

# The indirect effect of Bt maize (Cry1Ab) on *Cotesia sesamiae* (Hymenoptera: Braconidae)

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## ABSTRACT

*Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) are major pests of maize and sorghum in sub-Saharan Africa. The main larval endoparasitoids of these economically important lepidopteran stemborer species are *Cotesia flavipes* Cameron and *Cotesia sesamiae* (Cameron) (Hymenoptera: Braconidae). *Cotesia flavipes* has successfully been introduced into several countries in eastern and southern Africa, including South Africa where it could not be recovered after the first winter following its release despite its initial temporary establishment. This species was recently found in Botswana, where it was never released. Releases made in neighbouring countries are assumed to be the reason for the presence of *C. flavipes* in this country. The first aim of this study was to determine if *C. flavipes* has now also established in South Africa. *Busseola fusca*, *C. partellus* and *S. calamistis* larvae were collected from 15 localities in South Africa. *Cotesia* spp. recovered from these larvae were identified by means of morphological identification as well as molecular analyses. The only *Cotesia* species recovered from all localities were *C. sesamiae*, which confirms previous reports that no *C. flavipes* has been recovered to date in South Africa. Genetically modified Bt maize was planted in South Africa for the first time during the 1998/99 growing season for control of stemborers. The first Bt maize resistant *B. fusca* larvae in South Africa was reported during the 2006/07 growing season. *Cotesia sesamiae* is indirectly exposed to Bt proteins that are consumed by stemborer larvae when parasitising Bt-resistant *B. fusca* larvae. The second aim of this study was to determine the effect of indirect third-trophic level exposure to Cry1Ab proteins on the fitness (in terms of reproduction) and survival of *C. sesamiae*. Bt-resistant *B. fusca* larvae were reared on Bt maize stems until the 3<sup>rd</sup>/4<sup>th</sup> instar and parasitised with *C. sesamiae*. The number of cocoons, number of wasps emerging from cocoons and the sex ratio (females:males) of wasps were recorded during the first experiment, while the mass of host larvae and developmental time of parasitoid larvae were recorded in addition to this during the second experiment. Results obtained during both experiments showed that Bt exposure had no significant effect on *C. sesamiae* life history parameters. Significantly higher numbers of female wasps did, however, emerge from parasitised *B. fusca* larvae that fed on Bt maize compared to those that fed on non-Bt maize ( $t=2.93$ ;  $df=55$ ;  $P<0.01$ ) during experiment 1. Males can mate more than once and the increase in the number of females emerging from larvae that fed on Bt maize are therefore beneficial to the biological control of *B. fusca*. The use of *Cotesia* specimens, both *C. sesamiae* and *C. flavipes*, is therefore recommended in integrated pest management programmes for control of *B. fusca* on maize.

### **Keywords**

Bt maize, *Cotesia flavipes*, *Cotesia sesamiae*, molecular analyses, morphological identification, non-target effects, stemborers, tritrophic interactions.

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# Chapter 1: General introduction and literature review

## 1.1. Introduction

According to the United Nations (2017) the current world population is estimated to increase from 7.6 to 9.8 billion in 2050. An estimated 95% of the population will then live in developing countries (Cohen, 2005). The projection is that the populations of 26 African countries will at least double by 2050 (United Nations, 2017). Although this increase in the world population will cause food to be in great demand, human consumption in South Africa is expected to decline locally to 4.58 million tons in 2020 (Syngenta, 2016). An increase of close to 6.4 million tons in animal feed is however estimated by 2020 (Syngenta, 2016). The estimated increase in world population will also result in the overall global demand for maize (*Zea mays* L.) to grow by 45% by the year 2020, of which 72% will be in developing countries and the remaining 28% in developed countries (James, 2003).

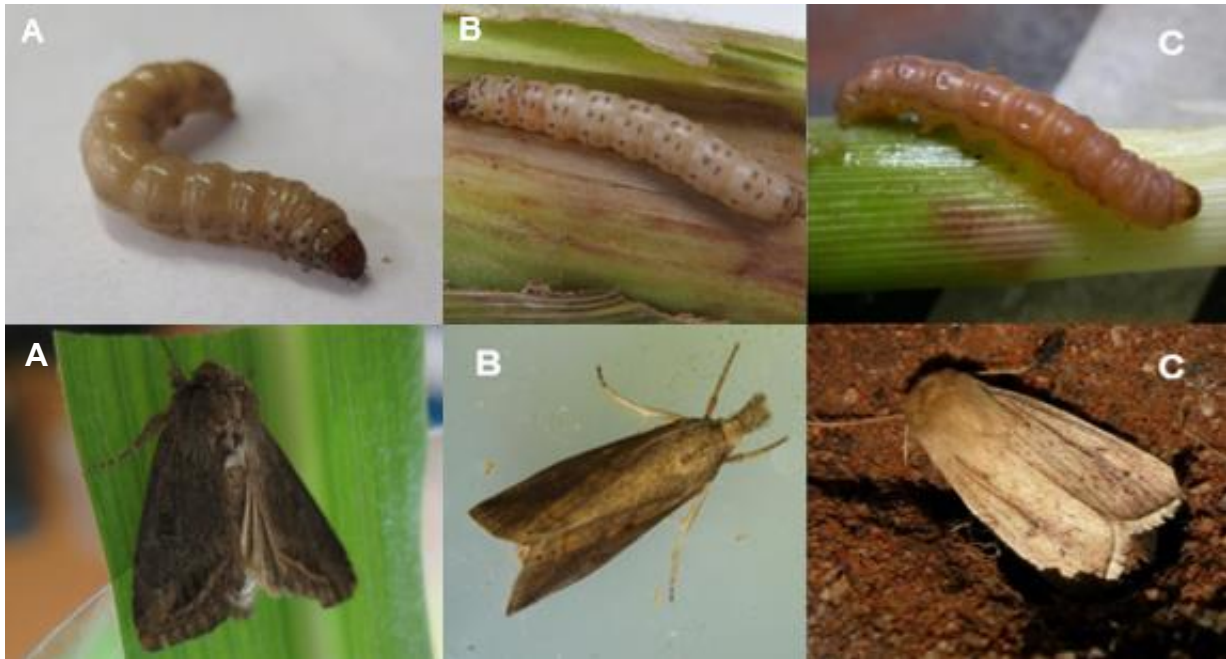
Maize is one of the three most important cereal crops along with rice (*Oryza sativa* L.) and wheat (*Triticum* spp. L.). It is grown worldwide in a range of agro-ecological environments and serves as a staple food for millions of people (Courteau, 2012). It has the highest worldwide production of all grain crops (Ensembl Plants, 2016), reaching a global production of 869 million tons during 2012 (Ferreira *et al.*, 2016). A third of this production (274 million tons) was from America with China, Brazil, Mexico, Argentina, India and Ukraine also producing significant amounts (Courteau, 2012; Ranum *et al.*, 2014). Maize is generally produced in temperate, sub-tropical and tropical regions (Adeyemo, 1984) where the mean daily temperature is higher than 19 °C, the mean temperature of the summer months are higher than 23 °C, and where the critical temperature of approximately 32 °C is reached. The distribution of rainfall required for maize production should be equal and more than 350 mm per year, mainly acquired from soil moisture reserves (Du Plessis, 2003).

Maize has been the largest contributor towards the gross value of field crops in South Africa for the past five seasons, followed by sugarcane, wheat, soybean and sunflower seed. About 59% of the maize produced in South Africa is white maize primarily used for human consumption, while the remaining 41% is yellow maize mostly used for animal feed. The gross value of maize in South Africa produced in the 2016/17 production season was R29 659 million, with 2.63 million hectares planted and 16.74 million tonnes produced (DAFF, 2017). It is therefore the most important summer grain crop in South Africa (Klopper, 2008) and the country is currently the main maize producer in the southern African region (DAFF, 2017).

In South Africa approximately 2.5 million hectares of farmland (about 25% of the land area), mostly concentrated in the main maize-growing provinces of the Free State, Mpumalanga and North-West (DAFF, 2017), is used for the production of maize. Maize production is limited by several biotic (arthropods, nematodes, diseases, weeds, rodents and/or birds) and abiotic (drought, soil fertility and/or mineral toxicity) factors (Kfir *et al.*, 2002). An estimated mean yield loss of 35% is caused by pests on agricultural crops worldwide, with losses in Africa recorded as the highest in the world (Klopper, 2008). Food security, the production of enough healthy food for all people (Pinstrup-Andersen, 2009), is therefore a major challenge in South Africa. In order to deal with the demand for maize needed for feed (directly and indirectly) and fuel (Syngenta, 2016), new production methods should be applied while the existing ones should be reinforced to better manage the constraints faced by maize farmers in South Africa (FAO, 2002).

## 1.2. Important pests of maize

Maize is known to have a wide diversity of pests and diseases present during all its developing stages which puts a constraint on its production (DAF, 2012). Lepidopteran stemborers are generally considered to be the most important (Nye, 1960; Van Rensburg *et al.*, 1988) and destructive pests (Seshu Reddy, 1998; Overholt *et al.*, 2001) of maize causing severe damage and yield losses (Ingram, 1958; Kfir *et al.*, 2002). The major success of these stemborer species results from their widespread distribution (Calatayud *et al.*, 2006). In the latest survey by Moolman *et al.* (2014) on the stemborer species present in southern Africa, 50 species of stemborers were reported in South Africa and 39 species in Mozambique. According to Maes (1997) only 20 of these species are of economic importance, while Kfir *et al.* (2002) found 21 of these species to be of economic importance. These include *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Sesamia calamistis* (Hampson) (Figure 1.1). These three stemborer species are regarded as the most important, widespread and destructive field insect pests of maize in southern Africa (Cugala & Omwega, 2001; Kfir *et al.*, 2002). *Sesamia calamistis*, even though considered a major pest of maize in Africa, is however not as widespread as *B. fusca* and *C. partellus* in eastern and southern Africa (Hill, 1973; Matama-Kauma *et al.*, 2001; Kfir *et al.*, 2002; Van den Berg & Van Wyk, 2007; Ong'amo *et al.*, 2016). The dominance and importance of a particular stemborer species vary and depend on the ecological conditions that prevail in different agro-ecological zones (Cherry *et al.*, 2004). The annual maize yield losses in South Africa caused by these stemborers averages around 10%, but losses ranging between 25 and 78% have also been reported (Sylvain *et al.*, 2015).



**Figure 1.1:** Larval and adult stages of the lepidopteran stemborer species (A) *Busseola fusca*, (B) *Chilo partellus* and (C) *Sesamia calamistis*, the most important pests of maize in southern Africa.

### 1.2.1. *Busseola fusca*

The African stemborer *B. fusca* is an indigenous species in tropical Africa (Mohyuddin & Greathead, 1970; Harris & Nwanze, 1992). In South Africa, it is responsible for higher crop losses than any other insect pest (Mally, 1920; Matthee, 1974; Walters *et al.*, 1975; Kfir, 1997a; 1998). *Busseola fusca* occurs widespread throughout sub-Saharan Africa (Harris & Nwanze, 1992), except on the islands of the Indian Ocean, including Madagascar and Zanzibar (Kfir *et al.*, 2002), and outside the African continent (Kfir, 1997b).

*Busseola fusca* occurs at most altitudes in central Africa (Cardwell *et al.*, 1997). Although it was reported to occur predominantly in areas >1500 m a.s.l. in eastern Africa (Sezonlin *et al.*, 2006), it was also recently reported to occur at high and low altitudes (Calatayud *et al.*, 2014) in most agricultural systems in East Africa, including mountain forests in the highlands as well as in the semi-arid and arid lowlands (Ndemah *et al.*, 2001). Tams and Bowden (1953) reported *B. fusca* to occur at sea level to 2000 m a.s.l. in West Africa. In southern Africa this species usually occurs at relatively low altitudes, for example in Zimbabwe, in areas higher than 600 m a.s.l. (Nye, 1960; Sithole, 1989), in the cooler eco-zones of coastal areas (Ebenebe *et al.*, 1999; Waladde *et al.*, 2001; Kfir *et al.*, 2002) as well as in the mountain areas with altitudes of up to 2131 m a.s.l. (Krüger *et al.*, 2008). The geographical distribution of *B.*

*fusca* on maize and sorghum in certain areas in Africa can therefore not be ascribed to the elevation where it is found, but rather to the effect of temperature (most important factor), rainfall and humidity (Sithole, 1987).

Eggs of *B. fusca* are laid under the inner surfaces of leaf sheaths in clusters of 10-80 eggs per batch (Walters *et al.*, 1975; Harris & Nwanze, 1992). The number of eggs laid by a single moth varies greatly, but it generally ranges between 100 and 800 eggs (Mally, 1920; Ingram, 1958; Harris, 1962; Van Rensburg *et al.*, 1987). After hatching, neonate *B. fusca* larvae migrate to the whorl of the plant where they feed and cause damage to the young whorl leaves (Walker & Hodson, 1976; Kfir, 1997b; Van den Berg & Van Wyk, 2007). According to Van Rensburg *et al.* (1988) up to 70% of the larvae migrate to other plants within five weeks, and up to 67% of them will occur individually per plant (Van Rensburg *et al.*, 1987). Older larvae, usually from the third instar onwards (Calatayud *et al.*, 2014), leave the whorl after approximately 10-14 days to tunnel into the stem of the plant where they feed for 3-5 weeks until pupation (Walker & Hodson, 1976; Kfir, 1997b). Larvae create emergence windows for moths before pupation by tunnelling towards the outside of the stem, leaving the outer epidermal layer intact. Depending on the temperature, moths which are ready to mate, emerge from the pupae approximately 9-14 days later (Harris & Nwanze, 1992). Not all of the larvae pupate, as some of them enter into diapause - a form of developmental arrest in insects that enables them to circumvent adverse conditions (Denlinger, 2009). During the dry winter season in South Africa, *B. fusca* overwinters as diapause larvae inside the lower dry stalks found just beneath the soil surface (Van Rensburg *et al.*, 1987).

*Busseola fusca* larvae cause most of its damage by feeding on whorl leaves and tunnelling into the stems of plants (Appert, 1970; Bosque-Perez & Mareck, 1991; Kfir, 1998). Larvae that feed in the whorl of young maize plants not only causes the distinctive shot hole damage (Harris & Nwanze, 1992), but can also destroy the growth points of the plant (Appert, 1970; Bosque-Perez & Mareck, 1991; Kfir, 1998) to such an extent that it will not be able to grow any further (Tilahun & Azerefegne, 2013). This is known as the "dead heart" syndrome (Harris & Nwanze, 1992) as seen in Figure 1.2. Stem tunnelling weakens the stem which causes it to break and plants to lodge (Appert, 1970; Bosque-Perez & Mareck, 1991; Kfir, 1998). Stem damage interferes with the translocation of nutrients and metabolites in the plant and it also decreases the functionality of the plant (Van Rensburg *et al.*, 1988; Tilahun & Azerefegne, 2013). This damage has an effect on the growth of the plant and development of grains (Appert, 1970; Bosque-Perez & Mareck, 1991; Kfir, 1998), subsequently leading to low quality maize being produced (Fandohan *et al.*, 2003). Maize ears that are directly damaged by larvae (Bosque-Perez & Mareck 1991; Kfir, 1998), as well as secondary fungal infections e.g.

*Fusarium* spp. due to the activity of stemborers (Fandohan *et al.*, 2003), cause substantial crop losses (Harris & Nwanze, 1992). Stemborer injury to plants also result in increased incidence and severity of maize stalk rots (Bosque-Perez & Mareck, 1991).



**Figure 1.2:** Images of maize depicting the presence of the “dead heart syndrome” (Pioneer, 2010; Wangai, 2013).

Yield losses caused by *B. fusca* depend on the plant growth stage, number of larvae per plant (infestation level) and the reaction of the plant to the stemborer injury (Appert, 1970; Bosque-Perez & Mareck, 1991; Van Rensburg & Flett, 2008). The pest status of *B. fusca* therefore varies between regions and agro-ecological zones (Ndemah *et al.*, 2001; Sezonlin *et al.*, 2006; Calatayud *et al.*, 2014). Damage caused by *B. fusca* is estimated to result in a yield reduction of 5-75% in conventional maize (non-Bt) (Matthee, 1974). Before genetically modified Bt maize was planted for the control of *B. fusca*, yield losses of between 10 and 100% were reported in South Africa (Mally, 1920; Matthee, 1974; Barrow 1987). The pest status of *B. fusca* is, however, unpredictable due to annual fluctuations in population sizes (Kruger *et al.*, 2009). In the previous century, Dabrowski (1985) reported Kenyan maize yield losses caused by *B. fusca* to be between 15 and 78% but according to De Groote (2002), *B. fusca* is generally responsible for an average of 14% of the loss in Kenya’s maize production. In the humid forest areas of Cameroon yield losses of around 40% are experienced (Cardwell *et al.*, 1997), while Usua (1968) found that in Nigeria the presence of only one or two *B. fusca* larvae per plant caused a reduction in the yield by as much as 25%. A study done in South Africa showed that an infestation with 20 first instar *B. fusca* larvae caused yield losses ranging between 39-100% on different maize genotypes (Barrow, 1987). *Busseola fusca* has a low economic impact on maize in West Africa (Sezonlin *et al.*, 2006), but in Zimbabwe, Sithole (1987) estimated yield

losses between 30-70% where no insecticides were applied, but less than 30% where insecticides were applied.

### 1.2.2. *Chilo partellus*

*Chilo partellus* is an exotic stemborer species in Africa which originates from the southeastern and southern Asian region (Sallam *et al.*, 1999). It was introduced into Malawi before the 1930's (Tams, 1932) and reported by Duerden (1953) in Tanzania twenty years later. It has since spread to nearly all the lowland areas of the countries in eastern and southern Africa. This species appeared in South Africa for the first time in 1958 as a pest of grain sorghum and has since 1970 also heavily infested maize (Van Rensburg & Van Hamburg, 1975). Although *C. partellus* is generally considered to be present in low to mid altitudes (<1500 m) and warmer areas (Overholt *et al.*, 1997; Cugala & Omwega, 2001), it was also reported to have expanded its geographical ranges to cooler areas with higher altitudes (Kfir, 1997b; Ebenebe *et al.*, 1999; Mwalusepo *et al.*, 2015; Mutamiswa *et al.*, 2017) including Israel (Ben-Yakir *et al.*, 2013) and Turkey (Bayram & Tonğa, 2016). There is a risk that *C. partellus* can invade other regions such as the Americas, Australia, China, Europe, New Zealand and West Africa (Yonow *et al.*, 2017). It has the potential to, as observed in areas where it has invaded, displace indigenous stemborer species (Kfir, 1997a;b; Overholt *et al.*, 1994a; Ofomata *et al.*, 2000; Kfir *et al.*, 2002).

Eggs of *C. partellus* are usually laid on the underside of leaves in batches of 10-80 flattened, imbricated eggs (Nye, 1960; Maes, 1998). One moth can lay on average 343 eggs (Ofomata *et al.*, 2000). Four to eight days after oviposition, larvae begin to hatch in the early morning (Panchal & Kachole, 2013) and these newly hatched *C. partellus* larvae enter the leaf whorls where they feed on the younger leaves (Maes, 1998). Larvae of *C. partellus* are often also found to feed behind the leaf sheaths of maize and sorghum (Van den Berg & Van Rensburg, 1996). They may also tunnel into the mid-ribs of young leaves or into leaf sheaths (Nye, 1960). The older larvae tunnel into the stems, where they pupate for 5-12 days after 2-3 weeks of feeding (Panchal & Kachole, 2013). When conditions are favourable, the life cycle of *C. partellus* takes between 25-50 days to complete, which allows for five or more successive generations to develop during a growing season (Maes, 1998). This is up to three times faster than the completion of the life cycle of *B. fusca* causing *C. partellus* to be more competitive than *B. fusca* (Kfir, 1997b; Panchal & Kachole, 2013). *Chilo partellus*, similar to *B. fusca*, also enters into diapause during cold and/or dry conditions in stubbles, stems and other crop residues for up to six months before pupating during favourable conditions (Maes, 1998). The

occurrence of a rest-phase (aestivation), however, has also been reported in *C. partellus* (Kfir, 1991).

According to Polaszek (1998) damage by *C. partellus* includes leaf feeding, extensive tunnelling in stems and maize ears which disrupts the flow of nutrients and it also causes the death of growing points which produce “dead heart” symptoms. Tunnelling into the stems may cause the plant to break at the weakened point which results in lodging (Chandy, 1955). Yield reductions in grain crops by *C. partellus* has been reported from many countries, e.g. maize yield losses up to 40% in East Africa (Seshu Reddy, 1998) and up to 50% in sub-Saharan countries, South East Asia as well as in India (Sharma & Sharma, 1987; Sharma *et al.*, 2010). Kfir *et al.* (2002) reported maize yield losses exceeding 50% in southern Africa and an 88% yield loss in sorghum. In Nepal, a maize yield loss of 28% was recorded when insecticides were not applied for control of *C. partellus* (Sharma & Gautam, 2011).

### **1.2.3. *Sesamia calamistis***

The African pink stemborer, *S. calamistis*, although not as an important crop pest in eastern and southern Africa as *B. fusca* and *C. partellus* (Harris, 1962), may cause serious damage if crop management is performed ineffectively (Van den Berg & Drinkwater, 2000). This species has the widest distribution of all stemborer species on the African continent (Van den Berg & Van Wyk, 2007), being widely distributed at all altitudes (Ingram, 1958; Nye, 1960) over East-, West-, Central- and southern Africa as well as the islands of Madagascar, Mauritius and Reunion (Jepson, 1954). All host plants of *S. calamistis* belong to the family Poaceae (Matthee, 1974) and include finger millet, maize, rice, sorghum and sugarcane (Nye, 1960).

Eggs of *S. calamistis* are laid between the lower leaf sheaths and stem of host plants, in batches of approximately 10-40 eggs per batch (Nye, 1960; Holloway, 1998). A single moth lays an average of 300 eggs in 3-5 days (Harris, 1962) which, depending on abiotic factors, generally hatch within 6-9 days (Ingram, 1958; Holloway, 1998). Several hours after hatching, the majority of neonate larvae leave the site of oviposition to penetrate the stem either directly or after feeding on the leaf sheath for a short period of time (Holloway, 1998; Van den Berg & Van Wyk, 2007). It was, however, also reported that neonate *S. calamistis* larvae feed in the whorl of the plant for approximately one week after which they penetrate the stem (Ingram, 1958). These larvae generally remain inside the stem or ears of the host plant until pupation (Harris, 1962; Holloway, 1998), unless they migrate to another plant (Harris, 1962; Matthee *et al.*, 1974). Moths emerge 10-12 days after pupation, ready to mate (Sithole, 1989). Unlike other stemborer species, larvae of *S. calamistis* do not enter into diapause and therefore

develop continuously throughout the year, even when conditions for development are unfavourable (Nye, 1960; Harris, 1962; Matthee *et al.*, 1974; Van den Berg & Drinkwater, 2000).

The larvae of *S. calamistis* attack and tunnel into the main stems, tassels and ears of maize. The damage done by these feeding activities includes the killing of young plants which causes a poor plant stand, hollowing of the main stems of older plants caused by larvae tunnelling into the stem, and malformation and stunting of younger plants. Wilting and lodging of older infested plants has also been found in some instances, as well as the destruction of maize ears (Matthee *et al.*, 1974). *Sesamia calamistis*, like other stemborer species, may therefore also cause 'dead heart' symptoms (Sithole, 1989). *Sesamia calamistis* infestations are most severe during the second cropping season in areas with bimodal rainfall and can reach almost 100% in the forest zones of Ghana and Cameroon (Cardwell *et al.*, 1997). Yield losses of up to 100% have also been recorded in West Africa where *S. calamistis* was found in mixed populations with *B. fusca* (Gounou & Shulthess, 2004). The highest infestation levels in South Africa are found late in the season, where infestation levels of up to 75% were reported in the Eastern Cape province (Waladde *et al.*, 2001). Infestation levels of between 30-70% have also been reported in sweetcorn stems and 30-40% in sweetcorn ears in South Africa (Matthee *et al.*, 1974). Losses caused by *S. calamistis* do, however, not exceed 5% of the potential yield in Kenya (Ong'amo *et al.*, 2016).

### **1.3. Stemborer control**

A wide range of methods have been researched, tested and implemented to alleviate, manage and control the stemborer pest species in maize (Obonyo, 2009a). These pest control strategies involve chemical, cultural and biological control as well as host plant resistance (Van Emden, 1983; Kfir *et al.*, 2002). When used in combination with one another, these different components or elements (also known as the four pillars of Integrated Pest Management (IPM)), provide successful solutions to pest and environmental problems (Ehler, 2006; Calatayud *et al.*, 2014). These strategies are holistic approaches to manage all classes of pests (insects, weeds, pathogens and vertebrates) by using an appropriate selection of methods, singly or in combination, to provide benefits to the environment, economy and society (Kogan, 1998). IPM programs must not only meet local needs, but should also be adapted to local conditions and resources (Harris & Nwanze, 1992).

### 1.3.1. Chemical control

Chemical control is a highly effective means of controlling target pests by either killing them or inhibiting their development. This is done by using chemicals such as pesticides, which are usually classified according to the pest they are intended to control i.e. insecticides (Dent, 2000). According to Harris and Nwanze (1992) and Kfir (1998) the first reports of successful chemical control of stemborers were in the 1920's in South Africa and Zimbabwe, where maize crops were treated with Derris(ol)<sup>®</sup> and carbolic dip/sheep dip, hycol solution, Pulvex<sup>®</sup>, Kymac<sup>®</sup>, Cryolite<sup>®</sup> and several other botanical insecticides. These insecticides were based on rotenone, a product of the leguminous plant *Derris chinensis* (Fabales: Fabaceae) (Harris & Nwanze, 1992). Rotenone inhibits the process of cellular respiration, which converts nutrient compounds into energy at cellular level, primarily on nerve and muscle cells in insects. This leads to the rapid cessation of feeding and subsequent death of insects several hours to a few days after exposure (EI-Wakeil, 2013). Today, a wide range of insecticides are available to control economically important stemborer species (Slabbert & Van den Berg, 2009).

Insecticides have to be applied into the whorls of maize plants, due to the cryptic feeding habitat of stemborers inside the whorls of the plant (Slabbert & Van den Berg, 2009). The use of contact insecticides has proven to be an effective control method against the first instars of both *B. fusca* and *C. partellus*. This is due to newly hatched larvae migrating upwards on the outside of the plant and into the whorl of the plant where they feed on the young leaves for several days (Nye, 1960; Walker, 1960; Hill, 1973; Walker & Hodson, 1976). Older larvae penetrate the more closely packed leaves in the whorl, migrate to neighbouring plants or/and tunnel into the stems and ears of plants (Critchley *et al.*, 1997). These larvae may therefore not be controlled as effectively as they are protected from the insecticides (Kfir, 2001; Slabbert & Van den Berg, 2009). It is therefore crucial for insecticide applications to be timed correctly in order for it to be effective (Slabbert & Van den Berg, 2009). For stemborers, this timing should be as close to egg-hatching as possible. Effective control of the first generation larvae is also important, since it reduces the numbers that give origin to the second generation as well as the overwintering population (Walker, 1960). *Sesamia calamistis* is more difficult to control than *B. fusca* and *C. partellus* (Nye, 1960) since the newly hatched larvae tunnel directly into the stem, therefore escaping the effects of contact insecticides (Harris, 1962).

Chemical control should be applied correctly and rationally to avoid risks such as pest resistance, resurgence of target pests, outbreaks of secondary pests and overall environmental contamination (Ehler, 2006; Minja, 1990). In addition to this, the relatively short period that stemborer larvae are exposed to insecticides, before tunnelling into the stems and

ears, necessitates repeated pesticide application (Obonyo, 2009a). This not only causes chemical control to be a short term solution (Van den Berg *et al.*, 1998), but also to be impractical and not always economically feasible for the majority of small-scale farmers due to it being time consuming and expensive (Bonhof *et al.*, 1997). Chemical control should therefore be used in combination with the other approaches such as biological and cultural control to optimise its effectiveness (Kfir, 1995; Van den Berg & Nur, 1998; Van den Berg *et al.*, 1998; Van Rensburg, 1999; Dent, 2000).

### **1.3.2. Cultural control**

Cultural control is a long-term, preventive strategy defined as the manipulation of the environment where pests live, to render it unfavourable for their survival (Dent, 2000) while making it favourable for crop production (Oka, 1979). This is done by using various methods which cause pests to be unable to locate their hosts and colonise crops leading to a reduction in their survival, reproduction and dispersal (Dent, 2000). Cultural control methods for stemborer management include crop rotation, planting of trap crops (Khan *et al.*, 2008), intercropping, tillage and destruction of crop residues after harvest to prevent diapausing populations from carrying over to the next cropping season (Hill, 1973; Harris & Nwanze, 1992; Van den Berg *et al.*, 1998).

Mally (1920) suggested the destruction of crop residues by the means of ploughing the maize stubble as deeply as possible into the soil. The burning of stalks or spreading of stems on the ground during the dry season may help to control stemborer larvae as it exposes them to extreme temperatures and predators (Adesiyun & Ajayi, 1980). The destruction of crop residues by burning can create problems on farms where organic matter is low and soil erosion from wind and rains is severe (Van den Berg *et al.*, 1998). It however has to be implemented on a wide scale to be effective and farmers may have competing uses for their old stalks (Minja, 1990). The manipulation of planting dates (planting earlier or later in seasons), which has an impact on the intensity of stemborer attack, has a limited impact on stemborer populations. It is not always practical in areas where water is a major constraint and intercrop planting of cereals with other crops commences after the first rains (Van den Berg *et al.*, 1998).

For intercropping to be successful the correct combination of crops (a host plant and a non-host companion plant) needs to be used. For example, planting maize and sorghum together for the control of *C. partellus* would increase the intensity of the pest in both crops, as both plants are host plants of *C. partellus* (Ogwaro, 1983). The intercropping of sorghum with pearl millet would on the other hand result in a decrease in larval infestation of sorghum stems,

because *B. fusca* larval survival on pearl millet is low (Adesiyun, 1983). Gounou and Schulthess (2006) also reported lower infestation levels of stemborers in maize/rice intercropping systems.

Cultural control is regarded as the most relevant method of stemborer control for the majority of resource-poor farmers in Africa (Obonyo, 2009a). It is economical, ecologically safe and non-polluting (Oka, 1979; Van den Berg *et al.*, 1998; Kfir *et al.*, 2002). Cultural control should therefore be the first approach around which other control strategies should be build (Coaker, 1987). This approach does however not attract the same interest as the other IPM approaches. The reason for that being that it is labour intensive and cannot lower pest infestations below the economic damage threshold when used independently. It also needs the co-operation of farmers within a particular region to be effective since moths emerging from an untreated field can infest adjacent crops (Van den Berg *et al.*, 1998; Dent, 2000). Cultural control in combination with other control approaches such as the use of pest resistant varieties is therefore recommended to optimise its effectiveness (Dent, 2000). Thorough knowledge of the eco-biology of the crops as well as their pests is needed before new cultural control strategies can be introduced (Oka, 1979), to ensure that they are compatible (Dent, 2000).

### **1.3.3. Host plant resistance**

According to Beck (1965), host plant resistance is defined as the collective heritable characteristics by which a plant species, race, clone or individual may reduce the possibility of successful utilization of that plant as a host by an insect species, race, biotype or individual. It therefore aims to reduce the intrinsic rate of population growth below zero, at which point the pest population will reduce over time or decrease to a level below the economic threshold level (Thomas & Waage, 1995). In terms of crop production, it is the inherent or intrinsic ability of crop plants to prevent, restrict, retard and overcome infestations of pests and improve the yield and quality of their harvest (Dent, 2000). Host plant resistance is considered to be the most promising and ideal method for the control of pests, since it is economically acceptable to farmers, effective, poses no environmental hazard and is generally compatible with other control methods (Bosque-Perez & Schulthess, 1998). Host plant resistance can be achieved by either conventional breeding or genetically modifying crops (Fontes *et al.*, 2002; Hilbeck, 2002).

### **1.3.3.1. Genetically modified (GM) crops**

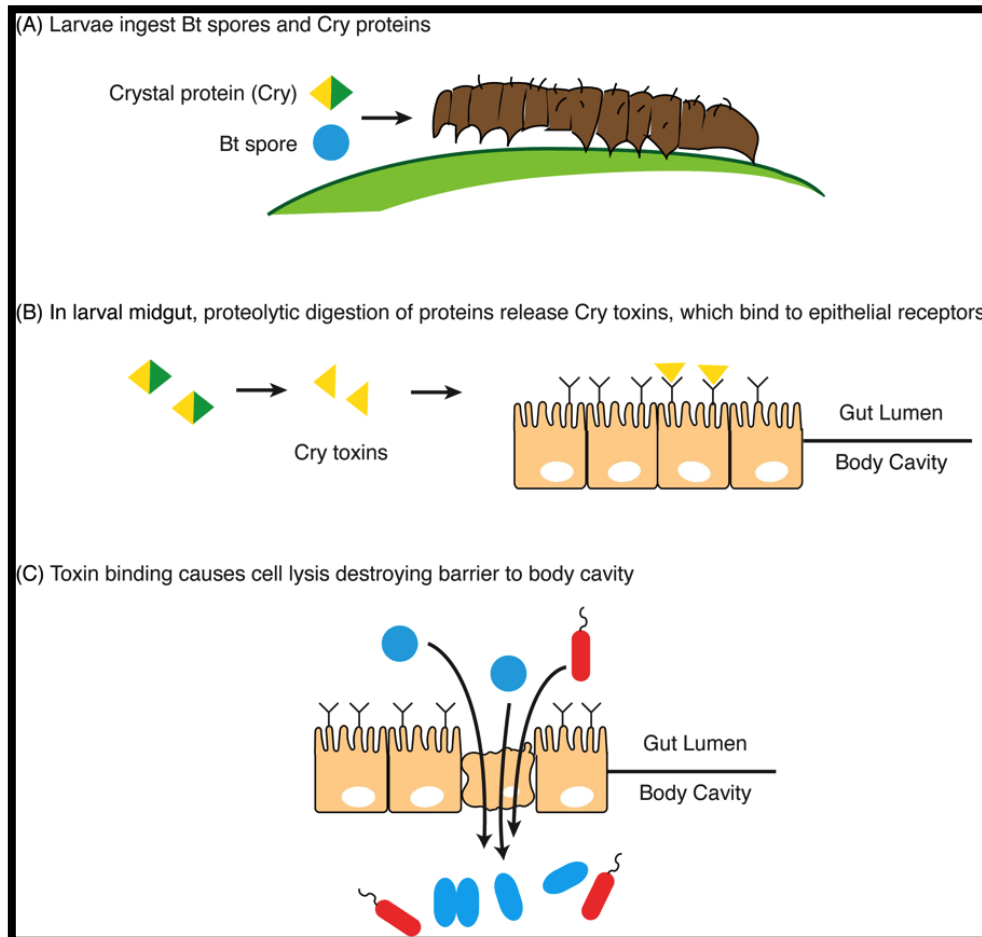
A genetically modified organism (GMO) refers to either plants or animals, in which their genetic material has been altered with genes that confer certain properties (Anklam *et al.*, 2002). Genetically engineered plants are usually classified into one of three groups. First-generation GM crops feature enhanced input traits such as herbicide tolerance, resistance to insect pests and environmental stress. Second generation GM crops feature value-added output traits such as nutrient-enhanced seeds, while third-generation crops feature traits that allow the production of pharmaceuticals and products beyond traditional food (Fernandez-Cornejo & McBride, 2002).

South Africa was the first country in Africa to commercially produce transgenic crops (Bt cotton) in 1997 (Gouse *et al.*, 2005). GM maize and cotton with insect resistance and herbicide tolerance as well as GM soybean with herbicide tolerance are currently cultivated in South Africa (Brookes & Barfoot, 2010). These GM food crops provide the opportunity to increase the amount of food available, by addressing any inherent limitations within the crop, which in turn increases food security (Mannion & Morse, 2013). South Africa, with a GM crop production of 2.7 million hectares, ranked ninth out of 26 countries in terms of area planted to GM crops (James, 2016). It is estimated that approximately 70% of the maize planted in South Africa is genetically modified, with 43% of that maize having traits that provide protection against maize stemborers and the remaining 57% having traits that provide herbicide tolerance (Falck-Zepeda *et al.*, 2013).

#### **1.3.3.1.1. Bt transgenic maize**

Insect-resistant Bt maize, is genetically engineered to encode pre-activated crystal protein toxins (Cry toxins) from the common soil-borne bacterium *Bacillus thuringiensis* Berliner (Bt) (Bøhn *et al.*, 2010; Székács *et al.*, 2012). This is achieved by inserting various transgenes from the bacterium into the genome of the plant (De Maagd *et al.*, 1999; Székács *et al.*, 2012). These pre-activated toxins, which are truncated forms of the bacterial protoxins, are continuously produced in plants (De Maagd *et al.*, 1999; Then, 2010). When ingested by a susceptible larva they are proteolytically activated in the insect midgut by enzymes (Whalon & Wingerd, 2003; Székács *et al.*, 2012; Van der Hoeven, 2014). The activated toxins then go through a complex sequence of events binding to specific midgut membrane receptors on the surface of the columnar epithelial cells to exert toxicity. This leads to the toxin being inserted into the membrane, aggregation of toxins, and the subsequent formation of pores (Schnepf *et al.*, 1998; Whalon & Wingerd, 2003; Tabasnik *et al.*, 2015). The cells are destroyed in this

way, leading to osmotic lysis which causes the larva to be killed by subsequent starvation or septicemia (Whalon & Wingerd, 2003; Székács *et al.*, 2012) as shown in Figure 1.3. Bt maize is currently used worldwide to control crop-feeding pests in the orders Coleoptera, Diptera, Hymenoptera and Lepidoptera (De Maagd *et al.*, 2001).



**Figure 1.3:** Action of Bt-proteins in an insect's midgut (Niederhuber, 2015).

Yellow Bt maize, which contains a Cry1Ab gene (single-gene Event MON810), was planted in South Africa for the first time during the 1998/99 growing season to control the two target stemborer species *B. fusca* and *C. partellus* (Van Rensburg, 1999). South Africa was therefore the first African country to plant Bt maize on a commercial scale (Van Rensburg, 2001). White Bt maize was first introduced into South Africa in 2001, but was planted during the 2002/03 growing season for the first time (Gouse *et al.*, 2005). With maize being a staple food in Africa and stemborers causing significant damage to maize crops, planting of Bt maize could have substantial positive impacts on the livelihood and food security of small-holder farmers (Fischer *et al.*, 2015). Protection of maize against stemborer damage may therefore result in increases in the quality and quantity of yields as well as a reduction in the use of expensive and environmentally hazardous insecticides (Gouse *et al.*, 2005; 2006).

*Busseola fusca* larvae resistant to Bt maize (MON 810) in South Africa, were found and reported during the 2006/07 growing season in the Christiana region (27°57'S, 25°05'E) (Van Rensburg, 2007). Resistance to Bt maize has since spread to many areas in the maize production region of the country (Kruger *et al.*, 2012). Proposed mechanisms for resistance to Bt maize include the modification of the site where the toxin binds, the quick replacement of the cells affected by Bt toxins and variations in the pH of the gut lumen (Martínez-Ramírez *et al.*, 1999; Oppert, 1999; Ma *et al.*, 2005). Another proposed mechanism suggests a change in the midgut's microbial content (Broderick *et al.*, 2006). Due to this resistance development, the emphasis was again placed on the implementation of alternative strategies to manage and control these pests.

#### **1.3.3.1.1.1. Non-target effects of Bt maize**

Debates regarding the use of GM crops, such as Bt maize, have been ongoing since it was first commercialised in 1996 (James, 2016). According to Mannion and Morse (2013), claims concerning the advantages and disadvantages of GM crops are based on the history of GM crops, results from laboratory and field experiments as well speculation. Advantages and disadvantages of GM crops are considered in four overlapping categories, namely agronomic issues, economic issues, environmental issues and social issues (Mannion and Morse, 2013).

The major advantages of using Bt maize in agricultural production systems include the significant reduction in the use of insecticides, improved suppression of target pests, season-long protection irrespective of weather conditions, improved yields and reductions in production input costs which in turn leads to an increase in profitability. The use of Bt maize may, however, also be disadvantageous as it can lead to the crossing out of non-transgenic plants (pollen drift), horizontal transfer of transgenes to unassociated organisms, development of Bt resistance in target pests and disruption of ecosystem processes (Naranjo *et al.*, 2005; Brookes & Barfoot, 2010). Bt maize is also more expensive than comparable non-Bt seed (Van Rensburg *et al.*, 1985).

Although Bt maize is generally thought to be environmentally safe to humans and animals, concerns have been raised about the non-target effects in trophic interactions (Fontes *et al.*, 2002). Tritrophic interactions, as the name suggests, involves the interactions between the three trophic levels, namely the plant (primary level), the pest (secondary level) and the natural enemy (tertiary level) (Price *et al.*, 1980). According to Hilbeck (2002) the term “non-target effects” refers to any unintended effects of transgenic, insecticidal plants (first trophic level) on organisms other than the target species itself. These unintended target species may

include pollinators, detritivores, and other herbivores (second trophic level) as well as organisms from higher trophic levels (third trophic level) such as the natural enemies of both the original target species and the non-target herbivores (Hilbeck, 2002). Due to the presence of Bt-toxins in a Bt maize field most of the target herbivores colonising Bt maize fields will ingest plant tissue containing Bt proteins. This may then be passed on to their natural enemies in a more or less processed form (Hilbeck & Bigler, 1999).

Many glasshouse, laboratory, field and semi-field studies that investigated the potential effects of Bt crops on non-target, beneficial species have been done (Groot & Dicke 2002; Romeis *et al.*, 2006). The effects of the Bt-toxin (Cry1Ab protein produced in Bt-maize) on the survival, population growth and reproduction of the water flea (*Daphnia magna* Straus (Cladocera: Daphniidae)), a crustacean arthropod commonly used as a model organism in ecotoxicological studies, was investigated by Bøhn *et al.* (2010). *Daphnia magna*, that fed on pollen and detritus from drainage water coming from agricultural fields planted with Bt crops, was negatively influenced by the Bt toxins which caused negative long-term effects on their fitness parameters (Bøhn *et al.*, 2010). Vojtech *et al.* (2005) investigated the effect of Bt transgenic maize on *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), a non-target host, and *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), a parasitoid of *S. littoralis*. Bt maize was found to have a significant effect on the developmental times and larval mass of *S. littoralis*, and it also negatively affected the survival, developmental times and cocoon mass of *C. marginiventris* if its host was reared on Bt maize (Vojtech *et al.*, 2005).

In tritrophic bioassays conducted by Garcia *et al.* (2010) to assess the prey-mediated effects of Cry1Ab (Bt maize) on the performance and digestive physiology of *Atheta coriaria* (Kraatz) (Coleoptera: Staphylinidae) using its prey *Tetranychus urticae* Koch (Acari: Tetranychidae) (Bt-fed), no differences were found between any of the parameters analysed which included the duration of the immature stages, sex ratio, survival, fecundity, egg fertility and proteolytic activities when reared on Bt-fed and Bt-free prey. Lundgren and Wiedenmann (2005) assessed the effect of rootworm resistant maize (MON863 expressing Cry3Bb1 protein) on the predator *Coleomegilla maculata* (DeGeer) (Coleoptera: Coccinellidae) by feeding it Bt-reared *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae). They found the fitness parameters of *C. maculata* to be similar when reared on Bt-reared or Bt-free prey, despite the aphids having a 33% reduction in mass (Lundgren & Wiedenmann, 2005).

In studies where adverse effects on life-table parameters of different parasitoids and predators were reported, the parasitoids and predators were fed herbivores that were sensitive to Cry toxins and were therefore sub-lethally affected (Wang *et al.*, 2017). These adverse effects,

also referred to as 'prey quality-mediated effects', were observed in numerous tritrophic systems with parasitoids or predators and Bt-transgenic plants (Li *et al.*, 2013; 2014; Han *et al.*, 2015). It may, in some cases, be explained not by the direct effect of the plant-produced Cry toxin on the parasitoids or predators, but rather by the reduced prey/host quality (Romeis *et al.*, 2006; Naranjo, 2009). The direct effects of Bt could therefore not be excluded, even though all of the adverse effects on parasitoids and parasites could be indirect (Wang *et al.*, 2017).

*Macrocentrus cingulum* (Brischke) (Hymenoptera: Braconidae) is not sensitive to the Cry1Ac toxin at concentrations higher than the concentrations encountered in Bt maize fields (Wang *et al.*, 2017). It is therefore necessary to use resistant hosts (Bt-resistant herbivores) or non-susceptible hosts (herbivores not susceptible to Cry toxins produced by plants) when assessing the possible adverse effect of GM plants on non-target organisms (Wang *et al.*, 2017). Using Cry-protein resistant hosts such as larvae of certain Lepidoptera species, ensures that the hosts are healthy and that the quality of the host does not affect the outcome of results regarding the parasitoids or predators that feed on these hosts (Chen *et al.*, 2008; Tian *et al.*, 2014a; Wu *et al.*, 2014; Su *et al.*, 2015).

Tian *et al.* (2014b) used Cry1F resistant *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) larvae to evaluate the effects of Cry1F on *C. marginiventris*, a larval endoparasitoid of *S. frugiperda*, over five generations. In doing so, they overcame the possible prey-mediated effects, as well as concerns about potential differences in laboratory- or field-derived Bt resistance. The developmental time, parasitism success, survival, sex ratio, longevity and fecundity of *C. marginiventris* was not affected when *S. frugiperda* larvae which were reared on Cry1F maize was parasitised. The findings of previous studies that Bt proteins are harmful to *C. marginiventris* were therefore refuted and it was suggested that those findings could rather be ascribed to prey-mediated effects through the use of Bt-susceptible lepidopteran hosts (Tian *et al.*, 2014b). Prey-mediated effects were also overcome in a laboratory feeding experiment in which the effects of the Bt-fed prey *Anaphothrips obscurus* (Müller) (Thysanoptera: Thripidae) (not sensitive to Cry1Ab toxins from Bt) on the predator *Orius majusculus* (Reuter) (Heteroptera: Anthocoridae) were studied. No significant differences in mortality or developmental time of *O. majusculus* were recorded between *A. obscurus* individuals that were reared on Bt and non- Bt plants (Zwahlen *et al.*, 2000).

### **1.3.4. Biological control**

#### **1.3.4.1. Defining biological control**

Biological control is the action of living organisms as pest control agents (Thomas & Waage, 1996). In a more elaborate definition, DeBach and Rosen (1991), defined it as the control and regulation of pest populations. This occurs either when alien pests are introduced into new geographic areas where their natural enemies are absent or chemicals have destroyed their natural enemy populations (Price, 1987), or when habitat modifications that differentially favours the pest (e.g. habitat simplification with monoculture) occurs, resulting in pests becoming dissociated from their natural enemies (Dent, 2000). Biological control is achieved by introducing exotic natural enemies or by using native natural enemies such as pathogens (bacteria, fungi, viruses, nematodes and protozoa), predators (entomophagous insects or vertebrates), parasites (nematodes) and parasitoids (DeBach & Rosen, 1991; Thomas & Waage, 1996). The interactions between insects and their natural enemies are therefore essential ecological processes contributing to the regulation of insect populations (Dent, 2000). It should be integrated into IPM strategies to reduce infestation levels to below economic injury levels (Van den Berg *et al.*, 1998).

#### **1.3.4.2. Different biological control approaches**

All insect pests in crops are under some degree of biological control, which is often limited by various factors. These factors include low plant diversity and the consequences thereof for the populations of natural enemies, the use of pesticides and the highly seasonal nature of field crops which makes it difficult for natural enemies to “catch up” with pests when they enter and start to grow rapidly on the abundant food of the early season (Thomas & Waage, 1996). Adding to these limiting factors is climate change which shifts host-natural enemy phenologies and consequently effects ecosystem services. The focus should therefore not only be on the impact of climate change on target organisms in ecological networks but also on the interactions between these organisms (Walther, 2010). There are five methods of biological control, namely classical biological control (most emphasized), inundation, augmentation, inoculation and natural enemy conservation (Dent, 2000).

##### **1.3.4.2.1. Classical biological control**

Classical biological control which started in the 1800's is the process where natural enemies of exotic insect pests are imported from their area of origin to control them (Ehler, 1998). It is

highly cost-effective and has the ability to suppress pest populations permanently through a single introduction, and have few risks associated with it (Thomas & Waage, 1996; Dent, 2000). Since natural enemies are imported, they need time to adapt to the environment which may take a long time (Ehler, 1998), but once adapted and established natural enemies can cause declines in pest populations. As the number of pests decrease, so do those of the number of the natural enemies, until a balance is reached where a low number of pests persist and any local resurgence is checked by the density-dependent natural enemy action. This may leave the pest population well below damaging levels (Thomas & Waage, 1996). An example of classical biological control is the introduction of *Neochetina eichhorniae* Hustache (Coleoptera: Curculionidae) (weevil) in South Africa. This species was imported and released for control of the aquatic weed, *Eichhornia crassipes* Mart. (Solms) (Commelinales: Pontederiaceae) (water hyacinth), for which chemical and mechanical control proved to be unsuccessful (Cilliers, 1991).

#### **1.3.4.2.2. Inundation**

Inundation is a biological control process which involves the release of massive numbers of natural enemies within a very short period of time. The natural enemy released is usually not very persistent, kills the pest relatively quick and is usually only relevant to the use of pathogens. These pathogens which include viruses, bacteria, fungi and entomopathogenic nematodes (EPN's) are formulated as biopesticides, which can be utilized as alternatives to chemical insecticides (Dent, 2000). An example of inundation is the use of the Green Muscle, as a mycoinsecticide, for locust and grasshopper control (Neethling & Dent, 1998).

#### **1.3.4.2.3. Augmentation and inoculation**

Augmentation and inoculation is used in situations where natural enemies are absent or population levels are too low to be effective (Dent, 2000). Augmentation involves the mass release of local natural enemies, reared in laboratories (Thomas & Waage, 1996; Dent, 2000). There usually is no interest in long-term sustainability but only in the suppression of pest numbers below the economic threshold level (Van Lenteren, 1986). This leads to multiple releases being needed, because the control is only temporary. Control with inoculative releases on the other hand will be seasonal or for the duration of the crop. Inoculative releases of natural enemies are used in situations where a native pest has extended its range, causing it to be separated from its natural enemies, or when an introduced natural enemy species is unable to survive indefinitely. An example of augmentation is the release of *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) to control the greenhouse whitefly, *Trialeurodes*

*vaporariorum* Westwood (Homoptera: Aleyrodidae) (Dent, 2000). An example of inoculation is the release of *Rhizophagus grandis* Gyllenhal (Coleoptera: Rhizophagidae) in Great Britain to control the greater European spruce beetle, *Dendroctonus micans* (Kugelann) (Coleoptera: Scolytidae) (Grégoire *et al.*, 1990). It established in infested areas of Britain and regulates the endemic levels of *D. micans* (Fielding and Evans, 1997).

#### **1.3.4.2.4. Natural enemy conservation**

Natural enemy conservation, also known as conservation biological control, is the process by which indigenous natural enemies of pests are conserved (Ehler, 1998; Bale *et al.*, 2008). This is done by various measures including the manipulation of the microclimate of crops, increasing the availability of alternative hosts and prey, creation of overwintering refuges and providing essential food resources (Gurr *et al.*, 2000; Wäckers, 2003; Winkler *et al.*, 2005). Even though conservation biological control has the disadvantage of being dependent on naturally occurring enemies of pests, it still offers the advantage of being more adapted to control their targets unlike classical biological control (Ehler, 1998). An example of conservation biological control is the conservation of native species of mite predators, to control the European red mite, *Panonychus ulmi* (Koch) (Acari: Tetranychidae) and two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) (Fulekar, 2010).

#### **1.3.4.3. Biological control of stemborers**

Biological control has been used against *B. fusca* and other maize stemborers in Africa (Harris & Nwanze, 1992), since these agents have effectively been used against stemborers on sugarcane in the Caribbean (Klopper, 2008). Interest in the use of biological control agents to reduce the density of stemborer populations has been renewed after Ingram (1983) stressed that little was known about the predation on stemborers, other than the occasional references to the eggs and first instar larvae of stemborers being attacked by ants. These biological control agents include ants, earwigs and spiders (all of which are believed to cause high mortalities of stemborer eggs and young larvae). Exotic parasitoids have also been introduced when indigenous larval and pupal parasitoids were not abundant enough to keep stemborer populations below economic injury levels (Mohyuddin & Greathead, 1970).

Stemborer larvae are mainly parasitised by hymenopteran or dipteran parasitoids (Kfir *et al.*, 2002). *Cotesia* spp. (Hymenoptera: Braconidae), *Goniozus* spp. (Hymenoptera: Bethylinidae), *Syzeuctus* spp. (Hymenoptera: Ichneumonidae), *Enicospilus* spp. (Hymenoptera: Ichneumonidae), *Pediobius furvus* (Gahan) (Hymenoptera: Eulophidae), *Sturmiopsis*

*parasitica* (Curran) (Diptera: Tachinidae) and *Descampsina sesamiae* Mesnil (Diptera: Tachinidae) are all examples of such parasitoids (Gounou & Schulthess, 2006; Hassanali *et al.*, 2008).

The effectiveness of parasitoids as biological control agents has been questioned by numerous authors (Kfir, 1995; Chabi-Olaye *et al.*, 2001; Kfir *et al.*, 2002; Van Rensburg & Flett, 2008). Their effectiveness may be influenced by their inability to regulate the population numbers of stemborers effectively, as well as poor establishment of newly introduced control agents (Kfir *et al.*, 2002).

#### **1.4. *Cotesia* species**

*Cotesia* spp. are highly effective biological control agents of stemborers of the Crambidae, Pyralidae and Noctuidae families (Mohyuddin, 1971; Beg & Inayatullah, 1980; Mohyuddin *et al.*, 1981). These parasitoids belong to an extremely species rich genus in the subfamily Microgastrinae of the family Braconidae (Hymenoptera) (Walker, 1993). This subfamily has an estimated number of almost 1 000 species distributed worldwide. They are significant for both their practical application in the biological control of pests, as well as their role as key model organisms in basic physiology and molecular biology studies of host-parasitoid interactions and ecological research (Michel-Salzat & Whitfield, 2004).

The two most common *Cotesia* species used against medium to large-sized tropical stemborers such as *B. fusca*, *C. partellus* and *S. calamistis* are the larval parasitoids *Cotesia sesamiae* (Cameron, 1906) [= *Apanteles sesamiae* (Cameron)] (Hymenoptera: Braconidae) and *Cotesia flavipes* (Cameron, 1891) [= *Apanteles flavipes* (Cameron)] (Hymenoptera: Braconidae) (Walker & Overholt, 1993). They are gregarious, koinobiont larval endoparasitoids (Walker, 1994) which are taxonomically closely related parasitoid wasps with a similar biology. These species are difficult to distinguish morphologically (Walker, 1993; Kimani-Njogu & Overholt 1997). Males can be distinguished from females by their longer antennae (Walker & Overholt, 1993) (Figure 1.4).



**Figure 1.4:** (A) A male *Cotesia* spp. wasp, identified by its longer antennae; (B) A female *Cotesia* spp. wasp; (C) A male and female *Cotesia* spp. wasp mating.

Koinobiont larval endoparasitoids lay their eggs in the larval host and larvae develop inside the larval hosts, with the final instar parasitoid larvae emerging from the host to pupate (Waage & Hassell, 1982) (Figure 1.5). Identification is mainly based on the reliable, but time consuming process of the examination of the male genitalia (Walker, 1993). The tip of the aedeagus of *C. sesamiae* has a more pointed penis valve than that of *C. flavipes* (more truncated). Identification is further backed up by the characteristic external morphology of the shape of the face and *C. sesamiae* has more hair on their face than *C. flavipes* (Sigwalt & Pointel, 1980; Polaszek & Walker, 1991; Kimani-Njogu & Overholt, 1997; Muirhead *et al.*, 2008). This is however difficult to distinguish when no specimens for comparison are available (Sigwalt & Pointel, 1980; Walker, 1993). Molecular identification of these parasitoids is also used, especially in addition to morphological identification to confirm the morphological identification (Dupas *et al.*, 2006; Assefa *et al.*, 2008; Getu, 2008).



**Figure 1.5:** Developmental stages of a *Cotesia* spp. parasitoid. (A) A final instar larva emerging from the larval host, (B) Final instar larvae pupate into cocoons after leaving the larval host, (C) *Cotesia* spp. wasp emerging from a cocoon.

Females mate only once during their lifetime unlike males which can mate several times. *Cotesia* spp. females inject and deposit multiple eggs (15-65) into the larval hosts (Walker &

Overholt, 1993). After three days these eggs hatch into small grub-like first instar larvae that feed on tissues inside the host for a week or two. The final (third) instar larvae chew small holes through the integument of their host and emerge through it (Figure 1.5A). The egg and larval period lasts 10-15 days (Walker & Overholt, 1993; NCSU, 2010). After emergence from the host larvae, the final instar larvae immediately start spinning silk cocoons and pupate (Walker & Overholt, 1993). These cocoons, which are often mistaken for eggs, are attached to the outside of their host which are still alive and dies within a day or two (Figure 1.5B). The cocoons can also be found inside the feeding tunnels made inside stems of poaceous host plants of Lepidoptera larvae. The cocoons darken and the next generation of wasps emerge within a week after pupation of the larvae (Figure 1.5C) (Walker & Overholt, 1993; NCSU, 2010). The adult parasitoids usually have a short lifespan of approximately 34 hours at 25°C if adults are not fed. The lifespan of these wasps can, however, be prolonged to approximately 51 hours by providing them with a 20% honey solution (Walker & Overholt, 1993).

*Cotesia flavipes* and *C. sesamiae*, similar to many other hymenopteran species, have a haplodiploid sex determination system. Males which are haploid (one chromosome), develop from unfertilised eggs and females which are diploid (two chromosome sets), develop from fertilised eggs (Walker & Overholt, 1993; Van Wilgenburg *et al.*, 2006). Unmated females, although still capable of oviposition, are therefore only able to produce male offspring while mated females are able to produce both male and female offspring (Walker & Overholt, 1993). This is the case as they do not have complementary sex determination (CSD), a biological process present in species with a genotype at one single locus with multiple alleles (Niyibigira *et al.*, 2004). This leads to diploid individuals developing into females when heterozygous and into males (generally either inviable or sterile) when homozygous, and therefore circumvent the genetic load by avoiding diploid male production altogether (Elias *et al.*, 2009).

Successful parasitism by these parasitoids require a sequence of distinct and consecutive processes (Vinson, 1975), including host habitat location, host location, host selection and acceptance, and host suitability and regulation (Smith *et al.*, 1993). During foraging these parasitoids use plant volatile chemical cues (semiochemicals) as well as host frass (Walker & Overholt, 1993; Obonyo *et al.*, 2010a) to guide them to their specific host habitat (infested plant) and eventually their host (inside the stem of the infested plant) (Vinson, 1975). Certain maize plant odours may also attract searching female *C. flavipes* parasitoids (Walker & Overholt, 1993). This is especially the case with stemborer infested plants (producing richer volatile profiles mainly comprising of C5-C6 alcohols, aromatic and aliphatic compounds and terpenoids) compared to uninfested plants (Ngi-Song *et al.*, 1996; Jembere *et al.*, 2003; Obonyo *et al.*, 2008). Hosts from their natural diet (maize or sorghum) were found to be more

attractive to *C. flavipes* parasitoids than hosts from an artificial diet (Walker & Overholt, 1993). The ability to perceive semiochemicals is therefore an important factor in successful parasitisation by these parasitoids (Dicke & Vet, 1999).

*Cotesia flavipes* and *C. sesamiae* display a similar hierarchy of behavioural events during host selection and acceptance. This includes the use of the antennae and particularly the distal antennomeres for host recognition, as well as both antennae and the tarsi for host acceptance and oviposition (Obonyo *et al.*, 2010b; Obonyo *et al.*, 2011). Both *C. flavipes* and *C. sesamiae* females share the same types and distribution of sensory receptors which allow them to detect volatiles as well as contact chemical stimuli from their hosts. This includes four types of sensilla on the three terminal antennomeres, namely non-porous sensilla trichodea likely to be involved in mechanoreception, uniporous sensilla chaetica with porous tips that have a gustatory function, multiporous sensilla placodea likely to have an olfactory function and sensilla coeloconica known to have a thermo-hygroreceptive function. The tarsi possess a few uniporous sensilla chaetica with porous tips which may have a gustatory function, as well as the distal end of the ovipositor which has numerous dome-shaped sensilla. No styloconica or sensilla coeloconica, known in other parasitoid species to have a gustatory function, are present in the ovipositors of these two species (Obonyo *et al.*, 2011).

After introduction of a suitable host larva to female *C. flavipes* and *C. sesamiae* parasitoids, a 16-17 seconds latency period follows after which the wasp walks quickly while drumming the surface with its antennae until it locates the larva. Location of the host lasts approximately 60-70 seconds while the antennal examination of the host lasts 30 seconds, followed by stinging with the ovipositor for a period of 5-6 seconds for successful oviposition. In the presence of non-host larvae, the latency period is between 25-70 seconds. The parasitoids also spend significantly more time walking and antennal drumming on non-host larvae without ovipositing. The decision whether to oviposit or not is therefore dependent on the use of tactile and contact-chemoreception stimuli from the hosts (Obonyo *et al.*, 2010b). Water soluble chemicals present on the surface of the larval cuticle were found to stimulate oviposition in both *C. flavipes* and *C. sesamiae* (Obonyo *et al.*, 2010a).

*Cotesia flavipes* and *C. sesamiae* together with *Cotesia chilonis* (Matsumura) (Hymenoptera: Braconidae), indigenous to Japan and China (Kimani-Njogu & Overholt, 1997), are generally believed to be morphologically similar species of *Cotesia* and is often referred to under the name the “*C. flavipes* complex” (Sigwalt & Pointel, 1980; Walker & Overholt, 1993). All three members of this complex, which have been redistributed from their native areas for use in classical biological control, are economically important worldwide as biological control agents

of cereal and sugarcane stemborers (Polaszek & Walker, 1991). They have similar ecological roles and overlapping host ranges, and are therefore likely to compete in nature (Ngi-Song *et al.*, 1995; 1996). A fourth species, *Cotesia nonagriæ* (Olliff) (Hymenoptera: Braconidae) indigenous to Australia, was also recently added to this species complex. This species was removed from its previous synonymy with *C. flavipes* due to it being found to represent a cryptic species on the basis of mitochondrial DNA phylogeography and biology (Muirhead *et al.*, 2006; 2008). The *C. flavipes* complex is therefore now classified as a monophyletic complex consisting of four allopatric sister species (Kimani-Njogu & Overholt 1997; Muirhead *et al.*, 2012).

#### **1.4.1. *Cotesia flavipes***

The biology of the exotic *C. flavipes* was initially studied and recorded by Gifford & Mann (1967) and later by Mohyuddin (1971). The adult is about 3-4 mm in length and lives for only a few days (Obonyo, 2009a). *Cotesia flavipes* should therefore mate quickly after they emerge and begin their search for suitable hosts (Walker & Overholt, 1993). The location of host larvae by *C. flavipes*, unlike *C. sesamiae*, is also mediated by a water soluble substance present in dried or rehydrated faeces of larvae (Botelho & Macedo, 2002). The female parasitoid, when in contact with stemborer faeces, is induced to begin its searching behaviour. This is characterised by a reduction in the rhythm of the motion of the parasitoid as well as by the touching of the faeces with its antennae. *Cotesia flavipes* also uses regurgitated material and the gallery system caused by borer feeding in the stem of the host plant, in addition to larval faeces, to locate their host. It is therefore believed that *C. flavipes* females not only use olfactory stimuli, as discussed above (see 1.4), but also use tactile stimuli to locate plants infested by host larvae (Da Silva *et al.*, 2012).

*Cotesia flavipes* parasitises several species of crambid and noctuid stemborers, including *Chilo orichalcociliellus* (Strand) (Lepidoptera: Pyralidae), *C. partellus* and *S. calamistis* in Africa (Dejen *et al.*, 2013). It also attacks *B. fusca* and the sugarcane stemborer *Eldana saccharina* Walker (Lepidoptera: Pyralidae), but these hosts are not suitable for development of the parasitoid (Ngi-Song *et al.*, 1996; Jiang *et al.*, 2004). This is due to the ability of the larval host to mount an immune response, known as encapsulation, against the invading species (Godfray, 1994). Encapsulation, the defence mechanism employed by *B. fusca* against a strain of the indigenous hymenopteran parasitoid *C. sesamiae*, is a cellular response process that involves the aggregation of hemocytes around the parasitoid eggs, eventually killing/melanising them and enabling larvae to survive (Ngi-Song *et al.*, 1998). Successful parasitism of *B. fusca* by *C. flavipes* was found to be possible only when *C. sesamiae* had

oviposited first and lowered the immune response of the host, thereby preventing encapsulation of the eggs of *C. flavipes* (Ngi-Song *et al.*, 1995). For multiparasitism to occur, a nonvirulent parasitoid needs to oviposit very soon after a virulent parasitoid oviposited (Sallam *et al.*, 2002). *Busseola fusca* larvae are therefore often found to be parasitised by *C. flavipes* under field conditions (Matama-Kauma *et al.*, 2007).

*Cotesia flavipes* occurs from Pakistan in the west to northern Australia in the east and it has established successfully in Madagascar (verification not available). It was also successfully introduced into the Caribbean and Mauritius (Walker & Overholt, 1993). According to Overholt *et al.* (1994b) and Matama-Kauma *et al.* (2007) the Commonwealth Institute for Biological Control (CIBC) introduced *C. flavipes* during 1968-72 from Pakistan into Uganda and other East African countries for the control of *C. partellus*. It did however not establish until much later in spite of multiple releases (CIBC, 1968–1972).

A new attempt was then initiated in 1991 by the International Centre of Insect Physiology and Ecology (ICIPE) with introductions into the coastal area of Kenya from North and South Pakistan and later India (Overholt *et al.*, 1994a; Overholt, 1998; Getu, 2008). This was done as part of the renewing of ICIPE's emphasis on biological control, and in 1993 releases were done in the coastal areas of southern Kenya (Kipkoech *et al.*, 2006) from Asia and Australasia (Polaszek & Walker, 1991). This introduction, which complemented the action of the closely related *C. sesamiae* (Overholt *et al.*, 1994a;c; Overholt *et al.*, 1997; Obonyo *et al.*, 2010a), was done against its more preferred host the invasive stemborer *C. partellus*. It was done based on the assumption that coevolved natural enemies, such as *C. flavipes* being the coevolved natural enemy of *C. partellus*, are best adapted to locating and successfully attacking their target hosts (Cugala, 2007). *Cotesia flavipes* was also selected as the preferred candidate due to its history of being a successful and important control agent of stemborers in its area of origin, Asia (Overholt *et al.*, 1994a). *Cotesia flavipes* became permanently established on the coast of Kenya and also spread to other areas (Omwega *et al.*, 1995).

The program was thereafter expanded to involve eleven other countries in eastern and southern Africa (Omwega *et al.*, 2006). These were Mozambique in 1996 (Cugala & Omwega, 2001), Somalia and Uganda in 1997, Uganda again in 1998 (Matama-Kauma *et al.*, 2001), and Ethiopia, Zanzibar (Niyibigira, 2003), Zimbabwe, Malawi (Chinwada *et al.*, 2001), Zambia (Sohati *et al.*, 2001) and Tanzania in 1999, and finally Eritrea in 2003. *Cotesia flavipes* established successfully in all of these countries where releases were made, except for Eritrea (Omwega *et al.*, 2006). Successful establishment of *C. flavipes* was also reported in Ethiopia where releases were never made. This was ascribed to possible invasions from Somalia (Getu

*et al.*, 2003). The successful introduction of *C. flavipes* is demonstrated by the establishment and spread from its point of release, increasing parasitism rates, a decrease in the density of stemborers and a positive impact in the economy associated with its introduction (Jiang *et al.*, 2006; Kipkoech *et al.*, 2006).

Over the years, *C. flavipes* has been introduced into at least 40 tropical and subtropical countries worldwide (Polaszek & Walker, 1991), including South Africa for the biological control of *B. fusca*, *C. partellus* (Skoroszewski & Van Hamburg, 1987) and *E. saccharina* (Conlong, 1994). Although *C. flavipes* became temporarily established on both *B. fusca* and *C. partellus* on maize in South Africa, it could not be recovered later (Skoroszewski & Van Hamburg, 1987). This was believed to be because of the relatively harsh winter conditions experienced in South Africa and the long diapause period of its hosts (Ngi-Song *et al.*, 1995; Kfir, 2001).

#### **1.4.2. *Cotesia sesamiae***

The indigenous generalist species *C. sesamiae* (Mailafiya *et al.*, 2010), which is endemic to central and southern Africa (Polaszek & Walker, 1991; Kimani-Njogu & Overholt, 1997; Dupas *et al.*, 2006), is one of the most important and abundant larval parasitoid species of indigenous lepidopteran stemborers in many countries of sub-Saharan Africa (Bonhof *et al.*, 1997). It has a wider host range than *C. flavipes*, and attacks mid- to late larval instars of both exotic and indigenous stemborer species especially noctuids such as *B. fusca* and *S. calamistis* (Mohyuddin, 1971; Overholt *et al.*, 1994c; Kfir *et al.*, 2002). *Cotesia sesamiae* does, however, not appear to be a very effective natural enemy against *C. partellus* (Overholt *et al.*, 1994c). The occurrence of *C. sesamiae* extends from the Sahelian region to South Africa and its establishment may also extend into the southeast Asian region and even Japan. It has been reported to have established successfully in Madagascar and Mauritius (verification not available) (Walker & Overholt, 1993). *Cotesia sesamiae* is the only *Cotesia* species which has been consistently recovered in South Africa (Van Rensburg *et al.*, 1988; Overholt, 1998) and may account for up to 90% of parasitised larvae (Kfir, 1995), although it typically never exceeds 5% parasitism (Sallam *et al.*, 1999).

At least two biotypes of *C. sesamiae* exist in Kenya, namely inland and coastal biotypes, each expressing differential abilities to develop in *B. fusca* larvae (Ngi-Song *et al.*, 1995; Mochiah *et al.*, 2001). The inland biotype originated from the highlands of central and western Kenya and successfully develops inside *B. fusca*. The coastal biotype originates from the lowlands and the eastern province and does not develop successfully inside *B. fusca* (Obonyo, 2009a;

Gitau *et al.*, 2010). These two strains of *C. sesamiae* are consequently termed the virulent and avirulent strains (Ngi-Song *et al.*, 1995; 1998; Mochiah *et al.*, 2001; 2002; Dupas *et al.*, 2008). This variation in the two biotypes is attributed to the ability of the inland type to develop in *B. fusca* due to it being physiologically suitable to mount an immune response against the encapsulation mechanism of the host larvae (Ngi-Song *et al.*, 1995; 1998; Mochiah *et al.*, 2002). Immune suppression of the host by *C. sesamiae* may therefore lead to successful super- or multiparasitism in an otherwise unsuitable host, as discussed in section 1.4.1. According to Mochiah *et al.* (2002) these two biotypes of *C. sesamiae* are, however, still able to interbreed.

Research has been done on *C. sesamiae* as part of the classical biological control program at ICIPE since 1993. It was assumed that its biology and behavioural attributes resembles that of *C. flavipes*. For this reason, less information is available on the biology of *C. sesamiae* as opposed to that of *C. flavipes* (Obonyo, 2009a).

## **1.5. Conclusion**

One of the main environmental concerns with the development of genetically modified Bt maize is the effect that unintended Bt exposure will have on the non-target insect *C. sesamiae*, which is indirectly exposed to Bt proteins as it develops inside *B. fusca* hosts that feed on Bt maize (Tounou *et al.*, 2005; Obonyo, 2009b; Ndolo *et al.*, 2018). Due to South Africa being the only country in the world having *B. fusca* larvae that have evolved resistance against Bt maize (Kruger *et al.*, 2011), no real-life evaluation of the indirect effect of Bt exposure on *C. sesamiae* has been done before. Since resistant *B. fusca* larvae feed on Bt maize without a negative influence on their health, it is for the first time possible to determine the indirect effect of Bt exposure, using *B. fusca* larvae that fed on Bt maize, on the fitness and survival (in terms of reproduction) of *C. sesamiae*. This study therefore addresses a multi-trophic level interaction between the primary level (Bt maize), secondary level (Bt maize-resistant *B. fusca* larvae) and the tertiary level (*C. sesamiae*).

## **1.6. Aims and objectives**

### **1.6.1. Aims**

The aims of this study were to determine if *C. flavipes* has established in South Africa, as well as to determine the indirect effects of Bt maize (Cry1Ab) on *C. sesamiae*.

### 1.6.2. Objectives

The specific objectives of this study were to determine:

- if *C. flavipes* has established in South Africa using morphological identification and molecular analyses to determine the species composition of *Cotesia* spp. (*C. flavipes* and *C. sesamiae*) sampled from field collected *B. fusca*, *C. partellus* and *S. calamistis*, respectively.
- the indirect effects of Bt exposure on *C. sesamiae* fitness (in terms of reproduction) and survival.

The results of this study are presented in the form of chapters with the following titles:

- **Chapter 2:** Morphological and molecular identification of *Cotesia* spp. reared from maize stemborer larvae in South Africa
- **Chapter 3:** The effects of indirect exposure to Cry1Ab protein on fitness and survival of *Cotesia sesamiae*
- **Chapter 4:** Conclusion and recommendations

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## Chapter 2: Morphological and molecular identification of *Cotesia* spp. reared from maize stemborer larvae in South Africa

### 2.1. Abstract

*Cotesia flavipes* Cameron and *Cotesia sesamiae* (Cameron) (Hymenoptera: Braconidae) are the main larval parasitoids of the economically important lepidopteran stemborer species *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae). *Cotesia sesamiae* is endemic to the Afrotropical region, while *C. flavipes* is indigenous to the Indo-Australian region. Both species have been redistributed worldwide for their use in classical biological control programs. *Cotesia flavipes* has successfully been introduced into several countries in eastern and southern Africa, including South Africa: it could not be recovered after the first winter following its release in South Africa, despite its temporary establishment. *Cotesia flavipes* was recently found in Botswana where releases made in neighbouring countries are assumed to be the reason for the presence of this species, since it was never released in this country. The aim of this study was to determine if *C. flavipes* has now also established in South Africa. *Busseola fusca*, *C. partellus* and *S. calamistis* larvae were collected from various localities in South Africa. *Cotesia* spp. recovered from these larvae were identified by means of morphological identification. Since these two closely related sibling parasitoids are difficult to distinguish, the morphological identifications were confirmed molecularly. The only *Cotesia* species recovered from all localities were *C. sesamiae*, which confirms previous reports that no *C. flavipes* has established in South Africa to date.

### **Keywords**

*Cotesia flavipes*, *Cotesia sesamiae*, CrV1, male genitalia, molecular identification, morphological identification, stemborers.

### 2.2. Introduction

Classical biological control is the effective process whereby natural enemies of exotic insect pests are imported from their area of origin into a new environment where it did not exist to control the target pest (Ehler, 1998; Lazarovitz *et al.*, 2007). According to Dupas *et al.* (2006) this method of biological control not only aims to stabilise ecosystems that have been destabilised by the introduction of these exotic invaders or pests but also by intensive agriculture. This is based on the theory that the introduction of natural enemies, which were

previously absent, will decrease the ecological success of invaders or pests causing the equilibrium of the system to be returned (Dupas *et al.*, 2006). It is however important to take into account that natural enemies themselves are also invaders in the system, and can also be unsafe for the ecosystem (Holt & Hochberg, 2001; Waage, 2001). The success of biological control programs have therefore been found to be dependent on the accurate identification and biosystematics of natural enemies as well as target pests (Debach & Rosen, 1991; Overholt, 1998). It is especially the case as closely related antagonist species are often difficult to distinguish morphologically from the introduced agent (Perdikis *et al.*, 2003). This is done by taxonomic diagnostic tools, developed to not only follow up on the success of introductions but to also better evaluate the effect of biological control agents on the environment (Hinz *et al.*, 2001).

*Cotesia flavipes* (Cameron 1891) and *Cotesia sesamiae* (Cameron 1906) (Hymenoptera: Braconidae) are the main gregarious koinobiont larval endoparasitoids of various economically important lepidopteran pests of maize and sorghum in sub-Saharan Africa (Walker, 1994; CABI, 2018). These include the most cited medium to large-sized tropical stemborer species, the noctuids *Busseola fusca* (Fuller) and *Sesamia calamistis* (Hampson), and the crambid, *Chilo partellus* (Swinhoe) (Walker & Overholt, 1993). Both *B. fusca* and *S. calamistis* are indigenous to Africa (Mohyuddin & Greathead, 1970; Harris & Nwanze, 1992), while *C. partellus* was introduced into southern Africa from south eastern and southern Asia (Sallam *et al.*, 1999) somewhere before the 1930's (Tams, 1932). The monophyletic *C. flavipes*-complex consists of *C. flavipes*, *C. sesamiae*, *Cotesia chilonis* (Matsumura) (Hymenoptera: Braconidae), indigenous to Japan and China (Kimani-Njogu & Overholt, 1997), and *Cotesia nonagriæ* (Olliff) (Hymenoptera: Braconidae), indigenous to Australia (Sigwalt & Pointel, 1980; Walker & Overholt, 1993; Muirhead *et al.*, 2006; 2008). These species are four allopatric sister species which are morphologically similar (Kimani-Njogu & Overholt 1997; Muirhead *et al.*, 2012).

*Cotesia flavipes* was introduced into Uganda and other East African countries between 1968 and 1972 for the control of *C. partellus*, as part of a program established by the Commonwealth Institute for Biological Control (CIBC). It did, however, not establish until much later in spite of multiple releases (CIBC, 1968-1972). A new attempt was then initiated by the International Centre of Insect Physiology and Ecology (ICIPE) in 1991 as part of the renewing of ICIPE's emphasis on biological control, where *C. flavipes* was introduced into the coastal area of Kenya from North and South Pakistan as well as from India (Overholt *et al.*, 1994; Overholt, 1998; Getu, 2008). Introductions were also later made in 1993 into the coastal areas of southern Kenya (Kipkoech *et al.*, 2006). *Cotesia flavipes* became permanently established on

the coast of Kenya and was even found to have spread to other areas (Omwega *et al.*, 1995). The program was thereafter expanded to cover 11 other countries in eastern and southern Africa, where they established successfully in all of the countries except Eritrea (Omwega *et al.*, 2006).

Over the years, *C. flavipes* has been introduced into at least forty tropical and subtropical countries worldwide (Polaszek & Walker, 1991). This includes the 1983/84 South Africa introduction for the biological control of *B. fusca* and *C. partellus* (Skoroszewski & Van Hamburg, 1987) as well as *Eldana saccharina* Walker (Lepidoptera: Pyralidae) (Conlong, 1994). Although *C. flavipes* became temporarily established in South Africa on both *B. fusca* and *C. partellus*, it could not be recovered after the first winter (Skoroszewski & Van Hamburg, 1987). This was believed to be due to the relatively harsh conditions experienced in South Africa during the winter months, as well as the long diapause period of its larval hosts (Ngi-Song *et al.*, 1995; Kfir, 2001). Getu *et al.* (2004) demonstrated that the establishment of *C. flavipes* is significantly affected by the host population, temperature and relative humidity, as well as by the interaction of these factors.

In recent field surveys conducted by Mutamiswa *et al.* (2017) to determine the diversity and relative abundance of the natural enemies of stemborers and their associated host plant species on natural and cultivated habitats in Botswana, *C. flavipes* was found to be dominating in cultivated habitats. *Cotesia flavipes* was never released in Botswana, but releases were made in neighbouring countries – including Mozambique (Cugala & Omwega, 2001), Zimbabwe, Malawi (Chinwada *et al.*, 2001) and Zambia (Sohati *et al.*, 2001). This could account for the presence of this species in Botswana (Mutamiswa *et al.*, 2017) as this exotic parasitoid has been reported to be capable to disperse >2000 km away from its release sites (Assefa *et al.*, 2008). Successful establishment of *C. flavipes* has also been found in Ethiopia where releases were never made, probably due to the invasion of *C. flavipes* from Somalia (Getu *et al.*, 2003).

The two congeneric parasitoid species, *C. flavipes* and *C. sesamiae* are difficult to distinguish morphologically (Walker, 1993; Kimani-Njogu & Overholt, 1997). Morphological identification should therefore preferably be confirmed by molecular identification, as chances of miss identification are higher when non-molecular methods of identification are used (Getu, 2008). Polymerase Chain Reaction (PCR) amplification is one of the most powerful techniques for species identification. Although the PCR-RFLP technique based on the specific digestion of a diagnostic amplicon has proven to be highly reliable and it is used widely, the technique based on allele-specific PCR and primer-induced fragment-length variation has proven to be more

cost effective and powerful. This technique uses one forward primer that is communal to all targets and one reverse primer that anneals specifically at different positions for each target. The PCR test using the polydnavirus gene (CrV1) proved to be a rapid and reliable method to distinguish between *C. flavipes* and *C. sesamiae*. Polydnaviruses are therefore suitable markers for low phylogenetic levels within species, and particularly for distinguishing between species that are closely related (Dupas *et al.*, 2006). This is due to the substitution of the virus, which is transmitted on wasp chromosomes from one generation to the next, being accelerated by positive Darwinian selection as well as increased mutation rates (Dupas *et al.*, 2003). In addition, pre-amplified PDV markers in the wasps' ovaries makes detection by PCR easier (Dupas *et al.*, 2006).

Polydnaviruses (PDV's) are obligate symbiotic viruses associated with parasitic wasps belonging to many of the Braconidae and Ichneumonidae families (Webb *et al.*, 2000; Volkoff *et al.*, 2010). The virus has double-stranded segmented circular DNA integrated stably into the genome of the parasitoid (Strand & Burke, 2014). Polydnaviruses exclusively attack larval stages of their lepidopteran hosts where they manipulate the physiology and immune responses of the herbivore (Webb & Strand, 2005; Pennacchio & Strand 2006; Strand & Burke 2013). They replicate only in the calyx region of the wasp's ovaries (Strand, 2010; Strand & Burke, 2014; 2015), where their circular genome is generated from linear DNA copies of the wasp's chromosomes (Volkoff *et al.*, 2010) and is vertically transmitted to the next generation of the wasp (Strand, 2010). During a parasitism event these viral particles are injected into the host when the wasp deposits her egg(s) within the host. This prevents the encapsulation of the eggs of the parasitoid by suppressing the immune response of the host (Edson *et al.*, 1981; Shelby & Webb 1999), enabling the hatching of eggs as well as development and survival of the larvae of the wasp (Beckage, 1998; Kroemer & Webb, 2004) to finally pupate in silken cocoons (Strand & Burke, 2014).

Due to the high migration potential and resultant spread and establishment of *C. flavipes* in new environments (Mutamiswa *et al.*, 2017), the main objective of this study was to determine if *C. flavipes* has established in South Africa since the previous survey by using morphological identification as well as molecular analyses.

## 2.3. Materials and Methods

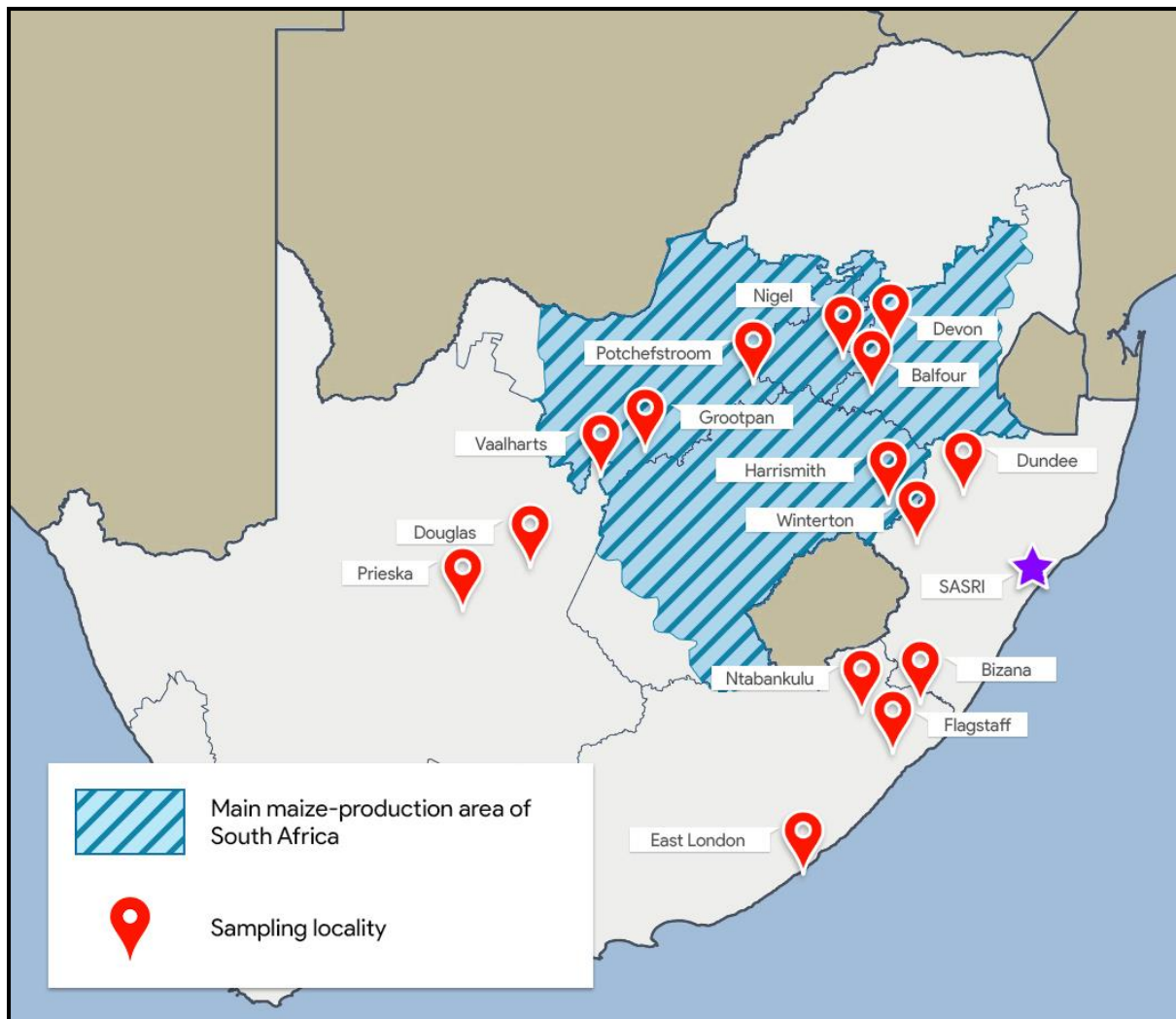
### 2.3.1. Insect sampling

*Cotesia* specimens used in this study were reared and recovered from *B. fusca*, *C. partellus* and *S. calamistis* larvae collected from 15 maize fields in South Africa (Table 2.1).

**Table 2.1:** Localities in South African where stemborer larvae parasitised by *Cotesia* spp. were collected from.

Locality	GPS coordinates	Stemborer host species
Balfour	26°43'27.7"S 28°31'07.3"E	Unknown
Bizana	30°55'58.8"S 29°34'10.4"E	Unknown
Devon	26°22'56.8"S 28°43'45.8"E	<i>B. fusca</i>
Douglas	29°03'30.8"S 23°53'21.5"E	<i>C. partellus</i> and <i>S. calamistis</i>
Dundee	28°02'31.2"S 30°22'45.5"E	<i>B. fusca</i>
East London	32°51'28.4"S 27°58'15.7"E	<i>B. fusca</i> and <i>C. partellus</i>
Flagstaff	31°05'57.6"S 29°38'10.9"E	Unknown
Grootpan	26°06'47.4"S 26°42'22.3"E	<i>B. fusca</i>
Harrismith	28°12'54.0"S 29°04'51.3"E	<i>B. fusca</i>
Nigel	26°24'47.9"S 28°16'41.5"E	<i>B. fusca</i>
Ntabankulu	31°04'57.8"S 29°19'30.1"E	Unknown
Potchefstroom	26°40'23.7"S 27°06'22.7"E	<i>B. fusca</i> and <i>C. partellus</i>
Prieska	29°29'48.8"S 23°00'24.4"E	<i>C. partellus</i>
Vaalharts	27°49'00.0"S 24°47'0.00"E	<i>B. fusca</i> and <i>S. calamistis</i>
Winterton	28°49'41.0"S 29°28'21.4"E	<i>B. fusca</i>

*Cotesia sesamiae* was also reared and recovered from infected *S. calamistis* larvae obtained from the South African Sugarcane Research Institute (SASRI), KwaZulu-Natal (Figure 2.1). Dead *C. flavipes* specimens preserved in absolute ethanol were obtained from ICIPE, Kenya.



**Figure 2.1:** Localities in South Africa where stemborer larvae parasitised by *Cotesia* spp. were collected from.

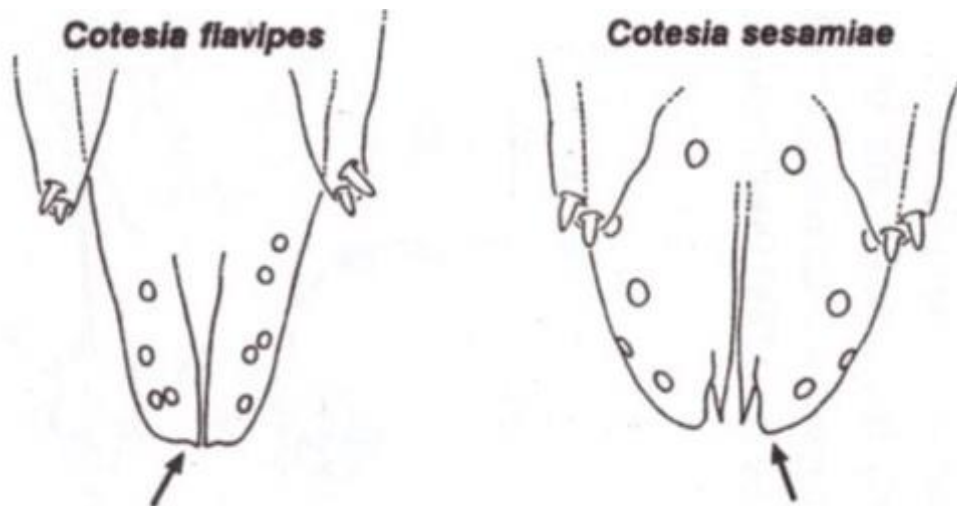
### 2.3.2. Morphological identification

Parasitoid wasps that emerged from field collected larvae were first identified to genus level using an identification key by Smith *et al.* (1993) to ensure that only *Cotesia* specimens were used during this study. The main characteristics for distinguishing *Cotesia* specimens from other parasites of lepidopteran stemborers are provided in Table 2.2. The characteristics used for identification of *Cotesia* specimens were photographed under a Nikon AZ100 stereo microscope equipped with a Nikon DS-Fi1 camera using NIS Elements microscope imaging software (Nikon Instruments Inc., Tokyo).

**Table 2.2:** Key used to distinguish *Cotesia* specimens from other parasites of lepidopteran stemborers (Smith *et al.*, 1993).

Characteristic	Description
Antennae	Filiform Arising near middle of head or higher.
Behaviour	Gregarious
Body	Mostly black
Emergence of adult parasite(s) or mature larva(e)	From stemborer larva
Feeding	Endoparasitically
Hind coxa	Not enlarged
Ovipositor	Shorter than body length
Wings	Two pairs of wings. Costal cell is absent in forewing. Forewing lacks second recurring vein Hindwing has at least one closed cell Radial vein weak, not reaching tip of wing, with only two submarginal cells

Adding to the characteristics mentioned in Table 2.2, the *Cotesia* specimens were further identified morphologically to species level based on the general shape of the male genitalia following the method of Kimani-Njogu and Overholt (1997). The studied specimens were compared to previous studies based on drawings made by Kimani-Njogu and Overholt (1997) as well as electron microscope images by Sigwalt and Pointel (1980) and Muirhead *et al.* (2008) of the apex of the aedeagus (Figure 2.2). The male genitalia were dissected under a stereo and light microscope and scanning electron microscope (SEM) images of the genitalia were taken to identify the *Cotesia* specimens.



**Figure 2.2:** Apex of the aedeagus of *Cotesia flavipes* compared to that of *Cotesia sesamiae* (Walker, 1993).

#### 2.3.2.1. Dissection of male genitalia

The male genitalia from *Cotesia* specimens (three male specimens per cocoon package, where available) sampled from the respective host species (Table 2.1) were removed under a Nikon SMZ1500 stereo microscope to study the morphology of the genitalia. The male genitalia of three known *C. sesamiae* specimens obtained from SASRI and one known *C. flavipes* specimen obtained from ICIPE were also dissected and used as references for identification purposes.

#### 2.3.2.2. Light microscope

Dissected male genitalia were photographed under a Nikon Eclipse 50i light microscope equipped with a Nikon DS-Fi1 camera using NIS Elements microscope imaging software (Nikon Instruments Inc., Tokyo).

#### 2.3.2.3. Scanning Electron Microscope (SEM)

Scanning electron microscopy images were taken of the dissected male genitalia of the known *C. sesamiae* and *C. flavipes* specimens (see 2.3.3.1). Preparation for SEM included dehydration of the dissected male genitalia in a graded series of ethanol. Dehydration started in 70% ethanol for 30 minutes, followed by 30 minutes in 90% ethanol and then overnight in 100% ethanol. The dehydrated male genitalia were transferred to small containers fitted with fine gauze on both sides in 100% ethanol. These samples were transferred to a critical point dryer under high pressure, where the ethanol inside the samples was replaced with liquid

carbon dioxide. The dried samples were mounted on a stub with double-sided adhesive carbon tape (NEM Tape, Nisshin Em. Co. Ltd., Tokyo, Japan), and placed in an ion coater (IB2 Ion Coater, EIKO Engineering Co. Ltd., Ibaraki, Japan) to sputter-coat the samples with a layer of gold/palladium. A field emission scanning electron microscope (FE-SEM) (FEI Quanta FEG 250, FEI Company, Hillsboro, USA) was used to take images of the coated samples.

### **2.3.3. Molecular analyses**

#### *2.3.3.1. DNA extraction from wasp tissue*

Genomic DNA was extracted from one whole *Cotesia* specimen using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) as recommended for purification of total DNA from insects using an electric homogeniser. The protocol was as follows: An individual whole *Cotesia* specimen was crushed with a metal pestil in a 1.5 ml Eppendorf tube, after which four BashingBeads™ (ZYMO) and a volume of 180 µl PBS (50 mM potassium phosphate and 150 mM NaCl [pH 7.2]) was added and the sample homogenised using a Retch mixer mill MM 400. A volume of 20 µl Pro-K and 200 µl Buffer AL (without added ethanol) was added, mixed by vortexing and incubated at 56 °C for 10 minutes. Two hundred microliter ethanol (96-100%) was added to the sample and mixed thoroughly by vortexing. The mixture (including any precipitate) was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for one minute at 8000 rpm. The flow-through as well as collection tube was discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW1 added and centrifuged for one minute at 8000 rpm. The flow-through as well as collection tube was discarded. The DNeasy Mini spin column was again placed in a new 2 ml collection tube and 500 µl Buffer AW2 added and centrifuged for three minutes at 13 500 rpm to dry the DNeasy membrane. The flow-through was discarded, the DNeasy Mini spin column placed back inside the collection tube and centrifuged for an additional 1 minute at 13 500 rpm to ensure that there was no residual ethanol carry over during the elution stage. The DNeasy Mini spin column was then placed in a new 1.5 ml Eppendorf tube, while the flow-through was discarded. Genomic DNA was initially eluted in 50 µl of 10 mM Tris buffer (Buffer AE) by pipetting the Buffer AE onto the membrane. The sample was incubated at room temperature for 5 minutes and then centrifuged for one minute at 8000 rpm to elute.

All specimens, which were either stored in ethanol or dried, were cleaned before the initiation of the extraction process by adding three alternations between ultrapure water and absolute ethanol (99.9%), whilst vortexing between alterations. All extracted DNA samples were stored

at -20 °C. Genomic DNA was extracted from three wasps per stemborer host for each locality, resulting in a total number of 60 wasps being extracted.

### 2.3.3.2. Polymerase Chain Reaction (PCR)

Polymerase Chain Reactions were carried out for identification of *Cotesia* specimens as well as to determine their phylogenetic position when compared with other related taxa. Two sets of primers were used for PCR amplification: i) one set of mitochondrial cytochrome oxidase c subunit I (COI) primers (LCO1490F and HCO2198R) to confirm the species obtained by ICIPE and SASRI as *C. flavipes* and *C. sesamiae*, respectively, and ii) one species-specific set of forward primers (CsV1394F and CfV1634F), which amplifies the CrV1 gene for *C. sesamiae* and *C. flavipes* at different positions, accompanied by one reverse primer (Csf1955R) to determine the species of the *Cotesia* specimens collected during this study (Table 2.3).

**Table 2.3:** Locus, sequence and amplification size of different primers used for molecular identification of *Cotesia* spp. collected from parasitised stemborer larvae in maize fields in South Africa.

Locus	Sequence (5'-3')	Source	Fragment size (bp)
COI	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	Folmer <i>et al.</i> (1994)	556-669
CrV1	AACGAACACTTTTCGATGAA GAGTATTTTCCGAAAATGG ACTCCTTCAACGCTGGGTTTCCTTG	Dupas <i>et al.</i> (2006)	511 271 -

DNA amplification was performed in a 25 µL volume of PCR mix that was prepared by adding 12.5 µL DreamTaq PCR Master Mix (2x) (ThermoScientific™, South Africa), 1 µL of each primer (10 µM), 4 µL of genomic DNA and subsequent nuclease free water to make the final volume. For each primer, the following conditions were used i) **COI**: initial denaturation for 1 minute 30 seconds at 95 °C, 35 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 47 °C, extension for 1 minute at 72 °C and finally an extension cycle of 7 minutes at 72 °C followed by a holding temperature of 4 °C, ii) **CrV1**: initial denaturation for 5 minutes at 94 °C, 40 cycles of denaturation for 50 seconds at 94 °C, annealing for 1 minute 20 seconds at 60°C, extension for 1 minute 20 seconds at 72 °C and finally an extension cycle of 10 minutes at 72 °C followed by a holding temperature of 4 °C. The morphologically confirmed *C. flavipes* and *C. sesamiae* specimens obtained from ICIPE and SASRI, respectively, were

used as positive controls and ddH<sub>2</sub>O as a negative control for the respective species identified during this study.

#### 2.3.3.3. Gel electrophoresis

After DNA amplification was completed, 4 µL of the PCR product from each sample mixed with 2 µL of 6x Purple loading dye (BioLabs, New England) was loaded on a 1.5% agarose gel prepared with 1 x TAE buffer (40 mM Tris, 20 mM Acetic acid, 1mM EDTA, at pH 8.0) to confirm PCR amplification. A volume of 2 µL of 100 bp molecular weight marker (GeneRuler ThermoScientific™, South Africa) was used for confirming the amplified product size. The gel was stained with ethidium bromide and visualised using a UV transilluminator. Gel electrophoresis was performed in a 1% TAE buffer during a 45 minutes run at 80 V using a mini-sub cell GT electrophoresis system (Bio-Rad, UK). Gel images were captured using the ENDURO™ GDS image system (Labnet International, Inc., US). The remaining products of each sample were stored at -20 °C prior to sequencing by the genomic company Inqaba BioTec™, South Africa ([www.inqaba-southafrica.co.za](http://www.inqaba-southafrica.co.za)). Primers used during the sequencing reaction process were the same as those used in the amplification step.

#### 2.3.3.4. Taxonomic and phylogenetic analyses

DNA sequences obtained from Inqaba Biotec™ for both the COI and CrV1 gene were viewed and edited using Chromas version 2.6.5 (Technelysium Pty Ltd, USA) and confirmed in a forward direction. During the editing of sequences degenerate nucleotides were replaced with a suitable nucleotide determined by colour peaks produced by the chromatogram and saved as a FASTA format. Basic Local Alignment Search Tool for nucleotides (BLASTn) was used to compare the sequences obtained during this study with the available DNA sequences of wasps in the National Center for Biotechnical Information (NCBI) database to confirm their identification. Homologous sequences of other related wasp species were obtained from the NCBI GenBank database for the CrV1 gene only. All of the sequences were added to the Alignment Explorer implemented in Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 and aligned using the ClustalW alignment tool (Larkin *et al.*, 2007). The aligned sequences were renamed, trimmed to remove uneven ends from the aligned sequences and transferred to MEGA 7 for Maximum Likelihood (ML) analysis at 10 000 bootstrap replicates. Published sequences used were from other species of the *Cotesia* genera including *C. congregata* (Say) (Hymenoptera: Braconidae), *C. flavipes*, *C. plutellae* (Kurdjumov) (Hymenoptera: Braconidae), *C. rubecula* (Marshall) (Hymenoptera: Braconidae) and *C. sesamiae* with *Delminichthys adspersus* (Heckel 1843) used as an outgroup (Figure 2.9). The nucleotide

substitution model, Tamaru 3-parameter, was determined as the most appropriate model by using the lowest Bayesian Information Criterion (BIC) score. Rates among sites were treated as uniform rates and gaps or missing data were deleted during the construction of the phylogenetic tree.

## 2.4. Results

### 2.4.1. Morphological identification

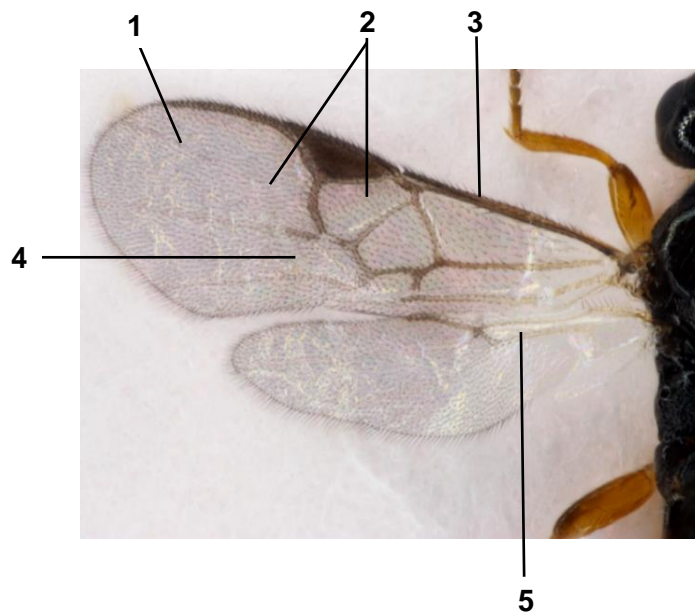
Parasitoid wasps were collected from 15 localities located in seven of the nine provinces of South Africa. Morphological identification of collected parasitoid wasps to genus level revealed that only one genus, *Cotesia*, was collected. The characteristics are indicated in Figure 2.3 with the body mostly black, the ovipositor  $369.6 \pm 9.2 \mu\text{m}$  long (blue circle) (Obonyo *et al.*, 2011) and the hind coxa, not enlarged (red circle). The filiform antennae protrudes from the middle of the head (Figure 2.4) and the veins on the wings used for identification are shown in Figure 2.5. The radial vein of the forewing does not reach the tip of the wing but it does have two submarginal cells, while the costal cell in the forewing as well as the recurrent vein in the forewing are absent. The hindwing contains a closed cell (Figure 2.5).



**Figure 2.3:** Light microscope image of a *Cotesia* specimen, displaying the characteristically mostly black body, short ovipositor (blue) and hind coxa – not enlarged (red).



**Figure 2.4:** Light microscope image of a *Cotesia* specimen, showing the filiform antennae protruding from the middle of the head.





**Figure 2.5:** Light microscope image of the wing of a *Cotesia* specimen, 1: radial vein of forewing not reaching the tip of the wing, 2: two submarginal cells, 3: absence of costal cell in forewing, 4: absence of recurrent vein in forewing, 5: closed cell in hindwing.

#### 2.4.1.1. Light microscope






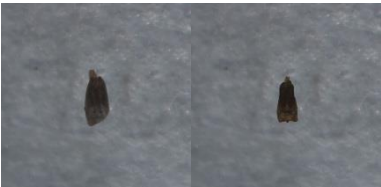


The dissected male genitalia from the *C. flavipes* specimen obtained from ICIPE and the three *C. sesamiae* specimens obtained from SASRI, served as reference specimens. Images of

these genitalia are provided in Table 2.4. A total of 46 *Cotesia* specimens from 13 localities were dissected and morphologically identified to species level. Only one species, *C. sesamiae*, was identified from the collected specimens by means of morphological identification (Table 2.5). No males were present in the *Cotesia* specimens sampled from Balfour, East London (*C. partellus*), Ntabankulu and Prieska (*B. fusca*), and therefore no identification was possible for specimens from these localities. The morphology results showed the substantial variation of the genitalia of *C. sesamiae* depends on the locality as well as the stemborer host species from where they were sampled from (Tables 2.4 and 2.5).

**Table 2.4:** Light microscope images of dissected male genitalia from *Cotesia flavipes* and *Cotesia sesamiae* to serve as reference for identification of sampled specimens.

<i>Cotesia</i> spp.	Image of male genitalia
<i>C. flavipes</i>	
<i>C. sesamiae</i>	

**Table 2.5:** Light microscope images of dissected male genitalia from *Cotesia* specimens sampled at 13 localities in South Africa.

Locality	Stemborer host	Image of male genitalia
Bizana	Unknown	
Devon	<i>B. fusca</i>	
Douglas	<i>C. partellus</i>	
	<i>S. calamistis</i>	
Dundee	<i>B. fusca</i>	
East London	<i>B. fusca</i>	
Flagstaff	Unknown	
Grootpan	<i>B. fusca</i>	

Harrismith

*B. fusca*



Nigel

*B. fusca*



Potchefstroom

*B. fusca*



*C. partellus*



Prieska

*C. partellus*



Vaalharts

*B. fusca*



*S. calamistis*



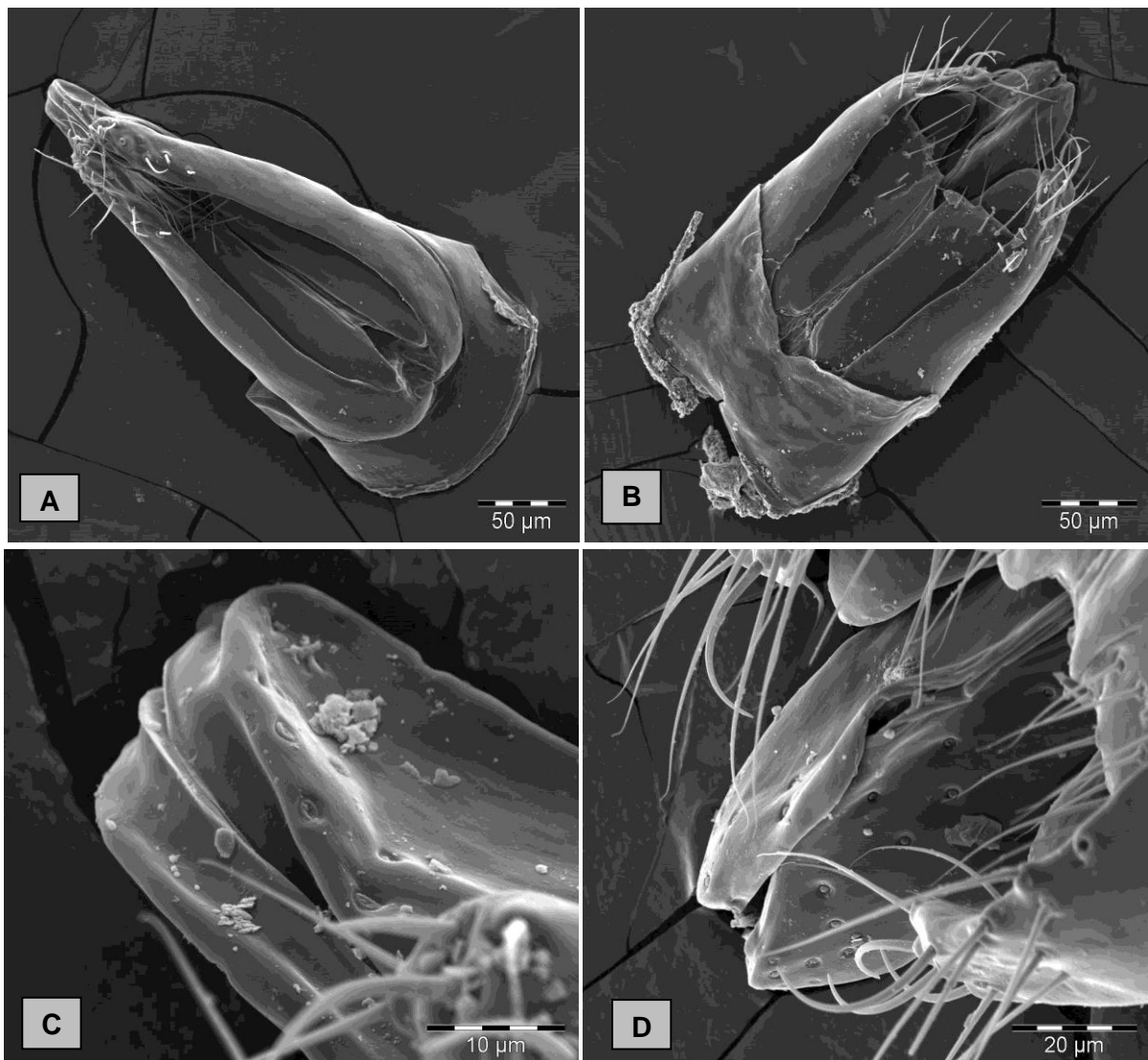
Winterton

*B. fusca*



#### 2.4.1.2. Scanning Electron Microscope (SEM)

The SEM images displayed in Figure 2.6 show the general morphology of the male genitalia as well as the apex of aedeagus of a known *C. flavipes* (specimen obtained from Kenya) and *C. sesamiae* (specimen obtained from SASRI). Confirmation of the identification of *Cotesia* spp. was done by using the descriptions of Sigwalt and Pointel (1980) and Kimani-Njogu and Overholt (1997). The male genitalia of *C. flavipes* are slender and elongated while the genitalia of *C. sesamiae* are short and robust. The shape of the apex of the aedeagus is truncated in *C. flavipes* and pointed in *C. sesamiae*.



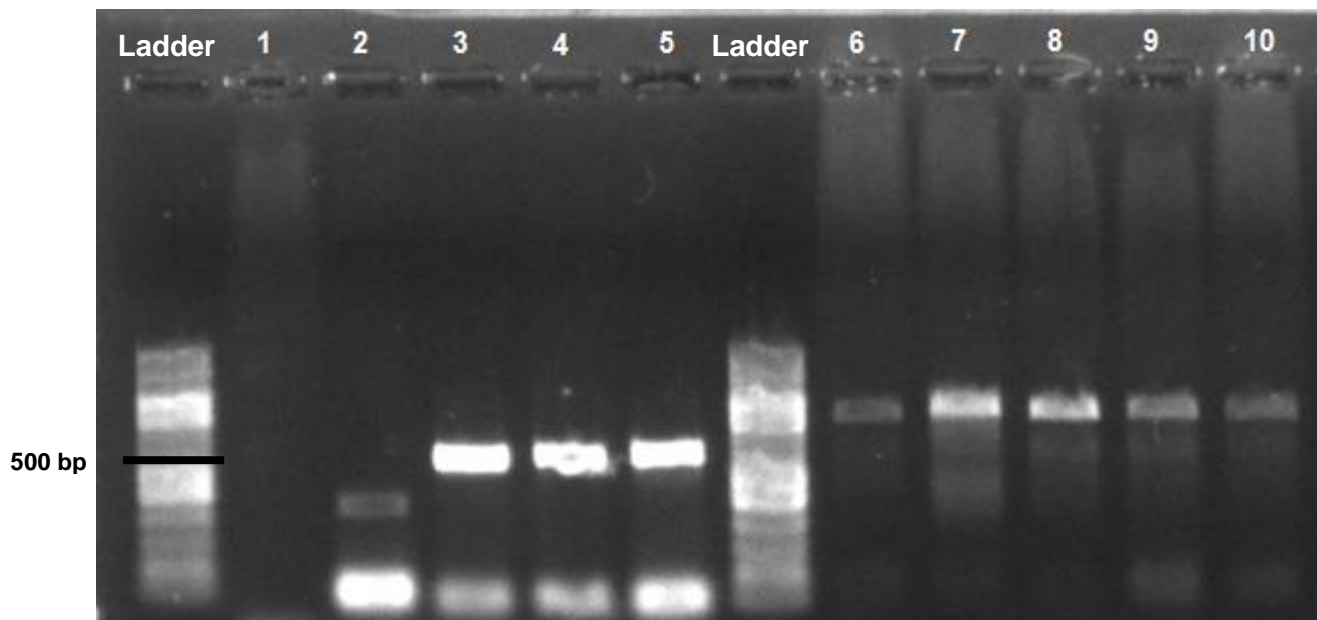
**Figure 2.6:** SEM images of the general morphology of the male genitalia of (A) *Cotesia flavipes* (slender and elongated) (B) *Cotesia sesamiae* (short and robust), as well as the tip of the aedeagus of a (C) *Cotesia flavipes* (truncated) and (D) *Cotesia sesamiae* (pointed).

## 2.4.2. Molecular analyses

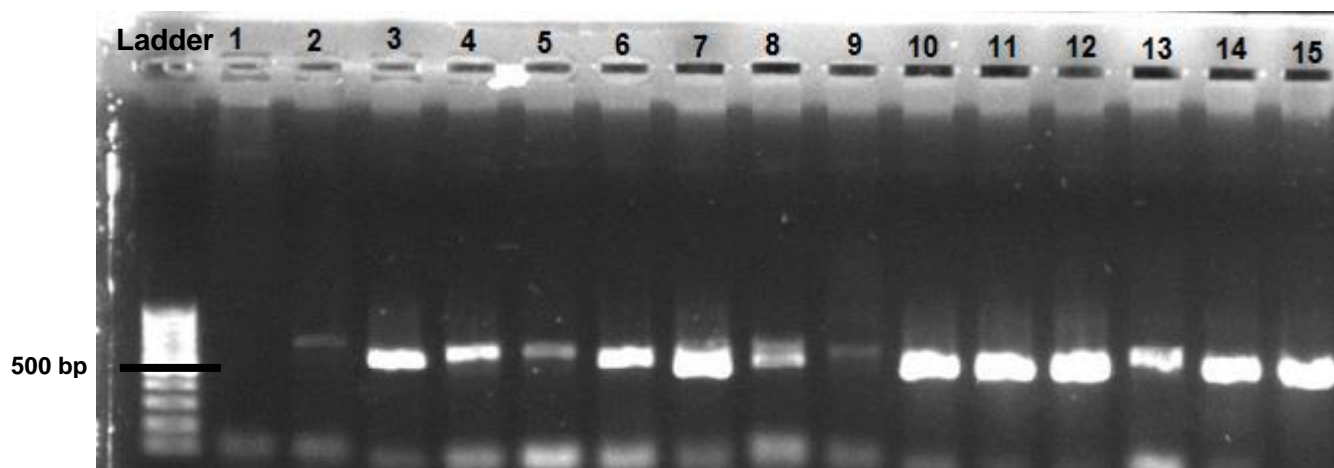
### 2.4.2.1. Polymerase Chain Reaction (PCR)

The COI gene was successfully amplified from the identified *C. flavipes* and *C. sesamiae* specimens obtained from ICIPE and SASRI, respectively. Amplification revealed positive bands between 556 and 669 bp for the COI gene (Figure 2.7). The information regarding the identity of the specimens obtained from ICIPE and SASRI, respectively, was therefore positively confirmed. These two populations of *Cotesia* were used throughout the rest of the study as reference specimens. Molecular analyses, using the COI gene primers, were also done for the samples collected during this study. Sequence results obtained for these samples were however consistently found to be *Wolbachia* spp., and another gene was therefore investigated.

Only 53 of the collected wasp samples were successfully amplified by species-specific PCR assays using the CrV1 gene. Amplification revealed positive bands at approximately 271 bp for *C. flavipes* and approximately 511 bp for *C. sesamiae* (Figures 2.7 and 2.8). All of the amplified samples were found to be *C. sesamiae*.



**Figure 2.7:** Gel electrophoresis of amplified COI (lanes 6-10) and CrV1 (lanes 1-5) gene products [Ladder = 100 bp; Lane 1 = negative control; Lanes 2 and 7 = *C. flavipes* obtained from ICIPE; Lanes 3 and 8 = *C. sesamiae* obtained from SASRI (200  $\mu$ l elution buffer); Lanes 4 and 9 = *C. sesamiae* obtained from SASRI (100  $\mu$ l elution buffer); Lanes 5 and 10 = *C. sesamiae* obtained from SASRI (50  $\mu$ l elution buffer); Lane 6 = *Cotesia* specimen collected from Vaalharts (*S. calamistis*)]



**Figure 2.8:** Gel electrophoresis of amplified CrV1 gene products [Ladder = 100 bp; Lane 1 = negative control (water); Lane 2 = positive control of *C. sesamiae* (*C. sesamiae* obtained from SASRI - 50 µl elution buffer seen in Figure 2.7); Lane 3 = Devon (*B. fusca*); Lane 4 = Douglas (*C. partellus*); Lane 5 = Douglas (*S. calamistis*); Lane 6 = Potchefstroom (*B. fusca*); Lane 7 = Potchefstroom (*C. partellus*); Lane 8 = Prieska (*B. fusca*); Lane 9 = Prieska (*C. partellus*); Lane 10 = Vaalharts (*B. fusca*); Lane 11 = Vaalharts (*C. partellus*); Lane 12 = Balfour; Lane 13 = Bizana; Lane 14 = Flagstaff; Lane 15 = Ntabankulu].

There was no major variation in the length of either set of primer sequences among the wasp specimens sampled from different stemborer species as well as from different localities.

#### 2.4.2.2. DNA characterisation

##### *Cotesia flavipes*

The length of the sequenced COI gene fragment for *C. flavipes* obtained from ICIPE was 684 bp. The sequence of the wasp matched 99% with published sequences of *C. flavipes* from GenBank (accession number: JF865973, JF865971, JF865972 and JF865973) when applied in BLASTn.

##### *Cotesia sesamiae*

The length of the sequenced COI gene fragment for *C. sesamiae* obtained from SASRI was 678 bp. The sequence of the wasp matched 97% with published sequences of *C. sesamiae* from Genbank (accession number: KJ882538, KJ882499, KJ100077, KJ100076) when applied in BLASTn.

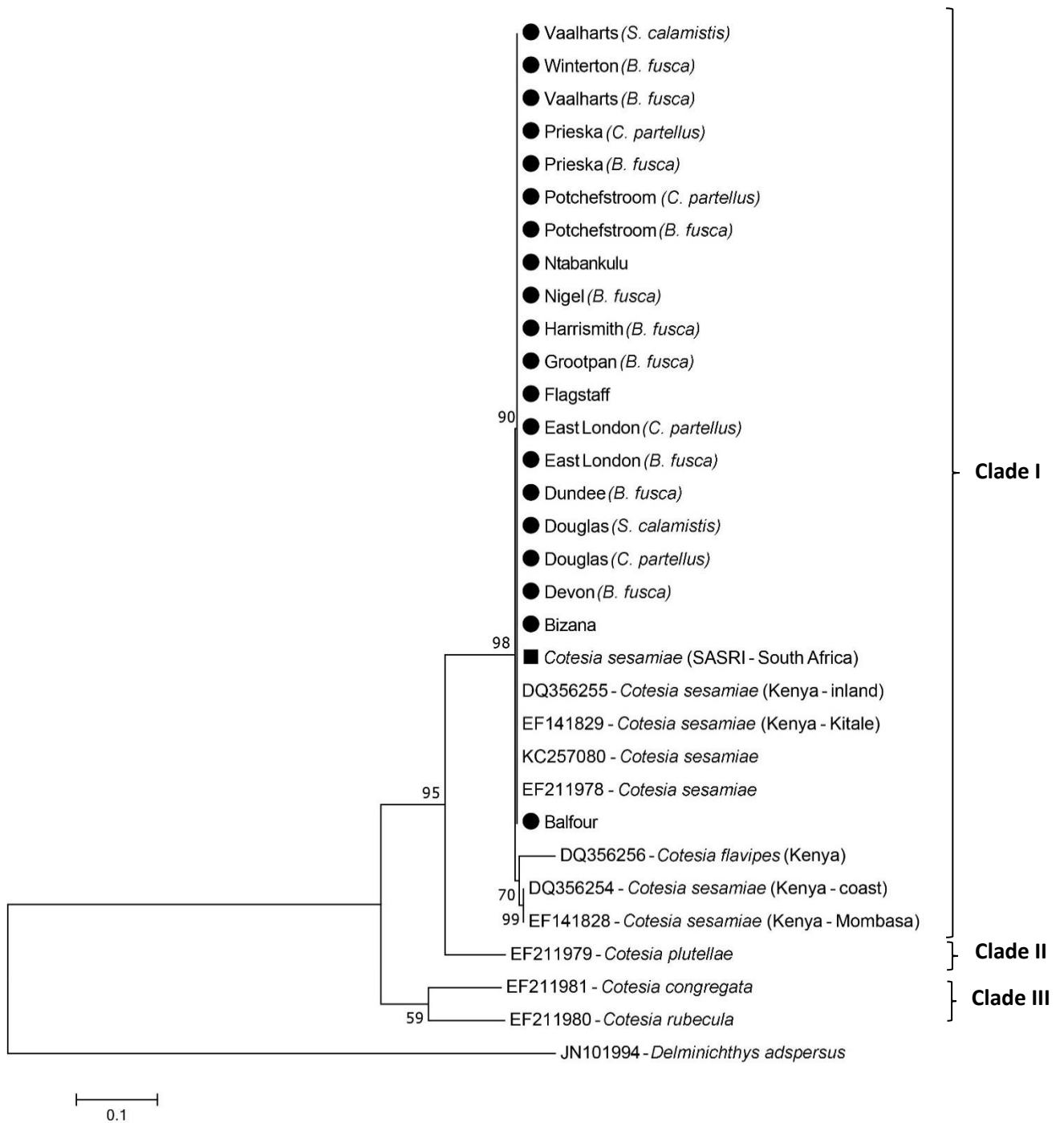
The average length of the sequenced CrV1 gene fragments for *Cotesia* spp. collected from the 15 maize fields located in South Africa was 520 bp. The BLASTn results for all the

sequences of the wasps from this gene showed a 97-100% similarity with published sequences of *C. sesamiae* from GenBank (Appendix 2 – Table 1).

#### 2.4.2.3. Phylogenetic analysis

The Maximum Likelihood (ML) phylogenetic tree (Figure 2.9) used a total number of 32 sequences, each with a total of 404 positions without gaps, and showed three major clades were resolved with high bootstrap support values. All 21 of the sequences generated during this study clustered with the sequences of *C. sesamiae* (inland and coast biotype) and *C. flavipes* from GenBank in the main clade (clade I) with a 98% bootstrap value. Furthermore, *C. plutellae* formed a well-supported clade (clade II; 95% bootstrap value), however, *C. rubecula* and *C. congregata* grouped together in clade III with a 59% bootstrap value. All of the CrV1 sequences generated during this study formed part of the *C. sesamiae* inland biotype group with a bootstrap support value of 90%, therefore indicating that *C. sesamiae* was the only species present. These sequences are represented by bullet symbols, as well as a filled square symbol.

The average nucleotide frequencies excluding gaps were 33.7% adenine, 19.9% cytosine, 18.1% guanine and 28.3% thymine. The distance of the Pairwise Maximum Composite Likelihood method (Table 2.6) among the taxa included in this study showed that there was no sequence divergence among the *C. sesamiae* generated during this study (0.000). This is also seen in Figure 2.10-2.16 displaying the nucleotide differences found in the CrV1 sequences of the *C. sesamiae* generated during this study per province. The sequence divergence between the *C. sesamiae* generated during this study and the inland and Kitale *C. sesamiae* biotype obtained from GenBank was also 0.000, while the sequence divergence between the *C. sesamiae* generated during this study and the coast and Mombasa *C. sesamiae* biotype from GenBank was 0.013.



**Figure 2.9:** Maximum Likelihood (ML) phylogenetic tree of the *Cotesia* specimens sampled in South Africa, using the Tamura 3-parameter method (Tamura, 1992). The stemborer species from which *Cotesia sesamiae* was sampled are indicated in brackets next to the respective localities.









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#Cotesia sesamiae (SASRI - South Africa)      TCG ATG AAA TAG ATG TTC ACA ATA GAA AAT TCT CGA TGA TTC CAA AGC ATC AIT CIG AAG
#DQ356255 - Cotesia sesamiae (Kenya - inland)  .....
#EF141829 - Cotesia sesamiae (Kenya - Kitale)  .....
#DQ356254 - Cotesia sesamiae (Kenya - coast)  ..... A.....G.....
#EF141828 - Cotesia sesamiae (Kenya - Mombasa) ..... A.....G.....
#KC257080 - Cotesia sesamiae                  .....
#EF211978 - Cotesia sesamiae                  .....
#DQ356256 - Cotesia flavipes (Kenya)          .....G.....A.....
#EF211979 - Cotesia plutellae                .....T CT.....A.....G.....C.....T.....T.....T.....
#EF211981 - Cotesia congregata                .....T CT.....A AAT.....G.....CT.....TC.....T.....CT.....CT GG.
#EF211980 - Cotesia rubecula                  .....T CT.....A GA.....G.....T.....T.....G.....T.....C.....T.....G.
#JN101994 - Delminichthys adspersus          ATA C A.....G.....CC.....GA.....AC.....T.....GG GTA GTG.....T.....CTT C.....T.....GIT.....A.....C.....T CGT
#Douglas (C. partellus)                      .....
#Douglas (S. calamistis)                    .....
#Prieska (B. fusca)                          .....
#Prieska (C. partellus)                      .....
#Vaalharts (B. fusca)                        .....
#Vaalharts (S. calamistis)                   .....

#Cotesia sesamiae (SASRI - South Africa)      CAC CTA GAA AGC AGT CTA ATT TTG AAG AIT TTC TAA GGT CTC AAC ATC CTT ACA TGC CTA
#DQ356255 - Cotesia sesamiae (Kenya - inland)  .....
#EF141829 - Cotesia sesamiae (Kenya - Kitale)  .....
#DQ356254 - Cotesia sesamiae (Kenya - coast)  .....A.....
#EF141828 - Cotesia sesamiae (Kenya - Mombasa) .....A.....
#KC257080 - Cotesia sesamiae                  .....
#EF211978 - Cotesia sesamiae                  .....
#DQ356256 - Cotesia flavipes (Kenya)          .....T.....A.....A.....T.....T.....C.....A.....
#EF211979 - Cotesia plutellae                A.....G.....G.....T.....TC.....G.....C.....T.....C.....A.....A.....G.....
#EF211981 - Cotesia congregata                .....C.....G.....GTC.....G.....A.....TGC.....G.....C.....T.....C.....G.....CT.....A.....A.....T.....G.....G
#EF211980 - Cotesia rubecula                  .....C.....G.....T.....T.....G.....G.....T.....T.....T.....A.....C.....A.....C.....C.....A.....A.....T.....A.....G
#JN101994 - Delminichthys adspersus          .GG T.....TGT.....CT TCC.....GG ACA.....T.....T.....G.....C.....GC.....C.....G.....A.....A.....AA.....T
#Douglas (C. partellus)                      .....
#Douglas (S. calamistis)                    .....
#Prieska (B. fusca)                          .....
#Prieska (C. partellus)                      .....
#Vaalharts (B. fusca)                        .....
#Vaalharts (S. calamistis)                   .....

#Cotesia sesamiae (SASRI - South Africa)      GAC ACC TTA AAA AAC ATA GAT CTA GCA CCA AAA CAA GAC CIT TTG AAA GAT ATA TTA TGA
#DQ356255 - Cotesia sesamiae (Kenya - inland)  .....
#EF141829 - Cotesia sesamiae (Kenya - Kitale)  .....
#DQ356254 - Cotesia sesamiae (Kenya - coast)  .....
#EF141828 - Cotesia sesamiae (Kenya - Mombasa) .....
#KC257080 - Cotesia sesamiae                  .....
#EF211978 - Cotesia sesamiae                  .....A.....G.....
#DQ356256 - Cotesia flavipes (Kenya)          C.....T.....G.....A.....TTG T.....C.....G.....C.....G.....
#EF211979 - Cotesia plutellae                .T.....T.....G.....A.....TTG T.....C.....G.....C.....G.....
#EF211981 - Cotesia congregata                A.T.....A.....G.....C.....G.....G.....C.....C.....T.....C.....T.....C.....T.....C.....C.....
#EF211980 - Cotesia rubecula                  .....A.....G.....C.....G.....G.....CGT T.G.....T.....C.....T.....G.....C.....T.....G.....C.....
#JN101994 - Delminichthys adspersus          CT.....G.....G.....C.....CCC T.....GA GAC A.C.....IT GTG.....TGG A.C.....GA GGC.....GC T.C.....C.....A
#Douglas (C. partellus)                      .....
#Douglas (S. calamistis)                    .....
#Prieska (B. fusca)                          .....
#Prieska (C. partellus)                      .....
#Vaalharts (B. fusca)                        .....
#Vaalharts (S. calamistis)                   .....

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**Figure 2.16:** Sequence alignment for *Cotesia sesamiae* from the Northern Cape province when compared to other related *Cotesia* specimens from GenBank. The dots indicate identical nucleotide sequences.

## 2.5. Discussion

The invasion of non-indigenous species, loss of habitat and landscape fragmentation is the biggest threat to global diversity (Walker & Steffen, 1997). Several morphological characteristics are used to distinguish between the species in the *C. flavipes*-complex, since morphological identification is challenging (Muirhead *et al.*, 2008). This causes the misidentification of these biological control agents (Getu, 2008) which should be avoided as it may lead to the introduction of non-native species into native areas. These characteristics include the colouration of the hind coxae, position of the antennae, width and length of the head, punctuation and pubescence of the mesosoma and metasoma, form of the scuto-scutellar sulcus, number of setae and shape of the male genitalia (Mohyuddin, 1971; Sigwalt & Pointel, 1980; Kimani-Njogu & Overholt, 1997; Muirhead *et al.*, 2008). Most of these characteristics were, however, proven to be unreliable (Sigwalt & Pointel, 1980; Polaszek & Walker, 1991) due to high levels of intraspecific variation

(Kimani-Njogu & Overholt, 1997; Muirhead *et al.*, 2008). Kimani-Njogu and Overholt (1997), however, found that the rugosity of the propodeum could be used to separate *C. flavipes* from *C. sesamiae* and *C. chilonis*. The propodeum was less rugose in *C. sesamiae* than in *C. flavipes* and *C. chilonis* (Kimani-Njogu & Overholt, 1997). The overall shape of the male genitalia is the only reliable and convenient characteristic which can be used to distinguish between *C. flavipes* and *C. sesamiae* (Sigwalt & Pointel, 1980; Polaszek & Walker, 1991; Kimani-Njogu & Overholt, 1997; Muirhead *et al.*, 2008).

The differences in the overall shape of the genitalia and the apex of the aedeagus (Sigwalt & Pointel, 1980; Walker, 1993; Kimani-Njogu & Overholt, 1997) were used in this study to identify the *Cotesia* specimens collected. The only *Cotesia* species identified, was *C. sesamiae*. Molecular analyses of these wasps confirmed the morphological identification and therefore proved that morphological identification using the male genitalia, is reliable. Morphological identification may therefore be adequate. Dupas *et al.* (2006), however, recommended that molecular analysis should be used especially in post-release surveys.

The Maximum Likelihood (ML) phylogenetic tree constructed with the CrV1 gene sequences revealed that the *C. sesamiae* generated during this study grouped with other *C. sesamiae* and *C. flavipes* specimens from GenBank in one clade. This shows that the species present in this clade are monophyletic species. The other two clades are, however, paraphyletic. The *C. sesamiae* sequences generated during this study inferred with the inland biotype of *C. sesamiae* with a score of 90, while *C. flavipes* inferred with the coastal biotype of *C. sesamiae* with a score of 70. This corresponds with the findings of Dupas *et al.* (2008) and Muirhead *et al.* (2012) who found two strains of *C. sesamiae* present in Kenya - one that can successfully develop inside *B. fusca* (virulent) and one that cannot develop inside *B. fusca* (avirulent). These authors found the virulent strain in western Kenya (inland at Kitale), while the avirulent strain was found in eastern Kenya (at the coast of Mombasa) (Dupas *et al.*, 2008; Muirhead *et al.*, 2012).

The tree is further backed up by the pairwise distance nucleotide differences. The sequence divergence between the *C. sesamiae* generated during this study and the inland and Kitale GenBank obtained *C. sesamiae* was 0.000, whereas the sequence divergence between the *C. sesamiae* generated during this study and the coast and Mombasa GenBank obtained *C. sesamiae* was 0.013. According to Gitau (2006) the virulent strain is found in the highlands, while the avirulent strain is found in the lowlands. When looking at the results obtained from this study,

where samples were collected predominantly in the Highveld region of the country, it corresponds with only finding the virulent strain. With *B. fusca* having been the predominant stemborer host from which the collected samples from this study were reared from, the presence of only the virulent strain in all collected samples corresponds as the avirulent strain would not have been able to successfully develop inside this host.

The Maximum Composite Likelihood method results based on the CrV1 sequences showed the low genetic divergence among the sequences. This was particularly evident for *C. sesamiae*, which can be due to the lack of enough variable regions in the sequence of this part of DNA. Ultimately, the high similarity between the *C. sesamiae* population obtained from SASRI and the *C. sesamiae* populations obtained during this study, indicated the low diversity among the *C. sesamiae* populations found within South Africa.

## 2.6. Conclusion

Morphological identification using the male genitalia prove to be accurate in this study, where all the morphologically identified *C. sesamiae* were also molecularly confirmed as *C. sesamiae*. Although *C. flavipes* has successfully been introduced into several countries in eastern and southern Africa, including South Africa, it does not seem to have established permanently in South Africa. This finding not only confirmed the findings of Skoroszewski and Van Hamburg (1987), but also proved that it did not spread from Botswana, where it was found recently, to South Africa. *Cotesia flavipes* was, however, not released in Botswana, but releases were done in neighbouring countries. Thus, the possibility that *C. flavipes* may spread from Botswana into South Africa in the near future seems very likely. A repeat of this study is therefore recommended in the near future.

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## Chapter 3: The effects of indirect exposure to Cry1Ab protein on fitness and survival of *Cotesia sesamiae*

### 3.1. Abstract

*Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) are major pests of maize in Africa. Genetically modified Bt maize was planted in South Africa for the first time during the 1998/99 growing season for control of these stemborers. The first Bt maize resistant *B. fusca* larvae in South Africa was reported during the 2006/07 growing season and resistance has since spread to many areas in the maize production region of the country. When parasitising Bt-resistant *B. fusca* larvae, the koinobiont larval endoparasitoid, *Cotesia sesamiae* (Cameron) (Hymenoptera: Braconidae) is indirectly exposed to Bt proteins that are consumed by these stemborer larvae. The aim of this study was to determine the effect of indirect third-trophic level exposure to Cry1Ab proteins on the fitness (in terms of reproduction) and survival of *C. sesamiae*. Bt-resistant *B. fusca* larvae were reared on non-Bt maize stems until the 3<sup>rd</sup>/4<sup>th</sup> instar, transferred to Bt maize stems and parasitised with *C. sesamiae* after 72 hours of feeding. The control treatment *B. fusca* larvae were left to continue feeding on non-Bt maize stems until experiments were conducted. Two laboratory experiments were conducted during which the number of cocoons (clutch size), number of wasps emerging from cocoons (brood size) and the sex ratio (females:males) of wasps were recorded. During the second experiment the mass of host larvae and developmental time of parasitoid larvae were also recorded. Results obtained during both experiments showed that Bt exposure had no significant effect on *C. sesamiae* life history parameters. However, during the 2<sup>nd</sup> experiment, significantly higher numbers of female wasps emerged from parasitised *B. fusca* larvae that fed on Bt maize compared to those that fed on non-Bt maize ( $t=2.93$ ;  $df=55$ ;  $P<0.01$ ).

#### Keywords:

Bt maize, Cry1Ab, non-target effects, stemborers, tritrophic interactions.

### 3.2. Introduction

Insect-resistant transgenic *Bacillus thuringiensis* (Bt) maize has been commercially produced in South Africa since the 1998/99 growing season for the control of the following lepidopteran pests: *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) (Van Rensburg, 1999; Van den Berg & Van Wyk, 2007). The insecticidal proteins produced by Bt crops confer resistance to certain phytophagous pests (Koziel *et al.*, 1993) since they produce these Cry toxins in most of their tissues throughout the growing season (Zwahlen *et al.*, 2000). Since crop plants and their associated arthropod complex form part of a complex agricultural ecosystem that involves multitrophic interactions (Poppy, 1997), Bt toxins may be encountered continuously and in high concentrations by target and non-target arthropods (Sisterson *et al.*, 2004). This has been shown in several studies where Bt toxins were reported to have moved through the arthropod food web into higher trophic levels (Harwood *et al.*, 2005; Obrist *et al.*, 2006a;b; Meissle & Romeis, 2009; Yu *et al.*, 2014).

Although the use of Bt maize provides many advantages to the economy, human health and the environment (Brookes & Barfoot, 2010) the potential impact of Bt crops on populations of non-target organisms continues to be debated as its cultivation and utilisation continues to increase (Ramirez-Romero *et al.*, 2007). Natural enemies which provide certain ecosystem services, especially parasitoids and predators, are of particular interest in most of these debates regarding non-target effects. Tritrophic interactions involve the interactions between the three trophic levels, namely the crop plant (first trophic level), the pest insect (herbivore, prey or host) (second trophic level) and the natural enemy (third trophic level) (Poppy, 1997; Powell *et al.*, 1996). The term “non-target effects” refers to any unintended effects of transgenic, insecticidal plants on organisms other than the target species itself (Dale *et al.*, 2002; Hilbeck, 2002).

Parasitoids are more likely to be affected than predators when their Bt-susceptible hosts are exposed to Bt toxins, since they are usually host specific and also complete their development in a single host individual. Predators on the other hand are often generalists and therefore feed on several different prey species (Vojtech *et al.*, 2005). The possible non-target effects of Bt toxins are especially of concern in koinobiont parasitoids since the intimate relationship between the parasitoid and host exposes young parasitoid life stages to the immune system of the host (Godfray, 1994; Pennacchio & Strand, 2006), as well as to the allelochemicals present in the diet

of the host (Barbosa *et al.*, 1986; 1990; Cortesero *et al.*, 2000; Sznajder & Harvey, 2003; Ode, 2006). Parasitoids are generally more susceptible to these compounds than their hosts, which often continue feeding and developing at the same rate despite being parasitised or not (Jiang *et al.*, 2004; Hailemichael *et al.*, 2008). This is ascribed to parasitoids often not being capable of metabolising the secondary compounds present in the host plants of their larval hosts (Campbell & Duffey, 1979; Quicke, 1997).

Several glasshouse, laboratory, field and semi-field studies investigating the potential effects of Bt crops on non-target, beneficial species have been done (Groot & Dicke, 2002; Romeis *et al.*, 2006). Although most studies have demonstrated that Bt crops do not harm natural enemies (Lundgren & Wiedenmann, 2005; Romeis *et al.*, 2006; Naranjo, 2009; Garcia *et al.*, 2010), some report that exposure to Cry proteins expressed in Bt crops do indeed harm certain natural enemies (Vojtech *et al.*, 2005; Lovei *et al.*, 2009).

Host-mediated effects, also known as “prey quality-mediated effects”, may also influence parasitoid performance (Schuler *et al.*, 1999). This happens when natural enemies (parasitoids and predators) are indirectly affected when feeding on Bt-susceptible or sub-lethally affected prey/hosts that were directly affected through exposure to Cry proteins produced by transgenic Bt crops (Schuler *et al.*, 1999; Romeis *et al.*, 2006). Observed adverse effects in such cases may therefore be explained not by the direct effect of the plant-produced Cry toxin on the parasitoids or predators, but rather by the reduced quality and availability of the prey/host (Romeis *et al.*, 2006; Naranjo, 2009).

A recognised method to eliminate the potential impact that reduced prey/host quality may have on bioassay results is to use Bt-resistant hosts or non-susceptible hosts (herbivores that can develop well on Bt crops) to ensure that the hosts are healthy and that the quality of the host does not affect the outcomes of studies (Chen *et al.*, 2008; Tian *et al.*, 2014; Wu *et al.*, 2014; Su *et al.*, 2015). It also ensures that ecologically relevant concentration of the Bt protein is present in the prey/host and that the natural enemy is exposed in a natural way (Romeis *et al.*, 2014). Van den Berg *et al.* (2017) demonstrated the necessity of using healthy prey when assessing third trophic level exposure of larvae of the lacewing, *Chrysoperla pudica* (Stephens) (Neuroptera: Chrysopidae), to Cry1Ab proteins produced by Bt crops. They reported that the feeding of lacewing larvae on Bt-resistant *B. fusca* larvae that consumed Cry1Ab protein had no adverse effects on the biology of *C. pudica* (Van den Berg *et al.*, 2017). Results of the latter study as well

as that reviewed by Romeis *et al.* (2014) contradict those of other studies and largely ascribe the observed differences to prey quality and methods of exposure (Van den Berg *et al.*, 2017). Due to the receptors for the Cry1Ab protein most likely being absent in Hymenoptera, Bt toxins are not expected to have direct lethal or sub-lethal effects on hymenopteran parasitoids (Ramirez-Romero *et al.*, 2007).

*Cotesia sesamiae* is a gregarious, koinobiont larval endoparasitoid (Walker, 1994) endemic to the Afrotropical region (Kimani-Njogu & Overholt, 1997). Host species of *C. sesamiae* include mid- to late larval instars of an exotic stemborer species (*C. partellus*) and indigenous stemborer species (*B. fusca* and *S. calamistis*) (Mohyuddin, 1971; Cugala *et al.*, 1999; Kfir *et al.*, 2002). This species is one of the most important natural enemies of indigenous lepidopteran pests of maize in many countries of sub-Saharan Africa (Bonhof *et al.*, 1997). *Cotesia sesamiae* does, however, appear not to be a very effective natural enemy of *C. partellus* (Overholt *et al.*, 1994). *Cotesia flavipes* Cameron (Hymenoptera: Braconidae), a closely related species, is however much more effective as a parasitoid of *C. partellus* (Sallam *et al.*, 1999). The economic impact of the release and spread of *C. flavipes* on stemborer management in three east and southern Africa countries - Kenya, Mozambique and Zambia - was reported to amount to \$1.4 billion dollars over a 20-year period (Mindingoyi *et al.*, 2016). If the future cultivation of Bt maize in Africa adversely affects these biocontrol agents, the ecosystem services provided by these biological control agents may be lost.

Many studies investigating the potential effects of Bt crops on the genus *Cotesia* have been done. These include species such as *C. plutellae* (Kurdjumov) (Hymenoptera: Braconidae) (Chilcutt & Tabashnik, 1999; Haseeb *et al.*, 2004; Schuler *et al.*, 2004), *C. marginiventris* (Cresson) (Hymenoptera: Braconidae) (Vojtech *et al.*, 2005; Ramirez-Romero *et al.*, 2007; Tian *et al.*, 2014; 2018) and even *C. flavipes* (Prütz & Dettner, 2004; Prütz *et al.*, 2004). The indirect effect of exposure of *C. flavipes* to purified Bt Cry toxins were evaluated in Cry protein-incorporated artificial diet bioassays (Tounou *et al.*, 2005) which showed no adverse effects in terms of egg clutch size or parasitism rates. Adverse effects were, however, observed in terms of the amount of females yielded where both groups, 0.016, 0.08 mg/mL CryIAc, yielded lower female-biased sex ratios than the control.. However, prior to this study, no real-life evaluation of the indirect effect of Bt exposure on *C. sesamiae* using Cry1Ab resistant *B.fusca* larvae that fed on Bt maize have been done. Using an indigenous and closely related parasitoid such as *C. sesamiae* as a

surrogate species for such an evaluation is facilitated by the presence of Bt maize-resistant *B. fusca* in South Africa.

Due to the presence of *B. fusca* larvae that evolved resistance against maize producing Cry1Ab proteins in South Africa (Kruger *et al.*, 2011; 2014), the rearing of these larvae on Bt maize with no observable negative effects can be facilitated. This resistance afforded me the opportunity to study the possible effects of the Cry1Ab protein on the fitness and survival of *C. sesamiae* without prey-mediated effects having a possible influence. The aim of this study was to evaluate the possible indirect effects of the feeding of *C. sesamiae* on healthy prey (Bt-resistant *B. fusca* larvae) that were reared on Bt maize plant tissue expressing the Cry1Ab protein.

### **3.3. Materials and methods**

#### **3.3.1. Insects**

##### *Busseola fusca* stock colony

Bt-resistant *B. fusca* larvae were collected from Bt maize plants at Devon (26°22'56.8"S 28°43'45.8"E) and Nigel (26°25'40.3"S 28°18'01.4"E), Gauteng province as well as in the Vaalharts region of the Northern Cape province (27°49'00.0"S 24°47'0.00"E). Larvae from the Vaalharts region is highly resistant to Cry1Ab maize (Kruger *et al.*, 2011). Resistance of larvae collected from the other two sites were confirmed during a laboratory trial where 50 neonate larvae (10 larvae per plant) of the first generation were artificially inoculated and reared on pieces of Bt maize plants for 14 days from inoculation, with food replaced at weekly-intervals. The number of live larvae were counted and showed a survival rate of above 60%. The collected larvae were reared on non-Bt maize in a rearing chamber for approximately 20 days until they reached the 3<sup>rd</sup>/4<sup>th</sup> instar, which were then used in the toxicity bioassays. Conditions of the rearing chamber were as follows: 26 ± 1 °C, 65 ± 10% relative humidity (RH) and a 14L: 10D photoperiod. Throughout the year the colony was rejuvenated with *B. fusca* larvae collected from Bt maize fields in the same areas.

### *Cotesia sesamiae* stock colony

The initial colony of *C. sesamiae* (morphologically and molecularly identified in Chapter 2) was obtained from parasitised *B. fusca* and *C. partellus* larvae collected from various maize fields in the maize production region of South Africa, including: Nigel, Devon, Potchefstroom (26°43'59"S 27°04'49"E) and Vaalharts. The parasitised *B. fusca* and *C. partellus* larvae were maintained in a rearing chamber at  $26 \pm 1^\circ\text{C}$ ,  $65 \pm 10\%$  RH and a 14L: 10D photoperiod until parasitoid emergence. These parasitoids were then reared in a rearing chamber using *B. fusca*, *C. partellus* and *S. calamistis* larvae as hosts under the same conditions as described above. Parasitoid cocoons were kept in small transparent plastic containers (55 mm in length x 25 mm in diameter) closed with a steel mesh-infused lid until emergence. After emergence the adults, which had access to a 20% sugar/water solution imbibed in cotton balls *ad libitum* throughout, were paired and kept under artificial light for 24 hours to mate.

### 3.3.2. Experimental procedure

Third and fourth instar *B. fusca* larvae reared on non-Bt maize were transferred to small transparent plastic containers closed with a steel mesh-infused lid. A 5-cm long piece of either non-Bt (control) or Bt maize stem (expressing Cry1Ab protein) was placed into each container and the larvae left for 72 hours to feed and produce frass. The Cry1Ab protein concentration and expression levels in the plant tissue used as larval food, were determined by means of ENVIROLOGIX® ELISA plates (detail provided under subheading 3.3.3).

#### Experiment 1 – Indirect exposure bioassay

Larvae that fed on the respective Bt and non-Bt maize treatments were transferred individually into small transparent plastic containers and covered with a steel mesh infused lid. One 1-day-old, naïve, putatively mated female *C. sesamiae* was transferred to each container. These larvae were left without food for 24 hours while they were exposed to the wasps to increase the likelihood of successful parasitisation. After 24 hours, the wasps were removed and the larvae were provided with Bt and non-Bt maize stem cuttings as food, depending on what they were originally fed with. They were inspected daily for mortality, pupation as well as parasitoid cocoon formation until no more pupa or parasitoid cocoons formed. Only parasitised larvae, characterised by cocoon formation, were considered in the analyses. The number of cocoons, number of wasps

emerging from cocoons and sex of these wasps were recorded. Experiments were carried out at  $26 \pm 1^\circ\text{C}$ ,  $65 \pm 10\%$  RH and a 14L: 10D photoperiod.

### Experiment 2 – Direct exposure bioassay

Larvae that fed on the Bt and non-Bt maize, respectively, were removed, weighed and individually transferred into glass Petri dishes (100 mm diameter x 10 mm height), placed on a white background, together with 2 to 4 1-day-old, naïve, putatively mated female wasps. The activity of these wasps were induced by placing them under bright artificial light (60 W Philips lamplight), since they are generally in nature found to be active throughout the photophase (Mohyuddin, 1971; Overholt, 1993; Smith *et al.*, 1993). These exposure bioassays were conducted in a laboratory maintained at  $26 \pm 1^\circ\text{C}$ . The experiment was closely observed and the larva was removed from the arena after it was stung once by a single female, and transferred back into another clean small transparent plastic container with a steel mesh infused lid where it was provided with either Bt or non-Bt maize stem tissue, depending on what it was originally fed with. The female wasp which stung the larva was also removed from the arena to ensure that a single female only stung one larva. In cases where more than one female parasitised the larva, data were discarded. Larvae were inspected daily for mortality, pupation as well as parasitoid cocoon formation until no more pupa or parasitoid cocoons formed. Only parasitised larvae, characterised by cocoon formation, were considered in the analyses. The number of cocoons, number of wasps emerging from cocoons, sex of these wasps, mass of the host larvae and developmental time of the wasps were recorded. The experiments were carried out at  $26 \pm 1^\circ\text{C}$ ,  $65 \pm 10\%$  RH and a 14L:10 D photoperiod.

### **3.3.3. Enzyme-Linked ImmunoSorbent Assay (ELISA)**

The plate-based immunoassay ELISA is a simple, fast and easy method used for detection of proteins, peptides, hormones and antibodies in genetically modified organisms (Dong *et al.*, 2018). This technique involves using an enzyme to detect the presence of an antigen or antibody in a sample (Zhu *et al.*, 2011). According to Goldsby *et al.* (2003) an ELISA test may either be run in a qualitative or quantitative format. ELISA data can be quantified when the optical density or fluorescent units of the samples are interpolated into a standard curve (a set of serial dilutions of a known antigen) (Goldsby *et al.*, 2003). For quantitative purposes, Cry1Ab analytical standard reference protein was acquired from Marianne P. Carey, Case Western Reserve University,

School of Medicine, Cleveland, USA. These proteins were purified by high pressure liquid chromatography (HPLC) from salt-free *Bacillus thuringiensis* full size proteins.

The plant tissue used as larval food during this study were sampled by cutting off a piece of maize stem from plants between the third and fifth vegetative stages. All samples were frozen in liquid nitrogen immediately after sampling and stored at -80 °C. The frozen maize stems were freeze dried and 5 - 10 mg of each sample of leaf material used. The mass of each sample was determined and noted before the sample was ground into a fine powder using a Retch mixer mill MM 400. Three steel beads were added per sample and the mixer mill placed on a 30 m/s setting for 3 minutes. Homogenisation of the leaf powder was done by adding 1.5 ml PBST-buffer (pH 7.4). After centrifugation, the supernatants were all diluted to a concentration of 1:100 with PBST-buffer. The level of Cry protein of the sampled stem material were determined by using antibody coated wells of Envirologix® (qualiplate combo kit AP 045) for Bt Cry1Ab. Standards of Cry1Ab at concentrations of 0.03, 0.06, 0.12, 0.24, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 ng/ml were used during the construction of standardised optical density curves used for estimating the protein content of samples. All standards were added in duplicate, while all samples were added in triplicate to a 96-well ELISA plate. Two standard curves were run per analysis. The positive control included in the Envirologix® kit, was added in sextuplet to the plate. A microplate reader (Berthold TriStar LB941) was used to measure the colour intensity at 450nm with a reference wavelength of 650nm. The content of the sample protein was calculated by using the linear regression equation for the standard curve. These concentrations were then calculated back to account for mass. Cry1Ab concentrations in the stem samples were expressed as µg/g dry mass (Envirologix, Portland, Me).

#### **3.3.4. Data analysis**

Data on the number of cocoons, number of wasps emerging from cocoons and sex ratio (females:males) of these wasps between Bt and non-Bt maize treatments were analysed by means of independent student t-tests using TIBCO Statistica™ 13.3 (TIBCO Software, Inc., 2017). The same analysis were done for the data on mass of host larvae and developmental time of the parasitoid larvae. The Cry1Ab protein concentration in Bt maize was analysed using descriptive statistics.

### 3.4. Results

The average concentration ( $\pm$ SD) of Cry1Ab toxins from 18 Bt maize stems was  $20.32 \pm 8.31$   $\mu$ g/g with a 8.2% coefficient of variation (CV).

#### Experiment 1 – Indirect exposure bioassay

The mean number of *C. sesamiae* cocoons that formed from parasitised *B. fusca* larvae that fed on Bt maize and those that fed on non-Bt maize, did not differ significantly. The mean number of wasps as well as mean number of male *C. sesamiae* wasps that emerged from these cocoons was also similar between Bt and non-Bt fed *B. fusca* larvae (Table 3.1).

Significantly more female wasps did, however, emerge from cocoons formed from parasitised *B. fusca* larvae that fed on Bt maize compared to *B. fusca* larvae that fed on non-Bt maize (Table 3.1). The sex ratio of *C. sesamiae* that emerged from Bt fed *B. fusca* larvae was 1 female (73%):0.36 male (27%) and from larvae fed on non-Bt maize, 0.88 female (47%):1 male (53%).

**Table 3.1:** Mean number of cocoons and *Cotesia sesamiae* that emerged from cocoons from parasitised *Busseola fusca* larvae that fed on Bt maize and non-Bt maize.

	<b>Bt maize</b> (mean number $\pm$ SE)	<b>Non-Bt maize</b> (mean number $\pm$ SE)	<b>t-value</b>	<b>Df</b>	<b>P value</b>
<b>Experiment 1 – Indirect exposure bioassay</b>					
<b>Cocoons</b>	59 $\pm$ 4.67	54 $\pm$ 4.75	0.68	55	NS
<b>Females</b>	36 $\pm$ 4.17	21 $\pm$ 2.96	2.93	55	<0.01
<b>Males</b>	13 $\pm$ 2.13	24 $\pm$ 4.64	-1.85	55	NS
<b>Total</b>	49 $\pm$ 4.79	46 $\pm$ 4.72	0.52	55	NS
<b>Experiment 2 - Direct exposure bioassay</b>					
<b>Cocoons</b>	43 $\pm$ 12.44	31 $\pm$ 8.64	0.87	13	NS
<b>Females</b>	16 $\pm$ 9.51	15 $\pm$ 5.46	0.11	13	NS
<b>Males</b>	19 $\pm$ 6.83	9 $\pm$ 2.74	1.47	13	NS
<b>Total</b>	35 $\pm$ 12.15	24 $\pm$ 7.82	0.77	13	NS
<b>*Days to larvae</b>	12 $\pm$ 1.02	12 $\pm$ 0.71	0	13	NS
<b>*Days to cocoons</b>	13 $\pm$ 1.02	13 $\pm$ 0.71	0	13	NS
<b>*Days to wasps</b>	20 $\pm$ 0.71	20 $\pm$ 0.41	1.01	13	NS

NS = no significant difference.

\*Developmental time of the parasitoids are represented additionally for experiment 2 presented as the number of days from parasitisation to each of the respective developmental parameters

#### Experiment 2 – Direct exposure bioassay

There was no significant difference between the mean number of cocoons, as well as the mean number of wasps that emerged from cocoons that formed from *B. fusca* larvae that fed on Bt maize and those that fed on non-Bt maize. The mean number of male and female wasps was also similar regardless of whether they emerged from cocoons that formed from *B. fusca* larvae that fed on Bt maize or from larvae that fed on non-Bt maize (Table 3.1). The sex ratio of *C. sesamiae* that emerged from Bt fed *B. fusca* larvae was 0.84 female (46%):1 male (54%) and from larvae fed on non-Bt maize, 1 female (63%):0.60 male (38%). The mean mass of the host larvae fed on Bt maize (0.25  $\pm$  0.04 g) did not differ significantly from those that fed on non-Bt maize (0.22  $\pm$  0.04 g) (t=0.48; df=13; P=0.64).

There was no significant difference between any of the *C. sesamiae* developmental parameters, namely the days from when the *B. fusca* larvae were parasitised to *C. sesamiae* larval emergence, cocoon formation as well as wasp emergence between *B. fusca* larvae that fed on Bt maize and those that fed on non-Bt maize. During both treatments, *C. sesamiae* larvae emerged 12 days after they parasitised *B. fusca* larvae. Cocoon formation occurred over the 24 hour period after emergence of *C. sesamiae* larvae from *B. fusca* larvae, and wasps emerged from these cocoons seven days later (Table 3.1).

### 3.5. Discussion

Smith *et al.* (1993) defined a foraging strategy as the set of behavioural and morphological adaptations that enables a parasite to effectively exploit a particular host. *Cotesia* species use the ingress and sting foraging strategy found within the larval parasite guild. These parasitoids gain access to the tunnels made by their larval hosts where they attack the host larvae feeding inside the tunnels. The parasitoids are guided to the immediate vicinity of the host by using cues such as the odour from larval frass as well as the kairomones present in the host plant tunnel itself (Smith *et al.*, 1993). The microhabitat described above does however not have to be similar for parasitisation to be successful and experiments of this nature can be conducted under laboratory conditions. Therefore, during experiment 1, the host larvae and parasitoids were put into a container where locating and stinging of the larval host was left to the wasp which was released in close proximity to the larva (Smith *et al.*, 1993). This method increases the likelihood of parasitisation, since Potting *et al.* (1997) reported that locating of host larvae inside the stemborer tunnel was time consuming for *C. flavipes* wasps which are short lived ( $\pm 34$  hours – unfed,  $\pm 51$  hours – provided with food (Walker & Overholt, 1993)). The host stinging activity is also high risk since 30-50% of females are killed by the aggressive spitting or biting behaviour of the defending host (Takasu & Overholt, 1997). The females in experiment 1 therefore wasted no time searching for their host and the release of host and wasp in close proximity lowered the mortality risk for the wasp, leading to a higher successful parasitisation rate.

The experimental procedure described above was however adapted for experiment 2 to ensure that each host larva that was exposed to a parasitoid was actually parasitised. Potting *et al.* (1997) found that the clutch size of *C. flavipes* decreased with each subsequent oviposition event, having a depletion period after only five or six hosts. This adaptation in experimental procedure also ensured that each host larva was stung only once by a naïve female and that it would therefore

have an approximately similar number of egg load deposited within them. Although several wasps were present in a single container, which could have resulted in over-parasitisation, this was not evident from the results which indicated similar numbers of cocoons and wasp emergence rates between the two experiments.

The mean number of cocoons as well as the mean number of wasps emerging from the cocoons obtained from Bt-fed *B. fusca* larvae compared to those obtained from non-Bt fed larvae were higher in both experiments, although not as significant as found by Tounou *et al.* (2005). These authors reported bigger clutch sizes in laboratory experiments conducted with *C. sesamiae* and *S. calamistis* larvae in Bt protein-incorporated artificial diets. In addition to the bigger clutch sizes, they also found that higher parasitism rates occurred between *S. calamistis* larvae which fed on Bt-incorporated diet after being parasitised when compared with those that fed on non-Bt diet as well as those that were never fed any Bt-incorporated diet (control) (Tounou *et al.*, 2005).

Tounou *et al.* (2005) proposed two possible explanations for these findings, the first being the paralyses and subsequent defencelessness of the larvae caused by the Cry toxins. It has been reported that successful parasitisation of *C. flavipes* is interrupted by the aggressive attacking of the larval stemborer host (Potting *et al.*, 1993; Takasu & Overholt, 1997). The second contributing factor was proposed to be the lower encapsulation rate of parasitoid eggs brought on by the weakened immune response of stemborer larvae caused by the Bt toxin (Tounou *et al.*, 2005). The observations by Tounou *et al.* (2005) should however be interpreted with care since it has been reported that poor larval food quality may result in poor host status which affects performance of parasitoids. Although the results reported by Tounou *et al.* (2005) for *B. fusca*, *S. calamistis* and *Eldana saccharina* Walker (Lepidoptera: Pyralidae) were similar to that observed in this study, the adverse effects of Cry1Ab toxins reported on *S. calamistis* could most likely be ascribed to less than optimal host quality that resulted from sub-lethal effects of the Cry protein in the artificial diet. This is the case as Van den Berg and Van Wyk (2007) as well as Van Wyk *et al.* (2009) reported that *S. calamistis* are highly susceptible to Bt toxins. The findings of Tounou *et al.* (2005) reporting that *S. calamistis* was the least susceptible stemborer species are therefore questioned. A possible explanation for the survival of the *S. calamistis* may be that of a weakened Bt expression level when provided in the form of an artificial diet. The possible effects of poor larval host quality on parasitoid performance was however not relevant in this study, since the *B. fusca* larvae used during this study was Bt-resistant and was therefore not sub-lethally effected.

Host quality is a limiting factor when determining the number of offspring produced by a parasitoid. The size and health status of larval hosts are good indicators of their host status and higher abundance of food sources is associated with healthy larvae (Charnov *et al.*, 1981). In a study by Mochiah *et al.* (2001) where the host suitability of four cereal stemborers for *C. sesamiae* was tested, the host size was found to be an important factor influencing parasitoid development as well as reproductive potential. They found that larvae of the noctuids, *B. fusca* and *S. calamistis*, which were much heavier than those of the crambids, *C. partellus* and *Chilo orichalcociliellus* (Strand) (Lepidoptera: Crambidae), resulted in higher *C. sesamiae* progeny production. *Cotesia sesamiae* was reported to deposit fewer eggs in smaller hosts, therefore suggesting that females are capable of allocating different numbers of eggs to larvae of different sizes (Mochiah *et al.*, 2001). A higher progeny production was also reported by Ngi-Song *et al.* (1995) in larger *C. partellus* larvae when parasitised by *C. flavipes*. Similar relationships between larval size and parasitism were reported by Jiang *et al.* (2004) for *C. partellus* parasitism under natural conditions by both *C. flavipes* and *C. sesamiae*. The indirect effect of host size was therefore also eliminated during this study since healthy, Cry1Ab resistant *B. fusca* larvae were used with no significant difference in the mass of host larvae which fed on either Bt or non-Bt maize.

In experiment 1, significantly higher numbers of female wasps emerged from cocoons that formed on *B. fusca* larvae that fed on Bt maize compared to larvae that fed on non-Bt maize. According to Hopper *et al.* (1993) the occurrence of more female wasps under laboratory conditions allows a higher production of female progenies in the next generation, therefore ensuring the establishment of a parasitoid population in their natural environment. This is due to a greater number of females which increases the chances of mating in the field (males are able to mate more than once), seeing that higher numbers of released females lead to increased parasitism and subsequently the production of higher numbers of females progenies (Hopper *et al.*, 1993). The emergence of more males from cocoons that formed from *B. fusca* larvae that fed on Bt maize in experiment 2 could be attributed to the presence of a *Wolbachia* spp. infection in *C. sesamiae* (Chapter 2). This was however not tested during this study. *Wolbachia* bacteria are reproductive manipulators, manipulating their own spread in a population by encouraging the reproduction of male *C. sesamiae* progeny (Delgado & Cook, 2009). Sweby *et al.* (2010) suggested that the presence of *Wolbachia* (Supergroup A) in *E. saccharina* in Uganda, Tanzania and Kenya causes cytoplasmic incompatibility in infected males, thereby leading to a reduction in the number of viable progeny produced.

Campos-Farinha *et al.* (2000) reported that release of *C. flavipes* at a ratio of 60% females to 40% males (1:0.67) is satisfactory for the establishment and eventual control of *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) populations in the field. At ratios of less than 60% females, parasitism has been found to be inefficient (Campos-Farinha *et al.*, 2000). The sex ratio of *C. sesamiae* recorded in this study (experiment 1) from cocoons that formed from *B. fusca* larvae that fed on Bt-maize was closer to that found by Campos-Farinha *et al.* (2000) than the sex ratio of *C. sesamiae* recorded from cocoons that formed on *B. fusca* larvae that fed on non-Bt maize. The opposite was however found for the sex ratios obtained in experiment 2, where the sex ratio of *C. sesamiae* recorded from cocoons that formed on *B. fusca* larvae that fed on non-Bt maize was found to have almost an identical sex ratio as that found by Campos-Farinha *et al.* (2000).

According to Walker and Overholt (1993) the egg-larval period lasts 10-15 days after which the final instar larvae emergence from the host larvae and immediately start spinning silk cocoons and pupate. The next generation of wasps emerge within a week after pupation (Walker & Overholt, 1993). The results on life history parameters of *C. sesamiae* observed in this study were similar to those reported for this species on stemborers in Kenya (Walker & Overholt, 1993). It therefore indicates that indirect exposure of *C. sesamiae* to Cry1Ab protein through feeding on healthy Bt maize-feeding stemborer larvae, do not have an adverse effect on their biology.

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## Chapter 4: Conclusion and recommendations

The current world population is estimated to reach 9.8 billion people by the year 2050, with the majority of growth occurring throughout the less developed countries of the African and Asian continents (United Nations, 2017). This places continuous pressure on the agricultural sector to provide an adequate food supply to an ever expanding population. A near doubling of the share of crop yields lost to insect pests were found by Pimentel *et al.* (1993), despite the 10-fold increase in both the toxicity as well as amount of synthetic insecticides used during the 40 years preceding 1993. No specific solution to ensure sustainable agriculture for the future exists, however, the integrated application of biological control and use of GM crops possess the potential to not only contribute largely to sustainable agriculture but to also provide the growing world population with food security (Christou & Cappel, 2009).

Maize is the dominant staple food throughout sub-Saharan Africa, and the largest produced field crop in South Africa (DAFF, 2017). An estimated annual yield loss of 10% is caused by stemborers throughout the maize production region of South Africa, with losses ranging between 25 and 78% also been recorded (Sylvain *et al.*, 2015). *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) is an insect pest of economic importance throughout sub-Saharan Africa (Van den Berg & Van Rensburg, 1996), and together with *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) it is regarded as the most important, widespread and destructive field insect pests of maize in southern Africa (Cugala & Omwega, 2001; Kfir *et al.*, 2002).

The two most commonly used biological control agents against medium to large-sized *B. fusca*, *C. partellus* and *S. calamistis* are the congeneric larval parasitoids *Cotesia sesamiae* and *Cotesia flavipes* Cameron (Hymenoptera: Braconidae) (Walker & Overholt, 1993). These gregarious, koinobiont larval endoparasitoids (Walker, 1994) are taxonomically closely related parasitoid wasps which are difficult to distinguish morphologically (Walker, 1993; Kimani-Njogu & Overholt 1997). *Cotesia sesamiae* is endemic to the Afrotropical region, while *C. flavipes* is indigenous to the Indo-Australian region (Kimani-Njogu & Overholt, 1997).

The use of male genitalia during this study proved to be accurate for use in the morphological identification of *Cotesia* specimens, where all the morphologically identified *C. sesamiae* were also molecularly confirmed as *C. sesamiae*. Although *C. flavipes* has successfully been

introduced into several countries in eastern and southern Africa (Omwega *et al.*, 2006), including South Africa, it does not seem to have established permanently in South Africa. This not only confirmed the findings of Skoroszewski and Van Hamburg (1987), but also showed that it has not spread from Botswana, where it was found recently, to South Africa.

Although *C. sesamiae* was the only species present in South Africa, the possible spread of *C. flavipes* from neighbouring countries to South Africa in the near future seems very likely. This is not only because of the presence of *C. flavipes* in Botswana as well as the ability of the parasitoid to disperse more than 2000 km away from its release site (Getu *et al.*, 2003), but also because of the highly competitive colonisation abilities of its co-evolved host *C. partellus*, allowing it to expand and establish in novel habitats. This is especially the case in Africa, where *C. partellus* outcompetes indigenous stemborer species (*B. fusca* and *S. calamistis*) in the changing climate experienced in Africa (Mutamiswa *et al.*, 2017). Mutamiswa *et al.* (2017) predicted an expansion of *C. partellus* to moist mid- and high-altitude areas. It is therefore possible that *C. partellus* can outcompete native stemborers in South Africa when the effect of Bt maize on *C. partellus* is left out of the equation. Future asynchrony was however predicted between *C. partellus* and its larval parasitoid *C. flavipes* due to the expected efficacy of *C. flavipes* to be better at low altitudes. With a 10% decrease in rainfall as well as more frequent droughts expected in southern Africa (Stathers *et al.*, 2013), a disruption in biological control is expected and it may significantly affect *Cotesia* species (Mwalusepo *et al.*, 2015). Therefore, although *C. partellus* has the ability to outcompete native stemborers in South Africa, leading to increased chances of parasitisation between the co-evolved host and natural enemy, the chances of disruption of the biological control as a result of a mismatch between the host and its main larval parasitoid *C. flavipes* are higher (Mutamiswa *et al.*, 2017). A repeat of this study is recommended in the near future. It is also recommended that parasitised stemborer larvae from more localities should be sampled, especially localities located close to the Botswana border.

The endosymbiont, *Wolbachia*, is an obligate intracellular bacteria that is maternally inherited by the wide range of hosts it infects (Murthy *et al.*, 2015). This host range includes parasitoids as well as their respective hosts (Mohammed *et al.*, 2017). The bacteria, belonging to the alpha-proteobacteria, infects the reproductive tissues (ovaries and testes) of arthropods (Murthy *et al.*, 2015). It leads to various host reproductive modifications including cytoplasmic incompatibility (CI), parthenogenesis, feminisation, and the killing of males (Werren *et al.*, 2008). Cytoplasmic incompatibility occurs when a *Wolbachia*-infected and a *Wolbachia*-uninfected form exists within

a species. Cytoplasmic incompatibility of the female type was found to be the most common effect of *Wolbachia* infections in *C. sesamiae* (Mochiah *et al.*, 2002). Mochiah *et al.* (2002) found that in crosses between cured and infected *C. sesamiae* specimens, all crosses produced both males and females while crosses between cured females and infected males produced only males. Due to incompatible eggs developing into uninfected males, the proportion of infected males largely determines the spread of the infection (Mochiah *et al.*, 2002). *Wolbachia* are therefore reproductive manipulators, manipulating their own spread in a population by encouraging the reproduction of male progeny (Delgado & Cook, 2009).

Werren and Windsor (2000) as well as Hilgenboecker *et al.* (2008) reported that about two thirds of all insect species are infected with *Wolbachia*. The high prevalence of *Wolbachia* within the samples from this study (Chapter 2) therefore does not come across as out of the ordinary. The compromise of insect barcoding by *Wolbachia* does however come across as out of the ordinary, as Smith *et al.* (2012) found that although it is possible to generate *Wolbachia* COI using the standard insect primers, the amplicon is almost never confused with the COI of the host. Although evidence of *Wolbachia* in the total genomic extracts of insects created for the DNA barcoding library construction was found in only 0.16% of cases, the unintended amplification of *Wolbachia* COI from insect genomic DNA extracts have been experienced by Smith and Fischer (2009). Authors who also used the COI gene to identify *Cotesia* specimens to species level, however, never reported this problem (Muirhead *et al.*, 2006; 2012; Assefa *et al.*, 2008). It was, however, found in this study and should be further investigated in future. This is especially the case as Mochiah *et al.* (2002) found that the release of *C. sesamiae* populations with *Wolbachia* infections in areas where a *Wolbachia*-free population occurs, will lead to a decrease in the reproductive potential of the native population due to sterile mating. Matings between males from *Wolbachia*-infected populations and females from *Wolbachia*-free populations therefore could theoretically contribute to the failure of biological control programs. Knowledge on the *Wolbachia* status of *C. sesamiae* is therefore important before release should be considered (Mochiah *et al.*, 2002).

Although Bt maize is generally thought to be environmentally safe to humans and animals, concerns have been raised about the non-target effects in tritrophic interactions (Fontes *et al.*, 2002). Lovei and Arpaia (2005) reported that 40% of parasitoid species and 30% of predator species used in studies where the effect of transgenic crops (including Bt and other crop traits) on the life history parameters of natural enemies were studied, significant negative effects were

recorded. According to Naranjo (2009) these numbers were exaggerated because of non-independence of multiple traits measured on a species within the same study, while Romeis *et al.* (2006) concluded that the negative effects reported on natural enemies were only present when they were provided with a host/prey that was susceptible to Bt proteins.

During this study the possible effects of the Cry1Ab protein on the fitness and survival of *C. sesamiae* could be studied without prey-mediated effects having a possible influence. This was due to the presence of *B. fusca* larvae in South Africa that evolved resistance against maize producing Cry1Ab proteins. Results obtained during this study showed that Bt exposure had no significant effect on the life history of *C. sesamiae*, but significantly more females emerged from parasitised *B. fusca* larvae that fed on Bt maize compared to those that fed on non-Bt maize. Males can mate more than once and therefore the increase in the number of females emerging from larvae that fed on Bt maize are beneficial to the biological control of *B. fusca* (Walker & Overholt, 1993). The integrated use of *Cotesia* specimens, both *C. sesamiae* and *C. flavipes*, is therefore recommended as the future cultivation of Bt maize in Africa does not appear to have adverse effects on these biocontrol agents. They are therefore able to provide their ecosystem services in synergy with Bt maize which uses their own mechanisms to also limit pests. This will result in increases in the quality and quantity of yields as well as a reduction in the use of expensive and environmentally hazardous insecticides (Gouse *et al.*, 2005; 2006).

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## Appendix 1

Five extraction methods were also evaluated and compared during this study to determine the optimal extraction method for extracting genomic DNA from whole *Cotesia* specimens. The modified Chelex-100 method was found to be the most effective method, while the Zymo ZR Quick-DNA™ Tissue/Insect Miniprep Kit was found to be the least effective method.

### Materials and methods

#### *DNA extraction from wasp tissue*

Various extraction methods were evaluated to obtain the optimal extraction method for extracting genomic DNA from whole *Cotesia* specimens. These methods included a modified salting out method, modified Chelex-100 method, Zymo Quick-DNA™ Tissue/Insect Miniprep Kit, Qiagen DNeasy Blood & Tissue Kit as well as a modified version of the Qiagen DNeasy Blood & Tissue Kit. All specimens, which were either stored in ethanol or dried, were cleaned before the initiation of the extraction process by adding three alternations between ultrapure water and absolute ethanol (99.9%), whilst vortexing between alterations. All extracted DNA samples were stored at -20 °C. Genomic DNA was extracted from five wasps per extraction method, resulting in a total number of 25 wasps being extracted.

#### 1. Modified salting out method

One whole *Cotesia* specimen was crushed with a metal pestil in a 1.5 ml Eppendorf tube, after which 500 µl of DNA extraction buffer (10 mM Tris-HCl [ph 8.0], 10 mM EDTA and 1% sodium dodecyl sulphate (SDS)) was added. A volume of 10 µl [10 mg/ml] Proteinase K (Pro-K) was added to the contents, mixed for 10 minutes using a tissue lyser, and incubated at 56 °C for 1 hour for DNA lysis and digestion. After an hour, an additional 10 µl of Pro-K was added and again incubated overnight at 56 °C to complete the digestion. On the second day, the reaction mixture was centrifuged for 5 minutes at 12 000 revolutions per minute (rpm) and the supernatant (upper aqueous phase) transferred into a new 1.5 ml Eppendorf tube. A volume of 180 µl of 5 M NaCl was added to the supernatant, vortexed for 30 seconds and then centrifuged at 13 500 rpm for 5 minutes. The supernatant was again transferred into a new 1.5 ml Eppendorf tube and 420 µl of ice cold isopropanol (Propan-2-ol) added. The reaction mixture was mixed by slowly inverting the

tubes 50 times, followed by centrifugation at 13 500 rpm for 15 minutes at 4 °C to precipitate the DNA. Subsequent to the centrifugation, the supernatant was discarded, the isopropanol removed with a pipette, and the DNA pellet (looks like a small tear drop at the base of the tube) washed with 250 µl of 75% ethanol, briefly vortexed and centrifuged at 13 500 rpm for 5 minutes. This step was repeated. The supernatant was discarded and the samples air dried on the bench with the caps covered with lint-free lab tissue for an hour at room temperature to evaporate the 75% ethanol. The DNA was dissolved with 200 µl of double distilled water (DDW) for resuspension of the DNA pellet and then incubated at 37 °C for one hour (Nasiri *et al.*, 2005).

## 2. Modified Chelex-100 method

One whole *Cotesia* specimen was crushed with a metal pestil in a 1.5 ml Eppendorf tube. Thirty microliter Chelex®100 (Bio-Rad, USA) and 10 µl Pro-K was added to the tube and centrifuged. The sample was then incubated for 2 hours at 56 °C followed by another incubation for 10 minutes at 95 °C, vortexed and the supernatant extracted (Musapa *et al.*, 2013).

## 3. Zymo Quick-DNA™ Tissue/Insect Miniprep Kit

Genomic DNA was extracted from one whole *Cotesia* specimen using the Zymo ZR Quick-DNA™ Tissue/Insect Miniprep Kit (ZYMO), recommended for the isolation of DNA (up to 25 µg) from fresh, frozen or stored insect and arthropod specimens. The protocol was as follows: An individual whole *Cotesia* specimen was crushed with a metal pestil in a 1.5 ml Eppendorf tube, and transferred to a ZR BashingBead™ Lysis Tube. A volume of 750 µl lysis solution was added, the lysis tube vortexed for 10 minutes at maximum speed and then centrifuged at 7 000 rpm for 1 minute. Up to 400 µl of the supernatant was transferred into a Zymo-Spin™ IV Spin Filter with orange cap in a collection tube and centrifuged again at 7 000 rpm for 1 minute. A total volume of 1200 µl of genomic lysis buffer was added to the filtrate in the collection tube from the previous step and mixed. About 800 µl of the previous mixture was transferred into a Zymo-Spin™ IIC Column placed in a new collection tube and centrifuged at 10 000 rpm for 1 minute. The flow through from the collection tubes was discarded and the previous step repeated. Two hundred microliters of DNA pre-wash buffer was added to the Zymo-Spin™ IIC Column placed in a new collection tube and centrifuged again at 10 000 rpm for 1 minute. A total volume of 500 µl of g-DNA wash buffer was added to the Zymo-Spin™ IIC Column and centrifuged at 10 000 rpm for

1 minute. The Zymo-Spin™ IIC Column was transferred to a clean 1.5 ml Eppendorf tube, 100 µl of DNA elution buffer added and then centrifuged at 10 000 rpm for 30 seconds.

#### 4. Qiagen DNeasy Blood & Tissue Kit

Genomic DNA was extracted from one whole *Cotesia* specimen using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) as recommended for purification of total DNA from animal tissue. The protocol was as follows: An individual whole *Cotesia* specimen was crushed with a metal pestil in a 1.5 ml Eppendorf tube, after which a volume of 180 µl Buffer ATL and 20 µl Pro-K was added, vortexed and the sample incubated at 56 °C until lysis was completed. A volume of 200 µl Buffer AL was added, mixed by vortexing and incubated at 56 °C for 10 minutes. Two hundred microliter ethanol (96-100%) was added to the sample and mixed thoroughly by vortexing. The mixture (including any precipitate) was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for one minute at 8000 rpm. The flow-through as well as collection tube was discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW1 added and centrifuged for one minute at 8000 rpm. The flow-through as well as collection tube was discarded. The DNeasy Mini spin column was again placed in a new 2 ml collection tube and 500 µl Buffer AW2 added and centrifuged for three minutes at 13 500 rpm to dry the DNeasy membrane. The flow-through was discarded, the DNeasy Mini spin column placed back inside the collection tube and centrifuged for an additional 1 minute at 13 500 rpm to ensure that there was no residual ethanol carry over during the elution stage. The DNeasy Mini spin column was then placed in a new 1.5 ml Eppendorf tube, while the flow-through was discarded. Genomic DNA was eluted in 200 µl of 10 mM Tris buffer (Buffer AE) by pipetting the Buffer AE onto the membrane. The sample was incubated at room temperature for 1 minute and then centrifuged for one minute at 8000 rpm to elute.

#### 5. Modified Qiagen DNeasy Blood & Tissue Kit

Genomic DNA was extracted from one whole *Cotesia* specimen using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) as recommended for purification of total DNA from insects using an electric homogeniser. The protocol was as follows: An individual whole *Cotesia* specimen was crushed with a metal pestil in a 1.5 ml Eppendorf tube, after which four BashingBeads™ (ZYMO) and a volume of 180 PBS (50 mM potassium phosphate and 150 mM NaCl [pH 7.2]) was added and the sample homogenised using a Retch mixer mill MM 400. A

volume of 20  $\mu$ l Pro-K and 200  $\mu$ l Buffer AL (without added ethanol) was added, mixed by vortexing and incubated at 56 °C for 10 minutes. Two hundred microliter ethanol (96-100%) was added to the sample and mixed thoroughly by vortexing. The mixture (including any precipitate) was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for one minute at 8000 rpm. The flow-through as well as collection tube was discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500  $\mu$ l Buffer AW1 added and centrifuged for one minute at 8000 rpm. The flow-through as well as collection tube was discarded. The DNeasy Mini spin column was again placed in a new 2 ml collection tube and 500  $\mu$ l Buffer AW2 added and centrifuged for three minutes at 13 500 rpm to dry the DNeasy membrane. The flow-through was discarded, the DNeasy Mini spin column placed back inside the collection tube and centrifuged for an additional 1 minute at 13 500 rpm to ensure that there was no residual ethanol carry over during the elution stage. The DNeasy Mini spin column was then placed in a new 1.5 ml Eppendorf tube, while the flow-through was discarded. Genomic DNA was eluted in 50  $\mu$ l of 10 mM Tris buffer (Buffer AE) by pipetting the Buffer AE onto the membrane. The sample was incubated at room temperature for 5 minutes and then centrifuged for one minute at 8000 rpm to optimise the elution step.

#### *Quantification and assessment of DNA purity*

The DNA concentrations for all the extraction methods were determined fluorometrically (Qubit™ - dsDNA HS assay, Life Technologies, Carlsbad CA), spectrophotometrically (Nanodrop™ ND-1000, ThermoFisher Scientific, USA) as well as by gel electrophoresis. The purity was assessed via 260/280 and 260/230 absorbance ratios, as determined via spectrophotometry.

#### *Statistical analysis*

Statistical analysis was performed using TIBCO Statistica™ 13.3 (TIBCO Software, Inc., 2017). Differences between the DNA extraction methods in total DNA concentration as well as purity were tested for homogeneity of variance (Levene's test) and normality (Shapiro-Wilk test), but these assumptions were not met. The data were log-transformed prior to analyses with one way ANOVA's. Means were separated using Tukey's HSD test ( $\leq 0.05$ ).

## Results

The modified Chelex-100 method was the cheapest method for extraction of DNA of *Cotesia* specimens, while the Qiagen DNeasy Blood & Tissue Kit as well as modified Qiagen DNeasy Blood & Tissue Kit was the most expensive method. In terms of time to conduct the respective methods, the Qiagen DNeasy Blood & Tissue Kit and modified Qiagen DNeasy Blood & Tissue Kit was less time consuming than the modified Chelex-100 method. This method and the Zymo Quick-DNA™ Tissue/Insect Miniprep Kit were the quickest methods for extraction of DNA of *Cotesia* specimens, while the modified salting out method was found to be the most time consuming method (Table 1).

Both the traditional “bench top” techniques, the modified salting out method and modified Chelex-100 method, as well as commercially available kit-based methods, Zymo Quick-DNA™ Tissue/Insect Miniprep Kit and Qiagen DNeasy Blood & Tissue Kit, proved to be successful in extracting DNA from *Cotesia* specimens. The modified Chelex-100 method produced the greatest total DNA yields in both Qubit and Nanodrop assays, with Nanodrop showing significantly higher yields when compared to all of the other extraction methods. Qubit showed the modified Chelex-100 method together with the modified Qiagen DNeasy Blood & Tissue Kit had significantly higher yields when compared to all of the other extraction methods. No statistically significant differences were found between the modified salting out method, Zymo Quick-DNA™ Tissue/Insect Miniprep Kit and Qiagen DNeasy Blood & Tissue Kit in the Qubit assay, but significant differences were found in the Nanodrop assay between all five of the methods. The Zymo Quick-DNA™ Tissue/Insect Miniprep Kit proved to be the least successful, having had the lowest total DNA yields in both Qubit and Nanodrop assays (Table 1). While a greater DNA yield may suggest a higher efficiency, DNA is normalised to a standard volume and concentration for amplification (Hart *et al.*, 2015). All of the methods examined in this study provided a sufficient quantity of DNA for PCR amplification.

The purity of extracted DNA is often assessed via 260/280 and 260/230 absorbance ratios, determined by spectrophotometry. The commercially available DNA extraction kits - Zymo Quick-DNA™ Tissue/Insect Miniprep Kit, Qiagen DNeasy Blood & Tissue Kit and modified Qiagen DNeasy Blood & Tissue Kit - provided more pure 260/280 values ( $\pm 1.8$  pure, lower = protein contaminated) when compared to those provided by the “bench top” techniques. The modified salting out method along with the Qiagen DNeasy Blood & Tissue Kit provided more pure 260/230

values ( $\pm 2.0$  pure, lower than 1.8 = contaminated) when compared to the other methods (Table 1).

**Table 1:** Comparison of DNA extraction methods. Cost of DNA extraction methods calculated on per sample basis of 50 samples. Time of extraction method determined from the start of specimen crushing to DNA elution. Mean 260/280 and 260/230 nm absorbance (as determined by spectrophotometry) and standard deviation for all DNA extraction methods. Number of amplified samples determined based on the successful visualisation of gel electrophoresis.  $n = 5$  per extraction method.

Extraction method	Cost ( $\pm$ Rand)	Time (hours)	Qubit concentration $\pm$ s.d.	Nanodrop concentration $\pm$ s.d.	Mean A260/280 $\pm$ s.d.	Amplification
					Mean A260/230 $\pm$ s.d.	
<b>Modified salting out</b>	980.00	28.5-29	0.77 $\pm$ 0.50a	2.16 $\pm$ 0.74a	1.30 $\pm$ 6.24b 1.48 $\pm$ 0.42c	4/5
<b>Chelex-100</b>	400.00	2.25-2.5	3.72 $\pm$ 0.63b	228.46 $\pm$ 24.37b	0.73 $\pm$ 0.02a 0.43 $\pm$ 0.06ab	4/5
<b>Zymo</b>	3448.57	1-1.25	0.35 $\pm$ 0.05a	0.68 $\pm$ 0.25c	2.08 $\pm$ 0.21b 0.38 $\pm$ 0.23a	2/5
<b>Qiagen</b>	3950.99	1-1.5	0.57 $\pm$ 0.32a	3.83 $\pm$ 0.63d	1.94 $\pm$ 0.13b 1.69 $\pm$ 0.27c	3/5
<b>Modified Qiagen</b>	3950.99*	1-1.5	3.22 $\pm$ 0.68b	12.36 $\pm$ 2.45e	2.06 $\pm$ 0.22b 0.71 $\pm$ 0.07b	5/5

\* No additional costs = re-used beads provided in Zymo Quick-DNA™ Tissue/Insect Miniprep Kit

Successful PCR-based amplification requires DNA templates containing little to no protein, RNA, polysaccharides or other contaminants (Hart *et al.*, 2015). The purity of the DNA assessed via 260/280 and 260/230 absorbance ratios, did however not accurately predict the success of amplification. The modified Chelex-100 method which produced the poorest absorbance ratios, produced the greatest total DNA yields in both Qubit and Nanodrop assays and had a successful amplification rate. Conversely, the Zymo Quick-DNA™ Tissue/Insect Miniprep Kit which produced an excellent 260/280 absorbance ratio but a poor 260/230 absorbance ratio, produced the lowest total DNA yields in both Qubit and Nanodrop assays and only had a few successful amplifications (Table 1). Hart *et al.* (2015) also found that neither high 260/280 or 260/230 absorbance ratios

accurately predicted the success of next-generation sequencing during a study where they did a comparative evaluation of DNA extraction methods from feces of multiple host species from downstream NGS.

## **Discussion**

In the current study, the performance of five DNA extraction methods were evaluated using *Cotesia* specimens collected in South Africa. All five extraction methods evaluated, *viz.* modified salting out method, modified Chelex-100 method, Zymo Quick-DNA™ Tissue/Insect Miniprep Kit, Qiagen DNeasy Blood & Tissue Kit as well as a modified version of the Qiagen DNeasy Blood & Tissue Kit successfully extracted genomic DNA from *Cotesia* specimens. While the modified Chelex-100 method consistently yielded higher levels of DNA, therefore indicating its effectiveness, this phenomenon could be ascribed to the method having used the lowest volume of elution buffer compared to the other methods. This is the case as the volume of elution buffer used determines how concentrated (low volume of elution buffer) or diluted (high volume of elution buffer) the finale DNA template is. The methods evaluated during this study were, however, evaluated according to standard protocols. The volume of elution buffer used during the methods were therefore not standardised between methods since it would have lead to the modification of the methods tested. Another possible reason for the higher levels of DNA yielded by the modified Chelex-100 method is the fact that the Chelex method, unlike the other methods tested, do not contain a retention step using a column. The extracted DNA therefore contained suspended impurities (Singh *et al.*, 2018).

The modified Chelex-100 method was found to be the most effective extraction method for extracting the DNA of *Cotesia* specimens, not only being the most cost-effective but also producing the highest DNA yields. The Zymo Quick-DNA™ Tissue/Insect Miniprep Kit was found to be the least effective extraction method for extracting the DNA of *Cotesia* specimens, not only providing low amounts of DNA yields but also being unsuccessful when it came to amplification. The modified salting out method and Qiagen DNeasy Blood & Tissue Kit also proved to be effective, with the modified salting out method having the downside of being time consuming and the Qiagen DNeasy Blood & Tissue Kit having the downside of being the most expensive method. The modified Qiagen DNeasy Blood & Tissue Kit provided the highest 260/280 and 260/230 values, second highest DNA yields and had a 100% successful amplification rate. This was the reason this method was used during this study to extract DNA from the samples collected. Various

authors optimised the Qiagen DNeasy Blood & Tissue Kit by freezing whole insect bodies in liquid nitrogen and then grinding them to a powder with a mortar and pestle (Dupas *et al.*, 2006; 2008; Assefa *et al.*, 2008).

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## Appendix 2

**Table 1:** BLASTn results for *Cotesia* specimens sampled from fifteen maize fields located within South Africa

Locality	Host	Repeat	Specimen identified	Max score	Total score	Query value	E value	Ident
Balfour	Unknown	1	<i>C. sesamiae</i>	902	902	98%	0.0	99%
		2		921	970	98%	0.0	99%
		3		830	889	95%	0.0	97%
Bizana	Unknown	1	<i>C. sesamiae</i>	911	960	98%	0.0	99%
		2		816	923	92%	0.0	99%
		3		810	810	99%	0.0	100%
Devon	<i>B. fusca</i>	1	<i>C. sesamiae</i>	915	960	98%	0.0	99%
		2		911	956	97%	0.0	99%
		3		910	969	96%	0.0	99%
Douglas	<i>C. partellus</i>	1	<i>C. sesamiae</i>	928	978	99%	0.0	99%
		2		888	888	97%	0.0	99%
		3		908	908	97%	0.0	99%
	<i>S. calamistis</i>	1	<i>C. sesamiae</i>	895	895	94%	0.0	99%
		2		830	938	92%	0.0	100%
		3		915	963	97%	0.0	99%
Dundee	<i>B. fusca</i>	1	<i>C. sesamiae</i>	810	810	87%	0.0	99%
		2		825	884	87%	0.0	100%
		3		829	888	88%	0.0	100%

East London	<i>B. fusca</i>	1	<i>C. sesamiae</i>	910	910	98%	0.0	99%
		2		919	978	98%	0.0	99%
		3		924	983	96%	0.0	100%
	<i>C. partellus</i>	1	<i>C. sesamiae</i>	897	897	95%	0.0	100%
		2		910	910	98%	0.0	99%
		3		904	949	97%	0.0	99%
Flagstaff	Unknown	1	<i>C. sesamiae</i>	809	916	92%	0.0	99%
		2		829	829	99%	0.0	100%
		3		861	861	99%	0.0	100%
Grootpan	<i>B. fusca</i>	1	<i>C. sesamiae</i>	902	902	97%	0.0	99%
		2		908	908	98%	0.0	99%
		3		827	886	87	0.0	100
Harrismith	<i>B. fusca</i>	1	<i>C. sesamiae</i>	919	965	97%	0.0	100%
		2		-	-	-	-	-
		3		-	-	-	-	-
Nigel	<i>B. fusca</i>	1	<i>C. sesamiae</i>	803	803	87%	0.0	99%
		2		825	825	99%	0.0	100%
		3		-	-	-	-	-
Ntabankulu	Unknown	1	<i>C. sesamiae</i>	895	895	98%	0.0	99%
		2		911	956	98%	0.0	99%
		3		854	913	95%	0.0	98%
Potchefstroom	<i>B. fusca</i>	1	<i>C. sesamiae</i>	910	910	97%	0.0	99%
		2		755	755	96%	0.0	98%
		3		930	989	97%	0.0	99%

	<i>C. partellus</i>	1	<i>C. sesamiae</i>	933	992	98%	0.0	99%
		2		827	886	87%	0.0	100%
		3		910	910	97%	0.0	99%
Prieska	<i>B. fusca</i>	1	<i>C. sesamiae</i>	895	943	97%	0.0	99%
		2		-	-	-	-	-
		3		-	-	-	-	-
	<i>C. partellus</i>	1	<i>C. sesamiae</i>	939	998	98%	0.0	100%
		2		823	930	92%	0.0	100%
		3		814	814	99%	0.0	100%
Vaalharts	<i>B. fusca</i>	1	<i>C. sesamiae</i>	919	967	97%	0.0	99%
		2		890	980	99%	0.0	100%
		3		-	-	-	-	-
	<i>S. calamistis</i>	1	<i>C. sesamiae</i>	902	902	97%	0.0	99%
		2		897	897	99%	0.0	100%
		3		-	-	-	-	-
		2		921	970	98%	0.0	99%
3	830	889	95%	0.0	97%			
Winterton	<i>B. fusca</i>	1	<i>C. sesamiae</i>	829	829	88%	0.0	100%
		2		740	740	99%	0.0	100%
		3		814	814	99%	0.0	100%

\* Accession number of top BLASTn result of all samples = HF562914