Diversity of stink bugs (Pentatomidae) and associated egg parasitoids in macadamia orchards in South Africa

by

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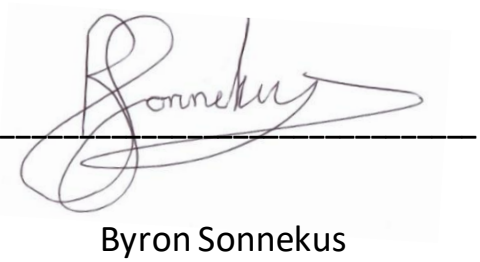
Declaration

I hereby declare that the dissertation, which I submit for the degree Magister Scientiae to the University of Pretoria, contains my own work and has not been submitted for any degree at any other University.



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Preface

Pentatomidae, commonly known as stink bugs, is an economically important and highly diverse insect group with around 4700 species described to date. Stink bugs feed via their piercing/sucking mouthparts (stylets) causing extensive damage on various fruit, vegetable, grain and nut crops worldwide leading to subsequent yield and economic loss. Pentatomidae display high diversity in morphology with variation in characteristics and the occurrence of colour morphs. Stink bug stylets differ considerably between species which determine the type of damage different stink bug species inflict in relation to host phenology.

Macadamia is one of the many nut crops that various stink bug species have been associated with in different growing regions globally. South Africa is currently the largest producer of macadamia worldwide, with KwaZulu-Natal, Limpopo and Mpumalanga serving as the three main production areas in the country. A complex of stink bug species including *Bathycoelia distincta* Distant, 1878, *Chinavia pallidoconspersa* (Stål, 1858), *Nezara viridula* (Linnaeus, 1758), *Parachinavia prunasis* (Dallas, 1851) and *Pseudatelus raptorius* Germar, 1838 are known to be present in macadamia orchards in South Africa. However, assemblage structure of stink bug species is known to fluctuate over time and the identities and potential damage of other stink bug species also present is currently unknown thus constant monitoring is required. There is currently a heightened search for alternative management strategies to control stink bug pest populations with the aim of reducing the reliance on broad-spectrum insecticides. The use of biological control agents is a promising option, however the identities of wasps (Hymenoptera: Scelionidae) known to be egg parasitoids in South Africa is currently limited.

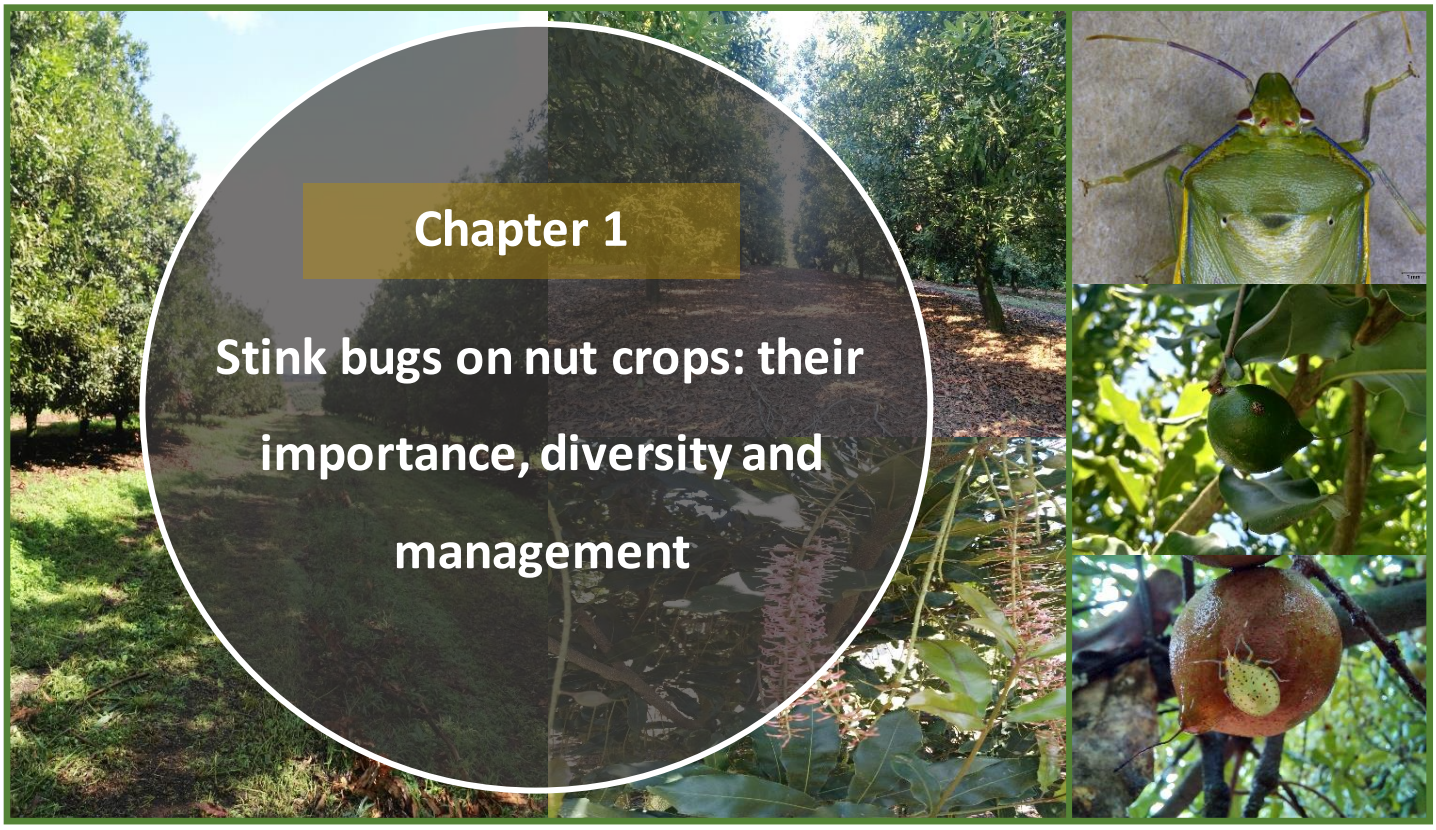
The work presented in this dissertation focused on pentatomid pests of macadamia, with an emphasis on species present in South Africa. Information on stink bug pests was synthesized, a survey of the stink bug species present in macadamia orchards between 2017 and 2020 in Limpopo and between 2019 and 2020 in KwaZulu-Natal, Limpopo and Mpumalanga was conducted, a DNA barcoding database linked to morphology (photographs) was developed for identification purposes, stink bug species presence was recorded over time and across

growing regions and a Pentatomidae phylogeny was constructed. Parasitized stink bug eggs were sampled from the Levubu growing region in Limpopo, parasitoids were identified and family-specific primers were tested against standard primers to determine the best molecular identification method.

Chapter 1 of this dissertation provides an overview of literature pertaining to Pentatomidae, with a focus on stink bugs associated with nut crops. Information such as the diversity, distribution, and damage of stink bug pests of nut crops as well as the impact of stink bugs on macadamia in South Africa is summarised. Current stink bug management strategies are considered with a specific focus on biological control.

Chapter 2 of this dissertation focuses on the identification stink bug pests in South African macadamia orchards based on the mitochondrial *cytochrome c oxidase subunit 1* (COI) gene linked to morphological identification and the development of a DNA barcoding database for future identifications. Sequence and morphological identification data were used to identify pentatomid species present from 2017-2020 in Limpopo and from 2019-2020 in KwaZulu-Natal, Limpopo and Mpumalanga. Sequence data was used to determine the phylogenetic relationships between these species and other closely related species and/or species previously associated with nut crops worldwide. Sequence divergence analysis was used to detect the presence of any cryptic stink bug species.

Chapter 3 of this dissertation aims to identify parasitoid wasps parasitising *B. distincta* eggs in the Levubu growing region of Limpopo. Mitochondrial COI sequence and morphological identification data was used to identify two native wasp species parasitizing and subsequently leading to mortality of *B. distincta* eggs. Phylogenetic relationships between these two species and well-studies scelionid wasps was determined. Finally, two molecular approaches (i.e., different primers for emerged and unhatched wasps) were assessed to determine the best identification method for native wasp parasitoids.



Chapter 1

Stink bugs on nut crops: their importance, diversity and management

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1.1. Introduction

Stink bugs (Hemiptera: Pentatomidae) are classified within the insect superfamily Pentatomoidea and suborder Heteroptera of the insect order Hemiptera. Pentatomidae is the largest and most diverse family of the superfamily, comprising of over 900 genera and approximately 4700 species (McPherson, 2018). Based on both molecular and morphological data (Grazia, Schuh & Wheeler, 2008), Pentatomidae is thought to be a monophyletic group of true bugs with nine recognised subfamilies (Grazia et al., 2008; Grazia & Schwertner, 2017). Pentatominae is considered the largest of the nine stink bug subfamilies within the Pentatomidae. This subfamily is estimated to contain around 600 genera and approximately 3000 species (Grazia et al., 2015). Pentatomidae display significant diversity in morphology with variation in characteristics such as the antennae, body (shape, size and texture), colour, genitalia, legs, wings and wing padding, and most importantly in their stylets. Stink bugs feed via their piercing/sucking mouthparts (stylets) and specific stylet segments have been found to play a role in the stylet penetration potential or the type of damage inflicted on certain crops (Esquivel, 2019).

Pentatomidae is regarded as the most important family in the Pentatomoidea as it contains the largest number of economically important pest species (Schuh & Slater, 1995; Schaefer & Panizzi, 2000). Since the economic importance of stink bug species is directly linked to damage, economic importance differs between stink bug species and the host plant species involved (Panizzi et al., 2000). Most stink bug species are phytophagous feeding on a wide variety of fruit, vegetable, grain, and nut crops, while also attacking numerous wild host plants, except for members of the predatory Asopinae subfamily. Stink bugs are known to follow food sources by shifting between plant hosts in relation to their peak production which poses more of a threat compared to other invasive pests that do not shift between hosts (McPherson & McPherson 2000). This was first observed for the cosmopolitan stink bug pest *Nezara viridula* (Linnaeus, 1758) (Todd, 1989). Another economically important species, *Halyomorpha halys* (Stål, 1855), has established as a major pest of a variety of crops in different non-native regions around the world (McPherson, 2018).

Stink bugs are important pests of nut crops such as almonds (*Prunus dulcis*, (Miller) D.A. Webb), hazelnuts (*Corylus* sp., Linnaeus), macadamia (*Macadamia integrifolia* Maiden & Betche and *Macadamia tetraphylla* L. Johnson), pecan (*Carya illinoinensis,* Koch) and pistachio (*Pistacia vera*, Linnaeus) in different growing regions globally (Table 1, Mitchell, Warner & Fukunaga, 1965; Daane *et al.,* 2005; Schoeman, 2013; Bosco, Moraglio & Tavella, 2018; Rijal & Gyawaly, 2018). Since 1917, *N. viridula* has been known to cause feeding damage on pecan and in 1965 the first damage on macadamia in plantings in Hawaii was reported (Turner, 1918; Mitchell et al., 1965). The stylet length of stink bug species is a limiting factor in the type of damage they inflict in relation to the development of the host nut crop, as observed in macadamia orchards (Schoeman, 2018; Bruwer et al., 2021). Feeding damage inflicted by stink bugs on nut crops differs as the season and nut development progresses, with multiple species that can cause damage on a single crop simultaneously or at different stages (Schoeman, 2018; Bruwer et al., 2021). Stink bug damage which typically results in early nut drop, malformed kernels, spotting or tissue necrosis subsequently leads to economic loss and therefore requires effective management.

The main control strategy employed thus far for stink bug management is pesticide application. The unsustainability, increase in the resistance of both target and non-target species and the decrease in insect biodiversity has led to the exploration and development of Integrated Pest Management (IPM) strategies for stink bug control. IPM can be defined as a decision-based process involving the coordinated use of multiple management strategies for optimizing the simultaneous control of all classes of pests in an ecologically and economically sound manner (Ehler, 2006; Prokopy, 2003). One of the main purposes of an IPM approach is the general reduction in pesticide application.

Biological control is a highly specific IPM strategy that has received a substantial amount of focus in terms of insect pest control and aims at reducing the pest population numbers. This involves the use of biological control agents such as natural predators and wasp egg parasitoids as well as entomopathogenic fungi and nematodes. Hymenopteran egg parasitoids (e.g., Scelionidae) and tachinid flies (Tachinidae) are the primary parasitoids of pentatomids and have been the focus of many biological control programs for stink bugs.

The aim of this review is to gain insight into management strategies employed to control stink bug pests, with a specific focus on the use of biological control agents. To gain insight into stink bug species associated with nut crops, their damage, distribution, economic impact and additional background information regarding stink bug species is provided.

1.2. Stink bugs (Pentatomidae) associated with nut crops and their impact on macadamia in South Africa

At least 19 stink bug species have been reported on different nut crops. These include some of the most agriculturally important and well-studied species such as *N. viridula, H. halys, Palomena prasina* (Linnaeus, 1761)*, Chinavia hilaris* (Say, 1832)*, Thyanta pallidovirens* (Stål, 1859)*, Chlorochroa uhleri* (Stål, 1872)*, Euschistus servus* (Say, 1832), *Euschistus tristigmus* (Say, 1832) and *Bathycoelia distincta* Distant, 1878 (Table 1), all in the Pentatominae. The stink bugs associated with nut crops are distributed throughout the Afrotropical, Indomalaya, Nearctic, Neotropic and Paleartic ecozones (Table 1). Some stink bug species are found in more than one of these ecozones and many stink bug species have been reported on multiple nut crop species. The proportion of molecular data (i.e., *cytochrome c oxidase subunit 1* gene sequences) available for these species is skewed towards well studied species (e.g., *N. viridula* and *H. halys*) and stink bugs found in North America (Table 1). The distribution, associated host plants and the damage inflicted on nut crops by well-studied stink bug species on nut crops is discussed below.

1.2.1. *Nezara viridula*

Nezara viridula (Figure 1A), otherwise commonly known as the southern green stink bug or green vegetable bug, is a cosmopolitan pest of numerous crops and non-cultivated plant species (Table 1, Todd, 1989). Due to the cosmopolitan distribution and economic importance, the species is well studied and is regarded as the 'model' species for invasive stink bugs. McPherson (2018) reviewed the cultivated and wild plants associated with *N. viridula* in North America. They found 43 plant families with 197 plant taxa in total, including 158 identified to species-level and 39 identified to genera, associated with this stink bug in North America. This polyphagous nature along with the increased global movement linked

with international trade has allowed this pest to be introduced and establish throughout the world. *Nezara viridula* is known to occur in tropical, subtropical and warm temperate regions of North and South America, Africa, Asia, Australia and Europe (McPherson & McPherson, 2000; Panizzi et al., 2000; McPherson, 2018), with the range of *N. viridula* still expanding.

Since the first report of green vegetable bug damage on nut crops in the USA (Turner, 1918), this pest has been reported to also cause damage to hazelnut and macadamia nut in various growing regions around the world (Table 1). In 1962, the first symptoms of *N. viridula* feeding damage was observed on macadamia nuts in Hawaii and has since become a major pest in this region (Mitchell et al., 1965; Jones & Caprio, 1992). Feeding damage was reported to result in abortion of small nuts and extensive reduction in quality due to necrosis in mature nuts, both in the tree canopy and on the ground (Jones & Caprio, 1994).

1.2.2. *Halyomorpha halys*

The brown marmorated stink bug (BMSB), *H. halys* (Figure 1B), is native to China, Japan, Korea and Taiwan (Hoebeke and Carter, 2003). With its broad range of more than 170 host plants, this stink bug species easily establishes as an agricultural pest in newly invaded areas. This is also because the BMSB has a strong dispersal capacity and reproductive output (Lee & Leskey, 2015; Leskey & Nielsen, 2018). This stink bug is thought to have been introduced into the USA through a single introduction from Beijing, China (Xu et al., 2014). In 1996, the first report of the BMSB was made from a region near Allentown, Pennsylvania and this bug has since become a major economic pest of numerous crops in the United States (Hoebeke and Carter, 2003). The BMSB has now spread to more than 40 states in the USA alone as well as in Chile, Canada, France, Russia, Italy and Switzerland among multiple other European countries (Rice et al., 2014; Bosco et al., 2018; Leskey & Nielsen, 2018).

The BMSB has been reported to feed on black walnut, hazelnut, pecan, pistachio and almond (Table 1). For example, the BMSB has become a threat to hazelnut plantations in newly invaded areas of Italy (Bosco et al., 2018). Stink bug damage on hazelnuts early in the season results in early nut drop or increased seed abortion while damage inflicted later in the season causes discolored spots, spongy tissue and depressions on the surface of the nut (Tavella et

al., 2001). When compared to the local hazelnut stink bug pest species in Turkey, namely *N. viridula* and *Palomena prasina*, it was found that *H. halys* was more harmful to the hazelnut crop (Bosco et al., 2018). In California, the BMSB has also been reported as an emerging pest of almonds with the first report of *H. halys* infestation and associated feeding damage emerging in the area in 2018 (Rijal & Gyawaly, 2018). *Halyomorpha halys* feeding damage on almond nut crops results in pinhole damage, internal and external gumming of the nuts as well as necrotic lesions (Rijal & Gyawaly, 2018). Additionally, pecan and pistachio have also been listed as known hosts of the BMSB with the same risk being posed on these nut crops (McPherson, 2018). The damage inflicted on pistachio nuts by *H. halys* results in the development of necrotic spots (Lara et al., 2017).

1.2.3. *Palomena prasina*

Palomena prasina, the green shield bug (Figure 1C), has a Palearctic distribution (Table 1). Suitable plant hosts of the genus *Palomena* Mulsant & Ray, 1866 include those of the Fabaceae and Solanaceae plant families (Grazia & Schwertner, 2017). In various parts of Europe, *P. prasina* is considered a minor pest of crops such as alfafa, apple, beans, pear, potato and hazelnut (Polajnar et al., 2013; Grazia & Schwertner, 2017). However, the green shield bug is a major pest of hazelnuts in Turkey which is considered the largest producer of hazelnuts globally. Additionally, *P. prasina* has also been reported as the dominant pest species present in Turkish hazelnut orchards (Erper et al., 2016; Bosco, Moraglio and Tavella, 2018). Damage associated with this species feeding on hazelnuts includes premature nut drop in the early season and extensive kernel damage towards the end of the hazelnut growing season.

1.2.4. *Chinavia hilaris, Thyanta pallidovirens* and *Chlorochroa uhleri*

The green stink bug, *Chinavia hilaris* (Figure 1D) (formerly known as *Acrosternum hilare*) is native to North America and known to have a Nearctic distribution (Panizzi et al., 2000; Grazia and Schwertner, 2017). The green stink bug is considered one of many agriculturally important stink bug species in the United States. This species of stink bug causes damage to several crops such as cotton, tomato, peach and soybean in the USA (Kamminga et al., 2012).

In terms of nut crops, the green stink bug has been associated with pecan and pistachio crops (Yates, Tedders & Sparks, 1991; Daane et al., 2005; Grazia & Schwertner, 2017). Characteristic early stink bug damage on pecan nuts results in the blackening of immature pecan nuts, commonly known as black pit, followed by premature nut drop (Yates et al., 1991). Similarly, the damage inflicted by *C. hilaris* feeding on pistachios early in the season results in the formation of epicarp lesions and subsequent premature nut drop (Uyemoto et al., 1986; Daane et al., 2005).

Thyanta pallidovirens and *Chlorochroa uhleri*, along with *C. hilaris*, have been reported to attack pistachios in California (Daane et al., 2005). Both *T. pallidovirens* and *C. uhleri* are also known to have a Nearctic distribution (Table 1; McPherson & McPherson, 2000; Daane et al., 2005). Like the damage caused by *C. hilaris*, the damage inflicted by *T. pallidovirens* and *C. uhleri* results in pistachio nut necrosis. *Thyanta pallidovirens* and *C. uhleri* are commonly known as the red shouldered stink bug and Uhler's stink bug, respectively. Importantly, the red shouldered stink bug should not be confused with the red banded stink bug, *Piezodorus guildinii* (Westwood, 1837). The red banded stink bug is reported to have a Neotropical distribution (Grazia & Schwertner, 2017). The extensive host range of *P. guildinii* was recently reviewed and it consists of about 75 plant species (McPherson, 2018). Despite the wide host range of this stink bug species, it has not yet been reported as a pest of nut crops. However, this stink bug species is a well-known major pest of soybean in South America, as well as other regions where this crop is cultivated (McPherson & McPherson, 2000). Hence, this species is well-studied and shows potential to become a pest of nut crops if introduced into the specific nut crop growing regions.

1.2.5. *Euschistus servus* and *Euschistus tristigmus*

The Nearctic brown stink bug, *Euschistus servus* (Figure 1E, Table 1), is considered the most economically important *Euschistus* pest species in North America when compared to other species such as *E. tristigmus,* commonly known as the dusky stink bug. Plants of the Fabaceae plant family, more specifically legumes, have been reported as the preferred host plants of stink bug species within the genus *Euschistus* (Grazia & Schwertner, 2017). *Euschistus servus* is known to inflict damage on a variety of crops including soybean, corn, cotton, alfalfa,

sorghum, tobacco, apples and other fruit (Panizzi et al., 2000). In terms of nut crops, both *E. servus* and *E. tristigmus* are known to feed on pecan. The feeding damage inflicted by these two species has been reported to cause (premature) fruit drop in pecan orchards in the United States (Panizzi et al., 2000; Grazia & Schwertner, 2017).

1.2.6. *Bathycoelia distincta* and the stink bug complex in South African macadamia orchards

Stink bugs are regarded as the most economically important pest of macadamia in South Africa. Contributing factors of unsound kernel recovery include early and late stink bug damage, pre-germination, immature nut drop, nut spotting, mould, and discolouration. Unsound kernel recovery percentage differs significantly between different growing regions and different growing seasons. Overall, stink bug damage was responsible for 50 % of the damaged kernel in 2020. Stink bug damage was the largest contributing factor to monetary loss in 2019 and 2020 resulting in losses of R 130 million and R 180 million, respectively (SAMAC, 2020a).

There is a complex of stink bug species that are causing significant damage in macadamia orchards in South Africa (van den Berg et al., 1999; Schoeman 2013, 2018). *Bathycoelia distincta*, the two spotted stink bug, is thought to be the most dominant species and contributing towards majority of the late season damage observed in macadamia orchards in South Africa (Schoeman 2013, 2020; Bruwer et al., 2021).The two spotted stink bug is native to Southern Africa and has an Afrotropical distribution (Figure 1F, Table 1). The complex of species on macadamia in South Africa includes other species such as *Chinavia pallidoconspersa* (Stål, 1858) (yellow-edged stink bug, previously *Nezara pallidoconspersa*), *N. viridula*, *Parachinavia prunasis* (Dallas, 1851) (previously *Acrosternum prunasis* and *Nezara prunasis*) and *Pseudatelus raptorius* Germar, 1838 (powdery stink bug, previously *Atelocera raptorial*) (Table 1; van den Berg et al., 1999; Schoeman, 2018). Of these, *B. distincta* and *P. raptorius* are the only stink bug species that have been reported to reproduce in macadamias in South Africa (Schoeman, 2018).

Schoeman (2018) assessed the relative seasonal abundance of stink bugs in macadamia orchards in South Africa and correlated abundance levels of stink bug species to the

phenological development of macadamia trees. He found that *B. distincta* and *P. raptorius* occurred throughout all the phenological stages, but that the abundance of *B. distincta* peaks in the summer months when mature/developed nuts are available and when oil accumulation in macadamia nuts has peaked. The abundance levels of *P. raptorius* were constantly high throughout the season. *Bathycoelia distincta* abundance levels decrease along with the availability of nuts over winter months. During this time the abundance levels of the other stink bug species mentioned previously, known as the winter complex, increase.

Relative seasonal abundance of stink bug species can be correlated to the type of stink bug damage observed in South African macadamia orchards (Schoeman, 2018). Early stink bug damage is thought to be caused by *B. distincta* and the winter complex of stink bug species (Schoeman, 2013). This damage occurs early in the season, due to the short mouthparts of the winter complex, prior to the development of a thick husk and shell (Schoeman, 2009; Bruwer et al., 2021). Early stink bug damage results in pitting marks present along the equator of the nuts (Schoeman, 2009). Late stink bug damage is thought to be caused by *B. distincta.* The species has a lengthier stylet in comparison to the other species, enabling this species to penetrate the thick husk and shell which develops as the season progresses (Schoeman, 2013; Bruwer et al., 2021). Characteristics of late stink bug damage are an oily kernel with a semi-transparent appearance and the presence of white feeding marks (Schoeman, 2009). A recent feeding damage trial performed by Schoeman (2020), on the most dominant cultivar in South Africa (Beaumont), confirmed that *C. pallidoconspersa* can also cause significant damage to macadamia nuts in the late season. This suggests that other species may also be involved in late damage, despite having shorter stylets compared to *B. distincta.*

1.3. Biological control of stink bugs

The significant damage caused by stink bug species associated with a range of nut crops emphasizes the need for effective strategies to manage these pests. Biological control involves the release of natural parasites or predators to control (reduce) pest populations and forms an integral part of an IPM approach. Various natural enemies of stink bugs are used in biological control programmes. These include hymenopteran egg parasitoids (e.g, Scelionidae), tachinid flies (Tachinidae) as well as entomopathogenic fungi and nematodes.

There are different approaches of biological control that are considered when attempting to manage pest species populations through natural enemy introductions. Classical biological control aims at controlling an exotic pest by introducing natural enemies of a pest, generally from the place of origin of the pest, into a location in which they do not occur naturally (van Lenteren et al., 2006; Hajek & Eilenberg, 2018). For example, the importation and subsequent release of parasitoids to control *N. viridula* has been implemented in most areas where this pest occurs, including New Zealand (Cumber, 1951), Australia (Wilson et al., 1960; Coombs and Sands, 2000), Hawaii and Taiwan (Su & Tseng, 1984). Augmentative biological control is a supplemental strategy that involves the frequent mass release of natural enemies that are either native or non-native to a specific area (van Lenteren et al., 2006; van Lenteren, 2012). An example includes augmentative releases of *Anastatus* sp. Motschulsky, 1859 (Eupelmidae) (non-native) against *H. halys* (native) in peach orchards in the Beijing Province of China, reportedly resulting in parasitism levels exceeding 60 % (Hou et al., 2009).

Another biological approach does not rely on the introduction of natural enemies but focuses on enhancing the effect of natural enemies already present. Conservation biological control aims to complement the management of pests by implementing strategies that support and enhance the populations of natural enemies present in the environment (Gurr, Wratten & Barbosa, 2000; Landis, Wratten & Gurr, 2000). For instance, the presence of buckwheat as a trap crop has been shown to both attract and increase fecundity in *Trissolcus basalis* (Wollaston, 1858) (Scelionidae) populations used to control *N. viridula* (Foti et al., 2017).

1.3.1. Wasp egg parasitoids

The genera *Trissolcus* Ashmead, 1893 and *Telenomus* Haliday, 1833 (Figure 2), within the family Scelionidae (Hymenoptera), are considered the most important egg parasitoids worldwide. In terms of stink bug management, egg parasitoids of the genus *Trissolcus* have been used as biocontrol agents of stink bugs in the *Bathycoelia* Amyot & Serville, 1843; *Brachynema* (Mulsant & Ray, 1852); *Chinavia* Orian, 1965*; Euschistus* Dallas*,* 1851; *Halyomorpha* Mayr, 1864 and *Nezara* Amyot & Serville, 1843 genera (Table 1). While those within the genus *Telenomus* have been used to control *P. guildinii*, *C. hilaris*, *P. prasina*, *Chlorochroa* sp. Stål, 1871 and *Euschistus* sp. Dallas, 1851 (Table 1). Several egg parasitoids have been reported for *H. halys* from its native range and these typically belong to the genera *Anastatus, Acroclisoides* Girault & Dodd*,* 1915; *Ooencyrtus* Ashmead*,* 1893; *Telenomus* and *Trissolcus* (Qiu, 2007). According to studies conducted in the invaded range of the United States, at least eight different species of egg parasitoids were found emerging from wild and sentinel BMSB egg masses (Jones et al., 2014; Cornelius et al., 2016). Hyperparasitoids have also been reported for natural enemies of stink bugs. In Australia, two species of *Acroclisoides* (Pteromalidae) have been reported to attack eggs previously parasitized by *T. basalis* (Clarke & Seymour, 1992). More recently, in Europe it has been shown that *Ooencyrtus telenomicida* (Vassiliev, 1904) (Encyrtidae) attacks eggs previously parasitized by the same *Trissolcus* sp. (Cusumano et al., 2013).

1.3.1.1. Trissolcus species

In 1983, *T. basalis* (Figure 2A) was imported from Hawaii and introduced into Taiwan to act as a biocontrol agent against *N. viridula* (Su & Tseng, 1984). The study reported high parasitism levels in the field, but there is no follow-up report available on the success of this biological control attempt. Following the introduction in Taiwan, the further spread of *T. basalis* to other non-native regions is evident as this wasp was recently reported to also be present in Japan (Mita et al., 2015).

In 1985, an augmentation biological control program involving *T. basalis* was initiated and implemented over four consecutive seasons in Brazil. The aim of the program in Brazil was to

develop and release mass-reared *T. basalis* to control *N. viridula* as well as *P. guildinii* and *E. heros* on soybean utilized as a trap crop (Correa-Ferreira, 1993; Corrêa-Ferreira & Moscardi, 1996; Correa-Ferreira & Panizzi, 1999). *Trissolcus basalis* reduced the average stink bug population by more than 50% within the soybean trap crop (Corrêa-Ferreira & Moscardi, 1996). The results of the study highlighted the importance of augmentative biocontrol for the control of stink bugs in soybean trap crops. Unfortunately, due to changes in farming practices and the increased use of insecticides to control these pests in Brazil, the program was terminated. Stemming from the success of using *T. basalis* to control *N. viridula*, further research led to the mass release of this egg parasitoid in other regions, for example in greenhouses in Almeria located in Spain (Cantón-Ramos & Callejón-Ferre, 2010).

The predominant natural enemy of *H. halys* is the egg parasitoid *Trissolcus japonicus* (Ashmead, 1904) (Yang et al., 2009). Collection of egg parasitoids of *H. halys* in China, Japan and South Korea, to search for biological control agents to introduce to North America, showed that at least five different *Trissolcus* species attack the BSMB in its native range of Asia, with the predominant species, *T. japonicus*, producing rates of parasitism of around 70 % in China (Yang et al., 2009; Lee et al., 2013). Talamas et al. (2015) reported the presence of natural populations of *T. japonicus* in wooded habitats in the region of Maryland, USA, suggesting an accidental introduction into the country. Molecular analyses proved these wasps to be genetically distinct from the *T. japonicus* populations held in quarantine facilities (Talamas et al., 2015; Milnes et al., 2016).

1.3.1.2. Telenomus species

The wasp parasitoid *Telenomus podisi* Ashmead, 1893 (Figure 2B) has been reported to parasitize the eggs of multiple stink bugs species, including *P. guildinii, N. viridula, E. heros* and *C. hilaris*, mostly in the Neotropical region (Table 1). In Brazil, *Te. podisi* was reported to parasitize more than 50 % of the egg masses of *P. guildinii* and *N. viridula* on soybean (Correa-Ferreira, 1994). Additionally, *Te. podisi* has previously been found parasitizing *P. guildinii* egg masses in Uruguay and Argentina (Cingolani, Greco & Liljesthröm, 2014; McPherson, 2018). The eggs of the green stink bug, *C. hilaris*, have reportedly been parasitized by two different *Telenomus* sp. in North America. In Virginia, this stink bug species is also attacked by *Te. podisi*

while in Louisiana it is attacked by *Telenomus cristatus* Johnson, 1984 (Orr et al., 1986; Koppel et al., 2009; Kamminga et al., 2012). *Telenomus ashmeadi* Morrill, 1907 has been reported as an egg parasitoid of both *Chlorochroa ligata* (Say, 1832) and *Chlorochroa uhleri* in North America (Table 1; Mehrnejad, Linnavuori & Alavi, 2013). In Europe, the eggs of *P. prasina* are reportedly parasitized by *Telenomus chloropus* Thomson, 1861 (Table 1).

1.3.1.3. *Anastatus bifasciatus*

The generalist egg parasitoid *Anastatus bifasciatus* (Geoffroy, 1785) (Eupelmidae) is native to Europe and has been used in biological control programs around the world with well-known examples occurring in its non-native ranges of Australia and China (Hou et al., 2009; Govender, 2015) . The high levels of parasitism observed in Beijing, China (Hou et al., 2009) has led to further exploration of utilizing *A. bifasciatus* as a biological control agent for the exotic BMSB in Europe (Stahl et al., 2018). This parasitoid's host range includes more than 50 different insect species (Stahl et al., 2018). This raises concern on how unpredictable non-target effects can be due to the broad host range of this egg parasitoid and emphasizes the need for further field tests.

Anastatus bifasciatus is the most prevalent native egg parasitoid with the capability of parasitizing *H. halys* eggs in both Switzerland and Italy. Initiated in both of these countries in 2016, the level of parasitism of *A. bifasciatus* on *H. halys* in the field was assessed in fruit orchards through an augmentative biocontrol approach conducted over three years (Stahl et al., 2019). The post-release monitoring of this study found that experimental releases of *A. bifastiacus* females resulted in moderate parasitism on *H. halys* in all the release sites. This parasitoid obtained better parasitism than a different European egg parasitoid, *O. telenomicida*, which only obtained parasitism in half of the release sites (Federico et al., 2017). Non-target parasitism was reported at all release sites with *A. bifasciatus.* Further research is required to fully understand the parasitism of *A. bifasciatus* on *H. halys* as well as possible non target effects in newly introduced environments (Stahl et al., 2019).

1.3.2. Tachinid flies

Stink bugs are frequently attacked by tachinid flies, including multiple species of which the majority are in the subfamily Phasiinae (McPherson, 2018). Tachinid flies are known to deposit their eggs on the surfaces of adult stink bugs, the eggs subsequently hatch and penetrate the adult stink bugs, leading to death. There has been speculation that the flies recognize the stink bugs as hosts but there is the possibility that stink bugs are physiologically unsuitable hosts. This is supported by the low rate of successful development and emergence rates of these flies from parasitized stink bug adults (Rice et al., 2014).

The feather-legged fly, *Trichopoda pennipes* (Diptera: Tachinidae) (Fabricius, 1781), was reported to have been used in an attempt to control *N. viridula* in South Africa (van den Berg & Greenland, 1996). This parasitoid fly species was reportedly introduced from Italy into South Africa in 1993. Following this introduction, it was reported that two separate releases of *Tr. pennipes* occurred in 1994 (van den Berg & Greenland, 1996). Adult stink bugs were collected in the beginning of 1995 and a third of these were found to be parasitized by *Tr. pennipes* (van den Berg & Greenland, 1996). There is no evidence for the establishment of this parasitoid and the effort was deemed unsuccessful. An attempt was made to use the tachinid fly species *Trichopoda giacomellii* (Diptera: Tachinidae) (Blanchard, 1966) imported from Australia to control stink bugs in South Africa (van den Berg, 1997). No follow up study was conducted to test the establishment and effectiveness of the biological control agent release.

1.3.3. Entomopathogenic fungi

Numerous entomopathogens have been reported from stink bugs and certain fungi have shown specificity to certain Pentatomidae species and could therefore be considered as potential biocontrol agents (Seiter, 2014). Laboratory and field studies to test the efficacy of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* (Hypocreales: Cordycipitaceae) (Pike, 2014) against *N. viridula*, *E. heros* (brown stink bug) and *P. guildinii* (Sosa-Gómez & Moscardi, 1998) found that *M. anisopliae* caused higher disease incidence compared *to B. bassiana*. However, despite causing morality in *N. viridula* and *P. guildinii*, the

rate of morality was not high enough for this to be considered an effective control strategy for stink bugs (Sosa-Gómez & Moscardi, 1998). Similarly, *M. anisopliae* was more effective than *B. bassiana* in controlling *N. viridula* in Egypt (Abdel-Raheem, Ragab & Abdel-Rhman, 2011), however, in that study both fungal species had a significant effect on the mortality *of N. viridula* and were thus deemed as promising biological control agents.

Only a few studies have focused on the use of fungal pathogens for control of the BMSB. In 2012, laboratory trials involving a strain of the entomopathogenic fungus *B. bassiana*, the active ingredient in Botanigard®, displayed strong efficacy against BMSB adults (Gouli et al., 2012). This study showed promise for the use of Botanigard® to control adult bugs but emphasized the need for research in controlling the other life stages of *H. halys*. A lower efficacy of strains of *M. anisopliae* against the BMSB was reported, possibly due to the release of defensive compounds released by the stink bugs (Pike, 2014). At low concentration these defense compounds, namely trans-2-octenal and trans-2-decenal, are known to prevent spore germination, as well as prevent fungal growth.

The efficacy of eight different isolates of fungi were lab tested in Turkey against the green shield bug, *P. prasina*, the predominant species causing damage in hazelnut orchards in this country (Erper et al., 2016). The fungal species used in this study included *Simplicillium lamellicola* (Cordycipitaceae), *Lecanicillium mauscarium* (Cordycipitaceae), *B. bassiana* and *Isaria fumosorosea* (Cordycipitaceae). All the isolates showed pathogenicity towards *P. prasina*. However, an isolate of *L. mauscarium* showed the highest rate of mortality with *B. bassiana* being the second most effective (Erper et al., 2016).

1.3.4. Entomopathogenic nematodes

Currently, the information available on the use of entomopathogenic nematodes to control stink bugs is very limited. Nematodes were found to be associated with several pentatomid species such as *P. guidlinii*, the red banded stink bug, and *Megacopta cribraria* (Hemiptera: Plataspidae) (Fabricius, 1798), the kudzu bug. However, these nematodes were not found to account for any significant mortality. The first report of a mermithid nematode, identified as a *Hexamermis* sp. Steiner, 1924 (Nematoda: Mermithidae) infecting *C. hilaris* worldwide, was

from the United States (Kamminga et al., 2012). The same nematode was, also for reported the first time to infect *P. guildinii* in the United States. The efficacy of three different species of entomopathogenic nematodes, from the genus *Steinernema* Travassos, 1927 (Rhabditida: Steinernematidae), was tested against *N. virirdula* (Pervez, Ali & Ahmad, 2008). The results were promising with all three species shown to be pathogenic to *N. viridula.* Field tests are required to evaluate the efficacy of these nematodes against stink bugs in the natural environment.

1.3.5. Predators

Predators could play an important role in controlling invasive stink bug species that may lack specialist natural enemies in the introduced area. Predaceous stink bugs within the Asopinae subfamily have been reported as predators of invasive stink bugs (McPherson, 2018). Predatory insects with chewing and sucking mouthparts have been reported to feed on various life stages of the BMSB in the United States (Biddinger et al., 2012; Morrison III, Mathews & Leskey, 2016). These insects were found to be members of at least 14 different families, including Pentatomidae. Predators that have been reported killing *H. halys* include *Orius* sp. Wolff, 1811 (Anthocoridae); *Arma chinensis* (Fallou, 1881) (Pentatomidae) and *Misumena tricuspidata* (Fabricius, 1775) (Thomisidae) (Qiu, 2007). Furthermore, ground beetles (Carabidae), earwigs (Forficulidae), jumping spiders (Salticidae) and crickets (Gryllidae) have also been reported to predate on BMSB eggs (Morrison III, Matthews & Leskey, 2016; Rice et al., 2014). Two species of ants, *Monomorium floricola* (Jerdon, 1851) (Formicidae) and *Pheidole megcephala* (Fabricius, 1793) (Formicidae) are known to feed on stink bug masses in trees and on the ground respectively (Jones, 1995). However, since most of these predators are generalists not many studies have been conducted to determine the effectiveness of certain predator species as biological control agents. Parasitoids have been found to follow their host insects to novel host plants, but generalist predators are much less likely to do so (Grosman et al., 2017). Additionally, birds (Exnerova, 2003) and bats (Galorio and Nuneza, 2014) have also been reported to feed on stink bugs including economically important species.

1.4. Other stink bug management practices

There are numerous management strategies used to control pest insects in agriculture, in addition to biological control. These include chemical control, behavioural interference control, cultural control and reproductive control (McPherson, 2018). The best approach towards effectively and sustainably managing stink bugs includes the incorporation of multiple different control strategies as part of an IPM approach and are discussed below.

1.4.1. Chemical control

Chemical control entails the use of pesticides, natural or synthetic, to reduce pest related damage. To date, the application of insecticides to control stink bugs has been the most successful and widely implemented strategy in most parts of the world, including South Africa (Schoeman, 2014a; Erper et al., 2016). Out of more than 100 insecticides registered in 2020 for insect pest control in macadamia orchards in South Africa, there are around 50 different registered insecticides for the control of stink bugs (www.samac.org). Despite the widespread use of pesticides, there are currently no chemicals available for the selective control of stink bugs. Farmers thus rely on broad spectrum insecticides such as carbamates, organophosphates, pyrethroids and neonicotinoids for stink bug control (Lee et al., 2013; Rice et al., 2014; McPherson, 2018). Farmers typically alternate the application of different insecticides according to their Insecticide Resistance Action Committee (IRAC) grouping which is based on pesticide's mode of action. The number of insecticide sprays per season is linked to stink bug numbers (i.e., abundance) in the field. Application of these sprays require careful consideration since the cost involved could range from R 4 000 to R 12 000 per hectare.

The continuous use of broad-spectrum pesticides can have various negative effects such as reduced efficacy, non-target effects on other insect species, a build-up of resistance in the pest population among other associated problems. Some species of stink bugs such as *P. guidlinii*, for example, are less sensitive to these pesticides and growers are forced to apply pesticides more frequently (Temple et al., 2013). In addition, the treatment with registered chemicals for stink bugs in the USA was ineffective against the newly introduced stink bug

species, *H. halys,* in fruit tree orchards in the mid-Atlantic region of the US in 2010 (Leskey et al., 2012). This failure in initial control was later supported by evidence of knockdown and recovery by *H. halys* when treated with certain pyrethroids and neonicotinoids (Leskey et al., 2012). Overall, these findings suggest the build-up of resistance in both target and non-target species due to the overuse of broad-spectrum insecticides. The extensive use of broad-spectrum insecticides also results in the occurrence of secondary pests such as thrips, mealy bugs and scale insects, currently of major concern in South Africa (Schoeman, 2018). This was also the situation in fruit tree orchards during the mid-Atlantic outbreak (Leskey et al., 2012).

1.4.2. Behavioural interference control

Behavioural interference control involves alterations to the environment through physical means to create an undesirable habitat or induce a specific behavioural response in insect pests. This can include the use of electricity, sound waves or vibratory signals, temperature and physical barriers (Banks, 1976; McPherson, 2018). Electric traps that lure and kill insects are commonly used to control flying insects, however these traps are non-selective and are ineffective in controlling targeted stink bug species. Temperature is known to play a vital role in insect physiology, development and biology, however manipulating temperature to control pest insects is better suited for pests of stored foods and not field crops (Fields, 1992).

Vibratory communication forms a vital part of stink bug reproductive behavior and substrate vibrations can be exploited to control stink bugs. This can be accomplished by using these substrate vibrations for stink bug monitoring or mating disruption (Grazia & Schwertner, 2017). In terms of monitoring, these vibratory signals can be used to attract a target pest species or the signals can be detected through a wide variety of sensors to confirm the presence of stink bugs (Grazia & Schwertner, 2017).

1.4.3. Cultural control

Cultural control can be defined as agricultural practices implemented to reduce insect populations by making environmental conditions unfavourable for pest establishment, subsequently decreasing insect-related injury to crops. Examples of general cultural control

methods include crop rotation, sanitation, tillage, plant density, row spacing, and trap and cover cropping (McPherson 2018). In terms of stink bug management in nut crops some of these strategies might not be possible as they are perennial crops.

Pruning of macadamia trees is recommended to reduce density and to improve pesticide spraying efficacy, air movement and increased sunlight. In terms of canopy management, there is a direct link between stinkbug prevalence and plant density. A study performed on the dispersal and distribution of stink bugs in macadamia orchards in South Africa found two important correlations between plant/tree density and stink bug incidence (Schoeman, 2014a). Firstly, there is a positive correlation between the percentage of kernel damage and tree density and secondly between the presence of *B. distincta* egg packets and tree density. The height of the trees was also reported to play a significant role in stink bug incidence in macadamia orchards with damage significantly increased towards the higher parts of the trees (Waite et al., 2000; Schoeman, 2014a). This can be linked to a limiting factor being that commercial chemical sprayers can only reach a height of about six meters. Overall, this emphasizes the fact that both pruning and tree height management should be considered as an important part of an IPM approach for the long-term control of stink bugs (Schoeman, 2014a, 2014b).

Another important cultural control strategy is the use of cover and trap crops which can be very useful as part of an integrated pest management approach. Trap crop management relies on the use of plant species that either attract or repel the pest of interest. Usually the trap crop is planted early in the growing season on a small portion of land to lure the pest away from the main crop (Hokkanen, 1991; Shelton & Badenes-Perez, 2006). Cover cropping aims at benefiting the agrosystem by improving soil quality, decreasing soil erosion, suppressing weed competition and improving natural enemy populations (Tillman et al., 2004). Trap crops also assist in recruiting beneficial insects and natural enemies due to an increase in the agrosystem biodiversity, therefore both methods link to conservation biological control.

Several researchers have demonstrated the effective management of stink bugs by implementing a trap crop strategy. In New Zealand, for example, it was shown that three different crop types can serve as effective trap crops for *N. viridula* present in organic maize

crops (Rea et al., 2002). These crops included white mustard (*Sinapsis alba*), pea (*Psium sativum*) and black mustard (*Brassica nigra*) (Rea et al., 2002). The use of trap crop management strategies to control stink bug species in macadamia orchards has been investigated in South Africa (Radzilani et al., 2012). This study included the evaluation of four different trap crops including rattle pod (*Crotalaria capensis*), sunnhemp (*Crotalaria juncea*), sunflower (*Helianthus annuus*) and cowpea (*Vigna unguiculate*). Amongst several unidentified pentatomids, *N. viridula, B. distincta* and *C. pallidoconspersa* were observed on all the crop varieties being assessed. Of the four different crops, it was found that sunflower was the most attractive trap crop for the stink bugs mentioned above. Stink bug populations were observed to increase in accordance with the development of the crops progressing towards flowering and fruit maturity. This highlights the importance of linking trap crop development with pest phenology to obtain the most effective results.

1.4.4. Reproductive control

Reproductive control involves a pest management strategy focused on disrupting insect pest reproduction (McPherson, 2018). This can be accomplished by either introducing sterile individuals to mate with wild type individuals or by preventing individuals from finding each other to mate. A general decrease in the ability to mate or an increase in the production of sterile eggs would lead to an overall decrease in the pest population over time.

The Sterile Insect Technique (SIT) involves the release of an overwhelming number of sterile insects, usually males, into the environment to mate with wild insects which results in the production of infertile offspring (Dyck, Hendrichs & Robinson, 2005). Sterilization strategies involve the partial or complete genetic sterilization of pest insects. This can be done by ionizing radiation, chemosterilants, RNA interference or CRISPR (Clustered Random Interspaced Short Palindromic Repeats) gene editing. Dyby & Sailer (1999) demonstrated that the low-level radiation treatment of fourth instar *N. viridula* female individuals resulted in the production of large numbers of nonviable eggs and reduced fecundity compared to non-treated individuals. However, SIT would not be feasible for the control of stink bugs due to the high cost of mass rearing these insects and because the males, which would be released in high numbers with this method, also cause damage to the crop (McPherson, 2018).

Semiochemicals are compounds, such as allelochemicals or pheromones, used during insect communication (Aldrich, 1988). Allelochemicals are compounds that mediate communication between individuals from different species. Pheromones mediate communication within the same species and can be classified as either aggregation, alarm or sexual pheromones. These pheromones have a wide range of applications such as insect population monitoring, mass trapping and mating disruption. Mating disruption involves the release of large amounts of synthetically produced sex pheromones into the environment to interfere with male and female individuals attempting to find each other to mate.

The use of semiochemicals and trap-based monitoring for the BMSB have been studied in both its native and invaded ranges, namely South Korea and the United States, respectively (Aldrich, Khrimian & Camp, 2007; Khrimian et al., 2007; Lee et al., 2013). It was found that traps baited with the BMSB male-produced aggregation pheromone and methyl (E,E,Z) -2,4,6-decatrienoate were most effective in producing a similar response in the nymphs and adults (Leskey et al., 2015; Morrison III et al., 2017). Ground-deployed pyramid traps baited with this chemical combination were used as scouting strategies to guide insecticide application for BMSB in apple orchards. This reportedly led to a drastic reduction in insecticide application of more than 40 % showing great promise as an “attract and kill” approach (Short, Khrimian & Leskey, 2017).

1.5. Conclusions

Stink bugs are a diverse group of insects of significant economic importance in agriculture and inflict significant damage on various nut crops. Numerous species have been reported on different nut crops in various growing regions worldwide. The type of damage inflicted by stink bugs varies between stink bug species, nut crop host type and the phenological development stage of the host (Schoeman 2013, 2018; Bruwer et al., 2021). The extent of economic loss due to stink bug damage differs between nut cropping systems and growing regions, but the consensus is that there is a clear need to successfully manage these pests on nut crops.

Despite the threat posed by these insects on nut crops there is currently a lack of sufficient IPM appropriate control methods. Farmers rely heavily on pesticides for the control of stink bugs in various cropping systems around the world. Unfortunately, the broad-spectrum nature and adverse non-target effects on both human and animal health makes this approach highly unsustainable (Upadhayay et al., 2020). This emphasizes the need to develop alternative control strategies that can be implemented in conjunction with pesticide application to ultimately leading to a reduction in the use of pesticides. The use of such alternative strategies in conjunction as an IPM approach could have a synergistic effect and ultimately lead to improved stink bug management.

Monitoring of stink bug species is an important step in determining the appropriate management strategy to implement and when to apply pesticides. South African growers are encouraged to base their spraying regime on pest monitoring results. This monitoring typically involves the use of knockdown sprays on a specific number of trees and subsequently identifying the insects collected to determine the presence of any pest species. The use of trap crops, pheromone lures and vibrational signals form part of an IPM approach but can also be used for the monitoring of pest species in a similar way. Replacing knockdown-spray application-based monitoring with one of these alternative methods would also result in the reduction of pesticide spraying.

Accurate pest identification is of utmost importance for an IPM approach to be effective. This highlights the importance in correctly identifying and understanding stink bug species presence. It assists in determining the role of damage that each species has so that the damaging species can be targeted to ensure that effective management results are obtained without over spraying pesticides. There is a strong relationship between relative seasonal abundance of stink bugs and tree phenology (Schoeman 2014b, 2018). Understanding this relationship in terms of different stink bug species population peaks is vital in controlling the correct pest species at a specific phase in the growing season and leads to a reduction in pesticide use due to timely pesticide application instead of continuously throughout the season.

Biological control is an important example of an IPM approach that shows great promise in stink bug management. In terms of entomopathogenic fungi, the species-specificity and mortality rates of certain entomopathogenic fungal strains towards some stink bug species suggests that some fungi can be used to control stink bugs. However, the lack of knowledge and proper field trials indicates that this still requires extensive future research along with determining the effectiveness of nematodes as biological control agents. The use of wasp egg parasitoids as biological control agents to control stink bugs has shown great promise with successful control via this method being reported in different crops globally. However, this also requires extensive knowledge on the identity, reproductive biology, dispersal capacity and host range of both the stink bug host and native or non-native parasitoids. There is currently a lack of some of this information for certain stink bug species and their associated parasitoids, therefore requiring extensive research in the future.

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1.7. Tables and figures

Table 1. Stink bug species (Hemiptera: Pentatomidae) associated with nut crops worldwide with potential biological control agents

Species name	Common name (s)	Crops	Origin	Distribution	Genetic ID (COI)	Potential biocontrol agents	References
Acrosternum breviceps	-	Pecan, pistachio	-	Middle East	No	See *C. hilaris*	(Mehrnejad et al., 2013)
***Antestia* species**	-	Macadamia	-	Afrotropical	No	-	(Bruwer et al., 2021; Schoeman, 2018; van den Berg et al., 1999)
Apodiphus amygdali	-	Pecan, pistachio	-	Europe, Middle East, Asia, Africa	No	**Egg parasitoid:** *Trissolcus radjabii*	(Mehrnejad et al., 2013; Iranipour & Johnson, 2010)
Bathycoelia distincta	Two-spotted stink bug	Macadamia	Africa	Southern Africa	No	-	(Schoeman 2013, 2018; van den Berg, 1997)
Brachynema germari	Pistachio green stink bug	Areca nut, pistachio	Middle East	Middle East	No	**Egg parasitoids:** *Trissolcus agriope, Trissolcus delucchii, Trissolcus deserticola, Trissolcus mitsukurii, Trissolcus niceppe, Trissolcus oobius, Trissolcus semistriatus, Psix saccharicola* and *Ooencyrtus telenomicida*	(Mohammadpour et al., 2016)
Carpocoris coreanus	-	Pistachio	-	Nearctic	No	-	(Mehrnejad et al., 2013)
***Chinavia hilaris* (previously *Acrosternum hilare*)**	Green (soldier) stink bug	Pecan, pistachio	North America	Nearctic	Yes	**Egg parasitoids**: *Trissolcus euschisti, Trissolcus basalis, Trissolcus edessae, Telenomus podisi* and *Telenomus cristatus*	(Kamminga et al., 2012; Daane et al., 2005)
Chinavia pallidoconspersa	Yellow edged stink bug	Macadamia	Africa	Afrotropical	No	-	(Schoeman, 2013, 2018; van den Berg, 1997; Orian, 1965)

Chlorochroa ligata	Conchuela bug	Pecan	North America	Nearctic	Yes	**Egg parasitoids**: *Telenomus ashmeadi*, *Gymnosoma fuliginosa*	(Grazia & Schwertner, 2017; Mehrnejad et al., 2013)
Chlorochroa uhleri	Uhler's stink bug	Almond, pistachio	North America	Nearctic	Yes	See *C. ligata*	(McPherson & McPherson, 2000)
Coenomorpha nervosa	Grey-brown stink bug	Pecan	(Southern) Africa	Afrotropical	No	-	(van Rooyen, 2017)
Euschistus servus	Brown stink bug	Pecan	North America	Nearctic	Yes	**Egg parasitoids:** *Te. podisi, Te. ashmeadi, T. basalis, T. euschisti* and *Trichopoda pennipes*	(Panizzi et al., 2000)
Euschistus tristigmus	Dusky stink bug	Pistachio, pecan	North America	Nearctic	Yes	**Egg parasitoids:** *T. euschisti* and *Te. podisi* **Tachinid fly:** *Euthera tentatrix*	(Mehrnejad et al., 2013; McPherson & McPherson, 2000)
Halyomorpha halys	Brown marmorated stink bug	Black walnut, hazelnut, pecan, pistachio, almond	East Asia	Asia, Europe, North and South America	Yes	**Egg parasitoids**: *Trissolcus japonicus, Anastatus bifasciatas*	(Bosco et al., 2018; Hamilton et al., 2018; Rijal & Gyawaly, 2018; Stahl et al., 2018; Lara et al., 2017; Rice et al., 2014)
Nezara viridula	Green vegetable bug	Pecan, macadamia	Ethiopia (East Africa)	Cosmopolitan	Yes	57 egg parasitoid species (Jones 1988)	(Bosco et al., 2018; Esquivel et al., 2018; McPherson & McPherson, 2000; Mitchell et al., 1965)
Palomena prasina	Green shield bug	Hazelnut	Europe	Palearctic	Yes	**Egg parasitoids**: *T. semistriatus* and *Telenomus chloropus* ***Entomopathogens****: Lecanicillium mauscarium and Beaevaria bassiana*	(Bosco et al., 2018; Erper et al., 2016)(Erper et al., 2016; Bosco, Moraglio & Tavella, 2018)
Parachinavia prunasis (formerly Acosternum prunasis* and *Nezara prunasis)	-	Macadamia	Africa	Afrotropical	No	-	(Schoeman, 2018; van den Berg et al., 1999)
Pseudatelus raptorius (formerly Atelocera raptorial)	Powdery stink bug	Pecan, pistachio, macadamia	Africa	Southern Africa	No	**Egg parasitoid:** *T. basalis*	(Haddad & Louw, 2006; Joubert & Neethling, 1994)
Thyanta pallidovirens	Red shouldered stink bug	Pistachio	North America	Nearctic	Yes	-	(Grazia & Schwertner, 2017; Daane et al., 2005)

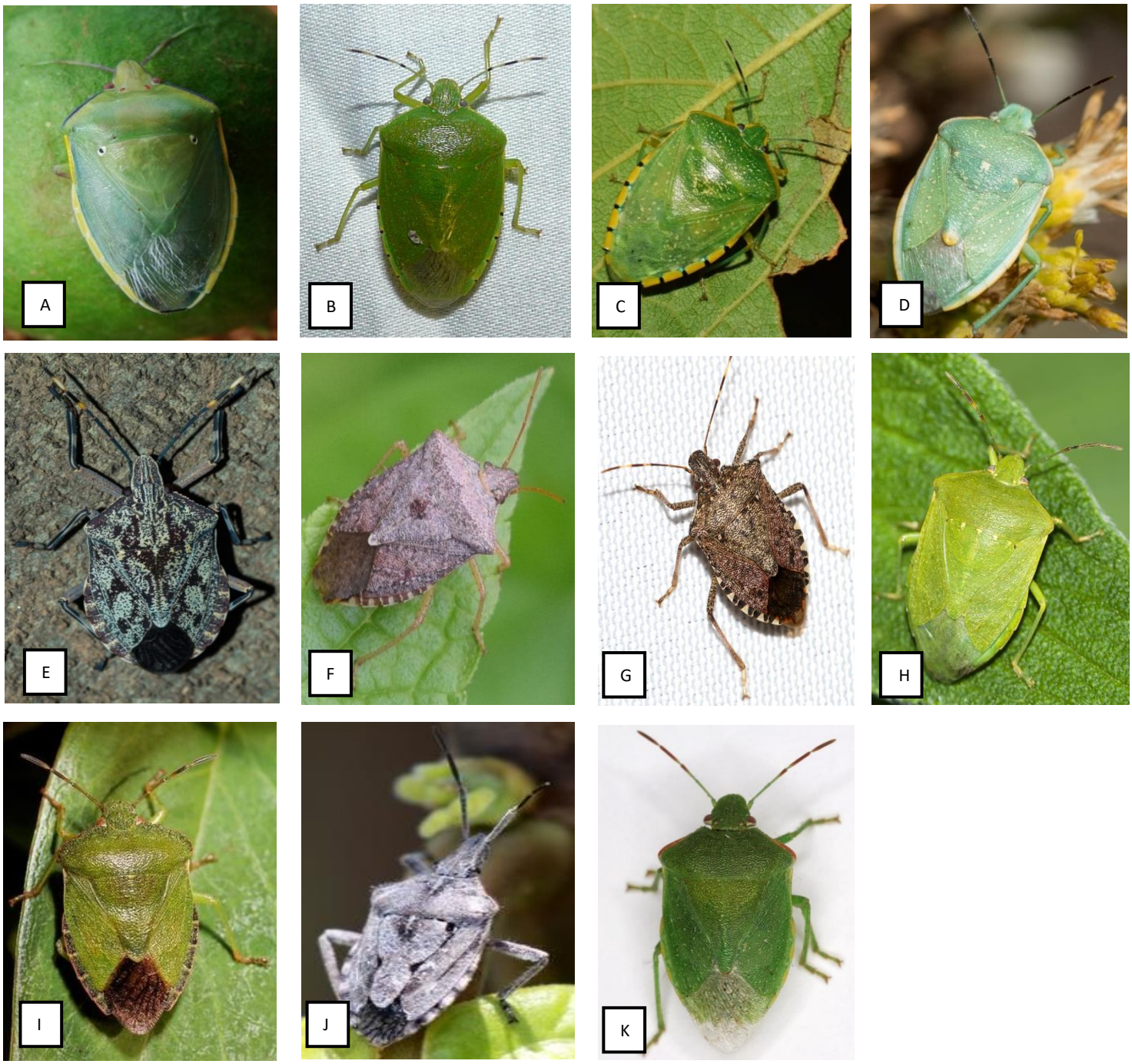


Figure 1. Most dominant and economically important stink bug species typically associated with nut crops. A. *Bathycoelia distincta* B. *Chinavia hilaris* C. *Chinavia pallidoconspersa* D. *Chlorochrpa uhleri* E. *Coenomorpha nervosa* F. *Euschistus servus* G. *Halyomorpha halys* H. *Nezara viridula* I. *Palomena prasina* J. *Pseudatelus raptorius* and K. *Thyanta pallidovirens* (Photos: www.inaturalist.org)

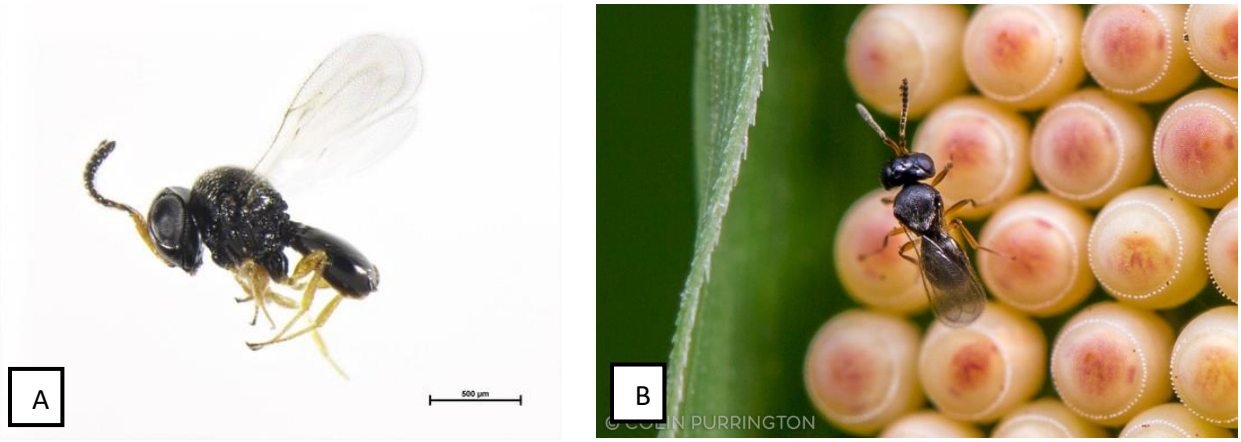
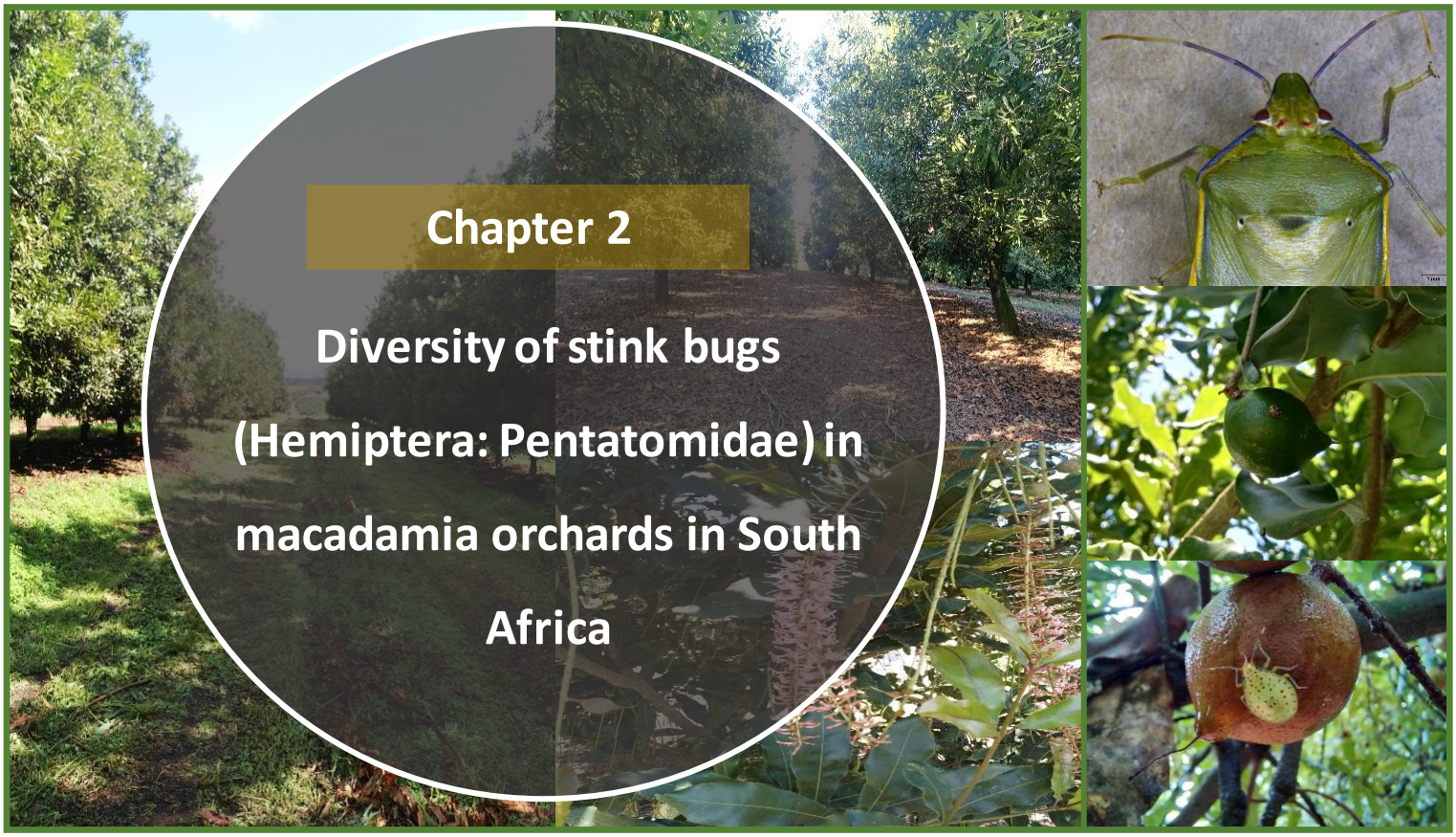


Figure 2. Two well-known egg parasitoid species of stink bugs. A. *Trissolcus basalis* and B. *Telenomus podisi* (Photos: www.inaturalist.org)



Chapter 2

Diversity of stink bugs (Hemiptera: Pentatomidae) in macadamia orchards in South Africa

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2.1. Abstract

Stink bugs are major pests of macadamia in South Africa. They inflict extensive damage with their piercing/sucking feeding strategy leading to significant yield and economic loss. Accurate identification and knowledge of species composition is important to inform management practices. The overall aim of this study was to identify stink bug species in macadamia orchards in South Africa using a DNA barcoding approach. A total of 20 stink bug species were found in macadamia orchards in KwaZulu-Natal, Limpopo and Mpumalanga. A DNA barcode database of the *cytochrome c oxidase subunit 1* for these and other available species was established to assist in future identifications. *Bathycoelia distincta* was the dominant species throughout all three growing regions. Species presence fluctuated over three growing seasons (2017-2020) in Limpopo and differed between the three growing regions during a growing season (2019-2020). *Boerias* sp. was the second most abundant species found in KwaZulu-Natal and this is the first report of this species being associated with macadamia. There also appears to be a second cryptic *Boerias* sp. found on macadamia in this region. The novel association of these species with macadamia and differences in species presence, both over time and across growing regions, highlights the need for ongoing monitoring of these pest species. Monitoring will allow for the implementation of species-specific control strategies, ultimately leading to a reduction in the use of broad - spectrum insecticides.

2.2. Introduction

Stink bugs (Hemiptera: Pentatomidae) represent a diverse and economically important group of insects in agriculture (McPherson & McPherson, 2000; McPherson, Scott Bundy & Wheeler, 2018). The Pentatominae is considered the most important sub family as it is the largest and contains the majority of the economically important stink bug species found in different ecozones globally (Grazia et al., 2015; Grazia & Schwertner, 2017). The type of damage inflicted via their piercing/sucking feeding strategy differs between the type of crop, the phenological stage of the host plant and the species of stink bug involved. Premature nut drop, malformed nuts, discoloured spots and nut necrosis are general symptoms of stink bug damage on nut crops such as almonds (*Prunus dulcis*, (Miller) D.A. Webb), hazelnuts (*Corylus* sp., Linnaeus), macadamia (*Macadamia integrifolia* Maiden & Betche and *Macadamia tetraphylla* L. Johnson), pecan (*Carya illinoinensis,* Koch) and pistachio (*Pistacia vera*, Linnaeus) (Jones & Caprio, 1994; Panizzi et al., 2000; Tavella et al., 2001; Daane et al., 2005; Rijal & Gyawaly 2018; Schoeman, 2018).

Macadamia trees are native to eastern Australia. These trees and their hybrids are planted commercially in various regions including Australia, Brazil, China, Colombia, Guatemala, Hawaii, Kenya, Malawi, South Africa and Vietnam (INC, 2020; SAMAC, 2020a). Production in South Africa has increased to such an extent that it is currently the largest producer of macadamia nuts globally. The three main growing regions in South Africa include parts of KwaZulu-Natal, Mpumalanga, and Limpopo with macadamia plantings also found in the Eastern Cape and Gauteng provinces. According to the International Nut and Dried Fruit Council (INC), South Africa contributed 29 % of the global macadamia kernel production in 2019, with an estimated total value reported to be around R 4.8 billion (INC, 2020).

Stink bugs are major pests of macadamia worldwide, including South Africa. Stink bug damage was reportedly responsible for almost half of the unsound kernel recovery percentage in South Africa in 2019, equating to a monetary loss of around R 181 million (SAMAC, 2020b). A complex of stink bug species has been reported to occur in macadamia orchards in South Africa

(Schoeman, 2013, 2018; van den Berg, Steyn & Greenland 1999). This complex includes species such as *Bathycoelia distincta* Distant, 1878 (two-spotted stink bug), *Nezara viridula* (Linnaeus, 1758) (Green vegetable bug), *Chinavia pallidoconspersa* (Stål, 1858) (yellow-edged stink bug, formerly *Nezara pallidoconspersa*), *Parachinavia prunasis* (Dallas, 1851) (formerly *Acrosternum prunasis* and *Nezara prunasis*), and *Pseudatelus raptorius* (Germar, 1838) (powdery stink bug, formerly *Atelocera raptorial*) (van den Berg *et al.,* 1999; Schoeman, 2018). Of these species, *B. distincta* is thought to be the most dominant species and contributes towards the majority of the damage observed (Schoeman, 2013). A clear fluctuation in the assemblage of stink bug species has been reported and the number of different species present in orchards also increased drastically during the last few years (van den Berg et al., 1999; Schoeman, 2018). The species identity of some of these are currently unknown.

The relative seasonal abundance of stink bugs in macadamia orchards has been correlated to the phenological development of macadamia trees (Schoeman, 2018). This also correlates to the type of stink bug damage observed in South African macadamia orchards. Early stink bug damage is typically caused by the 'winter complex' of stink bug species (Schoeman, 2013) such as *C. pallidoconspersa, P. prunasis* and *P. raptorius*. This damage occurs prior to the nut developing a thick husk and shell, by stink bugs with shorter mouthparts and *B. distincta* (Schoeman, 2009; Bruwer et al., 2021). Late stink bug damage is speculated to be caused by *B. distincta* which has a lengthier stylet, in comparison to the other species, enabling this species to penetrate a thick husk and shell which develops as the growing season progresses (Schoeman, 2009; Bruwer et al., 2021). Recent feeding damage trials conducted in South Africa suggest otherwise. Schoeman (2020) reported that *C. pallidoconspersa* can cause significant damage to macadamia nuts in the late season. Bruwer et al. (2021) reported *Farnya* sp. Schouteden, 1910 (synonymous with *Antestia* sp. Stål, 1865) being capable of causing low levels of late damage. These results suggest that other species may therefore also be involved in late season damage and this requires further investigation.

In terms of control, macadamia growers currently rely heavily on broad-spectrum insecticides, such as carbamates, organophosphates, pyrethroids and neonicotinoids, to manage stink bugs in their orchards. This strategy is however highly unsustainable due to, long-lasting residues, adverse health effects on humans, the build-up of resistance and the effects on non-target insects, including beneficial arthropods, ultimately leading to a decrease in biodiversity (Desneux et al., 2007; Pandian & Ramesh, 2020; Upadhayay et al., 2020). This highlights the need for effective alternative pest management strategies in macadamia orchards such as a biological control approach or the use of pheromones for trapping, mating disruption and/or attract-and-kill. Accurate identification and better knowledge on the host and pest species presence, abundance, biology and genetic diversity, among others, is however required to develop and implement such alternative control strategies (Dent & Binks, 2000; McPherson, 2018).

The aim of this study was to identify stink bug species present in macadamia orchards from the three main growing regions of South Africa. To achieve this, stink bugs were collected for three consecutive seasons from the Levubu area in Limpopo, and from Mpumalanga and KwaZulu-Natal for one season. Species presence was documented and compared to previous surveys. Species identity was firstly based on morphology and thereafter on the *cytochrome c oxidase subunit 1* (CO1) gene region of a representative set of specimens. This was done to develop a DNA barcoding database which will be valuable in terms of identification of cryptic or polymorphic species. The phylogenetic relationships of the species were also inferred and genetic divergence within and between abundant species was determined.

2.3. Materials and methods

2.3.1. Insect collection

Stink bug specimens were collected over three growing seasons, August/September (flowering) to July (post-harvest), from 2017 to 2020 via farmer scouting. Scouting is generally conducted prior to the spraying of pesticides to give an estimate of the number of stink bugs present in

orchards. Scouting involves the application of a knockdown spray to 10 trees in random blocks on the farm, usually at dawn. Plastic sheets are placed under the trees to collect stink bugs killed by the chemical spray. Stink bug specimens were sampled from trees of various cultivars and ages over the duration of this study. In both the 2017-2018 and 2018-2019 seasons, samples were collected from two farms in the Limpopo growing region, more specifically Levubu. For the 2019-2020 season, stink bug specimens were collected from two different farms from each of the three main growing regions, namely, Limpopo, KwaZulu-Natal, and Mpumalanga (Figure 1). Overall, the sampling strategy therefore allowed for the documentation of species presence throughout the season, the presence between different growing regions and across three consecutive growing seasons in Limpopo. Stink bugs from 342 scouting batches were included in this study. Stink bugs collected were preserved in 70 % ethanol at -20˚C for subsequent analyses.

2.3.2. Morphological identification

Scout batches obtained from KwaZulu-Natal (166), Limpopo (144) and Mpumalanga (32) were sorted by the date, farm name and block. In general, characteristics of stink bugs (Hemiptera: Pentatomidae) include a round/ovoid shape, with three-segmented-tarsi and five-segmented antennae, they also possess a short scutellum with a triangular shape that is usually narrowed posteriorly (McPherson & McPherson, 2000; Panizzi et al., 2000). Stink bug specimens were assigned to putative species groups based on external morphological features described in literature (Rolston, 1983; Staddon & Ahmad, 1995; Grazia & Schwertner, 2008; Kment & Jindra, 2009; Ferrari et al., 2010; Salini & Viraktamath, 2015). Vouchered specimens were pinned, accession numbers assigned and were deposited in the National Collection of Insects, Pretoria, Agricultural Research Council - Plant Health and Protection with the help of taxonomists. A representative from each species/morphological group was photographed and subsequent COI barcoding was used to confirm species groups.

2.3.3. DNA extraction, amplification and sequencing

To obtain DNA barcoding sequences, genomic DNA was extracted from the legs and shoulder area of each stink bug specimen using the NucleoSpin ® DNA insect kit (Separations, Johannesburg, South Africa). The DNA concentration was quantified using a Nanodrop spectrophotometer. Polymerase Chain Reactions (PCR) were performed in a 25 µl volume containing 5 µl of 5 mM MyTaq™ reaction buffer (Bioline, South Africa) containing $MgCl_2$, 0.5 µl of MyTaq™ DNA polymerase (Bioline, South Africa), 0.5 µl of 10 mM of LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAA ATCA -3') (Folmer et al., 1994), 16.5 µl of ddH_2O (Adcock Ingram, Bryanston, South Africa) and 2 µl of template DNA. Thermocycling conditions included an initial denaturation at 94 °C for 1 min, followed by 5 cycles of 94 °C for 1 min, 45 °C for 1 min 30 s and 72 °C for 1 min 30 s. This was followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min 30 s and 72 °C for 1 min with a final hold of 5 min at 72 °C. A negative control was added to ensure the absence of contamination. The amplicons obtained were subjected to agarose gel electrophoresis on a 2 % agarose gel in a 1x sodium borate buffer and visualized using GelRed (Biotium, Hayward, California, USA) and a 100 bp DNA ladder (Promega, USA) was used to determine the size of each amplicon. A Molecular Imager® GelDoc™ (Bio-Rad) was used to visualize the amplicons under ultraviolet light. Following the confirmation of the presence of the correct amplicons, a PCR clean up reaction was performed using ExoSAP-IT™ (Applied Biosystems, USA) by following the manufacturer's protocol.

The PCR products were sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The thermal cycler was programmed such that initial denaturation was at 96°C for 2 min, followed by 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The samples were precipitated using sodium acetate and sequenced using an ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystems, USA) at the Bioinformatics Sequencing facility at the University of Pretoria.

2.3.4. Phylogenetic analysis

Forward and reverse sequence reads were checked for base calling accuracy and manually edited using Biological Sequence Alignment Editor (BioEdit) (Hall, Biosciences & Carlsbad, 2011). Consensus sequences for each individual were generated by aligning the forward and reverse sequences. The generated sequences were then subjected to Basic Local Alignment Tool (BLAST) searches against the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) and Barcode of Life Data Systems (BOLD) (www.boldsystems.org) databases. Representative stink bug species sequences were downloaded from BOLD and NCBI representing 13 genera within the sub family Pentatominae containing at least 19 different species within the Pentatomidae family (Table 1, Park et al., 2011; Gariepy et al., 2014, 2019; Raupach et al., 2014; Tembe et al., 2014; Wanqing et al., 2015; Gwiazdowski et al., 2015; Tillman et al., 2015; Dhami et al., 2016; Barman et al., 2017; Valentin et al., 2017, Gowande et al., 2018). Sequences were also translated into amino acids in order to screen for the presence of premature stop codons. Thereafter, sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) version 7 (https://mafft.cbrc.jp/alignment/software) (Katoh, Rozewicki & Yamada, 2019) and trimmed to a length of 472 bp. The aligned sequences were subjected to phylogenetic analysis based on maximum likelihood (ML) with 1000 bootstrap replicates. Phylogenetic analysis was performed using Randomized Axelerated Maximum Likelihood (RaxML) version 8 (Stamatakis, 2014) and viewed/edited in Mega 7.0 (Kumar, Stecher & Tamura, 2016). *Podisus maculiventris* (MG940398) (Say, 1832) (Pentatomidae: Asopinae) was used as an outgroup.

2.3.5. Sequence divergence analysis

Sequence divergence was calculated for all the individuals that were found in all three growing regions or were found in a high number of scout samples. Interspecific divergence was used to determine whether the stink bug specimen groupings based on morphology were correct and represented distinct species groups. Intraspecific divergence was determined to assess the

possible presence of cryptic species. Nucleotide diversity (π), average number of nucleotide substitutions per site (D_{xy}), and net nucleotide substitutions per site (D_a) were calculated using DNA Sequence Polymorphism (DnaSP) version 5.10.01 (Librado & Rozas, 2009). The nucleotide diversity (π) value is the mean number of nucleotide differences per site between two sequences (Nei, 1987). For comparative purposes π, D_{xy} and D_a values were converted to percentages.

2.4. Results

2.4.1. Morphological identification

A total of 3670 stink bug specimens were collected from 342 scout batches from various farms in the three main growing regions of South Africa. The 3670 stink bug specimens were subsequently grouped, based on morphology, into 20 stink bug species, representing two Pentatomidae sub families (Pentatominae and Asopinae) (Figure 2 and Figure 3, Table 2).

2.4.2. Species presence

2.4.2.1. Species presence between Limpopo, Mpumalanga and KwaZulu-Natal

There was a clear distinction in species composition for the three different growing regions, KwaZulu-Natal showed species richness with 18 Pentatomidae species. A total of 8 species and 10 species were present in macadamia orchards in Limpopo and Mpumalanga for the 2019-2020 growing season, respectively (Figure 5). Based on the total number of specimens collected per species, *B. distincta* was the dominant species found in KwaZulu-Natal (1792, 75 %), Limpopo (500, 53 %) and Mpumalanga (423, 62 %) for the 2019 – 2020 season. Based on a total of 259 scout samples/batches collected during the 2019-2020 season, *B. distincta* represented the species observed most in Limpopo (50 of 55 batches, 91 %) and Mpumalanga (23 of 32 batches, 72 %) scout samples (Table 3). In KwaZulu-Natal, *Boerias* sp. Kirkaldy, 1909 (103 of 166 batches, 62%) and *B. distincta* (93 of 166 batches, 56%) were the most abundant species (Table 3).

Bathycoelia distincta, *C. pallidoconspersa*, *N. viridula*, *P. prunasis*, *Parantestia* sp. (Linnavuori, 1973) and *P. raptorius* were commonly found in scout batches from all three growing regions, including nymphs of *B. distincta* and *P. raptorius* (Figure 4; Table 3 and 4). *Bathycoelia distincta*, *Boerias* sp., *Parantestia* sp. and *P. raptorius* were present throughout the season. *Chinavia pallidoconspersa*, *N. viridula*, *P. prunasis* and *Piezodorus* sp. (Fieber, 1860) were observed in a higher proportion of scout samples obtained during the winter months and early season in either Limpopo or KwaZulu-Natal. *Boerias* sp. (KwaZulu-Natal only), *B. distincta* and *Parantestia* sp. were the species found in the highest proportion of scouting events for the late season. In Mpumalanga, *B. distincta* was observed in the highest proportion of scout samples followed by *C. pallidoconspersa* in the late season. *Nezara viridula, Parantestia* sp.*, P. prunasis* and *Piezodorus* sp. were also found in the late season in Mpumalanga.

Coenomorpha nervosa Dallas, 1851, *Antestia* sp. and *Platacantha lutea* (Westwood, 1837) were common between Mpumalanga and KwaZulu-Natal while *Piezodorus* sp. was common between Limpopo and Mpumalanga (Figure 5). A total of 12 species, namely *Agonoscelis versicoloratus* (Turton, 1802); *Antestia* sp., *Aspavia albidomaculata* (Stål, 1853), *Basicrytpus costalis* (Germar, 1838), *B. distincta*, *Coenomorpha nervosa*, *C. pallidoconspersa*, *Macrorhaphis acuta* Dallas, 1851, *N. viridula*, *P. prunasis*, *P. raptorius* and *Piezodorus* sp. represent stink bugs species associated with macadamia in South Africa from previous studies (Schoeman 2013, 2018; van den Berg et al. 1999; Bruwer 1992).

Caura rufiventris (Germar, 1838) was unique to Limpopo while *A. versicoloratus*, *Anolcus campestris* (Bergroth, 1893), *Antestiopsis thunbergii* (Gmelin, 1790), *A. albidomaculata*, *Boerias* sp., *B. costalis*, *Carbula recurva* Distant, 1915, *M. acuta* and *Tripanda signitenens* (Distant, 1898) were unique to KwaZulu-Natal (Figure 5). These species are considered as rare since they were only observed in a few scout samples with the exception of *Boerias* sp.

2.4.2.2. Species presence across growing seasons in Limpopo

There was a clear distinction in species composition across three growing seasons in the Levubu growing region (Limpopo). In total, 15 stink bug species were present across three seasons with 11 of them present in 2017-2018, 14 in 2018-2019 and 8 in 2019-2020 (Figure 5). *Bathycoelia distincta* was the species found in the highest proportion of scout samples obtained from Limpopo across two seasons, namely 2018-2019 (83% of the time) and 2019-2020 (91% of the time) (Table 4). *Nezara viridula* was the species observed the most in the scouting batches during 2017-2018 (78% of the time), followed by *B. distincta* (49%). *Parantestia* sp. and *N. viridula* were the second most abundant species in scout batches for 2018-2019 and 2019-2020, respectively. *Chinavia pallidoconspersa* and *Piezodorus* sp. were each observed in more than 40% of the scout batches obtained during the 2019-2020 season but were found in a lower proportion of scout batches for the previous two seasons. There was a clear fluctuation in the proportion of scout batches where each of the 'common' species was found between the three growing seasons and a fluctuation in the presence of the 'rare' species found in very low proportions of scout batches (Table 4, Figure 6).

2.4.3. Phylogenetic analysis

A 650 bp fragment of the mtDNA COI gene was sequenced for 62 stink bug specimens collected from various farms located in KwaZulu-Natal, Limpopo, and Mpumalanga (Table 2). These specimens included representatives from each morphological group. Based on percentage identity values of 98 % or higher, BLAST searches against the NCBI database identified some of the individuals as *Nezara viridula* (n=6) and *Piezodorus* sp. (n=4). A search against the Barcode of Life Database (BOLD) systems identified the same two species, as well as *Agonoscelis puberula* (Stål, 1853) (n=2) and *Macrorhaphis acuta* (n=2). Therefore, the majority (n= 48) of the specimens (16 stink bug species) could not be identified via a database search (NCBI and BOLD) due to the lack of availability of COI sequences.

Maximum likelihood analysis resolved the sequences into two distinct subfamily clades, namely Pentatominae and Asopinae (Figure 7). Within the Pentatominae clade, all 31 of the different species were separated into their individual clades, with strong bootstrap values (>90%), and represented 24 genera. Both of the Asopinae species included in the analysis, namely *M. acuta* and *P. maculiventris* (outgroup), grouped within the Asopinae sub family clade.

2.4.4. Sequence divergence analysis

Intraspecific and interspecific divergence was calculated for *B. distincta, Boerias* sp., *C. pallidoconspersa, N. viridula, P. prunasis*, *Parantestia* sp. and *Piezodorus* sp. Individuals of each species group clustered well within their respective branches. The π values for *B. distincta, C. pallidoconspersa, N. viridula, P. prunasis*, *Parantestia* sp. and *Piezodorus* sp. were 0.424; 0.085; 0.494; 0.11; 0.26 and 1.96 %, respectively (Table 5). The π values for the *Boerias* sp. and *P. raptorius* groups were found to be high with approximate values of 7.5 % and 3.8 %, respectively. The genetic divergence between species groups i.e., average number of nucleotide substitutions per site (D_{xy}) and the net nucleotide substitutions per site (D_a) between populations (Nei, 1987) for the same species groups ranged from 11.5 – 14.4 % (0.115 – 0.144) and 8.4 – 14.4 % (0.084 - 0.144), respectively (Table 6).

2.5. Discussion

Based on morphological identification and COI barcoding, 20 Pentatomidae species were found in macadamia orchards in KwaZulu-Natal, Limpopo, and Mpumalanga. This study serves as the most extensive species list to date, not only in terms of the number of species reported from macadamia orchards for the first time, but also because it is the first survey conducted in KwaZulu-Natal orchards. The latter survey led to the discovery of *Boerias* sp. occurring in a high proportion of scout batches. *Bathycoelia distincta* remained the most dominant stink bug species found in macadamia orchards in all three of the major growing regions of South Africa, as reported previously (van den Berg et al., 1999; Schoeman, 2013). However, species presence

differed between growing regions and across growing seasons. A COI DNA barcoding database was established for the Pentatomidae species found in this study as COI sequences were only available for four of the 20 stink bug species. Sequence divergence calculations were used to determine the accuracy of groupings and identifications based on morphology. All the species groups in this study represent distinct species with the exception of *Boerias* sp. and *P. raptrious* groups which contain potential cryptic species.

A high diversity of stink bug species was found across the main growing regions of South Africa with KwaZulu-Natal having the highest diversity with 18 species. The number of scout batches obtained from KwaZulu-Natal was over three and five times more than Limpopo and Mpumalanga, respectively, and could explain the higher diversity sampled in KwaZulu-Natal. Heteroptera populations are influenced by differences in tree size, tree and canopy density, surrounding natural vegetation and changes in husbandry practices between farms and growing regions (Schoeman, 2014). KwaZulu-Natal has a much higher ratio of young/establishing trees to old trees and the replacement of previous commonly grown crops with macadamia trees could also suggest that the 'rare' stink bug species are shifting host from these crops (SAMAC, 2020a). This highlights the possibility of surrounding crops/vegetation in different areas also determining stink bug species presence in macadamia orchards. A lower diversity of stink bug species was found in Limpopo (eight species) and Mpumalanga (ten species), both of which contain more older and established orchards that have experienced more chemical control over a long period of time (Desneux et al., 2007). Further monitoring is required to determine if species composition will change over time as the availability of nuts as a potential food source increases and the landscape changes with more orchards being established annually. Finally, the damage potential to macadamia nuts of a number of these species from KwaZulu-Natal is unknown and requires further investigation.

Differences in species composition between different growing regions and across three seasons in Limpopo was clearly evident in this study. The proportion of the dominant species, *B. distincta*, differed for each season while the proportion of the other species found in scout batches also

differed across different growing seasons. This was in agreement with previous studies that investigated stink bug species present in macadamia orchards in South Africa (Bruwer, 1992; van den Berg et al., 1999, Schoeman, 2018). The majority of the stink bug specimens from the 2017-2018 and 2018-2019 seasons were collected during the late season. The presence of *C. pallidocnspersa* and *P. raptorius* during the late season could suggest their involvement in late damage, but only *C. pallidoconspersa* has been shown to be capable of causing late damage (Schoeman, 2020). The specimens obtained from Limpopo during the 2019-2020 season were collected during the early season. Therefore, the change in species composition across seasons in Limpopo could be due to differences in host phenology at the time of sampling. The scout batches were not consistent between farms and growing seasons in Limpopo which could influence the results and limit the number of inferences that can be made. For example, less scout batches obtained in 2017-2018 could potentially have missed areas or time periods when *B. distincta* was present in high proportions resulting in *N. viridula* being found in higher proportions.

Bathycoelia distincta, *C. pallidoconspersa*, *N. viridula*, *P. prunasis*, *Parantestia* sp. and *P. raptorius* were commonly found in a higher proportion of scout batches from all growing regions and also represent the species listed in previous surveys from South Africa (Schoeman, 2018; van den Berg et al., 1999; Bruwer, 1992), with the exception of *Parantestia* species. *Bathycoelia distincta*, *Boerias* sp., *Parantestia* sp. and *P. raptorius* were present throughout the season, which suggests that macadamia is the preferred food source for these stink bug species, although the damage potential on macadamia nuts by *Parantestia* sp. requires further investigation. Nymphs of *B. distincta* and *P. raptorius* were found in all three growing regions during this study and thus remain the only two Pentatomidae species that have been reported to reproduce in macadamia orchards in South Africa (Schoeman, 2018). *Pseudatelus raptorius* is thought to be a bark-feeding stink bug species (van den Berg et al., 1999). It is considered a major pest of pistachio in the Northern Cape Province of South Africa (Haddad & Louw, 2006) and a pest of pecan, litchis and avocados in the Mpumalanga province (Joubert & Neethling, 1994; Schoeman & Morey, 2016).

There is a clear need for future studies to focus on the presence of the lesser studied/understood species such as the *Boerias* sp. and to determine their capability of inflicting damage based on their stylet penetration potential. *Boerias* sp. was the stink bug species found in the highest proportion of scout batches obtained during the KwaZulu-Natal region survey. This species was unique to the region, and this is the first report of *Boerias* sp. being associated with macadamia in South Africa. Little is currently known regarding the biology, management, and potential impact of this stink bug species in macadamia orchards. The presence of this species in a high proportion of KwaZulu-Natal scout batches throughout the growing season suggests that macadamia is the species' preferred food source, and that this species might cause nut damage. The stylet length of the *Boerias* sp. is shorter in comparison to *B. distincta* (Figure 2). Studies have reported late damage being inflicted by species with shorter stylets in South African macadamia orchards (Schoeman, 2020; Bruwer et al., 2021). Concerning stink bug feeding mechanics the hypothesis has been that stink bug species with lengthier rostrums are able to penetrate deeper and inflict damage on more well-developed fruits/nuts (Bruwer, 1992; Follett, Wright & Golden, 2009) . However, stylet penetration potential has been proven to be dependent on specific rostral segments and not total rostral length (Esquivel 2011, 2019).

A total of 13 species were considered as rare since they were found in low numbers in very few of the scout batches obtained from the different growing regions. *Agonoscelis versicoloratus*, *Antestia* sp., *B. costalis*, *C. recurva*, *C. nervosa*, *M. acuta* and *P. raptorius* are the only species that have been previously associated with macadamia in South Africa. The pest status of *B. costalis* and *C. recurva* are currently unknown. *Antestia* sp. and *A. versicoloratus* feed on flowers and fruits of *Jatropha curcas* L. (physic nut) in West Niger (Habou et al., 2014). *Coenomorpha nervosa* is a pest of avocado in Mpumalanga and a major pest of pecan in the Northern Cape and North West provinces in South Africa (van Rooyen, 2017). *Coenomorpha nervosa* was recently shown to not inflict significant damage while *P. raptorius* is only capable of early damage on macadamia (Schoeman, 2020). *Macrorhaphis acuta* is classified within the Asopinae subfamily of Pentatomidae and the members of this subfamily are considered as predatory stink bugs. *Macrorhaphis acuta* is a predator of *Achaea lienardi* (Boisduval, 1833) (Lepidoptera: Noctuidae)

larvae in South Africa (Taylor, 1965). This predatory stink bug species has been suggested as a biocontrol agent against *Boarmia* (*Ascotis*) *selenaria* (Schiffermiller 1775) (Lepidoptera: Geometridae), a pest of coffee in Kenya (Panizzi et al., 2000). Little is known regarding the effect of this species on other stink bug species and further investigation is required.

In addition to species identification based on morphology, COI sequences were generated to support morphological groupings. The generation of these sequences also allowed for the establishment of a COI sequence database for the stink bug species found in macadamia orchards in South Africa. A total of 62 COI sequences were produced for the 20 species, of which 16 of these stink bug species previously lacked sequence data. This database will be used as an identification/diagnostic tool for future identification of stink bug species and their relevant life stages. This is of particular importance since morphological identification of insects can be difficult and expert knowledge on the use of taxonomic keys is often required (Ball & Armstrong, 2006; Jinbo et al., 2011; Emam et al., 2020). In addition, the different life stages for a number of the stink bug species identified in this study are currently unknown and a COI barcode can help link an adult to a nymphal stage. COI sequences were generated for nymphs of *B. distincta* and *P. raptorius* and the nymphs grouped within their respective clades. Polymorphism is also known to occur in various stink bug species such as *N. viridula* (Kiritani, 1970) and DNA sequences have been used to determine species limits of polymorphic species in the genus *Halys* Fabaricius, 1803 (Memon et al., 2006). Three different colour morphs were observed for individuals of *N. viridula* collected during this study. These specimens were successfully resolved into their respective clades by using COI DNA barcoding and a phylogenetic approach.

The genetic divergence of the species analyzed in this study (*B. distincta, C. pallidoconspersa, N. viridula, P. prunasis*, *Parantestia* sp. and *Piezodorus* sp.) ranged from 0.085 to 1.96%, except for the *Boerias* sp. group with a sequence divergence of around 7% among individuals. Genetic divergence of ≥ 2% among individuals of an insect group may indicate the presence of cryptic species (Hebert et al., 2003a; Hebert et al., 2004) which suggest that at least two different species, belonging to *Boerias* genus, were sampled during this study. Mean COI divergence

between congeneric species pairs ranges from 6.6 to 11.5 % (Hebert et al., 2003b). Genetic diversity was high (>11.5%) between species in different genera in this study, indicating that all of the groups analysed for sequence divergence were in fact separate species and the grouping based on morphology was accurate.

Overall, a high diversity of stink bug species, with fluctuation of stink bug species presence, both over time and across growing regions, was observed in macadamia orchards in KwaZulu-Natal, Limpopo and Mpumalanga. This highlights the importance of continuous monitoring of stink bug presence and assessment of their damage potential. The DNA barcoding (COI) database established in this study will assist in future stink bug pest identification. Accurate pest identification of insect species is of utmost importance for effective pest management, especially when implementing species – specific control methods, such as biological control, sexual disruption and pheromone-based trapping. The novel association of *Boerias* sp. in high proportions of scouting samples from KwaZulu-Natal from both the early and late season indicates that this insect group plays a potential role in macadamia nut damage.

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2.8. Tables and figures

Table 1. *Cytochrome c oxidase subunit 1* (COI) sequences downloaded from Barcode of Life Data Systems (BOLD) and National Center for Biotechnology Information (NCBI) databases and included in the phylogenetic analysis.

Tribe	Species	Accession ID (NCBI/BOLD)	Location	Reference
Agonoscelidini	*Agonoscelis versicoloratus*	ETKH804-13	Durban, KZN	BOLD
Agonoscelidini	*Agonoscelis versicoloratus*	ETKH852-13	Pietermaritzburg, KZN	BOLD
Bathycoeliini	*Bathycoelia indica*	HQ236463	India	(Tembe et al., 2014)
Eyasarcorini	*Carbula biguttata*	GBMHH27000-19	Bengaluru, India	BOLD
Eyasarcorini	*Carbula biguttata*	GBMHH3121-14	India	BOLD
Eyasarcorini	*Carbula insocia*	HQ333534	India	(Tembe et al., 2014)
Eyasarcorini	*Carbula insocia*	GU247472	India	(Tembe et al., 2014)
Nezarini	*Chinavia hilaris*	MF679608	California, USA	(Barman et al., 2017)
Nezarini	*Chinavia hilaris*	MF679609	California, USA	(Barman et al., 2017)
Nezarini	*Chinavia hilaris*	HQ105392	British Columbia, Canada	(Park et al., 2011)
Nezarini	*Chlorochroa sayi*	MF679618	California, USA	(Barman et al., 2017)
Nezarini	*Chlorochroa sayi*	MF679619	California, USA	(Barman et al., 2017)
Nezarini	*Chlorochroa uhleri*	MF679634	California, USA	(Barman et al., 2017)

Nezarini	*Chlorochroa uhleri*	KR035090	Utah, USA	(Gwiazdowski et al., 2015)
Strachiini	*Eurydema dominulus*	KP190205	NA	(Zhao et al., 2015)
Strachiini	*Eurydema* sp.	KY214425	China	(Zhao et al., 2017)
Strachiini	*Eurydema* sp.	KY214424	China	(Zhao et al., 2017)
Carpocorini	*Euschistus servus*	MG939986	Ontario, Canada	(Gariepy et al., 2019)
Carpocorini	*Euschistus servus*	MG939985	Ontario, Canada	(Gariepy et al., 2019)
Carpocorini	*Euschistus tristigmus*	MG940023	Ontario, Canada	(Gariepy et al., 2019)
Cappaeini	*Halyomorpha halys*	KF273401	NA	(Gariepy, Haye, Fraser et al., 2014)
Cappaeini	*Halyomorpha halys*	MF537223	NA	(Valentin et al., 2017)
Cappaeini	*Halyomorpha picus*	MG838372	India	(Gowande et al., 2018)
Cappaeini	*Halyomorpha picus*	MG838371	India	(Gowande et al., 2018)
	Macrorhaphis acuta	ETKC565-13	KZN, South Africa	BOLD
Nezarini	*Nezara viridula*	JX548492	Georgia, USA	(Tillman et al., 2015)
Nezarini	*Nezara viridula*	SAFRA5341-1	KNP, Mpumalanga, South Africa	BOLD
Nezarini	*Nezara viridula*	KR044112	Orlando, Florida, USA	(Gwiazdowski et al., 2015)
Nezarini	*Nezara viridula*	KU601564	Canada	(Dhami et al., 2016)

Nezarini	*Palomena prasina*	KM022897	Berlin, Germany	(Raupach et al., 2014)
Nezarini	*Palomena prasina*	KM022107	Niedersachsen, Germany	(Raupach et al., 2014)
Piezodorini	*Piezodorus* sp.	ETKH174-12	KZN, South Africa	BOLD
Piezodorini	*Piezodorus* sp.	JX548501	USA	(Tillman et al., 2015)
Piezodorini	*Piezodorus* sp.	JX548496	USA	(Tillman et al., 2015)
Pentatomini	*Thyanta pallidovirens*	MF679646	California, USA	(Barman et al., 2017)
Pentatomini	*Thyanta pallidovirens*	HQ106419	British Columbia, Canada	(Park et al., 2011)
Pentatomini	*Thyanta pallidovirens setosa*	KR035886	British Columbia, Canada	(Gwiazdowski et al., 2015)
	Podisus maculiventris	MG940398	Ontario, Canada	(Gariepy et al., 2019)

Table 2. Collection details of specimens sequenced to determine species presence and composition of stink bugs in macadamia orchards in South Africa.

Sample code	Sub family	Tribe	Morphological identification	Date collected	Location	Farm/Town
B9	Pentatominae	Agonoscelidini	*Agonoscelis versicoloratus* (Turton, 1802)	11/2/2019	Limpopo	Bridelia
B19.7				26/2/2019	Limpopo	Bridelia
B6	Pentatominae	Halyini	*Anolcus campestris* Bergroth, 1893	14/2/2019	Limpopo	Bridelia
B17				5/3/2019	Limpopo	Bridelia
M15	Pentatominae	Antestiini	*Antestia* sp. Stål, 1865	20/7/2019	KwaZulu-Natal	Mattison
MP14				10/3/2020	Mpumalanga	Barberton
B9.2				31/10/2017	Limpopo	Bridelia
M25	Pentatominae	Antestiini	*Antestiopsis thunbergii* (Gmelin, 1790)	25/09/2019	KwaZulu-Natal	Mattison
J15				27/11/2019	KwaZulu-Natal	Jacaranda
J11	Pentatominae	Eysarcorini	*Aspavia albidomaculata* (Stål, 1853)	10/4/2020	KwaZulu-Natal	Jacaranda
J12				20/5/2020	KwaZulu-Natal	Jacaranda
M24	Pentatominae	Phyllocephalinae	*Basicryptus costalis* (Germar, 1838)	21/4/2020	KwaZulu-Natal	Mattison
J10				19/7/2019	KwaZulu-Natal	Jacaranda
B2	Pentatominae	Bathycoeliini	*Bathycoelia distincta* Distant, 1878	26/11/2019	Limpopo	Bridelia
B5.1				31/10/2017	Limpopo	Bridelia
B9.1				31/10/2017	Limpopo	Bridelia
M9.1				18/04/18	Limpopo	Muirhead and Roux
M2				24/9/2019	KwaZulu-Natal	Mattison

M18	Pentatominae	Cappaeni	*Boerias* sp. Kirkaldy, 1909	11/7/2019	KwaZulu-Natal	Mattison
J1				5/9/2019	KwaZulu-Natal	Jacaranda
M3.1				13/7/2019	KwaZulu-Natal	Mattison
M3.2				13/7/2019	KwaZulu-Natal	Mattison
M8				16/1/2020	KwaZulu-Natal	Mattison
M13				11/3/2020	KwaZulu-Natal	Mattison
J13	Pentatominae	Eyasarcorini	*Carbula recurva* Distant, 1915	5/12/2019	KwaZulu-Natal	Jacaranda
J14				15/4/2020	KwaZulu-Natal	Jacaranda
B7	Pentatominae	Cappaeni	*Caura rufiventris* (Germar, 1838)	26/2/2019	Limpopo	Bridelia
B10				19/02/2019	Limpopo	Bridelia
M1.1	Pentatominae	Nezarini	*Chinavia pallidoconspersa* (Stål, 1858)	20/7/2019	KwaZulu-Natal	Mattison
MP17				11/5/2020	Mpumalanga	Barberton
J8				3/6/2020	KwaZulu-Natal	Jacaranda
B12				20/8/2019	Limpopo	Bridelia
M20	Pentatominae	Halyini	*Coenomorpha nervosa* Dallas, 1851	20/5/2020	KwaZulu-Natal	Mattison
MP20				12/3/2020	Mpumalanga	Kiepersol
M14	Asopinae	-	*Macrorhaphis acuta* Dallas, 1851	12/6/2020	KwaZulu-Natal	Mattison
B7.3				25/7/2017	Limpopo	Bridelia
MP12	Pentatominae	Nezarini	*Nezara viridula* (Linnaeus, 1758)	11/5/2020	Mpumalanga	Barberton
MR2				Apr-19	Limpopo	Muirhead and Roux
MP3				12/3/2020	Mpumalanga	Kiepersol

J3				10/7/2019	KwaZulu-Natal	Jacaranda
B7.5				25/7/2017	Limpopo	Bridelia
B7.2				25/7/2017	Limpopo	Bridelia
MP8	Pentatominae	Nezarini	*Parachinavia prunasis* (Dallas, 1851)	16/4/2020	Mpumalanga	Barberton
MP10				4/6/2020	Mpumalanga	Barberton
B15.2				19/9/2017	Limpopo	Bridelia
MR4				01/2019	Limpopo	Muirhead and Roux
B4	Pentatominae	Antestiini	*Parantestia* sp. (Linnavuori, 1973)	12/2/2019	Limpopo	Bridelia
M9.2				18/04/18	Limpopo	Muirhead and Roux
MP5				20/06/06	Mpumalanga	Barberton
M16				30/4/2020	KwaZulu-Natal	Mattison
J7				20/3/2020	KwaZulu-Natal	Jacaranda
M15.2	Pentatominae	Piezodorini	*Piezodorus* sp. (Fieber, 1860)	18/04/18	Limpopo	Muirhead and Roux
B3.1				5/9/2017	Limpopo	Bridelia
MP18				6/6/2019	Mpumalanga	Barberton
B19.5	Pentatominae	Eurysaspidini	*Platacantha lutea* (Westwood, 1837)	5/3/2019	Limpopo	Bridelia
MP2				12/3/2020	Mpumalanga	Kiepersol
M9	Pentatominae	Halyini	*Pseudatelus raptorius* (Germar, 1838)	24/4/2020	KwaZulu-Natal	Mattison
M11				5/6/2020	KwaZulu-Natal	Mattison
MP6				12/3/2020	Mpumalanga	Kiepersol
MP7				12/3/2020	Mpumalanga	Kiepersol

M26	Pentatominae	Cappaeni	*Tripanda signitenens* (Distant, 1898)	6/8/2019	KwaZulu-Natal	Mattison
J20				6/3/2020	KwaZulu-Natal	Jacaranda

Table 3. Proportion of stink bug species in scout batches obtained in KwaZulu-Natal, Limpopo and Mpumalanga represented by the number of batches each species was found in and the percentage thereof.

Region	Total scout batches	*B. distincta*		*N. viridula*		*Parantestia* sp.		*P. prunasis*		*Piezodorus* sp.		*P. raptorius*		*C. pallidoconspersa*		*Boerias* spp.	
Limpopo	55	50	90.91	31	56.36	12	21.82	6	10.91	24	43.64	2	4	23	41.82	-	-
KwaZulu-Natal	166	93	54.07	22	12.79	45	26.16	6	3.49	0	0	24	25.81	16	9.3	103	59.9
Mpumalanga	32	23	71.88	14	43.75	12	37.5	7	21.88	6	18.75	2	8.70	14	43.75	-	-
Total	253	166	64.09	67	25.87	69	26.64	19	7.34	30	11.85	28	16.87	53	20.46	103	39.8

Table 4. Proportion of stink bug species in scout batches obtained in Limpopo over three seasons represented by the number of batches each species was found in and the percentage thereof.

Season	Total scout batches	*B. distincta*		*N. viridula*		*Parantestia* sp.		*P. prunasis*		*Piezodorus* sp.		*P. raptorius*		*C. pallidoconspersa*	
2017-2018	37	18	48.65	29	78.38	12	32.43	0	0	12	32.43	0	0	5	13.51
2018-2019	52	43	82.69	11	21.15	15	28.85	3	5.77	3	5.77	2	3.85	7	13.46
2019-2020	55	50	90.91	31	56.36	12	21.82	6	10.91	24	43.64	1	1.82	23	41.82

Table 5. Nucleotide diversity (π) values for 8 of the stink bug species from this study.

Stink bug species	Nucleotide diversity (π)	Percentage nucleotide diversity
Bathycoelia distincta	0.00424	0.424
Boerias sp.	0.07452	7.5
Chinavia pallidoconspersa	0.00085	0.085
Nezara viridula	0.00494	0.494
Parachinavia prunasis	0.00106	0.11
Parantestia sp.	0.00257	0.26
Piezodorus sp.	0.01961	1.96
Pseudatelus raptorius	0.03805	3.8

Table 6. Average number of nucleotide substitutions per site between populations (D_{xy}) and number of net nucleotide substitutions per site between populations (D_a) for some of the stink bug species from this study.

	Boerias sp.				*C. pallidonconspersa*				*N. viridula*				*P. prunasis*				*P. raptorius*				*Parantestia* sp.				*Piezodorus* sp.			
	Dxy	*%*	*Da*	*%*	*Dxy*	*%*	*Da*	*%*	*Dxy*	*%*	*Da*	*%*	*Dxy*	*%*	*Da*	*%*	*Dxy*	*%*	*Da*	*%*	*Dxy*	*%*	*Da*	*%*	*Dxy*	*%*	*Da*	*%*
1	0.124	12.4	0.084	8.4	0.127	12.7	0.124	12.4	0.130	13.0	0.126	12.6	0.144	14.4	0.141	14.1	0.175	17.5	0.154	15.4	0.145	14.5	0.141	14.1	0.126	12.6	0.121	12.1
2	0.136	13.6	0.098	9.8					0.135	13.5	0.133	13.3	0.143	14.3	0.142	14.2	0.170	17.0	0.151	15.1	0.130	13.0	0.128	12.8	0.125	12.5	0.122	12.2
3	0.138	13.8	0.099	9.9									0.131	13.1	0.129	12.9	0.166	16.6	0.146	14.6	0.115	11.5	0.112	11.2	0.141	14.1	0.137	13.7
4	0.144	14.4	0.106	10.6													0.173	17.3	0.153	15.3	0.134	13.4	0.133	13.3	0.144	14.4	0.106	10.6
5	0.162	16.2	0.106	10.6																	0.159	15.9	0.139	13.9	0.160	16.0	0.139	13.9
6	0.132	13.2	0.094	9.4																					0.118	11.8	0.114	11.4
7	0.126	12.6	0.086	8.6																								

Key	
1	*B. distincta*
2	*C. pallidoconspersa*
3	*N. viridula*
4	*P. prunasis*
5	*P. raptorius*
6	*Parantestia* sp.
7	*Piezodorus* sp.

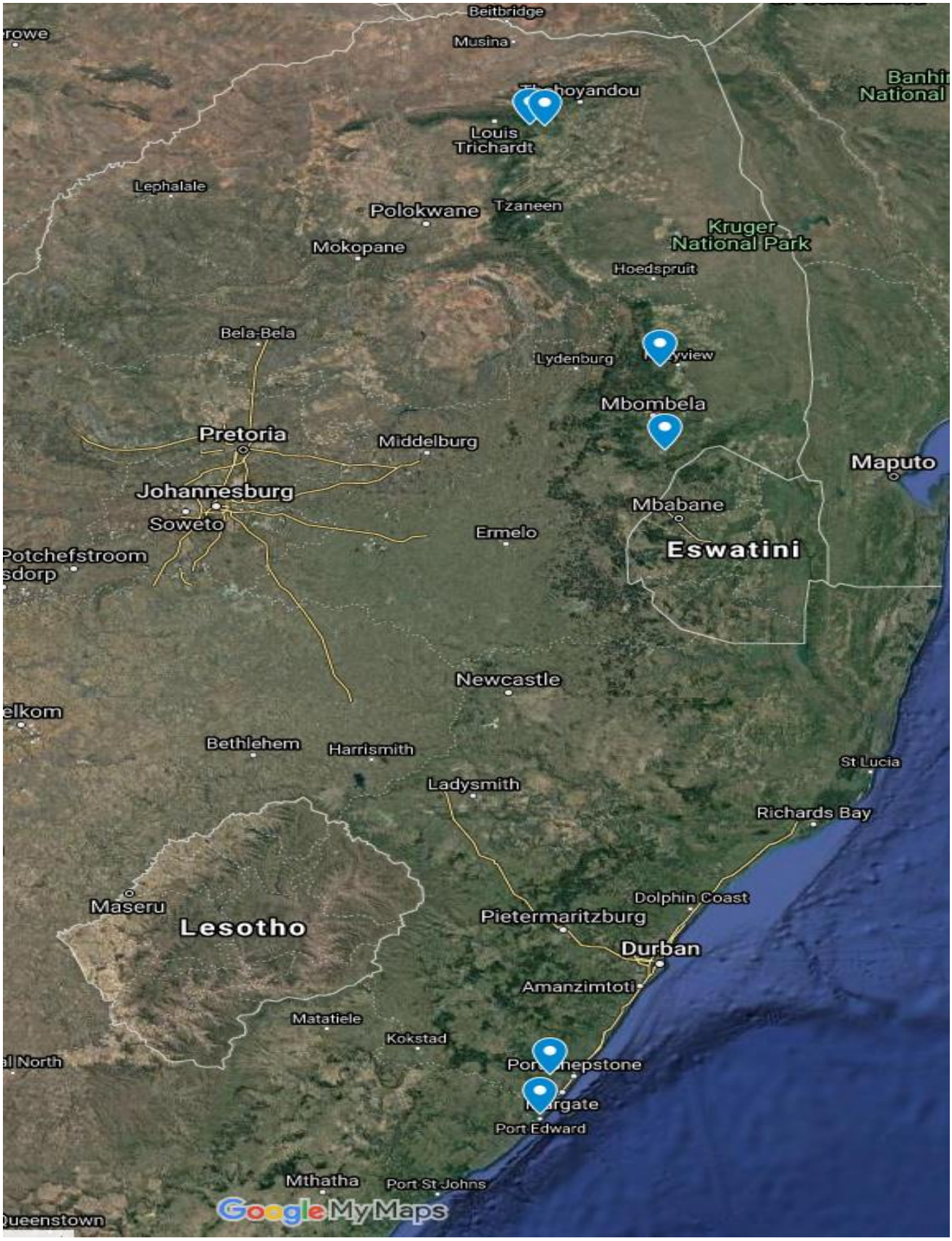


Figure 1. Map of the farm locations where stink bugs were sampled in KwaZulu-Natal, Limpopo and Mpumalanga (Google Maps 2020).

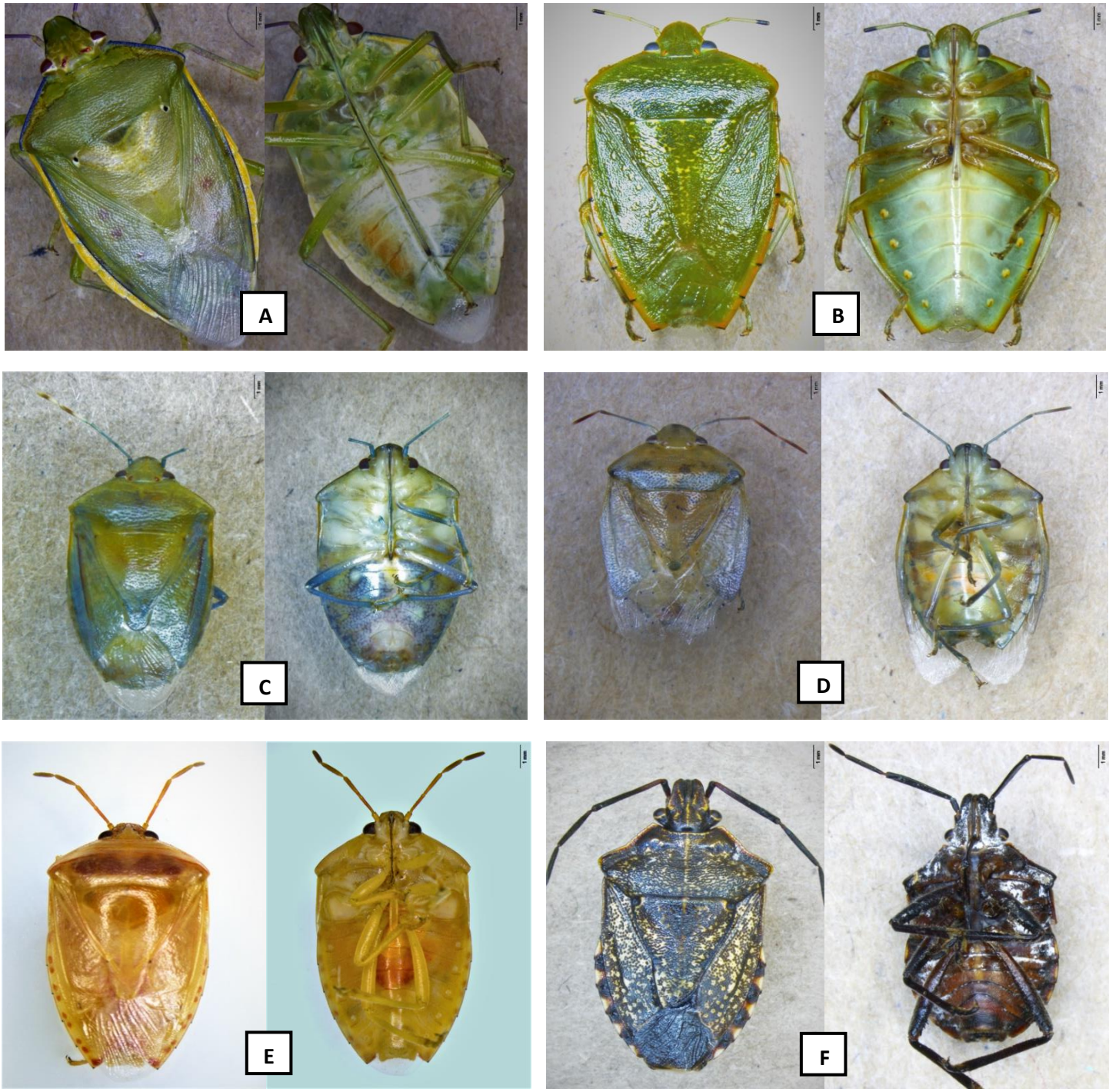


Figure 2. Pentatomidae species in common or high proportions in KwaZulu-Natal, Limpopo and Mpumalanga A. *Bathycoelia distincta* B. *Chinavia pallidoconspersa* C. *Parantestia* species. D. *Parachinavia prunasis* E. *Piezodorus* sp. F. *Pseudatelus raptorius*

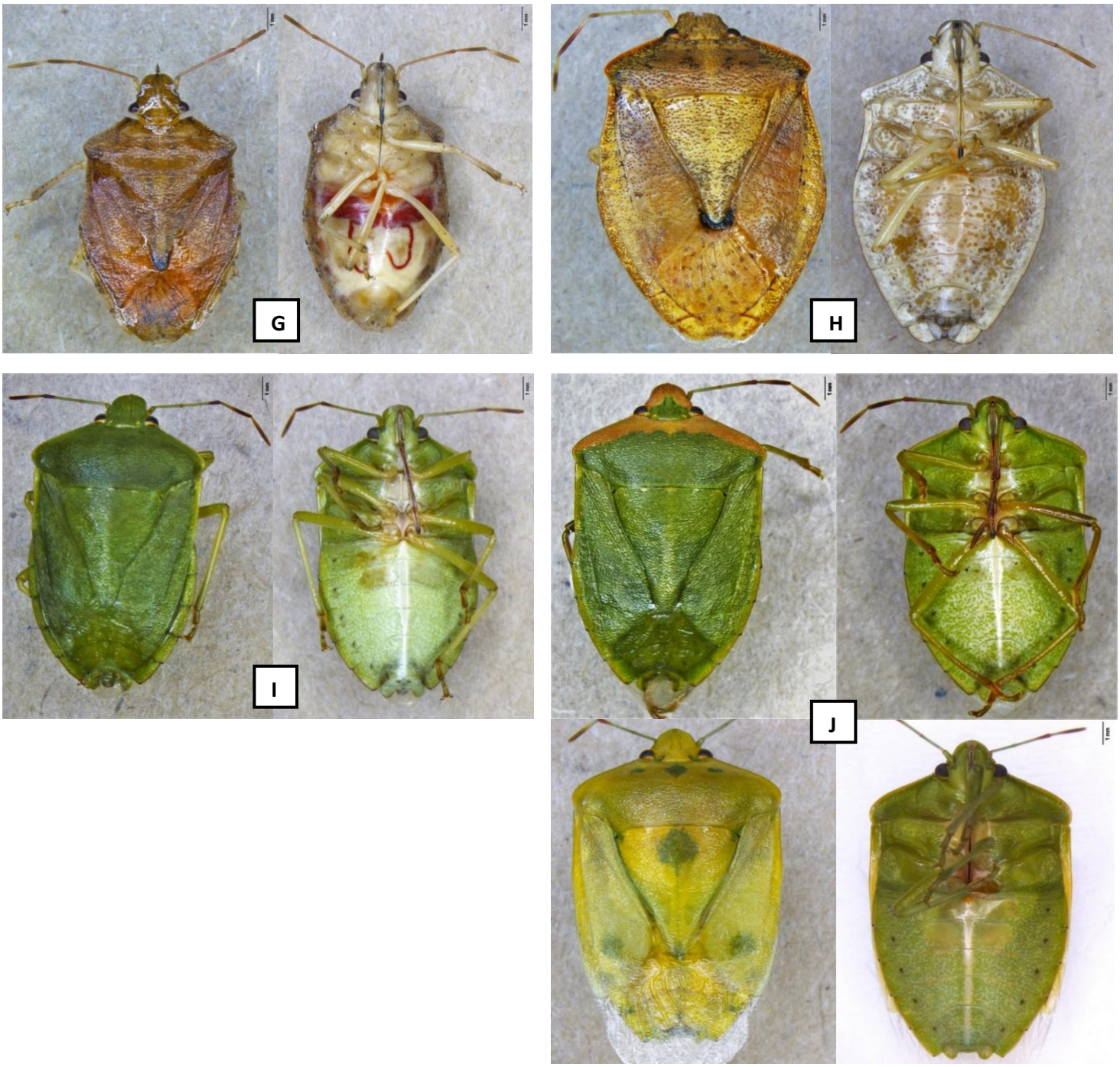


Figure 2 (continued). Pentatomidae species in common or high proportions KwaZulu-Natal, Limpopo and Mpumalanga G. *Boerias* sp. 1 H. *Boerias* sp. 2 I. *Nezara viridula* J. *Nezara viridula* colour morphs

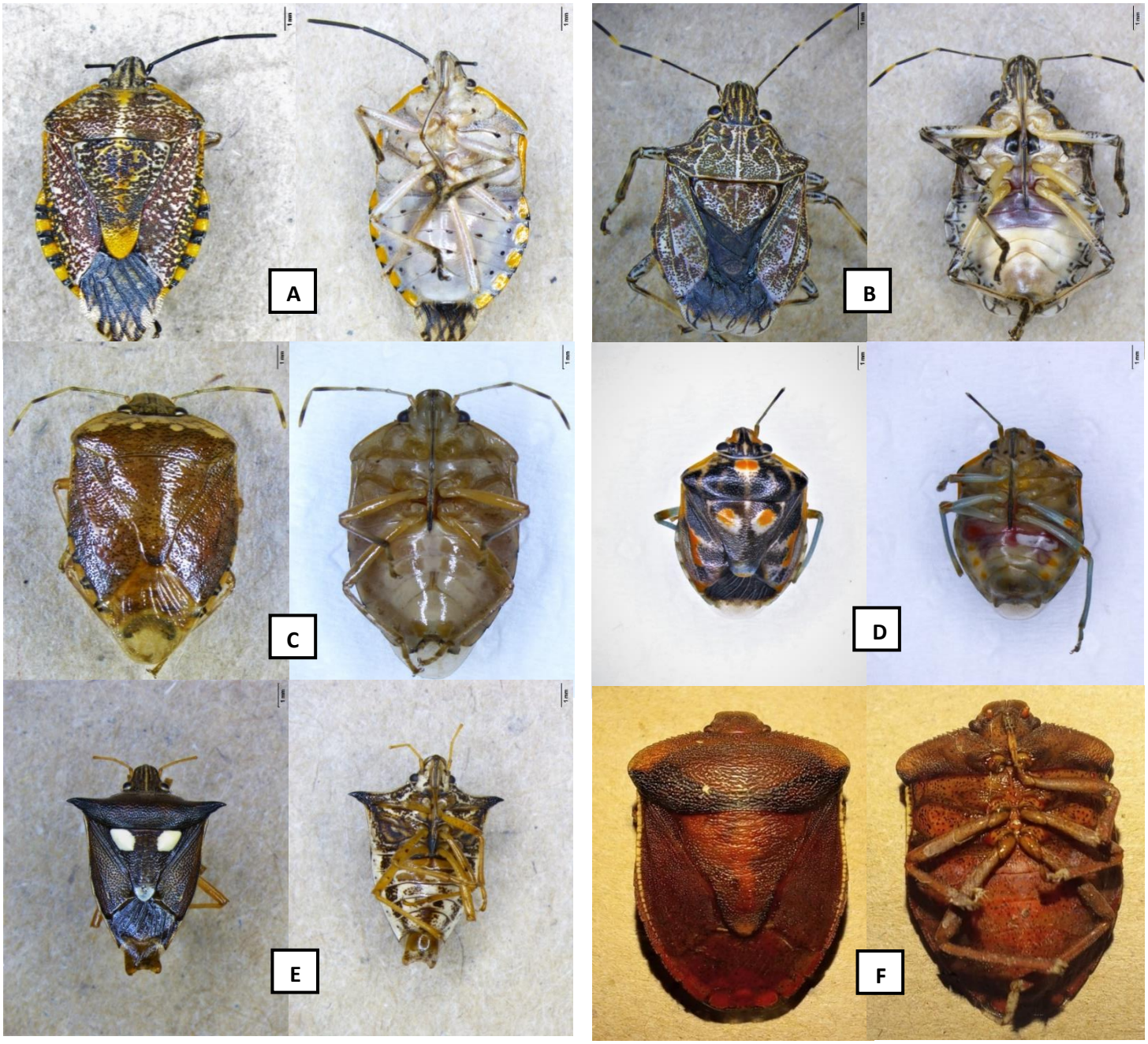


Figure 3. Pentatomidae species found in low proportions in KwaZulu-Natal, Limpopo and Mpumalanga A. *Agonoscelis versicoloratus* B. *Anolcus campestris* C. *Antestia sp.* D. *Antestiopsis thunbergii* E. *Aspavia albidomaculata* F. *Basicryptus costalis*

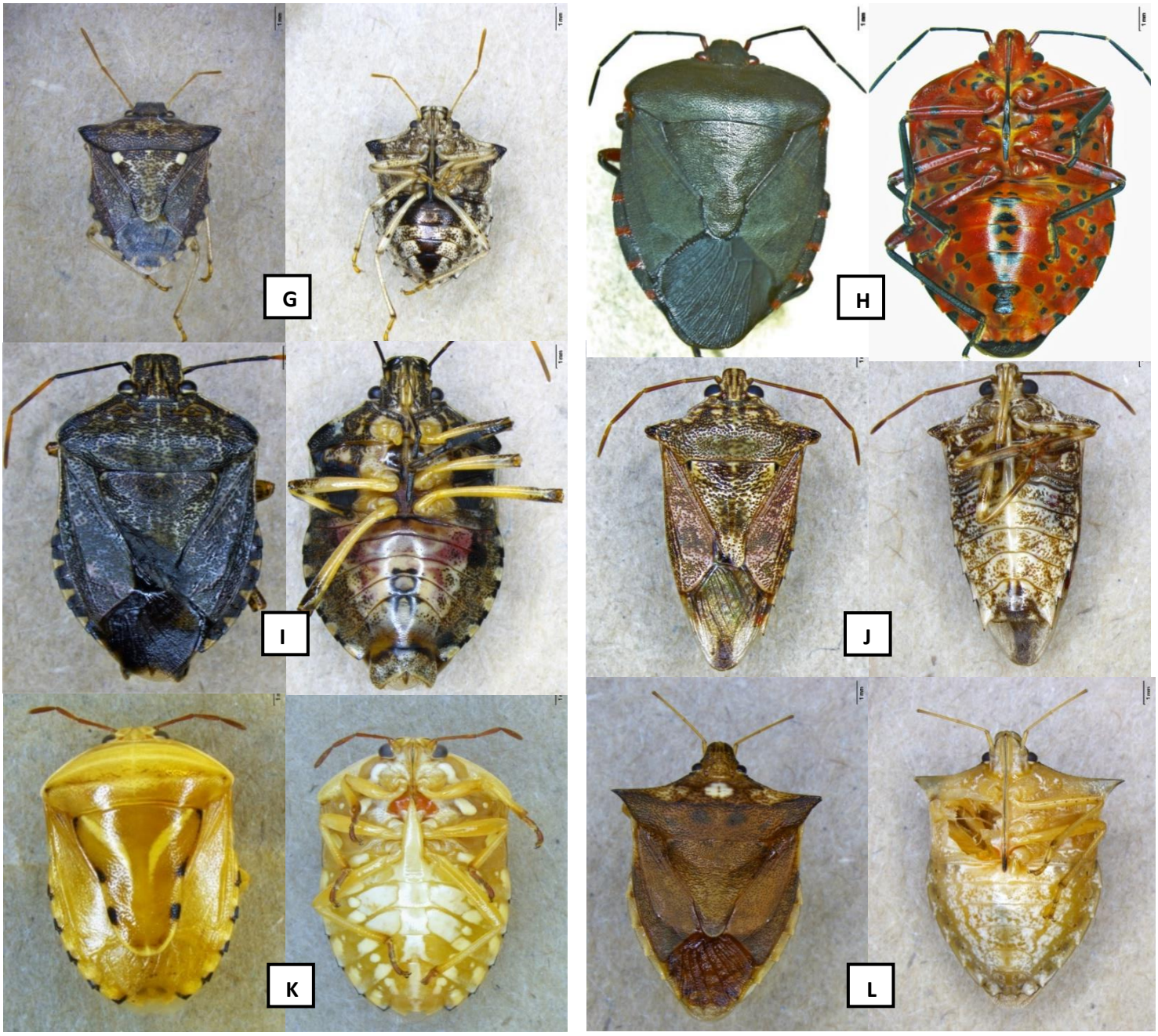


Figure 3 (continued). Pentatomidae species found in low proportions in KwaZulu-Natal, Limpopo and Mpumalanga G. *Carbula recurva* H. *Caura rufiventris* I. *Coenomorpha nervosa* J. *Macrorhaphis acuta* K. *Platacantha lutea* L. *Tripanda signitenens*

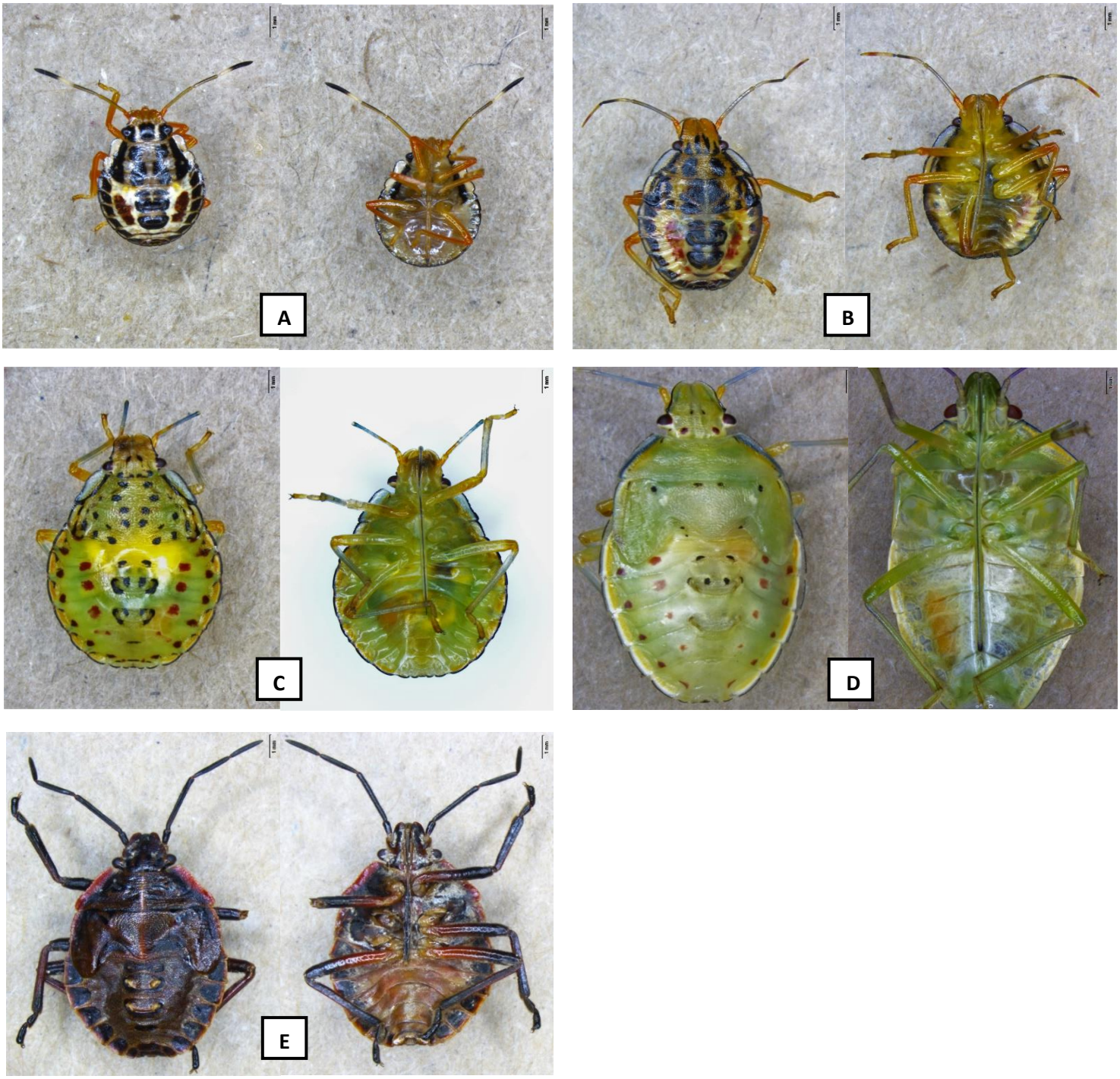


Figure 4. Nymphs found in this study. *B. distincta* A. Second instar B. Third instar C. Fourth instar D. Fifth instar and E. *P. raptorius*

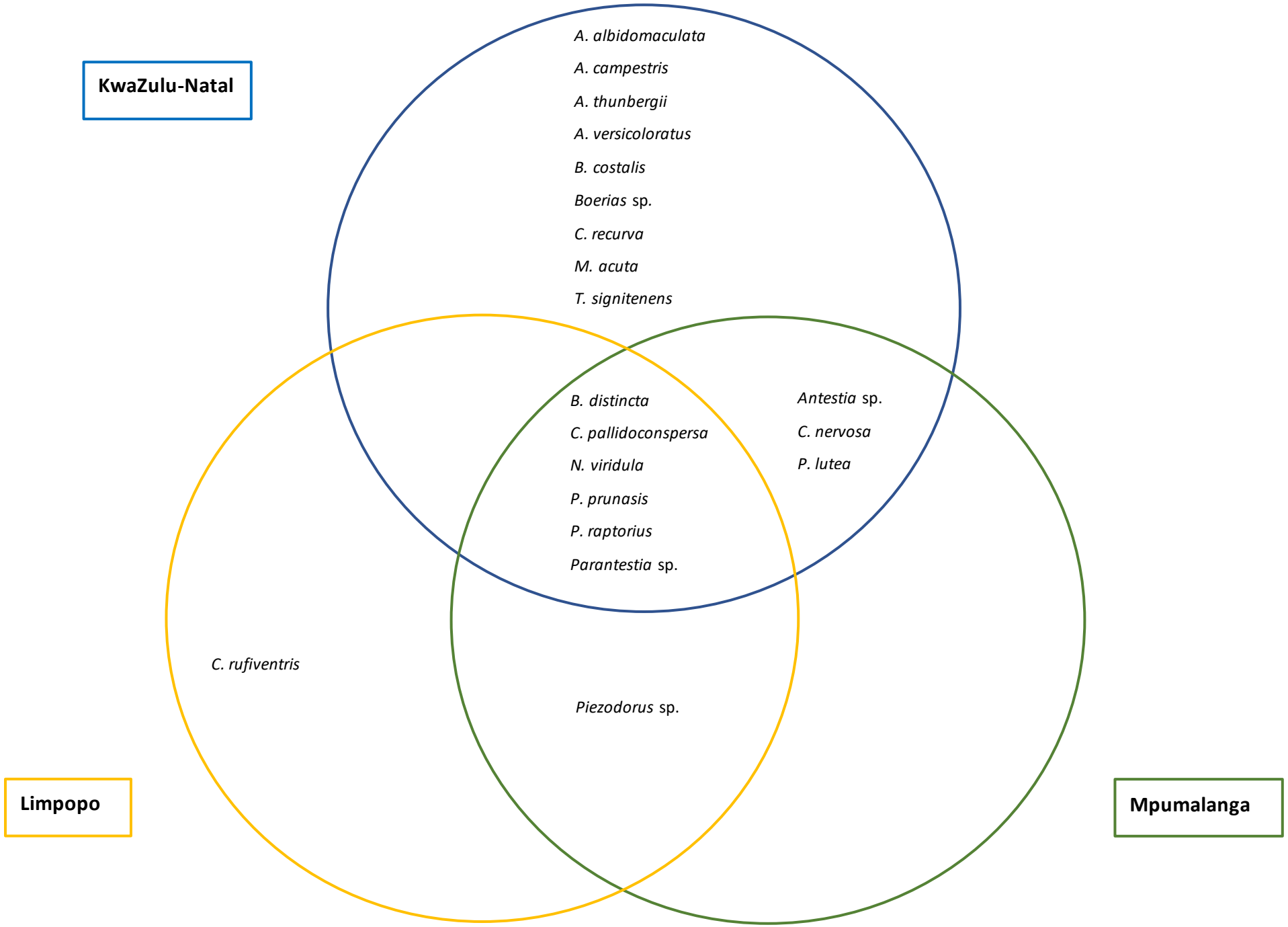


Figure 5. Stink bug species presence in KwaZulu-Natal, Limpopo and Mpumalanga for the 2019-2020 growing season.

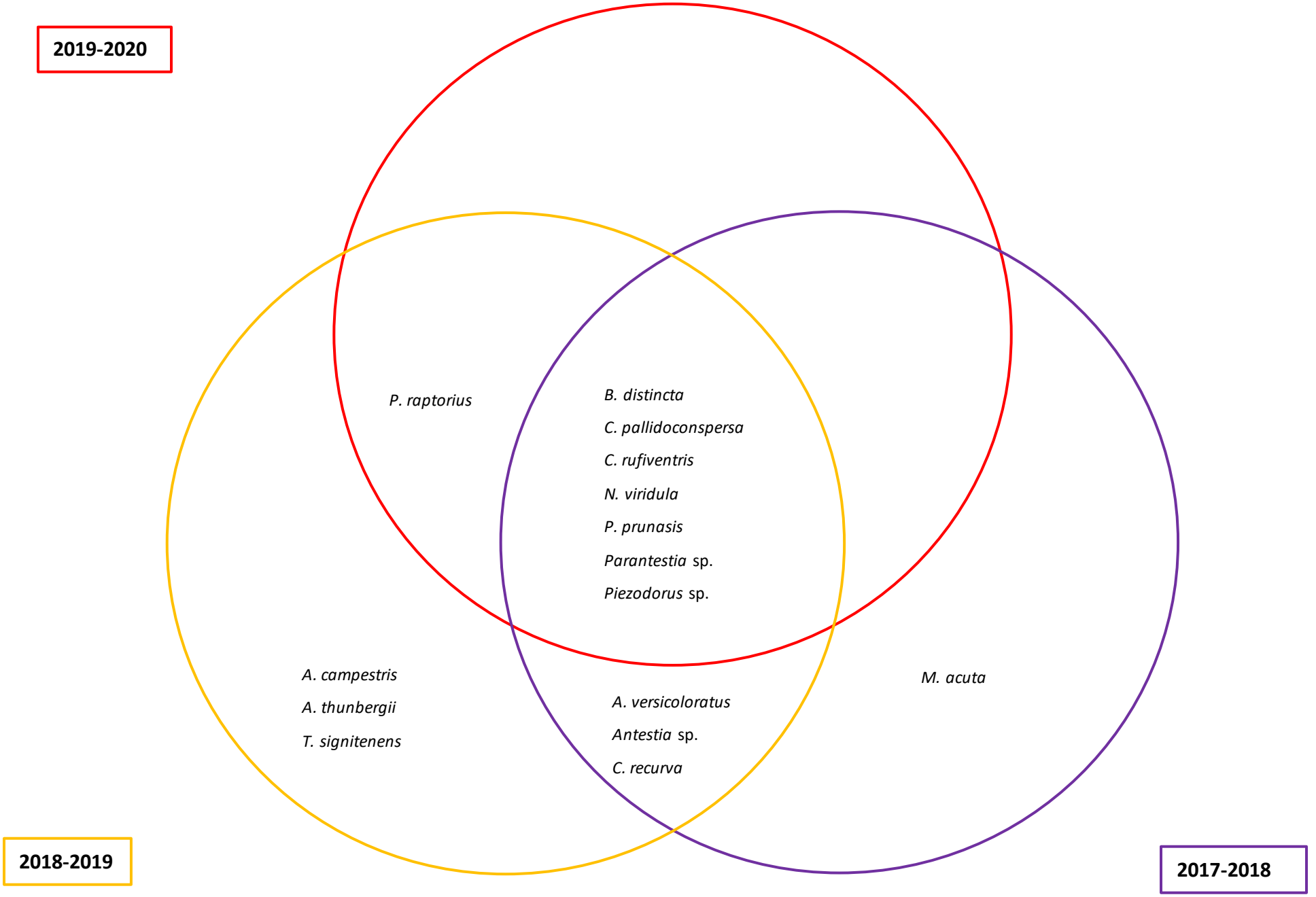


Figure 6. Stink bug species presence across three growing seasons (2017-2020) in the Limpopo growing region.

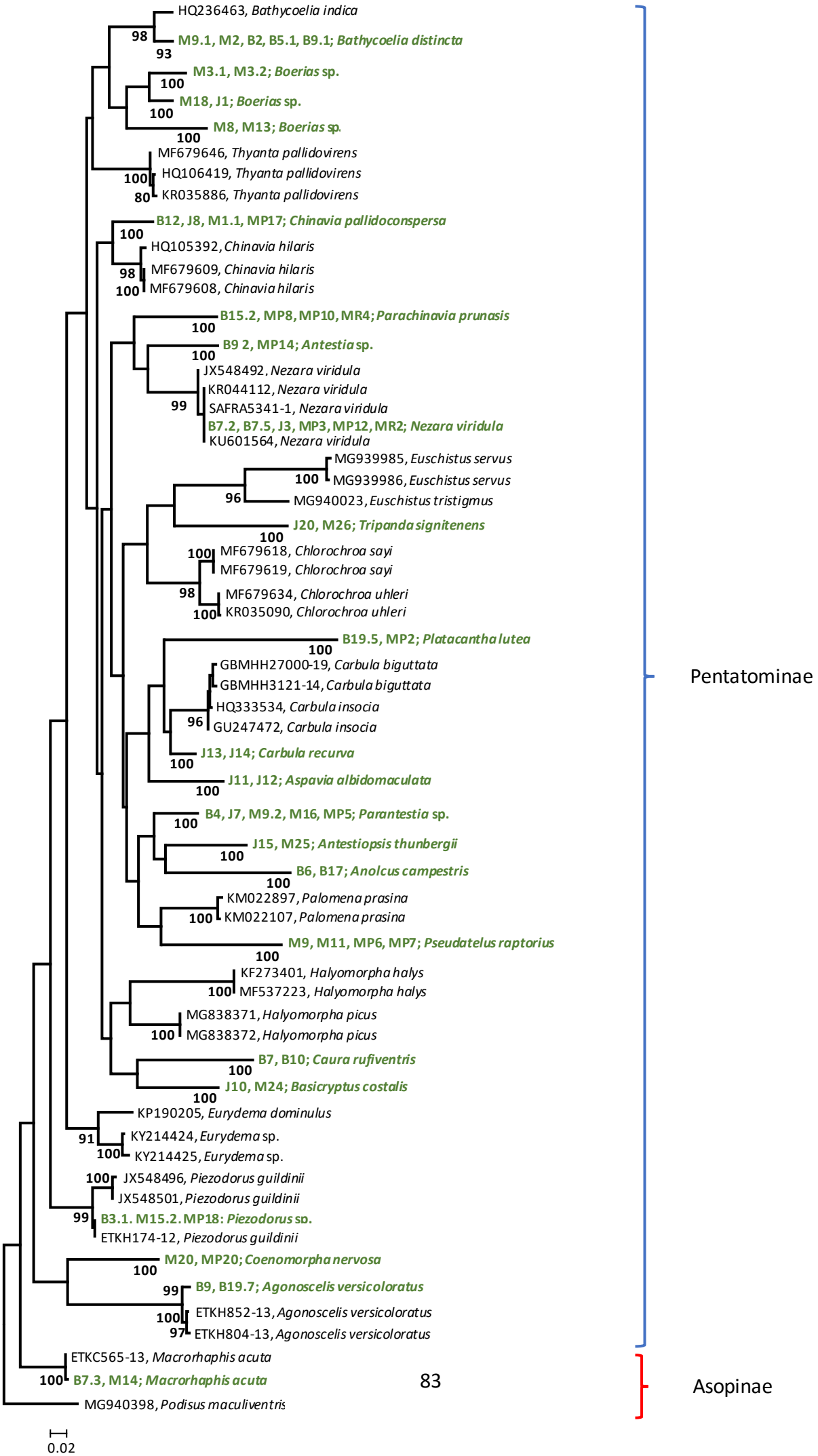
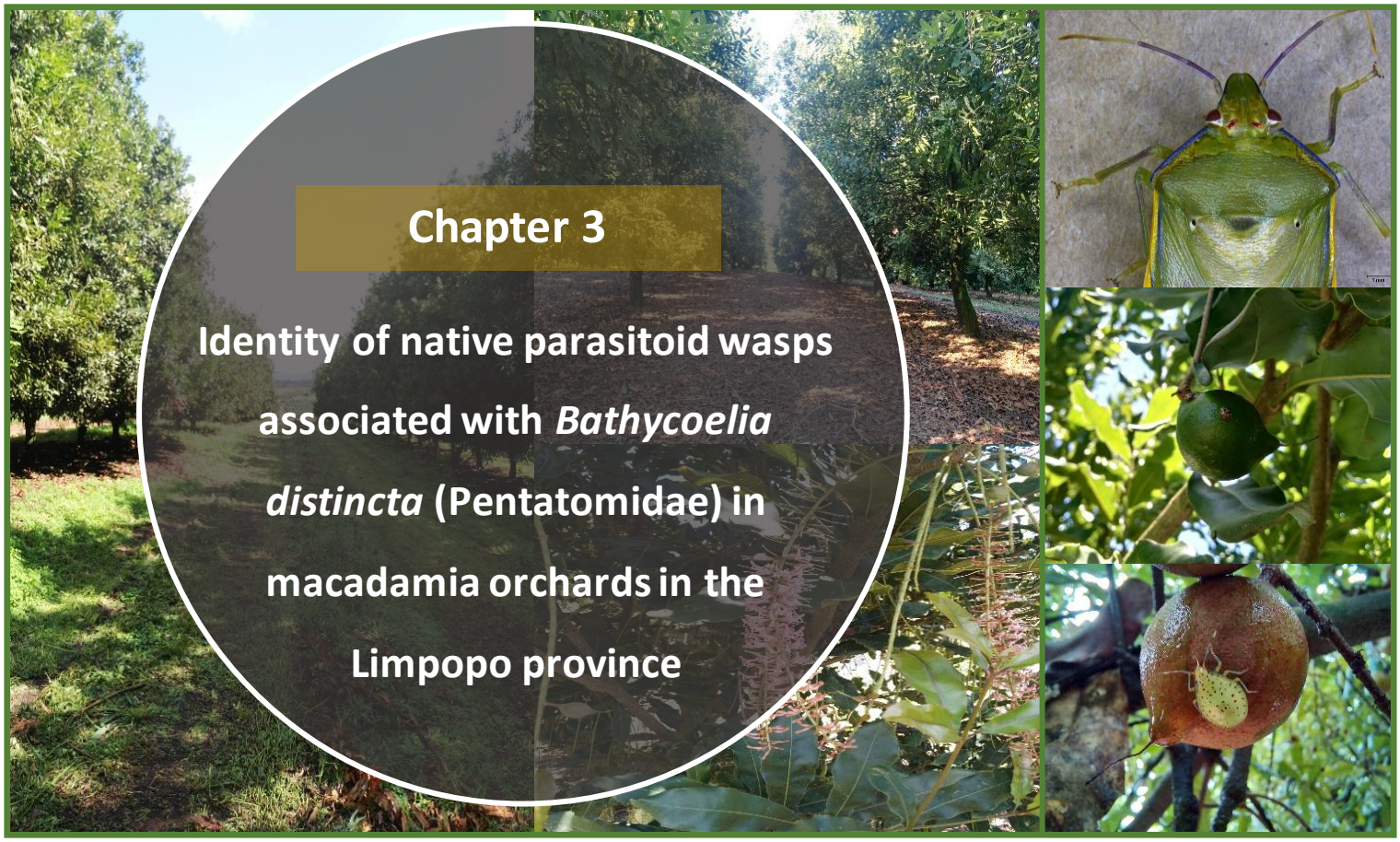

HQ236463, Bathycoelia indica
98
M9.1, M2, B2, B5.1, B9.1; Bathycoelia distincta
93
M3.1, M3.2; Boerias sp.
100
M18, J1; Boerias sp.
100
M8, M13; Boerias sp.
100
MF679646, Thyanta pallidovirens
HQ106419, Thyanta pallidovirens
100
KR035886, Thyanta pallidovirens
80
B12, J8, M1.1, MP17; Chinavia pallidoconspersa
100
HQ105392, Chinavia hilaris
MF679609, Chinavia hilaris
98
MF679608, Chinavia hilaris
100
B15.2, MP8, MP10, MR4; Parachinavia prunasis
100
B9 2, MP14; Antestia sp.
100
JX548492. Nezara viridula
KR044112, Nezara viridula
99
SAFRA5341-1, Nezara viridula
B7.2, B7.5, J3, MP3, MP12, MR2; Nezara viridula
KU601564, Nezara viridula
MG939985, Euschistus servus
100
MG939986, Euschistus servus
96
MG940023, Euschistus tristigmus
J20, M26; Tripanda signitenens
100
100
MF679618, Chlorochroa sayi
MF679619, Chlorochroa sayi
98
MF679634, Chlorochroa uhleri
100
KR035090, Chlorochroa uhleri
B19.5, MP2; Platacantha lutea
100
GBMHH27000-19, Carbula biguttata
GBMHH3121-14, Carbula biguttata
HQ333534, Carbula insocia
96
GU247472, Carbula insocia
J13, J14; Carbula recurva
100
J11, J12; Aspavia albidomaculata
100
B4, J7, M9.2, M16, MP5; Parantestia sp.
100
J15, M25; Antestiopsis thunbergii
100
B6, B17; Anolcus campestris
100
KM022897, Palomena prasina
100
KM022107, Palomena prasina
M9, M11, MP6, MP7; Pseudatelus raptorius
100
KF273401, Halyomorpha halys
100
MF537223, Halyomorpha halys
MG838371, Halyomorpha picus
100
MG838372, Halyomorpha picus
B7, B10; Caura rufiventris
100
J10, M24; Basicryptus costalis
100
KP190205, Eurydema dominulus
91
KY214424, Eurydema sp.
100
KY214425, Eurydema sp.
100
JX548496, Piezodorus guildinii
JX548501, Piezodorus guildinii
99
B3.1. M15.2. MP18: Piezodorus sp.
ETKH174-12, Piezodorus guildinii
M20, MP20; Coenomorpha nervosa
100
99
B9, B19.7; Agonoscelis versicoloratus
100
ETKH852-13, Agonoscelis versicoloratus
97
ETKH804-13, Agonoscelis versicoloratus
ETKC565-13, Macrorhaphis acuta
100
B7.3, M14; Macrorhaphis acuta
MG940398, Podisus maculiventris
0.02
Pentatominae
Asopinae

Figure 7. Maximum likelihood stink bug phylogeny based on the COI gene region. The specimens in green represent morphologically identified stink bugs obtained from macadamia orchards in the KwaZulu-Natal, Limpopo and Mpumalanga provinces of South Africa.



Chapter 3

Identity of native parasitoid wasps associated with *Bathycoelia distincta* (Pentatomidae) in macadamia orchards in the Limpopo province

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3.1. Abstract

The two spotted stink bug, *Bathycoelia distincta* (Hemiptera: Pentatomidae) is the dominant and most damaging stink bug species present in macadamia orchards in South Africa. The heavy reliance on pesticides to control stink bugs is of great concern and increases the need for alternative control methods such as biological control. One option for such a biological control programme is native parasitoids. Many parasitoids are known to be associated with stink bugs but accurate identification of these parasitoids can be difficult. The aim of this study was to identify native parasitoids of *B. distincta* present in the Levubu growing region of the Limpopo province of South Africa. We also test different molecular methods for their identification. Based on a DNA barcoding approach of the *cytochrome c oxidase subunit 1* (COI) gene, two parasitoid species were found parasitizing *B. distincta* eggs. These species were identified as *Psix striaticeps* (Hymenoptera: Scelionidae) and a *Trissolus* species (Hymenoptera: Scelionidae). Family-specific primers developed in a previous study were tested to determine if they can be used as a diagnostic tool to identify native wasp and stink bug DNA in parasitized eggs. These primers were effective for only two of the three parasitoid species tested and positively confirmed *B. distincta* as the host. COI sequencing of emerged wasp parasitoids was the most reliable approach to identify the three species.

3.2. Introduction

The need for alternative control methods against *Bathycoelia distincta* Distant, 1878 (Hemiptera: Pentatomidae) in macadamia orchards in South Africa is increasing. *Bathycoelia distincta*, the two-spotted stink bug, is considered the most important stink bug species in South Africa, due to the extensive damage it inflicts in macadamia orchards (*Macadamia integrifolia* Maiden & Betche and *Macadamia tetraphylla* L. Johnson) (Schoeman, 2013; Bruwer et al., 2021). Biological control using parasitoid wasps is a promising approach that can be implemented along with other stink bug control methods, such as the use of pheromones or vibrations that interrupt insect communication, as part of an Integrated Pest Management approach (Grazia & Schwertner, 2017). An important step when developing a biological control programme is to investigate native natural enemies that could prevent the unnecessary importation and release of exotic biocontrol agents, and thus avoid the costs, time and potential risks that are associated with importing exotic biocontrol agents (Kenis et al., 2019).

Stink bugs (Hemiptera: Pentatomidae) are a widespread pest of various crops worldwide. Parasitoids of the genera *Gryon* Haliday, 1833*, Psix* Kozlov & Lê, 1976*, Telenomus* Haliday, 1833 and *Trissolcus* Ashmead, 1893 (Scelionidae: Telenominae) are associated with many of these Pentatomidae species (Austin et al., 2005; Jones, 1988; Orr, 1988). The best-known example is the *Trissolcus basalis* (Wollaston, 1858) (Scelionidae: Telenominae) – *Nezara viridula* (Linnaeus, 1758) (Hemiptera: Pentatomidae) parasitoid-host association that has become the model system for research pertaining to egg parasitism of stink bugs. *Trissolcus basalis* was found to be the most common and closely associated parasitoid of *N. viridula* throughout its geographic distribution (Jones, 1988). Biological control programs utilizing *T. basalis* to control *N. viridula* have been implemented in areas such as Argentina, Australia, Brazil, Italy, New Zealand, Hawaii, the USA, Taiwan, South America, southern Africa and a number of Pacific islands (Waterhouse, 1998; McPherson, 2018).

Accurate identification of parasitoid species in question is a vital step in studies directed towards successful biological control. Failure to differentiate between closely related species of parasitoids due to the limitations of traditional identification methods (e.g. dissection and rearing) can result in inaccurate conclusions of host-parasitoid associations (Day, 1994; Gariepy et al., 2008). To overcome these challenges, molecular tools to detect and identify individuals in host-parasitoid associations have been developed (Gariepy et al., 2014; Gariepy et al., 2019; Stahl et al., 2019). These tools target the *cytochrome c oxidase subunit 1* (COI) gene region since Guz et al. (2013) concluded that the COI gene is more informative in differentiating species of *Trissolcus* than the 18S, 28S, ITS1 and ITS2 loci. The diagnostic tool makes use of family-specific forward primers nested within the COI gene region to distinguish between host (Pentatomidae) and parasitoid (Scelionidae) DNA within a parasitized egg (Gariepy et al., 2014, 2019). This molecular tool was further adapted to be more specific by designing species-specific primers for *Anastatus bifasciatus* (Geoffroy, 1785) (Hymenoptera: Eupelmidae) and *Trissolcus japonicus* (Ashmead, 1904) (Hymenoptera: Scelionidae) to study their associations with *Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae) (Stahl et al., 2019; Chen et al., 2021).

Knowledge regarding parasitoid wasp species associated with Pentatomidae in South Africa is currently limited. The species-level identities of the Scelionidae wasps attacking *B. distincta* in macadamia orchards in South Africa is currently unknown. A previous study reported *Pediobus* sp. (Walker 1846) (Hymenoptera: Eulophidae), *Pachyneuron* sp. Walker, 1833 (Hymenoptera: Pteromalidae), two species of *Trissolcus* and an undetermined Scelionidae species in Nelspruit, Mpumalanga and Levubu, Limpopo (Bruwer, 1992), however not much has been reported regarding the diversity and prevalence of parasitoids in orchards in the last 20 years. The aim of this study was to identify the native parasitoid wasp species present in Limpopo by developing a COI DNA barcoding approach supplemented by morphological identification. The molecular tool to detect and identify individuals in host-parasitoid associations developed by Gariepy et al. (2014) was also assessed for the *B. distincta* – parasitoid system in South Africa. This was to determine the best approach to identify native wasp parasitoids, either sequencing emerged adult parasitoids using the standard Folmer et al. primers (1994), or using family-specific primers to sequence unhatched parasitized stink bug eggs.

3.3. Materials and methods

3.3.1. Insect collection

Parasitized *B. distincta* egg batches were sampled from a farm in the Levubu growing region of the Limpopo province, South Africa in January 2019. Branches, leaves and trunks of macadamia trees in two orchards were inspected. Egg batches were placed in plastic tubes with a tight-fitting gauze lid and kept at 22˚C, 16L:8D and 85% RH, allowing the adult parasitoids to emerge. A second batch of parasitized egg batches were collected in February 2020. These egg batches were stored directly in absolute ethanol at -4˚C for molecular analysis.

3.3.2. Parasitoid identification

3.3.2.1. Morphology

Wasps of the Scelionidae family are generally well sclerotized with detailed sculpturing (Austin et al., 2005; Masner, 1993). They possess wings with greatly reduced venation while the antennae are positioned slightly above the mouth and close together and a prepectus located behind the lateral pronotum is absent in this group. Differences in the antennae, wing venation, body length and colour as well as distinguishing features present/absent on parts of the head, mesosoma and metasoma are used to differentiate species within the Telenominae subfamily including the *Psix* and *Trissolcus* genera (Johnson & Masner, 1985; Masner, 1993; Austin et al., 2005; Talamas et al., 2017). The minute size of scelionid wasps and the complexity of the distinguishing characteristics used to differentiate species makes species-level identification challenging for non-specialists. Representatives of the species found in this study were sent to Biosystematics Division, ARC-Plant Health and Protection, Roodeplaat, Pretoria for identification.

3.3.2.2. DNA extraction, amplification and sequencing

Genomic DNA was extracted from adult parasitoids using prepGEM® Insect kit (ZyGEM™, Hamilton, New Zealand) following the manufacturer's instructions. A total of 67 parasitoid wasps, representing 10 percent of the total wasp specimens collected, were selected based on morphology for sequencing. Genomic DNA was also extracted from *T. basalis,* obtained from Koppert Biological Systems as a positive control. A nanodrop spectrophotometer was used to measure the DNA concentration. To amplify the COI region, PCRs were performed using a mixture of 5 µl of 5 mM MyTaq™ reaction buffer (Bioline, South Africa) containing $MgCl_2$ and dNTPs, 0.5 µl of MyTaq™ DNA polymerase (Bioline, South Africa), 0.5 µl of 10 mM of LCO 1490 (5' -GGTCAACAAATCATAAAGATATTGG - 3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAA ATCA - 3') (Folmer et al., 1994), 16.5 µl of ddH_2O (Adcock Ingram, Bryanston, South Africa) and 2 µl of template DNA to make the final reaction volume of 25 µl. A negative control was added to ensure the absence of contamination. PCR amplification was done under the following cycling conditions: initial denaturation at 94 °C for 3 mins, followed by 35 cycles of 94 °C for 30 s, 52 °C for 1 min and 72 °C for 1 min with a final hold of 10 min at 72 °C.

Amplicons were visualized via agarose gel electrophoresis using GelRed (Biotium, Hayward, California, USA) and a 100 bp DNA ladder (Promega, USA) to determine amplicon size. A Molecular Imager® GelDoc™ (Bio-Rad) was used to visualize the amplicons under ultraviolet light. This was followed by a PCR product clean up reaction using ExoSAP-IT™ (Applied Biosystems, USA) according to the manufacturer's instructions. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was used to sequence the PCR products in both directions. The thermal cycler was programmed as follows: initial denaturation at 96°C for 2 min, followed by 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The samples were precipitated using sodium acetate and sequenced using an ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystems, USA) at the Bioinformatics Sequencing facility at the University of Pretoria.

3.3.2.3. Phylogenetic analysis

Consensus sequences were generated from aligned forward and reverse sequences that were manually edited using Biological Sequence Alignment Editor (BioEdit) (Hall, Biosciences & Carlsbad 2011). Basic Local Alignment Tool (BLAST) searches were conducted against the National Center for Biotechnology Institute (NCBI) GenBank (www.ncbi.nlm.nih.gov) and Barcode of Life Data systems (BOLD) (www.boldsystems.org) databases for each consensus sequence. Parasitoid wasp COI sequences mined from the NCBI GenBank database were used to supplement the sequence dataset (Table 1, Taekul et al., 2014; Hebert et al., 2016; Matsuo et al., 2018; Gariepy et al., 2019; Kenis et al., 2019; Talamas et al., 2019). These sequences represented 12 species belonging to three genera within the Telenominae (Hymenoptera: Scelionidae) subfamily. Sequences were also translated into amino acids in order to screen for the presence of premature stop codons. The consensus sequences and representative parasitoid sequences were aligned via multiple sequence alignment algorithms using Multiple Alignment using Fast Fourier Transform (MAFFT) version 7 (https://mafft.cbrc.jp/alignment/software) (Katoh et al., 2019). The aligned sequences were trimmed to a length of 357 bp and were subjected to phylogenetic analysis based on maximum likelihood with 1000 bootstrap replicates. Phylogenetic analysis was performed using Randomized Axelerated Maximum Likelihood (RaxML) version 8 (Stamatakis, 2014) and viewed/edited in Mega 7.0 (Kumar et al., 2016). *Probaryconus* sp. (Kieffer, 1908) (KC778425) (Platygastridae: Scelioninae) was used as an outgroup.

3.3.2.4. Sequence divergence analysis

Intraspecific divergence (i.e., divergence within species) was calculated for the two wasp species groups to determine the possible presence of cryptic species. Nucleotide diversity (π) was calculated using DNA Sequence Polymorphism (DnaSP) version 5.10.01 (Librado & Rozas, 2009). The nucleotide diversity (π) value is the mean number of nucleotide differences per site between two sequences (Nei, 1987).

3.3.3. Scelionidae and Pentatomidae diagnostic tool

3.3.3.1. DNA extraction, amplification and sequencing

Genomic DNA was extracted from a single parasitized stink bug egg in each reaction using prepGEM® Insect kit (ZyGEM™, Hamilton, New Zealand) according to the manufacturer's protocol. The DNA concentration was quantified using a Nanodrop spectrophotometer and diluted to between 100 – 300 ng/µl using ddH_2O (Adcock Ingram, Bryanston, South Africa).

3.3.3.2. Scelionidae-specific primers

Scelionidae specific PCRs were performed at a final volume of 25 µl consisting of 5 µl of 5 mM MyTaq™ reaction buffer containing $MgCl_2$ and dNTPs, 0.5 µl of MyTaq™ DNA polymerase (Bioline, South Africa), 0.5 µl of the Scelionidae family-specific primer SCEL_F1 (5'-GCAATAATTCGAATAGAATTAAGAGT-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAA ATCA - 3') (Folmer et al., 1994), 16.5 µl of ddH_2O (Adcock Ingram, Bryanston, South Africa) and 2µl of template DNA. The PCR cycling conditions for the wasp specific amplification was the following: initial denaturation at 94 °C for 40 s, followed by 30 cycles of 94 °C for 40 s, 51 °C for 40 s and 72 °C for 1 min with a final hold of 5 min at 72 °C (Gariepy et al., 2014). DNA obtained from adult wasps from the January 2019, as well as *T. basalis* were also included to test the suitability of the Scelionidae diagnostic tool to amplify and identify native parasitoid wasps. Amplicon visualization, PCR clean-up and DNA sequencing were performed as described above. Forward and reverse sequences obtained using the SCEL_F1 and HCO 2198 primers were aligned using Biological Sequence Alignment Editor (BioEdit) (Hall et al., 2011) to generate consensus sequences. The consensus sequences obtained using the SCEL_F1 forward and HCO-2198 reverse primer pair were trimmed to a length of 357 bp and included in the phylogenetic analyses of the adult parasitoid sequences (LCO1490 and HCO2198) described above.

3.3.3.3. Pentatomidae-specific primers

Stink bug specific PCRs were performed at a final volume of 25 µl consisting of 5 µl of 5 mM MyTaq™ reaction buffer containing $MgCl_2$ and dNTPs, 0.5 µl of MyTaq™ DNA polymerase (Bioline, South Africa), 0.5 µl of the Pentatomidae family-specific primer PENT_F2 (5'-TATTGAATTAGGACAACCTGGAAG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAA ATCA - 3') (Folmer et al., 1994), 16.5 µl of ddH_2O (Adcock Ingram, Bryanston, South Africa) and 2 µl of template DNA. A negative control was added. The PCR cycling conditions for the stink bug specific amplification was the following: initial denaturation at 94 °C for 40 s, followed by 30 cycles of 94 °C for 40 s, 53 °C for 40 s and 72 °C for 1 min with a final hold of 5 min at 72 °C (Gariepy et al., 2014). Amplicon visualization, PCR clean-up and DNA sequencing were performed as described above.

Forward and reverse sequences obtained using the PENT_F2 and HCO 2198 primers were aligned using Biological Sequence Alignment Editor (BioEdit) (Hall et al., 2011) to generate consensus sequences. The stink bug egg consensus sequences and representative stink bug sequences generated in Chapter 2 of this dissertation were aligned via multiple sequence alignment algorithms using Multiple Alignment using Fast Fourier Transform (MAFFT) version 7 (https://mafft.cbrc.jp/alignment/software) (Katoh et al., 2019). The aligned sequences were subjected to phylogenetic analysis based on maximum likelihood (ML) with 1000 bootstrap replicates. Phylogenetic analysis was performed using Randomized Axelerated Maximum Likelihood (RaxML) version 8 (Stamatakis, 2014) and viewed/edited in Mega 7.0 (Kumar et al., 2016). *Macrorhaphis acuta* Dallas, 1851 (Pentatomidae: Asopinae) was used as an outgroup.

3.4. Results

3.4.1. Parasitoid identification

A total of 80 parasitized stink bug egg batches were collected during January 2019 consisting of approximately 950 individual eggs. Parasitized stink bug eggs have a dark grey/black circular pattern in the middle compared to the unparasitized eggs which are light green in colour and develop a red pattern as the eggs mature (Figure 1). Parasitized stink bug egg packets were commonly located on the trunks of macadamia trees searched, however egg clusters were occasionally found on branches, leaves and developing nuts. Adult parasitoids were found to emerge from 24 hours up to 7 days after collection of parasitized stink bug egg batches due to varying ages of the stink bug eggs and the time at which they were parasitized. In total, 670 adult parasitoid wasps emerged from the collected eggs.

The native adult parasitoids represented two distinct species, based on BLAST searches of the 620 bp mtDNA COI gene fragment against the NCBI and BOLD databases as well as morphology. Species identification could not be determined by the Biosystematics Division, ARC-Plant Health and Protection, Roodeplaat, Pretoria. The first species group (Figure 2A), observed in a higher abundance, representing 51 of the parasitoid specimens sampled, had no BLAST searches with a percentage identity over 98 %. The other species group consisting of 16 individuals was putatively identified as *Psix striaticeps* (Dodd, 1920) with a percentage identity of around 99% against *P. striaticeps* voucher OSUC 266777 (KC778436) (Figure 2B). This vouchered specimen is linked to a verified morphological identification on the BOLD database (Taekul et al., 2014).

3.4.1.1. Phylogenetic analysis

Maximum likelihood analysis resolved the sequences into three distinct clades representing three genera, namely *Telenomus*, *Trissolcus* and *Psix* (Figure 3). The first group represents the *Trissolcus basalis* specimens obtained as a positive control from Koppert Biological Systems which successfully grouped within the *Trissolcus* clade and with other *T. basalis* sequences

(Figure 3). One of the species sampled in this study formed a unique lineage within the *Trissolcus* clade. The other species grouped with *P. striaticeps* within the *Psix* clade (Figure 3).

3.4.1.2. Sequence divergence analysis

Sequence divergence π values of 0 and 0.00230 (0.23 %) were obtained for the two groups in *Trissolcus* and *Psix*, respectively. These low sequence divergence values confirmed that no cryptic species were present within the two species groups.

3.4.2. Scelionidae and Pentatomidae diagnostic tool

A total of 10 parasitized stink bug egg batches, equating to 112 individual eggs, were collected during February 2020. Three individual eggs of each of the egg packets were used to test the Scelionidae and Pentatomidae diagnostic tool developed by Gariepy et al. (2014).

3.4.2.1. Scelionidae-specific primers

The SCEL_F1 and HCO 2198 primers were used to obtain sequences (represented in green, Figure 3) of about 600 bp in length. However, amplification was only successful for 13 out of a total of 30 (43%) parasitized eggs, despite testing various annealing temperatures. All sequences obtained from parasitized eggs grouped together as *Trissolcus* sp. (Figure 3). For the six adult parasitoid specimens representing three different species, the sequences obtained for the *T. basalis* positive control were resolved into the correct species group (bootstrap value = 100). Those generated for *Trissolcus* sp. specimens also grouped within the correct species group. Despite testing different annealing temperatures (50˚C - 55˚C) high-quality sequences could not be obtained for *P. striaticeps* specimens.

3.4.2.2. Pentatomidae-specific primers

A total of 20 sequences, out of the 30 parasitized eggs analysed (66%), were obtained using the PENT_F2 and HCO 2198 primer set. Searches against the NCBI (BLAST) and BOLD databases showed 95 % similarity with *Bathycoelia indica* (HG236463). Based on phylogenetic

analysis, the stink bug host egg sequences grouped, with high bootstrap support (100%), with *B. distincta* sequences that were generated in Chapter 2 of this dissertation (Figure 4).

3.5. Discussion

Two parasitoid species were identified as attacking *B. distincta* eggs in the Levubu growing region of South Africa using a COI barcoding approach. The Scelionidae-specific diagnostic primers were able to successfully amplify the parasitoid DNA present in parasitized *B. distincta* eggs, but not from *P. straiticeps* adult wasps. The Pentatomidae-specific primers were successfully used to amplify and sequence the *B. distincta* host DNA.

The parasitoid wasp species parasitizing *B. distincta* eggs in the Levubu growing region of the Limpopo province were identified as *P. striaticeps* and *Trissolcus* sp. Members of the *Trissolcus* genus are distributed globally. Over 160 species are described and many are parasitoids of economically important pests (Laumann et al., 2008; Yang et al., 2009). Some of these species, especially *T. basalis* are used in biological control programs in different areas worldwide (Su & Tseng, 1984; Corrêa-Ferreira & Moscardi, 1996; Cantón-Ramos & Callejón-Ferre, 2010).

The *Psix* genus is typically found in relatively arid climates such as central Australia, southeast Asia and savannas of Africa (Johnson & Masner, 1985). Specimens have, however, also been collected from humid forests similar to the conditions typical of macadamia orchards. *Psix striaticeps* is known from tropical Africa and India where it is a known parasitoid of *N. viridula.* The possibility of using *P. striaticeps* to control *Eocanthecona furcellata* (Wolff, 1811) (Hemiptera: Pentatomidae), a pest stink bug species of *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae) in sericulture, has been explored (Singh & Saratchandra, 2002). There are, however, no reports on whether biological control programs involving the species have been implemented.

The diversity of species found in this study is much lower than species diversity found by Bruwer (1992) that also collected parasitized egg masses from the Levubu growing region of

South Africa. Bruwer (1992) identified five hymenopteran parasitoid species emerging from *B. distincta* eggs and these included *Trissolcus* sp. A, *Trissolcus* sp. B, *Pediobus* sp., *Pachyneuron* sp. and an undetermined Scelionidae species. It was speculated that the *Pediobus* sp. is a hyperparasitoid as it was much smaller in comparison to the other species and occurred with other parasitoid species in some of the parasitized eggs collected. It is possible that the *Trissolcus* sp. found in this study could be one of the *Trissolcus* species found by Bruwer (1992) and the undetermined species could be *P. striaticeps.* This would, however, require further investigation and comparisons of the specimens found during the two different studies. More farms and different growing regions will need to be included in future surveys/studies to fully understand species presence of native parasitoids in macadamia orchards in South Africa.

Results from DNA sequencing/barcoding distinguished two wasp species parasitizing *B. distincta* eggs, but the species could not be identified through morphological features. There are various limitations when it comes to morphological identification of insect specimens and this is highly dependent on the level of expertise available, the amount of focus that has been placed on certain species in the past and the accessibility to literature used for morphological identifications (Ball & Armstrong, 2006; Jinbo et al., 2011; Emam et al., 2020). Also, certain similarities between specimens limit the identification of specimens to species and sub-species level. This highlights the importance for DNA databases to assist in future studies involving the counting of species and identifications as part of a diagnostic tool (Jalali et al., 2015; Hebert et al., 2016).

The family-specific forward primers (SCEL_F1 and PENT_F2) paired with HCO2198, produced variable results in their effectiveness to detect and identify the parasitoid and host DNA, respectively. The SCEL_F1 primer pair was successfully used to amplify and sequence parasitoid DNA in 43% of the stink bug eggs analysed which is higher than the 36 % detection rate reported by Gariepy et al. (2019). High-quality sequences were obtained for *T. basalis* and *Trissolcus* sp. adult specimens but sequences could not be generated for *P. striaticeps* adult specimens. Failure of SCEL_F1 to amplify and sequence DNA of *P. striaticeps* individuals is a limitation in this study, as it suggests that the parasitized eggs for which sequences could not be generated could have been parasitized by individuals of *P. striaticeps*. Future work

could involve the development of primers specifically for the identification of native wasp species DNA such as the species-specific primers developed for *A. bifasciatus* (Stahl et al., 2019). In the interim, since the standard LCO1490 and HCO2198 primers (Folmer et al., 1994) were successfully used to identify adult parasitoid specimens in this study, the best approach would be to collect parasitized stink bug eggs and allow the adult wasps to emerge before molecular analyses are performed. The PENT_F2 primer pair was successfully used to identify the host DNA in 66% of the eggs analyzed which is higher than the 42% reported by Gariepy et al. (2019).

This study provided information on the identity of native parasitoids of *B. distincta* in Limpopo, South Africa and tested different methods for their identification, thus laying an important foundation for future surveys and efforts towards developing a biological control programme for *B. distincta*. Surveys are required to determine the presence and prevalence of parasitoid species in all three of the main macadamia growing regions in South Africa. In addition, future studies should investigate the parasitism rates, host-specificity and functional response of these native parasitoids.

3.6. References

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3.7. Tables and figures

Table 1. COI sequences downloaded from the National Center for Biotechnology Information (NCBI) and included in the phylogenetic analysis.

Species	Accession number	Isolate/Voucher	Sequence length (bp)	Year	Country	Source
***Probaryconus* sp.**	KC778425	OSUC 181541	623	2013	Canada	Hebert et al., 2016
Psix striaticeps	KC778436	OSUC 266777	570	2013	Kenya	Taekul et al., 2014
Psix tunetanus	KC778435	OSUC 266779	623	2013	USA	Taekul et al., 2014
Telenomus podisi	MK188337	Tepo-0003	643	2019	Canada	Gariepy et al., 2019
Telenomus podisi	MK188336	Tepo-0002	643	2019	Canada	Gariepy et al., 2019
***Telenomus* sp.**	KC778456	OSUC 265220	611	2013	Panama	Taekul et al., 2014
Telenomus sp.	KC778458	OSUC 173839	658	2013	Canada	Taekul et al., 2014
***Telenomus* sp.**	KC778459	OSUC 173840	623	2013	Canada	Taekul et al., 2014
***Telenomus* sp.**	KC778479	OSUC 266781	564	2013	USA	Taekul et al., 2014
Trissolcus basalis	KC778495	OSUC 173848	666	2013	Italy	Taekul et al., 2014
Trissolcus basalis	KC778496	OSUC 173849	658	2013	Italy	Taekul et al., 2014
Trissolcus comperei	MN615659	USNM:ENT:01335780	611	2020	Japan	Talamas et al., 2019
Trissolcus comperei	MN615658	USNM:ENT:01335785	611	2020	Japan	Talamas et al., 2019
Trissolcus corai	MN615606	USNM:ENT:00977542	611	2020	China	Talamas et al., 2019
Trissolcus corai	MN615605	USNM:ENT:01059329	611	2020	China	Talamas et al., 2019
Trissolcus cultratus	MN615596	Tsp2	611	2020	Japan	Talamas et al., 2019

Trissolcus cultratus	MN615597	Tfla1	611	2020	Japan	Talamas et al., 2019
Trissolcus euschisti	MG939480	femAS-0015	496	2018	Canada	Gariepy et al., 2019
Trissolcus euschisti	MG939496	femAS-0016	562	2018	Canada	Gariepy et al., 2019
Trissolcus japonicus	MN615626	Tplau1	611	2020	Japan	Talamas et al., 2019
Trissolcus japonicus	MN615624	USNM:ENT:00989098	611	2020	China	Talamas et al., 2019
Trissolcus mitsukurii	MN615586	Tm1	611	2020	Japan	Talamas et al., 2019
Trissolcus mitsukurii	MN615587	USNM:ENT:00977533	611	2020	China	Talamas et al., 2019
Trissolcus plautiae	MN615615	USNM:ENT:01197808	611	2020	Japan	Talamas et al., 2019
Trissolcus plautiae	MN615616	USNM:ENT:01335789	611	2020	Japan	Talamas et al., 2019

Figure 1. A. Parasitized stink bug egg cluster. B. Unparasitized stink bug egg cluster

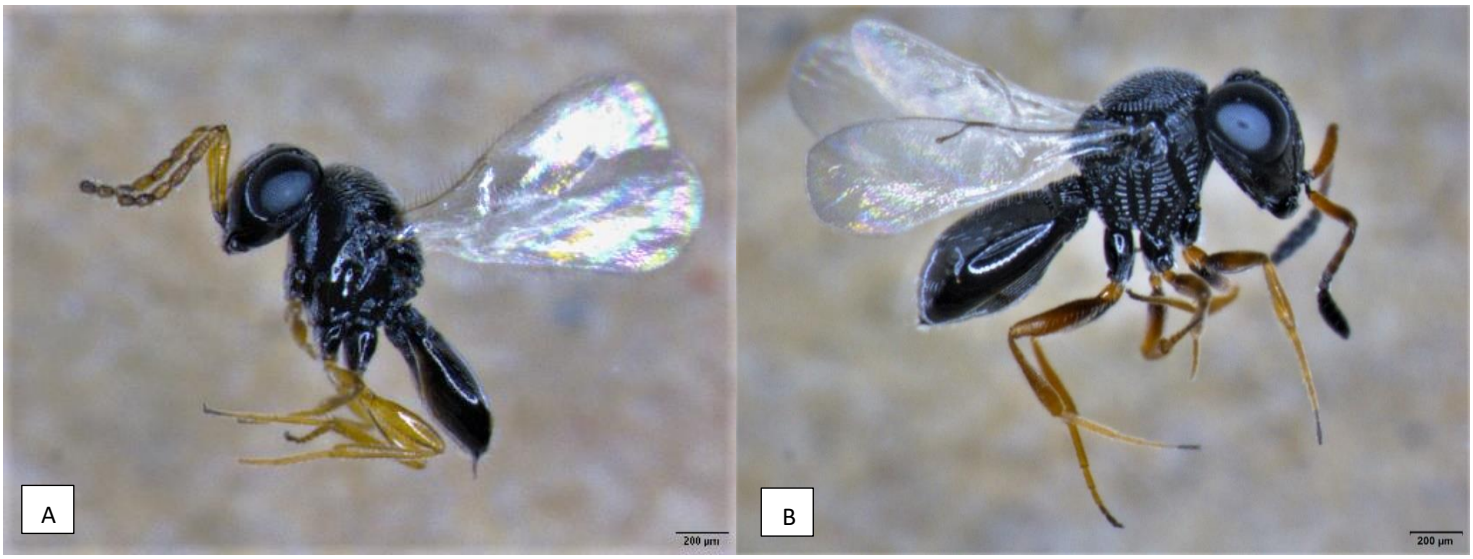

Figure 2. Parasitoid wasp species found attacking *Bathycoelia distincta* Distant (Hemiptera: Pentatomidae) eggs in Levubu in the Limpopo province A. *Trissolcus* sp. (Ashmead) B. *Psix striaticeps* (Dodd)

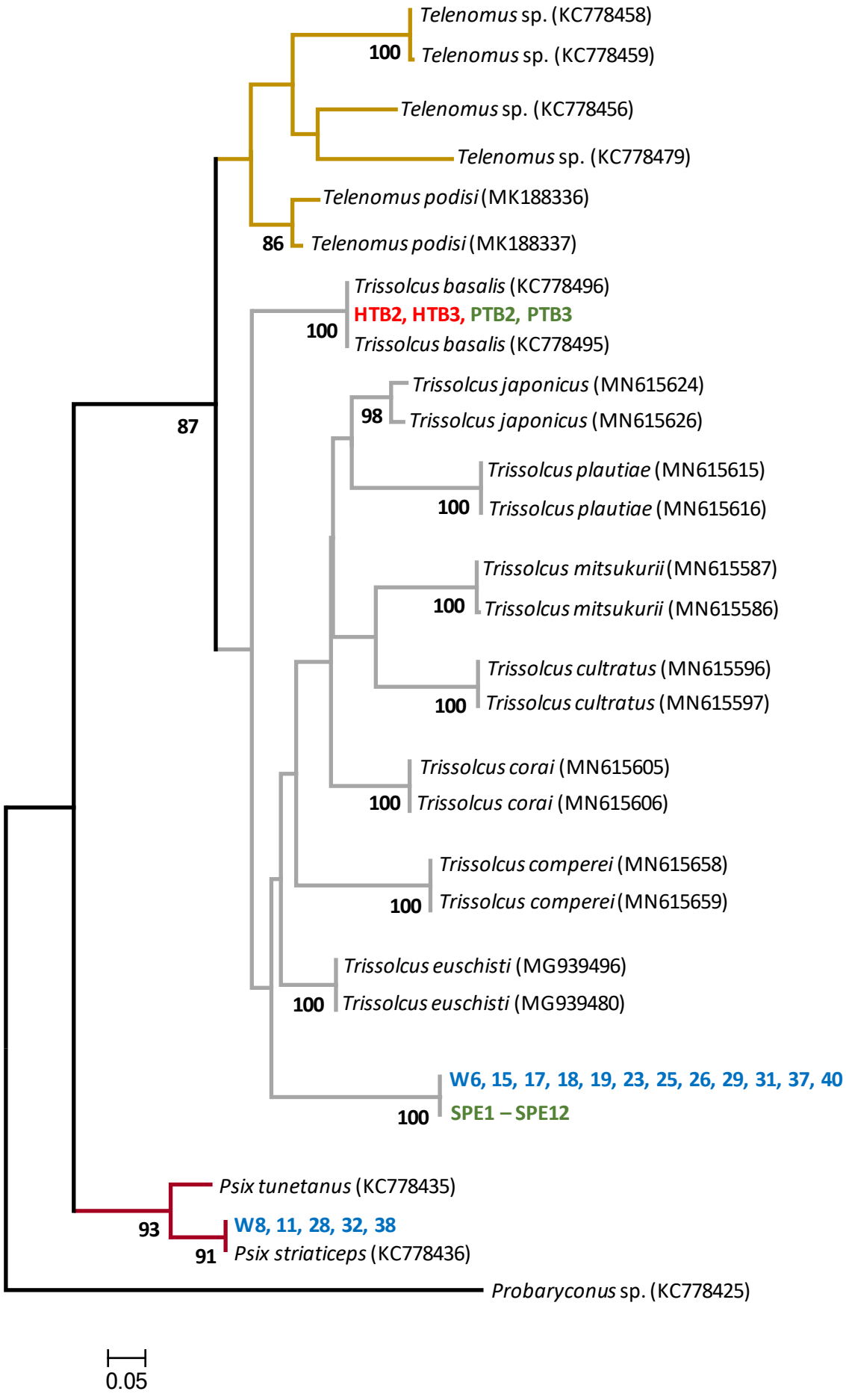
Telenomus sp. (KC778458)
100
Telenomus sp. (KC778459)
Telenomus sp. (KC778456)
Telenomus sp. (KC778479)
Telenomus podisi (MK188336)
86
Telenomus podisi (MK188337)
Trissolcus basalis (KC778496)
HTB2, HTB3, PTB2, PTB3
100
Trissolcus basalis (KC778495)
Trissolcus japonicus (MN615624)
98
Trissolcus japonicus (MN615626)
87
Trissolcus plautiae (MN615615)
100
Trissolcus plautiae (MN615616)
Trissolcus mitsukurii (MN615587)
100
Trissolcus mitsukurii (MN615586)
Trissolcus cultratus (MN615596)
100
Trissolcus cultratus (MN615597)
Trissolcus corai (MN615605)
100
Trissolcus corai (MN615606)
Trissolcus comperei (MN615658)
100
Trissolcus comperei (MN615659)
Trissolcus euschisti (MG939496)
100
Trissolcus euschisti (MG939480)
W6, 15, 17, 18, 19, 23, 25, 26, 29, 31, 37, 40
100
SPE1 – SPE12
Psix tunetanus (KC778435)
93
W8, 11, 28, 32, 38
91
Psix striaticeps (KC778436)
Probaryconus sp. (KC778425)
0.05

Figure 3. Maximum likelihood phylogeny of parasitoid wasps (Hymenoptera: Scelionidae) based on mtDNA *cytochrome c oxidase subunit 1* (COI) barcoding sequences. The three clades represent *Telenomus* (gold), *Trissolcus* (grey) and *Psix* (maroon) genera. The specimens in blue represent sequences obtained from emerged adult wasps using the LCO1490 and HCO2198 primers while those in green were obtained using the family-specific forward primer SCEL_F1 on parasitized eggs or emerged adult parasitoids. Both sets of sequences were obtained from parasitized *Bathycoelia distincta* eggs sampled from the Levubu growing region in Limpopo. The specimens in red represent verified *Trissolcus basalis* (Hymenoptera: Scelionidae) individuals.

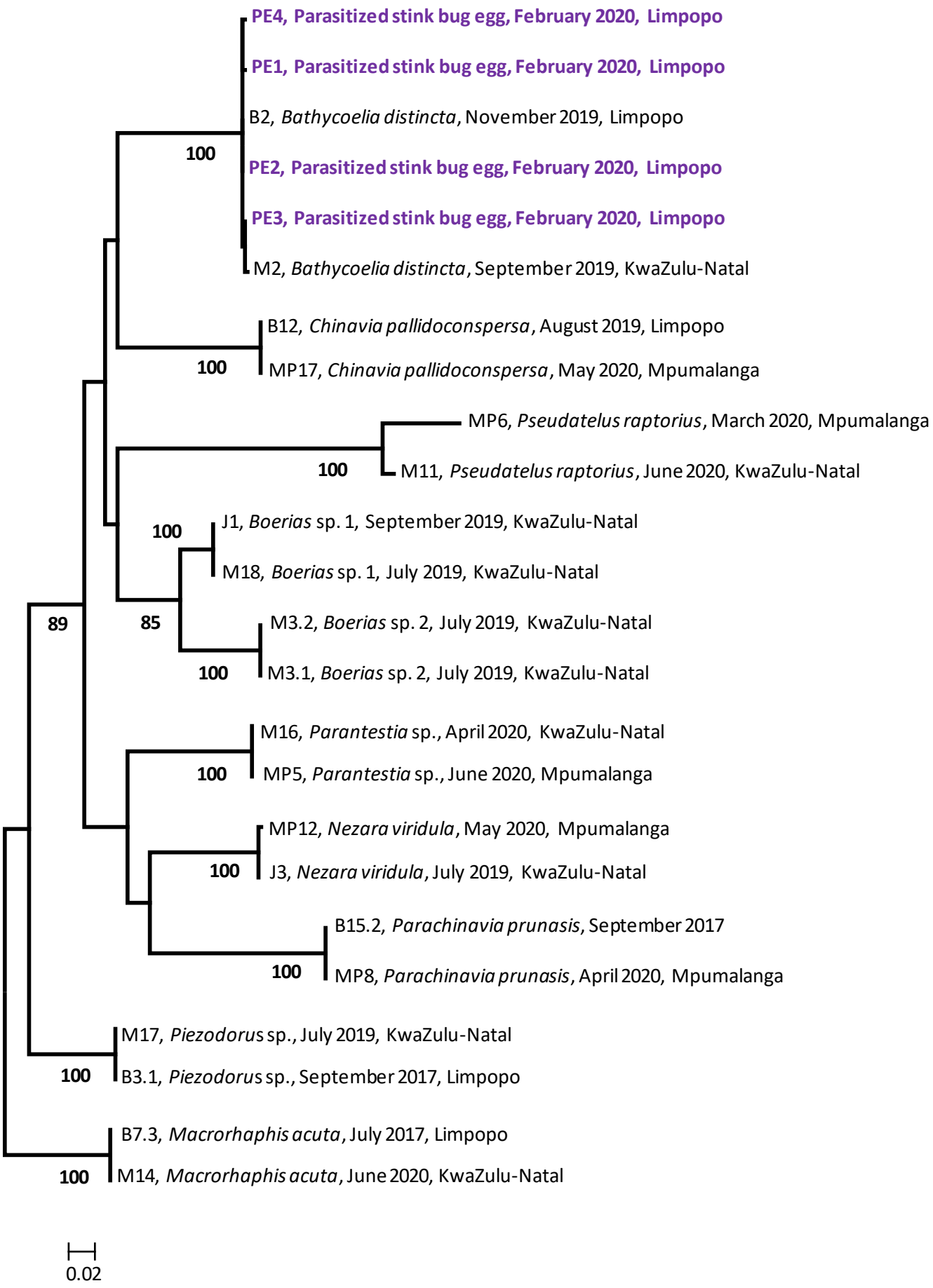

Figure 4. Maximum likelihood phylogeny based on stink bug (Hemiptera: Pentatomidae) mtDNA *cytochrome c oxidase subunit 1* (COI) barcoding sequences obtained from parasitized eggs using the PENT_F2 family-specific primers (represented in purple) and stink bug COI sequences obtained in Chapter 2 of this dissertation.

Summary

The aim of this dissertation was to synthesise information on stink bug diversity and their associated wasp egg parasitoids, with a focus on the species present in macadamia orchards in South Africa. The work presented in this dissertation includes an overview of the diversity, importance and management of stink bugs associated with nut crops. A survey in which the diversity of stink bug species present in macadamia orchards was determined over three seasons in Limpopo (2017-2020) and one season (2019-2020) in KwaZulu-Natal and Mpumalanga was also conducted. This included a phylogenetic analysis of these species and other closely related species and/or species previously associated with nut crops, a sequence divergence analysis and the development of a mtDNA *cytochrome c oxidase subunit 1* (COI) sequence and DNA database linked to morphology. Finally, a survey to identify wasp parasitoids parasitizing *Bathycoelia distincta* eggs in the Limpopo province and the testing two different molecular diagnostic approaches to identify these parasitoids was also conducted. Based on available literature, at least 19 Pentatomidae species have been associated with nut crops worldwide, with five species commonly associated with macadamia specifically in South Africa. Barcoding gene sequence availability on online databases is lacking for many of these species. The macadamia industry requires the implementation of effective integrated pest management (IPM) strategies and a reduction in yield loss due to stink bug damage to accomplish long-term sustainability. Successfully implementing strategies such as biological control using parasitoid wasps requires accurate identification of the stink bugs and their native parasitoids. A total of 20 stink bug species were identified across all three growing regions in this study and COI sequences for 16 of these species were made newly available on online databases. *Bathycoelia distincta* was the dominant species in all three regions and *Boerias* spp. were also found to be dominant in KwaZulu-Natal. Sequence divergence analysis confirmed the presence of cryptic species in the *Boerias* spp. and *Pseudatleus raptorius* groups. This is the first report of *Boerias* spp. on macadamia. The novel association of these species with macadamia and the fluctuation in species presence, both over time and across growing regions, highlights the need for ongoing monitoring of these pests. Monitoring will allow for the implementation of species-specific IPM strategies, ultimately leading to a reduction in the use of broad - spectrum insecticides. Monitoring relies

on accurate pest identification and the COI DNA, sequence and photo database developed in this study will assist in future identifications. Based on a COI DNA barcoding approach, two native wasp parasitoids parasitizing *B. distincta* eggs in Limpopo were identified as *Psix striaticeps* and *Trissolcus* sp. Family-specific primers to analyze unemerged parasitized eggs were tested against COI primers for emerged adult parasitoids to determine the best method for identification. The COI sequencing using the standard primers on emerged parasitoids was deemed the most reliable approach. Future work should focus on the impact and management of the *Boerias* spp. in KwaZulu-Natal, include surveys to determine parasitoid species presence in other growing regions and assess the effectiveness of native parasitoids as biological control agents of stink bugs.