			2023			Increase in number of records (%)	Increase in number of BINs (%)	Increase in number of species (%)
		Number of records	Number of BINs	Number of species	Number of records	Number of BINs	Number of species			
Insects	44088	37105	8238	513	733499	45420	5378	1876.8	451.3	948.3
Other	4195	-	-	-	65389	3540	9406	-	-	-
Arachnids	6630	1721	555	7	29919	2884	769	1638.5	419.6	10885.7
Molluscs	3154	734	214	22	2597	433	355	253.8	102.3	1513.6
Annelids	1110	357	99	3	1125	169	108	215.1	70.7	3500.0
Platyhelminthes	246	10	6	1	317	18	70	3070.0	200.0	6900.0
Cnidarians	932	15	3	1	112	15	19	646.7	400.0	1800.0
Echinoderms	410	197	6	2	842	122	104	327.4	1933.3	5100.0
Sponges	346	8	6	1	77	12	38	862.5	100.0	3700.0
Total	61111	40147	9127	550	833877	52613	16247	1977.1	476.5	2854.0

^ada Silva & Willows-Munro (2016)

^bHamer (Hamer, 2013)

SA = South Africa

Table S2. The number of invertebrate alien and biological control agent species represented on BOLD by phylum.

Phylum	Class	Alien			Biological control agent		
		Number of species	Number species BOLD	of in	Percentage species BOLD	of in	Number of species in BOLD
Arthropoda	Insecta	441	329		74.6	128	56
	Arachnida	97	50		51.5	4	0
	Malacostraca	54	42		77.8	0	0
	Collembola	27	25		92.6	0	0
	Diplopoda	9	6		66.7	0	0
	Maxillopoda	4	3		75.0	0	0
	Chilopoda	3	2		66.7	0	0
	Copepoda	2	2		100.0	0	0
	Branchiopoda	1	1		100.0	0	0
	Pycnogonida	1	1		100.0	0	0
	Thecostraca	1	0		0.0	0	0
Mollusca	Gastropoda	67	51		76.1	0	0
	Bivalvia	13	10		76.9	0	0

Annelida	Clitellata	55	39	70.9	0	0	-
	Polychaeta	13	7	53.8	0	0	-
	Unknown	1	0	0.0	0	0	-
Nematoda	Chromadorea	21	15	71.4	0	0	-
	Unknown	8	0	0.0	0	0	-
Platyhelminthes	Monogenea	13	2	15.4	0	0	-
	Cestoda	3	3	100.0	0	0	-
	Turbellaria	3	3	100.0	0	0	-
	Unknown	2	0	0.0	0	0	-
	Trematoda	1	0	0.0	0	0	-
Cnidaria	Hydrozoa	12	8	66.7	0	0	-
	Anthozoa	3	3	100.0	0	0	-
Chordata	Ascidacea	9	8	88.9	0	0	-
Bryozoa	Gymnolaemata	7	4	57.1	0	0	-
Echinodermata	Asteroidea	2	2	100.0	0	0	-
	Echinoidea	1	1	100.0	0	0	-
	Ophiuroidea	1	1	100.0	0	0	-
Brachiopoda	Lingulata	2	0	0.0	0	0	-
Ctenophora	Tentaculata	1	1	100.0	0	0	-
Euglenozoa	Kinetoplastea	1	0	0.0	0	0	-
Myxozoa	Dinophyceae	1	1	100.0	0	0	-
Porifera	Demospongiae	1	1	100.0	0	0	-
Total		881	621	70.5	132	56	42.4

Table S3. The number of invertebrate alien groups per province.

Group	Eastern Cape		Free State		Gauteng		KwaZulu-Natal		Limpopo		Mpumalanga		Northern Cape		North West		Prince Islands	Edward	Western Cape	
	Number		Number		Number		Number		Number		Number		Number		Number		Number	Number	Number	
	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD	of species in BOLD
Insects	34	28	23	21	81	74	58	50	45	40	55	52	15	13	16	14	13	8	72	59
Other	21	19	9	7	6	6	19	15	3	2	8	6	15	11	5	4	5	5	53	45
Arachnids	13	7	17	7	13	11	22	11	19	11	14	8	12	9	8	4	7	2	26	12
Molluscs	19	16	4	3	9	8	11	10	4	4	3	3	8	8	3	3	1	1	32	28
Annelids	23	20	10	7	16	13	37	30	19	14	10	9	4	3	11	9	0	0	27	24
Platyhelminthes	1	1	0	0	2	2	1	1	0	0	0	0	0	0	2	2	0	0	1	1
Cnidarians	3	2	0	0	0	0	3	2	0	0	0	0	2	2	0	0	0	0	3	3
Echinoderms	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Sponges	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
Total	114	93	63	45	127	114	152	120	90	71	90	78	57	47	45	36	26	16	214	172

R code

```

1  install.packages("ggplot2")
2  install.packages("tidyr")
3  install.packages("tidyverse")
4  install.packages("dplyr")
5  install.packages("treemap")
6  install.packages("rgbif")
7  install.packages("bold")
8  install.packages("ggpubr")
9  install.packages("tibble")
10 install.packages("remotes")
11 remotes::install_github("mrdwab/mrdwabmisc")
12
13 library(ggplot2)
14 library(tidyr)
15 library(tidyverse)
16 library(dplyr)
17 library(treemap)
18 library(rgbif)
19 library(bold)
20 library(ggpubr)
21 library(tibble)
22 library(remotes)
23
24 setwd("C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien Invertebrates")
25
26 inverts <- read.csv("alien_inverts_CURRENT.csv")
27
28 ##### CREATE ALIEN DA SILVA DATAFRAME #####
29
30 insects <-
31   inverts %>%
32   filter(is.na(legallyImported), class == "Insecta") %>%
33   select(-c(phylum, class)) %>%
34   add_column(Group = "Insects", .before = 1)
35 arachnids <-
36   inverts %>%
37   filter(is.na(legallyImported), class == "Arachnida") %>%
38   select(-c(phylum, class)) %>%
39   add_column(Group = "Arachnids", .before = 1)
40 molluscs <-
41   inverts %>%
42   filter(is.na(legallyImported), phylum == "Mollusca") %>%
43   select(-c(phylum, class)) %>%
44   add_column(Group = "Molluscs", .before = 1)
45 crustaceans <-
46   inverts %>%
47   filter(is.na(legallyImported), phylum == "Crustacea") %>%
48   select(-c(phylum, class)) %>%
49   add_column(Group = "Crustaceans", .before = 1)
50 annelids <-
51   inverts %>%
52   filter(is.na(legallyImported), phylum == "Annelida") %>%
53   select(-c(phylum, class)) %>%
54   add_column(Group = "Annelids", .before = 1)
55 cnidarians <-
56   inverts %>%
57   filter(is.na(legallyImported), phylum == "Cnidaria") %>%
58   select(-c(phylum, class)) %>%
59   add_column(Group = "Cnidarians", .before = 1)
60 echinoderms <-
61   inverts %>%
62   filter(is.na(legallyImported), phylum == "Echinodermata") %>%
63   select(-c(phylum, class)) %>%
64   add_column(Group = "Echinoderms", .before = 1)

```

```

65 sponges <-
66   invert %>%
67   filter(is.na(legallyImported), phylum == "Porifera") %>%
68   select(-c(phylum, class)) %>%
69   add_column(Group = "Sponges", .before = 1)
70 platyhelminths <-
71   invert %>%
72   filter(is.na(legallyImported), phylum == "Platyhelminthes") %>%
73   select(-c(phylum, class)) %>%
74   add_column(Group = "Platyhelminths", .before = 1)
75 other <- invert %>%
76   filter(is.na(legallyImported),
77         class != "Insecta",
78         class != "Arachnida",
79         phylum != "Mollusca",
80         phylum != "Crustacea",
81         phylum != "Annelida",
82         phylum != "Cnidaria",
83         phylum != "Echinodermata",
84         phylum != "Porifera",
85         phylum != "Platyhelminthes") %>%
86   select(-c(phylum, class)) %>%
87   add_column(Group = "Other", .before = 1)
88
89 invertsdaSilvaAlien <- rbind(insects,
90                             arachnids,
91                             molluscs,
92                             crustaceans,
93                             annelids,
94                             cnidarians,
95                             echinoderms,
96                             sponges,
97                             platyhelminths,
98                             other)
99
100 invertsdaPlot <- invertsdaSilvaAlien %>%
101   group_by(Group) %>%
102   summarise(n=n()) %>%
103   arrange(desc(n))
104
105 ## plot da Silva
106 ggbarplot(data=invertsdaPlot, x="Group", y="n",
107           width=0.8, fill="black",
108           xlab="Group", ylab=FALSE) +
109   rotate_x_text(angle=45)
110
111
112 ##### GET TAXON NAMES W/ GBIF #####
113
114 for(i in 1:nrow(inverts2019)){
115   tryCatch({
116     bb <- name_backbone(name=inverts2019[i,"species"])
117     if(max(dim(bb))!=-Inf){
118       inverts2019[i,"phylum"] <- bb[1,"phylum"]
119       inverts2019[i,"order"] <- bb[1,"order"]
120       inverts2019[i,"family"] <- bb[1,"family"]
121     }else{
122       bbg <- name_backbone(name=inverts2019[i,"genus"])
123       if(max(dim(bbg))!=-Inf){
124         inverts2019[i,"phylum"] <- bbg[1,"phylum"]
125         inverts2019[i,"order"] <- bbg[1,"order"]
126         inverts2019[i,"family"] <- bbg[1,"family"]
127       }
128     }
129   }, error = function(e){
130     message(paste("An error occurred in run #", i, ":\n"), e)

```

```

131   })
132 }
133
134 ## cross-check inverts2019 against inverts
135 inverts2019 <- inverts2019 %>%
136   mutate(crosscheck="",.after=species)
137
138 for(i in 1:nrow(inverts2019)){
139   if(inverts2019[i,"species"] %in% inverts$Species){
140     inverts2019[i,"crosscheck"] <- "MATCH"
141   }
142 }
143
144 write.csv(inverts2019, "C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien
Invertebrates\\alien_inverts_SANBI_CIB_2019_revised.csv")
145
146 ## cross-check inverts against inverts2019
147 inverts <- inverts %>%
148   mutate(crosscheck="",.after=Species)
149
150 for(i in 1:nrow(inverts)){
151   if(inverts[i,"Species"] %in% inverts2019$species){
152     inverts[i,"crosscheck"] <- "MATCH"
153   }
154 }
155
156 write.csv(inverts, "C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien
Invertebrates\\alien_inverts_checked_revised.csv")
157
158
159 ##### MINE BOLD FOR RECORD METADATA #####
160
161 for(i in 1:nrow(inverts)){
162   tryCatch({
163     records <- bold_specimens(taxon=inverts[i,"species"])
164     if(max(dim(records))!=-Inf){
165       ## boldRecords
166       inverts[i,"boldRecords"] <- nrow(records)
167       ## SAboldRecords
168       inverts[i,"SAboldRecords"] <- nrow(records %>%
169         filter(country=="South Africa"))
170       ## BINs
171       inverts[i,"BINs"] <- length(unique((records %>%
172         filter(bin_uri!=""))$bin_uri))
173       ## SABINs
174       inverts[i,"SABINs"] <- length(unique((records %>%
175         filter(country=="South Africa",
bin_uri!=""))$bin_uri))
176       ## boldImages
177       inverts[i,"boldImages"] <- nrow(records %>%
178         filter(image_ids!=""))
179       ## SAboldImages
180       inverts[i,"SAboldImages"] <- nrow(records %>%
181         filter(country=="South Africa", image_ids!=""))
182       ## province
183       provinceBOLD <- unique(records %>%
184         filter(country=="South Africa") %>%
185         select(province_state))
186       if(max(dim(provinceBOLD))!=-Inf){
187         inverts[i, "provincialDistribution"] <-
188           paste(c(inverts[i, "provincialDistribution"]), as.character(provinceBOLD),
collapse=", ")
189       }
190     }
191   }, error = function(e){
192     message(paste("An error occurred in run #", i, ":\n"), e)

```

```

193     }
194   )
195 }
196
197 write.csv(inverts, paste0("C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien
Invertebrates\\alien_inverts_BOLDDED_", Sys.Date(), ".csv"))
198
199 ##### PHYLA TABLE & PLOT #####
200
201 ## count the number of barcoded specimens per phylum
202 alienPhyla1 <- invert %>%
203   filter(is.na(legallyImported)) %>%
204   group_by(phylum) %>%
205   summarise(no_species=n())
206
207 alienPhyla2 <- invert %>%
208   filter(is.na(legallyImported)) %>%
209   filter(!is.na(boldRecords), boldRecords!=0) %>%
210   group_by(phylum) %>%
211   summarise(no_bold_records=n())
212
213 alienPhylaTable <- left_join(alienPhyla1, alienPhyla2) %>%
214   arrange(desc(no_species)) %>%
215   replace(is.na(.), 0)
216
217 alienPhylaTable <- rbind(alienPhylaTable, data.frame(phylum="Total", t(colSums(alienPhylaTable[, -
1]))))
218
219 write.csv(alienPhylaTable, "C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien
Invertebrates\\alienPhylaTable.csv")
220
221 ## BCA phyla table
222 bcaPhyla1 <- invert %>%
223   filter(legallyImported=="biocontrol") %>%
224   group_by(phylum) %>%
225   summarise(no_species=n())
226
227 bcaPhyla2 <- invert %>%
228   filter(legallyImported=="biocontrol") %>%
229   filter(!is.na(boldRecords), boldRecords!=0) %>%
230   group_by(phylum) %>%
231   summarise(no_bold_records=n())
232
233 bcaPhylaTable <- left_join(bcaPhyla1, bcaPhyla2) %>%
234   arrange(desc(no_species)) %>%
235   replace(is.na(.), 0)
236
237 bcaPhylaTable <- rbind(bcaPhylaTable, data.frame(phylum="Total", t(colSums(bcaPhylaTable[, -1]))))
238
239 write.csv(bcaPhylaTable, "C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien
Invertebrates\\bcaPhylaTable.csv")
240
241 ## PHYLA PLOT
242
243 ## alien phyla plot
244 ggbarplot(data=alienPhylaTable, x="phylum", y="n",
245           width=0.8, fill="black",
246           xlab="Phylum", ylab=FALSE) +
247   rotate_x_text(angle=45)
248
249 ## alien pie plot
250 ggplot(alienPhylaTable, aes(x="", y=n, fill=phylum)) +
251   geom_bar(stat="identity", width=1, color="white") +
252   coord_polar("y", start=0) +
253   theme_void()
254

```

```

255
256#### CLASS TABLE ####
257
258## alien class table
259alienClassTable1 <- invert %>%
260   filter(is.na(legallyImported)) %>%
261   group_by(phylum) %>%
262   group_by(class, add=TRUE) %>%
263   summarise(no_species=n())
264
265alienClassTable2 <- invert %>%
266   filter(is.na(legallyImported)) %>%
267   filter(!is.na(boldRecords), boldRecords!=0) %>%
268   group_by(class) %>%
269   summarise(no_bold_records=n())
270
271alienClassTable <- left_join(alienClassTable1, alienClassTable2) %>%
272   arrange(desc(no_species)) %>%
273   replace(is.na(.), 0)
274
275write.csv(alienClassTable, "C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien
Invertebrates\\alienClassTable.csv")
276
277## BCA class table
278bcaClassTable1 <- invert %>%
279   filter(legallyImported=="biocontrol") %>%
280   group_by(phylum) %>%
281   group_by(class, add=TRUE) %>%
282   summarise(no_species=n())
283
284bcaClassTable2 <- invert %>%
285   filter(legallyImported=="biocontrol") %>%
286   filter(!is.na(boldRecords), boldRecords!=0) %>%
287   group_by(class) %>%
288   summarise(no_bold_records=n())
289
290bcaClassTable <- left_join(bcaClassTable1, bcaClassTable2) %>%
291   arrange(desc(no_species)) %>%
292   replace(is.na(.), 0)
293
294write.csv(bcaClassTable, "C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien
Invertebrates\\bcaClassTable.csv")
295
296#### ARTHROPOD CLASS PLOT ####
297
298plotData <- invert %>%
299   filter(phylum=="Arthropoda", is.na(legallyImported)) %>%
300   group_by(class) %>%
301   summarise(n=n()) %>%
302   arrange(desc(n))
303
304## alien arthropod class plot
305ggbarplot(data=plotData, x="class", y="n",
306          width=0.8, fill="black",
307          xlab="Class", ylab=FALSE) +
308   rotate_x_text(angle=45)
309
310## pie chart of arthropod classes
311ggplot(plotData, aes(x="", y=n, fill=class)) +
312   geom_bar(stat="identity", width=1, color="white") +
313   coord_polar("y", start=0) +
314   theme_void()
315
316#### INSECT ORDER PLOT ####
317
318plotData <- invert %>%

```

```

319   filter(class=="Insecta", is.na(legallyImported)) %>%
320   group_by(order) %>%
321   summarise(n=n()) %>%
322   arrange(desc(n))
323
324## alien insect order plot
325ggbarplot(data=plotData, x="order", y="n",
326           width=0.8, fill="black",
327           xlab="Order", ylab=FALSE) +
328   rotate_x_text(angle=45)
329
330## alien insect pie plot
331ggplot(plotData, aes(x="", y=n, fill=order)) +
332   geom_bar(stat="identity", width=1, color="white") +
333   coord_polar("y", start=0) +
334   theme_void()
335
336## INSECT ORDER METRICS PLOT
337no_of_species <- invert %>%
338   filter(class=="Insecta", is.na(legallyImported)) %>%
339   group_by(order) %>%
340   summarise(no.of.species=n())
341
342records <- invert %>%
343   filter(class=="Insecta", is.na(legallyImported)) %>%
344   group_by(order) %>%
345   filter(!is.na(boldRecords), boldRecords!=0) %>%
346   summarise(records=n())
347
348BINS <- invert %>%
349   filter(class=="Insecta", is.na(legallyImported)) %>%
350   group_by(order) %>%
351   filter(!is.na(BINS), BINS!=0) %>%
352   summarise(BINS=n())
353
354images <- invert %>%
355   filter(class=="Insecta", is.na(legallyImported)) %>%
356   group_by(order) %>%
357   filter(!is.na(boldImages), boldImages!=0) %>%
358   summarise(images=n())
359
360daSilvaBOLD <- data.frame(group = rep(records$Group,4),
361                          metric   = c(rep("species",9), rep("records",9), rep("BINS",9),
362rep("images",9)),
363                          frequency = c(no_of_species$no.of.species, records$records, BINS$BINS,
364images$images))
365daSilvaBOLD$metric = factor(daSilvaBOLD$metric, levels=c("species", "records", "BINS", "images"))
366
367ggbarplot(data=daSilvaBOLD, x="group", y="frequency", fill="metric",
368          position=position_dodge(0.7),
369          xlab=FALSE, ylab=FALSE,
370          order = c("Insects", "Other", "Arachnids", "Molluscs", "Annelids", "Platyhelminths",
371"Cnidarians", "Echinoderms", "Sponges")) +
372   theme(legend.title=element_blank()) +
373   rotate_x_text(angle = 45)
374
375#### HEMIPTERA FAMILY PLOT ####
376
377plotData <- invert %>%
378   filter(order=="Hemiptera", is.na(legallyImported)) %>%
379   group_by(family) %>%
380   summarise(n=n()) %>%
381   arrange(desc(n))
382
383## alien hemipteran family plot
384ggbarplot(data=plotData, x="family", y="n",

```

```

382         width=0.8, fill="black",
383         xlab="Family", ylab=FALSE) +
384     rotate_x_text(angle=45)
385
386## alien hemipteran family pie plot
387ggplot(plotData, aes(x="", y=n, fill=family)) +
388    geom_bar(stat="identity", width=1, color="white") +
389    coord_polar("y", start=0) +
390    theme_void()
391
392
393
394
395##### BOLD QUALITY ANALYSES #####
396
397## count number of BINs
398barcodeTable <- data.frame(group=c(rep("alien invertebrates",6),rep("biological control agents",6)),
399                             metric=rep(c("records", "SA records", "BINs", "SA BINs", "images", "SA
images"),2),
400                             frequency=0)
401
402## count alien bold records
403barcodeTable[1,3] <- invert %>%
404    filter(is.na(legallyImported), !is.na(boldRecords), boldRecords!=0) %>%
405    summarise(n=n())
406## count alien SA bold records
407barcodeTable[2,3] <- invert %>%
408    filter(is.na(legallyImported), !is.na(SAboldRecords), SAboldRecords!=0) %>%
409    summarise(n=n())
410## calculate international records
411barcodeTable[1,3] <- barcodeTable[1,3] - barcodeTable[2,3]
412## count alien BINs
413barcodeTable[3,3] <- invert %>%
414    filter(is.na(legallyImported), !is.na(BINs), BINs!=0) %>%
415    summarise(n=n())
416## count alien SA BINs
417barcodeTable[4,3] <- invert %>%
418    filter(is.na(legallyImported), !is.na(SABINs), SABINs!=0) %>%
419    summarise(n=n())
420## calculate international BINs
421barcodeTable[3,3] <- barcodeTable[3,3] - barcodeTable[4,3]
422## count alien images
423barcodeTable[5,3] <- invert %>%
424    filter(is.na(legallyImported), !is.na(boldImages), boldImages!=0) %>%
425    summarise(n=n())
426## count alien SA images
427barcodeTable[6,3] <- invert %>%
428    filter(is.na(legallyImported), !is.na(SAboldImages), SAboldImages!=0) %>%
429    summarise(n=n())
430## calculate international images
431barcodeTable[5,3] <- barcodeTable[5,3] - barcodeTable[6,3]
432
433## count BCA bold records
434barcodeTable[7,3] <- (invert %>%
435    filter(legallyImported=="biocontrol", !is.na(boldRecords), boldRecords!=0) %>%
436    summarise(n=n()))
437## count BCA SA bold records
438barcodeTable[8,3] <- invert %>%
439    filter(legallyImported=="biocontrol", !is.na(SAboldRecords), SAboldRecords!=0) %>%
440    summarise(n=n())
441## calculate international records
442barcodeTable[7,3] <- barcodeTable[7,3] - barcodeTable[8,3]
443## count BCA BINs
444barcodeTable[9,3] <- invert %>%
445    filter(legallyImported=="biocontrol", !is.na(BINs), BINs!=0) %>%
446    summarise(n=n())

```

```

447## count BCA SA BINs
448barcodeTable[10,3] <- invert %>%
449  filter(legallyImported=="biocontrol", !is.na(SABINs), SABINs!=0) %>%
450  summarise(n=n())
451## calculate international BINs
452barcodeTable[9,3] <- barcodeTable[9,3] - barcodeTable[10,3]
453## count BCA images
454barcodeTable[11,3] <- invert %>%
455  filter(legallyImported=="biocontrol", !is.na(boldImages), boldImages!=0) %>%
456  summarise(n=n())
457## count BCA SA images
458barcodeTable[12,3] <- invert %>%
459  filter(legallyImported=="biocontrol", !is.na(SAboldImages), SAboldImages!=0) %>%
460  summarise(n=n())
461## calculate international images
462barcodeTable[11,3] <- barcodeTable[11,3] - barcodeTable[12,3]
463
464barcodeTable <- barcodeTable %>%
465  mutate(metric=rep(c("records", "records", "BINs", "BINs", "images", "images"), 2),
  .before=metric) %>%
466  mutate(origin=rep(c("international", "local"), 6), .after=group)
467
468write.csv(barcodeTable, "C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien
Invertebrates\\barcodeTable.csv")
469
470## plot BOLD quality metrics
471ggbarplot(data=barcodeTable, x="metric_", y="frequency", fill="origin",
472  position=position_dodge(0.7), facet.by = "group",
473  xlab=FALSE, ylab=FALSE) +
474  scale_y_continuous(breaks=c(0, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600))
475  theme(legend.title=element_blank())
476
477## DA SILVA BOLD QUALITY METRICS PLOT
478no_of_species <- invertSdaSilvaAlien %>%
479  group_by(Group) %>%
480  summarise(no.of.species=n())
481
482records <- invertSdaSilvaAlien %>%
483  group_by(Group) %>%
484  filter(!is.na(boldRecords), boldRecords!=0) %>%
485  summarise(records=n())
486
487BINs <- invertSdaSilvaAlien %>%
488  group_by(Group) %>%
489  filter(!is.na(BINs), BINs!=0) %>%
490  summarise(BINs=n())
491
492images <- invertSdaSilvaAlien %>%
493  group_by(Group) %>%
494  filter(!is.na(boldImages), boldImages!=0) %>%
495  summarise(images=n()) %>%
496  add_row(Group = "Sponges", images=0)
497
498daSilvaBOLD <- data.frame(group = rep(records$Group,4),
499  metric = c(rep("species",9), rep("records",9), rep("BINs",9),
  rep("images",9)),
500  frequency = c(no_of_species$no.of.species, records$records, BINs$BINs,
  images$images))
501daSilvaBOLD$metric = factor(daSilvaBOLD$metric, levels=c("species", "records", "BINs", "images"))
502
503ggbarplot(data=daSilvaBOLD, x="group", y="frequency", fill="metric",
504  position=position_dodge(0.7),
505  xlab=FALSE, ylab=FALSE,
506  order = c("Insects", "Other", "Arachnids", "Molluscs", "Annelids", "Platyhelminths",
  "Cnidarians", "Echinoderms", "Sponges")) +
507  theme(legend.title=element_blank()) +

```

```

508 rotate_x_text(angle = 45)
509
510## INSECT ORDER BOLD QUALITYMETRICS PLOT
511
512no_of_species_IO <- invert %>%
513   filter(class=="Insecta") %>%
514   filter(is.na(legallyImported)) %>%
515   group_by(order) %>%
516   summarise(no.of.species=n())
517
518records_IO <- invert %>%
519   filter(class=="Insecta") %>%
520   filter(is.na(legallyImported)) %>%
521   group_by(order) %>%
522   filter(!is.na(boldRecords), boldRecords!=0) %>%
523   summarise(records=n())
524
525BINS_IO <- invert %>%
526   filter(class=="Insecta") %>%
527   filter(is.na(legallyImported)) %>%
528   group_by(order) %>%
529   filter(!is.na(BINS), BINS!=0) %>%
530   summarise(BINS=n())
531
532images_IO <- invert %>%
533   filter(class=="Insecta") %>%
534   filter(is.na(legallyImported)) %>%
535   group_by(order) %>%
536   filter(!is.na(boldImages), boldImages!=0) %>%
537   summarise(images=n())
538
539alienInsectsBOLD <- data.frame(group = c(no_of_species_IO$order, records_IO$order, BINS_IO$order,
images_IO$order),
540                               metric = c(rep("species",nrow(no_of_species_IO)),
rep("records",nrow(records_IO)), rep("BINS",nrow(BINS_IO)), rep("images",nrow(images_IO))),
541                               frequency = c(no_of_species_IO$no.of.species, records_IO$records,
BINS_IO$BINS, images_IO$images))
542alienInsectsBOLD$metric = factor(alienInsectsBOLD$metric, levels=c("species", "records", "BINS",
"images"))
543
544ggbarplot(data=alienInsectsBOLD, x="group", y="frequency", fill="metric",
545          position=position_dodge(0.7),
546          xlab=FALSE, ylab=FALSE,
547          order = c("Hemiptera", "Coleoptera", "Diptera", "Lepidoptera", "Hymenoptera",
"Thysanoptera", "Psocodea", "Blattodea", "Dermaptera", "Siphonaptera", "Zygentoma", "Phthiraptera",
"Orthoptera", "Embioptera", "Phasmida")) +
548  theme(legend.title=element_blank()) +
549  rotate_x_text(angle = 45) +
550  scale_y_continuous(breaks=seq(0,150,10))
551
552#### PROVINCIAL PLOT ####
553
554ECsp <- invert %>%
555   filter(str_detect(RangeBroadAdmin, "EC")|str_detect(provincialDistribution, "Eastern Cape")) %>%
556   group_by(Group) %>%
557   summarise(n=n())
558
559ECbold <- invert %>%
560   filter(str_detect(RangeBroadAdmin, "EC")|str_detect(provincialDistribution, "Eastern Cape")) %>%
561   filter(!is.na(boldRecords), boldRecords != 0) %>%
562   group_by(Group) %>%
563   summarise(records=n())
564
565EC <- left_join(ECsp, ECbold)
566
567NCsp <- invert %>%

```

```

568 filter(str_detect(RangeBroadAdmin, "NC")|str_detect(provincialDistribution, "Northern Cape"))
%>%
569 group_by(Group) %>%
570 summarise(n=n())
571
572 NCbold <- invert_sdaSilvaAlien %>%
573 filter(str_detect(RangeBroadAdmin, "NC")|str_detect(provincialDistribution, "Northern Cape"))
%>%
574 filter(!is.na(boldRecords), boldRecords != 0) %>%
575 group_by(Group) %>%
576 summarise(records=n())
577
578 NC <- left_join(NCsp, NCbold)
579
580 KZNsp <- invert_sdaSilvaAlien %>%
581 filter(str_detect(RangeBroadAdmin, "KZN")|str_detect(provincialDistribution, "KwaZulu-Natal"))
%>%
582 group_by(Group) %>%
583 summarise(n=n())
584
585 KZNbold <- invert_sdaSilvaAlien %>%
586 filter(str_detect(RangeBroadAdmin, "KZN")|str_detect(provincialDistribution, "KwaZulu-Natal"))
%>%
587 filter(!is.na(boldRecords), boldRecords != 0) %>%
588 group_by(Group) %>%
589 summarise(records=n())
590
591 KZN <- left_join(KZNsp, KZNbold)
592
593 GPsp <- invert_sdaSilvaAlien %>%
594 filter(str_detect(RangeBroadAdmin, "GP")|str_detect(provincialDistribution, "Gauteng")) %>%
595 group_by(Group) %>%
596 summarise(n=n())
597
598 GPbold <- invert_sdaSilvaAlien %>%
599 filter(str_detect(RangeBroadAdmin, "GP")|str_detect(provincialDistribution, "Gauteng")) %>%
600 filter(!is.na(boldRecords), boldRecords != 0) %>%
601 group_by(Group) %>%
602 summarise(records=n())
603
604 GP <- left_join(GPsp, GPbold)
605
606 FSsp <- invert_sdaSilvaAlien %>%
607 filter(str_detect(RangeBroadAdmin, "FS")|str_detect(provincialDistribution, "Free State")) %>%
608 group_by(Group) %>%
609 summarise(n=n())
610
611 FSbold <- invert_sdaSilvaAlien %>%
612 filter(str_detect(RangeBroadAdmin, "FS")|str_detect(provincialDistribution, "Free State")) %>%
613 filter(!is.na(boldRecords), boldRecords != 0) %>%
614 group_by(Group) %>%
615 summarise(records=n())
616
617 FS <- left_join(FSsp, FSbold)
618
619 MPsp <- invert_sdaSilvaAlien %>%
620 filter(str_detect(RangeBroadAdmin, "MP")|str_detect(provincialDistribution, "Mpumalanga")) %>%
621 group_by(Group) %>%
622 summarise(n=n())
623
624 MPbold <- invert_sdaSilvaAlien %>%
625 filter(str_detect(RangeBroadAdmin, "MP")|str_detect(provincialDistribution, "Mpumalanga")) %>%
626 filter(!is.na(boldRecords), boldRecords != 0) %>%
627 group_by(Group) %>%
628 summarise(records=n())
629

```

```

630MP <- left_join(MPsp, MPbold)
631
632WCsp <- invertstdaSilvaAlien %>%
633   filter(str_detect(RangeBroadAdmin, "WC")|str_detect(provincialDistribution, "Western Cape")) %>%
634   group_by(Group) %>%
635   summarise(n=n())
636
637WCbold <- invertstdaSilvaAlien %>%
638   filter(str_detect(RangeBroadAdmin, "WC")|str_detect(provincialDistribution, "Western Cape")) %>%
639   filter(!is.na(boldRecords), boldRecords != 0) %>%
640   group_by(Group) %>%
641   summarise(records=n())
642
643WC <- left_join(WCsp, WCbold)
644
645NWsp <- invertstdaSilvaAlien %>%
646   filter(str_detect(RangeBroadAdmin, "NW")|str_detect(provincialDistribution, "North West")) %>%
647   group_by(Group) %>%
648   summarise(n=n())
649
650NWbold <- invertstdaSilvaAlien %>%
651   filter(str_detect(RangeBroadAdmin, "NW")|str_detect(provincialDistribution, "North West")) %>%
652   filter(!is.na(boldRecords), boldRecords != 0) %>%
653   group_by(Group) %>%
654   summarise(records=n())
655
656NW <- left_join(NWsp, NWbold)
657
658LPsp <- invertstdaSilvaAlien %>%
659   filter(str_detect(RangeBroadAdmin, "LP")|str_detect(provincialDistribution, "Limpopo")) %>%
660   group_by(Group) %>%
661   summarise(n=n())
662
663LPbold <- invertstdaSilvaAlien %>%
664   filter(str_detect(RangeBroadAdmin, "LP")|str_detect(provincialDistribution, "Limpopo")) %>%
665   filter(!is.na(boldRecords), boldRecords != 0) %>%
666   group_by(Group) %>%
667   summarise(records=n())
668
669LP <- left_join(LPsp, LPbold)
670
671PEIsp <- invertstdaSilvaAlien %>%
672   filter(str_detect(RangeBroadAdmin, "PEI")|str_detect(provincialDistribution, "Prince Edward
Islands")) %>%
673   group_by(Group) %>%
674   summarise(n=n())
675
676PEIbold <- invertstdaSilvaAlien %>%
677   filter(str_detect(RangeBroadAdmin, "PEI")|str_detect(provincialDistribution, "Prince Edward
Islands")) %>%
678   filter(!is.na(boldRecords), boldRecords != 0) %>%
679   group_by(Group) %>%
680   summarise(records=n())
681
682PEI <- left_join(PEIsp, PEIbold)
683
684combined <- rbind(
685   transform(EC, Dataset="EC"),
686   transform(NC, Dataset="NC"),
687   transform(KZN, Dataset="KZN"),
688   transform(GP, Dataset="GP"),
689   transform(FS, Dataset="FS"),
690   transform(MP, Dataset="MP"),
691   transform(WC, Dataset="WC"),
692   transform(NW, Dataset="NW"),
693   transform(LP, Dataset="LP"),

```

```

694   transform(PEI, Dataset="PEI")
695 )
696
697 write.csv(combined, "C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien
Invertebrates\\provinceGroupTable.csv")
698
699 ## produce province table
700 provinceTable <- combined %>%
701   reframe(Province = Dataset, Group = Group, Species = n, Records = records) %>%
702   group_by(Province) %>%
703   arrange(desc(Species), .by_group = TRUE) %>%
704   group_by(Group, .add = TRUE)
705
706 write.csv(provinceTable, "C:\\Users\\15tri\\Documents\\Admin\\Masters\\R Scripts\\Barcoding Alien
Invertebrates\\provinceTable.csv")
707
708 ## plot ALL
709 ggplot(combined, aes(x="", y=n, fill=Group)) +
710   geom_bar(stat="identity", width=1, color="white", position="fill") +
711   coord_polar("y", start=0) +
712   theme_void() +
713   facet_wrap(~ Dataset, ncol = 3)
714
715 ## plot NC
716 ggplot(NC, aes(x="", y=n, fill=Group)) +
717   geom_bar(stat="identity", width=1, color="white") +
718   coord_polar("y", start=0) +
719   theme_void()
720
721 ## plot KZN
722 ggplot(KZN, aes(x="", y=n, fill=Group)) +
723   geom_bar(stat="identity", width=1, color="white") +
724   coord_polar("y", start=0) +
725   theme_void()
726
727 ## plot GP
728 ggplot(GP, aes(x="", y=n, fill=Group)) +
729   geom_bar(stat="identity", width=1, color="white") +
730   coord_polar("y", start=0) +
731   theme_void()
732
733 ## plot FS
734 ggplot(FS, aes(x="", y=n, fill=Group)) +
735   geom_bar(stat="identity", width=1, color="white") +
736   coord_polar("y", start=0) +
737   theme_void()
738
739 ## plot MP
740 ggplot(MP, aes(x="", y=n, fill=Group)) +
741   geom_bar(stat="identity", width=1, color="white") +
742   coord_polar("y", start=0) +
743   theme_void()
744
745 ## plot WC
746 ggplot(WC, aes(x="", y=n, fill=Group)) +
747   geom_bar(stat="identity", width=1, color="white") +
748   coord_polar("y", start=0) +
749   theme_void()
750
751 ## plot NW
752 ggplot(NW, aes(x="", y=n, fill=Group)) +
753   geom_bar(stat="identity", width=1, color="white") +
754   coord_polar("y", start=0) +
755   theme_void()
756
757 ## plot LP

```

```

758 ggplot(LP, aes(x="", y=n, fill=Group)) +
759   geom_bar(stat="identity", width=1, color="white") +
760   coord_polar("y", start=0) +
761   theme_void()
762
763 ## plot PEI
764 ggplot(PEI, aes(x="", y=n, fill=Group)) +
765   geom_bar(stat="identity", width=1, color="white") +
766   coord_polar("y", start=0) +
767   theme_void()
768
769 ## count number of species with unknown provincial distributions
770 noProvince <- invertSdaSilvaAlien %>%
771   filter(is.na(RangeBroadAdmin), is.na(provincialDistribution))

```

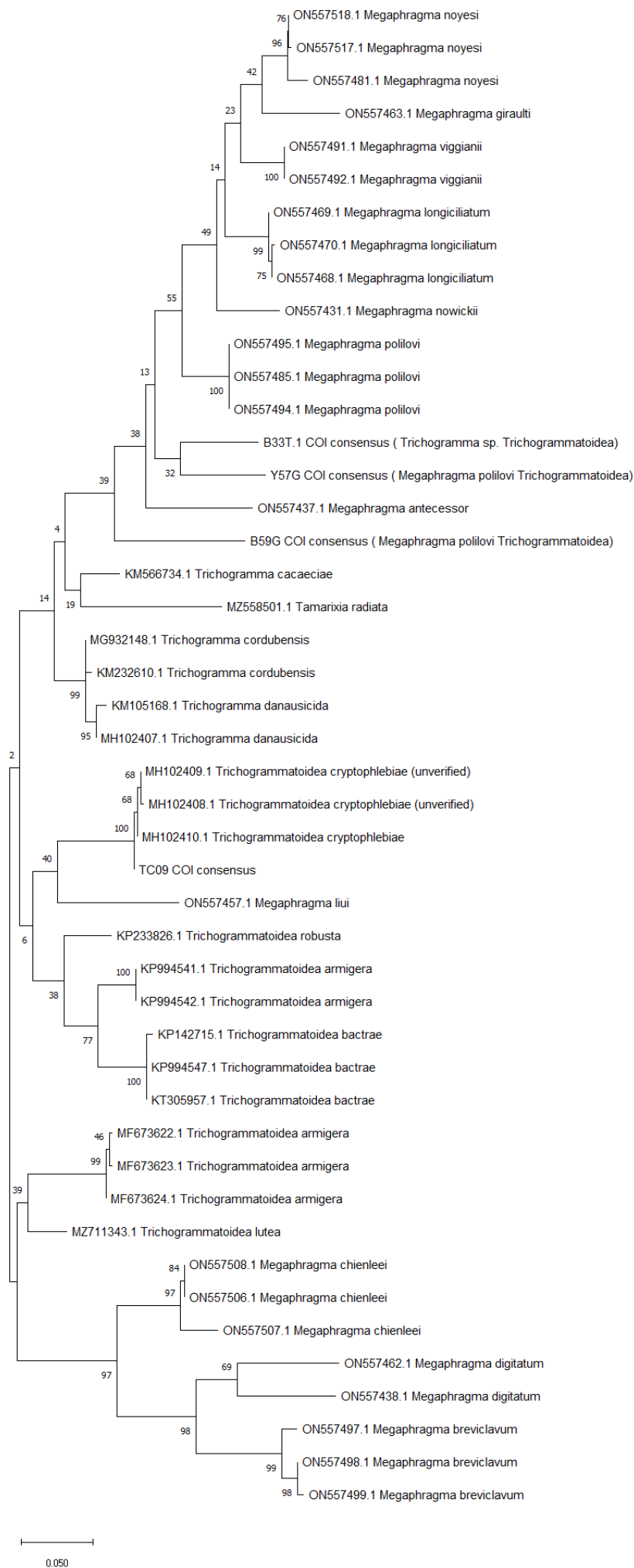


Figure S1. Maximum likelihood phylogeny with bootstrap values.

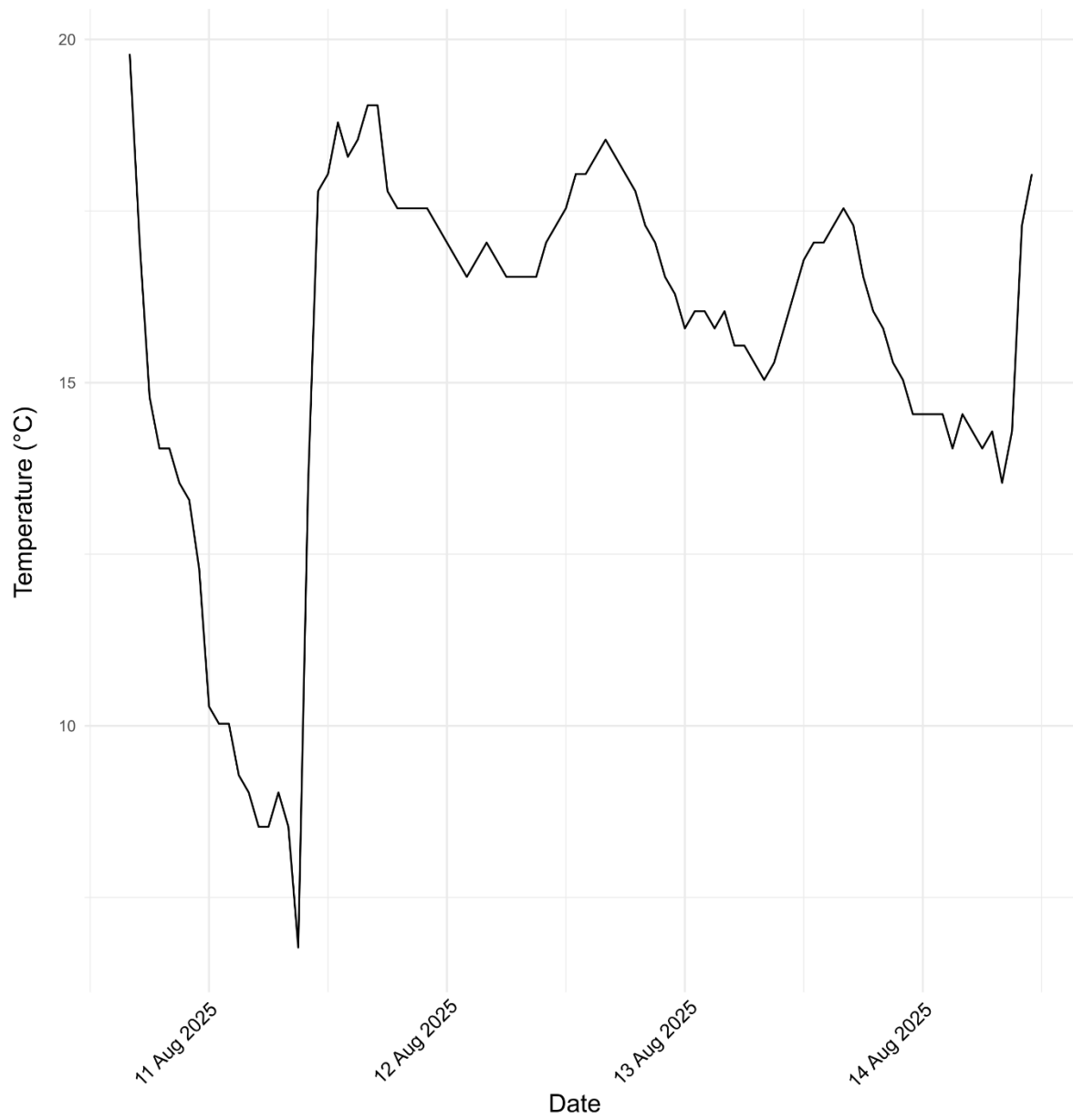


Figure S2. Temperature of cooler box used to transport *T. cryptophlebiae* eggs, where the first temperature data point is the moment of packing eggs at Vital Bugs (Tzaneen, Limpopo Province) and the last temperature data point is the moment of receipt at the University of Cape Town (Cape Town, Western Cape Province).