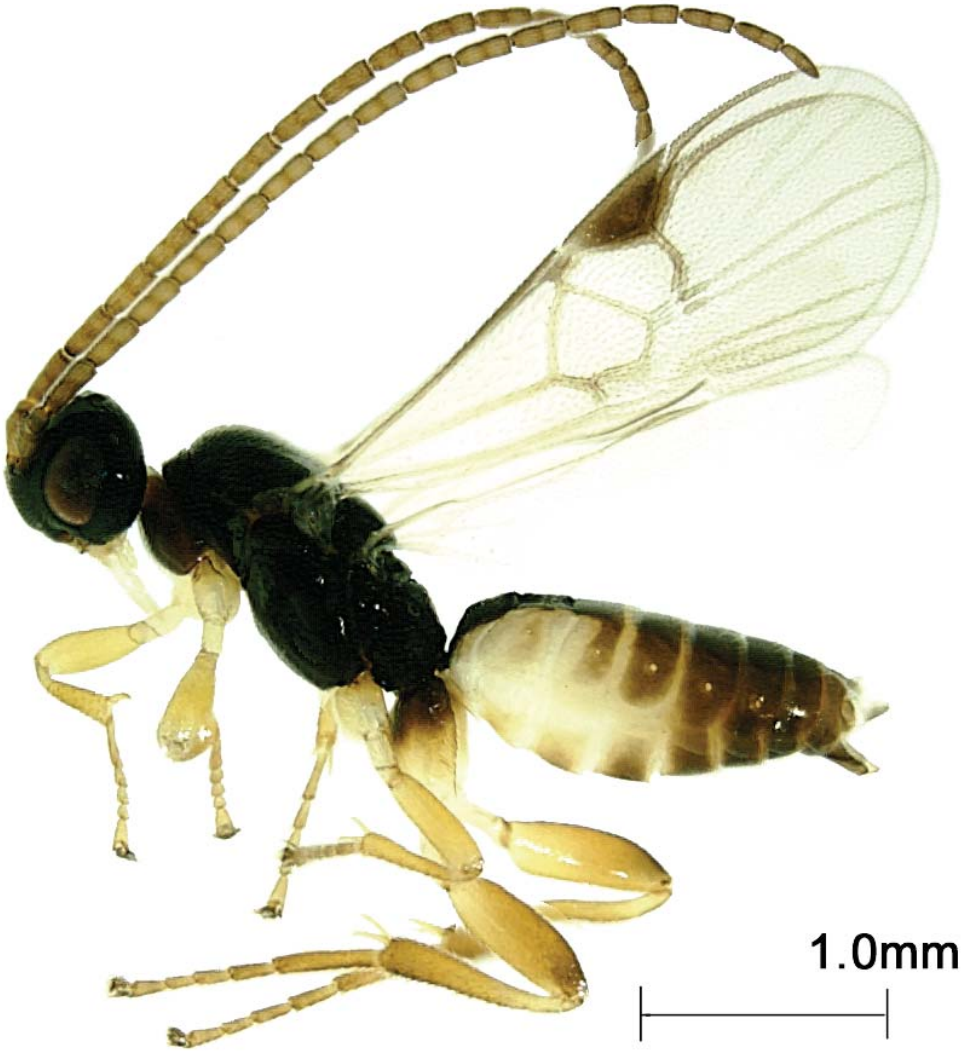
PHYLOGENETICS OF THE *COTESIA FLAVIPES* SPECIES COMPLEX: TOWARDS THE EFFECTIVE CONTROL OF STEMBORER PESTS IN AUSTRALIA



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DECLARATION

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Kate Muirhead
1 December, 2009

DEDICATION

To my dearly loved parents,
Valentina and Robb Muirhead

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ABSTRACT

The *Cotesia flavipes* species complex of parasitoid wasps are economically important worldwide for the biological control of lepidopteran stemborers. The complex currently comprises three species: *Cotesia flavipes* Cameron, *C. sesamiae* (Cameron) and *C. chilonis* (Matsumura), which appear morphologically similar. Despite their economic importance, considerable confusion surrounds the identity of species and host-associated biotypes. Differences in the biology and variation in host range of geographic populations have generally been interpreted as genetic divergence among strains, but direct genetic evidence is lacking. In Australia, several stemborer pests in neighbouring countries have been identified as significant threats to the sugar industry. However, the status of *C. flavipes* in Australia is unknown. To examine the genetic variation among worldwide populations of the *C. flavipes* complex and investigate the status of the Australian *C. flavipes*-like species, a pilot study based on 21 geographic populations of the complex and four outgroups was carried out using partial sequence data generated for mitochondrial gene regions, *16S rRNA* and *COI*. Phylogenetic analyses supported the monophyly of the complex and the existence of genetically divergent populations of *C. flavipes* and *C. sesamiae*. The geographically isolated Australian haplotypes formed a distinct lineage within the complex and were ~3.0% divergent from the other species.

Based on molecular, morphological and preliminary investigations into biological differences, the Australian species *Cotesia nonagriae* Olliff stat. rev. was redescribed and formally removed from synonymy with *C. flavipes*. Investigations of biology and behaviour of *C. nonagriae* on the native stem borer host, *Bathytricha truncata* (Walker) (Noctuidae) revealed that duration of the larval stages and adult longevity of *C. nonagriae* were longer than previously recorded for other members of the species complex. In addition, *C. nonagriae* oviposited an average of over 100 eggs into each host, almost three times more than for other species in the *C. flavipes* complex (30-40). During microhabitat location, both naïve and experienced females demonstrated a strong response towards the plant host complex, with experienced wasps benefiting by having a more rapid response time to infested than noninfested plants.

Genetic variation and relationships among the complex were further studied by generating nucleotide sequence data for two partial mtDNA gene regions (*COI, 16S*) and three anonymous nuclear loci (*CfBN*, *CfCN*, *CfEN*) among 42 worldwide populations within the *C. flavipes* complex and three outgroups. Phylogenetic reconstructions provided strong support for the monophyly of the complex and the presence of at least four species, *C. chilonis* (from China and Japan), *C. sesamiae* (from Africa), *C. flavipes* (originating from the Indo-Asia region but introduced into Africa and the New World), and *C. nonagriae* (from Australia and PNG). Although there was geographic variation within species, the analyses did not support the overall separation and monophyly of clades associated with different host species.

Members of the complex habour polydnavirus (PDV) symbionts, which play a key role in determining host range by host immune suppression during the course of parasitoid development. A worldwide phylogeny of the *C. flavipes* complex PDV *CrV1* locus was determined to investigate cophylogeny between wasps and their PDV symbionts. The results showed that there were numerous PDV *CrV1* haplotypes within worldwide populations. However, not all coevolutionary analyses supported the cophylogeny between wasp and PDV trees. Phylogenetic incongruence was most likely a result of the ability of PDVs to coevolve with host resistance through a process of natural selection, whereas the wasp genes were not under selection. The most important result of this study was the implication for the use of the *CrV1* locus as a virulence marker in biological control.

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CHAPTER I

General introduction

Genetic variation and biological control

Across a range of geographic scales, many insect species utilise diverse resources and populations may experience localised selection pressures. Thus, species are often subdivided into geographic populations that may be adapted to utilise specific resources and, therefore, display intraspecific variation in life history traits (Unruh & Messing 1993; Sunnucks 2000; Roderick & Naavajas 2003). Intraspecific variation in hymenopteran parasitoids is well documented and has been reported for ecological, behavioural and physiological traits, such as climatic adaptability, diapause, host selection and virulence (Hopper *et al.* 1993; Unruh & Messing 1993; Ngi-Song *et al.* 1995,1998; Alleyne & Wiedenmann 2001a,b; Heraty *et al*. 2007; Dupas *et al.* 2008; Phillips *et al.* 2008). Ruberson *et al.* (1989) alone listed over 65 studies that deal with intraspecific variation in hymenopteran parasitoid species, predominantly discovered from biological control introductions. The diversity of ecosystems in which a parasitoid species can exist and the differential use of resources that selects for variation in life history traits, raises the question of genetic variation among populations. From an evolutionary point of view, it is important to identify the forces that structure genetic differences among parasitoid populations relative to their host insects (Vaughn & Antolin 1998). In addition, the ability to discriminate between parasitoid genotypes on different hosts is crucial for biological control programs, which depend on accurate identification and adequate systematics of both natural enemies and target pests (Debach & Rosen 1991; Overholt 1998; Herty *et al.* 2007; Dupas *et al.* 2008).

Genetic variation among populations of several parasitoid species has been studied using a range of genetic markers (Goldson *et al.* 1997; Heimpel *et al.* 1997; Vaughn & Antolin 1998; Daza-Bustamante *et al.* 2002; Jensen *et al.* 2002; Vink *et al.* 2003; Herty *et al.* 2007; Phillips *et al.* 2008). Many of these studies indicate that large- and small-scale geographic populations display genetic divergence that often correlates with variation in host-use patterns. For example Vaughn and Antolin (1998) found that the aphid parasitoid, *Diaeretiella rapae* (McIntosh) (Braconidae), displayed low dispersal rates, and populations less than 1.0 km from each other were genetically

differentiated. Their results indicated that *D. rapae* populations were genetically subdivided on a small spatial scale that corresponded to host-use patterns. Likewise, on a much broader scale, populations of the parasitoid *Microctonus aethiopoides* Loan (Braconidae) were examined for the biological control of weevils in the genera *Sitona* and *Hypera* (Curculionidae) around the world. Nucleotide sequence data from populations in Australia, Iran, New Zealand, the United States and 10 European countries supported the presence of at least two *M. aethiopoides* strains, one associated with *Hypera* species and the other with *Sitona* species (Vink *et al.* 2003).

A major issue in biological control is defining and measuring genetic diversity among conspecific populations of natural enemies, especially if they utilise different host species. The fact that such variation is known to exist has led many biological control practitioners to accept intuitively that 1) any variation in features, such as host specificity, among samples of morphologically similar individuals, is likely to represent intraspecific differences among populations; 2) collection of conspecific individuals from different localities is likely to yield differentially adapted populations whose additional release is likely to enhance the chances of biological success; and 3) comparisons of major adaptations (such as host associations) between populations made under laboratory conditions are accurate reflections of that behaviour in the field (Clarke & Walter 1995).

Molecular systematics and biological control

Traditional morphological taxonomy is often the fastest and most cost-effective way of gathering data and is sometimes adequate for identification of biological control agents (Schauff & Lasalle 1998). However, many insect species are members of complexes of extremely closely related taxa, which are morphologically similar or indistinguishable and, thus, represent cyptic species (Wharton *et al.* 1990; Pinto *et al.* 1991; Hunter *et al.* 1996; Stouthamer *et al.* 1999, 2000; Kimani-Njogu *et al.* 2001; Molbo *et al.* 2003; Rincon *et al.* 2006; Herty *et al.* 2007). Within such complexes, reliance on traditional morphological characters for the identification of species and delineation of intraspecific variation among populations has been problematic (Polaszek & Walker 1991; Smith & Kambhampati 1999). As discussed above, defining and measuring genetic diversity can be imperative to biological control success since genetic divergence often correlates with variation in the host-use

patterns (Vaughn 1998). Thus, the use of DNA-based characters can provide valuable data to complement more traditional approaches (Hillis *et al.* 1996).

Advances in molecular systematics and phylogenetic reconstruction have revolutionised the field of biological control for understanding such aspects as, species identification population structure, genetic improvement and evolutionary change (Sunnucks 2000; Roderick & Naavajas 2003). The use of molecular markers for elucidating population structure provides essential information for reliable identification of species and genetic variants (Avise 1998). The organisation of genetic variation in natural populations is a result of ecological and evolutionary factors, such as migration, mutation, natural selection, drift, mating systems and geographical isolation (Moritz & Lavery 1996; Sunnucks 2000). Mitochondrial genes are useful molecular markers for diverse evolutionary studies, including phylogenetic inference, identification of species' origin, phylogeography, analysis of population structure and dynamics, and molecular evolution (Zhang & Hewitt 1997). In particular, several studies have shown that mitochondrial DNA (mtDNA) is a sensitive way to detect population structure for several reasons. It is maternally inherited and non-recombinant and therefore allows genetic relationships among individuals to be evaluated and traced back to an ancestral type (Simon *et al.* 1994). MtDNA evolves rapidly at the sequence level and most differences between sequences reflect point mutations (Hwang & Kim 1999). Its haploid copy number and maternal transmission make mtDNA less susceptible to interpopulation genetic homogenisation via gene flow and more susceptible to population bottlenecks (Hale & Singh 1987). Furthermore, previous studies show that mtDNA can elucidate genetic variation and species limits in a variety of insect species complexes (Hale & Singh 1987; Harrison *et al.* 1987; Sperling 1993; Armstrong *et al.* 1997; Sperling *et al.* 1999; Bogdanowicz *et al.* 2000).

The *Cotesia flavipes* species complex and biological control

This thesis examines genetic variation in the *Cotesia flavipes* complex of parasitoid wasps (Branconidae: Microgastrinae). The complex comprises three species; *C. flavipes* Cameron, *C. sesamiae* (Cameron) and *C. chilonis* (Matsumura) that appear morphologically similar, but as a group are characterised in part by a dorso-ventrally flattened body shape (Walker 1994) (Fig. 1.1). All three species are gregarious

endoparasitoids of graminaceous stemborers and have been used around the world for biological control. The species were grouped as the '*C. flavipes* complex' because *C. flavipes* has been the most successful and widely used biological control agent of the three species (Watanabe 1965; Sigwalt & Pointel 1980). It has been

FIGURE 1.1. Example of the dorso-ventrally flattened body shape of the *C. flavipes* complex compared to another species of *Cotesia, C. lunata* (from image database: http://www.life.illinois.edu/whitfield/cotesia/cotesia.htm).

introduced into over 40 countries and has been recorded from numerous stemborers, particularly species of *Chilo*, *Sesamia* and *Diatraea* (see Shenefelt 1972; Polaszek & Walker 1991; Walker 1994). However, lack of taxonomic knowledge and inaccurate identification of these wasps has complicated their use in biological control (Kimani-Njogu & Overholt 1997). Certain populations are recorded as having differences in host and/or habitat range, which may have potentially important implications for their use as biological control agents (Mohyuddin 1971; Shami & Mohyuddin 1992; Zhang & Hewitt 1996; Potting *et al.* 1997b; Ngi-Song *et al.* 1998). These differences may be indicative of genetic divergence among strains, but direct genetic evidence is lacking. To date, the complex has been studied intensively using morphology, but many of the characters that have been used to separate the species have proven unreliable due to intraspecific variation (Polaszek & Walker 1991; Smith & Kambhampati 1999). Two studies (Smith & Kambhampati 1999; Michel-Salzat & Whitfield 2004) have employed nucleotide sequence data to determine the phylogenetic relationships among the members of the complex, however the results are conflicting, which may be due to genetic divergence among strains and/or inadequate taxon sampling. Thus, there is an urgent need to develop techniques that can accurately identify populations among the complex and provide a phylogenetic framework as a prelude to their application for biological control.

Distribution

The species of the *C. flavipes* complex are thought to be endemic to the following areas: *C. flavipes* to the Indo-Australian region; *C. sesamiae* to central and southern Africa; and *C. chilonis* to the eastern Asia, including Japan (Polaszek & Walker 1991; Kimani-Njogu & Overholt 1997). However, all three species have been implemented in classical control programs against stemboring pests, resulting in their transport worldwide (Polaszek & Walker 1991). In some cases, a species of the complex has been introduced into an area indigenous to one of the other two species (Smith & Kambhampati 1999). For example, *C. flavipes* has been introduced several times into various countries in Africa (Overholt *et al.* 1994a,b), and is now established in Kenya and northern Tanzania (Omwega *et al.* 1995; Overholt *et al.* 1997) where it coexists with the native, *C. sesamiae*. Although *C. flavipes* and *C. sesemiae* can occupy a similar ecological niche it has been shown that these two species are not likely to compete because they prefer different host species (Rajabalee & Govendasamy 1988; Sallam *et al.* 2001; Sallam *et al.* 2002).

The earliest recorded attempt at biological control of stemborers using the *C. flavipes* complex was in 1951 when *C. sesamiae* was imported into Mauritius from Kenya (Anon 1954; Greathead 1971; Mohyuddin 1971). However, reports of attempted introductions have often remained unpublished, making it hard to track the movements of these species. For example, unconfirmed reports claim that *C. flavipes* was introduced into Mauritius, Reunion and Madagascar as early as 1917 (Appert 1973). On the other hand, it is also possible that *C. flavipes* may have arrived with its host around 1850 from India (see Greathead 1971; Mohyuddin 1971; Overholt 1998).

Cotesia flavipes has been purposely used as a biocontrol agent since 1950 against stemborers, such as *Chilo (Ch.) partellus* (Swinhoe), *Diatraea saccharalis* (Fabricius) and *Ch. sacchariphagus* Bojer (Crambidae) (Polaszek & Walker 1991; Overholt 1998). It now occurs in the Caribbean, major parts of North and South America, East Africa, the India Ocean islands of Madagascar, Mauritius and Reunion, and has also been redistributed within Asia (Mohyuddin *et al.* 1981). The establishment and success of *C. flavipes* in biological control programmes has been recorded on many occasions. For example Appert *et al.* (1969) reported a 60% parasitism rate on *Ch.*

sacchariphagus and a 2000 ton reduction in sugar losses in one state of Madagascar following the introduction of *C. flavipes* in the late 1950's. Similarly, *C. flavipes* was introduced into Barbados from India in 1966 and was recorded to have achieved parasitism levels of up to 80% against *D. saccharalis* (Simmonds 1969). More recently, *C. flavipes* was introduced into Kenya from Indian and Pakistan to target *Ch. partellus*, a major pest that had been introduced into Africa from India (Overholt *et al.* 1994a,b). The parasitoid is now established and successfully controlling this pest and other African native borers (Overholt *et al.* 1997). *Cotesia flavipes* has also been introduced and continuously mass released in several countries, including Brazil, Papua New Guinea and Indonesia (Sallam 2003). In Brazil, mass releases of *C. flavipes* for the control of *D. saccharalis* have sometimes involved over 180 million wasps (Anon 1980) and have resulted in a reduction in infestation levels of about 50% (Mecedo *et al.* 1993). It is often not known if mass release biological control programmes have resulted in the establishment of the species because they involve continuous introductions and inundative seasonal releases.

Status in Australia

Although *C. flavipes* is considered to be from the Indo-Australia region, its status within Australia has been unknown. Based on morphological comparison, the native *Apanteles nonagriae* Olliff was synonymised with *C. flavipes* (Wilkinson 1928a; Austin & Dangerfield 1992). However, the two species could be sibling taxa that have evolved similar characteristics due to comparable life history traits, such as host use and endoparasitism. Therefore, there are three possible scenarios regarding the status of *Cotesia flavipes* in Australia that require more research: 1) *Apanteles nonagriae* is correctly synonymised with *C. flavipes*, and the latter taxon is native to Australia; 2) *A. nonagriae* and possibly other related taxa are native *Cotesia* different from *C. flavipes*, and the latter does not occur in Australia, and 3) *A. nonagriae* is different from *C. flavipes* and both occur in Australia.

Due to the geographic isolation of Australia, major stemborer species do not occur on the continent. However, there is a minor pest of sugarcane, *Bathytricha truncata* (Noctuidae), which does not cause significant crop damage (Sallam & Allsopp 2002; Sallam 2003). A number of key stemborers inhabit neighbouring countries to the north of Australia and on several India Ocean islands and, therefore, the potential for

incursion is considered high by the Australian sugar industry (Sallam & Allsopp 2002; Sallam 2003). Pest species in the genera *Chilo*, *Sesamiae*, *Scirphphaga*, *Maliarpha*, *Acigona*, and *Aargyroploce* are widely distributed throughout southeast Asia, Papua New Guirnea and Indonesia and have caused considerable damage (Sallam & Allsopp 2002; Sallam 2003). Some stemborers are specific pests of sugarcane, whereas others will attack cereal crops such as maize, sorghum or rice. However many species can exploit various gramineous plants for their development (Sallam 2003). Consequently, the incursion of any of these pests into Australia is likely to impact detrimentally on the Australia sugar industry and other cereal growing industries in the country.

Taxonomic difficulties within the C. flavipes complex

As mentioned above, the *Cotesia flavipes* complex has been extremely successful for the biological control of stemborers in several countries. However, two major taxonomic issues have confounded their use in this respect: 1) the absence of clear diagnostic characters to separate the similar species (Polaszek & Walker 1991); and 2) the occurrence of geographic strains that exhibit differences in host range (Potting *et al*. 1997b).

The taxonomic history of the *C. flavipes* complex is somewhat confusing due to the difficulty in distinguishing the three species using external morphology. Debate regarding their status as full species, rather than geographic races has perpetuated for almost a century (e.g.Wilkinson 1928a,b; Watanabe 1932, 1965; Alam *et al.* 1972; Ingram 1983; Polaszek & Walker 1991). Over the years, several studies (Rao & Nagaraja 1967; Nagaraja 1971; Alam *et al.* 1972; Sigwalt & Pointel 1980) have used a range of morphological characteristics to separate the species, however, with the exception of male genitalia, these characters have proven unreliable and, thus, complicated the identification of species for biological control purposes. For example, *C. flavipes* was released for the biological control of *Ch. partellus* in East Africa from 1968-72. The identification of recovered *Cotesia* spp. was based on the coloration of the hind coxae and antennae, and the density of hairs on the antennae (Mohyuddin 1971). Likewise, in Mauritius, assessment of parasitisation by *C. flavipes* and *C. sesamiae* on *Ch. sacchariphagus* and *Sesamia calamistis* Hampson (Noctuidae), respectively, was based on similar morphological characters. These characters are

now known to be subject to intraspecific variation and unreliable for separating *C. flavipes* and *C. sesamiae* (Polaszek & Walker 1991). Furthermore, in South Africa, where *C. flavipes* was released against *Ch. partellus*, recoveries were recorded from both this species and *Busseola fusca* (Fuller) (Noctuidae). However, it is now known that *B. fusca* is not a suitable host for *C. flavipes*, but is suitable for *C. sesamiae* (Mohyuddin 1971; Ngi-Song *et al.* 1995, 1998), which suggests that there may have been misidentifications involved.

Male genitalia is the only characteristic that has proven partly reliable for separating species in the *C. flavipes* complex. Polaszek and Walker (1991) divided the complex into two morphospecies based on male genitalia; the *C. sesamiae*/*C. chilonis* subcomplex and *C. flavipes* (Fig. 1.2). However, no morphological characters have been found to sufficiently separate *C. sesamiae* and *C. chiloinis*. Kimani and Overholt (1995) carried out a series of experiments to determine if the species within the complex were reproductively isolated. Interspecific crosses showed that males exhibited courtship behaviour, copulated with females and transferred sperm, yet the only cross which resulted in viable female progeny involved *C. sesamiae* males x *C. chilonis* females. Investigations on male-female pheromone attraction indicated that *C. flavipes* males were attracted to conspecific females, but not to females of the other two species, suggesting that interspecific mating is unlikely to occur in nature. On the other hand, *C. sesamiae* males responded to pheromones emitted by *C. chilonis* females, indicating that mating between these two could occur in nature. Nonetheless, Kimani and Overholt (1995) argued that that the ability to interbreed can transcend well-defined species boundaries and does not, in itself establish evolutionary units or their components (Cracraft 1989). Therefore, the partial compatibility between the two populations is not sufficient evidence to support the assumption that they are very closely related or in fact the same species.

FIGURE 1.2. Male genitalia can be use to separate the *C. flavipes* complex into two morphospecies; the *C. sesamiae*/*C. chilonis* subcomplex and *C. flavipes* (Polaszek & Walker 1991).

The status of the *C. flavipes* complex has also been investigated using morphometrics, (Kimani-Njogu *et al.* 1997), allozyme electrophoresis and DNA sequence data (Smith & Kambhampati 1999; Michel-Salzat & Whitfield 2004), however the results have been inconclusive. Principal component analysis based on 16 morphometric parameters of the head, thorax, hind leg, and ovipositor separated the complex into three somewhat overlapping groups (Kimani-Njogu *et al.* 1997). Kimani-Njogu *et al.* (1998) proposed that variation in allozymes at 14 loci provided diagnostic characters for separating populations in the complex. However, cladistic analysis of the allozyme data indicated that allopatric populations currently included in *C. flavipes* may be polyphyletic, yet supported the monophyletic status of *C. chilonis* and *C. sesamiae.*

Nucleotide sequence data has been used in two studies to infer the phylogenetic relationships among members of the complex (Smith & Kambhampati 1999; Michel-Salzat & Whitfield 2004). Smith and Kambhampati (1999) used partial sequence data from mitochondrial genes *16S* and *ND1* to compare seven species of *Cotesia* and found the *C. flavipes* complex to be monophyletic. The results supported the morphological and behavioural data previously mentioned, indicating that *C. chilonis* and *C. sesamiae* were more closely related to each other with respect to *C. flavipes*. However, there was only 1% sequence divergence among the three species and they were unable to determine whether *C. chilonis* and *C. sesamiae* were separate species. Conversely, the sequence data from four genes (*16S, 28S, ND1, longwave opsin*),

indicated that *C. sesamiae* is the most basal, with *C. chilonis* and *C. flavipes* as sister species (Michel-Salzat & Whitfield 2004). Nevertheless, the phylogenies in both of these studies were inferred from one allopatric population of each member of the complex. Genetic divergence among conspecific populations of parasitoid wasp species is well documented (Goldson *et al.* 1997; Vaughn & Antolin 1998; Smith & Kambhampati 1999; Liu *et al.* 2000; Baker *et al.* 2003; Vink *et al.* 2003; Hufbauer *et al.* 2004). Therefore, it is possible that studies comparing different geographic populations may result in conflicting phylogenies. The relationships among members of the complex is an area that requires significantly more research. There is a need to validate the specific status of *C. sesamiae* and *C. chilonis* and to identify reliable characters/methods for species identification prior to biological control releases.

Another major issue that has complicated the use of the *C. flavipes* complex in biological control is the possible occurrence of host and/or plant specific strains among different geographic populations. This has been most studied in *C. flavipes*, but has also been shown for *C. sesemiae.* Although these species have been reported from numerous stemborer host species and graminaceous plants, certain populations may have a more restricted host or habitat range. Mohyuddin (1971, 1978) first suggested the possible occurrence of cryptic species and strains adapted to different hosts and host plants because of the discontinuous distribution of *C. flavipes* in southeast Asia. The theory that *C. flavipes* has habitat specific strains has been postulated by several authors (Mohyuddin 1971; Mohyuddin *et al.* 1981; Inayatullah 1983; Mohyuddin 1990; Shami & Mohyuddin 1992). Evidence of this is best shown by the biological control programme involving the introduction of *C. flavipes* into Pakistan in 1962 for release in sugarcane, rice and maize fields. The parasitoid was recorded to have established on *Ch. partellus* in maize (Alam *et al.* 1972), but was rarely recorded from stemborers in sugarcane. This led to the introduction of 'sugarcane-adapted strains' from Thailand, Indonesia and Barbados, which resulted in establishment of the parasitoid in sugarcane (Mohyuddin *et al.* 1981; Mohyuddin 1990; Shami & Mohyuddin 1992). Similarly, Shami and Mohyuddin (1992) found that different populations of *C. flavipes* varied in their preferences for frass produced by hosts feeding on sugarcane versus maize, and that those preferences could be switched through artificial selection in five generations.

However, the existence of habitat-adapted strains was challenged by Potting *et al.* (1997b), when they compared six geographic strains of *C. flavipes* that differed in plant/host complex for plant/host selection behaviour and physiological compatibility on various stemborers. The results indicated that there was no intraspecific variation in plant/host selection behaviour among the different strains, yet there was variation in reproductive success among strains. They argued that the reported existence of *C. flavipes* strains is based not on differences in plant/host selection behaviour, but on differences in physiological compatibility between local parasitoid and host populations. On this basis, they attributed the success of a parasitoid population on its ability to overcome the host's immune system.

The *Cotesia flavipes* complex and their polydnavirus symbionts

Successful development of these endoparasitoids is influenced by the wasp's ability to evade the host's defence system, which is obviously an important issue in biological control systems (Alleyne & Wiedenmann 2001a,b; Hufbauer 2002). One of the main defence mechanisms of insect larvae against endoparasitism is encapsulation (Alleyne & Wiedenmann 2001b). This involves recognition by host hemocytes of foreign particles, subsequently resulting in an increase in the number of circulating haemocytes, and eventually the development of a multicellular capsule that kills the parasitoid (Lackie 1988). However, endoparasitoids are able to evade this immune response of their habitual hosts via several means (Strand & Pech 1995; Pennacchio & Strand 2006). They often rely on active mechanisms to avoid the immune response, such as factors associated with the parasitoid progeny themselves (egg or larval surface features), ovarian proteins, polydnaviruses, venoms and possibly teratocytes (Edson *et al.* 1982; Dahlman 1991; Beckage & Kanost 1993; Webb & Luckhart 1994; Strand & Pech 1995; Lavine & Beckage 1996; Webb & Luckhart 1996; Pennacchio & Strand 2006). In physiologically compatible parasitoid-host populations, the female wasp blocks the host immune reaction, often introducing protective virus-like particles or polydnaviruses (PDVs) into the host hemocoel (Whitfield 2002; Whitfield & Sussan 2003; Web & Strand 2005). PDVs are classified as either bracoviruses or ichnoviruses, when associated with braconid or ichneumonid wasps. Phylogenetic studies have shown that bracovirus-associated wasps form a monophyletic group known as the microgastroid complex (Whitfield 2002; Murphy *et al.* 2008).

Recent studies have shown that members of the *C. flavipes* complex are known to habour different polydnavirus (PDV) variants (Gitau *et al.* 2006, 2007; Dupas *et al*. 2008). For example, in Kenya, *C. flavipes* and *C. sesamiae* carry different PDVs. Moreover, *C. sesamiae* varies in its developmental success on one of its major hosts, *B. fusca*. Two biotypes show variation in virulence that is correlated with average stem borer community composition (Gitau 2006, 2007; Dupas *et al*. 2008). In addition, the geographic distribution of PDV gene *CrV1* alleles in Kenyan *C. sesamiae* is correlated to the relative abundance of the native host, *B. fusca* (Dupas *et al.* 2008). These PDVs are integrated in the wasp genome and play an important role in host immune suppression and, in turn, successful parasitism. Thus, PDVs are considered as key factors in determining parasitoid host range (Whitfield 1994; Cui *et al.* 2000; Pennacchio & Strand 2006). Therefore, variation in PDV symbionts between populations may have potentially important implications for host utilisation and the diagnosis of appropriate strains for biological control against specific host species.

Research Objective

Biological control offers environmentally friendly and sustainable solutions to a variety of insect pest problems. A major obstacle that impedes biological control implementation is insufficient taxonomic information of both natural enemies and target pests (Danks 1988; Schauff & LaSalle 1998). Adequate systematics and accurate identification can prevent delays, wasted resources and failure of programs (Schauff & LaSalle 1998). Thus, the identification among species of the *C. flavipes* complex and the diagnosis of appropriate host strains can have a dramatic effect on the outcome of a biological control program against a specific host species. Prior to any release attempts of a natural enemy, a comprehensive study of its geographical distribution, host specificity, host range and history of introduction is required. Knowledge is also required of the type of association between a pest and candidate natural enemies for introduction. Important aspects to consider are whether a natural enemy has a long history of association with the pest, or whether it is a novel association (see Smith & Wiedenmann 1997; Wiedenmann & Smith 1997).

Although morphological and protein electrophoretic criteria have been employed to recognise strains among the complex, they have met with very little success and have been used to examine less than 20% of the total number of recognised strains worldwide (Kimani-Njogu & Overholt 1997; Kimani-Njogu *et al.* 1997; Kimani-Njogu *et al.* 1998; Smith & Kambhampati 1999). Clearly, there is a need to develop techniques that can accurately identify strains useful for biological control and clarify the relationships among the species within the complex. Molecular markers provide a diagnostic tool that lends itself to answering these types of questions and should assist in understanding the basis for behavioural and morphological differences among populations (Avise 1994).

The principal aims of this project were to determine the genetic variation in the *C. flavipes* complex of parasitoid wasps and their PDV symbionts, and investigate observed variation in respect to geographic origin and host range. Further, it was imperative to provide a phylogenetic framework to delineate the status of *C. flavipes* in Australia, as a crucial first step in the pre-emptive planning associated with future biological control in Australia and other countries. This is essential to facilitate a rapid biological control response when a stemborer incursion occurs. The project can be divided into four separate but closely related parts:

1. To determine the phylogenetic relationships among the species comprising the *C. flavipes* complex (Chapters II and V).

2. To characterise genetic differences among strains of *C. flavipes* on a worldwide basis using molecular markers, and examine host associations (Chapter V).

3. To clarify the status of the *C. flavipes*-like species in Australia (Chapters III, IV and V).

4. To examine polydnavirus haplotype diversity in worldwide populations of the *C. flavipes* complex, and hosts associated with these haplotypes (Chapter VI).

Thesis Outline

To address these aims the thesis comprises a series of stand-alone chapters that were written in a style suitable for publication. Chapters II – III are already published, Chapter IV is *in press*, and Chapters V – VI are being prepared for submission. However, for the purpose of this thesis, some formatting changes have been made to standardise stylistic differences between publishers, and all references have been compiled into a single list.

Genetic variation of the *C. flavipes* complex was first investigated in a pilot study (Chapter II) using 21 ingroup populations and four outgroups. The objectives of this study were to test different mtDNA markers to see whether they could be used to differentiate among worldwide populations, with a particular interest in the Australian species. This paper was published in a special issue of *Annales de la Société Entomologique de France,* 42 (3-4), for papers presented at the International Conference on Lepidopterous Cereal Stem and Cob Borers in Africa (ICLCBA), held 24-28th October 2005 at the International Centre of Insect Physiology and Ecology (ICIPE) in Duduville Nairobi, Kenya.

Chapter III investigated the taxonomy and preliminary biology of the Australian species. The Australian species *C. nonagriae* Olliff stat. rev. was redescribed and formally removed from synonymy with *C. flavipes* based on molecular (Chapter II), morphological (Chapter III) and biological differences (Chapter III – IV). This is now published in *Zootaxa*: Muirhead *et al.* 2008. Life history traits of *C. nonagriae*, such as egg load, potential and realized fecudity and longevity, are examined in detail in Chapter IV. In addition, foraging behavior of *C. nonagirae* involved with microhabitat location and host location were examined for naïve and experience wasp. Life history traits and behavior were compared with those previously recorded for *C. flavipes.* The paper from this work is now in press with the *Australian Journal of Entomology* (manuscript accepted for publication 4 July 2009).

The occurrence of cryptic species and biotypes is a main issue in biological control programs. The phylogenetic relationships and genetic variation of 42 worldwide populations of the *C. flavipes* complex were investigated in Chapter V. Sequence data was generated for mitochondrial gene regions, *16S rRNA* and *COI* and three

anonymous nuclear markers. Host data were mapped onto the phylogeny to determine if there were wasp lineages associated with particular stemborer species/groups.

Local parasitoid adaptation to a complex of hosts may drive natural variation in host-parasitoid virulence. The *Cotesia* PDV *CrV1* gene evolves through natural selection and plays a key role in determining host range by immune suppression during the course of parasitoid development. Variation at the *CrV1* gene was studied in Chapter VI and lineages were investigated for host associations, and the wasp and PDV phylogenies were analysed for coevolution.

CHAPTER II

Mitochondrial DNA phylogeography of the *Cotesia flavipes* complex of parasitoid wasps (Hymenoptera: Braconidae).

STATEMENT OF AUTHORSHIP

This chapter is a published research article (Appendix I):

Muirhead KA, Murphy NP, Sallam MN, Donnellan SC, Austin AD (2006) Mitochondrial DNA phylogeography of the *Cotesia flavipes* complex of parasitoid wasps (Hymenoptera: Braconidae). *Annales de la Société Entomologique de France,* 42 (3–4): 309–318

Muirhead KA (Candidate)

Corresponding author: Collection of species, maintained colony of *Bathytricha truncata* and *Cotesia* sp. from Australia, undertook all DNA sequencing, conducted all analyses, wrote manuscript and produced all figures.

Signed Date 1-12-09

Murphy NP

Supervised the direction of study, assisted with molecular techniques and analyses, evaluated manuscript.

Signed Date 27-11-9

Sallam MN

Sought and won funding, supervised the direction of study, collected overseas taxa and assisted in Australian fieldwork, assisted in the maintenance of colonies, evaluated manuscript.

Signed Date 1-12-09

Donnellan SC

Sought and won funding, supervised the direction of study, evaluated manuscript.

Signed Date 1-12-09

Austin AD

Sought and won funding, supervised the direction of study, evaluated manuscript.

Signed Date 1-12-09

DNA sequence data from this chapter was also used as part of another study (Appendix I):

Assefa Y, Mitchell A, Conlong DE, Muirhead KA (2008). Establishment of *Cotesia flavipes* (Hymenoptera:Braconidae) in sugarcane fields of Ethiopia and origin of founding population. *Journal of Economic Entomology*, 101:686–691.

Assefa1, Y., Mitchell, A., Conlong, D.E. and Muirhead, K.A. (2008) Establishment of *Cotesia flavipes* (Hymenoptera: Braconidae) in Sugarcane Fields of Ethiopia and Origin of Founding Population.
Journal of Economic Entomology, v. 101 (3), pp. 686-691, June 2008

CHAPTER III

The systematics and biology of *Cotesia nonagriae* (Olliff) stat. rev. (Hymenoptera: Braconidae: Microgastrinae), a newly recognised member of the *Cotesia flavipes* species complex

STATEMENT OF AUTHORSHIP

This chapter is a published research article (Appendix I):

Muirhead KA, Sallam MN, Austin AD (2008) The systematics and biology of *Cotesia nonagriae* (Olliff) stat. rev. (Hymenoptera: Braconidae: Microgastrinae), a newly recognized member of the *Cotesia flavipes* species complex. *Zootaxa*, 1846:35–46.

Muirhead KA (Candidate)
Corresponding author: Collection of species, maintained colony of *Bathytricha truncata* and *Cotesia nonagriae* from Australia, undertook all laboratory experimental techniques, conducted all analyses, prepared the taxonomic description, wrote manuscript and produced all figures.

Signed Date 1-12-09

Sallam MN
Sought and won funding, supervised the direction of study, assisted the collection of taxa, assisted in the maintenance of colonies, evaluated manuscript.

Signed Date 1-12-09

Austin AD
Sought and won funding, supervised the direction of study, assisted in preparting the taxonomic description, evaluated manuscript.

Signed Date 1-12-09

Abstract

The Australian species *Cotesia nonagriae* Olliff stat. rev. (Hymenoptera: Braconidae) is redescribed and formally removed from synonymy with *C. flavipes*, based on molecular, morphological and biological differences. The taxonomic history and phylogenetic relationships of *C. nonagriae* with other members of the *C. flavipes* complex are presented and underscore the importance of molecular-based identification within this group. The biology of *C. nonagriae* on the native stem borer host, *Bathytricha truncata* (Lepidoptera: Noctuidae), is compared with previously recorded *C. flavipes* life history traits. The implications of this taxonomic study relative to biological control and importation of stemborer parasitoids into Australia are discussed.

Introduction

The *Cotesia flavipes* complex of parasitioid wasps are natural enemies of lepidopterous stemboring pests associated with sugarcane and cereal crops (Walker 1994). Since these are staple crops in many countries, the complex is economically important worldwide as biological control agents. The complex currently consists of three species, *Cotesia flavipes*, *C. sesamiae* and *C. chilonis,* of uncertain taxonomic validity and relationships. Identifying the various species within the *flavipes* complex has been problematic in the past and has been usefully summarized by Kimani-Njogu and Overholt (1997). The monophyly of the complex is well supported by molecular (Smith & Kambhampati 1999; Michel-Salzat & Whitfield 2004; Muirhead *et al.* 2006) and morphological characters, such as a dorsoventrally compressed mesosoma (Watanabe 1965; Walker 1994). However, the species within the complex are morphologically similar, and many of the characters that have been used to separate species have proven unreliable due to intraspecific variation (Polaszek & Walker 1991; Smith & Kambhampati 1999). As a result, their use in biological control has been confounded by inaccurate identification, as well as the existence of host specific populations (Kimani-Njogu & Overholt 1997).

The species of the *C. flavipes* complex are thought to be endemic to the following areas: *C. flavipes* to the Indo-Australian region; *C. sesamiae* to central and southern

Africa; and *C. chilonis* to eastern Asia, including Japan (Polaszek & Walker 1991; Kimani-Njogu & Overholt 1997). However, all three species have been utilised for classical biological control of stemboring pests, resulting in their much broader inter-continental distribution (Polaszek & Walker 1991). In some cases, a species of the complex has been introduced into an area indigenous to one of the other two species (Smith & Kambhampati 1999). For example, *C. flavipes* has been introduced several times into various countries of Africa (Overholt *et al.* 1994) and is now established in several parts of sub-Saharan Africa (Omwega *et al.* 1995; Overholt *et al.* 1997) where it co-exists with the native *C. sesamiae*. Although *C. flavipes* and *C. sesamiae* can occupy a similar ecological niche, it has been shown that they prefer different host species and are not likely to compete (Rajabalee & Govendasamy 1988; Sallam *et al.* 2001; Sallam *et al.* 2002). Similarly, certain populations of the same species within the complex have differences in host range (Mohyuddin 1971; Shami & Mohyuddin 1992; Zhang & Hewitt 1996; Potting *et al.* 1997b; Ngi-Song *et al.* 1998), an indication of genetic divergence among strains (Muirhead *et al.* 2006) and the possible existence of cryptic species.

There has been ongoing confusion regarding the status and presence of *C. flavipes* in Australia, and this has the potential to impact the future importation of biological control agents. Over 80 years ago, the Australian native species *Apanteles nonagriae* Olliff, 1893 was synonymized with *A. flavipes* (Cameron, 1891) (Wilkinson 1929; Austin & Dangerfield 1992), thus indicating the presence of *C. flavipes* in Australia. However, recent molecular work suggests that the Australian populations represent a 'cryptic' species different from *C. flavipes* and other members of the species group (Muirhead *et al.* 2006). Records of *A. nonagriae* in Australia extend back to its original description when it was first recorded as a parasitoid of the native noctuid stemborer *Nonagria exitiosa* Olliff (= *Bathytricha truncata* (Walker)) in sugarcane in the Richmond and Clarence River Districts of north-eastern New South Wales (Olliff 1893). It was subsequently reared from *Phragmatiphila truncata* Walker (= *Bathytricha truncata*) in sugarcane at South Mulgrave, south of Cairns, Queensland (Jarvis 1927). The same report also indicated that the parasitoid had been previously recorded parasitising 50% of *B. truncata* larvae infesting rice in New South Wales. *Bathytricha truncata* is a stemborer recorded from sugarcane, rice, maize and a range of other plants (Sallam 2003). It has a distribution from Cairns to South Australia and

Tasmania (Common 1990) and is considered a minor pest that rarely causes substantial damage (Jones 1966). Bell (1934) recorded *Apanteles nonagriae* on *B. truncata* larvae at Mackay, Queensland. Similarly, Li (1970) recorded "*A. flavipes* (*A. nonagriae*)" from *Ch. suppressalis* (Walker) and *Ch. polychrysa* (Meyrick) (Pyralidae) in rice fields in the Northern Territory, but no voucher material was deposited in any collection to confirm this finding.

Apanteles nonagriae was originally described by Olliff (1893) along with *Tetrastichus howardi* (Olliff), a eulophid pupal parasitoid reared from *B. truncata* (Boucék 1988). In his study of Indo-Australian *Apanteles* s.l., Wilkinson (1928a, b) noted the strong similarity between *A. nonagriae* Olliff and *A. flavipes* but did not synonymise them until the following year (Wilkinson 1929). However, he did synonymise a second species of the same name, *A. nonagriae* Viereck, 1913, with *A. flavipes* that had been reared from *Sesamia* (*Nonagria*) *inferens* Walker from Taiwan (Wilkinson 1928a). Unfortunately, Olliff (1893) did not designate any type specimens in the original description of *C. nonagriae* and did not refer to any depository that might hold syntypes.

Based on morphological examination and biological data, supplemented by the previous molecular study (Fig. 3.1) (Muirhead *et al.* 2006), we formally recognise *Cotesia nonagriae* stat. rev. as a distinct species. In so doing, we redescribe the species and discuss its taxonomic history, relationships within the *flavipes* complex, its biology compared to *C. flavipes* and the implications of this taxonomic study to future biological control programs and importation of stemborer parasitoids into Australia.

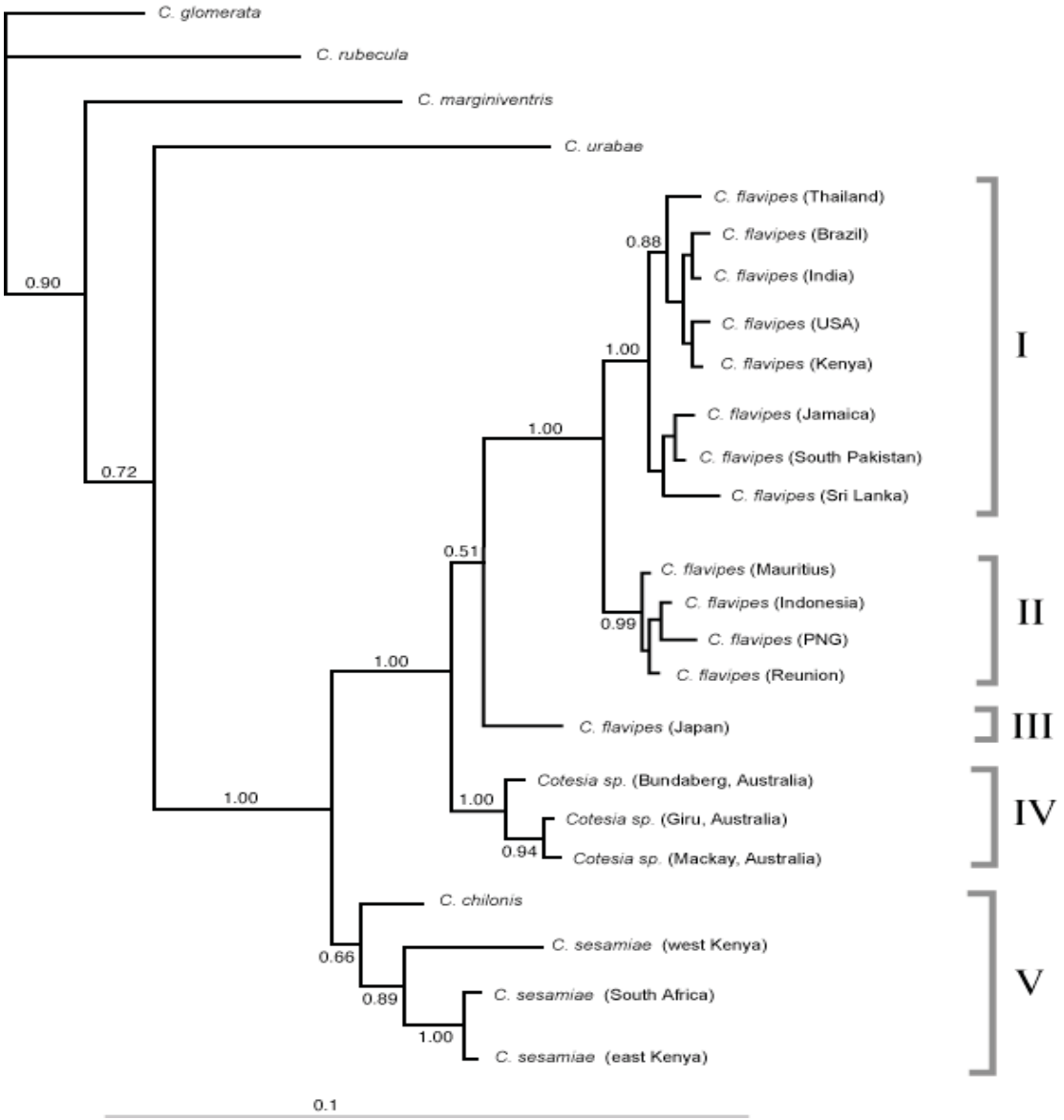

FIGURE 3.1. Bayesian tree derived from partial *16S rRNA* and *COI* mtDNA nucleotide sequence data from geographic populations of the *Cotesia flavipes* complex (Clades I-V) and four outgroups. Australian *Cotesia nonagriae* populations are shown in clade IV. The numbers represent Bayesian posterior probabilities $\geq$ 50% (from Muirhead *et al*. 2006).

Materials and methods

Taxonomy

Specimens of *C. nonagriae* used in this study were reared from *B. truncata* collected from three sugarcane-growing localities in Queensland, while material of *C. flavipes* (India, Thailand, Japan, Papua New Guinea, Kenya, Maritius), *C. sesamiae* (west Kenya, east Kenya, Tanzania) and *C. chilonis* (China, Japan) was accessed from the voucher material from Muirhead *et al.* (2006) deposited in the Waite Insect and Nematode Collection, Adelaide. Morphological terminology follow Sharkey and

Wharton (1997) for body structures and venation, Eady (1968) and Harris (1979) for sculpturing, and Kimani-Njogu & Overholt (1997) for male genitalia. Specimens were imaged using a Philips XL30 FEGSEM electron microscope at the Adelaide Microscopy and Microanalysis Research Facility, The University of Adelaide. Male genitalia were dissected from the metasoma of several specimens and mounted on carbon conductive adhesive tabs after overnight digestion in 140 µl of lysis buffer and 7 µl of proteinase K (20 mg/ml) at 55°C.

Abbreviations for collections in the text are: AM, Australian Museum, Sydney; ANIC, Australian National Insect Collection, Canberra; ASCT, Agricultural Scientific Collections Trust, Orange Agricultural Institute, Orange; QDPI, Queensland Department of Primary Industries, Brisbane; and WINC, Waite Insect and Nematode Collection, Adelaide.

Biology

Insect colonies. We maintained two colonies of *C. nonagriae* originating from field parasitised larvae of *B. truncata* infesting sugarcane in Mackay and Bundaberg, Queensland. Parasitoids were maintained on laboratory reared fourth instar *B. truncata* larvae in a temperature controlled room at 25°C, 60–70% RH under a 12L:12D photoperiod. Mated females were offered one host larva with some fresh larval frass to stimulate oviposition. Wasp cocoons were collected from host larvae and transferred to emergence cages where they were provided honey as a food source. Field collected *B. truncata* were reared to the pupal stage within cut sugarcane stems, whereas subsequent lab generations were maintained on an artificial diet adopted from Onyango and Ochieng-Odero (1994), replacing maize leaf powder with sugarcane leaf powder. See Songa *et al.* (2001) and Macqueen (1969) for more details on stemborer rearing procedures and the life history of *B. truncata*. Adult moths were kept in oviposition cages with waxed paper tubes to provide suitable oviposition sites. Egg masses were cut from the paper daily and transferred to a closed Petri dish containing moist cotton wool to maintain high humidity. Egg masses at the blackhead stage were transferred to 2.55 mm diameter containers with artificial diet. Larvae used in the experiments were removed from the artificial diet as fourth instars and fed 5 cm cuts of sugarcane stems.

Life history traits. To study the life history of *C. nonagriae* on the native host *B. truncata*, we employed the procedure used by Sallam *et al*. (2002) on *C. flavipes* and *C. sesamiae*. Thirty fourth instar host larvae were parasitised by newly emerged, mated female parasitoids. Adult female parasitoids were kept in individual vials and exposed to one host larva each for oviposition. Parasitised larvae were kept in vials containing cut sugarcane stems until the mature parasitoid progeny emerged and pupated. Ten parasitised hosts were dissected one to two days after oviposition to determine the number of parasitoid progeny allocated to each host. Cocoon masses from the remaining 20 larvae were counted, weighed and placed in vials. Duration of the parasitoid's immature stages, percent emergence, number of adult progeny, adult longevity and sex ratio were recorded. Three females from each progeny (n=60) were chosen randomly and dissected to count the number of eggs contained in the ovaries.

Descriptive taxonomy

***Cotesia nonagriae* (Olliff) stat. rev.**

(Fig. 3.2-3.4)

Apanteles nonagriae Olliff, 1893: 376 [original description]; Wilkinson (1928b): 136 [type data, biology, taxonomic status].

Apanteles flavipes (Cameron, 1891); Wilkinson (1928a): 93 [synonymy of *A. nonagriae* Viereck]; Wilkinson (1929): 108 [synonymy *A. nonagriae* Olliff]; Shenefelt (1972): 509 [complete taxonomic bibliography].

Cotesia flavipes Cameron, 1891: 185 [original description]; Mason (1981): 113 [resurrected the genus with *C. flavipes* as type]; Austin and Dangerfield (1992): 21 [status and hosts for Australia].

Material examined. Queensland: 21F 3M Bundaberg, 12-30.xi.2004, K. Muirhead (10F 1M ANIC, 11F 2M WINC); 16F 2M Mackay, 12-30.xi.2004, K. Muirhead (6F 1M ANIC, 10F 1M WINC); 14F 3M Giru [via Townsville], 5.x.2003, M. Sallam (9F 1M QDPI, 5F 2M WINC): 3F 1M '*parasite larva sugar-cane moth*' '*Apanteles nonagriae Olliff'*, no date or locality (1F AM, 2F 1M ASCT).

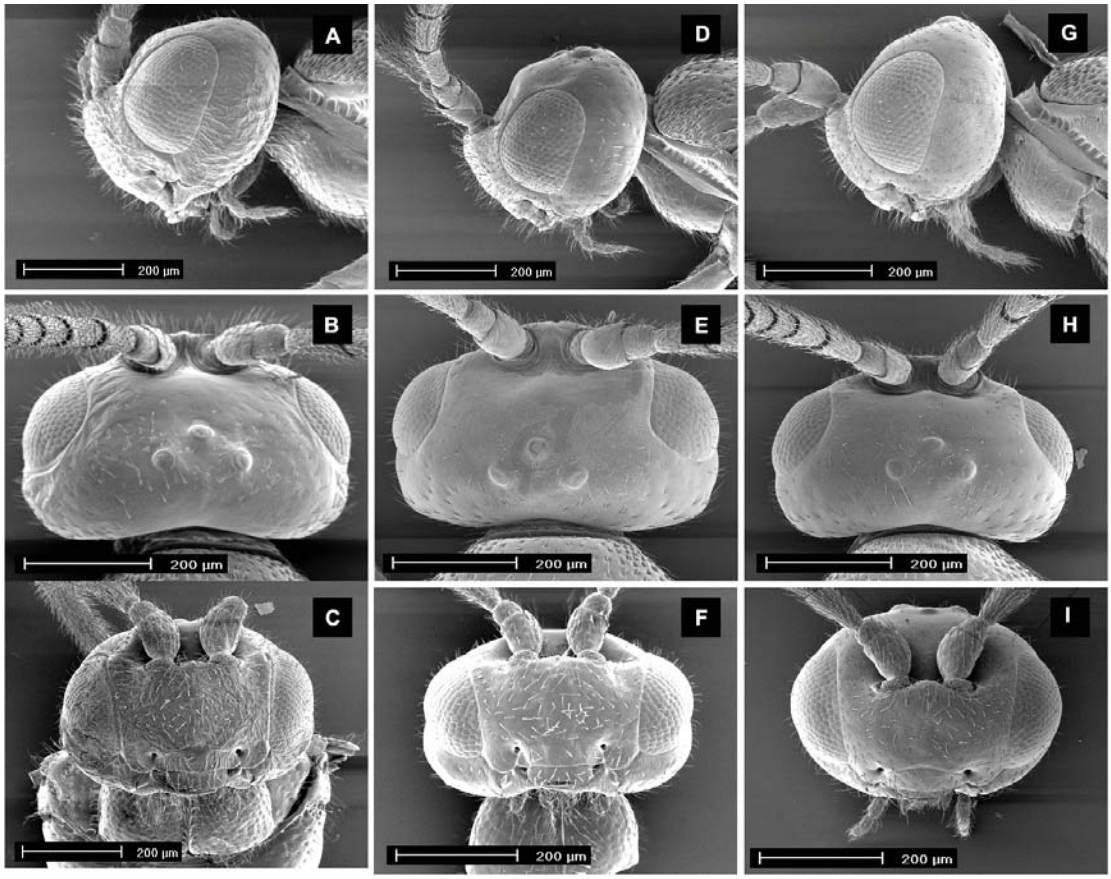

FIGURE 3.2. A–C: *Cotesia nonagriae* (Bundaberg, Australia), head. (A) lateral view, (B) dorsal view, (C) anterior view. D–F: *C. nonagriae* (Mackay, Australia), head. (D) lateral view, (E) dorsal view, (F) anterior view. G–I: *C. flavipes* (India), head. (G) lateral view, (H) dorsal view, (I) anterior view.

Female

Length. Body 2.1-2.4 mm

Colour. Body black, metasomal sterna including hypopygium dark brown to brown, antenna dark brown with scape lighter, palps yellow; legs yellow brown with tarsus slightly darker, mesocoxa pale brown, metacoxa dark brown to black basally grading to brown apically; forewing stigma brown, venation slightly lighter.

Head. In anterior view oval in shape, substantially wider than high, eyes slightly converging ventrally, face slightly rugulose-punctate to punctuate; in lateral view oval (globular) in shape, only slightly higher than long, gena and temples rugulose-punctate to punctuate, slightly more striate along posterior eye margin; in dorsal view vertex and occiput moderately smooth except for scattered fine punctures associated with sparse short setae, frons usually smooth but sometimes with faint striations along eye margin.

Mesosoma. Strongly flattened dorsoventrally so that posterior two-thirds of scutum, scutellum, anterior part of propodeum and ventral margin of mesopleuron horizontal

and parallel; in dorsal view scutum punctate anteriorly, mostly smooth posteriorly and along midline, notauli indicated by posterior extension of anterior punctuate area and smooth areas on either side but disappearing before reaching posterior margin; medial scutellum smooth with sparse setae, posterior margin broad; propodeum coarsely rugose-punctate, often with indistinct carina around spiracle and oblique lateral carina converging posteriorly; in lateral view mesopleuron smooth, sternaulus faintly indicated along dorsal margin by sparse punctures; metapleuron rugose-punctate in posterior part, smooth in anterior part; dorsal and outer surfaces of hind coxa punctuate; forewing veins r and 2RS usually meeting at distinct angle, sometimes with small stub of 3RS present; 2M 0.5 to almost 1.0X as
long as 2RS.

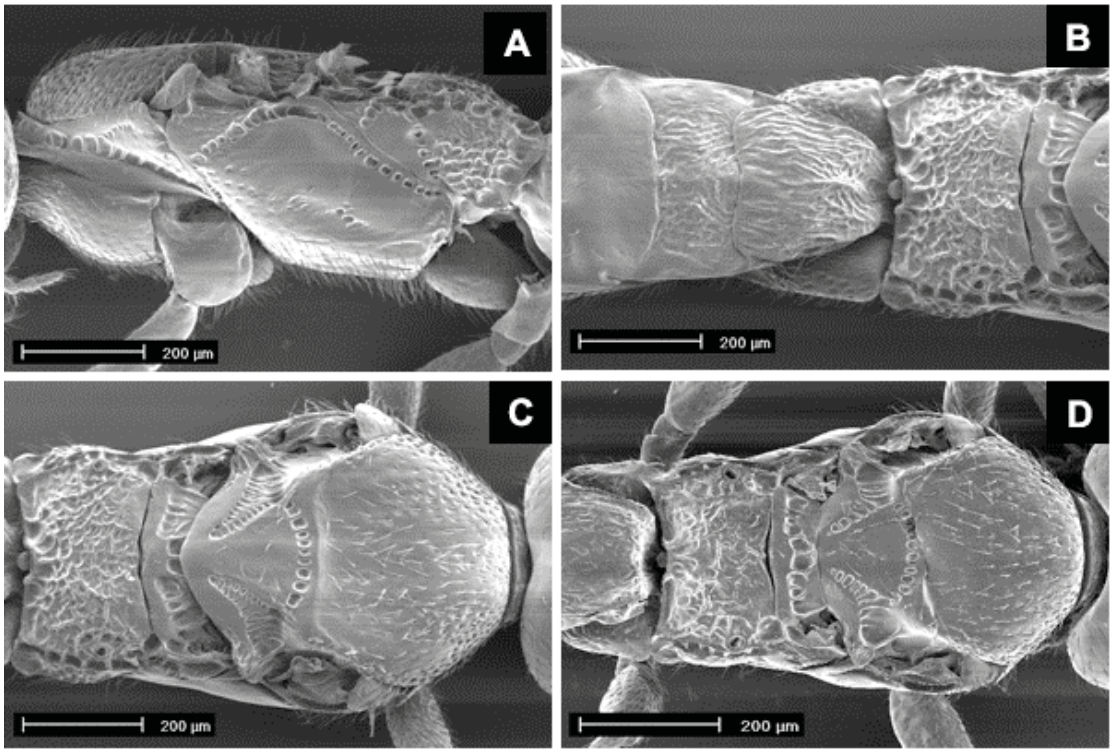

FIGURE 3.3. A–C: *Cotesia nonagriae* (Bundaberg, Australia): (A) mesosoma, lateral view, (B) posterior mesosoma and terga 1–3, dorsal view, (C) mesosoma, dorsal view. D: *C. flavipes* (India), mesosoma, dorsal view.

Metasoma. Tergum 1 almost as wide at posterior margin as long, lateral margins strongly diverging posteriorly; longitudinally striate-rugulose, often with an incomplete medial longitudinal carina; tergum 2 longitudinally striate-rugulose with smoother longitudinal area medially and at sides; remaining terga smooth with sparse longish hairs.

Male

As for female except: antennae slightly longer and lighter in colour; punctuate sculpturing on scutum, particularly in anterior part, slightly denser; genitalia very similar to *C. flavipes*; aedeagal-volsella shaft elongate; volsella more than 4.0 x as long as wide, digital (apical) teeth minute; aedeagus barely protruding past apex of parameres and volsella.

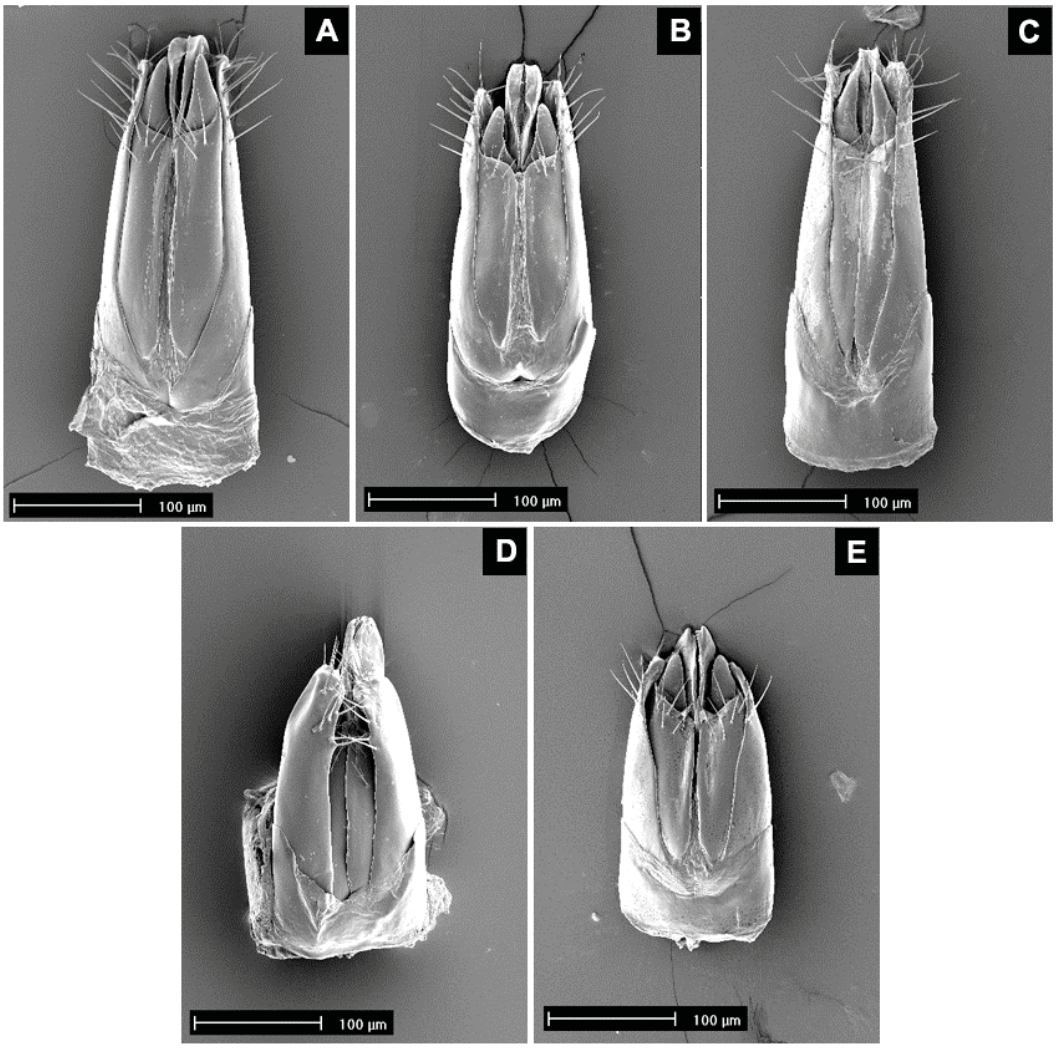

FIGURE 3.4. A–E: Male genitalia of *Cotesia flavipes* complex species. (A) *C. nonagriae* (Australia), (B) *C. flavipes* (India), (C) *C. flavipes* (Japan), (D) *C. sesamiae* (Kenya), (E) *C. chilonis* (China).

Comments

The description above is largely based on specimens from Bundaberg, while for material from Mackay and Giru the degree of sculpturing on the face and gena is less pronounced and the frons and temples are completely smooth. These populations also have the propodeum less coarsely sculptured and rugulose rather than rugulose punctuate, and tergite 1 lacking a medial longitudinal carina. As such they are more similar to *C. flavipes.* The specimens in AM and ASCT have identical labels and are clearly very old. We initially considered that they were part of Olliff's original material and therefore a likely syntype series. This was based on the age of the material and that there are several lectotypes of Olliff species in the AM, including that of *T. howardi* (designated by Boucék 1988) which was described by Olliff in the same paper as *C. nonagriae*. However, comparison of the labels on Olliff specimens in the AM shows that the handwriting is different to the AM and ASCT specimens, and so they cannot be directly associated with that used in the original description of *C. nonagriae*. The specimens in AM and ASCT have the face and gena smooth and are therefore more similar to the recently collected material from Mackay and Giru.

Results and Discussion

Species recognition

Based on the mitochondrial gene phylogeny of Muirhead *et al.* (2006), there are clearly two pairs of sister species within the *flavipes* complex: *C. sesamiae/C. chilonis* and *C. flavipes/C. nonagriae.* Although there are a number of morphological differences that distinguish these two pairs of species (ie., form of the scutoscutellar sulcus and propodeal sculpturing), they also display relatively high levels of intraspecific variation making it difficult to interpret these characters. Without doubt, the definitive difference between these species pairs is the structure of the male genitalia. In *C. sesamiae/C. chilonis* the major elements of the genitalia are relatively short and broad, while in *C. flavipes/C. nonagriae* they are more elongate (Kimani-Njogu & Overholt 1997; Fig. 3.4). Distinguishing between *C. nonagriae* and *C. flavipes* is more difficult if geographic location is not taken into account. The more sculptured head of *C. nonagriae* and to a lesser degree the courser sculpturing on the propodeum will distinguish most populations. However, levels of intraspecific variation that occur in both species will at times render identification difficult. Because of this, and until *C. flavipes* can be shown definitively not to occur in

Australia, we advocate the use of molecular diagnostic techniques using the phylogenetic framework generated by Muirhead *et al.* (2006) in cases where accurate identification is critical.

Biology

Life history traits for *C. nonagriae* assessed as part of this study are summarised in Table 3.1 and are compared with published data for *C. flavipes*. The potential fecundity of *C. nonagriae* females was similar to *C. flavipes* with an initial egg load of ~ 200 eggs. However, *C. nonagriae* females allocated an average of 111.6 (SD ± 25.32) eggs into each host, whereas *C. flavipes* is known to allocate a maximum of 30-40 eggs into at least two different hosts (Sallam *et al.* 2002). This high egg allocation suggests that *C. nonagriae* females will deplete their egg load after just two oviposition events, while *C. flavipes* females are depleted of eggs after they have parasitised four to five hosts (Potting *et al.* 1997c). In spite of the higher number of *C. nonagriae* progeny emerging from *B. truncata,* cocoon weight was not very different from that produced by *C. flavipes* parasitizing *Sesamia calamistis* in Africa (based on studies by Sallam *et al.* 2002). The total life cycle of *C. flavipes* is about 20 days, but is longer for *C. nonagriae* at 24 days. This is due to a longer duration of the larval stages (17 versus 21 days), which may also be influenced by the higher number of larvae competing for food. After 21 days *C. nonagriae* larvae emerged from the host and formed small white silken cocoons, which were usually surrounding the host cadaver within its tunnel. *Cotesia nonagriae*, like *C. flavipes* adults, generally lives for 1-3 days without food, however *C. flavipes* can live up to six days when provided honey (Potting *et al.* 1997c).

TABLE 3.1. Number of cocoons, adult progeny, cocoon weight duration of immature stages, sex ratio, adult longevity, emergence rate and potential fecundity (mean ± SD) of *C. nonagriae* on the native stemborer host *Bathytricha truncata* compared with the same biological traits for *C. flavipes* on *S. calamistis* (from Sallam *et al.* 2002).

Species	Number of cocoons/host	Cocoon Weight (mg)	Adult progeny/host	Duration of immature stages (days)	Sex ratio (% female/ total progeny)	Adult longevity (days)	% Emergence	Potential fecundity(egg load)
C. nonagriae	99.28 (21.8)	0.101 (0.023)	91.56 (20.9)	21.07 (1.2)	52.1 (5.8)	2.92 (0.35)	91.97 (4.92)	196.56 (12.2)
C. flavipes (Sallam *et al.* 2002)	34.3 (17.2)	0.106 (0.011)	32.0 (17.6)	17.2 (3.0)	53.0 (0.26)	3.6 (0.7)	92.9 (8.9)	203.6 (8.7)

Relevance to biological control

Accurate identification of both natural enemies and pest species is vital for research, quarantine and successful biological control (Clausen 1942; Debach 1960; Compere 1969; Danks 1988; Debach & Rosen 1991; Schauff & LaSalle 1998; Beard 1999). However, biocontrol programs are often confounded by intraspecific variation within complex taxonomic groups. Overlapping intraspecific variation in hymenopteran parasitoids is well documented and has been reported for ecological, behavioural and physiological traits, such as climatic adaptability, diapause, host selection and virulence (Hopper *et al.* 1993; Unruh & Messing 1993). Ruberson *et al.* (1989) alone listed over 65 studies that deal with intraspecific variation in hymenopteran parasitoids, predominantly revealed through biological control introductions. Species that are seemingly widespread and abundant in reality can represent several cryptic species. This may well be the case for the *C. flavipes* complex, where numerous authors have recorded geographic variation among *C. flavipes* populations in ecology, host-searching behaviour and host-parasitoid compatibility (Mohyuddin 1971; Mohyuddin *et al.* 1981; Inayatullah 1983; Polaszek & Walker 1991; Ngi-Song *et al.* 1995; Potting et al. 1997b; Ngi-Song et al. 1998; Mochiah *et al.* 2001). The ability to discriminate between genotypes on different hosts is crucial for biological control. Moreover, from an evolutionary perspective, it is important to identify the forces that structure genetic differences among parasitoid populations relative to their host insects (Vaughn & Antolin 1998; Heraty 2004)

Whereas this study underscores the need for molecular diagnostic techniques (e.g. Dupas *et al.* 2006; Muirhead *et al.* 2006) for reliable identification of cryptic species or closely related sister species, it also emphasizes the need for detailed comparative morphology and supplemental biological data to support critical taxonomic decisions. Templeton's Cohesive Species Concept stresses the importance of establishing species boundaries by examining phylogenetically distinct entities for reproductive incompatibility or ecological, behavioural, or morphological differences (Templeton 1989). The mtDNA sequence data of Muirhead *et al.* (2006) provided the first evidence for the monophyly of the Australia populations and, likewise, our results support the conclusion that *C. nonagriae* is a distinct species based on additional morphological and biological traits.

Although there are subtle morphological difference between *C. nonagriae* and *C. flavipes*, and the other members of the complex, it is not surprising that earlier authors confused these species, given their close similarity and intrinsic viability (Wilkinson 1928a; Watanabe 1932; 1965; Alam *et al.* 1972; Ingram 1983; Polaszek & Walker 1991). Male genitalia is certainly the most reliable character and clearly separates two morphospecies groups, *C. sesamiae*/*C. chilonis* and *C. flavipes/C. nonagriae* (Polaszek & Walker 1991) (Fig 3.4). Despite biological variation between *C. nonagriae* and *C. flavipes*, there is limited phenotypic diversity. Their similarity probably reflects not only recent common ancestry but also stabilising selection arising from ecological selection, while diversification within the complex is probably linked to biogeographic barriers and host use.

Independent of the conclusion that *C. nonagriae* is a distinct species associated with the native sugarcane pest *B. truncata*, we were unable to discern whether or not *C. flavipes* also occurs in Australia. Previous researchers reporting the occurrence of *C. flavipes* (= *A. flavipes*) over the last century have failed to lodge voucher material in recognized insect collections (e.g. Jarvis 1927; Macqueen 1969; Li 1970) and thus, no reliable material is available to verify the identity of *C. flavipes* referred to in the literature. In several cases these are very likely to be *C. nonagriae* when associated with *B. truncata* (e.g. Bell 1934). However, reference to *C. flavipes* associated with *Ch. suppressalis* and *Ch. polychrysa* in rice (Li 1970) is more problematic, given that

verified *C. flavipes* have been reared from these hosts in southeast Asia (Kajita & Drake 1969; Hattori & Siwi 1986; Khoo 1986; van Verden & Ahmadzabidi 1986). We were unable to access populations of either *Chilo* spp. in Australia to rear parasitoids for comparison. Thus, the question of whether *C. flavipes* occurs in Australia still needs to be addressed. This is crucial for future biological control projects in Australia because, if *C. flavipes* is not native, it will need to undergo pre- and post-release studies in order to assess its interaction with *C. nonagriae* and impact on non-target species (Howarth 1991; Messing 1992; Samways 1997; Sands 1997; Henneman & Memmott 2001)

Perhaps a more central issue for potential stem borer pest incursions into Australia is the host range of *C. nonagriae* and whether it will successfully parasitise host species not encountered during its evolutionary history. Interestingly, this was the case for *C. flavipes*, which formed a novel association with *Diatraea saccharalis* (F.) when introduced into the New World for biological control purposes (Simmonds 1969) Polaszek & Walker 1991). Thus, future work could profitably be directed towards the testing of *C. nonagriae* on high threat stem borer species from Indonesia and Papua New Guinea.

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CHAPTER IV

Life history traits and foraging behaviour of *Cotesia nonagriae* (Olliff) (Hymenoptera: Braconidae), a newly recognised member of the *Cotesia flavipes* complex of stemborer parasitoids.

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Muirhead KA (Candidate)
Corresponding author: Collection of species, maintained colony of *Bathytricha truncata* and *Cotesia nonagriae*, undertook all laboratory experimental techniques, conducted all analyses, wrote manuscript and produced all figures.

Signed Date 1-12-09

Sallam MN
Sought and won funding, supervised the direction of study, assisted the collection of taxa, assisted in the maintenance of colonies, evaluated manuscript.

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Austin AD
Sought and won funding, supervised the direction of study, evaluated manuscript.

Signed Date 1-12-09

Abstract

Cotesia nonagriae from Australia, a parasitoid of the incidental native pest of sugarcane, *Bathytricha truncata*, was previously thought to be a synonym of *Cotesia flavipes*. However, recent studies using DNA sequences, morphology and preliminary biological information show that this parasitoid is clearly a different species than *C. flavipes* and other members of the species complex. Here we further examine differences in the biology of these species by undertaking a detailed study of the life history traits of *C. nonagriae*, including adult longevity and the potential and realised fecundity of females. In addition, the influence of learning on microhabitat location and foraging behaviour were investigated. Duration of the larval stages and adult longevity of *C. nonagriae* were longer than previously recorded for other members of the species complex. The potential fecundity of females was similar to *C. flavipes* (~200 eggs), however *C. nonagriae* oviposited an average of over 100 eggs into each host, almost three times more than for other species in the *C. flavipes* complex (30-40). The propensity of *C. nonagriae* to allocate a large number of eggs to each host may be an evolutionary strategy due to the high mortality rate (50-57%) of ovipositing adult wasps. During microhabitat location, both naïve and experienced females demonstrated a strong response towards the plant host complex, with experienced wasps benefiting by having a more rapid response time to infested than noninfested plants.

Introduction

Lepidopterous stemborers are major pests of sugarcane and cereal crops worldwide (Polaszek & Walker 1991; Overholt *et al.* 1997). Each year crop yields are diminished by more than 50 species of stemborers belonging to the families Pyralidae, Crambidae and Noctuidae (see Smith *et al*. 1993). Although these pests naturally feed on a range of grasses, sedges and cat-tails, the development of subsistence farming and large-scale monocultures has provided extensive areas of suitable host plants, that allow for substantial population increases. Stemborers now attack an array of cash crops including maize (*Zea mays* L.), rice (*Oryza sativa* L.), sorghum (*Sorghum* spp.), millet (*Pennisetum* spp.) and sugarcane (*Saccharum* spp.) (Smith & Wiedenmann 1997). Although stemborers are a taxonomically diverse group, they exhibit similar

life history patterns (Smith *et al*. 1993) that render them highly successful pests. They feed internally on their host plants and have concealed larval and pupal stages. Thus, they are both physically protected by surrounding plant tissues and are generally not easily accessible to predators and parasitoids (Hawkins 1993).

Members of the *Cotesia flavipes* species complex are specialist parasitoids that have been extremely successful for the biological control of stemborers worldwide. These gregarious endoparasitoids have developed behavioural and morphological characteristics that allow them to utilise hosts with concealed lifestyles. This success relies heavily on their ability to use a variety of chemical and physical cues during habitat and host location (Mohyuddin *et al*. 1981; Shami & Mohyuddin 1992; Wiedenmann *et al.* 1992; Potting *et al*. 1995; Ngi-Song *et al.* 1996; Ngi-Song & Overholt 1997; Nwanze & Nwilene 1998; Rutledge & Wiedenmann 1999; Jembere *et al.* 2003; Gohole *et al.* 2005; Obonyo *et al.* 2008), as well as their ability to access hosts within stemborer tunnels. The *C. flavipes* complex has comprised three species, (*C. flavipes*, *C. sesamiae* and *C. chilonis*), which are polyphagous and recorded from many gramineous stemboring species, particularly species of *Chilo*, *Sesamia* and *Diatraea* (see Shenefelt 1972). However, variability in host compatibility and reproductive success among the species has been recorded (Potting *et al*. 1997b; Wiedenmann & Smith 1997; Mochiah 2001; Ngi-Song *et al.* 1995,1998; Alleyne & Wiedenmann 2001a; Chinwada *et al.* 2003; Gitau *et al.* 2006, 2007; Dupas *et al.* 2008).

In Australia, there has been ongoing confusion regarding the status and presence of *C. flavipes* on the continent. Over 80 years ago, the Australian native species *Apanteles nonagriae* was synonymised with *A. flavipes* (Wilkinson 1929; Austin & Dangerfield 1992), thus indicating the presence of *C. flavipes* in Australia. Recently, however, the Australian populations were formally removed from synonymy with *C. flavipes* and recognised as a distinct species, based on molecular, morphological and preliminary biological studies (Muirhead *et al*. 2006, 2008). Major stemborer pest species are not present in Australia (Allsopp *et al*. 2000) and there is only one recorded host, *Bathytricha truncata*, which is a sporadic native pest attacked by *C. nonagriae* (Sallam & Allsopp 2002; Muirhead *et al*. 2008). However, a number of serious pest species inhabit neighbouring countries to the north of Australia and Indian Ocean

islands and, thus, the potential for incursion is considered high by the Australian sugar industry (Sallam 2003). Pest species in the genera *Chilo*, *Sesamia*, *Scirpophaga*, *Maliarpha*, *Acigona* and *Argyroploce* are widely distributed throughout southeast Asia, Papua New Guinea and Indonesia and cause considerable damage (Sallam 2003). Given that many stemborers are polyphagous on gramineous plants (Sallam 2003), the incursion of any of these pests into Australia is likely to have a significant impact on the Australia sugar industry and other cereal growing enterprises.

Cotesia nonagriae is therefore considered a potentially important control agent and yet the biology of this species remains virtually unknown. The success of biological control programs depends on accurate identification and knowledge of the biology of both natural enemies and target pests (Debach & Rosen 1991; Overholt 1998). Therefore, the purpose of this study was to investigate the biology and behaviour of *C. nonagriae* in regard to its ability to suppress outbreaks of *B. truncata*, and as a precursor to its assessment as a potential control agent for more serious pests if they enter Australia. In this study, life history traits of *C. nonagriae*, including adult longevity and the realised fecundity of females were investigated. In addition, we used a wind tunnel arena to examine the influence of learning on microhabitat location and parasitoid foraging behavior in *C. nonagriae*. The results are compared with previous studies on *C. flavipes* and other members of the species complex, and the potential of *C. nonagriae* as a biological control agent is discussed.

Materials and methods

Insect colonies

Two colonies of *C. nonagriae* originating from Mackay and Bundaberg, Queensland were established at the University of Adelaide from field-collected individuals of *B. truncata* infesting sugarcane. *Bathytricha truncata* individuals were raised to the pupal stage within cut sugarcane stems (Fig. 4.1), whereas subsequent laboratory generations were maintained on an artificial diet adopted from Onyango and Ochieng-Odera (1994), replacing maize leaf powder with sugarcane leaf powder and generally following the procedures outlined by Songa *et al.* (2001) and MacQueen (1969). Adult moths were kept in oviposition cages with waxed paper tubes to provide suitable oviposition sites. Egg masses were cut from the paper daily and transferred to a closed Petri dish containing moist cotton wool to maintain high humidity. Egg

masses at the 'blackhead' stage were transferred to containers with artificial diet. Larvae used in experiments were removed from the artificial diet as fourth instars and fed on sugarcane stems for approximately three days.

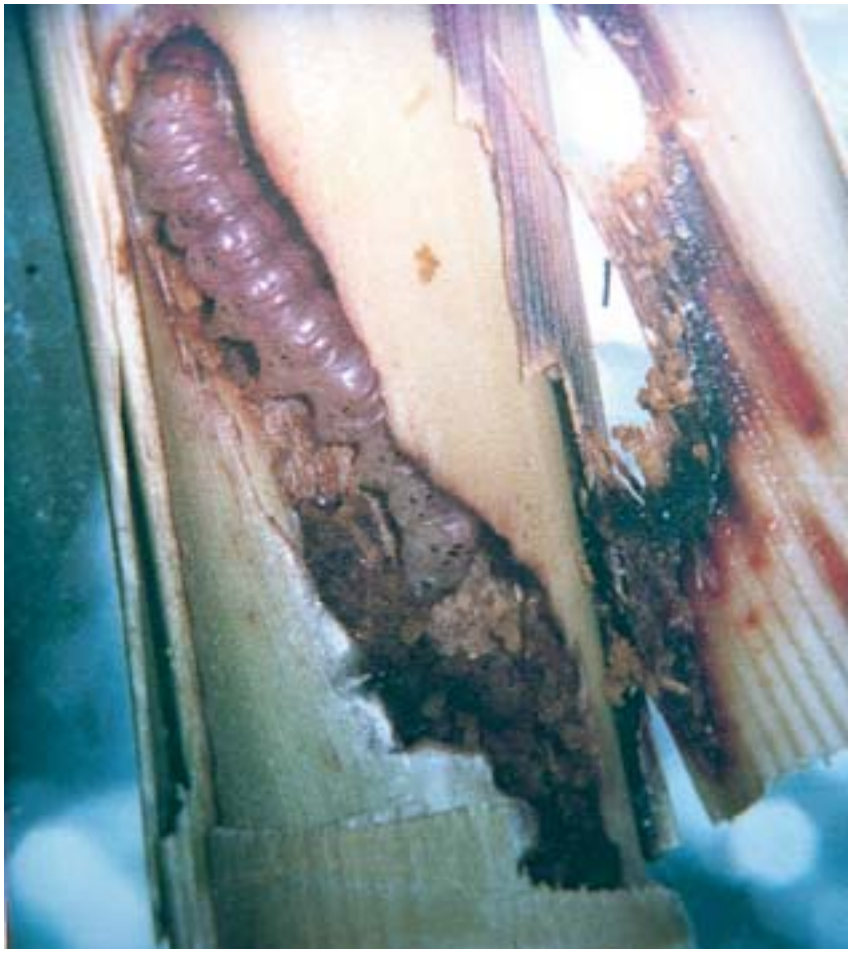

FIGURE 4.1. *Bathytricha truncata* late stage larva feeding inside sugarcane. BSES Limited.

Cotesia nonagriae colonies were maintained on laboratory reared fourth instar *B. truncata* larvae in a temperature controlled room at 25^{o}C, 60-70% RH under a 12L:12D photoperiod. Mated females were offered one host larva with some fresh larval frass to stimulate oviposition. Cocoons of *C. nonagriae* were collected from host larvae and transferred to 25 x 25 x 25 cm emergence cages, where emerging adult wasps were provided with water and honey as a food source. Wasp progeny were separated into vials with small holes in the lid to provide access to other progenies so mating behaviour could be observed.

Life history traits

To examine the life history traits of *C. nonagriae* on the native host *B. truncata* we employed the procedure used by Sallam *et al*. (2002) with *C. flavipes* and *C. sesamiae*; 30 fourth instar host larvae were parasitised by newly emerged, mated female wasps. Adult females were kept in individual vials and exposed to one host larva for oviposition. The host larva was removed immediately after the first oviposition. If the wasp had not oviposited after 5 min the host larva was removed and not used in the experiment. Parasitised larvae were kept in separate vials containing cut sugarcane stems until the mature parasitoid progeny emerged and formed cocoons. Subsets of 10 parasitised hosts were dissected 1-2 days after oviposition to determine the number of parasitoid progeny allocated to each host. Cocoon masses from the remaining 20 larvae were counted, weighed and placed in vials. Duration of the parasitoid's immature stages, percentage emergence of adults, number of adult progeny, adult longevity and sex ratio per clutch were recorded. Three females from each progeny (n = 60) were chosen randomly and dissected to determine the ovarian egg load.

Adult longevity

To allow for comparison, the methods used for adult longevity and fecundity experiments on *C. nonagriae* were similar to procedures used in previous studies on *C. flavipes* by Potting *et al*. (1997c). Adult longevity of mated females was measured at four temperatures (22^{o}C, 25^{o}C, 28^{o}C and 31^{o}C), two humidities (30-40% RH and 70-80% RH) and with or without food. Females were kept individually in plastic vials, with or without a drop of honey as food and with or without a layer of agar to maintain a high humidity. The lifespan of 10 individuals was measured for each treatment combination. Incubators were maintained at a photoperiodic regimen of 12L:12D and a light intensity of 600 lux. Mortality was recorded twice daily until all wasps had died. Treatment effects were tested using three-way ANOVAs performed in JMP 7 (Statistical Discovery from SAS) with Tukey HSD post hoc tests, with temperature, humidity and food as the main effects.

Oviposition and fecundity

The potential fecundity of individual females was measured by dissecting 1-2 day old, mated female wasps (n = 60) and recording the number of eggs in the ovaries. The

realised fecundity was determined by offering individual females two hosts (one in the morning and one in the afternoon) for three days. This was carried out by placing a forth instar *B. truncata* larva in a small vial (15 mm dia x 50 mm) with a single wasp. Individual wasps (n =20) that had oviposited were kept in a plastic vial at 25^{o}C, 70-80% RH and fed with a drop of honey. Wasps that were killed by a host larva during the experiment were excluded from the analysis. Host larvae were maintained individually on artificial diet and dissected 3-5 days after parasitisation to assess the number of parasitoid progeny. The clutch size was assumed to be equal to the number of parasitoid larvae, as *C. nonagriae* eggs and/or larvae are not encapsulated by *B. truncata* and there is no evidence of larval combat. Oviposition time was determined with a stopwatch and the oviposition period was defined as the duration during which the parasitoid's ovipositor remained inserted in the host body (after Potting *et al*. 1997c).

Influence of learning on microhabitat location and foraging behaviour

Microhabitat location experiments were conducted in an open wind tunnel (160 x 65 x 65 cm) arena. Balanced illumination was provided by two 36W fluorescent lamps on each side and two 18W lamps on each end of the test section. Average wind speed was 22.4 cm/s and temperature was 24-25^{o}C (50-70% RH). Details on the design of the wind tunnel used in these experiments are provided in Keller (1990). For each observation, a female wasp was released from a plastic vial (15 mm dia x 50 mm) with a plug of cotton wool restricting movement to the upper 5 mm. The vial was positioned 50 cm downwind of the experimental plants on a stand level in height to the middle of the plant. To examine the response of *C. nonagriae* to sugarcane plants infested with *B. truncata*, an experiment was conducted comparing a ~2 month old uninfested excised sugarcane stem with leaves as the control and a similar excised stem infested with one host as the treatment. Stems were placed 30 cm apart and the position of the two odour sources were periodically switched to negate any directional bias in the wind tunnel. Prior to the experiment, stemborer infested stems were obtained by introducing one host larva into a hole bored horizontally in the stem 24 h prior to the first experiment. During this time larvae constructed a small tunnel in the stem and many packed frass around the entrance hole.

To determine whether experience with a host larva or plant-host-complex (PHC) increased the responsiveness of female *C. nonagriae* towards infested plant odours and altered foraging behaviour on plants, two different experience groups were compared; 1) naïve females: one-day-old mated females that had no previous contact with a host or host product (n = 20), and 2) experienced females: one-day-old mated females that were exposed to a pre-flight treatment in the wind tunnel, where each was allowed to forage for about 20 min on a *B. truncata* infested sugarcane stem (n = 20). Experienced females were then recaptured, and tested in the wind tunnel within 2 h of this initial foraging experience. The latency of flight was measured by recording time before flight initiation, after the wasp first raised its antennae above the lip of the release vial (Keller 1990).

The following microhabitat location parameters and foraging behaviour were recorded: latency of flight, time from release to plant, duration of flight, time spent pointing (i.e. where the wasp raises the anterior portion of its body, faces upwind, and orientates its raised, spread antennae into the wind (Keller 1990)), time locating the entrance tunnel, time spent inside the stem and total time on plant. Experiments were terminated after 1h of reaching the plant or when the parasitoid left the plant. After each replicate the experimental sugarcane stems were replaced. If the parasitoid entered the stemborer tunnel, the host larva in that hole was removed and placed in a vial containing artificial diet then dissected 3 days later to determine if it was parasitised. For wasps that did not come out of the tunnel after 1h, the stem was opened to check if the wasp had been killed.

Results

Life history traits

Life history traits and developmental stages for *C. nonagriae* are summarised in Table 4.1 and Figure 4.2, respectively. Females had an initial egg load of 196.6 (SE ± 2.4) eggs and allocated an average of 111.6 (SE ± 3.4) eggs into the first host. This egg allocation pattern suggests that *C. nonagriae* females will deplete their egg load after just two oviposition events. There was no significant difference between mean egg allocation and mean number of cocoons ($P = 0.063$) per first oviposition. Likewise, no difference was found between mean number of cocoons and mean number of adult progeny ($P = 0.311$).

TABLE 4.1. Average number of eggs allocated, cocoons, adult progeny, cocoon weight duration of immature stages, sex ratio, adult longevity, emergence rate and potential fecundity of *C. nonagriae* clutches on the native stemborer host *Bathytricha truncata* (n = 18).

Cotesia nonagriae life history parameter	Mean (± SE)
Egg allocation	111.61 (3.49)
Number of cocoons	99.28 (5.37)
Cocoon weight (mg)	0.101 (0.01)
% Emergence	91.97 (1.04)
Durations of immature stages (days)	21.07 (0.17)
Adult progeny	91.56 (5.26)
Sex ratio (% fem./total progeny)	52.06 (1.38)
Adult longevity (unfed) (days)	2.92 (0.08)
Potential fecundity/ egg load	196.56 (2.38)

There was, however, significantly fewer surviving adult progeny than eggs allocated to each host ($t = 3.318$, $df = 34$, $P = 0.003$), indicating immature mortality in a number of eggs, larvae or cocoons. There was a positive correlation (Pearson $r = 0.88$, $P < 0.0001$, $r^2 = 0.77$) between the number of adult progeny and cocoon weight (Fig. 4.3), suggesting that larger progeny are not food limited. The total life cycle of *C. nonagriae* averaged 24 days, including 14-15 days in the egg and larval stages within the host. Wasp larvae exited the host's body and formed small white silken cocoons that usually surrounded the host cadaver within its tunnel. The rest of the life cycle comprised 6-7 days in the pupal stage and 1-3 days of adult life span (unfed with water provided). The adult sex ratio was generally 1:1 (F:M) (52%; SE ± 1.38, female) with males observed to emerge 2-6 h before females then mate with their sibs upon emergence.

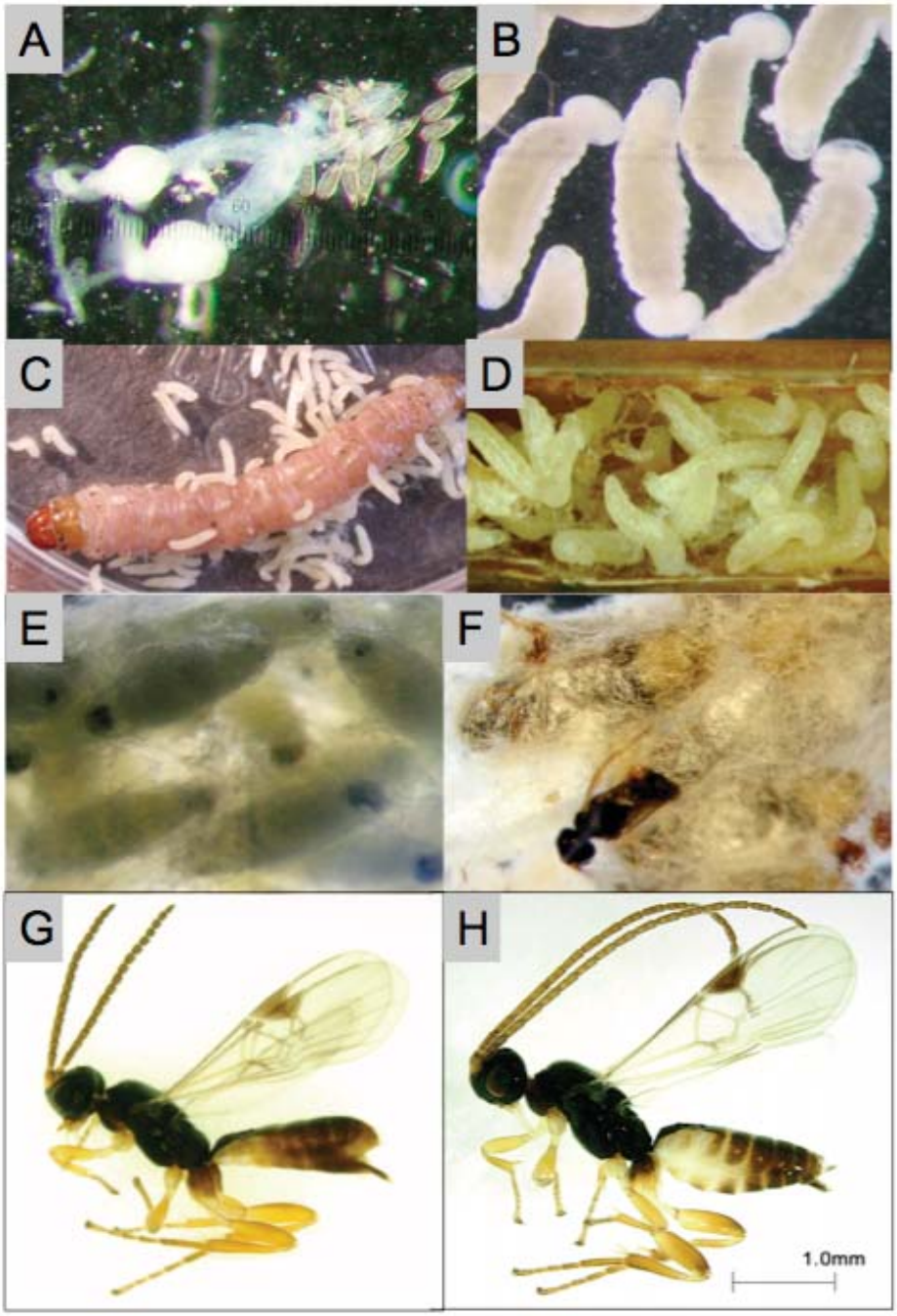

FIGURE 4.2. Life history stages of *C. nonagriae* from Mackay, Australia: A) ovaries and eggs; B) 12 day old larvae; C) larvae emerging from the native host *B. truncata*; D) larvae starting to form cocoons after emergence from host E) cocoons; F) adult wasp emerging from cocoon; G) adult female; H) adult male.

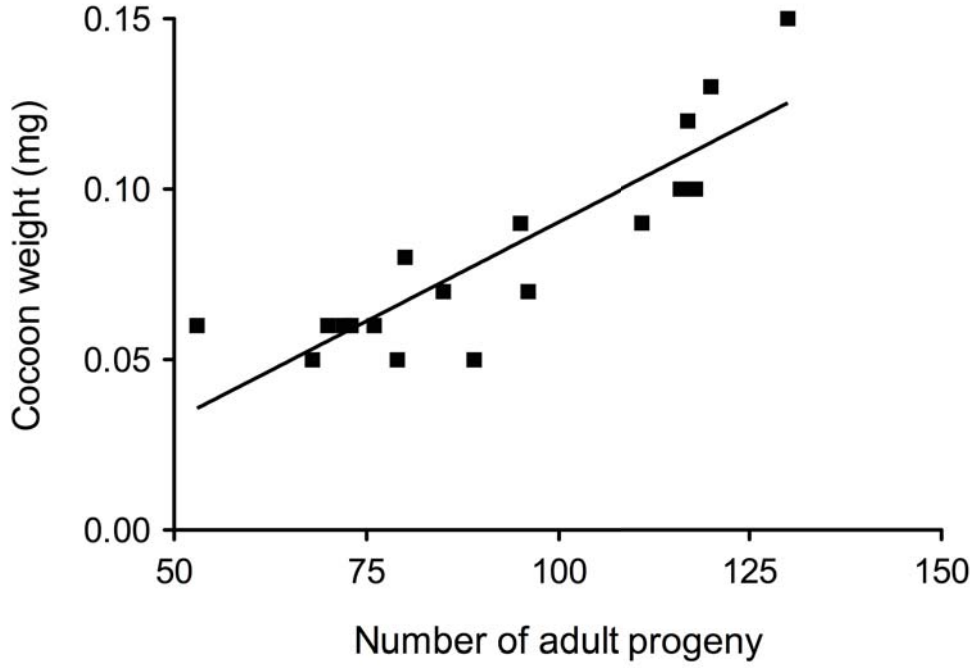

FIGURE 4.3. Correlation between the number of *C. nonagriae* adult progeny and cocoon weight per clutch. Pearson r = 0.8763, 95% CI (0.6928 to 0.9532), $P < 0.0001$, $r^2 = 0.7679$.

Adult longevity

Longevity of adults was investigated by exposing female wasps to four different temperatures, low or high humidity and food or no food (Fig. 4.4). There were significant effects of temperature (F = 3.35, df = 3, $P = 0.021$), humidity (F = 5.10, df = 1, $P = 0.0254$) and food availability (F = 376.89, df = 1, $P < 0.0001$) on the longevity of females, as well as significant interactions between temperature*food (F = 11.80, df = 3, $P < 0.0001$), humidity*food (F = 51.11, df = 3, $P < 0.0001$) and temperature*humidity*food (F= 2.96, df = 3, $P = 0.035$). Female wasps survived the longest at 25°C and 28°C, in high humidity and with food provided. Under these conditions adult life span was 8-14 days. At all temperatures, the availability of food under humid conditions significantly extended lifespan. However, the greatest effect was food availability, with wasps living only 1-3 days without food, regardless of temperature and humidity (Fig. 4.4).

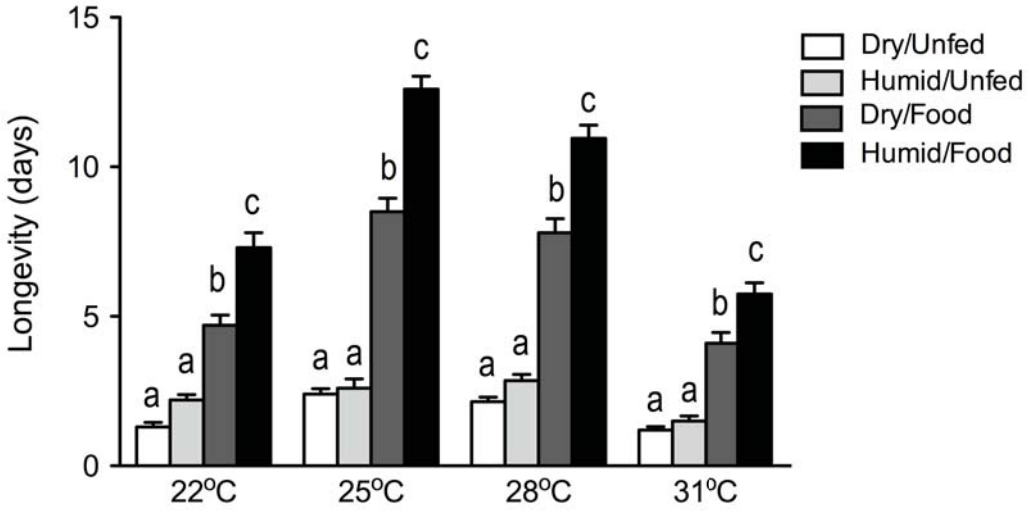

FIGURE 4.4. Average longevity (± SE, n = 10) of *C. nonagriae* females at four different temperatures and different experimental conditions. Different letters above error bars indicate significant differences among groups.

Fecundity

The mean oviposition time of *C. nonagriae* on the first host larva offered was 5.4s (SE ± 1.76, n = 17), resulting in a mean number of progeny of 111.6 (SE ± 3.4, n = 17). There was a positive correlation between the duration of oviposition and clutch size (r = 503, n = 17, *P* = 0.039) (Fig. 4.5A). The realised fecundity measured as the total number of progeny allocated to all attacked hosts was 194.6 (SE ± 7.04, n = 17). The clutch size allocated to hosts decreased with oviposition number (Fig. 4.5B). After oviposition into the second host larva most females were depleted of eggs or had allocated ~ 85% of their initial egg load. Although all females had depleted their initial egg load after three hosts, some wasps still accepted and attempted to oviposit into hosts, but were unable to produce eggs.

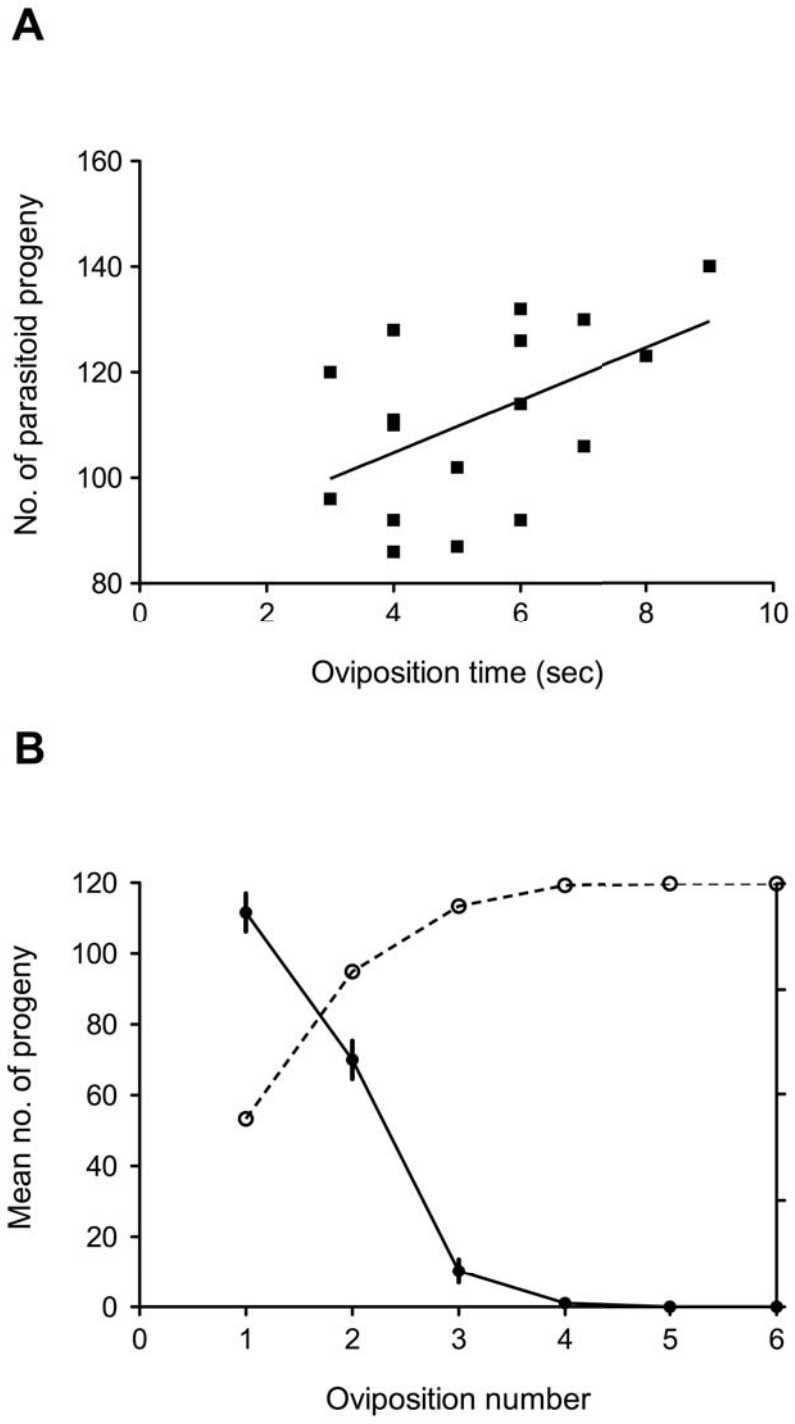

FIGURE 4.5. A) Correlation between clutch size and oviposition time (s) for *C. nonagriae*, Pearson r = 0.503, 95% CI (0.029 to 0.792), $P < 0.039$, $r^2 = 0.253$; B) Clutch size allocation of *C. nonagriae* in subsequently encountered hosts. Individual females were offered six hosts over three days. Solid line indicates the average number (± SE) of parasitoid larvae present 5-6 days after parasitization. Dashed line indicates cumulative percentage of individual realised fecundity. Numbers of females for first oviposition: 1 (n =20); 2 and 3 (n = 18); 4 (n = 16); 5 and 6 (n = 15).

Influence of learning on microhabitat location and foraging behaviour

Response level was defined as the percentage of tested females that flew upwind and made a choice between the uninfested control and infested treatment stems (Fig. 4.6A). In total, 55% of naïve wasps and 70% of experienced wasps flew to the odour source. In all tests both naïve ($P < 0.0001$) and experienced females ($P < 0.0001$) had a significant preference for the PHC, and there was no significant difference in the response level between the two groups (Fisher's exact test, 2-tail, $P = 0.46$). This strong response to the PHC indicates that wasps detected the presence of the host before initiating flight. Only one naïve wasp landed on an uninfested plant, staying for 143s before flying to the infested plant. The proportion of time spent pointing was the same in the presence of both infested and uninfested stems, although there was a tendency for each act of pointing to be shorter in the presence of a host larva. Experienced females had a much faster response time to the PHC than naïve wasps (Fig. 4.6B); the latency of flight was significantly shorter in experienced females ($t = 4.272$. $df = 23$, $P = 0.0002$), as was the total time from release to reaching the infested plant ($t = 4.644$, $df = 23$, $P < 0.0001$). However, there was no difference of flight duration between naïve and experienced parasitoids. Wasps that landed on the PHC were observed approaching it along a zigzag path.

After arriving on an infested plant, wasps immediately started walking up and down the stem searching for host cues. Although, no significant difference was found in the total time spent on the PHC or inside the stem between experienced and naïve wasps, females with prior experience took significantly less time to locate the host entrance tunnel ($t = 3.237$, $df = 22$, $P = 0.004$) (Fig. 4.6D). Only one naïve wasp that landed on the PHC flew off before finding the tunnel. The entrance tunnels were usually packed with frass by the feeding larva. After contacting the frass, the female started antennating it and either entered the tunnel immediately or started grooming or chewing on the frass.

Wasp mortality and post-oviposition behaviour

A large number of naïve (50%) and experienced wasps (57%) that entered the stemborer tunnel were killed inside the stem. These wasps were generally crushed, bitten or covered by caterpillar regurgitant inside the stemborer tunnel. However, 20%

of naïve wasps and 25% of experienced wasps still managed to oviposit into the host before they were killed (Fig. 4.6C). Only 60% of naïve wasps and 57% of experienced wasps that successfully parasitised a larva survived the host attack and exited the tunnel. The time spent inside host tunnels varied considerably (range 9.65-52.42 mins) for both experience groups, probably depending on the quantity of frass within the tunnel (Fig. 4.6D). After exiting most females remained on the plant grooming until the termination of the experiment.

Discussion

Several aspects of the biology of *C. nonagriae* differ when compared with *C. flavipes* and other members of the *C. flavipes* species complex. All members of the complex are pro-ovigenic and have a fixed complement of eggs upon emergence. *Cotesia flavipes* has about 150-200 eggs available for oviposition and a realised fecundity (the total number of progeny allocated to hosts) of around 150 eggs (Potting *et al*. 1997c; Sallam *et al*. 2002). The present study found a similar potential fecundity and realised fecundity (~200) for *C. nonagriae*, however females of the latter species allocated an average of 112 eggs into the first host, whereas *C. flavipes* introduced into Africa and North America is known to allocate a maximum of 30-60 eggs into each of at least three different hosts (Wiedenmann *et al.* 1992; Sallam *et al*. 2002). Under laboratory conditions, clutch size of *C. nonagriae* and *C. flavipes* decreased with each host encounter until they had laid all their eggs. However, it is likely that in their natural environment, *C. nonagriae* do not realise their potential fecundity, given that 50-57% of all females entering a stemborer tunnel were killed, a level of mortality greater than the 40% recorded for *C. flavipes* parasitising *Chilo partellus* and *Busseola fusca* (Potting *et al*. 1997c; Takasu 1997). By dividing the potential fecundity of *C. flavipes* by the optimal cutch size (the mean clutch size found in the first encounter host), Potting *et al*. (1997c) estimated that female host encounters would be limited to 3-4 hosts. Based on this, the expected host encounters of *C. nonagriae* would be limited to only two hosts, with half the available egg load being allocated to the host encountered first. In nature, the number of hosts encountered may be limited by factors such as parasitoid adult life span, host density and availability, the efficiency with which a parasitoid searches for hosts, time spent handling the host, and the risk of mortality pre- and post-oviposition in the host's tunnel.

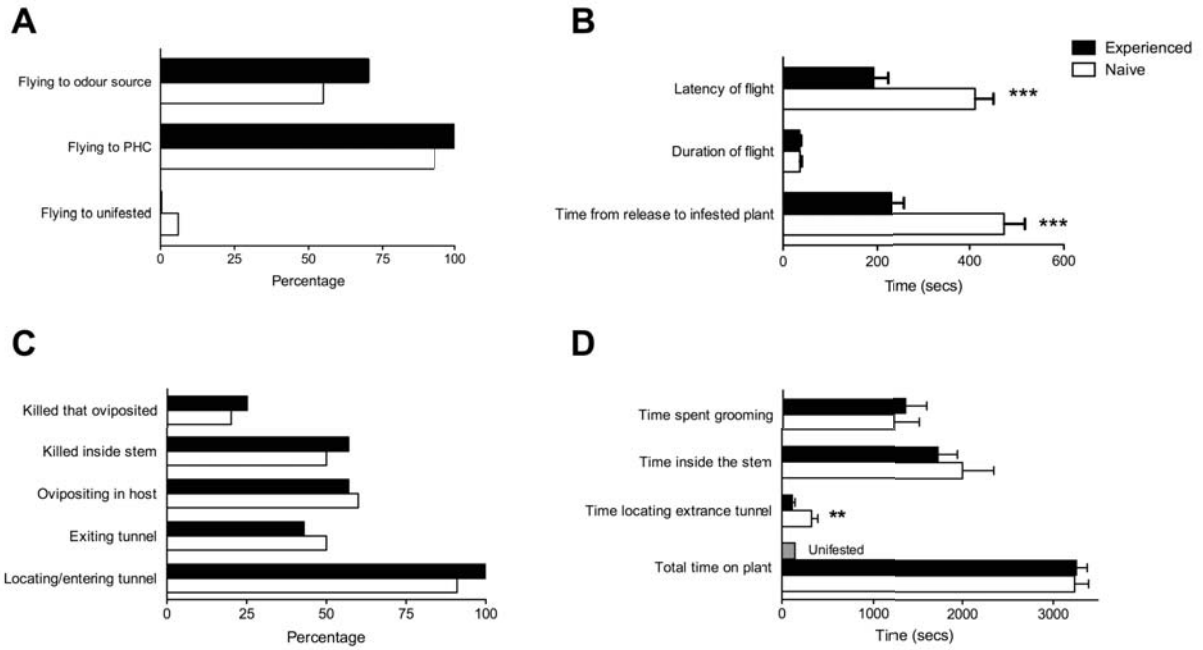

FIGURE 4.6. A, B) Microhabitat location; C, D) foraging behaviour parameters of naïve and experienced *C. nonagriae* females. * indicates a significant difference between groups.

The propensity of *C. nonagriae* to allocate a large number of eggs into each host encountered may be due to any one of these factors, however the high mortality risk at oviposition and the short life span may have contributed to the evolution of this strategy.

In spite of the larger number of *C. nonagriae* progeny emerging from *B. truncata,* cocoon weight was not substantially different from that of *C. flavipes* parasitising *Sesamia calamistis* and *Ch. partellus* in Africa (Sallam *et al*. 2002), suggesting that *C. nonagriae* individuals may be smaller. Although clutch sizes for *C. nonagriae* were much larger than recorded for *C. flavipes*, food resources were not restricted, as cocoon weight increased with larger progenies. The total life cycle of *C. flavipes* is about 20 days, but is slightly longer for *C. nonagriae* at 24 days. This is due to a longer duration of the larval and pupal stages (17 versus 21 days), which may be influenced by the higher number of larvae and resource availability. Adults of both species have a short life span of 1-3 days without food (Alleyne & Wiedenmann 2001a). However, with food and under high humidity conditions, *C. flavipes* has a life span of 5-6 days (Potting *et al*. 1997c; Mbapila & Overholt 2001). In this study, *C. nonagriae* adults lived up to two times longer (8-14 days) than *C. flavipes* under the same environmental conditions.

Although populations of *C. flavipes* have been found to have a female-biased sex ratio (Kajita & Drake 1969; Wiendenmann *et al.* 1992), this was not the case for *C. nonagriae* (52% female) in eastern Australia. A female-biased sex ratio suggests that *C. flavipes* exhibits local mate competition, however no male-male competition has been observed in mating trials (Joyce *et al.* 2009). In most gregarious parasitoids, sib-mating circumvents the problem of finding mates (Quicke 1997). *Cotesia flavipes* has been observed mating with siblings on the under surface of leaves after emergence from cocoon masses in sugarcane stems (Arakaki & Ganaha 1986), while in the current study *C. nonagriae* males emerged around 2-6 h before females and mated with their siblings upon female emergence (Muirhead *pers. observ.*).

The strong response of *C. nonagriae* females to infested sugarcane indicates that this parasitoid uses volatile chemicals emitted by the plant host complex (PHC) during habitat and host location. The use of such volatile semiochemicals in long-range host

microhabitat location has been demonstrated for numerous parasitoid species (e.g. Vet & Dicke 1992; Turlings *et al*. 1993), including other members of the *C. flavipes* complex (Mohyuddin *et al*. 1981; Shami & Mohyuddin 1992; Potting *et al*. 1995; Ngi-Song *et al.* 1996; Ngi-Song & Overholt 1997; Rutledge & Wiedenmann 1999; Jembere *et al.* 2003; Gohole *et al.* 2005). An infested plant will emit synomones (chemical signals that benefit the producer and the receiver) that attract parasitoids, thereby benefiting the plant and the parasitoid (Nordlund & Lewis 1976). Previous studies have found that a major source of attractant for females of the *C. flavipes* complex in the initial stages of host location are the volatiles emitted by infested plants and larval frass (Wiedenmann *et al.* 1992; Potting *et al*. 1995; Ngi-Song *et al.* 1996; Ngi-Song & Overholt 1997; Nwanze & Nwilene 1998; Rutledge & Wiedenmann 1999; Jembere *et al.* 2003; Gohole *et al.* 2005; Obonyo *et al.* 2008). This systemic plant response can be elicited when stemborer regurgitant is inoculated into the stem of an uninfested plant, thus eliciting the release of plant volatiles from the leaves that attract female *C. flavipes* (Dicke *et al.* 1990, Turlings & Tumlinson 1992; Potting *et al*. 1995, 1997c).

In the current study, wasps that landed on the PHC approached them along a zigzag path similar to that observed in other braconid species (Drost *et al*. 1986; Zanen *et al*. 1989; Keller 1990; Gu & Dorn 2000). The preference for the PHC in both naïve and experienced groups implies that this is an innate response that can be improved by experience. Like *C. flavipes*, previous experience with hosts or host-related cues did not increase the responsiveness (in terms of numbers) of *C. nonagriae* females. However, experienced wasps benefited by having a more rapid response time to host-induced volatiles and cues. This increase of responsiveness in parasitoids is often shown when test wasps have direct contact with their hosts and/or host cues (Tumlinson *et al*. 1993). In *C. glomerata* (Linnaeus), for example, the flight response of female wasps to a PHC significantly increased after walking on host-infested cabbage leaves with host silk and faeces for 20s or experiencing oviposition on the host (Steinberg *et al.* 1992). This learning ability in female wasps likely represents a more efficient foraging strategy that may be important for parasitoids with short life spans and long host-handling times.

Once a female has located a stemborer-infested plant, it has to find the concealed host larva inside the plant stem. Short-range cues used in host location are larval frass (Kajita & Drake 1969; Mohyuddin *et al*. 1981; Shami & Mohyuddin 1992; Wiedenmann *et al.* 1992; Potting *et al*. 1995; Ngi-Song *et al.* 1996; Ngi-Song & overholt 1997), larval regurgitant (Van Leerdam *et al*. 1985; Turlings *et al.* 1993; Mattiacci *et al*. 1994; Agelopoulos & Keller 1994; Potting *et al*. 1997c) and holes in the stem (Wiedenmann *et al.* 1992; Potting *et al*. 1997c). The present study found that *C. nonagriae* females with prior host experience were more efficient at finding stemborer tunnels and located them in about half the time of naïve females. Foraging behaviour of *C. nonagriae* was similar to *C. flavipes* on stemborer infested plants (Potting *et al*. 1997c; Takasu & Overholt 1997), with females searching the sugarcane stem for the entrance hole of the stemborer tunnel. Once the parasitoid found an entrance hole, it tried to reach the larva by entering the tunnel. This could be time consuming as the tunnel was often blocked by larval frass and the female wasp had to squeeze through the contracted entrance to reach the tunnel (Muirhead *pers. observ.*). However, the dorso-ventrally flattened body shape, that is typical of *C. nonagriae* and other *C. flavipes* complex members is arguably an adaptation to facilitate this behaviour (Walker 1994; Muirhead *et al.* 2008).

The time spent inside the tunnel is probably dependent on the position of the larva and the amount of frass in the tunnel. For *C. nonagriae*, this is partly demonstrated by the fact that there was no difference in the two experience groups. Attacking a host larva inside the confined space of a stemborer tunnel is not only time-consuming, but also risky for the parasitoid. Although it only takes a few seconds for *C. nonagriae* to oviposit around 100 eggs into its host, stemborer larvae are aggressive and will defend themselves against parasitism. Wasps are often crushed by the stemborer within the tunnel or killed by being bitten or covered with regurgitant. Subsequently, the mortality rate of both *C. nonagriae* (50-57%) and *C. flavipes* (30-40%) (Potting *et al*., 1997a; Takasu & Overholt 1997) is extremely high. Takasu and Overholt (1997) found that *C. flavipes* females had a higher probability of being killed by the stemborer, *C. partellus,* if the host larva was approached towards the head. However, the majority of parasitoids killed were able to parasitise their host successfully (Takasu & Overholt 1997). Results here showed that only 20-25% of wasps killed inside the stem were still able to oviposit before they were killed, and observations of

the stemborer attack generally showed that host aggressiveness started after the parasitoid had commenced ovipositing.

When an opportunistic forager is introduced into a new region, novel parasitoid-host associations can develop when related host species occur in the same niche as indigenous host species (Wiedenmann *et al.* 1992; Alleyne & Wiedenmann 2001b). Using natural enemies that have no coevolutionary history with a target pest is termed a 'novel association' (Hokkanen & Pimentel 1984; Wiedenmann *et al.* 1992; Wiedenmann & Smith 1997). The use of novel associations, especially with parasitoid wasps, has been highly effective against unmanageable pest problems in several situations (Alam *et al.* 1971; Hokkanen & Pimentel 1984; Macedo *et al.* 1984; Hokkanen 1985; Hokkanen & Pimentel 1989; Stiling 1990, Wiedenmann *et al.* 1992). This was the case for *C. flavipes* which formed a novel association with *Diatraea saccharalis* when introduced into the New World for biological control purposes (Simmonds 1969; Polaszek & Walker 1991; Wiedenmann *et al.* 1992). Although the scope of this study did not evaluate novel associations with *C. nonagriae,* a central issue for pest incursions into Australia is the host range of this species and whether it will successfully parasitise host species not encountered during its evolutionary history. Thus, future work could profitably be directed towards the testing of *C. nonagriae* on high threat stemborer species from Indonesia and Papua New Guinea.

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CHAPTER V

Molecular phylogeography and haplotype diversity of the *Cotesia flavipes* complex of parasitoid wasps (Hymenoptera: Braconidae).

Abstract

The *Cotesia flavipes* complex of parasitoid wasps are economically important for the biological control of lepidopteran stemboring pests associated with gramineous crops. Some members of the complex can successfully parasitise numerous stemborer pest species, however certain geographic populations have demonstrated variation in host range. In addition, the morphology of the complex is highly conserved and considerable confusion surrounds the identity of species and host-associated biotypes. This study generated nucleotide sequence data for two partial mtDNA gene regions (*COI*, *16S*) and three anonymous nuclear loci (*CfBN*, *CfCN*, *CfEN*) to analyse genetic variation and relationships among worldwide populations within the *C. flavipes* complex. Partitioned Bayesian and maximum parsimony analyses of the molecular data provide strong support for monophyly of the complex and the presence of at least four species, *C. chilonis* (from China and Japan), *C. sesamiae* (from Africa), *C. flavipes* (originating from the Indo-Asia region but introduced into Africa and the New World), and *C. nonagriae* (from Australia and PNG). Strong discordance was found between the mitochondrial and nuclear markers in the PNG haplotypes, which may be an outcome of hybridisation and introgression of *C. flavipes* x *C. nonagriae.* Haplotype diversity of geographic populations relate to historical biogeographic barriers and biological control introductions, and reflects previous reports of ecological variation in these species. The phylogenetic analyses did not support the overall separation and monophyly of clades associated with different host species, although some clades did show specific host associations, possibly due to localised host availability, rather than host specificity. The results provide a framework for assessing whether distinct lineages represent cryptic species, and for examining parasitoid-host evolution and compatibility more generally. Given the limitations of morphology, molecular-based identification is recommended for members of this complex prior to any biological control introductions.

Introduction

The success of biological control programs depends on accurate identification and biosystematics of both natural enemies and target pests (Rosen 1986, Debach & Rosen 1991; Overholt 1998). However, biological control programs are often confounded by cryptic species and intraspecific groupings, often termed strains, biotypes or races (Hopper *et al.* 1993; Unruh & Messing 1993; Goldson *et al.* 1997; Beard 1999, Vink *et al.* 2003). Cryptic species complexes are composed of closely related species that appear similar, making taxonomic identification based on morphology problematic. Likewise, variation among populations of organisms that can interbreed can be defined as strains or biotypes. Such taxonomic issues as defining cryptic species and assessing genetic diversity within species is imperative to biological control success since differences often represent phenotypic variation in behaviour, ecology and physiology, such as climatic adaptation, host specificity and host range (DeBach, 1969; Darling & Werren 1990; Clarke & Walter 1995). The advent of molecular systematics and phylogenetics have revolutionised the field of biological control for understanding such aspects as population structure, genetic diversity and evolutionary change (Sunnucks 2000; Roderick & Naavajas 2003) and, when employed for taxonomic studies, have often led to the discovery of cryptic species and genetic variants (eg. Campbell *et al.* 1993; Wiedenmann & Smith 1997; Chinwada *et al.* 2003; Kankare *et al.* 2005a,b; Rincon *et al.* 2006; Gitau *et al.* 2006, 2007; Phillips *et al.* 2008)

Hymenopteran parasitoids, which are free-living as adults, but have parasitic larvae, are arguably the most important group utilised as biological control agents. Yet, historical success rates for biological control are relatively low. The occurrence of cryptic species (eg. Wharton *et al.* 1990; Clarke & Walter 1995; Kazmer *et al.* 1996; Stouthamer *et al.* 1999, 2000; Molbo *et al.* 2003; Herty *et al.* 2007) and intraspecific variation (eg. Ngi-Song *et al.* 1995,1998; Alleyne & Wiedenmann 2001b; Mochiah 2001; Dupas *et al.* 2008) in wasp parasitoids is well documented and undoubtedly an underlying cause for low success rates in some biocontrol programs. Since parasitoids are often closely coevolved with their hosts, it is essential to understand the ecological and evolutionary processes involved in parasitoid-host associations for successful biological control. Moreover, understanding these processes minimises the potential risk of biological control introductions impacting non-target hosts (Howarth 1983;

Simberloff and Stiling 1996; Hufbauer 2002).

This study examines genetic variation in a taxonomically difficult group, the *Cotesia flavipes* species complex of parasitoid wasps. The species in this complex are gregarious endoparastiods of stemborer pests associated with sugarcane and cereal crops (Walker 1994). Since these are staple crops in many countries, members of the complex are economically important worldwide as biological control agents. Until recently, the complex was thought to comprise three species: *C. flavipes*, *C. sesamiae* and *C. chilonis.* Recently, however, Australian populations associated with the sugarcane borer pest, *Bathytricha truncata*, were found to represent a cryptic species on the basis of mtDNA phylogeography and biology (Muirhead *et al.* 2006, Muirhead *et al.* 2008). Thus, *Cotesia nonagriae* in Australia was removed from synonym with *C. flavipes*, and is now recognised as a distinct species (Muirhead *et al.* 2008). So far *C. nonagriae* is the only *C. flavipes* complex species recorded in Australia, where the other species in the complex are thought to be endemic to the following areas: *C. flavipes* to the Indo-oriental region, *C. sesamiae* to central and southern Africa, and *C. chilonis* to eastern Asia, including Japan (Polaszek & Walker 1991; Kimani-Njogu & Overholt 1997). However, all three species have been utilised for classical biological control of stemboring pests, resulting in their much broader inter-continental distribution (Polaszek & Walker 1991).

Although these species have been reported from numerous stemborer species, certain populations appear to have more restricted host ranges (Rajabalee & Govendasamy 1988; Potting *et al.* 1997b; Mochiah 2001; Ngi-Song *et al.* 1998, 2001; Chinwada *et al.* 2003; Gitau *et al.* 2006). Several studies have recorded variation in the ecology and host-searching behaviour of geographic populations of the *C. flavipes* complex, suggesting the existence of host and/or plant associated biotypes (Mohyuddin 1971; Mohyuddin *et al.* 1981; Inayatullah 1983; Polaszek & Walker 1991; Ngi-Song *et al.* 1995; Ngi-Song *et al.* 1998; Rutledge & Wiendenmann 1999; Mochiah *et al.* 2001). In addition, variation in host-parasitoid physiological compatibility and reproductive success among species and populations has been recorded (Ngi-Song *et al.* 1995,1998; Potting *et al.* 1997; Wiedenmann & Smith 1997; Alleyne & Wiedenmann 2001b; Mochiah 2001; Chinwada *et al.* 2003; Gitau *et al.* 2006, 2007; Dupas *et al.* 2008).

To date, genetic variation within and among species of the *C. flavipes* complex has been investigated using allozyme electrophoresis (Kimani-Njogu *et al.* 1998) and limited DNA sequence data (Smith & Kambhampati 1999; Muirhead *et al.* 2006). These studies have demonstrated the monophyly of the complex, yet relationships within the ingroup change (Smith & Kambhampati 1999; Michel-Salzat & Whitfield 2004, Muirhead *et al.* 2006) because of insufficient sequence data, limited taxon sampling, or both. More recently, preliminary studies on a restricted number of samples have found intraspecific mtDNA variation among geographic populations of *C. flavipes* and *C. sesamiae* (Muirhead *et al.* 2006; Muirhead *et al.* 2008).

The ability to distinguish cryptic species, biotypes and relationships among them is crucial for biological control success and highlights the need for a 'cohesive' approach to establishing species and population boundaries (see Templeton 1989). Examining phylogenetically distinct entities for reproductive incompatibility or ecological, behavioural or morphological differences will not only improve the likelihood of success rates of biological control programs, but will eliminate potential impacts of introduced natural enemies on non-target species (Hufbauer & Roderick 2005; Phillips *et al.* 2008). In this study, we present the first comprehensive molecular phylogeny of the *C. flavipes* complex using both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) markers. Specifically, we examined genetic variation in worldwide populations of the complex with an aim to 1) investigate relationships among geographic populations; 2) provide a framework to identify genetically divergent haplotypes that may represent biotypes or cryptic species; and 3) evaluate host associations within lineages.

Materials and Methods

Taxonomic sampling

Specimens from *C. flavipes* complex populations were sourced internationally from 15 countries and all have associated host/habitat information. The Australian populations were directly collected from three sugarcane-growing localities in Queensland. Parasitoids were either reared from wild collected hosts or sampled from colonies in research laboratories. The origin and host/habitat information of the 45 populations sampled and the three outgroups is provided in Table 5.1. All outgroups were source from the alcohol-preserved parasitoid collection of A.D. Austin at the University of Adelaide. Voucher specimens of most haplotypes have been lodged in the Waite Insect and Nematode Collection, the University of Adelaide.

TABLE 5.1. The initial identity, collection locations, host/habitat information, and number of individuals, source for taxa analysed in this study. Source = lab colony or field collection; '-' indicates unavailable information.

Species	Locality	Host	Habitat	Source	Number
C. flavipes	Thailand	*Chilo tumidicostalis*	Sugarcane	field	3
	Piracicaba, Brazil	*Diatraea saccharalis*	Sugarcane	colony	2
	India	*Chilo partellus*	Maize	field	5
	Florida, USA	*Diatraea saccharalis*	Sugarcane	colony	2
	Mombasa, Kenya	*Chilo partellus*	Maize	field	2
	Mandeville, Jamaica	*Diatraea saccharalis*	Sugarcane	field	1
	Mozambique	*Chilo partellus*	-	field	1
	south Pakistan	*Chilo partellus*	Maize	colony	2
	Sri Lanka	*Chilo sacchariphagus*	Sugarcane	field	1
	Labour-donnais, Mauritius	*Chilo sacchariphagus*	Sugarcane	field	1
	south Sumatra, Indonesia	*Sesamia inferens*	Sugarcane	field	2
	Ramu, PNG (Hap. 1)	*Sesamia grisecens*	Sugarcane	field	3
	Ramu, PNG (Hap. 2)	*Sesamia grisecens*	Sugarcane	field	1
	Reunion	*Chilo sacchariphagus*	Sugarcane	field	1
	Okinawa Prefecture, Japan	*Sesamia inferens*	Sugarcane	field	2
	Mbulamuti, Uganda (Hap. 1)	*Chilo partellus*	-	field	1
	Nyamomgo, Uganda	*Busseola fusca*	-	field	1
	Manyame, Zanzibar	*Chilo partellus*	-	field	1
C. nonagriae	Giru, Australia	*Bathytricha truncata*	Sugarcane	field	5
C. nonagriae	Bundaberg, Australia	*Bathytricha truncata*	Sugarcane	field	16

C. nonagriae	Mackay, Australia	*Bathytricha truncata*	Sugarcane	field	12
C. chilonis	Illinois, USA (originated from Japan)	*Diatraea saccharalis* (*Chilo supressalis*)	Sugarcane (rice)	colony	1
	China	*Chilo supressalis*	-	field	1
C. sesamiae	Ethopia	*Chilo partellus*	Sorghum	field	1
	Mombasa, E. Kenya (Hap. 1)	*Sesamia calamistis*	Maize	field	1
	Mombasa, E. Kenya (Hap. 2)	*Chilo partellus*	Sorghum	field	1
	Mombasa, E. Kenya (Hap. 3)	*Chilo partellus*	Sorghum	field	1
	Mombasa, E. Kenya (Hap. 4)	*Chilo partellus*	Sorghum	field	1
	Mombasa, E. Kenya (Hap. 5)	*Chilo partellus*	Sorghum	field	1
	Kitale, W. Kenya (Hap. 1)	*Busseola fusca*	Soghum	field	1
	Kitale, W. Kenya (Hap. 2)	*Busseola fusca*	Soghum	field	1
	Kitale, W. Kenya (Hap. 3)	*Busseola fusca*	Soghum	field	1
	Mutane, South Africa (Hap. 1)	*Sesamia calamistis*	Maize	field	1
	Pietersburg, South Africa (Hap. 2)	*Chilo partellus*	Maize	field	1
	Cedara, South Africa (Hap. 3)	*Busseola fusca*	Maize	field	1
	Pietersburg, South Africa (Hap. 4)	*Chilo partellus*	Maize	field	1
	Pietersburg, South Africa (Hap. 5)	*Busseola fusca*	Maize	field	1
	Miyambo,Tanzania	*Chilo partellus*	-	field	1
	Kiwengwa, Zanzibar (Hap. 1)	*Chilo partellus*	-	field	1
	Many, Zanzibar (Hap. 2)	*Sesamia calamistis*	-	field	1
	Kizimba, Zanzibar (Hap. 3)	*Sesamia calamistis*	-	field	1
	Musikavanu, Zimbabwe	*Chilo partellus*	-	field	1
C. glomerata	University of Adelaide, Australia	-	-	-	1
C. rubecula	University of Adelaide, Australia	-	-	-	1
C. urabae	University of Adelaide, Australia	-	*Eucalyptus* sp.	field	1

Molecular markers

Regions of the mitochondrial *cytochrome c oxidase subunit I* (*COI*) and *16S ribosomal RNA* genes and three single-copy anonymous nuclear loci were used for phylogenetic analysis. Partial *16S rRNA* and *COI* mtDNA nucleotide sequence data for 21 populations and the three outgroups have previously been used in a preliminary study (Muirhead *et al.* 2006) (Table 2.1: Chapter II), and the extracted DNA from these specimens was used here to sequence the three anonymous nuclear loci plus a further 24 populations were added to the ingroup. Primers pairs used for amplification and sequencing are shown in Table 5.2. Universal primers C1-J-1718 and C1-N-2329 (Simon *et al.* 1994) were used to amplify a 550 bp fragment of the *COI* gene, and a 375 bp fragment of *16S rRNA* was amplified using 16SWb (Dowton & Austin 1994) and 16S outer (Whitfield 1997). Three anonymous nuclear loci were developed specifically for this study and included *CfBN* (728 bp), *CfCN* (757 bp) and *CfEN* (847 bp).

TABLE 5.2. Primers used in this study

Gene/Locus	Primer name	Sequence (5′−3′)	T_a (°C)	Reference
COI			50	
Forward	C1-J-1718	GGAGGATTTGGAAATTGATTAGTTCC		Simon *et al.* 1994
Reverse	C1-N-2329	ACTGTAAATATATGATGAGCTCA		Simon *et al.* 1994
16S rRNA			50	
Forward	*16S*Wb	CACCTGTTTATCAAAAACAT		Dowton & Austin 1994
Reverse	*16S* outer	CTTATTCAACATCGAGGTC		Whitfield 1997
Anonymous nuclear locus	*CfBN*		44	
Forward		AAGGCTGGATTAAGAGAC		Present study
Reverse		CCACGGCTGTATAAAATC		Present study
Anonymous nuclear locus	*CfCN*		49	
Forward		CACACTTCATTTGACTCCG		Present study
Reverse		CTATACTATGTGATGCCAAGAG		Present study
Anonymous nuclear locus	*CfEN*		49	
Forward		GCAAGAAGCCGCTGTTAC		Present study
Reverse		GTTTCATCGTGTCATATTTCG		Present study

Anonymous nuclear marker isolation

Three single-copy anonymous nuclear loci were developed by sequencing 20 single-copy clones from a genomic DNA library constructed for *C. flavipes*. The applicability of these loci was tested on the *C. flavipes* complex and other species of *Cotesia*. The protocols used to construct the genomic library were adapted from Glenn and Schable (2005). Total genomic DNA extracted from 10 *C. flavipes* individuals of a single progeny (total of ~2 µg DNA) was digested with restriction enzymes *Rsa*I and *Sna*BI. Digested DNA was size selected for ≥ 1000 fragments by agarose gel electrophoresis and excised and ligated to a double stranded adaptor (with adaptor sequences from protocol i.e. S475 5'-GTTTAAGGCCTAGTCTAGCAGAATC-3' and S476 5'-GATTCTGCTAGCTAGGCCTTAAACAAAA-3'). Adaptor-ligated DNA fragments were amplified using the Expand Long Template PCR System (Roche) and gel-purified using agarose gel electrophoresis with crystal violet (TOPO XL PCR Cloning

Kit, Invitrogen), size selecting again for ≥ 1000 fragments. Purified PCR product was ligated into the PCR®-XL-TOPO® vector and transformed into One Shot® TOP10 competent cells (TOPO XL PCR Cloning Kit, Invitrogen). Randomly selected colonies were screened for single copy loci using Photobiotin™ acetate (GeneWorks) under the labelling and hybridisation conditions described in the GeneWorks protocol. Twenty clones were amplified and sequenced, and Blasted to GenBank to verify as anonymous regions. Primers were designed from the clone insert sequences using the program Oligo 7 Primer Analysis Software (Molecular Biology Insights, Inc.). Amplification of 20 loci was attempted on the four *C. flavipes* complex species and three *Cotesia* outgroup taxa to test their cross-species applicability.

DNA extraction, amplification and sequencing

All specimens were stored in 100% ethanol and preserved at –20°C until DNA was extracted. For all sequenced specimens, total genomic DNA was extracted from headless, whole wasps using the Gentra Systems Puregene® DNA Purification Kit (GentraSystems 2005). Polymerase chain reaction (PCR) amplification was carried out in an Eppendorf thermal sequencer. Each 25 μl reaction comprised of PCR buffer, 0.2 mM of each dNTP, 0.5 μM of each forward and reverse primers, 2 mM $MgCl_2$, 0.5 units of AmpliTaq Gold® DNA Polymerase (Applied Biosystems Inc.) and 25-100 ng of genomic DNA. PCR conditions were: denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 45 sec, annealing at 50°C for 45 sec, and extension at 72°C for 30 sec. Final extension was at 72°C for 3 min. PCR products were purified using the Ultraclean™ PCR Clean-up™ Kit (MoBio Laboratories inc.) and sequenced using ABI Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA, USA) in 20 μl reaction volumes. Fragments were resolved on an ABI 3700 capillary sequencer (Applied Biosystems).

Sequence alignment

Mitochondrial DNA (mtDNA) sequences were edited and aligned manually using BioEdit Sequence Alignment Editor version 7.0.1. (Hall 1999). Regions of the *16S rRNA* alignment were highly conserved, thus gaps were easily inferred by eye and confirmed using ClustalX v.1.83 (Chenna *et al.* 2003). Protein-encoding *COI* nucleotide sequences were translated into amino acid sequences using the toggle

translation option. The presence of nucleotide saturation in the *COI* 3rd codon position was examined by plotting observed transitions and transversions against genetic divergence. Nuclear DNA sequences were viewed and edited in Codon Code Aligner 2.0.4. (CodonCode Corporation). Forward and reverse consensus sequences were assembled and contigs were aligned with ClustalW in CodonCode.

Phylogenetic analyses

Phylogenetic reconstructions were conducted for the two mtDNA genes and three nuclear loci separately using Bayesian criteria of optimality. Concatenated trees were evaluated using Bayesian and Maximum parsimony (MP) analysis. MP analyses, base frequency and pairwise distance calculations were performed using PAUP* version 4.0b10 (Swofford 2000), employing equal weights for all positions, with gaps treated as missing data. For MP methods the heuristic search algorithm options were used with stepwise addition and 100 random taxon addition sequence replicates. Support values for each node (BSV) were examined by bootstrap analysis (Felsenstein 1985) from 1000 pseudoreplicated data sets with full heuristic searches.

Bayesian analyses was implemented using MrBayes version 3.0b4 (Huelsenbeck & Ronquist 2001), incorporating the models chosen by Modeltest (Table 5.3). The model parameters were unlinked and estimated separately for each locus. Analyses were run for five million generations sampling the four Markov chains every 100 generations. Stationarity was determined from an examination of log likelihood scores and model parameters. Trees recovered prior to stationarity being reached were discarded and Bayesian posterior probabilities of each bipartition, representing the percentage of times each node was recovered, were calculated from a consensus of the remaining trees. Multiple runs were performed to assess that all parameters were not considerably different at stationarity based on alternate prior probabilities.

TABLE 5.3. Models chosen for data partitions by the Akaike information criterion in Modeltest (Posada and Crandall 1998).

Partition	Model chosen
COI	GTR+I+G
16S rRNA	GTR+I
CfBN	GTR
CfCN	HKY+G
CfEN	GTR+G

Results

Mitochondrial gene phylogeny

A total of 925 base pairs (bp) were sequenced for phylogenetic analysis, including partial *16S rRNA* and *COI* mtDNA nucleotide sequence data (*COI* = 550 bp, *16S rRNA* = 375 bp). Separate Bayesian analyses of the two mtDNA genes found similar topologies, however there was incongruence in the relationship between haplotypes of *C. chilonis* and *C. sesamiae.* The *COI* phylogeny (Fig. 5.1) supported the monophyly of *C. sesamiae* haplotypes and showed weak support for a sister relationship between *C. chilonis* and the *C. sesamiae* clade. This was not found in the *16S* tree (Fig. 5.2), where *C. sesamiae* haplotypes were not monophyletic and a low support was found for a sister relationship between *C. chilonis* and the *C. sesamiae* haplotypes from Ethiopia, West Kenya, and East Kenya 4; the other *C. sesamiae* haplotypes being monophyletic and sister to *C. flavipes* + *C. nonagriae* (Pbay <0.5).

The placement of the Japanese *C. flavipes* also differed between the two trees; it was sister to all other *C. flavipes* haplotypes in the *COI* phylogeny, but was sister to *C. flavipes* (PNG 1) + *C. nonagriae* haplotypes (Australia) in the *16S* phylogeny. Likewise, in the *16S* tree there was strong support for a sister relationship between the Thailand *C. flavipes* haplotype and all remaining *C. flavipes*, but this was not found in the *COI* phylogeny.

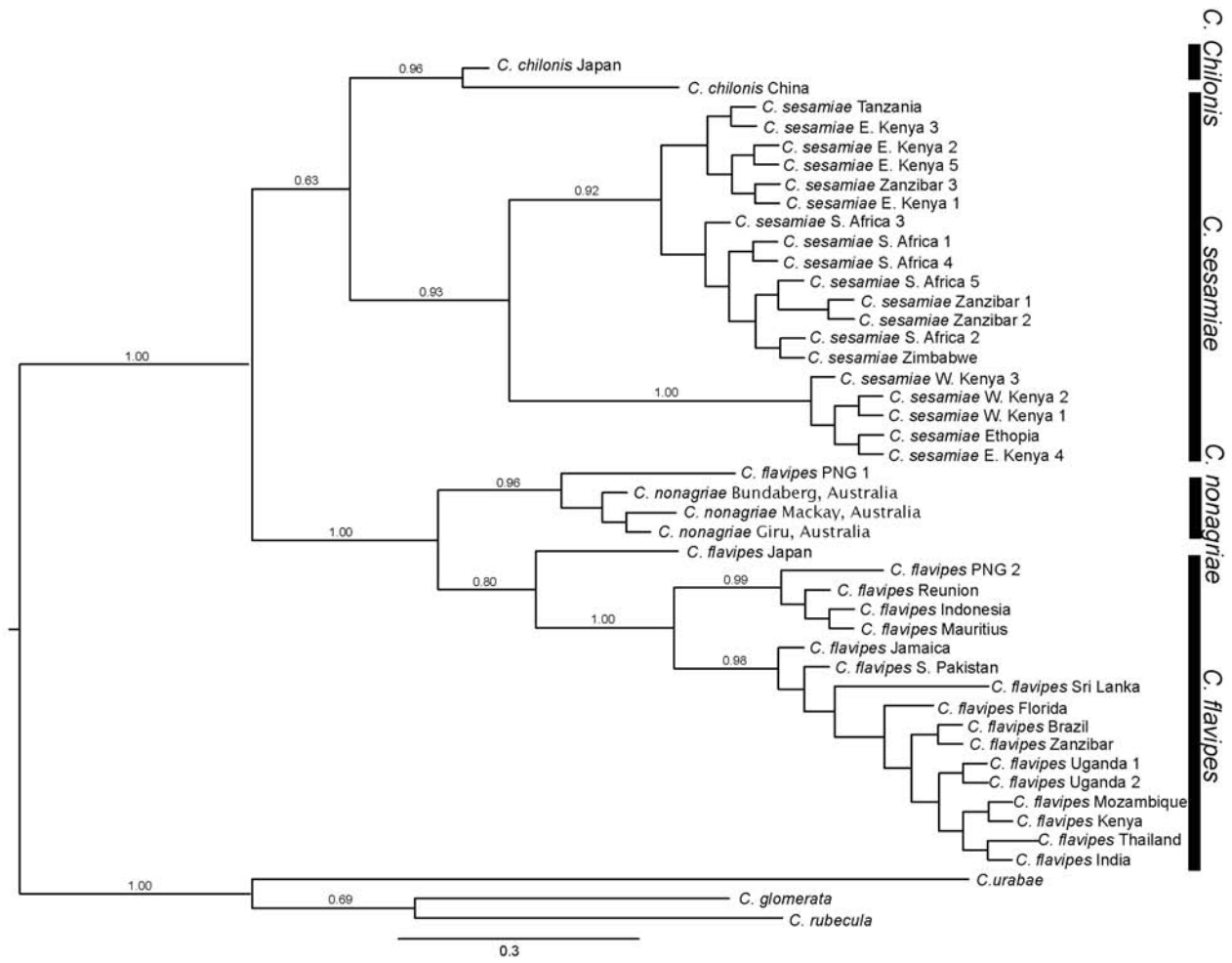

FIGURE 5.1. Bayesian trees derived from *COI* sequence data for geographic populations of the *Cotesia flavipes* complex and three outgroups. The numbers above the nodes represent Bayesian posterior probabilities $\geq$ 50.

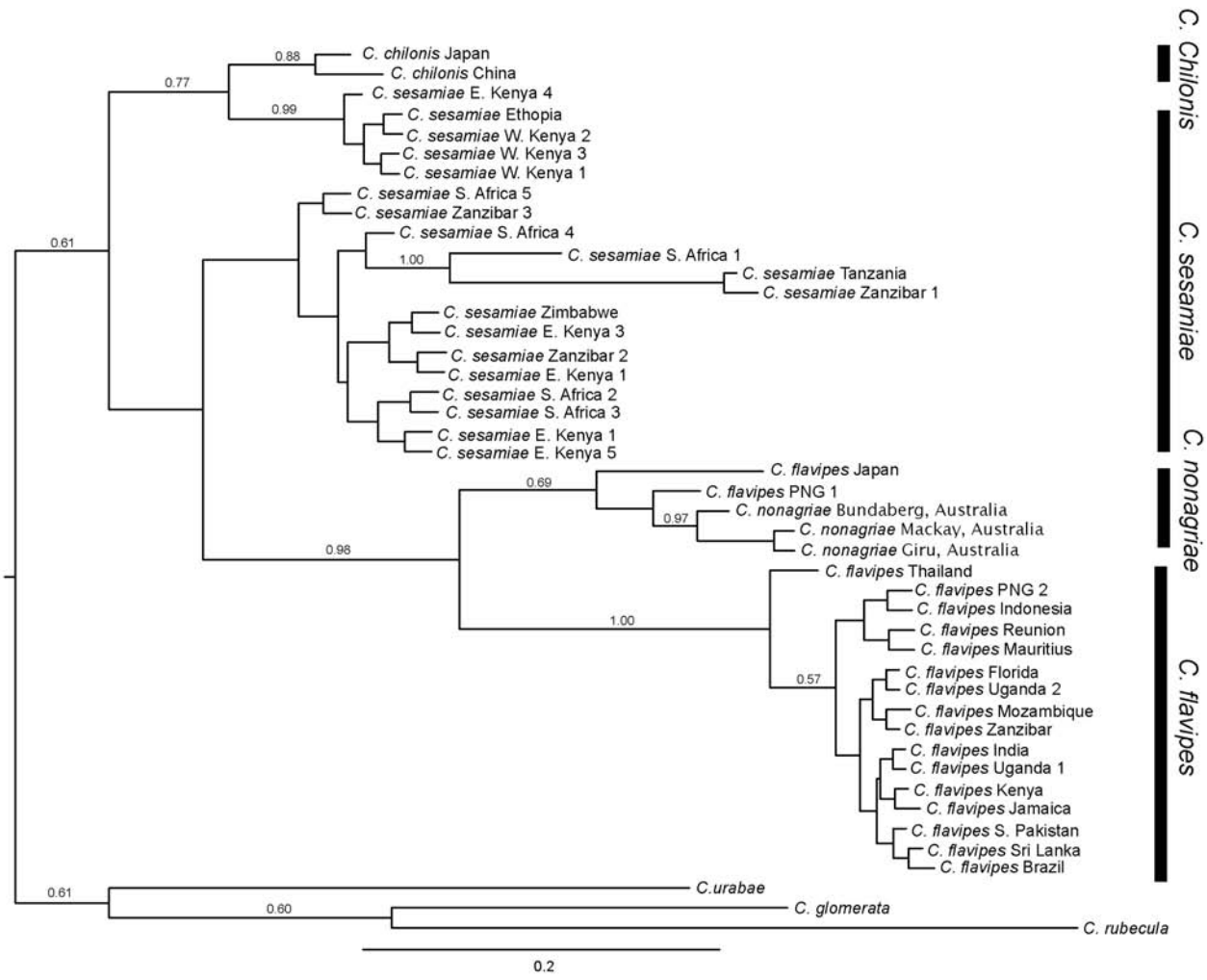

FIGURE 5.2. Bayesian trees derived from *16S* sequence data for geographic populations of the *Cotesia flavipes* complex and three outgroups. The numbers above the nodes represent Bayesian posterior probabilities $\geq$ 50.

MP analysis of the combined mtDNA (925 bp) resulted in 30 most-parsimonious trees of 222 steps (CI = 0.75 and RI = 0.83), with 63 variable and 89 parsimony-informative characters. The MP bootstrap consensus and Bayesian analyses produced trees with similar topologies, however the MP analysis (not shown) recovered the Thailand *C. flavipes* haplotype as sister to *C. flavipes* from Africa, India and the New World (BSV = 83%), where the Bayesian analysis (Fig. 5.3) did not resolve any relationships within this clade. The mtDNA tree topology was most similar to the *COI* gene tree and strongly supported a monophyletic group showing four major species level clades, with a relationship of (*C. chilonis* + *C. sesamiae*) + (*C. flavipes* + *C. nonagriae*).

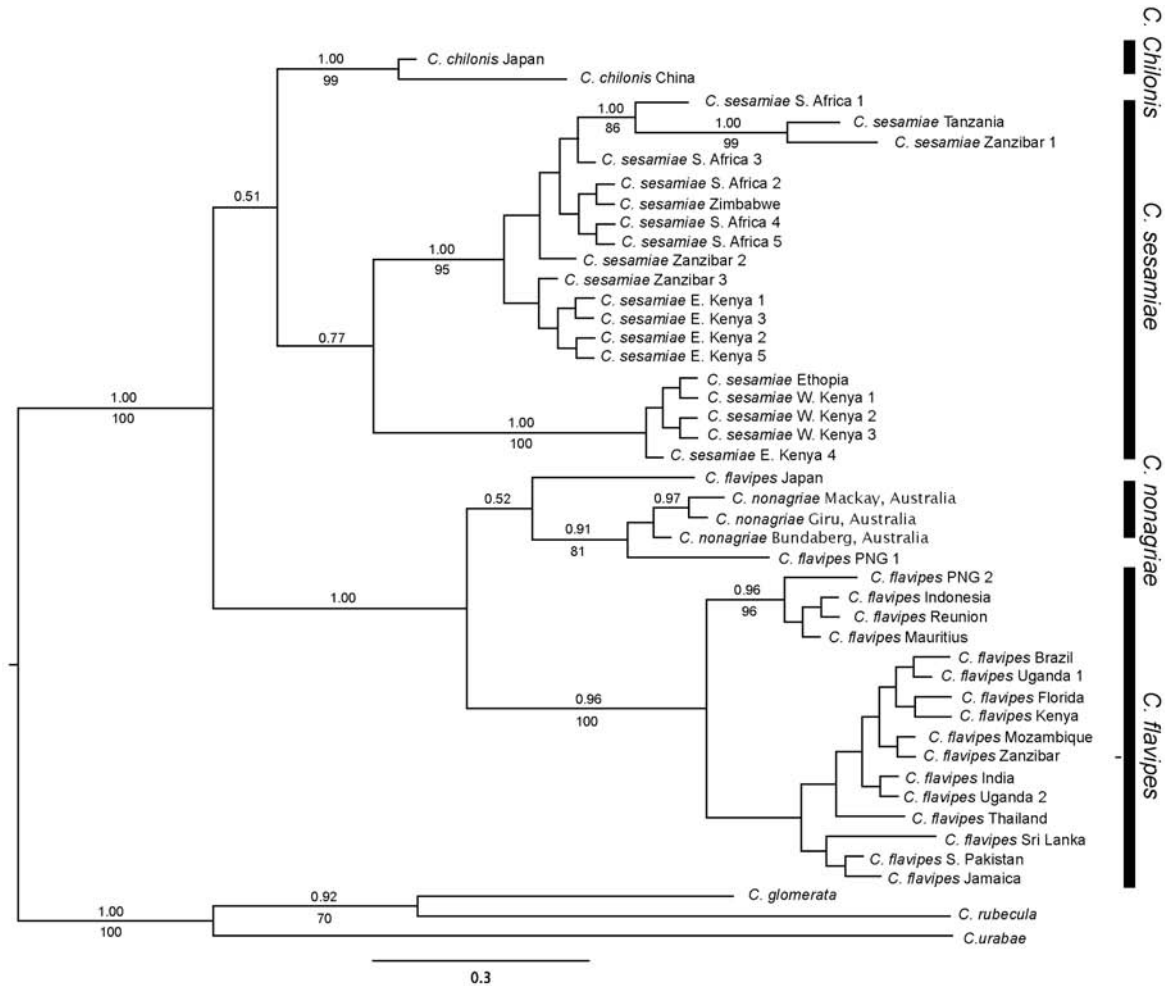

FIGURE 5.3. Bayesian trees derived from partial *16S* and *COI* sequence data for geographic populations of the *Cotesia flavipes* complex and three outgroups. Bayesian posterior probabilities ≥ 50%; numbers below the nodes indicate bootstrap proportions ≥ 70% values from 1000 pseudoreplicates of the MP analysis.

Nuclear gene phylogeny

Three of the 20 anonymous nuclear loci sequenced for *C. flavipes* were variable and homologous amongst all seven *Cotesia* species tested (*CfBN* = 728 bp, *CfCN* = 757 bp, *CfEN* = 847 bp). Baysian reconstructions from the three nuclear loci resulted in differing topologies (Figs. 5.4-5.6) The resolution of trees from separate analysis of *CfBN* and *CfCN* loci was low. The *CfBN* phylogeny (Fig. 5.4) showed support for the monophyly of the complex, the *C. flavipes* clade and the *C. nonagriae* group. Likewise, the *CfCN* phylogeny (Fig. 5.5) showed support for the monophyly of the complex, *C. chilonis*, *C. sesamiae* and the *C. flavipes* + *C. nonagriae* group, but poor resolution at the shallower nodes. However, in the *CfEN* phylogeny (Fig. 5.6) the two *C. chilonis* haplotypes were paraphyletic with respect to the remaining ingroup and *C. sesamiae* East Kenya 4 fell out within the major *C. flavipes* clade, but generally this

analysis showed the most resolution of the three nDNA loci.

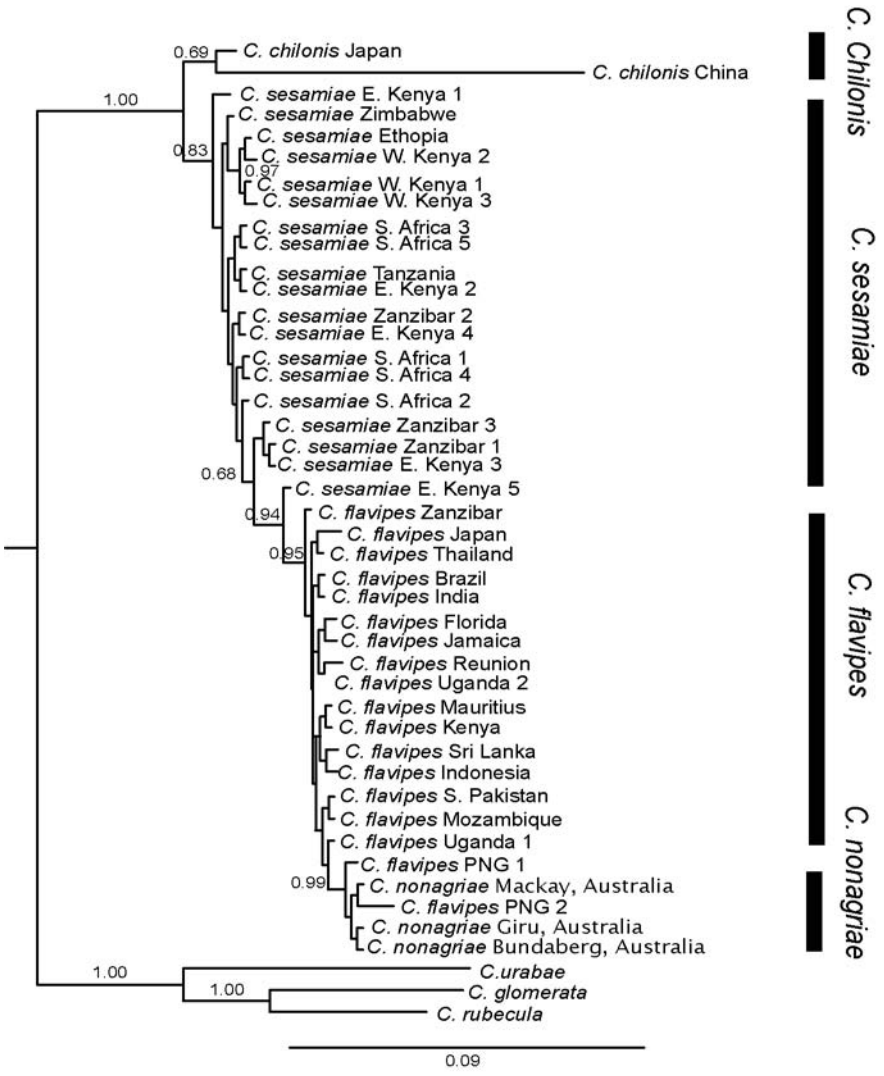

FIGURE 5.4. Bayesian trees derived from anonymous nuclear locus, *CfBN* for geographic populations of the *Cotesia flavipes* complex and three outgroups. The numbers above the nodes represent Bayesian posterior probabilities $\geq$ 50.

The *CfCN* tree showed weak support for a sister relationship between *C. chilonis* and *C. sesamiae* (as in the 16S tree), whereas the *CfEN* phylogeny strongly supported a sister relationship between *C. sesamiae* and *C. flavipes* + *C. nonagriae*. Both *CfCN* and *CfEN* gene trees showed strong support for a sister relationship between *C. flavipes* and *C. nonagriae,* however the *CfEN* tree recovered the PNG 2 haplotype as sister to all *C. flavipes/C. nonagriae*, where it fell out within the *C. nonagriae* group in the *CfCN* tree.

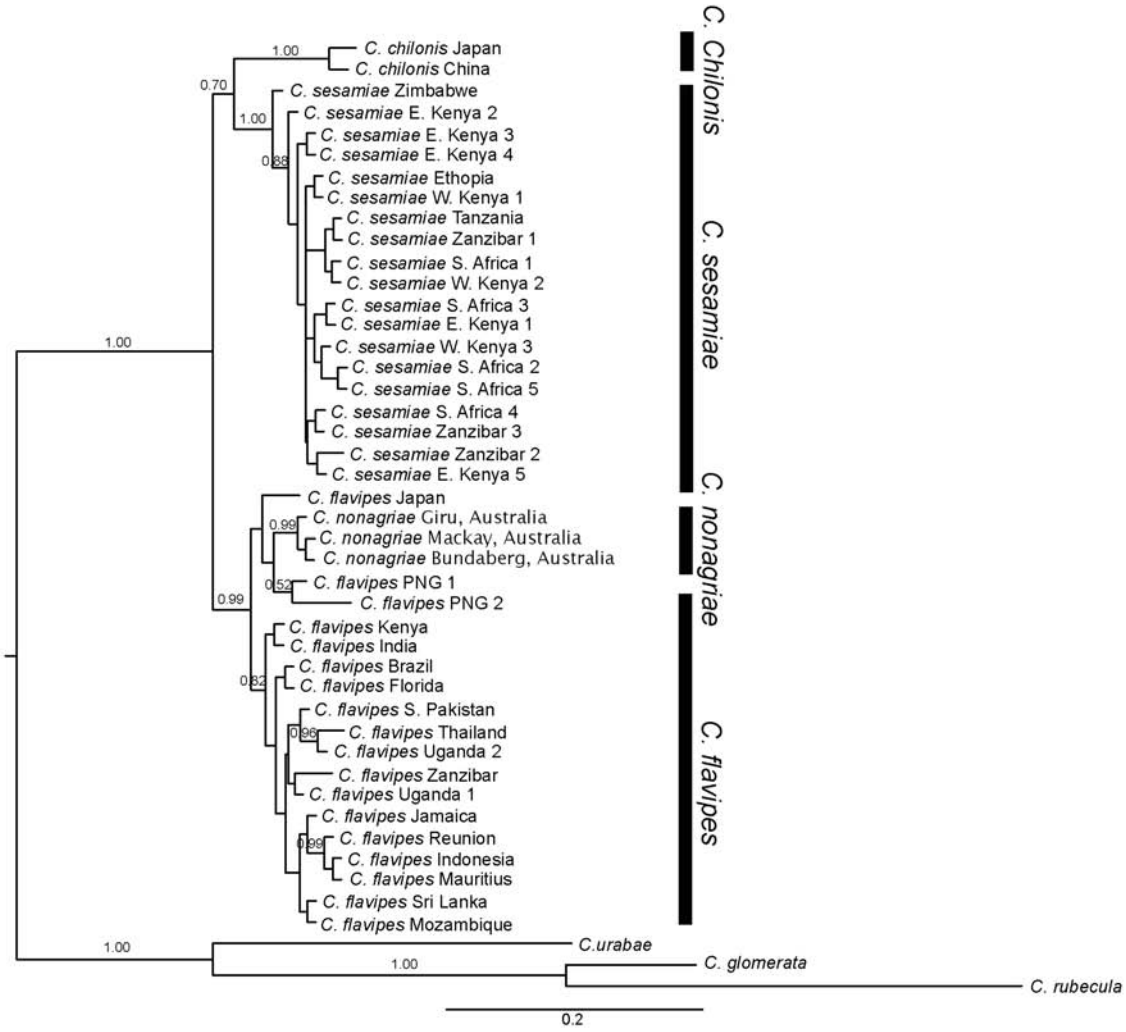

FIGURE 5.5. Bayesian trees derived from anonymous nuclear locus, *CfCN* for geographic populations of the *Cotesia flavipes* complex and three outgroups. The numbers above the nodes represent Bayesian posterior probabilities $\geq$ 50.

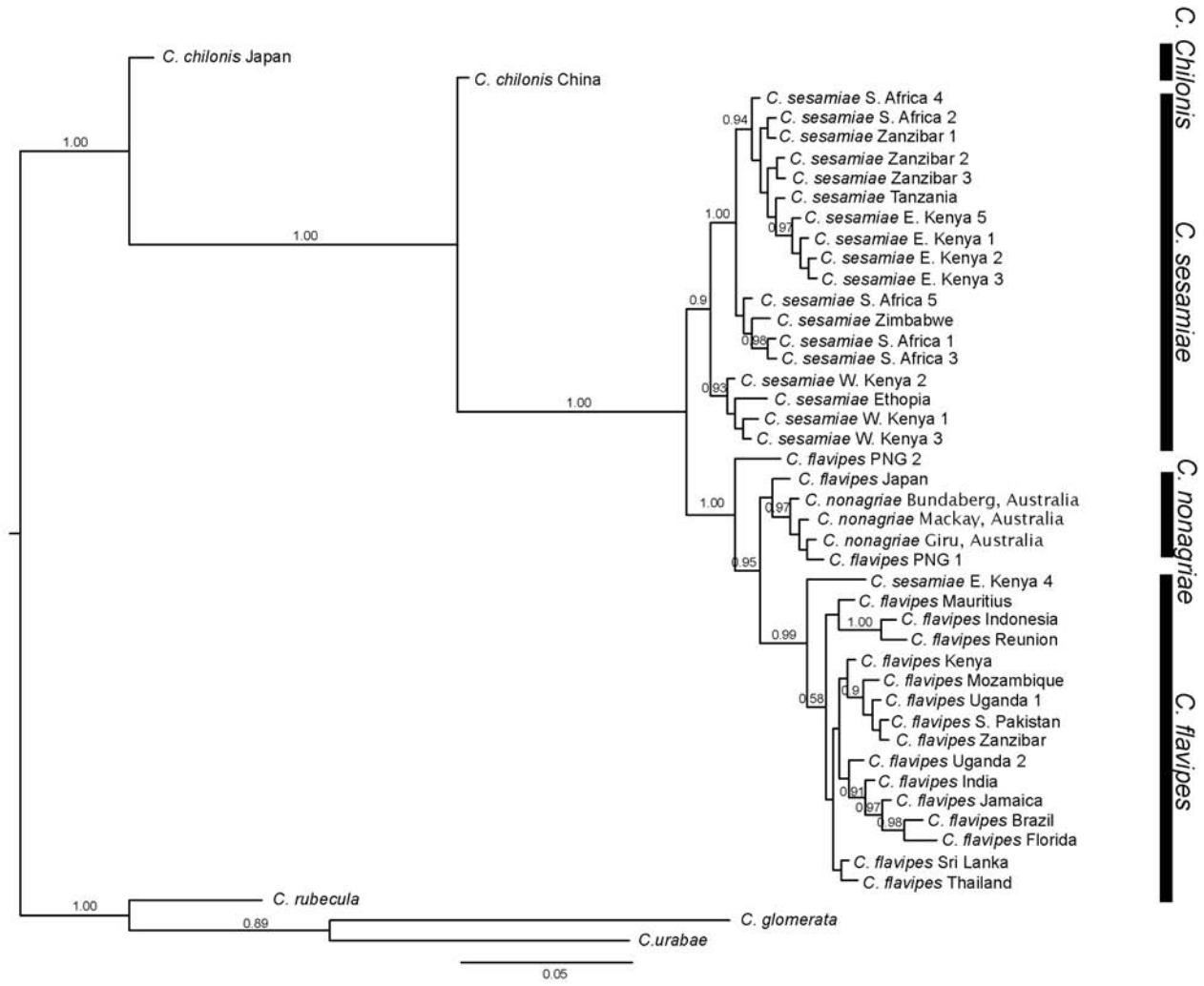

FIGURE 5.6. Bayesian trees derived from anonymous nuclear locus, *CfEN* for geographic populations of the *Cotesia flavipes* complex and three outgroups. The numbers above the nodes represent Bayesian posterior probabilities ≥ 50.

MP analysis of the combined nDNA data used 2332 nucleotides, of which 228 were variable and 245 were parsimony-informative, and resulted in 178 most-parsimonious trees of 598 steps (CI = 0.35 and RI = 0.83). The MP bootstrap consensus and Bayesian analyses produced trees with similar topologies, however the relationships recovered by the MP analysis (not shown) rof *C. flavipes* haploypes from PNG 2 and Japan were unresolved and formed a polytomy with the *C.flavipes* + *C. nonagriae* clade. The Bayesian analysis (Fig. 5.7) resolved a monophyletic *C. chilonis, C. sesamiae* with *C. chilonis* as sister to the rest of the complex but, in contrast to the MP tree, showed a strong sister relationship between the Japanese haplotype and the main *C. flavipes* clade, and weaker support (Pbay = 92) for a sister relationship between PNG2 and *C. nonagriae* + PNG1.

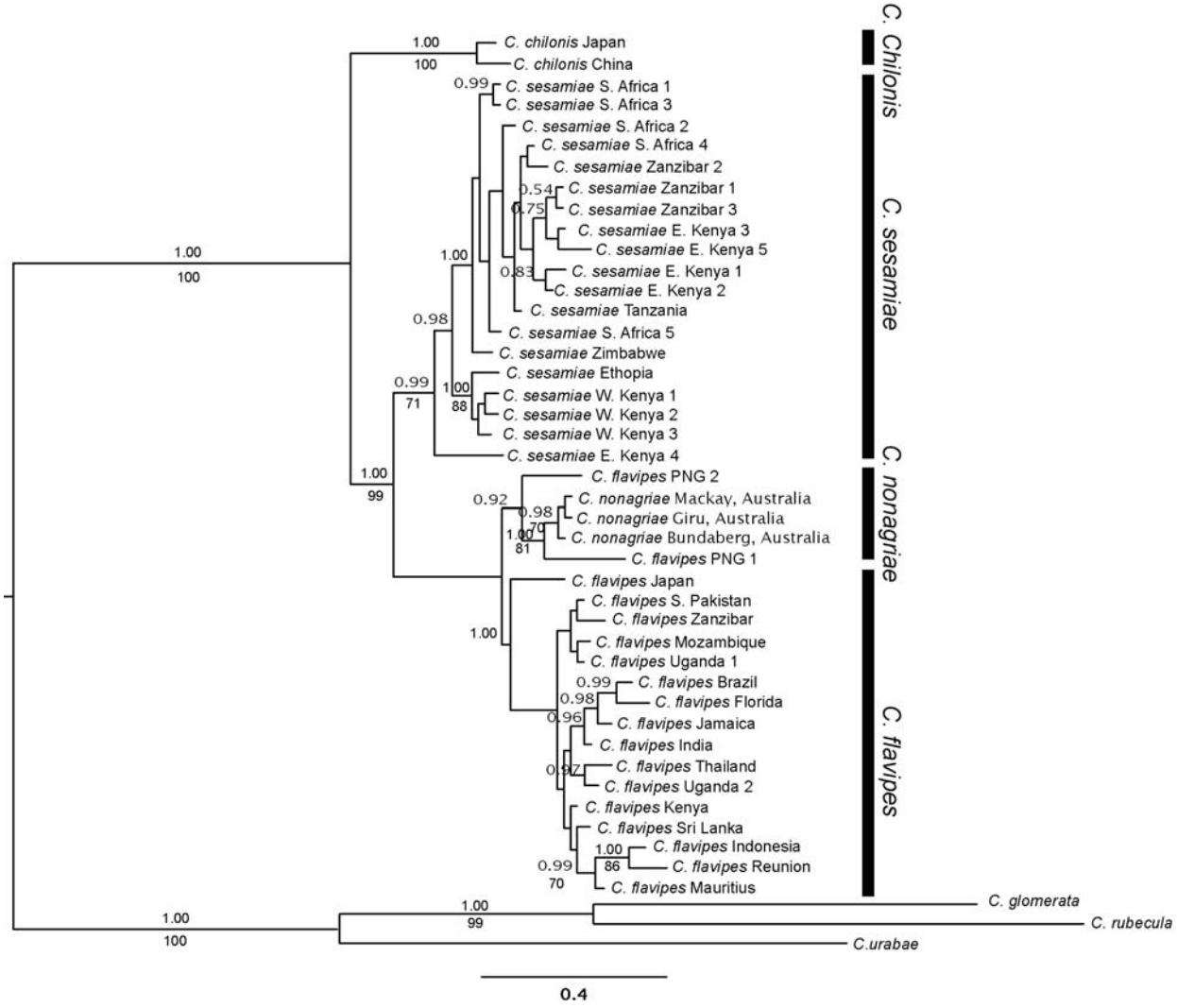

FIGURE 5.7. Bayesian trees derived from three anonymous nuclear loci for geographic populations of the *Cotesia flavipes* complex and three outgroups. Bayesian posterior probabilities ≥ 50%; numbers below the nodes indicate bootstrap proportions ≥ 70% values from 1000 pseudoreplicates of the MP analysis.

Incongruence between mitochondrial and nuclear data sets

Phylogenetic reconstructions based on the two mtDNA regions and three anonymous nuclear loci yielded trees with some incongruence. The main difference between the topologies was found in the *C. chilonis* + *C. sesamiae* relationship. The mtDNA phylogeny (Fig. 5.2) showed low support for a sister relationship between these taxa, while the nDNA tree (Fig 5.7) strongly supported a sister relationship between *C. sesamiae* and *C. flavipes* + *C. nonagriae*. Other differences were found in the placement of PNG 2 and the Japanese haplotype. The concatenated mtDNA phylogeny and the *COI* gene tree showed strong support for PNG2 within the *C.*

flavipes clade, whereas the nDNA tree found PNG 2 was more closely related to haplotyes of *C. nonagriae* + *C. flavipes* PNG 1. In contrast to the nDNA tree, the combined mtDNA phylogeny suggests that the Japanese *C. flavipes* haplotype falls within the *C. nonagriae* clade, a relationship also found in the gene trees for *16S*, *CfCN* and *CfEN*.

Combined mtDNA and nDNA analysis

Overall a total of 3257 base pairs (bp) were sequenced for the combined analysis, including partial *16S rRNA* and *COI* mtDNA nucleotide sequence data (*COI* = 550 bp, *16S rRNA* = 375 bp) and three anonymous nuclear loci (*CfBN* = 728, *CfCN* = 757, *CfEN* = 847). Maximum parsimony (MP) analysis of the combined data resulted in 5816 equally parsimonious trees, each with a tree length of 832 steps, with 334 parsimony informative characters. The MP bootstrap consensus (not shown) and Bayesian analysis (Fig. 5.8) yielded trees with similar topologies. Both analyses recovered the *C. flavipes* complex as a strongly supported monophyletic group showing four major species level clades, with a relationship of *C. chilonis* + (*C. sesamiae* + (*C. flavipes* + *C. nonagriae*)). Within *C. flavipes* there were five clades; I) PNG 1, II) Japan, III) PNG 2, IV) Mauritius + (Indonesia + Reunion) and V) the remaining 12 haplotypes distributed globally. There was also strong support for two major clades within the *C. sesamiae* group; haplotypes from South Africa, East Kenya, Tanzania, Zanzibar and Zimbabwe formed clade I, whereas haplotypes from west Kenya, Ethopia, and another from east Kenya formed clade II. Interestingly, the *C. flavipes* PNG 1 haplotype (Clade I) grouped with *C. nonagriae* in this analysis and all individual gene trees, whereas the PNG 2 haplotype (Clade III) was sister to the remaining *C. flavipes* in the combined tree but its position was variable among the other analyses.

When host data for individual haplotypes were mapped onto the combined analysis (Fig. 5.8) there were no obvious host specific clades. Most obvious is that many unrelated haplotypes of *C. flavipes* and *C. sesamiae* from East Africa utilized the same host species, *Chilo partellus*. Some clades, however, did show putatively specific host associations, for example between *C. chilonis* and *Chilo suppressalis, C. nonagriae* and *B. truncata*, and North and South American *C. flavipes* haplotypes with *Diatraea saccharalis*.

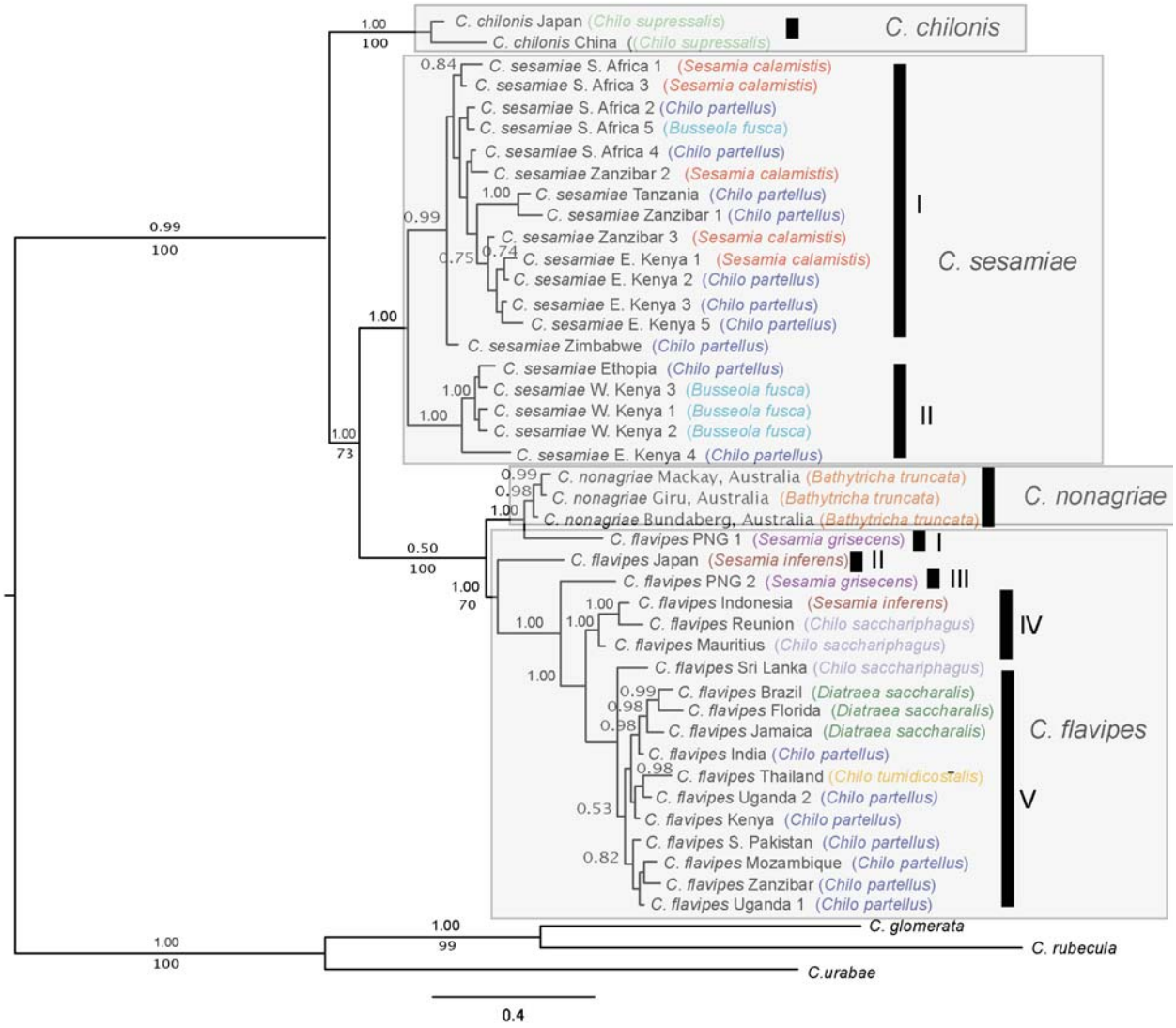

FIGURE 5.8. Bayesian tree derived from partial *16S rRNA* and *COI* mtDNA nucleotide sequence data and three single copy anonymous nuclear loci from geographic populations of the *Cotesia flavipes* complex and three outgroups. The numbers above the nodes represent Bayesian posterior probabilities $\geq$ 50%; numbers below the nodes indicate bootstrap proportions $\geq$ 70% values from 1000 pseudoreplicates of the MP analysis and parasitoid host data is shown in parentheses.

Discussion

Phylogenetic relationships

Phylogenetic reconstructions of the *C. flavipes* complex populations supported the monophyly of the complex, as have the previous more limited studies (Muirhead *et al.* 2006, Kimani-Njogu *et al.* 1998; Smith & Kambhampati 1999; Michel-Salzat & Whitfield 2004). The additional taxon sampling and sequencing of five molecular markers including both nuclear and mitochondrial genomes has resulted in the most detailed analysis of the complex to date. The combined analysis strongly supported a sister relationship between *C. sesemiae* and *C. flavipes* + *C. nonagriae*, with *C. chilonis* being basal to the rest of the complex. Although there were topological differences among datasets, they were mainly limited to a few haplotypes, in particular *C. flavipes* PNG 2 and Japan, and *C. sesamiae* East Kenya 4 and Zimbabwe.

Previous research has questioned the validity of *C. chilonis* as a discrete species to *C. sesamiae*, as no definite morphological characters have been established to distinguish the two species, whereas *C. flavipes* can be separated based on male genitalic morphology (Polaszek & Walker 1991; Muirhead *et al*. 2008). However, based on the molecular results here, *C. chilonis* and *C. sesamiae* were distinct taxa, with *C. chilonis* being sister to the remaining species in the complex.

Haplotype diversity and cryptic species

The combined mtDNA and nDNA analysis indicated haplotype divergence in geographic populations of *C. flavipes* and *C. sesamiae* that corresponded to biogeographic barriers and may represent the occurrence of cryptic speciation (e.g. '*C. flavipes'* from Japan). Furthermore, as discussed in Chapter II, the close relationships and low genetic diversity found among populations from the Asia, Africa, North and South America, and the Caribbean corroborated the movement history of *C. flavipes* for biological control introductions in these regions. For example, India and Pakistan were the source countries of several biological control introductions, including the New World (Mohyuddin *et al.* 1981; Mecedo *et al.* 1993; Potting 1996) and East Africa (Overholt *et al.* 1997) and thus, these haplotypes formed a major lineage within *C. flavipes* (Fig. 5.8; Clade V).

Likewise, this study showed strong support for a clade formed by haplotypes from Mauritius, Réunion and Indonesia (Fig. 5.8; Clade IV). This is a direct reflection of biological control introductions into these localities. *Cotesia flavipes* was reportedly introduced into Mauritius, Réunion and Madagascar in 1917 from Java (Breniere *et al.* 1985) and it is postulated that *C. flavipes* arrived in Mauritius from southeast Asia with its host *Ch. sacchariphagus* (Mohyuddin 1971; Rajabalee & Govendasamy 1988).

Perhaps the most significant finding of the study was the close relationship found between *C. flavipes* PNG 1 and Australian *C. nonagriae*, and the incongruent position of *C. flavipes* PNG 2, in the mtDNA and nDNA analyses. The position of PNG 1 strongly indicated that it was in fact a haplotype of *C. nonagriae* that occurs in New Guinea, but has been misidentified as *C. flavipes*. This is certainly feasible given the lack of morphological variation between the species (Muirhead *et al.* 2008) (Chapter III). Since the host of *C. nonagriae, B. truncata*, is apparenetly absent from New Guinea, an important question arises as to whether the host associations of PNG 1 is more similar to that of *C. flavipes*. For PNG 2, one possibility is that hybridisation and introgression of *C. flavipes* x *C. nonagriae* has occurred and has resulted in the strong discordance between the mitochondrial and nuclear markers for this population. There is also a possibility that *C. flavipes* is not endemic to New Guinea, but a hybridisation or introgression event has occurred with *C. flavipes* from Indonesia. However, much further sampling would be required to test these scenarios. The possibility of a natural hybrid zone to the north of Australia provides potential for future work on the issue of speciation and host specialisation. However, many taxa are threatened by hybridisation and introgression, and these are considered the greatest biological threats to native populations (Rhymer & Simberloff 1996). Thus, the possibility of hybridsation between *C. flavipes* x *C. nonagriae* requires further research, as the introduction of *C. flavipes* into Australia for biological control has the potential to greatly impact populations of *C. nonagriae*.

Host associations and biotypes

Phylogenetic analysis shows, overall, that members of the *C. flavipes* complex have not diversified into separate host specific lineages. However, the absence of a host associated population structure does not necessarily indicate lack of differential

fitness or behavioural variation in host preference by local populations. The success of a parasitoid is dependent of its ability to overcome the host's immune system. Encapsulation of parasitoid eggs and larvae by non-permissive host hemocytes is the primary cellular defensive mechanisms used by lepidopteran larvae when they are invaded by endoparasitoids. One primary mechanism used by *Cotesia* species is an endosymbiotic polydnavirus (PDV). PDVs are mutualistic hereditary viruses that are integrated in the wasp genome and play an important role in host immune suppression and, in turn, successful parasitism. The virus is injected into the host along with the parasitoid's eggs, allowing the wasp to disrupt the host's immune response (Whitfield 1994; Webb & Strand 2005). PDV genes have evolved through natural selection and are genetically linked to factors of suppression of local host resistance (Dupas *et al.* 2008).

The present study supports previous research on the presence of host-associated biotypes in *C. flavipes and C. sesamiae.* For example, the combined phylogenetic analyses revealed two lineages within *C. sesamiae*, showing divergence among haplotypes from West Kenya and Ethiopia, compared with East Kenya, South Africa, Tanzania, Zanzibar and Zimbabwe. Two wasp biotypes with different PDV genotypes have also been found for *C. sesamiae* in Kenya. A virulent *C. sesemiae* population originating from western Kenyan highlands can successfully develop on the native stemborer, *Busseola fusca.* However, avirulent populations from lowlands and the Eastern Province, where *B. fusca* does not occur, cannot complete development in this host (Ngi-Song *et al.* 1995; Ngi-Song *et al.* 1998; Mochiah *et al.* 2001, 2002; Gitau *et al.* 2006, 2007; Dupas *et al.* 2008). Moreover, virulent and avirulent *C. sesamiae* populations can interbreed (Mochiah *et al.* 2002). A similar trend has been reported in Zimbabwe between two reproductively compatible *C. sesemiae* populations occurring in the highveld (> 1200 m) and the lowveld (< 600 m) regions (Chinwada *et al.* 2003).

Likewise, *C. flavipes* haplotypes from Mauritius in this study formed a separate lineage (with Reunion and Indonesia), and have previously exhibited differences in morphology and biology from other populations of *C. flavipes*. Kimani-Njogu & Overholt (1997) found the morphology of the male genitalia from Mauritius to be slightly different from other populations. Moreover, the Mauritius strain is unable to

develop on the sympatric host *Sesamia calamistis* in sugarcane and maize (Rajabalee & Govendasamy 1988), whereas populations in Kenya can successfully utilise this host (Ngi-Song *et al.* 1995). Further work investigating variation in virulence for this strain may prove interesting, as host suitability exerts a diversifying selection pressure that can be the basis for genetic divergence and indeed speciation (Roush 1990).

Relevance to biological control

The existence of several genetically distinct and recognisable populations in this study should facilitate exploration into the coevolution of host-parastioid compatibility. Our results are immediately applicable to on-going work on the biological control efforts against stemborer pests worldwide. This work supports previous research that *C. sesamiae* and *C. flavipes* have populations that are locally adapted to certain hosts. Thus, research efforts could probably address the question of host compatibility in this complex via behavioural and ecological experiments. If local host-parasitiod compatibility can be established experimentally, then further work on reproductive isolation would be warranted to identify cryptic species. Together, such studies may provide additional insights into both the evolution of host use and diversification in this parasitoid complex and may be of great practical importance for the selection of the effective parasitoid populations for management of key stemborer pests.

Determining the potential and actual host range of a natural enemy is crucial before it can be imported and released for biological control purposes. The success of these parastioids is influenced by the host's immune response and the parasitoid ability to evade this system (Strand & Pech 1995). Haplotype divergence among immune-suppressing PDV symbionts between species and populations within the *C. flavipes* complex may have potentially important implications for host utilisation and, thus, the diagnosis of appropriate strains for biological control against specific host species. In this respect, further studies on polydnaviruses associated with these wasps may provide a better understanding of microevolution on different host species in this group.

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CHAPTER VI

Polydnavirus haplotype diversity in the *Cotesia flavipes* complex and phylogenetic incongruence between wasp and PDV phylogenies

Abstract

The *Cotesia* Polydnavirus *CrV1* gene evolves through natural selection and plays a key role in determining host range by immune suppression during the course of parasitoid development. A worldwide phylogeny of the PDV *CrV1* locus for the *Cotesia flavipes* complex was determined and cophylogeny between these wasps and their PDV symbionts was investigated. The results showed that there were numerous PDV *CrV1* haplotypes within populations of this small species complex. The overall cophylogeny fit between both trees was significant according to the topology-based program (TreeMap 1.0), but not according to the distance-based method (ParaFit), and few individual links were found to be significant. The diversity of *CrV1* strains is likely to be associated with adaptation to host community structure, with phylogenetic incongruence probably being a result of the ability of the PDV to coevolve with host resistance via natural selection, whereas the wasp genes are not under selection. The most important result of this study is the implication for the use of the *CrV1* locus as a virulence marker in biological control.

Introduction

The success of an endoparasitoid is influenced by numerous factors, including the host's immune response and a parasitoid's ability to evade the host's defence system (Strand & Pech 1995, Alleyne & Wiedenmann 2001a,b; Hufbauer 2002). One of the main defence mechanisms of insect larvae against endoparasitism is encapsulation (Alleyne & Wiedenmann 2001a). Encapsulation involves recognition by host hemocytes of foreign particles, subsequently resulting in an increase in the number of circulating haemocytes and, eventually, a multicellular capsule that kills the parasitoid (Lackie 1988). Parasitoids are able to evade this immune response of their habitual hosts via several means (Strand & Pech 1995; Pennacchio & Strand 2006), including ovarian proteins, polydnaviruses, venoms and possibly teratocytes (Edson *et al.* 1982; Dahlman 1991; Beckage & Kanost 1993; Webb & Luckhart 1994; Lavine & Beckage 1996; Webb & Luckhart 1996).

Polydnaviruses (PDVs) are virus-like symbionts that are integrated into the genome of some wasp groups that are endoparasitoids of lepidopteran larvae (Webb *et al.* 1998;

Belle *et al*. 2002). The viral genome is composed of multiple double-stranded DNA moecules that habour immunosuppressive genes that are expressed in the host and are essential for successful parasitism (Espagne *et al.* 2004; Kroemer & Webb 2004). When eggs are artificially injected into caterpillars without the viral particles, they are encapsulated by the host, indicating that the particles are essential for successful parasitism (Edson *et al.* 1981; Alleyne & Wiedenmann 2001). The PDV particles replicate in the ovarian clayx gland of the female wasp and there is no viral DNA replication in the parasitised host (Volkoff *et al.* 1995; Webb 1998; Wyler & Lanzrein 2003). PDVs are injected along with eggs during oviposition into the caterpillar host hemocoel. Following infection of cells in host tissues, PDVs interfere with the host endocrine system, which regulate host development, causing developmental arrest (Strand & Pech 1995; Pennacchio *et al.* 2001; Web *et al.* 2001; Beckage & Gelman 2004). The PDV genes express proteins that disrupt host physiology and are responsible for immune suppression during the course of parasitoid development (Shelby & Webb 1999; Turnbull & Webb 2002; Whitfield & Asgari 2003).

PDVs are classified as either bracoviruses or ichnoviruses, when associated with braconid or ichneumonid wasps. Recently, bracovirus particles have been shown to be derived from ancestral nudiviruses (Bézier *et al.* 2009), which are a group of insect viruses that have been shown to replicate in the reproductive tissues and cause sterility of insect pests (Burand 1998; Raina *et al.* 2000). Phylogenetic studies have shown that bracovirus-associated wasps form a monophyletic group known as the microgastroid complex (Whitfield 2002; Murphy *et al.* 2008). The PDV genome of *Cotesia congregata* (Braconidae) has been sequenced (Espagne *et al.* 2004) and encodes several products resembling virulence factors used by various parasites and pathogens, which has allowed for a better understanding of host-parasitoid virulence (Thoetkiattikul *et al.* 2005; Espagne *et al.* 2005).

Although PDVs are considered as key factors determining parasitoid host range (Whitfield 1994), phylogenetic separation of PDVs from closely related wasp species and populations has rarely been demonstrated (but see Whitfield 2000, Gitau *et al.* 2007; Dupas *et al*. 2008). The *Cotesia flavipes* species complex comprises a group of lepidopteran endoparasitoids that offer an excellent opportunity to explore PDV haplotype diversity and possible coevolution with their wasp carriers. Although

described as generalist parasitoids, certain *C. flavipes* populations display variation in host range that is associated with local adaptation to host community structure (Gitau *et al.* 2007; Dupas *et al*. 2008).

The PDV gene *CrV1*, was first detected in *Pieris rapae* (Linnaeus), the host larvae of the parasitoid *C. rubecula* Marshall. This gene is one of four major bracovirus gene products expressed in *P. rapae* tissues after parasitization (Asgari *et al.* 1996; Glatz *et al.* 2004). The gene product is a secreted glycoprotein that has been implicated in depolymerization of the actin cytoskeleton of the host haemocytes leading to haemocyte inactivation (Asgari *et al.* 1996). Haplotype variation at the *CrV1* locus has been found in several *Cotesia* species and cophylogeny has been demonstrated between the PDV *CrV1* gene phylogeny and the mitochondrial DNA phylogeny of these *Cotesia* species (Whitfield 2000).

To our knowledge, this is the first study to generate a phylogeny of the PDV *CrV1* locus and investigate cophylogeny among wasps and their PDV symbionts within and among closely related species. In Chapter V, we provide a wasp phylogeny that established the existence of several genetically distinct and recognizable geographic haplotypes (Fig 5.8). If parasitoid–host immune interactions vary geographically and are locally adapted to a different suite of hosts, we predict similar levels of haplotype diversity at the *CrV1* locus. Furthermore, since species-level cophylogeny was found in members of *Cotesia* and their PDVs (Whitfield 2000), we aim to determine if there is phylogenetic congruence at the population level. The results are discussed in relation to the evolution of host range and the use of PDV genes as potential markers for the diagnosis of appropriate strains for biological control against specific pest species.

Materials and Methods

Taxonomic sampling

Specimens from *C. flavipes* complex populations were sourced internationally from 15 countries and all have associated host/habitat information. The Australian populations were directly collected from three sugarcane-growing localities in Queensland. Parasitoids were either reared from wild collected hosts or sampled from colonies in research laboratories. The origin and host/habitat information of the 42

populations sampled and the three outgroups is provided in Table 5.1. All outgroups were sourced from the alcohol-preserved parasitoid collection at the Waite Insect and Nematode Collection at the University of Adelaide. Voucher specimens of most haplotypes have been lodged in this collection.

DNA extraction, amplification and sequencing

The PDV phylogeny was constructed using sequence data from a region of the *CrV1* gene. Genomic DNA was extracted from headless, whole wasps using the Gentra Systems Puregene® DNA Purification Kit (GentraSystems 2005). PCR amplification of the CrV1 gene (bp) was performed in 25 μl reactions using primers CrV1087F (5'-ATGTCACTCGTCAAAAGTGC-3') and CrV2107R (5'-AAAGTTTGCGATGGGGTTGT- 3') (Dupas *et al.* 2006). Each reaction comprised of PCR buffer, 0.2 mM of each dNTP, 0.5 μM of each forward and reverse primers, 2 mM $MgCl_2$, 0.5 units of AmpliTaq Gold® DNA Polymerase (Applied Biosystems Inc.) and 25-100 ng of genomic DNA. PCR conditions were: denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 45 sec, annealing at 50°C for 45 sec, and extension at 72°C for 30 sec. Final extension was at 72°C for 3 min. PCR products were purified using the Ultraclean™ PCR Clean-up™ Kit (MoBio Laboratories inc.) and sequenced using ABI Big Dye Terminator Chemistry (Applied Biosystems, Foster City, CA, USA) in 20 μl reaction volumes. Fragments were resolved on an ABI 3700 capillary sequencer (Applied Biosystems). DNA sequences were viewed and edited in Codon Code Aligner 2.0.4. (CodonCode Corporation). Forward and reverse consensus sequences were assembled and contigs were aligned with ClustalW in CodonCode.

Phylogenetic analyses

Phylogenetic reconstructions were conducted for the *CrV1* gene using Bayesian criteria of optimality and Maximum parsimony (MP) analysis. MP analyses, base frequency and pairwise distance calculations were performed using PAUP* version 4.0b10 (Swofford 2000), employing equal weights for all positions, with gaps treated as missing data. For MP methods the heuristic search algorithm options were used with stepwise addition and 100 random taxon addition sequence replicates. Support

values for each node (BSV) were examined by bootstrap analysis (Felsenstein 1985) from 1000 pseudoreplicates with full heuristic searches.

Bayesian analysis was implemented using MrBayes version 3.0b4 (Huelsenbeck & Ronquist 2001), incorporating the GTR+G model chosen by the Akaike information criterion in Modeltest (Posada and Crandall 1998). Analyses were run for five million generations sampling the four Markov chains every 100 generations. Stationarity was determined from an examination of log likelihood scores and model parameters. Trees recovered prior to stationarity being reached were discarded and Bayesian posterior probabilities of each bipartition, representing the percentage of times each node was recovered, were calculated from a consensus of the remaining trees. Multiple runs were performed to assess that all parameters were not considerably different at stationarity based on alternate prior probabilities.

Cophylogenetic analyses

The *C. flavipes* complex phylogeny based on two mtDNA and three anonymous nuclear loci (Chapter V) was used to examine cophylogeny of PDVs with their wasp hosts. The tree-based reconciliation method implemented in TreeMap 1.0 (Page 1994) reconciles the wasp and the PDV tree by introducing four types of events: cospeciation (C), host-switching (H), duplication (D), and sorting (S). Using parsimony the program attempts to explain the differences between both phylogenies by postulating the fewest possible number of events and maximizing the number of cospeciation events. A Randomisation test was performed with the proportional-to-distinguishable option on the reconstruction to determine whether the two phylogenies contain more copeciation events than expected by chance alone.

The distance-based method ParaFit (Legendre 2001; Legendre *et al.* 2002) was used to test the null hypothesis of random association between wasp and PDV distance sets. Distance matrices for wasp and PDVs were derived from ML estimates of pairwise genetic distances using model parameters derived from both hLRTs and the AIC, as selected by ModelTest. The programs DistPCoA (Legendre & Anderson 1998) and the R Package (Casgrain & Legendre 2001) were used to convert distance matrices into principal coordinate matrices. Tests of random association were performed with 999 permutations globally across both matrices and for each

individual host-parasitoid association.

Results

PDV phylogeny

A total of 618 base pairs (bp) of the *CrV1* gene were sequenced, of which 142 were parsimony informative. Maximum parsimony (MP) analysis resulted in five equally parsimonious trees, with a tree length of 324 steps. The MP bootstrap consensus (not shown) and Bayesian analysis (Fig. 6.1) yielded trees with similar topologies. Both analyses recovered the *C. flavipes* complex PDV as a strongly supported monophyletic group (P_{bay} = 0.97, BSV = 100), and also revealed significant haplotype variation among the four wasp species. The MP analysis recovered *C. chilonis* as sister to *C. sesamiae* PDV (Clade II) (BSV = 51%) instead of sister to all other *C. flavipes* + *C. nonagriae* PDV haplotypes (P_{bay} = 0.97), as in the Baysian analysis, and did not resolve the position of PNG 2 (Clade II), which fell between *C. nonagriae* (Clade VI) and *C. sesamiae* PDVs (Clade II).

Both analyses showed intraspecific variation for the *CrV1* gene with two major clades recovered within *C. sesamiae* haplotypes, i.e. Clade 1 (South Africa, Ethopia, Zimbabwe and East and West Kenya) and Clade II (Tanzania, Zanibar and East Kenya). *CrV1* variation also occurred in five clades recovered within *C. flavipes* haplotypes, i.e. PNG 1 (Clade I), PNG 2 (Clade II), Japan (Clade III), India, Pakistan, East Africa and the New World (Clade IV) and Thailand, Sri Lanka, Indonesia, Mauritius and Reunion (Clade V).

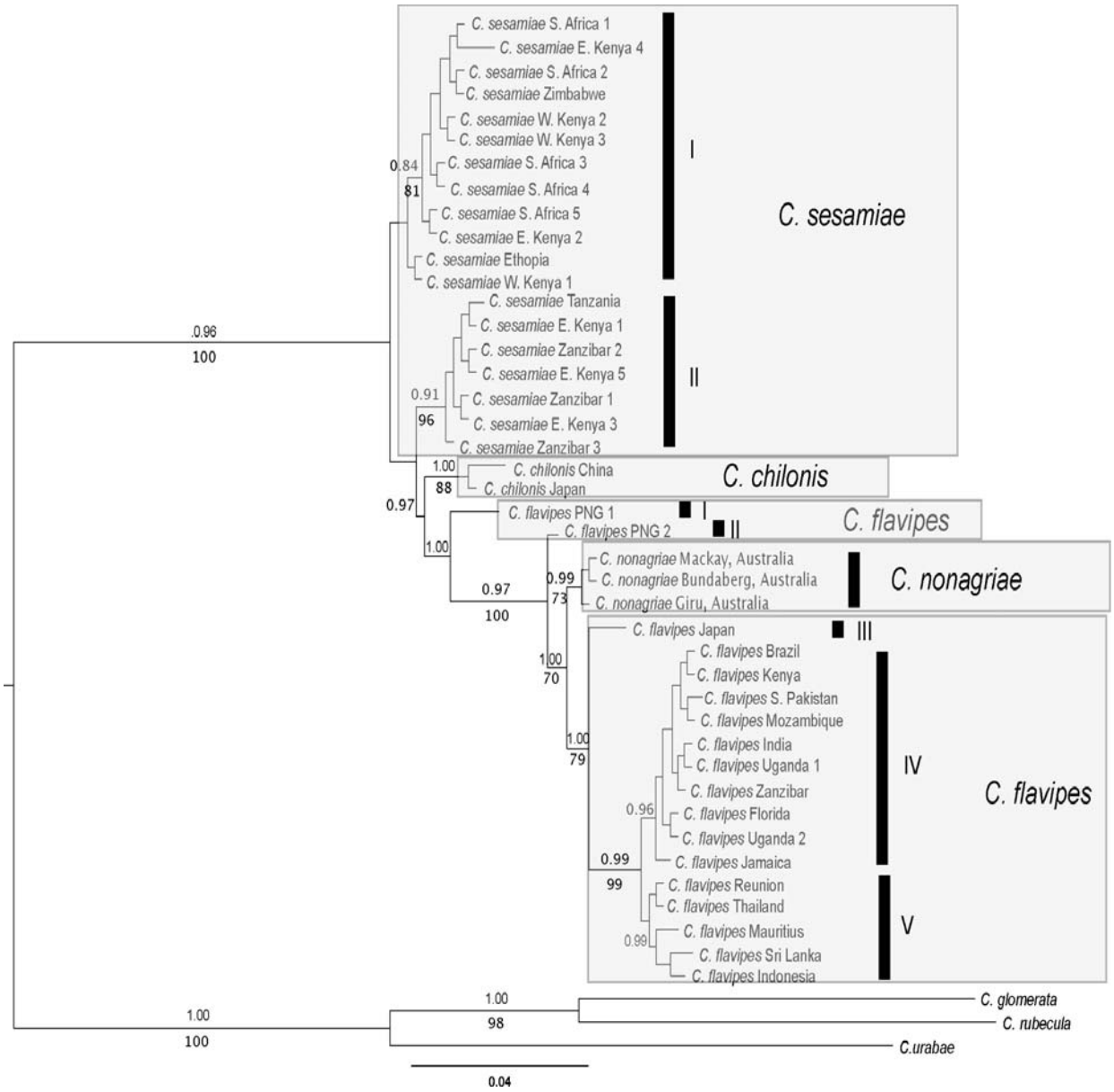

FIGURE 6.1. Bayesian tree derived from partial PDV *CrV1* nucleotide sequence data from geographic populations of the *Cotesia flavipes* complex and three outgroups. The numbers above the nodes represent Bayesian posterior probabilities ≥ 50%; numbers below the nodes indicate bootstrap proportions ≥ 70% values from 1000 pseudoreplicates of the MP analysis

When host data for individual haplotypes were mapped onto the PDV tree (Fig. 6.2) some host specific clades could be identified. Most obvious was the association between *C. chilonis* and *Chilo suppressalis, C. nonagriae* and *Bathytricha truncata*, and PNG *C. flavipes* with *Sesamia grisecens.* Although many unrelated haplotypes of *C. flavipes* and *C. sesamiae* from East Africa utilised *Chilo partellus*, haplotypes of *C. sesamiae* found on *Busseola fusca*, were all found in Clade I. Also notable was the

close relationship among haplotypes from Sri Lanka and the India Ocean islands utilizing *Chilo sacchariphagus* (Clade V).

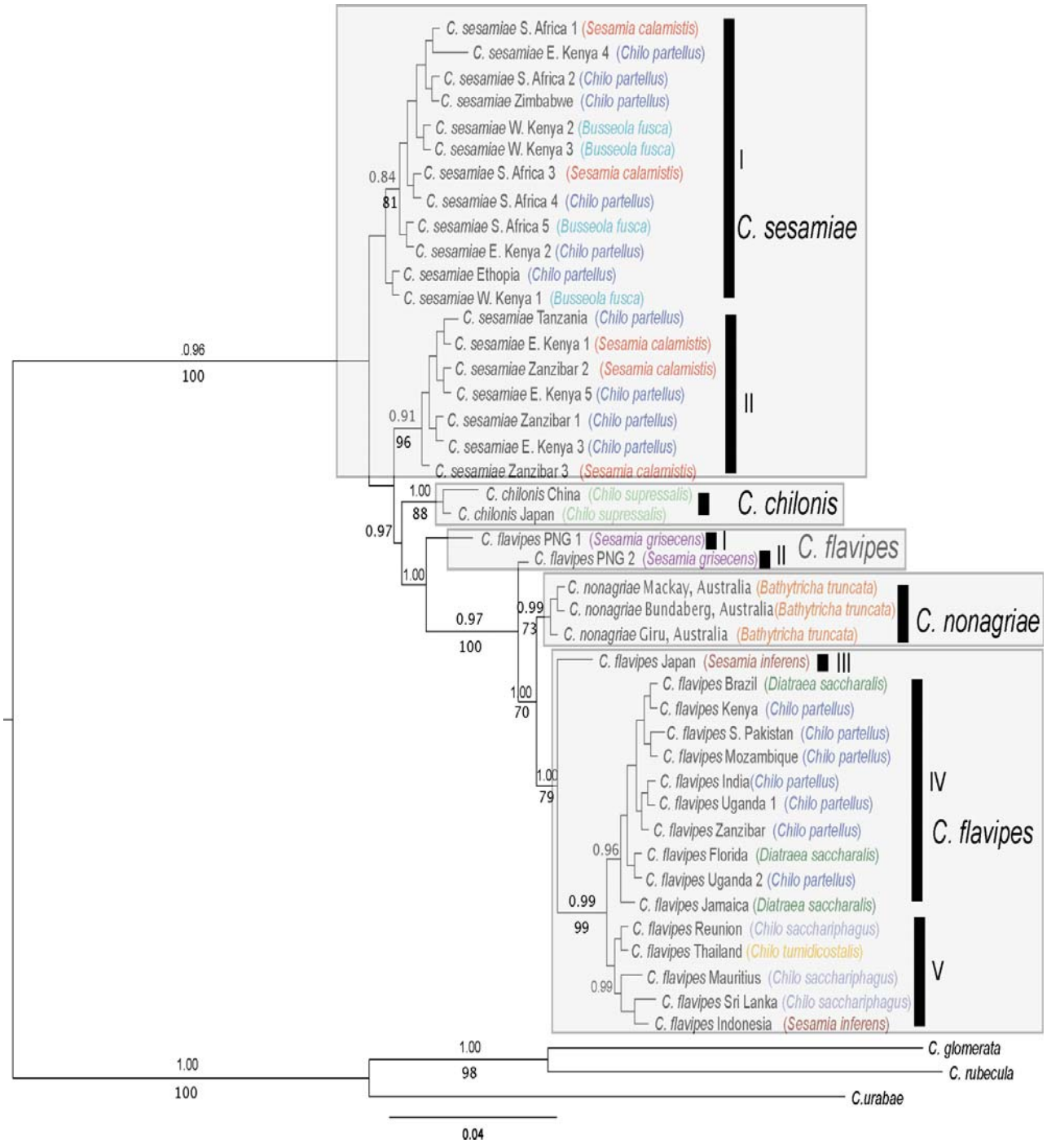

FIGURE 6.2. Bayesian tree derived from partial PDV *CrV1* nucleotide sequence data from geographic populations of the *Cotesia flavipes* complex and three outgroups (as in Fig. 6.1), showing parasitoid host data in parentheses.

Differences between wasp and PDV relationships

Variation was observed between wasp (Fig. 5.8) and PDV *CrV1* (Fig. 6.1) phylogenies in the relationships among the main species clades. Relationships among the wasp phylogeny were *C. chilonis* + (*C. sesamiae* + (*C. flavipes* + *C. nonagriae*)), whereas the PDV phylogeny recovered *C. sesamiae* Clade I (South Africa, Ethopia, Zimbabwe and East and West Kenya) as sister to all members of the species complex. In the PDV tree, *C. sesamiae* (Clade II) (Tanzania, Zanibar and East Kenya) was more closely related to *C. chilonis*, *C. nonagriae* and *C. flavipes* haplotypes than to *C. sesamiae* (Clade I). In the wasp phylogeny the relationships between the *C. sesamiae* haplotypes were also different, with Clade I comprising East Kenya, South Africa, Tanzanian, Zanibar and Zimbabwe haplotypes and Clade II formed by Ethopia, West Kenya and a single East Kenya haplotype.

Relationships with *C. flavipes* also differ between wasp and PDV trees; the wasp tree (Fig. 5.8) recovered PNG 1 (Clade I) with *C. nonagriae* haplotypes from Australia and PNG 2 (Clade III) as a separate lineage, but this was not the case in the PDV tree (Fig. 6.1) where both PNG haplotypes (Clades I & II) form separate lineages relative to all other *C. nonagriae* and *C. flavipes* haplotypes. There was also strong support for a clade comprising Indonesia, Reunion and Mauritius (Clade IV) in the wasp tree (Fig. 5.8) as sister to African, Thailand, New World and Asian haplotypes, whereas there was strong support for a clade formed by Reunion, Thailand, Mauritius, Sri Landa and Indonesia in the PDV tree (Clade V) (Fig 6.1).

Cophylogeny analyses

Without invoking any host-switching events, TreeMap 1.0 had to introduce 26 cospeciation events, 18 duplications, and 101 sorting events to reconcile the trees (Fig 6.3). Adding host-switching events (using a heuristic search) lead to 27 cospeciation events, 16 duplications, one host-switching, and 97 sorting events. By randomising the PDV tree, a null frequency distribution was generated, with the observed number of cospeciations significantly higher (P <0.001) than expected by chance (Table 6.1). The percentage of cospeciating nodes (i.e. the number of cospeciating nodes divided by the total number of nodes in the PDV phylogeny, x 100) was 31%.

The global test of cospeciation using Parafit (Table 6.1) on the complete data set of raw distances did not reveal a global association between the wasps and their PDVs (P = 0.27). Considering the individual wasp-PDV links, only 10 of the 45 links between wasps and PDVs were significant, including *C. flavipes* from Sri Lanka and India, and *C. sesamiae* from South Africa 1 & 3, Ethopia, Zanzibar 4, West Kenya 1 & 2, East Kenya 1 and the outgroup, *C. rubecula.* To determine if there was cospeciation at the population level for more closely related species, separate matrixes were constructed from the data set of *C. flavipes*/*C. nonagriae* haplotypes (21 haplotyes) and *C. sesamiae*/*C. chilonis* (21 haplotypes), the result of the global test was significant (P = 0.046) for *C. flavipes*/*C. nonagriae* haplotypes, but not significant for *C. sesamiae*/*C. chilonis* (P = 0.107). There were 10 significant wasp-PDV links for *C. flavipes/C. nonagriae* including, Sri Lanka, Indonesia, Japan, Mauritius, Brazil, South Pakistan, Mozambique, Thailand, Zanzibar and Uganda 2. In contrast, when taking *C. sesamiae*/*C. chilonis* separately, there were only five significant links, i.e. between *C. sesamiae* from South Africa 1, East Kenya 3 & 5, Zanzibar 3 and *C. chilonis* (Japan).

TABLE 6.1. Probabilities computed by ParaFit. The H_o hypothesis of the global test is that the wasps and PDVs evolve at random. Probabilities with a * are significant at a level of 5%.

Test	**Probaility**
TreeMap	0.001 *
Parafit: whole data set	0.270
Parafit: *C. flavipes C. nonagriae*	0.046 *
Parafit: *C. sesamiae/C. chilonis*	0.107

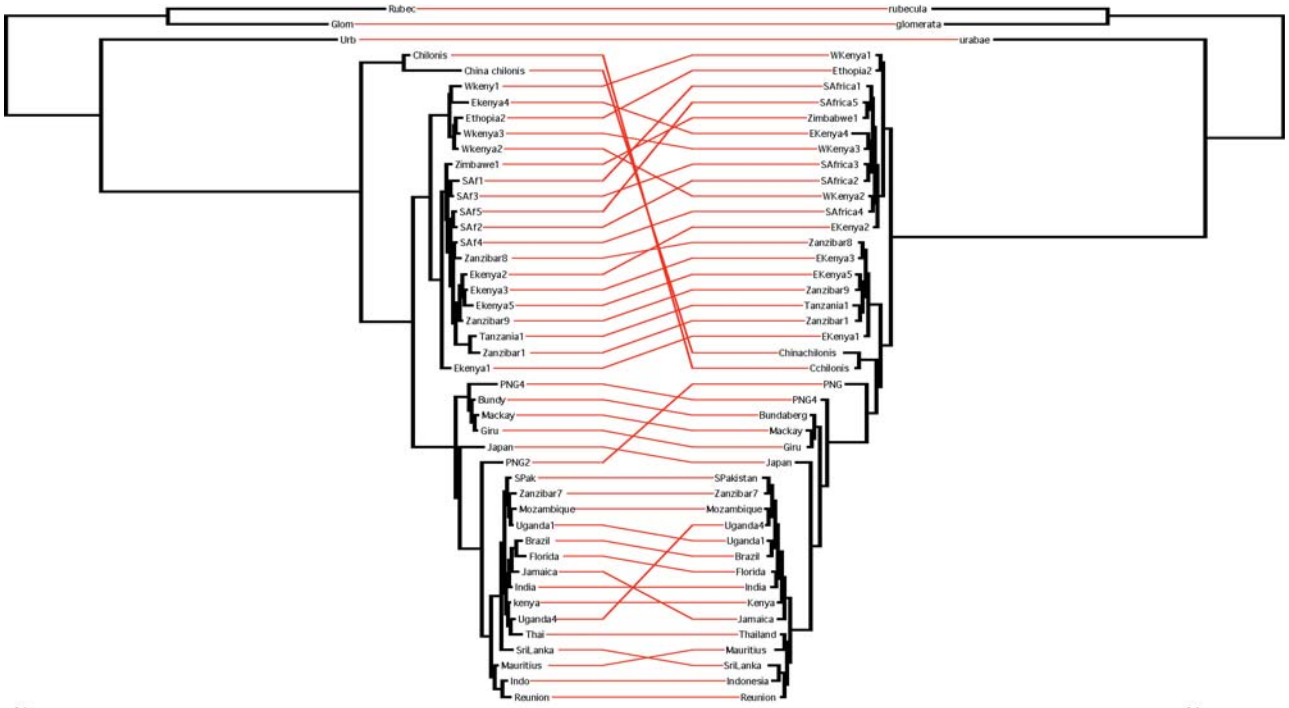

FIGURE 6.3. Tangle from TreeMap 1.0 showing wasp phylogeny on the left (from Fig. 5.8) and PDV tree on the right (from Fig. 6.1) with lines connecting coexisting wasps and PDVs.

Discussion

This study is the first to demonstrate the occurrence of numerous PDV lineages among closely related populations of a small species complex. Previous studies have shown that the PDV *CrV1* gene evolves through natural selection and is genetically linked to factors of suppression of local host resistance (Gitau *et al.* 2006, 2007; Dupas *et al.* 2008) and, thus, is a key factor determining host range (Whitfield 1994). For example, in Kenya, *C. sesamiae* has been shown to vary its developmental success on one of its major hosts, *B. fusca*. Two biotypes have demonstrated variation in virulence that was correlated with average stem borer community composition (Gitau 2006, 2007). In addition, the geographic distribution of *CrV1* alleles in Kenyan *C. sesamiae* was correlated with the relative abundance of the native host, *B. fusca.* (Dupas *et al.* 2008).

The existence of two major PDV clades in *C. sesamiae* in the present study corresponded to that documented for Kenyan *C. sesamiae* variants (Dupas *et al.* 2008). When host data was mapped onto the PDV tree (Fig 6.2), the *CrV1* haplotypes utilising *B. fusca* are grouped together in Clade I, while *B. fusca* does not appear as a host in Clade II. This host was found in both *C. sesamiae* clades in the wasp phylogeny (Fig 5.8), suggesting that wasp DNA may not be an appropriate diagnostic tool for determining host strains in these populations. Moreover, in the wasp tree (Fig. 5.8) there was a sister relationship between *C. sesamiae* clades, whereas in the PDV tree (Fig. 6.2) Clade II *C. sesamiae* and *C. flavipes* were more closely related, a result also shown for Kenyan populations of *C. sesamiae* PDVs (Dupas *et al.* 2008). This is an interesting result considering previous studies have shown that *C. flavipes* is avirulent to *B. fusca* (Ngi-Song *et al.*1995; Ngi-Song *et al.* 2001). Further, crossing experiments have demonstrated that virulent and avirulent *C. sesamiae* represent a single interbreeding species (Mochiah *et al.* 2002), however *C. sesamiae* and *C. flavipes* do not interbreed (Kimani & Overholt 1995). Therefore, the relationship of avirulent PDV strains from *C. sesamiae* and *C. flavipes* at the *CrV1* locus may be ancestral and where virulence was acquired (Dupas *et al.* 2008).

Phylogenetic reconstruction of the *CrV1* locus showed grouping of host specific lineages (Fig. 6.2), some of which were not found when host data was mapped onto the wasp phylogeny (Fig 5.8). As mentioned above, the association between *B. fusca*

and Clade I of *C. sesamiae* was not found in the wasp-host associations. In addition, in the PDV tree (Fig 6.2) haplotypes from Sri Lanka and the India Ocean islands utilising *Ch. sacchariphagus* fell into Clade V, a relationship not found in the wasp tree (Fig. 5.8). One explanation for this is that the PDV is under local selection, whereas the patterns of variation in the wasp phylogeny correspond to biogeographic barriers of dispersal and the movement history of these species for biological control introductions (Chapter V). In the *CrV1* phylogeny, however, there are shared hosts among some lineages, due to the movement of these wasps and the development of novel parasitoid-associations. One example of this is the use of *C. flavipes* against *Diatraea saccharalis*, which is a novel parasitoid-host association (Alam *et al.* 1971; Fuchs *et al.* 1979; Rossi & Fowler 2003). Unlike classical biological control, which reunites coevolved natural enemies with their native hosts, novel parasitoid-host associations can develop when related host species occur in the same niche as the ancestral host species (Hokkanen & Pimentel 1989). Potting *et al.* (1997b) investigated reproductive success among strains of *C. flavipes* from Pakistan, Texas and Thailand on *Ch. partellus* and the New World host, *D. saccharalis.* All strains had a lower survival rate on *D. saccharalis* compared to the ancestral host, *Ch. partellus*. However, the Texas strain, which had the longest period of co-existence with the new host, had the highest survival rate on *D. saccharalis* compared to other strains tested, suggesting the existence of adaptive evolution. In the present study, the *CrV1* phylogeny *C. flavipes* haplotypes from the Asia, Africa and the New World formed a lineage and have a host association with *Ch. partellus* and *D. saccharalis*, whereas all other *CrV1 C. flavipes* populations diversify to other lineages and utilise different hosts.

Another host that was associated with different lineages is *Sesamia inferens* from Japan and Indonesia. One explanation for this variation may be that the *CrV1* gene is coevolved with a suite of different hosts in these regions. Three species that have been recorded in Indonesia but do not occur in Japan are *Ch. auricilius, Ch. infuscatellus* and *Ch. polychrysus* (Sallam 2003). However, an alternative explanation is that the Japanese *C. flavipes* is a cryptic species, and the wasp-PDV integrated genome may be evolving at different rates. Selection can also occur at other PDV loci that are genetically linked with *CrV1* (Asgari *et al*. 2003). This may also be the case for two haplotypes of *CrV1* from PNG. Although they are both from the same region and

utilise the host *Sesamia grescens*, PNG 1 was more closely related with *C. nonagriae* and PNG 2 may be a hybrid between *C. flavipes* and *C. nonagriae* (see discussion in Chapter V). However, the divergence of both PNG haplotyes from Australian *C. nonagriae* is most likely because the Australian host, *B. truncata*, does not occur in PNG.

Phylogenetic congruence between wasps and PDVs was imperfect or absent in some interactions at the population level, in contrast to the perfect cophylogeny found for several species of *Cotesia* (Whitfield 2000). This may be because the wasp species/populations from the present study are very closely related and parasitise only stemborer hosts from two families, Crambidae and Noctuidae, whereas the wasps previously studied by Whitfield (2000) are more diverged and most utilise different host groups. The species in the *C. flavipes* complex also share host species, such as *C. flavipes* and *C. sesamiae* on *Ch. partellus* in Africa, although this is a novel association for *C. sesamiae*. Phylogenetic incongruence may also be a result of the ability of the PDV to coevolve with host resistance via natural selection, where the wasp genes may not be under host selection.

It is also worth noting that it is unknow whether these tests of coevolution are appropriate for wasp-PDV data, given that they were developed for independent host-parasite associations, such as mammals and lice (e.g. Hafner *et al.* 1994; Light & Hafner 2008), whereas PDVs are integrated into the wasp genome. In addition, no horizontal transfer of PDVs occurs during *Cotesia* evolution, whereas parasites can diverge by host-switching.

The most important result of this study is the implication for the use of the *CrV1* locus as a virulence marker in biological control. The occurrence of multiple *CrV1* lineages within these species may explain previously reported failures in biological control programs, where different strains were needed for the parasitoid to establish (Mohyuddin 1971; Mohyuddin *et al.* 1981; Inayatullah 1983; Mohyuddin 1990; Shami & Mohyuddin 1992). Identifying genetic variation in these parasitoids is useful for establishing evolutionary history and geographic populations. Nonetheless, wasp genes appear unsuitable as diagnostic tools for identifying correct host-specific strains. The use of the *CrV1* for identifying host strains requires further research, since

the *CrV1* locus coevolves with host resistance via natural selection. However, future studies would benefit from a more complete knowledge of local host community structure. Given the tight linkage between PDV and caterpillar host, coevolution at the population level is more likely to appear in PDV-caterpillar associations, rather than between the PDV and their wasp carrier. Thus, investigating coevolution between PDVs and stemborer hosts and examining the success of different *CrV1* haplotypes on a range of host species is likely to be a profitable avenue of research.

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CHAPTER VII

General Discussion

Successful biological control is dependent on reliable taxonomy and the correct identification of both the target pest and its natural enemies. Likewise, the recognition of cryptic species or intraspecific variation is of utmost importance when selecting source populations, as complexes of narrowly specific parasitoids may be incorrectly viewed as polyphagous species (Sands & van Driesche 2004). This thesis addressed the need for sound taxonomy in the *C. flavipes* complex, an important group of biological control agents with an intricate taxonomic and ecological history. This study has provided the first phylogeny and information on molecular genetic variation among multiple geographic populations of this complex and their PDV symbionts. Moreover, the research investigates the taxonomy and biology of an Australian member of the complex, which is directly applicable to the threat of stemborer incursions into Australia, where major pest species do not occur.

Phylogenetics and geographic structure

As outlined in the introduction to this thesis, the failure to distinguish between closely related species and host-specific strains is the main cause of repeated failures in past efforts to use these parasitoids in the biological control of stemborer pests. With the exception of a few well-studied populations of *C. sesamiae* from Kenya (Gitau *et al.* 2006; 2007; Dupas *et al.* 2008), phylogenetic relationships and genetic variation in the *C. flavipes* complex have been poorly understood. Work presented here in chapters II and V is the first step towards providing a robust phylogeny of these species. This study has shown that these parasitoids, which are difficult to differentiate on the basis of their morphology due to intraspecific variation (Polaszek & Walker 1991; Muirhead *et al.* 2008), are clearly separate genetically, based on mtDNA and nDNA. DNA sequence analysis has confirmed the species status of the four members of the complex, *C. flavipes, C. sesamiae, C. chilonis* and *C. nonagriae*. Using the phylogeny from this study it should be possible to use DNA markers to identify any morphologically indeterminate specimens. Phylogenetic reconstructions from this study demonstrated the value of the *COI* gene for distinguishing species of this complex. The topology of the *COI* phylogeny (Fig. 5.1) was very similar to the relationships found in the multigene phylogeny (Fig 5.8). Analysis of mtDNA *COI* sequences confirmed the species-level separation of *C. chilonis* from *C. sesamiae* and

C. nonagriae from *C. flavipes*. The use of a single marker barcode relies on a solid taxonomic foundation, including adequate sampling of variation within species (Moritz & Cicero 2004). Using *COI* sequence data could rapidly and accurately recognize most species and may help resolve problematic classifications of closely related taxa. Moreover, within *C. flavipes* complex species complex there is sufficient structure that it will be possible to allocate an individual to a particular geographic population using *COI*. Although this holds true for most populations, those from PNG are the exception. The *COI* barcode would not resolve the possible existence of *C. flavipes* x *C. nonagriae* hybridisation in PNG. The strong discordance between the mitochondrial and nuclear markers for this population is one example of how a single marker would not suffice for delineating species boundaries. This reflects a real need to use multiple markers at the onset of a study so that problem cases and errors can be detected. The possible hybridisation scenario in PNG remains problematic and requires further research and extensive sampling.

Species boundaries

Delineating species boundaries in parasitic Hymenoptera is often difficult due to the prevalence of cryptic species (Campbell *et al*. 1993; Wiedenmann & Smith 1997; Chinwada *et al.* 2003; Kankare *et al.* 2005a,b; Rincon *et al.* 2006). Species determinations should make use of a range of evidence, including molecular systematics, crossing experiments and an examination of life history traits (Templeton 1989). Speciation involves a certain proportion of a population becoming isolated from the original population in such a way that gene flow is interrupted. In this study, the isolated Australian species, *C. nonagriae,* was recognised as a distinct species based on phylogenetic separation and variation in life history traits (Chapter VI). Most notable of these differences was the propensity of *C. nonagriae* females to allocate an average of 100 eggs into each host, three times more than that recorded for other species in the *C. flavipes* complex. Females of all species in the complex have a potential fecundity (initial egg load) of ~200 eggs, however *C. nonagriae* allocates most of her eggs into just two hosts, whereas other members utilise four to five. The tendency of *C. nonagriae* to allocate a large number of eggs to each host may be an evolutionary strategy due to the high mortality rate (50-57%) of ovipositing adult wasps, however high mortality rates have also been found in other members of the complex. It may also be an evolutionary strategy due to the utilisation of only a single

moderately rare host in Australia, whereas overseas there are multiple hosts and significant pest outbreaks. This may also explain why the adult life span is longer in these species, as wasps may spend more time searching for hosts.

Previous studies have shown that male genitalia is the only characteristic that has proven reliable for separating species in the *C. flavipes* complex into two morphospecies; the *C. sesamiae*/*C. chilonis* subcomplex and *C. flavipes* (Fig. 1.2) (Polaszek & Walker 1991). Taxonomy research as part of this study showed that the male genitalia of *C. nonagriae* was very similar to *C. flavipes* (Chapter III). This begs the question as to whether *C. flavipes* and *C. nonagriae* would be able to interbreed in nature, as appears to be a possibility in PNG. Interspecific crosses of *C. sesamiae* males x *C. chilonis* have previously resulted in viable female offspring (Kimani & Overholt 1995). In addition, *C. sesamiae* males responded to pheromones emitted by *C. chilonis* females, indicating that mating between these two could occur. However, prior to the movement of these species for biological control, these species were allopatric and did not overlap in their distribution. The question of whether a *C. flavipes* x *C. nonagriae* hybrid is possible requires more research and is an important issue in biological control. Although these species have similar genitalia, there may be other pre-zygotic isolating mechanisms, such as courtship behaviours, or post-zygotic mechanisms, such as *Wolbachia* induced cytoplasmic incompatibility (Breeuwer & Werren 1990; Bordenstein *et al.* 2001; Branca & Dupas).

Recent studies have found differences among members of the complex in the duration and frequency of wing fanning vibration in the courtship song (Joyce *pers. comm.*). Furthermore, certain populations of these species are infected with *Wolbachia* (Branca & Dupas 2006), including the Australian populations (Muirhead *unpublished*) *Wolbachia*-infected males are unable to successfully reproduce with uninfected females. Moreover, certain populations of *C. sesamiae* have displayed bidirectional cytoplasmic incompatibility, involving a male and a female carrying different strains of *Wolbachia* (Branca & Dupas 2006). Such a situation could isolate populations which are carrying different strains of *Wolbachia*. This was shown in two wasp species, *Nasonia giraulti* and *N. longicornis,* which harbour different *Wolbachia* strains. The incompatibility caused by this bacterium resulted in total reproductive isolation and speciation of infected and uninfected populations (Breeuwer & Werren

1990; Bordenstein *et al.* 2001).

Biotypes and polydnavirus

The coevolution between hosts and parasitoids is a classic evolutionary arms race, with hosts evolving morphological, behavioural, biochemical or immunological defence mechanisms. Parasitoids, on the other hand, have to adapt to these defences or, as an alternative strategy, attack related susceptible host species. Understanding these adaptive processes will contribute to better management practices in biological control. Larval survival in the host may be the most discriminatory stage in determining the host range of a parasitoid (Van Driesche & Murray 2004). Members of the *C. flavipes* complex must manipulate and overcome the immune response to maintain a favourable environment for their developing offspring. The complex mechanisms by which these parasitoids influence host defences include symbiosis with polydnaviruses (Shelby & Webb 1999; Beckage & Gelman 2004). This study was the first to investigate haplotype diversity and phylogenetic relationships in geographic populations of members of the *C. flavipes* complex. Results indicate that there are numerous PDV variants within and among species. Most PDV strains were linked with different host species or groups of hosts, except those where novel associations had formed. Novel associations in this study include *C. flavipes* and New World *D. saccharalis*, and the native African parasitoid, *C. sesamiae*, and the introduced pest, *Ch. Partellus*. In both the wasp and PDV phylogenies *C. flavipes* populations from the New World, Asia and Africa formed distinct lineages within these trees. In the wasp phylogeny, there was geographic structure within this *C. flavipes* lineage, separating New World populations from Old World. This structure was not found in the PDV phylogeny among haplotypes on *Ch. partellus* and *D. saccharalis*, which suggests that these novel hosts were physiologically equivalent to ancestral hosts (Wiedenmann & Smith 1997). Similarly, both *C. sesamiae* and *C. flavipes* utilised the host *Ch. partellus* so this host was found in two *C. sesamiae* clades and one *C. flavipes* lineage.

In Sub-Saharan Africa, the main pest are the native species, *B. fusca* and *S. calamistis*, and the introduced pest, *Ch. partellus*. *Cotesia sesamiae* attacks all these borer species, however, two biotypes exist, a virulent biotype that successfully develops on *B. fusca* and an avirulent biotype that is unable to avoid encapsulation by *B. fusca*

(Gitau *et al.* 2007; Dupas et al. 2008). This study confirmed the findings of these previous studies, as two alleles were found at the *CrV1* locus, an inland allele linked to virulence on *B. fusca* and the Coast allele linked to avirulence. Moreover, avirulent strains of *C. sesamiae* were more closely related to *C. flavipes*, which is also avirulent to *B. fusca* (Ngi-Song *et al.*1995; Ngi-Song *et al.* 2001).

Populations that deserve further examination are Japanese and Mauritius *C. flavipes*. Depending on the marker employed, the Japanese haplotype fell out as sister to *C. nonagriae* or, as found in the combined data set (Fig. 5.8), as sister to all other haplotypes of *C. flavipes.* Like the Australian species, this population was isolated, utilized different hosts and had a different PDV variant. Thus, the Japanese taxon may represent a cryptic species or a distinctive biotype. Investigations into the life history and ecology of this population would help to clarify this matter. The Mauritius haplotype formed a clade with Reunion and Indonesia (and sometimes PNG) in the wasp phylogeny. This clade was also found in the PDV phylogeny, with the inclusion of Thailand and Sri Lanka. *Cotesia flavipes* was introduced into Mauritius and Reunion with its host, *Ch. sacchariphagus* in 1917 (Rajabalee & Govendasamy 1988; Ganeshan & Rajabalee 1997). The separations of these haplotypes from other *C. flavipes* haplotypes is an interesting result considering previous studies investigating Mauritius populations have found differences in the morphology of the male genitalia (Kimani-Njogu & Overholt 1997). Moreover, the Mauritius strain is unable to develop on the sympatric host *Sesamia calamistis* in sugarcane and maize (Rajabalee & Govendasamy 1988), whereas populations in Africa have been found to successfully utilise this host (Ngi-Song *et al.* 1995).

Future directions

Understanding the factors that influence diversification is a major challenge in biological control and evolutionary biology. Members of the *C. flavipes* complex have been studied intensively for their role in biological control of stemborer pests. However, much remains to be learned about the immune suppression role of polydnaviruses and the formation of host-specific strains in these species. Only one study to date has demonstrated the role of PDV variation in natural populations of these wasps. Dupas *et al.* (2008) observed positive Darwinian selection at specific amino acid sites among *CrV1* variants between virulent and avirulent *C. sesamiae*

strains on the stem borer host, *B. fusca*. The phylogenetic structure seen within PDV haplotypes in this study suggests that other populations may have narrower host ranges. Previous experiments on host suitability of *C. flavipes, C. sesamiae* and *C. chilonis* indicate that, although these wasps are taxonomically, behaviorally and ecologically very similar, they differ in their ability to utilise host species (Allyene & Wiedenmann 2001b). Moreover, parasitism in related hosts can produce different outcomes in success (Potting et al. 1997b; Allyene & Wiedenmann 2001b).

The PDV phylogeny presented in this project can be used as a framework for the identification of unrecognised biotypes. Previous studies have shown that the PDV *CrV1* gene evolves through natural selection and is genetically linked to factors of suppression of local host resistance (Dupas *et al.* 2008). Thus, the use of PDV *CrV1* locus may prove to be a valuable diagnostic tool for identifying host strains within the complex. Future studies investigating coevolution between *CrV1* variants and their stemborer hosts will broaden our understanding of the host specificity in these parasitoids. These studies will require an extensive knowledge of host community structure to determine if geographic PDV strains are coevolving to a suite of hosts.

Biological control in Australia

Australia has remained free of serious stemborer pests due to its isolation and strict quarantine procedures. However, maintenance of this pest-free status is being threatened by a number of stemborers from the neighboring countries, such as PNG and Indonesia. FitzGibbon *et al.* (1998) identified 12 stemborer species in the genera *Chilo*, *Sesamia* and *Scirpophaga* to the immediate north of Australia that pose threats to the Australian sugar industry. The research described in this thesis was directly related to the incursion management strategies for these pests.

Further research on the Australian species, *C. nonagriae*, is necessary to facilitate its use in future biological control programs. Prior to any release attempts of a natural enemy, a comprehensive study of its geographical distribution, host specificity and host range is required. Laboratory tests will help to establish the physiological host range of *C. nonagriae* on certain extralimital pest species. These tests need to determine if this parasitoid can form novel host associations and complete

development on them. Moreover, host range testing determines if there is variability in parasitoid-host compatibility and reproductive success.

Host specificity assessments have to focus not only on physiological host range, but also on ecological host range (McEvoy 1996; Strand & Obrycki 1996). Ecological host range is influenced by factors including climate, phenology and behaviour of both the host and natural enemy. Successful oviposition by parasitoids is dependent on the ability of the female to locate and accept the host. Female parasitoids locate hosts in complicated and heterogeneous environments via chemical and physical cues emitted by the host insect and/or the host's food plant (Vet and Dicke 1992). Experiments carried out in Chapter IV showed that during microhabitat location, both naïve and experienced *C. nonagiae* females demonstrated a strong response towards the sugarcane/*B. truncata* plant host complex. However, tests are needed to determine if *C. nonagriae* females will respond to the cues elicited by other stemborers species on sugarcane.

If *C. nonagriae* is incompatible with high threat species, compatible strains of *C. flavipes* may need to be imported into Australia for biological control if a serious pest incursion occurs. Before this can happen, tests will need to be carried out to determine if *C. flavipes* and *C. nonagriae* are able to interbreed and produce viable female offspring. This has the potential to greatly impact on the conservation of this native parasitoid, and may also affect the natural control of *B. truncata* in Australia. Likewise, host range testing of *C. flavipes* will need to investigate the suitability of *B. truncata* as a host, which will help to determine whether there will be competition between *C. flavipes* and *C. nonagriae,* as well as the potential of *C. flavipes* to parasitize non-target native hosts. The host range of a biological control agent in the area of origin is potentially useful as a guide to likely host range in new areas of introduction. If available, this information can assist in risk assessment and prediction of post-release impacts, and is an important first step in the process of biological control agent host specificity testing, particularly selection of test species (Kuhlmann *et al.* 2005).

In conclusion, this is the first study to investigate the status of *C. flavipes* in Australia and geographic populations of the *C. flavipes* complex using wasp DNA and PDV

sequence data. Results show that geographical barriers and biological control introductions play a key role in structuring wasp populations, whereas PDV variants are likely to be coevolving with host community structure. The data suggests that local variation in the use of resources has produced genetically divergent PDV strains that may be useful as a diagnostic tool for identifying host specific wasp strains. The phylogeny presented here can be used to examine the presence of cryptic species and biotypes and investigate species boundaries. Additional studies are needed to determine the reproductive success of *C. nonagriae* on high threat stemborer pests to the Australian sugar industry. This will help to facilitate a rapid biological control response at the onset of any future pest incursion.

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APPENDIX I

Published papers

Muirhead, K.A., Murphy, N.P., Sallam, M.N., Donnellan, S.C. and Austin, A.D. (2006) Mitochondrial DNA phylogeography Of The Cotesia flavipes complex of parasitic wasps (Hymenoptera: Braconidae).
Annals of the Entomological Society in France, v. 42 (3/4), pp. 309-318, 2006

Assefa1, Y., Mitchell, A., Conlong, D.E. and Muirhead, K.A. (2008) Establishment of *Cotesia flavipes* (Hymenoptera: Braconidae) in Sugarcane Fields of Ethiopia and Origin of Founding Population.
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The systematics and biology of *Cotesia nonagriae* (Olliff) stat. rev. (Hymenoptera: Braconidae: Microgastrinae), a newly recognized member of the *Cotesia flavipes* species complex

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Abstract

The Australian species *Cotesia nonagriae* Olliff stat. rev. (Hymenoptera: Braconidae) is redescribed and formally removed from synonymy with *C. flavipes* based on molecular, morphological and biological differences. The taxonomic history and phylogenetic relationships of *C. nonagriae* with other members of the *C. flavipes* complex are presented and underscore the importance of molecular-based identification within this group. The biology of *C. nonagriae* on the native noctuid stemborer host, *Bathytricha truncata* (Walker), is compared with previously recorded *C. flavipes* life history traits. The implications of this taxonomic study relative to biological control and importation of stemborer parasitoids into Australia are discussed.

Key words: parasitoid, taxonomy, biology, stemborer, Australia, biological control

Introduction

The *Cotesia flavipes* complex of parasitioid wasps are natural enemies of lepidopterous stemboring pests associated with sugarcane and cereal crops (Walker 1994). Since these are staple crops in many countries, the complex is economically important worldwide as biological control agents. The complex currently consists of three species, *Cotesia flavipes* Cameron, *C. sesamiae* (Cameron) and *C. chilonis* (Matsumura), of uncertain taxonomic validity and relationships. Identifying the various species within the *flavipes* complex has been problematic in the past and has been usefully summarized by Kimani-Njogu and Overholt (1997).

The monophyly of the complex is well supported by molecular (Smith & Kambhampati 1999; Michel-Salzat & Whitfield 2004; Muirhead *et al*. 2006) and morphological characters, such as a dorsoventrally compressed mesosoma (Watanabe 1965; Walker 1994). However, the species within the complex are morphologically similar, and many of the characters that have been used to separate species have proven unreliable due to intraspecific variation (Polaszek & Walker 1991; Smith & Kambhampati 1999). As a result, their use in biological control has been confounded by inaccurate identification, as well as the existence of host specific populations (Kimani-Njogu & Overholt 1997).

The species of the *C. flavipes* complex are thought to be endemic to the following areas: *C. flavipes* to the Indo-Australian region; *C. sesamiae* to central and southern Africa; and *C. chilonis* to eastern Asia, including Japan (Polaszek & Walker 1991; Kimani-Njogu & Overholt 1997). However, all three species have been utilized for classical biological control of stemboring pests, resulting in their much broader inter-continental distribution (Polaszek & Walker 1991). In some cases, a species of the complex has been introduced into an area indigenous to one of the other two species (Smith & Kambhampati 1999). For example, *C. flavipes* has been

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introduced several times into various countries of Africa (Overholt *et al*. 1994) and is now established in several parts of sub-Saharan Africa (Omwega *et al*. 1995; Overholt *et al*. 1997) where it co-exists with the native *C. sesamiae*. Although *C. flavipes* and *C. sesamiae* can occupy a similar ecological niche, it has been shown that they prefer different host species and are not likely to compete (Rajabalee & Govendasamy 1988; Sallam *et al*. 2001; Sallam *et al*. 2002). Similarly, certain populations of the same species within the complex have differences in host range (Mohyuddin 1971; Shami & Mohyuddin 1992; Zhang & Hewitt 1996; Potting *et al*. 1997b; Ngi-Song *et al*. 1998), an indication of genetic divergence among strains (Muirhead *et al*. 2006) and the possible existence of cryptic species.

There has been ongoing confusion regarding the status and presence of *C. flavipes* in Australia, and this has the potential to impact the future importation of biological control agents. Over 80 years ago, the Australian native species *Apanteles nonagriae* Olliff, 1893 was synonymized with *A. flavipes* (Cameron, 1891) (Wilkinson 1929; Austin & Dangerfield 1992), thus indicating the presence of *C. flavipes* in Australia. However, recent molecular work suggests that the Australian populations represent a 'cryptic' species different from *C. flavipes* and other members of the species group (Muirhead *et al*. 2006). Records of *A. nonagriae* in Australia extend back to its original description when it was first recorded as a parasitoid of the native noctuid stemborer *Nonagria exitiosa* Olliff (= *Bathytricha truncata* (Walker)) in sugarcane in the Richmond and Clarence River Districts of north-eastern New South Wales (Olliff 1893). It was subsequently reared from *Phragmatiphila truncata* Walker (= *Bathytricha truncata*) in sugarcane at South Mulgrave, south of Cairns, Queensland (Jarvis 1927). The same report also indicated that the parasitoid had been previously recorded parasitising 50% of *B. truncata* larvae infesting rice in New South Wales. *Bathytricha truncata* is a stemborer recorded from sugarcane, rice, maize and a range of other plants (Sallam 2003). It has a distribution from Cairns to South Australia and Tasmania (Common 1990) and is considered a minor pest that rarely causes substantial damage (Jones 1966). Bell (1934) recorded *Apanteles nonagriae* on *B. truncata* larvae at Mackay, Queensland. Similarly, Li (1970) recorded "*A. flavipes* (*A. nonagriae*)" from *Chilo suppressalis* (Walker) and *Chilo polychrysa* (Meyrick) (Pyralidae) in rice fields in the Northern Territory, but no voucher material was deposited in any collection to confirm this finding.

Apanteles nonagriae was originally described by Olliff (1893) along with *Tetrastichus howardi* (Olliff), a eulophid pupal parasitoid reared from *B. truncata* (Boucěk 1988). In his study of Indo-Australian *Apanteles* s.l., Wilkinson (1928a, b) noted the strong similarity between *A. nonagriae* Olliff and *A. flavipes* but did not synonymize them until the following year (Wilkinson 1929). However, he did synonymize a second species of the same name, *A. nonagriae* Viereck, 1913, with *A. flavipes* that had been reared from *Sesamia* (*Nonagria*) *inferens* Walker from Taiwan (Wilkinson 1928a). Unfortunately, Olliff (1893) did not designate any type specimens in the original description of *C. nonagriae* and did not refer to any depository that might hold syntypes.

Based on morphological examination and biological data, supplemented by the previous molecular study (Fig. 1) (Muirhead *et al*. 2006), we formally recognize *Cotesia nonagriae* stat. rev. as a distinct species. In so doing, we redescribe the species and discuss its taxonomic history, relationships within the *flavipes* complex, its biology compared to *C. flavipes* and the implications of this taxonomic study to future biological control programs and importation of stemborer parasitoids into Australia.

Materials and methods

Taxonomy

Specimens of *C. nonagriae* used in this study were reared from *B. truncata* collected from three sugarcane-growing localities in Queensland, while specimens of *C. flavipes* (India, Thailand, Japan, Papua New Guinea, Kenya, Mauritius), *C. sesamiae* (west Kenya, east Kenya, Tanzania) and *C. chilonis* (China, Japan) were accessed from the voucher material from Muirhead *et al*. (2006) deposited in the Waite Insect and Nem-

atode Collection, Adelaide. Morphological terminology follows Sharkey and Wharton (1997) for body structures and venation, Eady (1968) and Harris (1979) for sculpturing, and Kimani-Njogu and Overholt (1997) for male genitalia. Specimens were imaged using a Philips XL30 FEGSEM scanning electron microscope at the Adelaide Microscopy and Microanalysis Research Facility, The University of Adelaide. Male genitalia were dissected from the metasoma of several specimens and mounted on carbon conductive adhesive tabs after overnight digestion in 140μl of lysis buffer and 7μl of proteinase K (20mg/ml) at 55°C.

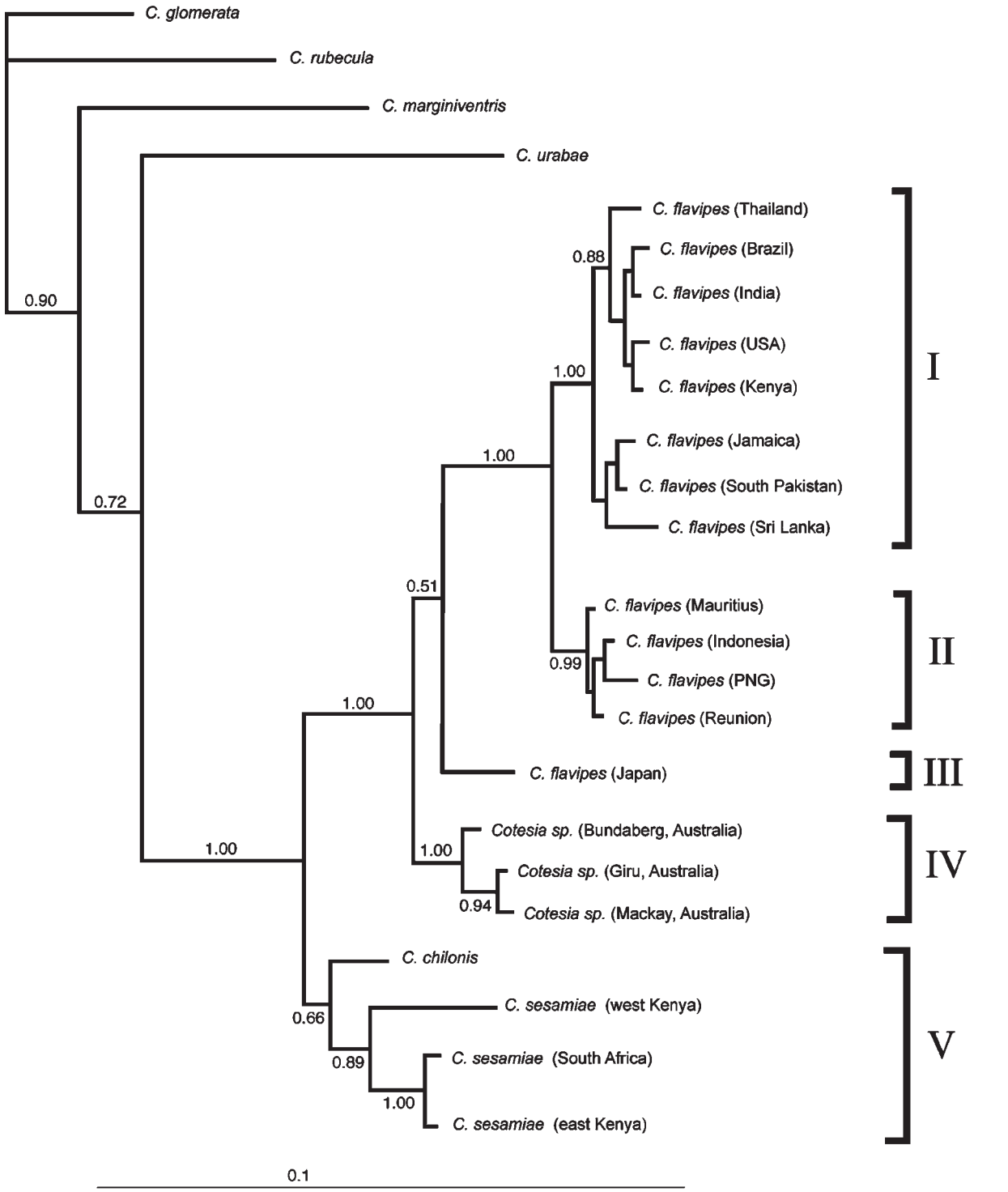

FIGURE 1. Bayesian tree derived from partial *16S rRNA* and *COI* mtDNA nucleotide sequence data from geographic populations of the *Cotesia flavipes* complex (Clades I-V) and four outgroups. Australian *Cotesia nonagriae* populations are shown in clade IV. The numbers represent Bayesian posterior probabilities $\geq$ 50% (from Muirhead *et al*. 2006).

Abbreviations for collections in the text are: AM, Australian Museum, Sydney; ANIC, Australian National Insect Collection, Canberra; ASCT, Agricultural Scientific Collections Trust, Orange Agricultural Institute, Orange; QDPI, Queensland Department of Primary Industries, Brisbane; and WINC, Waite Insect and Nematode Collection, Adelaide.

Biology

Insect colonies. We maintained two colonies of *C. nonagriae* originating from field parasitized larvae of *B. truncata* infesting sugarcane in Mackay and Bundaberg, Queensland. Parasitoids were maintained on laboratory reared fourth instar *B. truncata* larvae in a temperature controlled room at 25ºC, 60–70% RH under a 12L:12D photoperiod. Mated females were offered one host larva with some fresh larval frass to stimulate oviposition. Wasp cocoons were collected from host larvae and transferred to emergence cages where they were provided honey as a food source.

Field collected *B. truncata* were reared to the pupal stage within cut sugarcane stems, whereas subsequent lab generations were maintained on an artificial diet adopted from Onyango and Ochieng-Odero (1994), replacing maize leaf powder with sugarcane leaf powder. See Songa *et al*. (2001) and Macqueen (1969) for more details on stemborer rearing procedures and the life history of *B. truncata*. Adult moths were kept in oviposition cages with waxed paper tubes to provide suitable oviposition sites. Egg masses were cut from the paper daily and transferred to a closed Petri dish containing moist cotton wool to maintain high humidity. Egg masses at the blackhead stage were transferred to 2.55 mm diameter containers with artificial diet. Larvae used in the experiments were removed from the artificial diet as fourth instars and fed 5 cm cuts of sugarcane stems.

Life history traits. To study the life history of *C. nonagriae* on the native host *B. truncata*, we employed the procedure used by Sallam *et al*. (2002) on *C. flavipes* and *C. sesamiae*. Thirty fourth instar host larvae were parasitised by newly emerged, mated female parasitoids. Adult female parasitoids were kept in individual vials and exposed to one host larva each for oviposition. Parasitised larvae were kept in vials containing cut sugarcane stems until the mature parasitoid progeny emerged and pupated. Ten parasitised hosts were dissected one to two days after oviposition to determine the number of parasitoid progeny allocated to each host. Cocoon masses from the remaining 20 larvae were counted, weighed and placed in vials. Duration of the parasitoid's immature stages, percent emergence, number of adult progeny, adult longevity and sex ratio were recorded. Three females from each progeny (n=60) were chosen randomly and dissected to count the number of eggs contained in the ovaries.

Descriptive taxonomy

***Cotesia nonagriae* (Olliff) stat. rev.**
(Figs. 2–4)

Apanteles nonagriae Olliff, 1893: 376 [original description]; Wilkinson (1928b): 136 [type data, biology, taxonomic status].

Apanteles flavipes (Cameron, 1891); Wilkinson (1928a): 93 [synonymy of *A. nonagriae* Viereck]; Wilkinson (1929): 108 [synonymy *A. nonagriae* Olliff]; Shenefelt (1972): 509 [complete taxonomic bibliography].

Cotesia flavipes Cameron, 1891: 185 [original description]; Mason (1981): 113 [resurrected the genus with *C. flavipes* as type]; Austin and Dangerfield (1992): 21 [status and hosts for Australia].

Material examined. Queensland: 21♀ 3♂ Bundaberg, 12-30.xi.2004, K. Muirhead (10♀ 1♂ ANIC, 11♀ 2♂ WINC); 16♀ 2♂ Mackay, 12-30.xi.2004, K. Muirhead (6♀ 1♂ ANIC, 10♀ 1♂ WINC); 14♀ 3♂ Giru [via Townsville], 5.x.2003, M. Sallam (9♀ 1♂ QDPI, 5♀ 2♂ WINC): 3♀ 1♂ '*parasite larva sugar-cane moth*' '*Apanteles nonagriae Olliff'*, no date or locality (1♀ AM, 2♀ 1♂ ASCT).

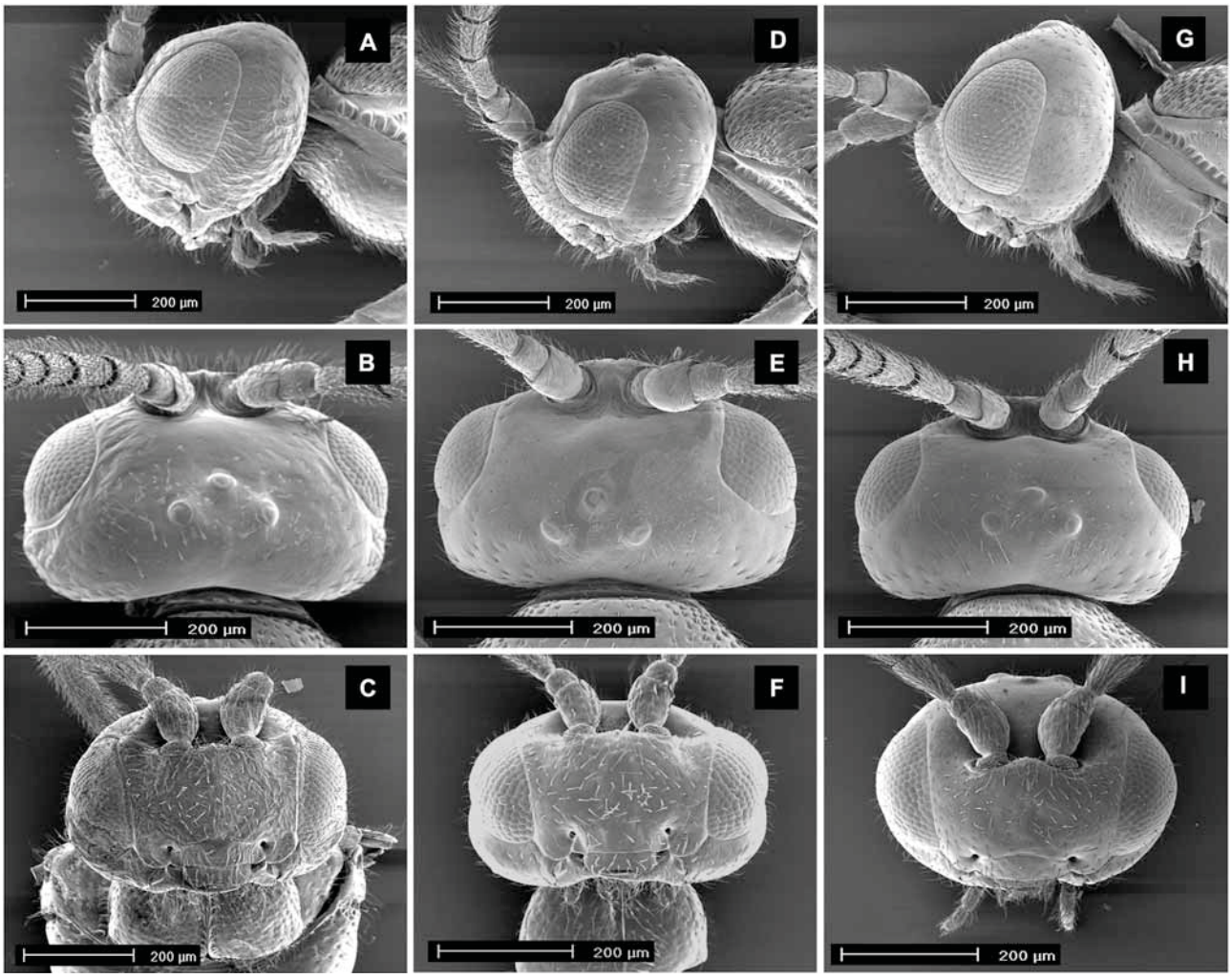

FIGURE 2. A–C: *Cotesia nonagriae* (Bundaberg, Australia), head. (A) lateral view, (B) dorsal view, (C) anterior view. D–F: *C. nonagriae* (Mackay, Australia), head. (D) lateral view, (E) dorsal view, (F) anterior view. G–I: *C. flavipes* (India), head. (G) lateral view, (H) dorsal view, (I) anterior view.

Female. *Length*. Body 2.1–2.4 mm

Colour. Body black, metasomal sterna including hypopygium dark brown to brown, antenna dark brown with scape lighter, palps yellow; legs yellow brown with tarsus slightly darker, mesocoxa pale brown, metacoxa dark brown to black basally grading to brown apically; forewing stigma brown, venation slightly lighter.

Head. In anterior view oval in shape, substantially wider than high, eyes slightly converging ventrally, face slightly rugulose-punctate to punctuate; in lateral view oval (globular) in shape, only slightly higher than long, gena and temples rugulose-punctate to punctuate, slightly more striate along posterior eye margin; in dorsal view vertex and occiput moderately smooth except for scattered fine punctures associated with sparse short setae, frons usually smooth but sometimes with faint striations along eye margin.

Mesosoma. Strongly flattened dorsoventrally so that posterior two-thirds of scutum, scutellum, anterior part of propodeum and ventral margin of mesopleuron horizontal and parallel; in dorsal view scutum punctate anteriorly, mostly smooth posteriorly and along midline, notauli indicated by posterior extension of anterior punctuate area and smooth areas on either side but disappearing before reaching posterior margin; medial scutellum smooth with sparse setae, posterior margin broad; propodeum coarsely rugose-punctate, often with indistinct carina around spiracle and oblique lateral carina converging posteriorly; in lateral view mesopleuron smooth, sternaulus faintly indicated along dorsal margin by sparse punctures; metapleuron rugose-punctate in posterior part, smooth in anterior part; dorsal and outer surfaces of hind coxa punctuate; forewing veins r and

2RS usually meeting at distinct angle, sometimes with small stub of 3RS present; 2M 0.5 to almost 1.0X as long as 2RS.

Metasoma. Tergum 1 almost as wide at posterior margin as long, lateral margins strongly diverging posteriorly; longitudinally striate-rugulose, often with incomplete medial longitudinal carina; tergum 2 longitudinally striate-rugulose with smoother longitudinal area medially and laterally; remaining terga smooth with sparse longish setae.

Male. As for female except: antenna slightly longer and lighter in colour; punctuate sculpturing on scutum, particularly in anterior part, slightly denser; genitalia very similar to *C. flavipes*; aedeagal-volsella shaft elongate; volsella more than 4.0X as long as wide, digital (apical) teeth minute; aedeagus barely protruding past apex of parameres and volsella.

Comments. The description above is largely based on specimens from Bundaberg. For specimens from Mackay and Giru, the degree of sculpturing on the face and gena is less pronounced, and the frons and temples are completely smooth. These populations also have the propodeum less coarsely sculptured and rugulose rather than rugulose-punctate and tergum 1 lacking a medial longitudinal carina. As such they are more similar to *C. flavipes*. The specimens in AM and ASCT have identical labels and are clearly very old. We initially considered that they were part of Olliff's original material and therefore a likely syntype series. This was based on the age of the material and that there are several lectotypes of Olliff species in the AM, including that of *T. howardi* (designated by Boucěk 1988) which was described by Olliff in the same paper as *C. nonagriae*. However, comparison of the labels on Olliff specimens in the AM shows that the handwriting is different to the AM and ASCT specimens, and so they cannot be directly associated with that used in the original description of *C. nonagriae*. The specimens in AM and ASCT have the face and gena smooth and are therefore more similar to the recently collected material from Mackay and Giru.

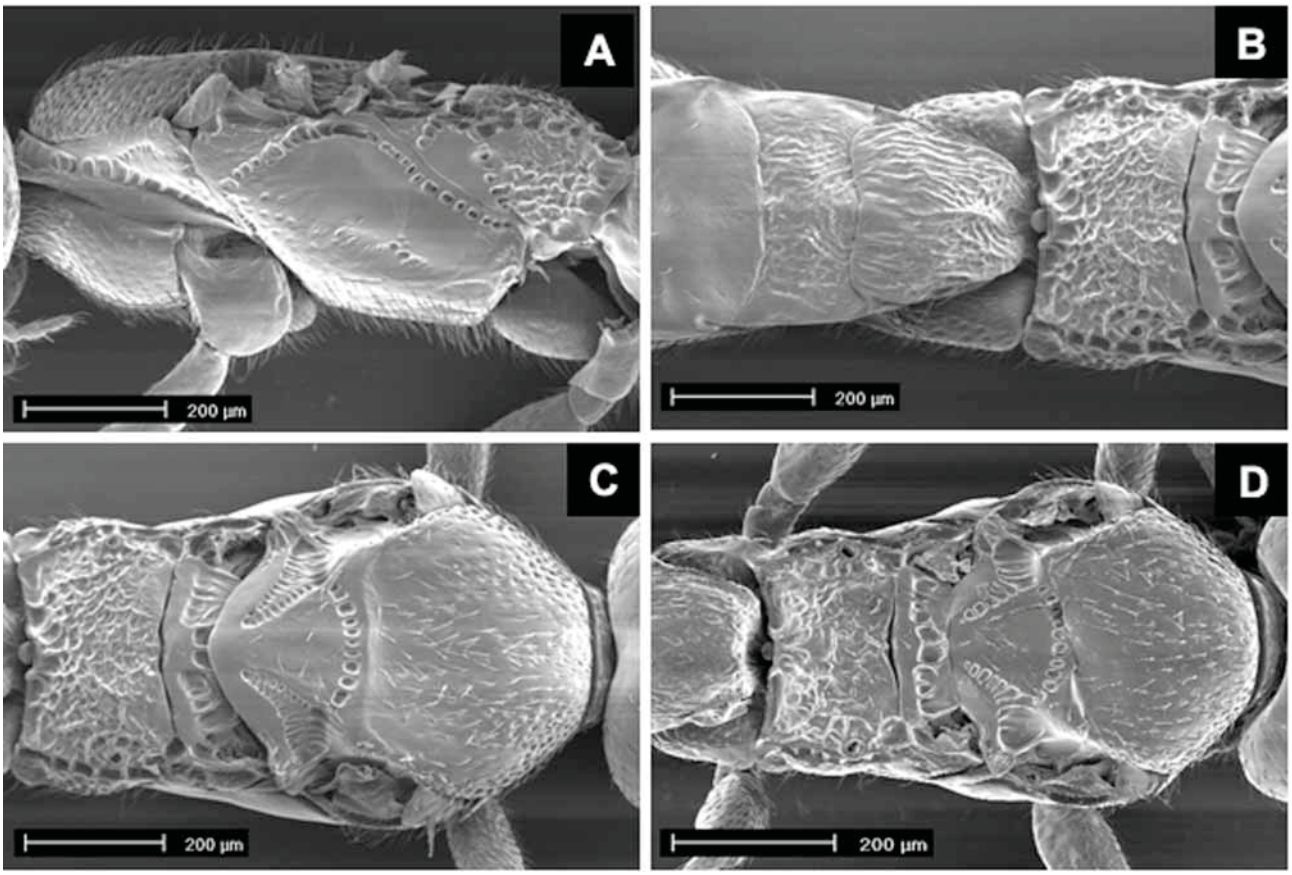

FIGURE 3. A–C: *Cotesia nonagriae* (Bundaberg, Australia): (A) mesosoma, lateral view, (B) posterior mesosoma and terga 1–3, dorsal view, (C) mesosoma, dorsal view. D: *C. flavipes* (India), mesosoma, dorsal view.

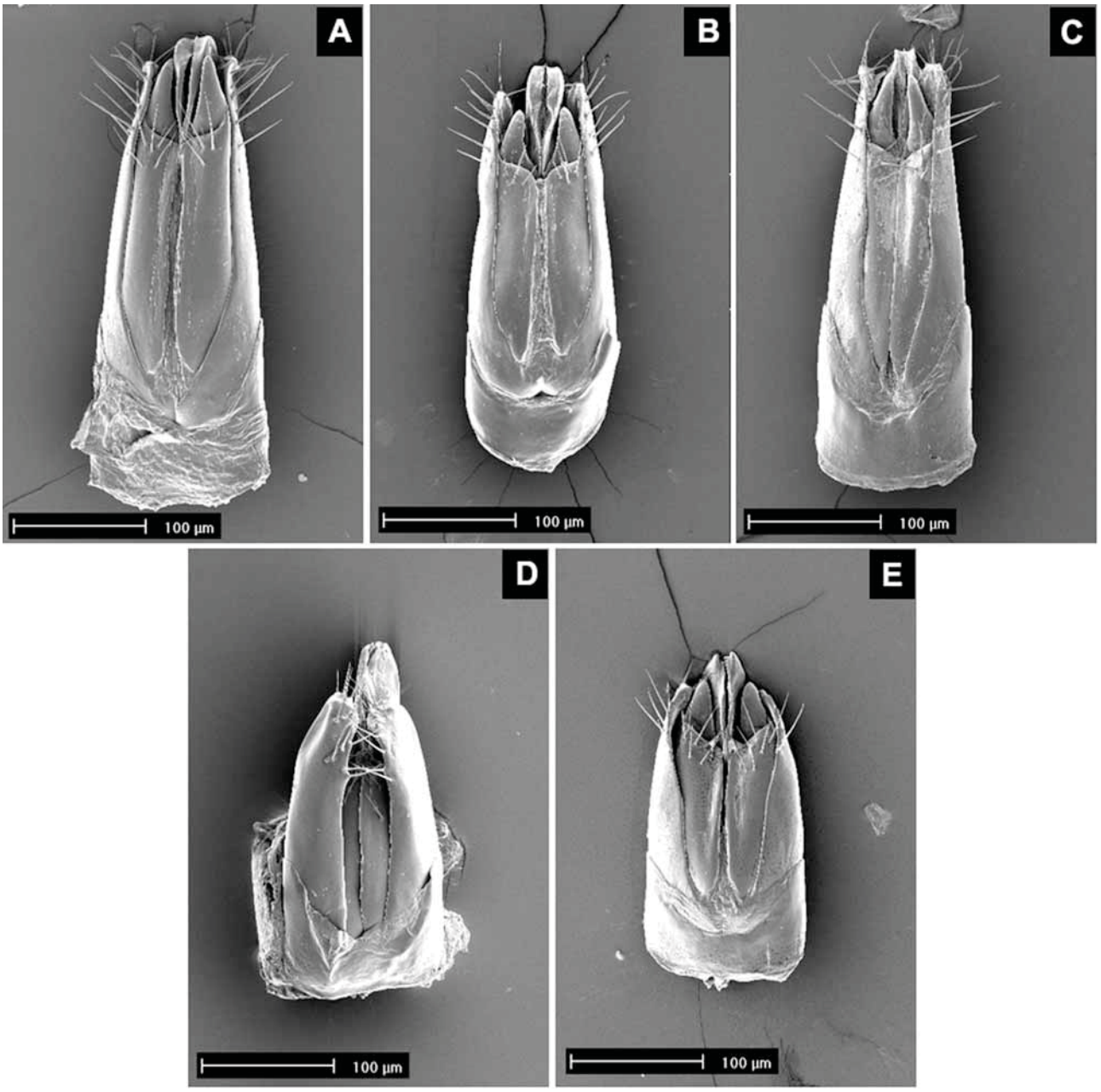

FIGURE 4. A–E: Male genitalia of *Cotesia flavipes* complex species. (A) *C. nonagriae* (Australia), (B) *C. flavipes* (India), (C) *C. flavipes* (Japan), (D) *C. sesamiae* (Kenya), (E) *C. chilonis* (China).

Results and discussion

Species recognition

Based on the mitochondrial gene phylogeny of Muirhead *et al*. (2006), there are clearly two pairs of sister species within the *flavipes* complex: *C. sesamiae*/*C. chilonis* and *C. flavipes*/*C. nonagriae*. Although there are a number of morphological differences that distinguish these two pairs of species (e.g., form of the scuto-scutellar sulcus and propopeal sculpturing), they also display relatively high levels of intraspecific variation making it difficult to interpret these characters. Without doubt, the definitive difference between these species pairs is the structure of the male genitalia. In *C. sesamiae*/*C. chilonis* the major elements of the genitalia are relatively short and broad, while in *C. flavipes*/*C. nonagriae* they are more elongate (Kimani-Njogu & Over-

holt 1997; Fig. 4). Distinguishing between *C. nonagriae* and *C. flavipes* is more difficult if geographic location is not taken into account. The more sculptured head of *C. nonagriae* and to a lesser degree the courser sculpturing on the propodeum will distinguish most populations. However, levels of intraspecific variation that occur in both species will at times render identification difficult. Because of this, and until *C. flavipes* can be shown definitively not to occur in Australia, we advocate the use of molecular diagnostic techniques using the phylogenetic framework generated by Muirhead *et al*. (2006) in cases where accurate identification is critical.

Biology

Life history traits for *C. nonagriae* assessed as part of this study are summarized and compared with published data for *C. flavipes* in Table 1. The potential fecundity of *C. nonagriae* females was similar to *C. flavipes* with an initial load of ~ 200 eggs. However, *C. nonagriae* females allocated an average of 111.6 (SD ± 25.32) eggs into each host, whereas *C. flavipes* is known to allocate a maximum of 30–40 eggs into at least two different hosts (Sallam *et al*. 2002). This high egg allocation suggests that *C. nonagriae* females will deplete their egg load after just two oviposition events, while *C. flavipes* females are depleted of eggs after they have parasitized four to five hosts (Potting *et al*. 1997a). In spite of the higher number of *C. nonagriae* progeny that emerged from *B. truncata,* cocoon weight was not very different from that produced by *C. flavipes* parasitizing *Sesamia calamistis* Hampson (Noctuidae) in Africa based on studies by Sallam *et al*. (2002). The total life cycle of *C. flavipes* is about 20 days but is longer for *C. nonagriae* at 24 days. This is due to a longer duration of the larval stages (17 versus 21 days), which may also be influenced by the higher number of larvae competing for food. After 14–15 days *C. nonagriae* larvae emerged from the host and formed small white silken cocoons, which usually surrounded the host cadaver within its tunnel. Like *C. flavipes* adults, *C. nonagriae*, generally lives for one to three days without food; however *C. flavipes* adults can live up to six days when provided honey (Potting *et al*. 1997a).

TABLE 1. Number of cocoons, cocoon weight, adult progeny, duration of immature stages, sex ratio, adult longevity, emergence rate and potential fecundity (mean (± SD)) of *C. nonagriae* on the native stemborer host *Bathytricha truncata* compared with the same biological traits for *C. flavipes* on *S. calamistis* (from Sallam *et al*. 2002).

Species	Number of cocoons/host	Cocoon Weight (mg)	Adult progeny/host	Duration of immature stages (days)	Sex ratio (% female/ total progeny)	Adult longevity (days)	% Emergence	Potential fecundity(egg load)
C. nonagriae	99.28 (21.8)	0.101 (0.023)	91.56 (20.9)	21.07 (1.2)	52.1 (5.8)	2.92 (0.35)	91.97 (4.92)	196.56 (12.2)
C. flavipes (Sallam *et al*. 2002)	34.300 (17.2)	0.106 (0.011)	32.00 (17.6)	17.20 (3.0)	53.00 (0.26)	3.60 (0.7)	92.97 (8.9)	203.60 (8.7)

Relevance to biological control

Accurate identification of both natural enemies and pests is vital for research, quarantine and successful biological control (Clausen 1942; Debach 1960; Compere 1969; Danks 1988; Debach & Rosen 1991; Schauff & LaSalle 1998; Beard 1999). However, biocontrol programs are often confounded by intraspecific variation within complex taxonomic groups. Overlapping intraspecific variation in hymenopteran parasitoids is well documented and has been reported for ecological, behavioural and physiological traits such as climatic adaptability, diapause, host selection and virulence (Hopper *et al*. 1993; Unruh & Messing 1993) Ruberson *et al*. (1989) alone listed over 65 studies that deal with intraspecific variation in hymenopteran parasitoids, predominantly revealed through biological control introductions. Species that are seemingly widespread and abundant in reality can represent several cryptic species. This may well be the case for the *C. flavipes* complex,

where numerous authors have recorded geographic variation among *C. flavipes* populations in ecology, host-searching behaviour and host-parasitoid compatibility (Mohyuddin 1971; Mohyuddin *et al*. 1981; Inayatullah 1983; Polaszek & Walker 1991; Ngi-Song *et al*. 1995; Potting *et al*. 1997b; Ngi-Song *et al*. 1998; Mochiah *et al*. 2001). The ability to discriminate between biotypes on different hosts is crucial for biological control. Moreover, from an evolutionary perspective, it is important to identify the forces that structure genetic differences among parasitoid populations relative to their host insects (Vaughn & Antolin 1998; Heraty 2004)

Whereas this study underscores the need for molecular diagnostic techniques (e.g., Dupas *et al*. 2006; Muirhead *et al*. 2006) for reliable identification of near cryptic species, it also emphasizes the need for detailed comparative morphology and supplemental biological data to support critical taxonomic decisions. The Cohesive Species Concept stresses the importance of establishing species boundaries by examining phylogenetically distinct entities for reproductive incompatibility or ecological, behavioural or morphological differences (Templeton 1989). The mtDNA sequence data of Muirhead *et al*. (2006) provided the first evidence for the monophyly of the Australia populations, and likewise, our results support the conclusion that *C. nonagriae* is a distinct species based on morphological and biological traits.

Although there are subtle morphological differences between *C. nonagriae*, *C. flavipes* and the other members of the complex, it is not surprising that earlier authors confused these species given their close similarity and intrinsic variability (Wilkinson 1928a; Watanabe 1932, 1965; Alam *et al*. 1972; Ingram 1983; Polaszek & Walker 1991). Male genitalia are certainly the most reliable character system (Polaszek & Walker 1991) and clearly separate the two morphospecies groups: *C. sesamiae*/*C. chilonis* and *C. flavipes*/*C. nonagriae* (Fig 4). Despite biological variation between *C. nonagriae* and *C. flavipes*, there is limited phenotypic diversity. Their similarity probably reflects not only recent common ancestry but also stabilizing selection arising from ecological selection, while diversification within the complex is probably linked to biogeographic barriers and host use.

Independent of the conclusion that *C. nonagriae* is a distinct species associated with the native sugarcane pest *B. truncata*, we were unable to discern whether or not *C. flavipes* also occurs on the continent. Previous researchers reporting the occurrence of *C. flavipes* over the last century have failed to deposit voucher specimens in recognized insect collections (e.g., Jarvis 1927; Macqueen 1969; Li 1970) Thus, no reliable specimens are available to verify the identity of *C. flavipes* referred to in the literature. In several cases these are very likely to be *C. nonagriae* when associated with *B. truncata* (e.g., Bell 1934). However, reference to *C. flavipes* associated with *C. suppressalis* and *C. polychrysa* in rice (Li 1970) is more problematic given that true *C. flavipes* have been reared from these hosts in southeast Asia (Kajita & Drake 1969; Hattori & Siwi 1986; Khoo 1986; van Verden & Ahmadzabidi 1986). We were unable to access populations of either *Chilo* species in Australia to rear parasitoids for comparison. Thus, the question of whether *C. flavipes* occurs in Australia still needs to be addressed. This is crucial for future biological control projects in Australia because if *C. flavipes* is not native, it will need to undergo pre- and post-release studies in order to assess its interaction with *C. nonagriae* and impact on non-target species (Howarth 1991; Messing 1992; Samways 1997; Sands 1997; Henneman & Memmott 2001).

Perhaps a more central issue for potential stemborer pest incursions into Australia is the host range of *C. nonagriae* and whether it will successfully parasitize host species not encountered during its evolutionary history. Interestingly, this was the case for *C. flavipes*, which formed a novel association with *Diatraea saccharalis* (F.) when introduced into the New World for biological control purposes (Simmonds 1969; Polaszek & Walker 1991). Thus, future work could profitably be directed towards the testing of *C. nonagriae* on high threat stemborer species from Indonesia and Papua New Guinea.

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