Sex determination and symbiont transmission in the *Sirex-Amylostereum* mutualism

By

Amy Lorraine Wooding

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Supervisor:	**Prof. Bernard Slippers**
Co-supervisors:	**Prof. Michael J. Wingfield**
	Prof. Jaco M. Greeff
	Dr. Brett P. Hurley
	Dr. Jeffrey R. Garnas

Declaration

I, the undersigned, declare that this thesis/ dissertation, which I hereby submit for the degree of *Magister Scientiae* to the University of Pretoria, contains my own independent work and has not been previously submitted for any degree at this or any other tertiary institution.

Amy Lorraine Wooding

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I dedicate this thesis to my late grandmother, *Patricia Ann Glenday* and my grandfather *Bruce Stuart Glenday*.

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The woodwasp *Sirex noctilio* is one of the most serious invasive pests of pine-based forestry throughout the Southern Hemisphere. The pest status of this woodwasp and its obligate fungal symbiont *Amylostereum areolatum*, result from the woodwasps' ability to attack and kill healthy pine trees during population outbreaks. These population outbreaks have caused major economic losses to the pine forestry industry across this region over the past century. The woodwasps' pest status has led to a large body of research on the biology of *S. noctilio*, *A. areolatum* and its natural enemies in an effort to control this pest. Despite almost a century of research on *S. noctilio* and *A. areolatum*, many questions pertaining to the evolution of the *Sirex-Amylostereum* mutualism, transmission of *A. areolatum,* and reproduction in *S. noctilio* remain unanswered.

This thesis explores two key aspects pertaining to the population dynamics and biology of *S. noctilio*. First the interactions between native North American and invasive European woodwasps are investigated with respect to the influence of this novel interaction on the existing mutualism between *Sirex* and *Amylostereum* species. Second causes of skewed sex ratios in invasive populations are examined as these can have a large impact on the on the population dynamics in invasive populations. Increased knowledge in both of these areas will increase our understanding of the current and potential impact of invasive *S. noctilio* populations as well as allow for the development of improved management strategies for these populations.

The evolution of stable obligate mutualisms, where interacting organisms exhibit behaviours which reciprocally benefit one another, is considered to a conundrum. This is because altruistic behaviour towards non-relatives does not promote the direct propagation of an organism's genes to the next generation, and is thus expected to be selected against. Altruistic behaviour is also predicted to be vulnerable to cheating, where one organism (or certain individuals in the population) could exploit the altruistic behaviour of another organism without behaving in a reciprocal manner. Despite this, obligate mutualisms

between organisms from different kingdoms of life have evolved and remained stable over evolutionary time. **Chapter one** reviews the literature pertaining to the evolution of stable, obligate mutualisms, focusing on the strategies and mechanisms in these mutualisms to prevent cheating. The review focuses in particular on mutualisms between the fungus–farming insects and their mutualistic fungi, which are among the best characterised insect–fungal mutualisms. This information, together with insights from other mutualisms, are used as a framework to explore the ecological and evolutionary factors important in the evolution of the *Sirex–Amylostereum* mutualism.

An important factor in the maintenance of stable obligate insect–fungal mutualisms is the manner in which the fungal partner is transmitted between generations. When female *S. noctilio* oviposit into the sapwood of trees, they also deposit oidia of *A. areolatum* which grow into the wood and act as a source of digestive enzymes for the degradation of wood for the developing larvae. Fragments of fungal mycelia are then collected by the adult female into specialised internal fungus carrying organs called mycangia. Hence, in *S. noctilio,* transmission of *A. areolatum* is thought to be vertical, from mother to daughter. For this reason the relationship between *Sirex* and *Amylostereum* species is thought to be highly specific. In **chapter two** the specificity of the mutualism between *Sirex* and *Amylostereum* species is examined in populations of *S. noctilio* in Canada. This region was chosen as the *Sirex* species native to it are known to be associated only with *A. chailletii*. We determine the identity of *Amylostereum* isolates from a collection of native and invasive *Sirex* woodwasps, including from areas where they infest the same trees, to ascertain whether, and at what frequency these wasps might acquire *Amylostereum* strains and species horizontally.

As a member of the order Hymenoptera, *S. noctilio* is haplodiploid. Under haplodiploidy fertilized eggs develop into females, and unfertilised eggs develop into males. The ancestral genetic mechanism for sex determination in the Hymenoptera is complementary sex determination where heterozygosity at a specific sex determination locus (loci) results in

female development. Homozygosity results in the development of diploid males. To study this phenomenon ploidy needs to be determined. Therefore, in **chapter three** we examine the use of flow cytometry for the determination of ploidy in populations of *S. noctilio*.

In the **final chapter** the phenomenon of extremely male–biased sex ratios in newly established populations of *S. noctilio* is investigated. Potential causal factors for this phenomenon, and its persistence in the KwaZulu–Natal region of South Africa, are explored. These factors include the presence of the reproductive parasite *Wolbachia*, preferential oviposition of one sex into wood with different perceived resource quality and host tree susceptibility as measured by wood moisture, and a high frequency of constrained sex allocation (unmated females) in the population. We also explore the influence of low genetic diversity on the haplodiploid sex determination system, where low diversity at the sex determination locus could lead to higher frequencies of diploid males.

The importance of reproductive and dispersal mechanisms in the evolution of the *Sirex–Amylostereum* mutualism

Chapter 1

Abstract

Interactions among living organisms fall along the mutualism-parasitism continuum; where mutualistic interactions benefit one or both organisms and parasitic interactions harm them. Mutualisms are a particularly interesting form of interaction as their evolutionary stability is constantly at risk of destabilisation by cheaters which take greater advantage of their partners than other symbionts in the population. This has resulted in organisms involved in mutualistic interactions have evolved many mechanisms to prevent destabilisation by cheaters. Insects are involved in mutualistic interactions with a myriad of organisms, in particular micro-organisms. Some of the most well documented insect–micro–organism interactions are those of the fungus–farming insects; Attine ants, termites and ambrosia beetles, and the obligate mutualistic fungi they cultivate. These mutualisms have remained stable over millions of years. Another, less well studied, apparently stable, obligate insect-fungus mutualism is the interaction between *Sirex* woodwasps and *Amylostereum* fungi. In this review we examine the evolution of mutualisms from initial interaction, through to maintenance of a stable obligate interaction, and explore the mechanisms that act to stabilise them. We explore the evolutionary and ecological factors necessary for the maintenance of the *Sirex–Amylostereum* mutualism in the context of work that has been done on the evolution of other more extensively studied insect-fungus mutualisms.

Introduction

Co-evolved organisms interact with one another in a myriad of ways. These interactions can range from highly beneficial to detrimental to one or all of the organisms involved. When these interactions are persistent they are known as symbioses; originally defined as, 'the living together of unlike named organisms' (deBary (1879) as quoted in Klepzig*, et al.* 2009; Little & Currie 2007; and Margulis 1990). Symbioses can be further divided into three broad classes of interaction, namely; mutualism (mutually beneficial), commensalism (beneficial to one organism, benign to the other) and parasitism (beneficial to one organism, harmful the other).

Symbioses were originally thought of as interactions between only two organisms. More recently they have been recognised as being highly complex, at times involving multiple organisms, that are often from different kingdoms, with the ability to interact with each other in different ways (Little & Currie 2007; Mueller 2012). The nature of the interactions between the organisms can also change during their association from being beneficial, neutral, to parasitic, and vice versa. These changes can occur due to shifts in behaviour, population density of one or both interacting partners, the stage of the life-cycle of the interacting partners, and environmental changes (Brown*, et al.* 2012; Parmentier & Michel 2013). Shifts in the nature of interacting organisms are known as the parasitism-mutualism continuum (Ewald 1987). This complexity, along with the growing notion of symbiosis as a driver of biological diversification has led to intensified interrogation of these fascinating relationships (Janson*, et al.* 2008; Margulis 1990).

This review concentrates on mutualistic interactions, with a focus on mutualistic interactions in insect-fungal symbioses. Mutualisms are generally described as interactions in which different organisms gain fitness benefits by interacting with each other (Sachs*, et al.* 2004; West*, et al.* 2007), and are sometimes described as examples of reciprocal exploitation

(Herre*, et al.* 1999). Mutualisms are delineated as being obligate or facultative associations among organisms based on the degree of interdependence that is exhibited by the associated organisms. Where the organisms cannot survive outside of the mutualism, the interaction is considered to be obligate, in contrast to the situation where they can survive outside of the mutualism and the interaction is considered to be facultative. These mutualisms are often characterised by either vertical or horizontal transmission of the mutualists respectively (Dale & Moran 2006).

There is a vast variety of fitness benefits that can be derived from microbial mutualists in insect-microbe mutualisms. Bacterial mutualists can influence a wide variety of biological processes including insect development, nutrition, reproduction, immunity/ defence and speciation (Dale & Moran 2006). Fungal mutualists of insects most often play a role in host insect nutrition, providing digestive enzymes or food for the insects (Kukor & Martin 1983; Mueller*, et al.* 2005). Microbial mutualists can exist as endosymbionts; carrying out their life cycles within insect host cells (Sasaki*, et al.* 1996). The insect host cells provide a suitable, protected environment for microbe reproduction. These microbes are also provided with the necessary transport to new hosts because they are vertically transmitted (Dale & Moran 2006). Microbial mutualists can also exist as ectosymbionts, completing their life-cycles on external host insect surfaces, including the cuticle and lining of the gut, or within specialised internal organs (Mueller*, et al.* 2005). Ectosymbiotic microbes gain benefits by being transported to suitable substrates, often in areas protected from competing micro-organisms and environmental stresses. Insect partners can also actively provide the microbes with a suitable substrate as is seen in Attine ants and termites (Mueller*, et al.* 2005).

Siricid woodwasps exist in a highly specific, obligate mutualistic relationship with fungi (Gaut 1970; Nielsen*, et al.* 2009; Talbot 1977). The biology of the association between Siricid woodwasps and their mutualistic fungi has been best characterised in the woodwasp *Sirex noctilio* (Slippers, *et al*. 2012). This wasp and its fungal symbiont, *Amylostereum areolatum*,

are native to Europe and North Africa (Spradbery & Kirk 1978). This mutualistic association has been intensely researched as a result of its pest status in pine-based forestry throughout the Southern Hemisphere, where it was introduced in the early 1900s (Hurley*, et al.* 2007; Slippers & Wingfield 2012). *Sirex noctilio* has recently become established in North America where it poses a potential threat to natural and commercial pine forests (Ciesla 2003; Hoebeke*, et al.* 2005). This invasion marks the first time that *S. noctilio* and *A. areolatum* have been introduced into an area where native *Sirex* and *Amylostereum* species exist. Studies on this introduced population have shown that the mutualism between *Sirex* and *Amylostereum* species is not as specific as was previously believed (Hajek*, et al.* 2013; Wooding*, et al.* 2013). These findings will influence our understanding of the evolution and maintenance of this mutualism.

While the *S. noctilio–A. areolatum* mutualism has been studied in detail in terms of the biology of the system and management strategies against this insect-fungus complex (Slippers*, et al.* 2012), less research has been carried out on the evolution of the mutualism; including factors governing its evolutionary stability. The aim of this review is to outline the importance of reproduction and transmission in the evolution and maintenance of mutualisms and to reflect on how these aspects could influence the *Sirex-Amylostereum* mutualism. Topics discussed include (i) the establishment and evolutionary stability of mutualisms, (ii) the evolution and stability of insect-fungal mutualisms, with a focus on the fungus-farming insects, (iii) reproduction and transmission in the *Sirex-Amylostereum* mutualism, and (iv) the role of reproductive and transmission strategies in the evolution of the *Sirex-Amylostereum* mutualism.

The establishment and evolutionary stability of mutualisms

The establishment and evolution of mutualisms represents a complex field of study. Mutualistic interactions rarely occur amongst only two partners. Secondary relationships with other organisms can and do influence system dynamics within the mutualism being studied, the evolutionary stability of the mutualism, as well as the nature of the interactions between mutualists. Most work in this field has been performed on pair-wise relationships because limitations often prevent the study of all interacting partners (Aanen & Hoekstra 2007; Hoeksema & Bruna 2000). This is done with the knowledge that investigating only two interacting species provides only a partial representation of the interaction, but that it can establish a foundation on which to carry out further investigations. It is also necessary to study the interacting individuals independently of one another in order to understand the selective pressures acting on the individuals that will determine the nature of the association (Ewald 1987; Sachs*, et al.* 2004).

Important avenues of inquiry into understanding the evolution of mutualisms include understanding what stimulates the initial interactions among potential partners and what causes organisms to continue interacting. This is related to the costs and benefits that are likely to affect the fitness of the interacting partners, and the mechanisms that are used to overcome conflicts of interest between interacting partners. It is also necessary to consider the external factors that are likely to influence the interaction such as abiotic factors and interactions with other organisms, which may be mutualistic, commensal or parasitic (Foster & Wenseleers 2005; Herre*, et al.* 1999; Hoeksema & Bruna 2000; Jones*, et al.* 2012; Sachs*, et al.* 2004).

In order to understand the mechanisms involved in the establishment and maintenance of mutualisms, it is first necessary to understand the types of mutualisms that exist (table 1). These differ in the amount of investment (costs incurred by the investor) and the nature of

the fitness benefits that are obtained by one or both partners (Bshary & Bergmuller 2007; Bshary & Bronstein 2011). Benefits and costs can be in the form of resources and/ or services obtained from or provided to a mutualistic partner. In this review, benefits are defined as any factor which increases an individual's chance of survival and reproductive success (fitness). Costs are defined as any factors or behaviours that decrease fitness by allocating resources to other individuals rather than to increased survival and reproductive success.

Mutualisms in which no direct investment is made by either partner are known as by-product mutualisms. In these mutualisms, the normal fitness-increasing behaviours of one partner produce resources that are useful to the other partner (Sachs*, et al.* 2004). By-product mutualisms are often characterised by short term interactions (table 1). An example of a by-product mutualism is the development of Müllerian mimicry. In this system, individuals of different but not necessarily related species develop the same warning signals for use as deterrents against their common predators. This co-ordinated predator defence is a by-product of existing colouration in insects that are repellent to the predator. The similarity of the warning signals among these species reduces the costs to all the species of educating predators of their distastefulness (Sherrat 2008).

Mutualisms in which both partners have evolved behaviours by which they appear to actively invest in the production of resources that benefit the interacting partner are known as reciprocal mutualisms (Sachs*, et al.* 2004). This apparent investment of resources by the interacting partners is not altruistic, but increases the fitness of the organism performing the action. These mutualisms are the opposite extreme of by-product mutualisms in that resources are specifically invested in a behaviour that benefits the interacting partner (table 1). They are consequently characterised by repeated or continuous interaction among the partners involved. One of the best studied examples of this type of mutualism is the relationship that exists between leaf-cutter ants and the mutualistic fungi which they farm. In

this mutualism the ants perform a costly service by providing the fungus with nutrition (leaves), as well as ensuring no competing fungi become established in the fungal garden. The fungus produces specialised nutritional structures (gonglydia), which are the sole source of nutrition for developing ant larvae, and provide a significant proportion of the adults' nutritional needs (Mueller*, et al.* 2005).

An intermediate type of mutualism is known as pseudo-reciprocity, or by-product reciprocity, in which the by-products of behaviours that increase one partner's fitness (the passive partner) benefit the other partner (the active partner) (Sachs*, et al.* 2004). The active partner develops behaviours that provide the passive partner with some beneficial resource, which increases the abundance of the by-product it produces, thereby increasing the fitness of the active partner (table 1). An example of such a relationship is that between the Greater Honeyguide and humans. This bird guides humans to bee hives, humans then forage the honey leaving sufficient food for the bird to eat. This relationship is thought to have evolved between the birds and early hominids in Africa (Dean*, et al.* 1990).

Mechanisms involved in the establishment and maintenance of mutualisms

The establishment of mutualisms is dependent on repeated interactions among the participating individuals. These interactions will occur by chance at first, and be repeated due to the fitness benefits obtained by one or both interacting partners or due to the spatial structure of the interacting populations (Leimar & Hammerstein 2010; Yamamura*, et al.* 2004). There are also theoretical models of mutualism evolving from initially parasitic interactions (Pannebakker*, et al.* 2004; Yamamura 1993). The most basic requirement for the initial establishment of a mutualism is that the fitness benefits received by the interacting individuals outweigh the costs incurred by those individuals. This allows for positive selective pressure to increase the frequency of mutualistic traits, which can be morphological,

chemical or behavioural, in a population (Bshary & Bergmuller 2007; Foster & Wenseleers 2005; Leigh 2010).

By-product mutualisms involve no costs because the resources used are natural by-products of actions that increase the fitness of the actor (Bshary & Bergmuller 2007; Sachs*, et al.* 2004). These chance interactions, where there are no costs involved, are believed to be the initiation points of many mutualisms (Hamilton 1971; Martin 1992; Mueller*, et al.* 2001; Yamamura*, et al.* 2004). Mutualisms involving pseudo-reciprocity are an intuitive "stepping stone" between by-product and reciprocal mutualisms. Initially one partner invests in the interaction to accrue greater fitness benefits, after which the other partner can, in turn, invest for increased fitness benefits. In both pseudo-reciprocal and reciprocal mutualisms, positive selection for increased inclusive fitness will ensure that the mutualistic interaction is continued over generations (Bshary & Bergmuller 2007; Doebeli & Knowlton 1998; Foster & Wenseleers 2005; Leigh 2010; Leimar & Hammerstein 2010; Schwartz & Hoeksema 1998).

One of the commonly asked questions regarding pseudo-reciprocal and reciprocal mutualisms is how they remain stable over evolutionary time, given the benefits of cheating (Herre*, et al.* 1999; Sachs 2006; Sachs*, et al.* 2004). Cheating occurs when one partner uses the benefits provided by the other partner without reciprocating, essentially acting as a parasite. This maximises the benefits received, and minimises the costs incurred by the cheater, placing the cheater at a selective advantage over non-cheaters (Douglas 2008; Ferriere*, et al.* 2002; Herre*, et al.* 1999; Sachs 2006). Three mechanisms help to maintain a high benefit to cost ratio for the organisms involved in the mutualism, namely partner fidelity feedback, partner choice and host sanctions.

In the case of partner fidelity feedback, individuals involved in the mutualism rely on the benefits derived from their partners. Thus changes in the fitness of one partner directly affect the fitness of the other partner, creating a fitness feedback loop. This mechanism ensures

that cheating individuals have a lower fitness as a result of cheating, and are consequently less likely to successfully produce offspring (Bshary & Bergmuller 2007; Bull & Rice 1991; Leimar & Hammerstein 2010; Sachs*, et al.* 2004). Partner fidelity feedback is found in a number of mutualisms, including the mutualism that exists between fungus growing termites in the sub-family Macrotermitinae and their cultivated fungi in the genus *Termitomyces*. Termites can acquire their fungal symbionts either as spores from the environment or as vertically transmitted clones (Aanen*, et al.* 2009a; Korb & Aanen 2003; Nobre*, et al.* 2011). They then cultivate this fungus, providing it with nutrients and a suitable environment for growth. The fungus in turn provides nutrients to termite larvae and adults (Mueller*, et al.* 2005). In this mutualism, the lifetime association of the termite colony and a single fungal cultivar, maintained by the termites, ensures that neither partner cheats because this would result in decreasing the fitness of both organisms (Aanen*, et al.* 2009a).

Partner choice is the conditional provision of resources by one partner based on the behaviour of the other partner during the interaction (Foster & Wenseleers 2005). Using this control mechanism, interacting organisms can prevent cheating by choosing not to provide, or reducing the provision of, resources (benefits) from partners that are cheaters, thereby decreasing their fitness (Bshary & Bergmuller 2007; Bull & Rice 1991; Leimar & Hammerstein 2010; Sachs*, et al.* 2004). This mechanism can be used prior to, or after the interaction has been initiated. One of the better known mutualisms using partner choice prior to interaction as a control mechanism is that of the bobtail squid, *Euprymna scolopes*, and the bioluminescent bacterium *Vibrio fischeri*. In this mutualism the bacterial partners are acquired horizontally from the surrounding sea water after the squid hatches. *Vibrio fischeri* passes through a rigorous series of physical and chemical hurdles before being able to colonise the squids' light organs. This ensures that only the appropriate strain of *V. fischeri* is able to colonise the light organs, where the bacterial cells remain for the duration of the host squids' life. The bacteria multiply within the light organ, using resources provided by the

squid. Daily, 95% of the bacterial cells are ejected from the light organ increasing the pool of acceptable mutualists for the squid's offspring (Nyholm & McFall-Ngai 2004).

Host sanctions are often viewed as a form of partner choice in that they are effectively choices imposed after the initial interaction between mutualists (Bshary & Bergmuller 2007; Foster & Wenseleers 2005; Kiers & Denison 2008; Leimar & Hammerstein 2010). This mechanism acts to control cheating behaviours by withholding resources or services to partners which are exhibiting cheating behaviour, resulting in decreased fitness of the cheating partner (Bshary & Bergmuller 2007; Foster & Wenseleers 2005; Sachs*, et al.* 2004). Sanctions are used to minimise cheating in the fig tree-fig wasp mutualism, where figs are dependent on fig wasps for pollination. Fig wasps lay their eggs in the flowers of the figs that they pollinate, using the resources provided for larval development. In the fig tree *Ficus nymphaeifolia*, sanctions are imposed on non-pollinating wasps whose larvae are not provided with resources from the fig tree because the fruit in which the eggs are laid are abscised (Jander*, et al.* 2012). In this mutualism, as in plant-rhizosphere mutualisms, sanctions do not completely prevent cheating (Jander*, et al.* 2012; Kiers & Denison 2008; Simms*, et al.* 2006). Multiple wasps can enter a fig, and when one pollinating and one non-pollinating wasp enter the fig, no sanctions are imposed by the fig tree (Jander*, et al.* 2012).

Many mutualisms display elements of two or all of the above-mentioned anti-cheater mechanisms. An example of this is the obligate pollination mutualism that exists between yucca plants and yucca moths. In this mutualism, the plant has the ability to abscise flowers in which too many yucca moth eggs are deposited, preventing seed formation and depriving the larvae of their only food source (Huth & Pellmyr 2000; Marr & Pellmyr 2003; Pellmyr & Huth 1994). Selective abscission in flowers has been described as both a form of partner choice (Bull & Rice 1991; Foster & Wenseleers 2005; Sachs*, et al.* 2004) and host sanctioning (Bshary & Bronstein 2011; Leimar & Hammerstein 2010). Weyl *et al.* (2010) have suggested that partner fidelity feedback is a better explanation for the maintenance of

stability in this mutualism (Weyl*, et al.* 2010). This apparent confusion in the literature is a result of overlapping definitions of the mechanisms , particularly those of partner choice and host sanctions (Bshary & Bergmuller 2007; West*, et al.* 2007).

Factors aligning the interests of mutualists

Cheating behaviour arises due to a conflict of interest between the partners in a mutualism, which results from each partner aiming to maximise its' own reproductive success (Ferriere*, et al.* 2002; Herre*, et al.* 1999). In light of this, mutualisms have also been described as mutual exploitations rather than cooperative associations (Bronstein 1994; Herre*, et al.* 1999; Sachs*, et al.* 2004). The persistence of cheating in mutualisms, despite the presence of mechanisms that prohibit it, lends further support to the idea of mutualisms being mutual exploitations. This has led to the exploration of other factors that act in concert with stabilising mechanisms to further discourage cheating. These are factors that act to align the reproductive interests of the interacting partners.

The primary factor capable of aligning reproductive interest of partners in a mutualism is the vertical transmission of one partner (the symbiont) between generations of the other partner (the host). This is because symbiont reproductive success is completely dependent on host reproductive success (Douglas 2008; Herre*, et al.* 1999; Leigh 2010). Vertical transmission is common in insect-microbe mutualisms, including the mutualisms between fungus-farming insects and their fungi (Mueller*, et al.* 2005). Vertical transmission of symbionts has also been described as one of the main mechanisms allowing parasitic symbioses to evolve into mutualisms (Ewald 1987; Sachs & Wilcox 2006; Yamamura 1993). This happens because natural selection favours the less harmful symbionts, which are passed on to the next host generation at a higher frequency than those which harm their hosts to the extent that the host has reduced reproductive capabilities (Herre*, et al.* 1999).

Vertical transmission promotes the reduction of genetic diversity within symbionts by creating a symbiont population bottleneck during transmission (Herre*, et al.* 1999). In some mutualisms, where bacterial symbionts spend their entire lifecycle within host cells, reduction in genetic diversity has occurred via genome reduction. In these mutualists, functional genes that allow the bacteria to survive outside the host cell have been lost, making the bacteria completely dependent on their hosts for reproduction (Moran & Wernegreen 2000). This can be seen in the aphid nutritional mutualist *Buchnera sp.* that has a ~600Kb genome (Charles & Ishikawa 1999; Shigenobu*, et al.* 2000). This is an extreme form of reduced genetic diversity in that the bacterial symbionts are unable to survive outside the host, and may be a factor leading to the mutualism becoming obligate. A less extreme form of reduced genetic diversity can be seen in the fungal symbionts of Attine ants and Siricid woodwasps. In these mutualisms asexual spores of the fungal symbionts are vertically transmitted across generations (Chapela*, et al.* 1994; Gilmour 1965). The reduction in genetic diversity observed in these mutualisms prevents variation in traits, which could be harmful to the host and increase the symbionts competitive abilities (Aanen*, et al.* 2009a; Herre*, et al.* 1999; Vasiliauskas*, et al.* 1998).

Another factor that can align the reproductive interests of mutualistic partners is a lack of options of partner species outside of the mutualism, which are capable of providing the necessary benefits (Douglas 2008; Herre*, et al.* 1999). This is ensured in mutualisms with vertical transmission of symbionts, because there is no choice involved in the inheritance of symbionts. Another means by which partner choice outside of the mutualism can be limited is by the spatial structure of the populations in which the mutualistic partners are found (Leimar & Hammerstein 2010). Limited dispersal of mutualists as well as repeated interactions amongst potential mutualistic partners caused by population spatial structure can act to stabilise mutualistic interactions. This spatial structure can also be a result of environmental heterogeneity limiting dispersal opportunities of potential mutualists (Boza & Scheuring 2004; Doebeli & Knowlton 1998; Yamamura*, et al.* 2004). While limited dispersal

is generally considered to promote cooperation, there are cases where limited partner choice due to spatial structure does not promote cooperation. Verbruggen *et al.* (2012) found that in some cases beneficial arbuscular mycorrhizal fungi are at a competitive advantage when spatial structure is reduced.

The mechanisms and factors that align the interests of mutualistic partners are not sufficient to completely prevent cheating behaviour in evolutionarily stable mutualisms. Ferriere *et al.* (2002) proposed a model to explain the maintained presence of cheating in mutualisms. They suggest that the use of excess resources by cheating individuals provides a competitive background against which better mutualists can be selected (Ferriere*, et al.* 2002). This concept was developed further by Jones *et al.* (2012), who viewed mutualisms in terms of resource exchange, rather than net costs and benefits. This approach also highlighted the importance of cheaters in providing a background for the selection of more effective mutualists (Jones*, et al.* 2012). All of the mechanisms and factors outlined above act in concert to ensure that mutualisms are able to withstand the selective forces that also act to destabilise them (Herre*, et al.* 1999; Sachs*, et al.* 2004; Sachs & Simms 2006).

The evolution and stability of insect-fungal mutualisms: the fungus-farming insects

Insect-fungal mutualisms are widespread in nature, with the best characterised examples being those between bark beetles, ambrosia beetles, termites and Attine ants, and their respective fungal mutualists. These mutualisms have a number of common features, whereby the fungal symbiont provides benefits in the form of nutrition for their insect hosts. The insect host provides benefits by transporting the fungal symbiont to suitable substrates (bark and ambrosia beetles), or to areas where suitable substrate is provided (ants and termites) (Aanen*, et al.* 2009a; Mueller*, et al.* 2005; Six 2012). Attine ants and termites also actively maintain fungal monocultures of their symbionts. These areas of overlap have

allowed for generalisations to be made regarding the evolution of obligate insect-fungal mutualisms, making the fungus-farming insect-fungal mutualisms excellent model systems for investigations into the evolution of these mutualisms (Mueller*, et al.* 2005).

The Attine ant, termite and bark beetle-fungus mutualisms are thought to have evolved in one of two ways: the traditional 'consumption first' strategy and the recently-described 'transmission first' strategy (Fig. 1). The consumption first strategy is used in seven explicit models that follow the same basic strategy but differ in the substrate that is thought to have been used in initial cultivation of the fungus. These substrates are stored seeds, nest walls, rotting wood, mycorrhizae, arthropod corpses, ant faeces and infrabuccal pellets (Mueller*, et al.* 2001). More recently Sánchez-Peňa (2005) added an eighth model to the consumption first strategy. He suggested that the Attine ant-fungus mutualism could have arisen from an association with ambrosia beetles or Siricid woodwasps (Sánchez-Peňa 2005). This model has been refuted in that the evolution of fungal agriculture in ambrosia beetles is more recent than in ants (Mueller*, et al.* 2005; Rabeling*, et al.* 2005). The possibility of an interaction with (now extinct) Siricid woodwasps has yet to be explored.

The 'transmission first' model was proposed by Mueller *et al* (2001). This model suggests that the ancestors of ant-cultivated fungi utilised ants to disperse spores. Here, a possible reward system similar to eliosomes was used to ensure that ants were attracted to the fungal species that they were dispersing. The fungus subsequently grew within the nests of ants from regurgitated infrabuccal pellets after which the ants began to feed on the fungus in their nests and eventually cultivated a fungal garden (Mueller*, et al.* 2001). Both the transmission first and consumption first models can be framed in terms of the mechanisms necessary for the evolution of mutualisms described above. In the consumption first model, the continuous contact between the ants and fungi allows for the establishment of a partner choice situation, where ants can select the fungi with the best nutritional value. In the transmission first model constant transmission of beneficial fungal spores to the nest results in the greatest fitness

benefit to the ants creating a partner-fidelity feedback loop. The ants however do posses the ability to transport spores from different fungi allowing for partner choice.

Role of reproductive and transmission strategies in the evolution and stability of insect-fungal mutualisms

The fungus-farming insect-fungal mutualisms have been evolutionarily stable for the last 20-65 million years (Aanen*, et al.* 2002; Farrell*, et al.* 2001; Mueller*, et al.* 2001). The evolutionary stability of these mutualisms is maintained by many of the mechanisms and factors described in the previous section. The mechanism thought to be of foremost importance in maintaining this stability is partner fidelity feedback (Mueller*, et al.* 2005). This is facilitated by the lifetime association of the mutualists, because any cheating behaviour by the host or symbiont, could be detrimental to the survival of both mutualists (Aanen*, et al.* 2009a; Mueller*, et al.* 2005; Sachs*, et al.* 2004). Attine ants and termites maintain fungal monocultures in their gardens. Bark beetles attempt to maintain a monoculture of their vertically transmitted fungi, but invariably their gardens are assemblages of the dominant, vertically transmitted fungus, and secondary beneficial fungi and bacteria (Mueller*, et al.* 2005). The fact that fungal gardens are maintained as monocultures helps to minimise the conflicts of interest between host insects and fungi because fungi do not need to compete with close relatives for resources. This prevents selection for competitive traits that could harm the host (Aanen*, et al.* 2009a; Frank 1996; Herre*, et al.* 1999; Mueller*, et al.* 2001; Six 2012).

Vertical transmission of the fungal symbiont in Attine ants, some termites and bark beetles discourages cheating behaviour because the reproductive interests of the insects and fungi are entwined (Frank 1996; Herre*, et al.* 1999; Mueller*, et al.* 2005). This vertical transmission decreases symbiont genetic diversity, minimising within-host conflict, as well as ensuring that symbionts are completely dependent on their hosts for reproduction (Douglas 2008;

Frank 1996; Herre*, et al.* 1999; Leigh 2010). The majority of fungal symbionts are able to exist outside of the mutualism, despite their dependence on hosts for transmission (Mueller*, et al.* 2005), indicating that vertical transmission alone is not sufficient to result in complete fungal dependence on hosts. This is illustrated by cases where horizontal transmission has occurred in ant lineages (Mehdiabadi*, et al.* 2012; Mikheyev*, et al.* 2006; Mikheyev*, et al.* 2007). The majority of termites obtain garden inoculates as fungal spores from the environment, making the main mode of symbiont transmission horizontal in these insects (Korb & Aanen 2003). This indicates that partner choice may be playing a greater role in the maintenance of evolutionary stability in the fungus-farming insect-fungus mutualisms than previously thought (Mikheyev*, et al.* 2007).

The biology of reproduction and transmission in the *Sirex-Amylostereum* mutualism

Sirex woodwasps (family Siricidae) are xylophagous sawflies commonly known as horntails (Schiff*, et al.* 2006). These wasps are native to the Northern Hemisphere where they are generally considered secondary pests of angiosperms and conifers (Goulet & Huber 1993; Spradbery & Kirk 1978). All members of the Siricidae have wood boring larvae (Smith 1979), and most are thought to be involved in species-specific, obligate mutualisms with basidiomycete wood decay fungi that are dispersed as asexual spores by the wasps (Gilmour 1965). The mutualism is facultative for the fungi that are capable of dispersing as windblown sexual basidiospores (Wermelinger & Thomsen 2012).

Wood decay fungi are essential to the lifecycle of siricid wasps, even in wasps that do not carry the fungus themselves (Fukuda & Hijii 1997). The ingestion of fungal mycelia by developing larvae allows them to retain digestive enzymes produced by the fungus. These enzymes facilitate digestion of ingested wood, providing nutrients for the developing larvae (Kukor & Martin 1983). Fungal mutualists benefit from the association as female woodwasps

transport asexual spores to suitable sporulating environments; namely the sapwood of stressed, dying or dead host trees (Goulet & Huber 1993). When they oviposit, female woodwasps simultaneously deposit phytotoxic venom and spores of their symbiotic fungus into trees. The venom and fungus are carried in specialised venom glands and mycangia, respectively. The venom kills the wood at the drill site and releases the fungal odia from the wax packet surrounding them, allowing the fungus to grow into the wood (Gilmour 1965; Ryan & Hurley 2012).

Reproduction in Sirex noctilio

Sirex woodwasps are members of the order Hymenoptera (suborder Symphyta). Sex determination mechanisms within the Hymenoptera are extremely diverse and sparsely identified, particularly in the Symphyta. Only four species within the Symphyta have been studied in terms of their sex determination mechanisms, while in the sub-order Apocrita, over 60 species have been studied (van Wilgenburg*, et al.* 2006). While genetic mechanisms of sex determination are highly varied, the order Hymenoptera has the defining characteristic that all its members follow a haplodiploid sex determination system (Cook & Crozier 1995). In this system, males are haploid and females are diploid (Cook 1993; Heimpel & de Boer 2008).

In the Hymenoptera, arrhenotokous parthenogenesis (arrhenotoky) is the dominant form of sex determination. This form of sex determination evolved 300 million years ago and is found in all families in the order (Heimpel & de Boer 2008). In this system, females develop from fertilised and males from unfertilised eggs (Cook 1993). Many models have been developed to explain the genetic mechanisms underlying arrhenotokous development; however, only two have empirical support. The genetic mechanisms with experimental support are complementary sex determination (CSD) (Beye*, et al.* 2003) and genetic imprinting sex determination (Verhulst*, et al.* 2010).

The large variety of possible sex determination systems found in the Hymenoptera makes identifying the mode of sex determination in a particular species difficult. Several criteria have been identified to identify populations which may follow a CSD model, as this is the most commonly identified model in the order (van Wilgenburg*, et al.* 2006). The Symphyta are likely to follow the CSD model of arrhenotokous parthenogenesis (Asplen*, et al.* 2009). Hence *Sirex noctilio* is also expected to follow a CSD model, as this is the accepted ancestral model of sex determination in the literature (de Boer*, et al.* 2012; Schmieder*, et al.* 2012).

Reproduction and transmission in Amylostereum areolatum

The life-cycle of *Amylostereum* follows that of a typical Basidiomycete (Fig. 2). Nuclear fusion is spatially and temporally separated in the Basidiomycetes (Kues 2000). The monokaryotic phase, also called the homokaryon, is haploid, however cells can contain more than one genetically identical nucleus (Casselton & Olesnicky 1998; Kues 2000; Raudaskoski & Kothe 2010). During mating, these monokaryotic hyphae, containing nuclei of different genetic types, fuse allowing reciprocal nuclear migration. This leads to the formation of dikaryotic (heterokaryotic) hyphae containing two genetically distinct haploid nuclei.

Clamp connections make it possible to retain the dikaryotic state in developing mycelium. This is a complex form of cell division ensuring the maintenance of one copy of each haploid nucleus in each dikaryotic cell. The resulting fertile dikaryotic mycelium is able to form fruiting bodies on which protected basidiospores are produced. In the immature fruiting body, karyogamy occurs between the nuclei present in the dikaryon in specialised cells known as basidia, followed by meiosis and migration of the resulting haploid nuclei into specialised basidiospores once the fruiting body has matured (Hawksworth*, et al.* 1995; Kues 2000).

Basidiospores are spread by various means, and germinate to form monokaryotic mycelia. This is the start of the less dominant part of the Basidiomycete lifecycle. In this phase of the lifecycle, homokaryotic hyphae form vegetative spores (known as arthrospores) on aerial structures (odiophores)that germinate to form homokaryotic hyphae genetically identical to their parents (Casselton & Olesnicky 1998; Kues 2000).

Amylostereum areolatum associated with woodwasps is heterokaryotic, forming clamp connections in culture. There is, however, little evidence of sexual reproduction of the fungus in its native range, where fruiting bodies are rare (Vasiliauskas*, et al.* 1998). No evidence of sexual reproduction has been found in any of the areas where *A. areolatum* is invasive (Slippers*, et al.* 2003). This results from the fact that this fungus is spread only as oidia (heterokaryotic mycelia fragments) when associated with *S. noctilio* and other *Sirex* species. Clones of these asexually reproducing mycelia are widely distributed across the native and invasive ranges of *S. noctilio* (Slippers*, et al.* 2001; Vasiliauskas & Stenlid 1999; Vasiliauskas*, et al.* 1998).

The role of reproductive and transmission strategies in the evolution of the *Sirex-Amylostereum* mutualism

While the evolutionary relationships between symbionts in the fungus-farming insects have been described in detail, relatively little is known about the evolution of the *Sirex-Amylostereum* mutualism. The presence of co-evolved characters suggests that the mutualism has been stable over a long period of evolutionary time. These characters include mycangia in *Sirex* species and the extended asexual phase in *Amylostereum* species. This mutualism is likely to be stabilised over evolutionary time by many of the factors and mechanisms outlined in the previous sections.

Mechanisms and factors stabilising the Sirex-Amylostereum *mutualism*

Partner fidelity feedback is likely to be the main mechanism maintaining the stability of the *Sirex-Amylostereum* mutualism. Partner fidelity feedback is defined as a fitness feedback loop that minimises cheating in mutualisms (Bshary & Bergmuller 2007; Bull & Rice 1991; Leimar & Hammerstein 2010; Sachs*, et al.* 2004). This mechanism is particularly effective in preventing the wasp from cheating, as failing to transport *Amylostereum* will result in the death of the *S. noctilio* larvae. *Amylostereum* is also subject to partner fidelity feedback as failing to contribute to wood digestion, which would result in larval death, would minimise opportunities for reproduction by preventing fungal transmission to new host trees. This transmission to host trees is essential to *A. areolatum* as sexual reproduction (as evidenced by fruiting bodies) is rare and reliant on the interaction of genetically dissimilar monokaryotic hyphae (Vasiliauskas & Stenlid 1999; Vasiliauskas*, et al.* 1998). This feedback loop is most likely also strengthened by the fact that *Sirex* wasps spend the majority of their life-cycle in constant contact with the *Amylostereum*-infested wood on which they feed as larvae. This lifetime association between wasps and their fungal symbionts minimises possible cheating behaviour which would decrease the fitness of both partners (Aanen*, et al.* 2009a).

Partner choice was until recently not expected to play a major role in the prevention of cheating within the mutualism. This is because vertical transmission of fungal symbionts precludes the ability of both *Sirex* and *Amylostereum* to choose between symbionts and hosts respectively. There is, however, some evidence of partner choice in the Eastern United States. After the invasion of *S. noctilio* into this area, it was found that specimens of the native *S. nigricornis* were carrying *A. areolatum* (Bergeron*, et al.* 2011; Hoebeke*, et al.* 2005; Nielsen*, et al.* 2009, Wooding, *et al.* 2013). This was unexpected as *A. areolatum* was not known to occur in North America prior to the invasion of *S. noctilio* and all native *Sirex* species were thought to carry *A. chailletii*. A recent study by Hayek *et al.* (2013) found that native Eastern North American *S. nitidus* and *S. nigricornis* were carrying *A. areolatum.* The

native wasps were found to be carrying one of three strains of *A. areolatum* designated by intergenomic spacer (IGS) region profiles. These profiles were IGS-BE, IGS-D and IGS-BD. IGS-D and IGS-BD, and were isolated from both native *Sirex* species and the invasive *S. noctilio.* IGS-BE was isolated only from native *Sirex* species (Hajek*, et al.* 2013). Wooding *et al.* (2013) also identified *A. areolatum* with profile IGS–BE only from *S. nigricornis* (Wooding*, et al.* 2013). These findings support previous work by Nielsen *et al.* (2009) and Bergeron *et al.* (2011) who identified an *A. areolatum* strain unique to Eastern North America (Bergeron*, et al.* 2011; Nielsen*, et al.* 2009). This lends credence to the hypothesis of a native strain of *A. areolatum* in Eastern North America. If this is the case, then partner choice could be an important mechanism in the prevention of cheating in native *Sirex* species in Eastern North America.

The discovery of fungal symbiont switching in Eastern North America means that partner fidelity feedback is unlikely, at least in these populations, to be able to minimise cheating to the extent that it ostensibly does in European populations. If a cheater strain were to arise that has a reasonable chance of being picked up by co-occurring woodwasps, this strain could rapidly spread through woodwasp populations. The spread of the cheater strain could potentially destabilise the mutualism via shifting benefit: cost ratios or potential shifts along the parasitism mutualism continuum (Sachs & Simms 2006). Another outcome would be altered selective pressures on non-cheater strains due to the presence of the cheater strains (Ferriere*, et al.* 2002; Jones*, et al.* 2012). Fungal strain switching between woodwasp species will also result in fungal strains being exposed to different selective pressures, whether as a product of host tree mismatches due to woodwasp preferences or potential mismatches with the woodwasp larvae and/ or adults. Fungal species switching among woodwasps has many potential knock-on effects, including the possibility of mismatches with natural parasites and parasitoids (Slippers*, et al.* 2012). Whether these knock-on effects will be ecologically significant needs to be carefully examined in future studies.

Vertical transmission of *Amylostereum* aligns the reproductive interests of wasp and fungal species (Douglas 2008; Herre*, et al.* 1999; Leigh 2010). This ensures that *Amylostereum* reproduction is completely dependent on *Sirex* transmission when the fungus is associated with the wasp. This theoretically prevents selection for cheating traits; particularly given the possibility that *A. areolatum* associated with *Sirex* spp may have lost the ability to form fruiting bodies (van der Nest, personal communication). Populations of *A. chailletii* in Northern Europe have genetic structures similar to sexually reproducing populations of Basidiomycetes, indicating that these populations are transmitted both vertically by woodwasps and by abiotic factors such as wind (Vasiliauskas & Stenlid 1999; Vasiliauskas*, et al.* 1998). Populations of *A. areolatum* are less genetically diverse, as is expected with strict vertical transmission of asexual mycelia fragments (Herre*, et al.* 1999; Vasiliauskas & Stenlid 1999; Vasiliauskas*, et al.* 1998). This reduced diversity can also be a function of reduced frequency of sexual reproduction in *A. areolatum* populations in Northern Europe. This reduced genetic diversity in *A. areolatum* is likely to prevent selection for competitive traits that could harm its wasp host (Frank 1996; Herre*, et al.* 1999).

An important concept that has only recently been considered in the ecology of the *Sirex-Amylostereum* mutualism is its interaction with other co-habiting phloem- and wood-boring insects and the fungi (Ryan*, et al.* 2012; Ryan*, et al.* 2011). Many of these insects, such as bark beetles and their mutualistic fungi, have mutualisms that are as old as that of the Siricids and their mutualistic fungi. The organisms involved in these mutualisms could have interacted with one-another for millions of years (Mueller *et al* 2005). Characterizing the ecology of these interactions would help understand some of the selective pressures that have shaped the evolution of Siricid-fungal mutualisms. For example, competing fungi introduced into the phloem of stressed trees significantly affect the fitness of *Amylostereum*, and thus the Siricid wasp it is associated with. Selection for behaviour related to host choice, inoculation of the symbiont (time and position) and tunnelling might have been affected by the presence of such competing insect-fungal mutualisms in the phloem. It also has

important implications for understanding and managing current population dynamics and outbreaks of Siricids.

Conclusion

The study of the evolution of mutualisms continues to gain momentum, continuously expanding our understanding of these important interactions. Recent studies have highlighted the importance of mutualism in the creation of diversity, building on its importance in the origin of eukaryotic life (Gray*, et al.* 2001; Janson*, et al.* 2008). The emerging complexity of these interactions, with multiple interacting partners and environments implies that there will be many fascinating questions and components to be analysed and unravelled. It also demonstrates the need to examine mutualisms as important associations in ecosystems, particularly in light of the rapid global climate changes that are being experienced at present (Aanen*, et al.* 2009b; Wermelinger & Thomsen 2012).

This review highlights a number of fundamental questions regarding the evolutionary dynamics within the *Sirex-Amylostereum* mutualism that remain to be answered. These include an understanding of the specificity of the relationships between *Sirex* and *Amylostereum* species, elucidation of the evolutionary origins of the mutualism, and the identification of all the other organisms involved in the mutualism (Slippers*, et al.* 2006). While empirical data are still needed, a number of aspects of the evolution of the mutualism and its stability can be inferred from what is known about the specificity of the relationship, as well as from co-evolved characters which exist in both partners.

The availability of advanced molecular techniques, along with the recent invasion of *Sirex noctilio* into North America present a unique opportunity to study the diversity of the *Amylostereum* and *Sirex* species, the spatial structure of that diversity, and to identify shared

genotypes as signatures of strain exchange. Understanding the evolutionary origins and dynamics of the *Sirex-Amylostereum* mutualism will increase our understanding of the relationships that exist between other insects in obligate associations with fungi. While the fungus-farming insects provide a useful foundation from which to better to understand insect-fungal mutualisms, an additional and unrelated mutualistic system such as that typified by *S. noctilio* and *A. areolatum* would provide outstanding opportunities for comparison.

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Table 1 Types of interactions involved in different mutualisms

Type of mutualism		By-Product	Pseudo-reciprocal	Reciprocal
Organism 1	**Partner type**	Passive	Passive	Active
	Costs	None	Minimal; Specific behaviour increases benefits received from organism 2	Resource provided to organism 2
	Benefits	None	Resources obtained from organism 2	Benefit received from organism 2
Organism 2	**Partner type**	Passive	Active	Active
	Costs	None	None	Resource provided to organism 1
	Benefits	Benefits from natural by-product of organism 1	Behaviour of organism 1 increases resources available to organism 2	Benefit received from organism 1
Example		Müllerian mimicry	Greater Honeyguide and humans	Attine ants and their fungal gardens

Fig. 1 Models of evolution of the Attine ant-fungus mutualism. **Consumption first strategy:** This model suggests that a fungus growing accidentally in ant nests was incorporated into the ants' diet. The ants then evolved the ability to promote fungal growth by addition of substrate. The final evolutionary step was the development of a mechanism by which fungal cultivars could be transmitted from parent to daughter nests. **Transmission first strategy:** This model suggests that ants were initially used by specialised fungi as transport vectors. The ants subsequently incorporated these fungi into their diets. Finally, they evolved the ability to cultivate the fungi by addition of substrate (Modified from Mueller*, et al.* 2001)

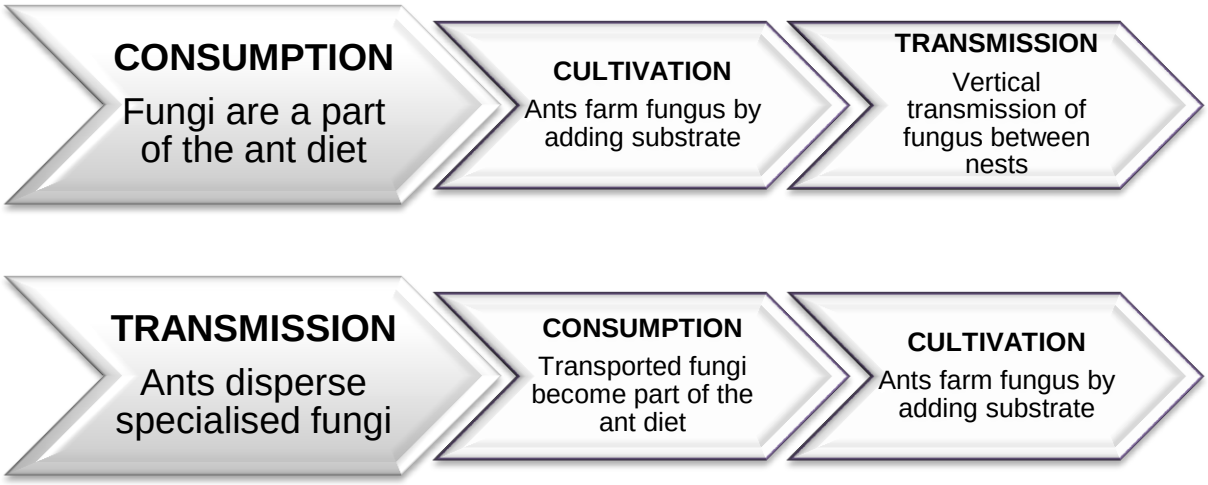
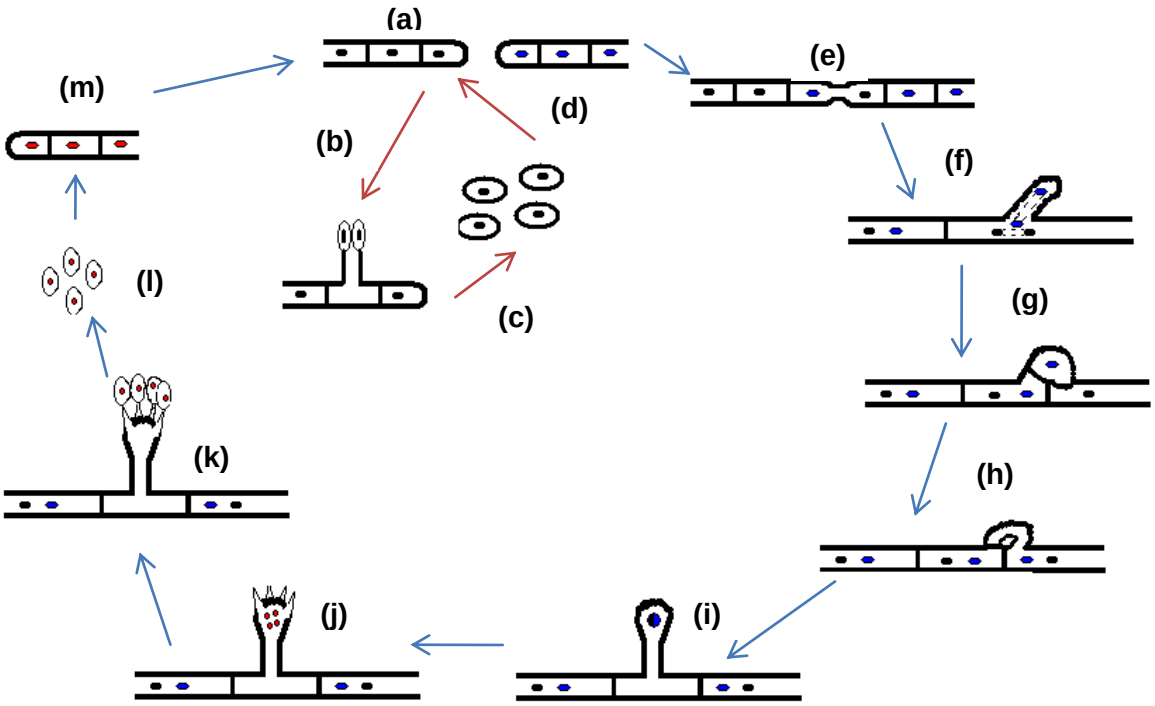


Fig. 2 Life-cycle of a typical Agaricomycete. **Asexual phase** (red arrows) (a) Monokaryotic hyphae (b) Odiophore formation (c) Odia (d) Odia germination into monokaryotic hyphae. **Sexual phase** (blue arrows) (a) Monokaryotic hyphae of different genotypes attracted to one-another (e) Hyphal fusion and reciprocal nuclear migration (f-h) Dikaryotic cell division and clamp connection formation (i) Karyogamy (j) Meiosis and probasidium formation (k) Basidium with four basidiospores (l) Basidiospore release and germination (m) Monokaryotic hyphal growth.



Lack of fidelity revealed in an insect-fungal mutualism after invasion

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Abstract

Symbiont fidelity is an important mechanism in the evolution and stability of mutualisms. Strict fidelity has been assumed for the obligate mutualism between *Sirex* woodwasps and their mutualistic *Amylostereum* fungi. This assumption has been challenged in North America where a European woodwasp, *Sirex noctilio*, and its fungal symbiont *Amylostereum areolatum*, have recently been introduced. We investigate the specificity of the mutualism between *Sirex* and *Amylostereum* species in Canada, where *S. noctilio* co-infests *Pinus* with native *S. nigricornis* and its mutualist *A. chailletii*. Using phylogenetic and culture methods, we show that extensive, reciprocal exchange of fungal species and strains is occurring, with 75.3% of *S. nigricornis* carrying *A. areolatum* and 3.5% of *S. noctilio* carrying *A. chailletii*. These findings show that the apparent specificity of the mutualism between *Sirex* spp. and their associated *Amylostereum* spp. is not the result of specific biological mechanisms that maintain symbiont fidelity. Rather, partner switching may be common when shifting geographic distributions driven by ecological or anthropogenic forces bring host and mutualist pairs into sympatry. Such novel associations have potentially profound consequences for fitness and virulence. Symbiont sharing, if it occurs commonly, may represent an important but overlooked mechanism of community change linked to biological invasions.

Introduction

A frequently cited consequence of globalisation is the growing homogenisation of biotic communities, commonly driven by biological invasions. Invasive species can have serious negative impacts on the ecosystems in which they become established. Such impacts include invasive species altering existing mutualisms among native species, or acquiring novel symbionts that affect virulence in one partner (Battisti*, et al.* 1999; Hulcr & Dunn 2011; Richardson 2011).

In the Southern Hemisphere the invasive wood-boring wasp *Sirex noctilio* and its obligate nutritional fungal mutualist *Amylostereum areolatum*, is a highly aggressive pest complex infesting and killing healthy plantation pines (Hurley*, et al.* 2007). In its native range in Eurasia and North Africa this complex is a secondary pest, infesting dead or dying conifers, primarily in the genus *Pinus* (Spradbery & Kirk 1978). The complex has recently been introduced into eastern North America (ENA), where it poses a potential threat to planted and natural pine forests (Slippers*, et al.* 2012).

The introduction of *S. noctilio* into ENA provides an opportunity to study the specificity of the mutualism between *Sirex* and *Amylostereum* species. This mutualism was until recently assumed to be highly specific as a result of fungal mutualists being vertically transmitted as asexual spores (Sachs*, et al.* 2004). This dogma has been questioned recently with the discovery of specimens of native *S. nigricornis* and *S. nitidus* carrying *A. areolatum* (Nielsen*, et al.* 2009).

We investigate the specificity of the *Sirex-Amylostereum* mutualism in invasive and native populations in Canada. In this study we question whether native *S. nigricornis* and invasive *S. noctilio* are strictly associated with their known symbionts, *A. chailletii* and *A. areolatum* respectively. We further examine evidence for recent exchange of *A. areolatum* strains

between *S. nigricornis* and *S. noctilio* by identifying shared clonal lineages of *Amylostereum* in native and invasive wasp populations.

Materials and Methods

Sample collection, species identification, multilocus genotyping, and sequencing

A collection of 134 *Sirex* woodwasps and their mutualistic fungi, isolated from female mycangia (as described in Thomsen & Harding 2011), was obtained from collaborators in Canada (see appendix A1 and A2 for sampling locations and storage details). Wasp and fungal samples were identified to species using sequence data from the mitochondrial cytochrome *c* oxidase subunit I (COI) and mitochondrial small subunit (mtSSU) genes respectively (appendix A3 and A4). The internal transcribed spacers (ITS) and intergenomic spacer (IGS) regions of the rRNA locus were sequenced for representative samples of each *A. areolatum* mtSSU haplotype (appendix A5), to compare them with isolates from previous studies which produced multilocus genotypes (MLG's). Where cloning was necessary, the Promega pGEM®-T Easy Vector System was used. PCR products were sequenced on an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems) at the sequencing facility of the University of Pretoria.

Sequence assembly, haplotype analyses and phylogenetic analyses

Bidirectional sequences were assembled and edited in CLC Main Workbench 6.6.2 (CLC Bio Inc. Denmark) and aligned using MEGA 5 (Tamura*, et al.* 2011) and MAFFT (Katoh & Toh 2008). Sequence Evolution Models were selected using AIC in jModelTest 0.1.1 (Posada 2008). Species identification was based on group membership in neighbour-joining trees constructed in PAUP 4.0 (Swofford 2002). Haplotype analysis was carried out using

Splitstree4 (Huson & Bryant 2006). Maximum likelihood (PhyML (Guindon & Gascuel 2003)) and Bayesian (MrBayes (Huelsenbeck & Ronquist 2003)) approaches were used for phylogenetic analysis of ITS sequence data from representative samples. Laboratory methodologies, sequence evolution models and programme parameters can be found in appendix A6-A8.

Vegetative incompatibility assays

Vegetative incompatibility assays using randomly selected representative samples from each identified *A. areolatum* mtSSU haplotype were performed using established methods to determine vegetative compatibility groups (VCG's; appendix A9) (Slippers*, et al.* 2001). VCG richness was then used as an additional measure of genetic diversity.

Results

Species identification and haplotype analysis

COI sequencing identified 77 *S. nigricornis* and 57 *S. noctilio* specimens. Fungal species switching has occurred in Canadian siricid populations with 75.3% of *S. nigricornis* females carrying *A. areolatum* (n=58), and 3.5% of *S. noctilio* females carrying *A. chailletii* (n=2; appendix A10). We detected a single mtSSU haplotype for *A. chailletii* and three mtSSU haplotypes for *A. areolatum,* which differed by a maximum of two base pairs (Fig. 1). Two of the *A. areolatum* haplotypes, H3 and H2, were uniquely detected in *S. noctilio* and *S. nigricornis* respectively, whereas the third (H1) was carried by both species.

Multilocus genotype analysis

Representative *A. areolatum* isolates for which we obtained MLGs grouped into two clades, (A and B), based on ITS sequence data (table 1, appendix A12). Both MLG1 and MLG3 corresponded with previously isolated samples (MLG3 and MLG2 in Bergeron*, et al.* 2011 respectively). MLG2 was unique in this study, although ITS (MLG2a and MLG2b) and IGS sequences (MLG2a) have been previously isolated (Hajek*, et al.* 2013; Nielsen*, et al.* 2009).

Vegetative incompatibility assays

Multiple VCG's were identified within each mtSSU haplotype. VCG richness was high; 14 VCG's were identified from 27 isolates of *A. areolatum.* Ten isolates were incompatible with all others, and up to 5 distinct VCGs were isolated from wasps emerging from the same tree. One VCG was shared between *S. noctilio* and *S. nigricornis,* confirming recent lateral transfer of strains between invasive and native Canadian *Sirex* populations.

Discussion

The identification of a shared MLG and VCG of *A. areolatum* between newly sympatric *Sirex* species strongly supports direct lateral transfer of symbionts. This transfer is bi-directional, as ~3% of *S. noctilio* females carried *A. chailletii*, but is skewed towards *A. areolatum* transfer to *S. nigricornis*. This skewed directionality of transfer could result from temporal patterns in oviposition and emergence, or disproportionate utilization of *S. noctilio*-weakened trees by *S. nigricornis*.

The lack of host-symbiont fidelity detected in this study, also shown in an independent concurrent study (Hajek*, et al.* 2013), calls into question the mechanisms maintaining the

apparent fidelity of symbiont associations in the native range of *S. noctilio*. It is possible that geographic, host, or temporal segregation among native siricids may be sufficient to maintain the low rates of transfer observed in Europe and elsewhere (Spradbery & Kirk 1978). Alternatively, fungal and or wasp mis-identification and sparse sampling across Europe could have led to underestimates of symbiont switching (Slippers*, et al.* 2012) We propose that the process of invasion of *S. noctilio* into Canada has facilitated symbiont switching among *S. noctilio* and *S. nigricornis*. The sequence data produced in this study (GenBank; appendix A3-A5) will serve as an important reference for further studies examining the mechanism of horizontal symbiont transfer among siricid woodwasps.

Symbiont transfer is stable over at least one generation; 54 of 58 *S. nigricornis* females carrying *A. areolatum* emerged from trees where no *S. noctilio* emerged in the sampling season. Similarly, *S. noctilio* specimens carrying *A. chailletii* emerged from logs that were not infested with *S. nigricornis*. However, these wasps could have entered trees pre-infected via wind dispersed spores (as can occur with *A. chailletii* in Europe (Vasiliauskas*, et al.* 1998)), un-emerged woodwasp infestation, or aborted woodwasp attacks. Important questions which should be addressed by further studies are whether carrying the “wrong” symbiont influences wasp or fungal fitness, and whether novel symbiont associations are stable over longer time periods. It is evident however, that fungal switching increases opportunities for the fungi to spread by increasing the pool of potential vectors.

Previous studies have shown that diversity of *A. areolatum* in the Southern Hemisphere is low, with only two VCG’s identified (Slippers*, et al.* 2001). We identified 14 VCG’s demonstrating higher than expected diversity in an invaded area, even higher than in the putative native range in Northern Europe (Vasiliauskas*, et al.* 1998). This diversity could reflect more than one introduction of the fungus into ENA, including the possibility of introduction prior to the *S. noctilio* invasion, together with *S. juvencus* (Benson 1962). The high amount of VCG diversity could also be influenced by sexual reproduction of the fungus

in ENA. However, fruiting bodies of *A. areolatum* have not been reported in North America, and are rare in the native European range (Slippers*, et al.* 2001).

The detection of an *A. areolatum* MLG unique to Canada supports the results of Nielsen *et al.* and Bergeron *et al.* (Bergeron*, et al.* 2011; Nielsen*, et al.* 2009), who identified *A. areolatum* isolates unique to ENA. These findings suggest that this genotype could have been introduced from a previously unsampled *S. noctilio* source population, as identified by a recent analysis of a global *S. noctilio* collection (Boissin*, et al.* 2012), or that ENA harbours an unsampled native *A. areolatum* population. A concurrent study of woodwasp-fungal fidelity in eastern USA revealed that two native woodwasps, *S. nigricornis* and *S. nitiudus,* carried *A. areolatum* in their mycangia (Hajek*, et al.* 2013). The majority of these fungal isolates were shown to contain IGS type BE, which was identified in MLG2 in this study. MLG2 was associated with 55% of *A. areolatum* carrying *S. nigricornis* specimens. This IGS type, and IGS type E unique to the present study, have been identified exclusively from ENA. This lends further support to the hypothesis of a native population *A. areolatum* in ENA.

The ecological and evolutionary consequences of symbiont switching in the *Sirex-Amylostereum* mutualism are not known, but could be significant (Richardson 2011). One dramatic possibility is that symbiont swapping could induce changes in wasp virulence with respect to their ability to attack and kill healthy host trees (Hulcr & Dunn 2011). Undoubtedly, the potential threat of the *S. noctilio – A. areolatum* complex to native and commercial forest ecosystems in ENA is more complex than might previously have been anticipated. Given the specificity of interactions between native Siricids and their parasites, these relationships could also be altered by symbiont switching [6]. The discovery of symbiont switching at considerable frequency also calls into question the wisdom of importing foreign strains of the nematode *Deladenus siricidicola*, which feeds on *A. areolatum* during part of its life-cycle, as a biological control agent (Slippers*, et al.* 2012), as the nematode could easily escape into

native siricid populations. The lack of specificity observed between *Sirex* and *Amylostereum* species after the invasion of *S. noctilio* into ENA highlights a need to reassess the specificity of the mutualism in Eurasia and North Africa.

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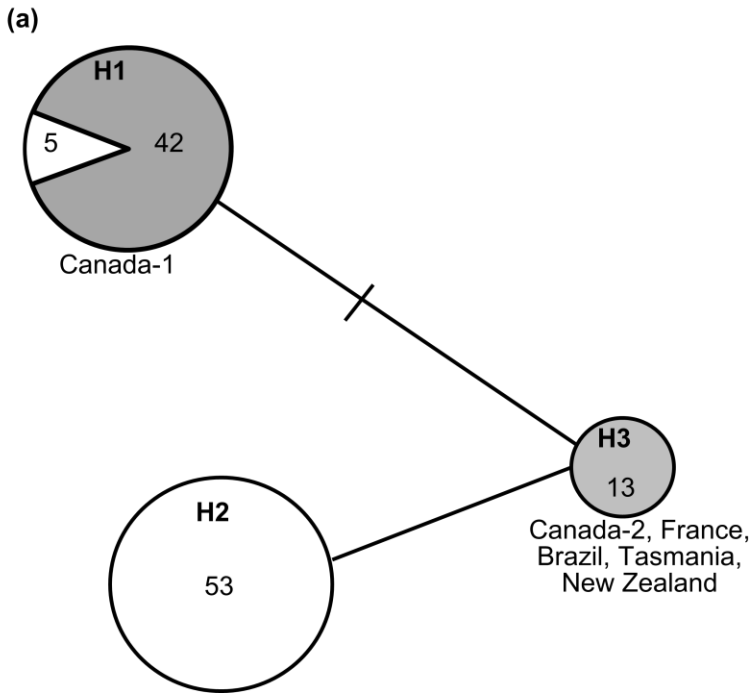
Table 1 Multilocus genotypes (MLGs) of representative *Amylostereum areolatum* samples in comparison to previously sampled *A. areolatum* isolates

Genotype	mtSSU-ITS profile	IGS profile	mtSSU previously sampled	ITS previously sampled	IGS previously sampled	Region
MLG1	H1-A	D	Bergeron *et. al.* (2011)	Bergeron *et. al.* (2011)	Bergeron *et. al.* (2011) and Hayek *et al*. (2013)	ENA, Southern Hemisphere
MLG2a*	H2-B	BE	**No**	Nielsen *et. al.* (2009)	Nielsen *et. al.* (2009) and Hayek *et al*. (2013)	USA
MLG2b*	H2-B	E	**No**	Nielsen *et. al.* (2009)	**No**	USA
MLG3a*	H3-A	BD	Bergeron *et. al.* (2011)	Bergeron *et. al.* (2011)	Bergeron *et. al.* (2011) and Hayek *et al*. (2013)	ENA
MLG3b*	H3-A	D	Bergeron *et. al.* (2011)	Bergeron *et. al.* (2011)	Bergeron *et. al.* (2011) and Hayek *et al*. (2013)	ENA

*a and b represent IGS profiles

Fig. 1 (a) Haplotype network of *Amylostereum areolatum* (n=113). Numbers of isolates per wasp species are shown within haplotype nodes. Dark grey indicates samples isolated from *S. noctilio*, white from *S. nigricornis*. (b) Haplotype network of *A. chailletii* isolates. Connecting lines specify one substitution, cross-bars specify additional substitutions separating haplotypes. Below the nodes are countries from which the haplotypes have previously been sampled (accession numbers in appendix A11).



Appendices

A1: Sampling locations of trees felled for collection of Sirex *wasps are indicated by white balloons. The black scale bar indicates a distance of 100km.*

A2: Storage collection information for wasp (EntoStock Collection) and fungal (CMW* *) samples used in this study*

Sample Number	EntoStock number*	CMW number of fungal isolate**	Sampling site	Tree
92	296	36936	202	1
95	297	36937	202	1
98	298	36938	202	1
105	299	37078	L6	2
107	300	37032	202	2
121	301	36989	L6	3
123.1	302	37053	202	1
123.2	303	36939	202	1
123.3	304	37033	202	1
124	305	37054	202	1

125	306	36940	202	1
126	307	36941	203	2
127	308	37055	203	2
128	309	37056	203	1
141	310	36942	L6	2
142	311	36990	L6	3
144	312	37057	T	3
146	313	36943	202	1
150	314	37058	L6	3
151	315	39644	L6	2
152	316	36991	L6	2
153	317	36945	L6	2
154	318	36992	L6	2
161	319	36993	203	2
163	321	37035	202	1
166	323	36946	L6	2
167	324	36947	L6	2
168	325	37206	L6	1
169	326	36994	L22	1
170	327	36995	T	2
192	328	36996	L22	1
193	329	36997	L6	2
200	331	36998	202	2
208	333	36999	203	2
209	334	37000	202	2
213	335	37079	202	2
214	336	37060	L6	2
215	337	37061	H2	1
220	338	37062	202	2
222	339	37001	L6	2
228	340	36949	L6	3
229	341	37002	L6	2
230	342	37003	L6	2
231	343	36950	202	1
232	344	36951	202	2
250	345	37063	56	1
251	346	37064	202	2
254	347	37005	201	2
260	348	37006	202	2
265	349	37036	201	1
266	350	37080	201	1
267	351	36952	203	2
277	353	37065	56	1
278	354	36954	203	2
283	355	36955	56	1
284	356	36956	203	2

285	357	36957	200	1
286	358	36958	200	1
287	359	36959	200	1
288	360	36960	200	1
289	361	37007	200	1
290	362	37037	200	1
291	363	37081	200	1
292	364	36961	200	1
293	365	37066	200	1
294	366	37067	200	1
295	367	36962	200	2
296	368	37038	200	2
309	369	37008	200	1
310	370	36963	200	1
311	371	36964	200	1
312	372	36965	200	1
313	373	37009	200	2
314	374	37010	203	2
315	375	37011	56	1
324	376	36966	200	1
325	377	37207	201	1
332	378	37012	200	1
334	379	37039	200	1
335	380	36967	200	1
337	381	36968	158	1
338	382	37068	200	2
348	383	37013	56	1
357	384	36969	200	1
358	385	36970	200	1
359	386	36971	200	1
360	387	36972	200	1
361	388	37040	200	2
362	389	37041	203	2
375	390	37014	158	2
376	391	36973	158	1
378	392	36974	T	2
380	393	37015	158	1
382	394	36975	200	1
384	395	36976	200	1
385	396	37208	200	1
386	397	36977/37016	200	1
387	398	37017	158	2
388	399	37083	158	2
389	400	37069	158	1
390	401	36978	158	1
393	402	36979	L22	1

407	403	36980	200	1
408	404	37084	158	2
412	405	37085	158	1
414	407	37043	158	1
421	408	37070	158	2
422	409	37071	158	2
423	410	37072	158	2
424	411	37018	158	2
425	412	37073	158	2
426	413	37209	158	2
427	414	36981	158	2
428	415	37074	158	1
429	416	37086	158	1
430	417	37019	158	1
431	418	37075	158	1
432	419	37087	158	1
433	420	37020	158	1
434	421	37044	158	1
437	422	37021	158	1
440	423	37076	201	1
441	424	36982	201	1
442	425	36983	201	1
443	426	37077	L22	1
444	427	36984	T	2
459	428	37022	158	2
460	429	36985	158	2
462	430	36986	158	1
464	431	36987	158	1
476	432	37045	L22	1
477	433	37023	L22	1
514	434	37024	158	1
517	435	36988	L22	1

* Permanent collections of the Forestry and Agricultural Biotechnology Institute

A3: Representative COI samples submitted to GenBank

Sample number	**Wasp species**	**COI accession number**	**MS haplotype**	**Location**
EB296	*Sirex noctilio*	KC310477	1	202
EB335	*Sirex noctilio*	KC310480	3	202
EB336	*Sirex noctilio*	KC310481	3	L6
EB319	*Sirex noctilio*	KC310478	1	203
EB328	*Sirex noctilio*	KC310479	3	L22

EB337	*Sirex noctilio*	KC310482	1	H2
EB342	*Sirex noctilio*	KC310483	1	L6
EB345	*Sirex nigricornis*	KC310484	1	56
EB347	*Sirex noctilio*	KC310485	1	201
EB362	*Sirex nigricornis*	KC310486	2	200
EB365	*Sirex nigricornis*	KC310487	1	200
EB375	*Sirex nigricornis*	KC310488	2	56
EB392	*Sirex noctilio*	KC310489	1	T
EB408	*Sirex nigricornis*	KC310490	2	158
EB424	*Sirex nigricornis*	KC310491	2	201
EB432	*Sirex nigricornis*	KC310492	1	L22

A4: Representative mtSSU samples submitted to GenBank

Sample number	**mtSSU accession number**	**Fungal species**	**Wasp species isolated from**	**mt SSU haplotype**	**Location**
36936	KC296918	*A. areolatum*	*S. noctilio*	1	202
36993	KC296895	*A. areolatum*	*S. noctilio*	1	203
36994	KC296896	*A. areolatum*	*S. noctilio*	1	L22
37061	KC296899	*A. areolatum*	*S. noctilio*	1	H2
37003	KC296900	*A. areolatum*	*S. noctilio*	1	L6
37063	KC296901	*A. areolatum*	*S. nigricornis*	1	56
37005	KC296902	*A. areolatum*	*S. noctilio*	1	201
37066	KC296904	*A. areolatum*	*S. nigricornis*	1	200
36974	KC296908	*A. areolatum*	*S. nigricornis*	1	T
36975	KC296910	*A. areolatum*	*S. noctilio*	1	T
37077	KC296916	*A. areolatum*	*S. nigricornis*	1	L22
37037	KC296903	*A. areolatum*	*S. nigricornis*	2	200
37013	KC296907	*A. areolatum*	*S. nigricornis*	2	59
37015	KC296909	*A. areolatum*	*S. nigricornis*	2	158
36982	KC296914	*A. areolatum*	*S. nigricornis*	2	201
37019	KC296913	*A. areolatum*	*S. nigricornis*	2	158
36940	KC296893	*A. areolatum*	*S. noctilio*	3	202
36945	KC296894	*A. areolatum*	*S. noctilio*	3	L6
36996	KC296897	*A. areolatum*	*S. noctilio*	3	L22
37060	KC296898	*A. areolatum*	*S. noctilio*	3	L6
37010	KC296905	*A. chailletii*	*S. noctilio*	-	203
36968	KC296906	*A. chailletii*	*S. nigricornis*	-	158
36979	KC296911	*A. chailletii*	*S. nigricornis*	-	222

36980	KC296912	*A. chailletii*	*S. nigricornis*	-	200
36983	KC296915	*A. chailletii*	*S. nigricornis*	-	201
36984	KC296917	*A. chailletii*	*S. noctilio*	-	T

A5: Representative samples used to assess diversity

Sample number	**ITS accession number(s)***	**IGS Accession number(s)***	**IGS Type**	**mtSSU Haplotype**	**Location****
CMW36936	KC329719 / KC329720 / KC329721	KC296875	D	1	202-1
CMW36940	KC329722	KC296876 / KC296877	BD	3	202-1
CMW36945	KC329723	KC296878 / KC296879	D	3	L6-2
CMW36993	KC329724 / KC329725 / KC329726 / KC329727	KC296880	D	1	203-2
CMW37060	KC329748 / KC329749	KC296891 / KC296892	BD	3	L6
CMW36996	KC329728 / KC329729 / KC329730 / KC329731	KC296881	D	3	L22-1
CMW36974	KC329751 / KC329750	KC296890	D	1	T-2
CMW37006	KC329732 / KC329733 / KC329734	KC296882	D	1	202-2
CMW37037	KC329745 / KC329746 / KC329747	KC296887	E	2	200-1
CMW37009	KC329736 / KC329735	KC296883 / KC296884	BE	2	200-2
CMW37015	KC329739 / KC329740	KC296885 / KC296886	BE	2	158-1
CMW37019	KC329741 / KC329742 / KC329743 / KC329744	KC296888 / KC296889	BE	2	158-1

*Multiple accession numbers are given where multiple alleles were found

**-1 and -2 indicate which tree wasps emerged from at each site

A6: PCR and DNA extraction conditions

Wasp DNA was extracted from thorax tissue using the *prep*GEM™ Insect DNA extraction kit from ZyGEM Corporation Ltd, as per manufacturers' instructions. Fungal DNA was extracted using a modified phenol:chloroform extraction method (Slippers*, et al.* 2004)

All PCRs were carried out in either a BIO RAD iCycler or Veriti (Life Technologies Corporation) thermocycler.

PCR of the COI gene had a total volume of 25µl, 5µl of 5x MyTaq™ Reaction Buffer (Bioline), 0.1µM of both LCO1490 and HCO 2198 (Folmer*, et al.* 1994), 0.5µl MyTaq™ DNA polymerase (Bioline), 30-100ng of template DNA was used. Cycling conditions were 95°C for 5 minutes, followed by 41 cycles of 95°C for 30 seconds, 46.5°C for 60 seconds and 72°C for 60 seconds, with a final extension step of 72°C for 30 minutes.

PCR of the mtSSU gene had a total volume of 25µl, 2.5µl 10x FastStart Taq DNA Polymerase PCR Buffer (Roche Ltd), 50nM magnesium chloride (Roche Ltd), 400ng of each dNTP, 0.1µM of both MS1 and MS3 (White*, et al.* 1990) and 2.5U FastStart Taq DNA Polymerase (Roche Ltd), 30-100ng of template DNA was used. Cycling conditions were 95°C for 3 minutes, followed by 35 cycles of 95°C for 45 seconds, 58°C for 30 seconds and 72°C for 60 seconds, with a final extension step of 72°C for 10 minutes.

PCR of the IGS rDNA gene had a total volume of 25µl, 5µl of 5x MyTaq™ Reaction Buffer (Bioline), 0.1µM of both P-1 and O-1 (Coetzee*, et al.* 2001), 0.5µl MyTaq™ DNA polymerase (Bioline), 30-100ng of template DNA was used. Cycling conditions were 94°C for 1 minute, followed by 35 cycles of 95°C for 30 seconds, 60°C for 20 seconds and 72°C for 30 seconds, with a final extension step of 72°C for 7 minutes.

PCR of the ITS rDNA gene had a total volume of 25µl; 5µl of 5x MyTaq™ Reaction Buffer (Bioline), 0.1µM of both ITS1 and ITS4 (White*, et al.* 1990), 0.5µl MyTaq™ DNA polymerase (Bioline), 30-100ng of template DNA was used. Cycling conditions were 95°C for 5 minutes, followed by 13 cycles of 95°C for 35 seconds, 55°C for 55 seconds and 72°C for 45 seconds, 13 cycles of 95°C for 35 seconds, 55°C for 55 seconds and 72°C for 2 minutes, 13 cycles of

95°C for 35 seconds, 55°C for 55 seconds and 72°C for 3 minutes, with a final extension step of 72°C for 10 minutes.

PCR of cloning products had a total volume of 50µl; 5µl 10x FastStart Taq DNA Polymerase PCR Buffer with magnesium chloride (Roche Ltd), 400ng of each dNTP, 0.1µM of both SP6 and T7 and 2.5U FastStart Taq DNA Polymerase (Roche Ltd). Cycling conditions were 95°C for 3 minutes, followed by 25 cycles of 95°C for 30 seconds, 51°C for 30 seconds and 72°C for 30 seconds, with a final extension step of 72°C for 10 minutes.

PCR and sequencing success rate was 100% for all reactions for all samples.

References

Coetzee, M. P., Wingfield, B. D., Harrington, T. C., Steimel, J., Coutinho, T. A. & Wingfield, M. J. 2001 The root rot fungus Armillaria mellea introduced into South Africa by early Dutch settlers. *Molecular Ecology.* **10,** 387-396.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. 1994 DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology.* **3,** 294-299.

Slippers, B., Crous, P. W., Denman, S., Coutinho, T. A., Wingfield, B. D. & Wingfield, M. J. 2004 Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea*. *Mycologia.* **96,** 83-101.

White, T. J., Lee, S. & Taylor, J. 1990 *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*. In PCR protocols: a guide to methods and applications (eds. D. H. G. Innis, I. J. Sninsky& T. J. White) 315-322. San Diego Academic Press

A7: Modified Promega pGEM®-T Easy Vector System Protocol

1. **Ligation reaction:** Ligation reactions contained 2.5µl 2X Ligation Buffer, 0.5µl T4 DNA Ligase, 0.5µl pGEM®-T Easy vector and 1.5µl clean PCR product.
2. **Colony Screening:** Colony screening was performed using a colony PCR (conditions described in S2 above). DNA was obtained by using a pipette tip to 'pick' a colony, this was touched onto a replica plate and then placed in the PCR reaction mix. The tip was then removed and the reaction placed in a thermocycler.

A8: Evolutionary models and analysis parameters for phylogenetic analyses

Models of nucleotide substitution used for COI and mtSSU analyses are; (i) a three-parameter model with unequal base frequencies and gamma-distributed rate variation and (ii) a Felsenstein 1981 model with no invariable sites respectively. Node support for neighbour joining analyses was estimated using non-parametric bootstrapping.

The model of nucleotide substitution used for ITS analyses was a Tamura-Nei nucleotide substitution model with no invariable sites. Node support for maximum likelihood analyses was estimated using non-parametric and nearest neighbour interchange – subtree pruning and regrafting bootstrapping. In the Bayesian analysis the Markov chain Monte Carlo was run for 10 million generations, sampled every 100 steps with the first 25% of samples discarded as burnin.

A9: Representative Amylostereum areolatum *isolates and their VCG groupings*

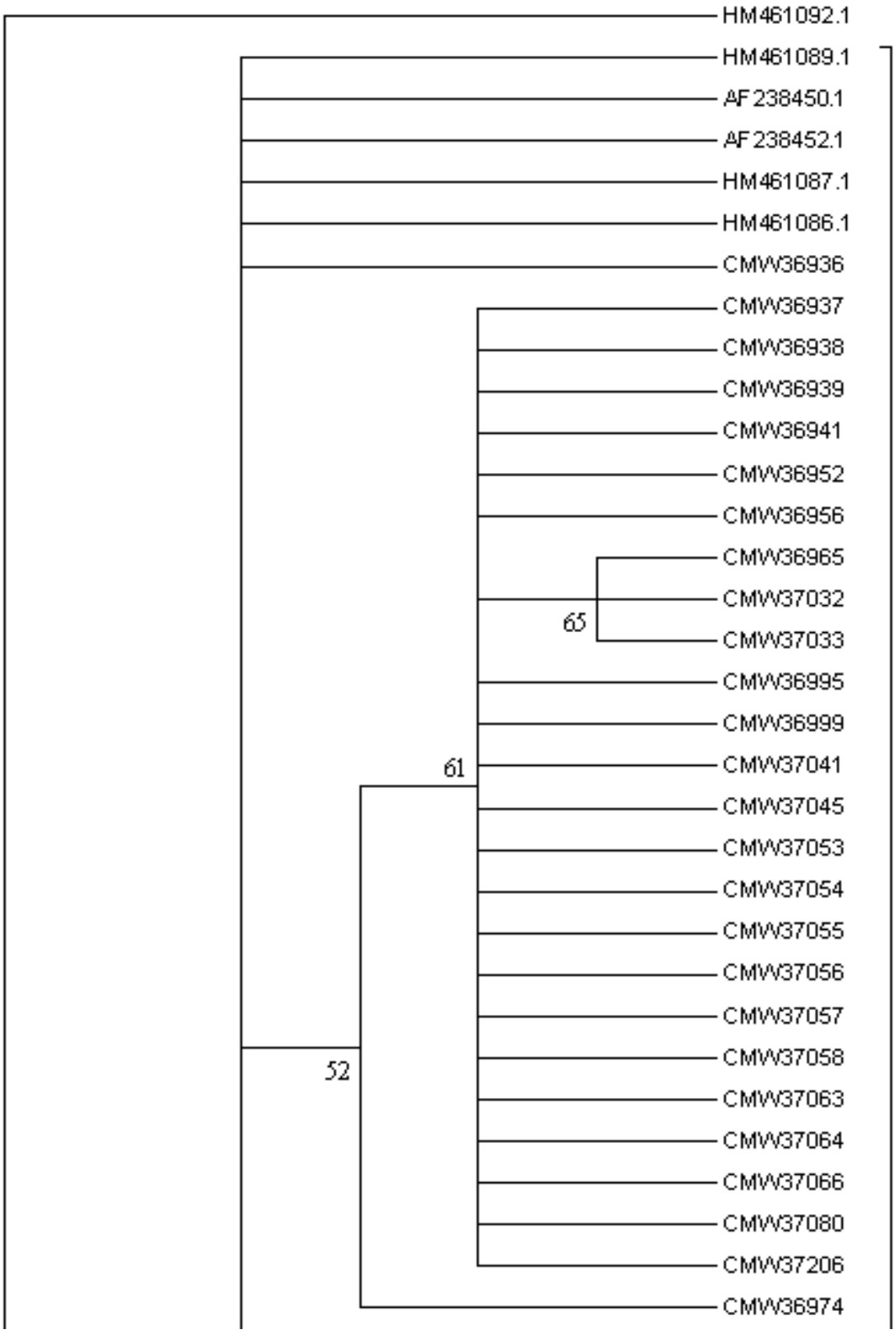
Sample number	mtSSU Haplotype	VCG*	Sampling Location**	Wasp species
37041	1	1.1	203-2	*S. noctilio*
37023	1	1.1	L22-1	*S. nigricornis*
36937	1	1.1	202-1	*S. noctilio*
36993	1	1.1	203-2	*S. noctilio*
37054	1	1.1	202-1	*S. noctilio*
37055	1	1.1	203-2	*S. noctilio*
37063	1	1.2	56-1	*S. nigricornis*
37020	1	1.2	158-1	*S. nigricornis*
37066	1	1.3	200-1	*S. nigricornis*
36965	1	1.4	200-1	*S. nigricornis*
37003	1	1.5	L6-2	*S. noctilio*
37006	1	1.6	202-2	*S. noctilio*
37037	2	2.1	200-1	*S. nigricornis*
37068	2	2.1	200-2	*S. nigricornis*
37018	2	2.1	158-2	*S. nigricornis*
36961	2	2.2	200-1	*S. nigricornis*
37017	2	2.2	158-2	*S. nigricornis*
37009	2	2.3	200-2	*S. nigricornis*
36955	2	2.4	56-1	*S. nigricornis*
37019	2	2.5	158-1	*S. nigricornis*
39644	3	3.1	L6-2	*S. noctilio*
36991	3	3.1	L6-2	*S. noctilio*
36947	3	3.1	L6-2	*S. noctilio*
36997	3	3.1	L6-2	*S. noctilio*
37060	3	3.1	L6-2	*S. noctilio*
37079	3	3.2	202-2	*S. noctilio*
37001	3	3.3	L6-2	*S. noctilio*

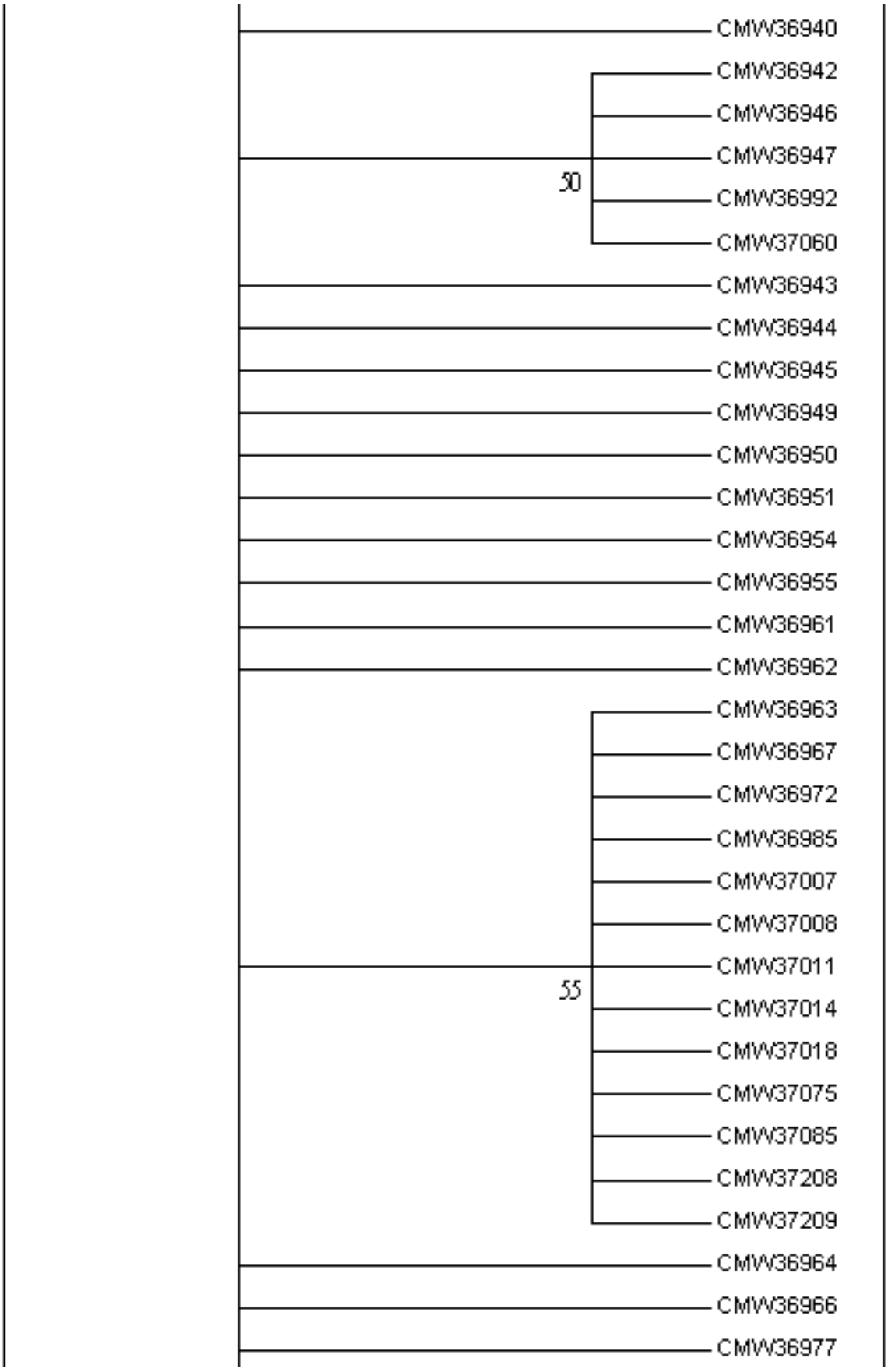
* VCGs are named first according to mtSSU haplotype and second VCG number identified within that haplotype. These numbers are separated by a '.'

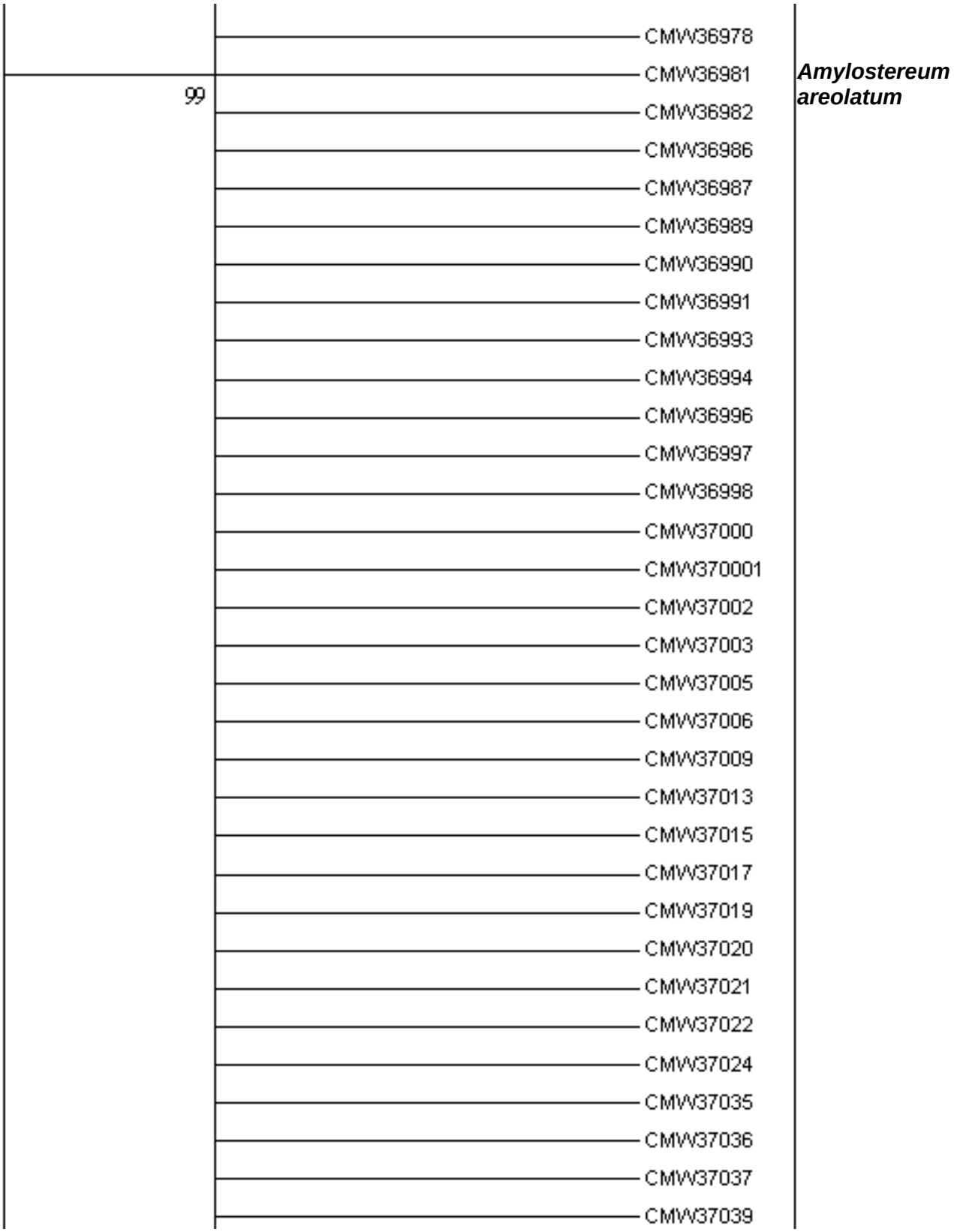
**-1 and -2 indicate which tree wasps emerged from at each site

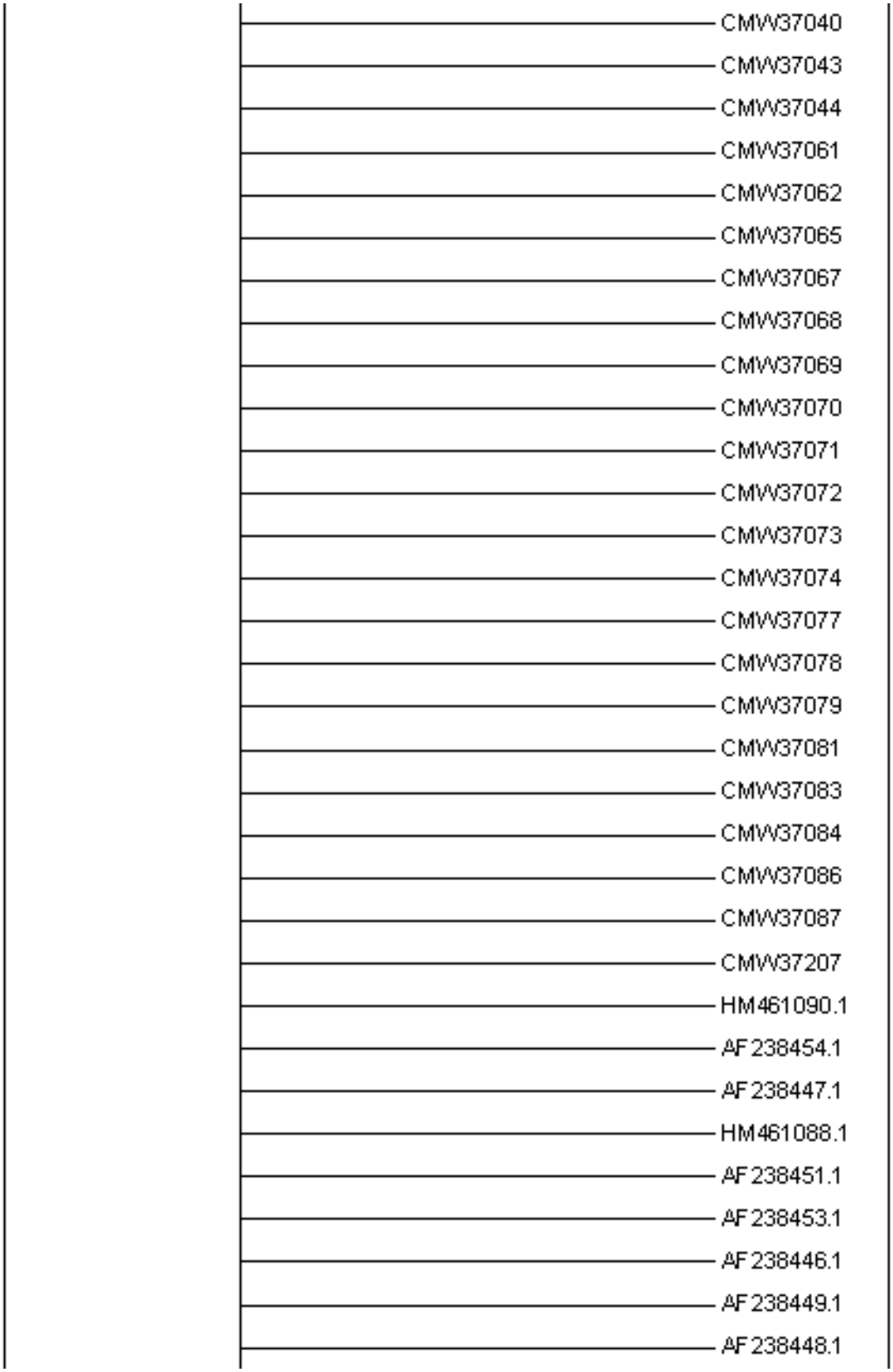
A10: (a) Rooted neighbour-joining trees of mitochondrial small subunit gene for fungal species identification. (b) Rooted neighbour-joining cytochrome oxidase c subunit 1 gene for wasp species identification. (c) Table of extent of fungal symbiont switching based on wasp and fungal species identification

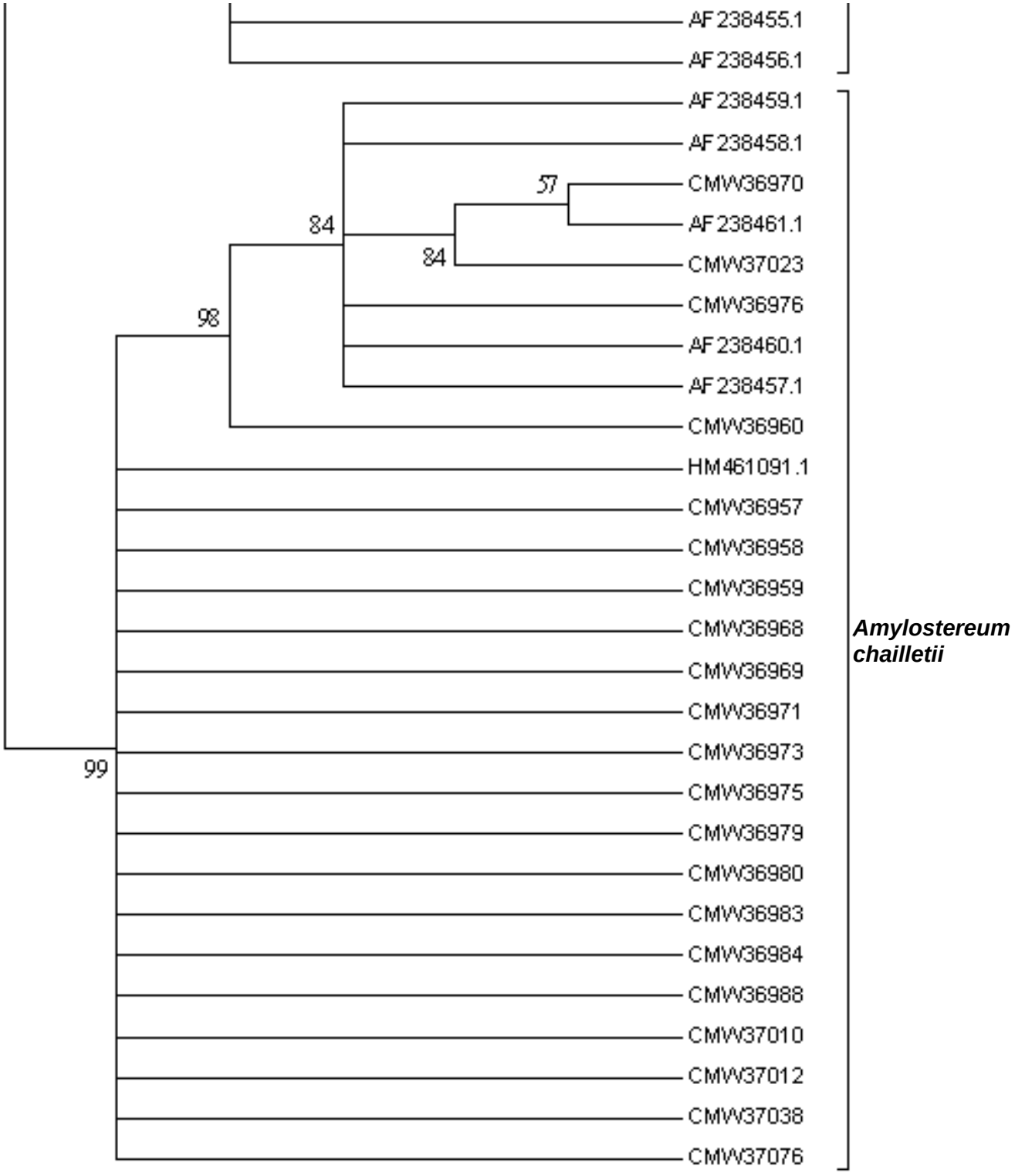
(a)



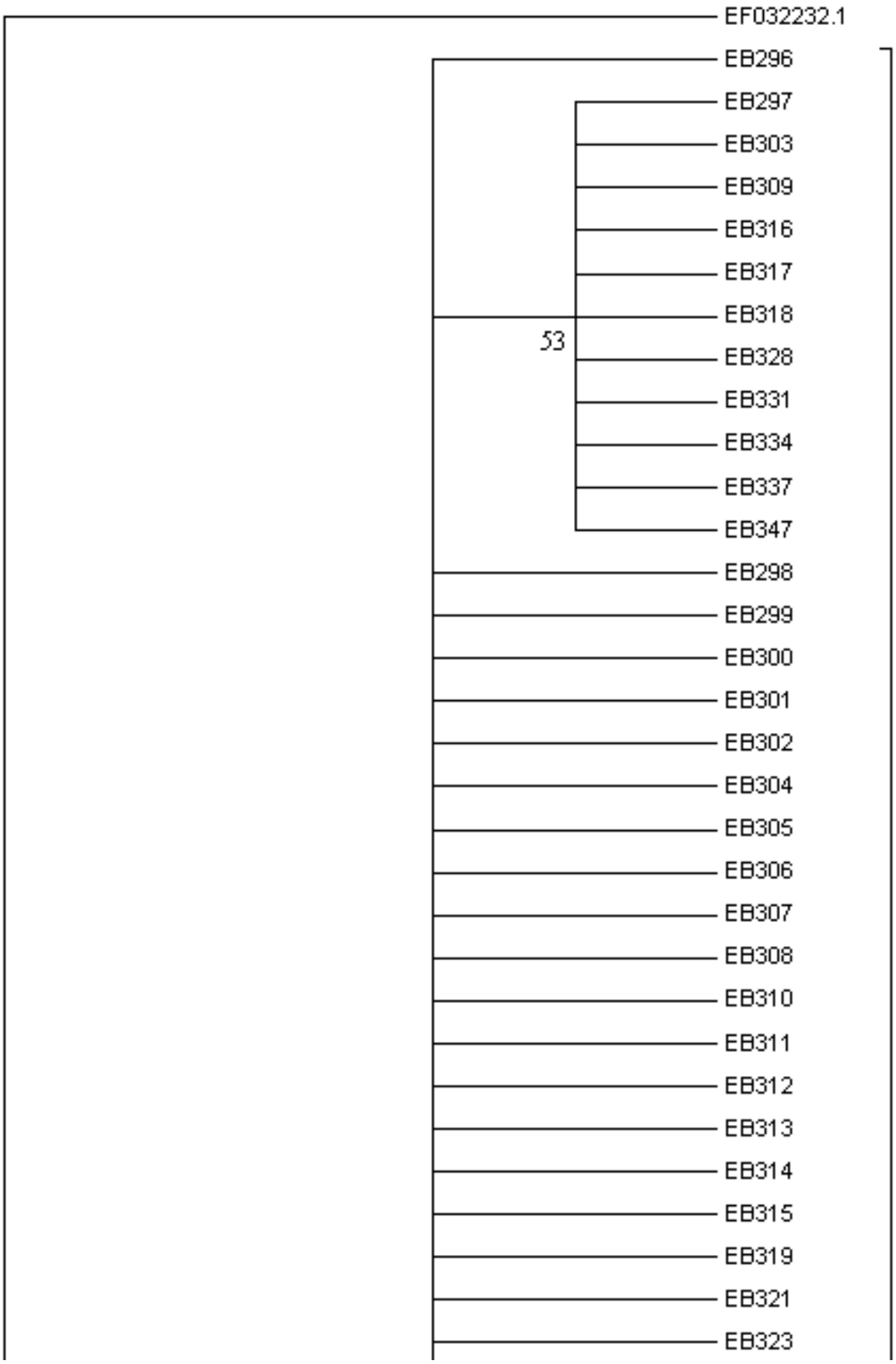


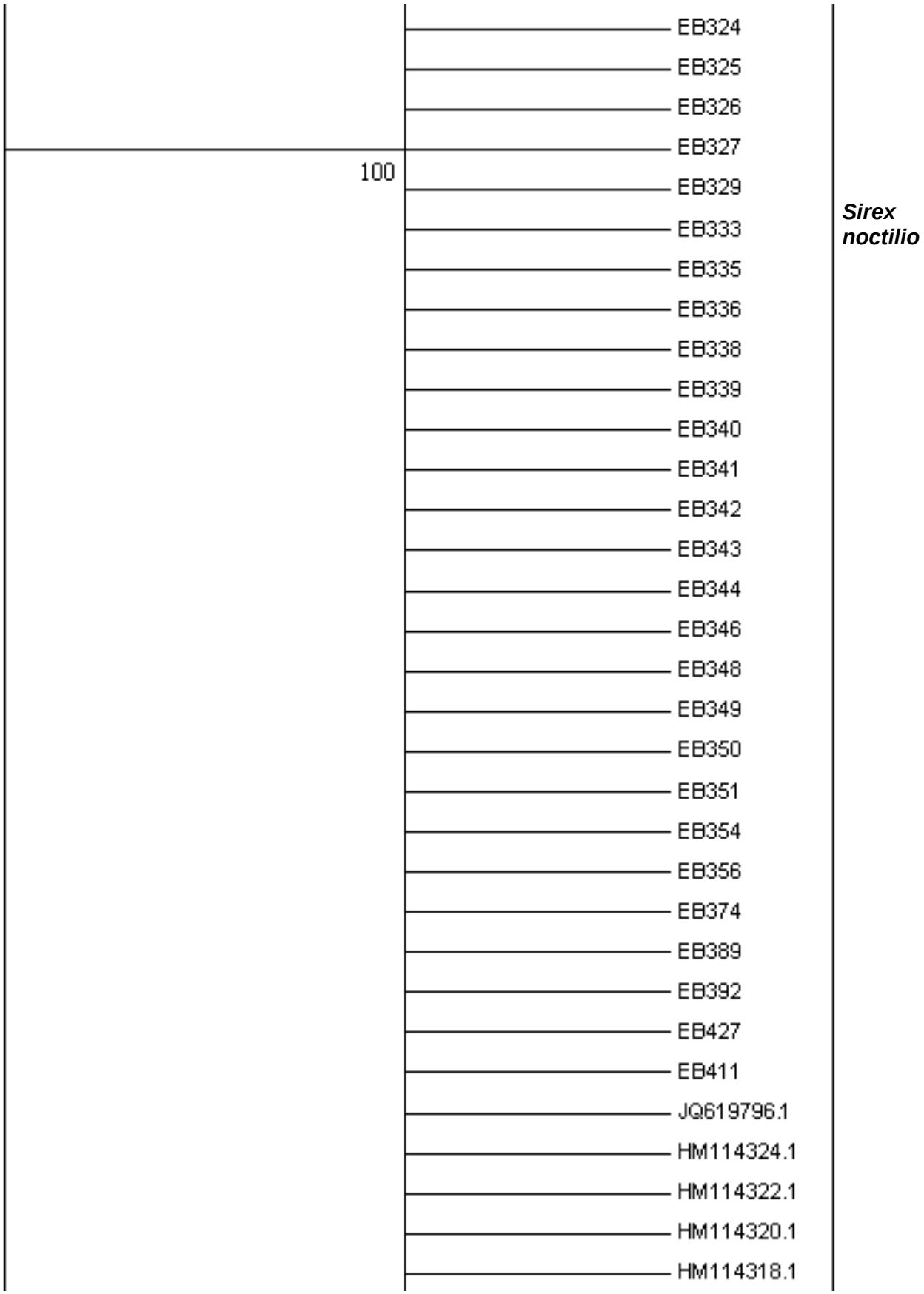


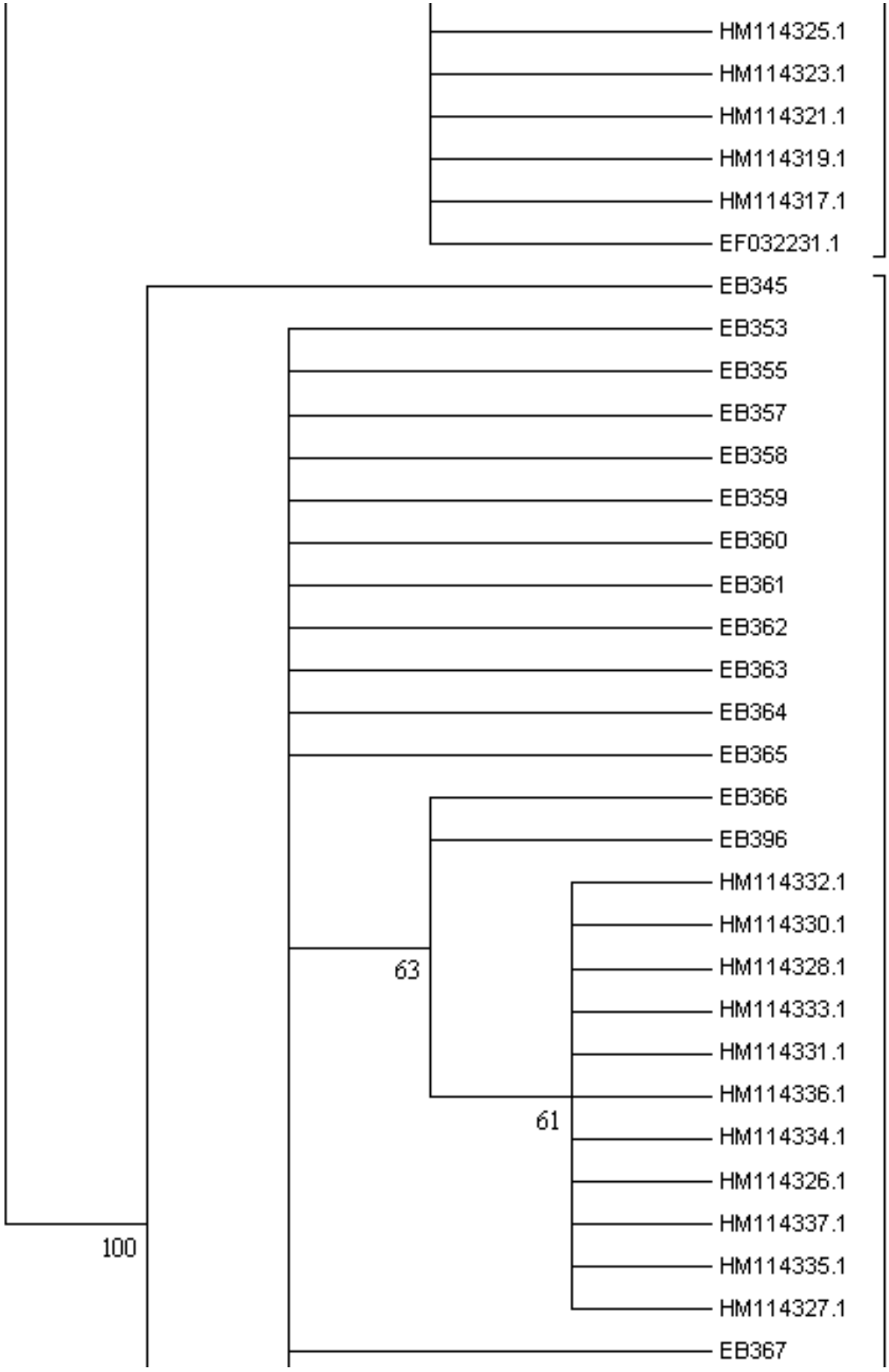


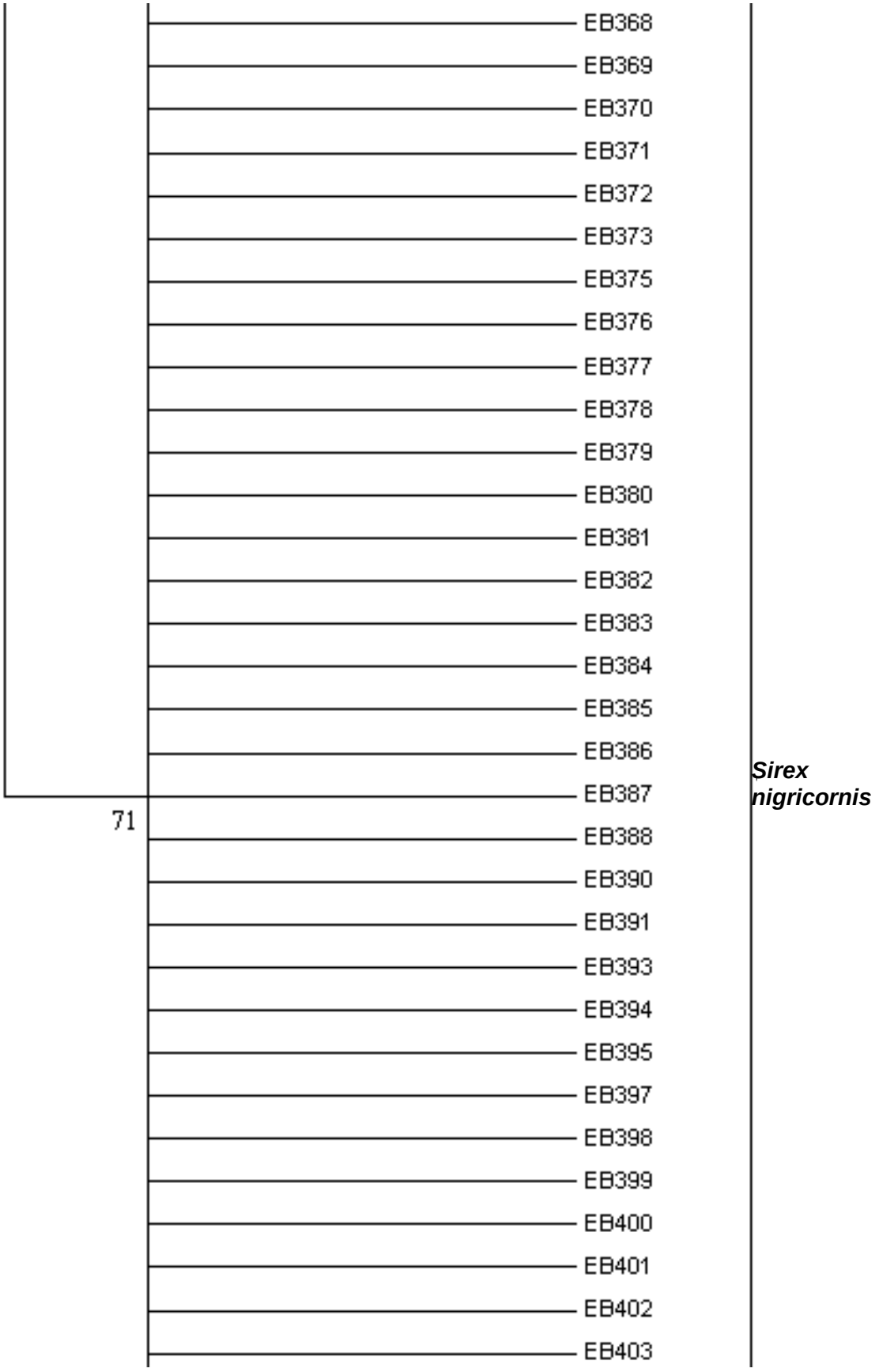


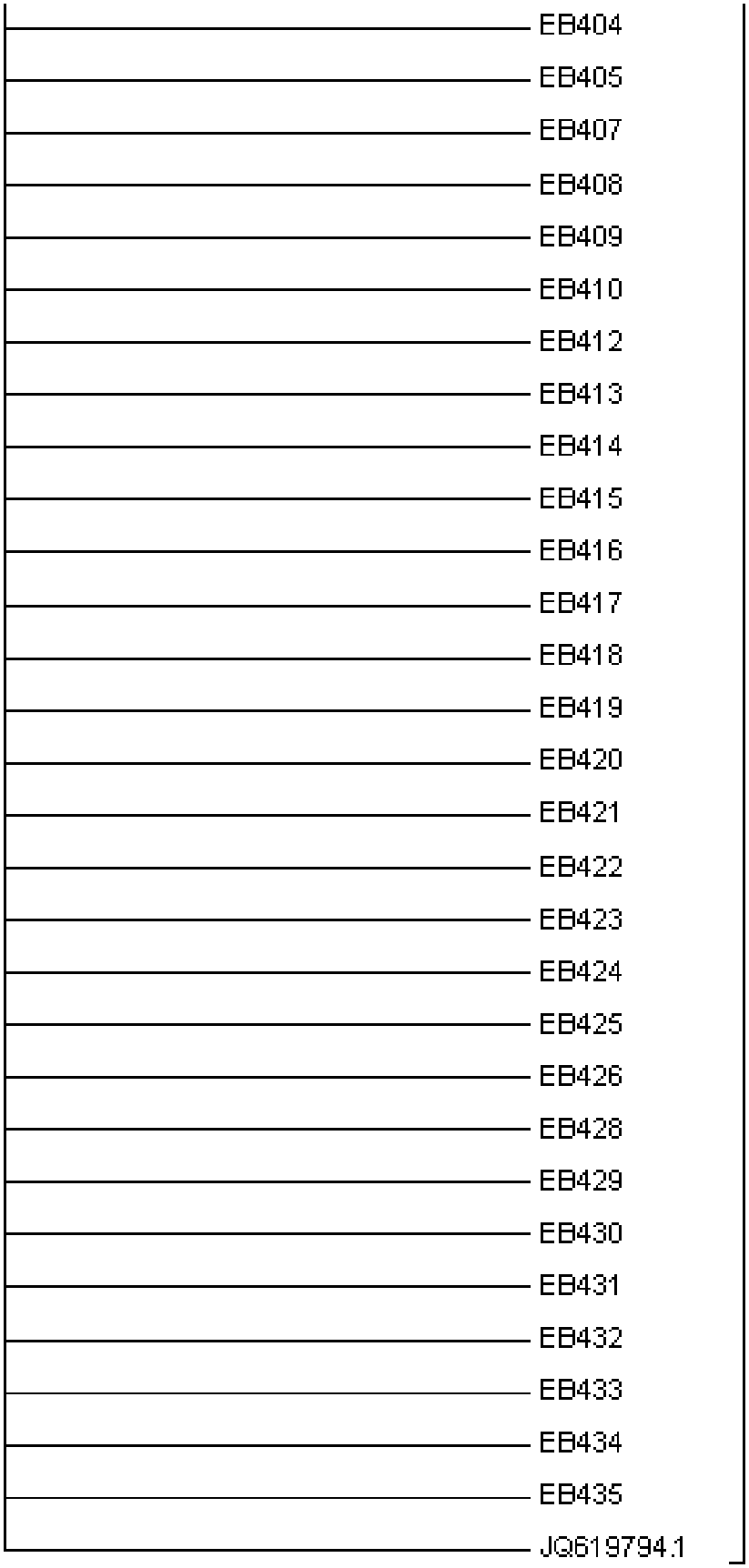
(b)











(c)

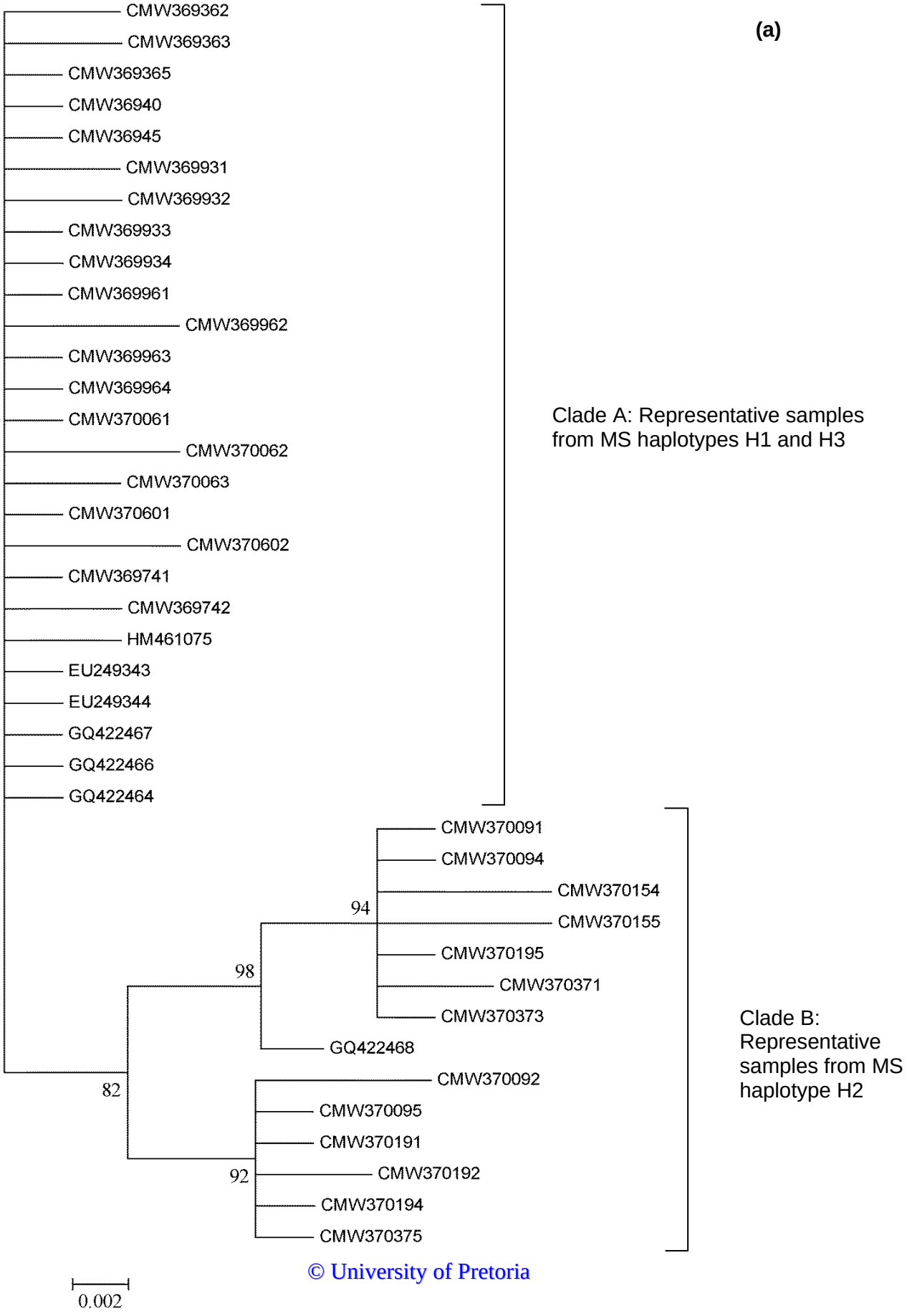
	Number of wasps	Number of wasps carrying incorrect* fungal symbiont	Percentage of symbiont switching
Sirex nigricornis	77	58	75.3
Sirex noctilio	57	2	3.5

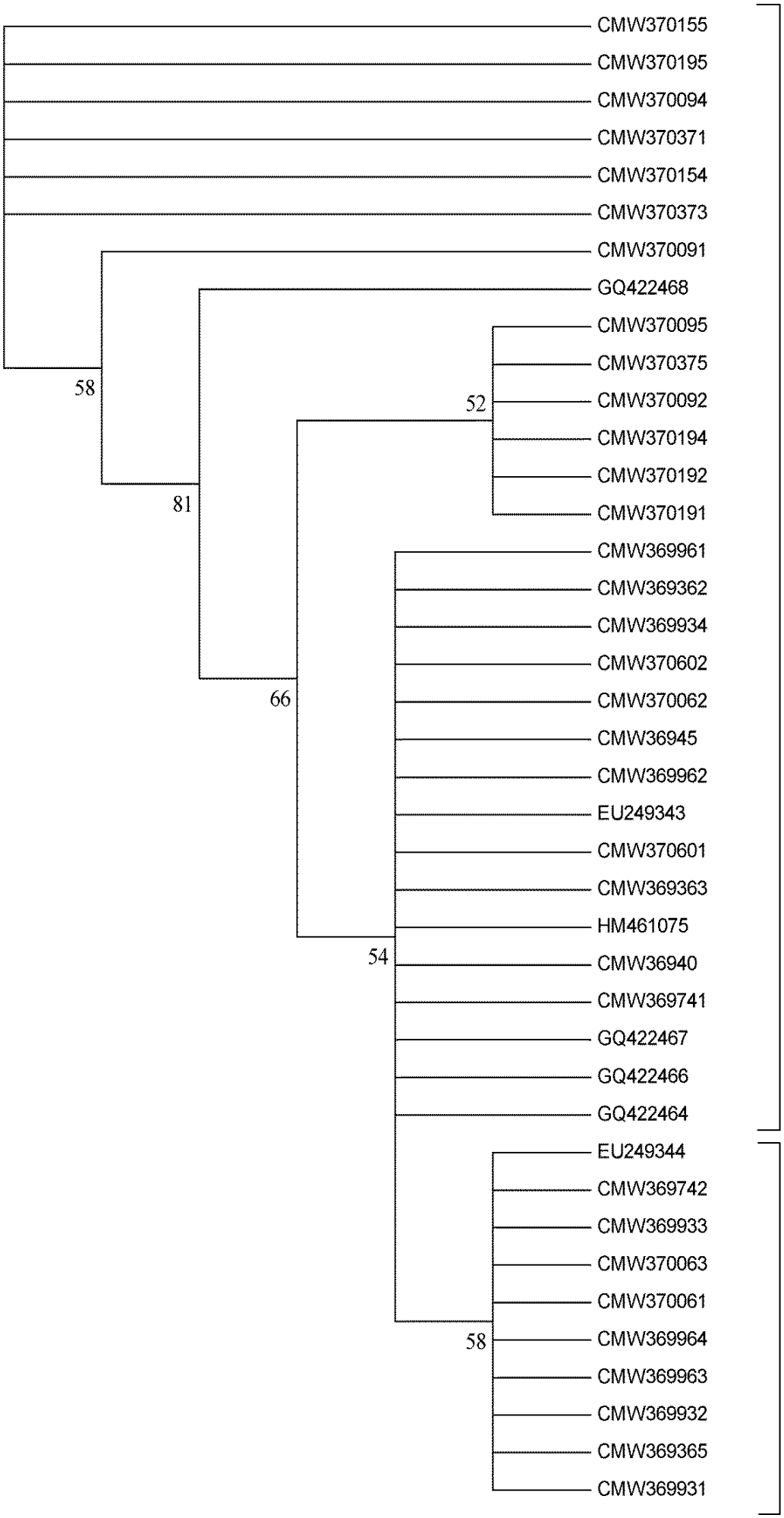
**Sirex nigricornis* is expected to carry *Amylostereum chailletii*, and *S. noctilio* is expected to carry *A. areolatum*

A11: Accession numbers of previously isolated Amylostereum areolatum *mtSSU used for identification of mtSSU haplotypes and comparison to previously identified* A. areolatum *multilocus genotypes*

Accession number	**Species**	**Location**	**Figure 1 Reference**
HM461091.1	*A. chailletii*	Canada	Canada-5
HM461087.1	*A. areolatum*	Canada	Canada-1
HM461086.1	*A. areolatum*	Canada	Canada-2
AF238459.1	*A. chailletii*	Canada	Cananda-3
AF238457.1	*A. chailletii*	Scotland	Scotland-1
AF238447.1	*A. areolatum*	France	France-1
AF238460.1	*A. chailletii*	Lithuania	Lithuania
AF238458.1	*A. chailletii*	Canada	Cananda-4
AF238456.1	*A. areolatum*	Tasmania	Tasmania
AF238452.1	*A. areolatum*	Brazil	Brazil-1
AF238446.1	*A. areolatum*	New Zealand	New Zealand
AF238461.1	*A. chailletii*	Scotland	Scotland-2

A12: (a) Unrooted Bayesian inference tree of ITS sequences. (b) Unrooted, Maximum Likelihood tee of ITS sequences. These are condensed trees where nodes with bootstrap values of less than 50% have been collapsed into polytomies.





Clade A: Representative samples from MS haplotypes H1 and H3

Clade B: Representative samples from MS haplotype H2

(b)

Flow cytometry for ploidy determination in *Sirex noctilio*

Abstract

Sirex noctilio is a highly invasive woodwasp which has achieved pest status in many parts of the Southern Hemisphere where *Pinus* spp. are grown. Like all Hymenopteran insects, *S. noctilio* exhibits a haplodiploid sex determination system where fertilised eggs develop into females and unfertilised eggs develop into males. The ancestral genetic mechanism for haplodiploid development is complementary sex determination (CSD) in which heterozygosity at a sex determination locus (or loci) is required for the development of female individuals. Homozygosity at the sex determination locus results in diploid male development. Here we report on the development of methodology for using flow cytometry to generate male (haploid) and female (diploid) specific fluorescence profiles for ploidy determination in *S. noctilio*. These profiles can be used as templates for comparison in further studies into the levels male diploidy in *S. noctilio* populations.

Introduction

Sirex noctilio is a member of the sub-order Symphyta in the order Hymenoptera. One of the characteristics of the order Hymenoptera is that the all of its members have a haplodiploid sex determination system. Under haplodiploidy, females develop from fertilised eggs and males from unfertilised eggs (Cook & Crozier 1995). A number of models have been proposed to explain the genetic mechanism underlying this phenomenon, including single and multiple locus complementary sex determination (Snell 1935; Whiting 1933), maternal imprinting (Verhulst*, et al.* 2010), paternal genome elimination and thelytoky (Heimpel & de Boer 2008). The only model that has been experimentally demonstrated in both Hymenopteran suborders, the Apocrita and Symphyta, is the complementary sex determination (CSD) model (Heimpel & de Boer 2008). In this model, heterozygosity at a specific locus or loci is necessary to initiate female development, while homozygosity at this locus or loci results in male development (Snell 1935; Whiting 1933).

It is generally accepted that the most likely ancestral sex determination mechanism in the Hymenoptera is CSD (Asplen*, et al.* 2009; de Boer*, et al.* 2012; Schmieder*, et al.* 2012). As a member of the basal Symphyta, *S. noctilio* is therefore also expected to follow the CSD model. There are a number of ways in which CSD can be identified. Indicators of CSD include extreme male-bias in natural populations (Johns & Whitehouse 2004), the presence of diploid males in natural and laboratory breeding populations (Aron*, et al.* 2003), predictable sex ratios under conditions of inbreeding (Salin*, et al.* 2004), linkage mapping, molecular characterisation of the sex determination locus, or a combination of some or all of these indicators (Naito & Suzuki 1991; van Wilgenburg*, et al.* 2006).

Invasive populations of *S. noctilio* are characterised by extremely male-biased sex ratios which decline towards a 2:1 male to female sex ratio over time (Beèche*, et al.* 2012; Hurley*, et al.* 2008; Taylor 1981; Zondag & Nuttall 1977). Long established populations of *S. noctilio*

have sex ratios resembling those of native European populations of *S. noctilio*, which are reported to be slightly male-biased with 1.82 males for every female (Spradbery & Kirk 1978). This trend has been observed throughout the Southern Hemisphere other than in the KwaZulu-Natal populations of *S. noctilio* in South Africa, where ratios remained above highly male-biased for 8 years after initial establishment in 2002. Early estimates, however, from late 2012 and 2013 suggest that the male-bias has decreased to ~3 males: 1 female (Dr Brett Hurley, unpublished data and Philip Croft, personal communication).

Prolonged male-bias observed in the KwaZulu–Natal *S. noctilio* populations have led to investigations into possible causes for the observed bias. A number of mechanisms were hypothesised to be capable of producing this bias. Infection with the sex ratio distorting bacterial reproductive parasite *Wolbachia*, which is known to be ubiquitous (Jiggins*, et al.* 2001), could skew the sex ratio of *S. noctilio* populations toward males (Engelstädter*, et al.* 2004). Constrained sex allocation among females could also skew sex ratios towards males, because unmated females are incapable of fertilising eggs to produce female offspring (Whiting 1933). It is also possible for abiotic factors such as host quality to influence sex ratios (Johns*, et al.* 2010). Finally, reduced genetic diversity may arise from a recent population bottleneck associated with newly established populations. This could result in the production of diploid male offspring as predicted in CSD.

A common test for CSD lies in the identification of diploid males within a population. Diploid males are predicted to be present in all populations but increase in frequency as genetic diversity decreases and/ or population size decreases. Molecular techniques that rely on diversity, such as microsatellite markers, are not well suited to identifying diploid males in less genetically diverse populations as there is a higher likelihood that individuals identified as haploid are actually homozygous diploids. Rather, cytological techniques for testing ploidy can more accurately represent the levels of male diploidy within these populations.

Flow cytometry was originally developed for identification and characterisation of cancerous cells however, this powerful tool is increasingly being used by evolutionary and population biologists to answer a wide range of questions (Kron*, et al.* 2007), including ploidy determination (Aron*, et al.* 2003). Flow cytometry has been used to confirm single locus CSD in the ichneumonid parasitoid wasp *Diadegman chrysostictos* and to differentiate between diploid females and haploid males and assess brood sex ratios in the ant *Linepithema humile* (Aron*, et al.* 2003; Butcher*, et al.* 2000). Here we test the use of flow cytometry as a cytological method to identify diploid males, as a means of confirming the presence of a CSD system in *S. noctilio*.

Materials and Methods

Sample collection

Initially specimens preserved in ethanol from wasps collected from in the 2009–2011 emergence periods were used for flow cytometric analyses. These wasps were collected from the Linwood depot in KwaZulu-Natal; a site used by industry to store infested pine logs and monitor emergent wasp sex ratios and levels of parasitism with the biocontrol agent *Deladenus siricidicola*. During these analyses it was found that preserved specimens were not suitable for a ploidy profile to be developed, hence live specimens were used. Two months prior to adult emergence in September 2012, trees infested with *S. noctilio* were felled, sectioned and stored in emergence drums at the Linwood depot in KwaZulu-Natal. Emerging male and female wasps were collected in plastic jars and placed in cooler boxes for transport to the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. Wasps from these deliveries are dissected for the parasitic nematode *Deladenus siricidicola*. Dissected wasps were used for flow cytometric analyses, however the tissue

from these wasps was also not suitable for analyses. For this reason additional wasps were collected specifically for flow cytometry analyses which were not dissected.

Sample preparation

Wasps were examined and those that died prior to preparation were discarded. Live wasps were rated in terms of relative vigour (very vigorous, moderately vigorous, and least vigorous) before sample preparation. For each wasp body length was measured from the mandibles to the end of the last abdominal segment and head diameter was measured at the widest point. Wasps were killed by removing the heads using dissecting scissors, and flame sterilised using 96% ethanol. Heads were halved and placed in 250 µl of buffer solution (dimethyl sulfoxide in sucrose-sodium citrate) from the Becton Dickinson CycleTEST^{TM} PLUS DNA reagent kit. Head tissue was used as other tissues are known exhibit elevated ploidy in Hymenopterans (Aron*, et al.* 2005). The halves were then macerated using forceps to allow the tissue to come into contact with the buffer and the tissue–buffer solution was incubated at room temperature overnight.

After incubation samples were aspirated with a 21 gauge needle in order to separate possible cell and nuclei clumps. The suspension was filtered through the gauze lid of a BD Falcon 5 ml polystyrene round-bottom tube with 35µm cell-strainer cap in order to remove debris, such as exoskeleton fragments from the cell suspension (Becton Dickinson, New Jersey, USA). Prior to flow cytometric analysis, 100µl of solution A (trypsin in spermine tetrahydrochloride detergent buffer), and 75µl of both solution B (RNase A and trypsin inhibitor in spermine buffer) and C (propidium iodide in spermine buffer), were added to the filtered sample. The samples were swirled gently to homogenise and incubated on ice in the dark for 30 minutes.

Sample analysis

All flow cytometric analyses were performed on a BD FACS ARIA flow cytometer (Becton Dickinson, California, USA). Fluorescence excitation was conducted at a wavelength of 488 nm and a band pass filter of 556 nm was used. Ten thousand events were recorded per sample at a low flow rate, and measured in a PE-W/ PE-A gated region which contained haploid, diploid and tetraploid nuclei. The acquisition protocol was designed to measure forward scatter (FSC) at 0V and side scatter (SSC) at 270V on a linear scale and with propidium iodide fluorescence at 520V and 0.4 area scaling. Resulting data were analysed using FlowJo 10.0.5 (TreeStar, California, USA).

Results

Fresh tissue was essential for successful flow cytometric analysis. Runs using wasps preserved in ethanol resulted in no detectable populations of nuclei. Wasps which had been previously dissected for the parasitic nematode *Deladenus siricidicola* also resulted in unusable fluorescence profiles where no recognisable populations of nuclei could be found. When live wasps were used, evidence of discrete populations of nuclei could readily be identified (Fig. 1). These populations of nuclei were transformed into histograms of relative DNA content (fluorescence peaks) which corresponded to different ploidy levels (Fig. 2).

A standard DNA content profile histogram was established for female and male *S. noctilio* woodwasps (Fig. 2). Profiles consistent with this standard were produced for 15 female and 89 male wasps. For females, a single profile was obtained with one major fluorescence peak with a relative mean DNA content of 150 corresponding to a ploidy of 2N (Fig. 2a). A major peak is defined as containing the largest percentage of the events recorded in the parent population (Fig. 3). Males had one of two profiles. Male profile 1 was observed for 61% of

males and had a major peak in channel number 75 which corresponds to a ploidy of 1N (Fig. 2b). Male profile 2 was observed in 39% of male samples. This profile had a major peak in channel number 150 (Fig. 2c). There is no apparent relationship between the presence of these 2N peaks and wasp head diameter, body length, or vigour (Fig. 4). Minor peaks in all profiles correspond to nuclei with a double DNA content as a result of being in the synthesis (S) phase of the cell cycle (Pierce 2012).

Discussion

Flow cytometry was chosen for cytological ploidy determination in *S. noctilio* because the method is direct, does not rely on breeding, linkage mapping or detailed knowledge of the sex determination genes, and has been used successfully in other Hymenopterans (Aron*, et al.* 2003). In this study we successfully use flow cytometry to develop ploidy profiles for male and female *S. noctilio* woodwasps, provided live specimens are used and after some optimisation. These profiles will be useful tools in analysing level of male diploidy in invasive populations of *S. noctilio.*

Fluorescence peak profiles for *S. noctilio* females were found to be consistently diploid in all of our samples. Large peaks in channel number 150 (ploidy = 2N) were observed in 39 % of male profiles, but it is unlikely that this corresponds to the presence of diploid males. The expectation for diploid males is a 2N major peak and a 4N minor peak, with no evidence of significant frequency of 1N nuclei. The presence of prominent haploid peaks in all of our male samples suggests that they are in fact haploid, with the 2N peaks likely deriving from mandibular muscle tissue present in the samples (Serge Aron, personal communication). The haploid designation for males was supported by the absence of haploid peaks in any of the female fluorescence profiles.

The presence of the prominent 2N peak in male profile 2 suggests that further refinement of the sample preparation technique is required. Rather than macerating the entire head, it is possible to remove brain tissue from the head prior to the maceration step (personal observation). If no muscle tissue, which often exhibits elevated ploidy in insects (Aron*, et al.* 2005), is present in samples, it is likely that diploid peaks could be avoided in male samples. This would also reduce the amount of debris from the insect head capsule, which can compromise accuracy. The removal of brain tissue would be difficult for smaller specimens and could potentially result in too little tissue being obtained for proper analyses.

The elucidation of sex determination mechanisms using controlled inbreeding and measurement of offspring sex ratios is difficult in the Hymenoptera due to long life cycles and barriers to captive rearing. Flow cytometry represents an ideal technology to screen for diploid males in natural populations, rapidly and easily. The development of a standardised flow cytometric technique to be used for *S. noctilio* would allow rapid screening of woodwasp populations, and would lead to an improved understanding of the mechanisms of sex determination in this group of insects. Elucidation of sex determination mechanisms within this group may help to clarify some of the proximate causes of elevated male-bias observed in newly established populations of *S. noctilio*. Furthermore, it could positively influence control programmes against this highly invasive forest pest.

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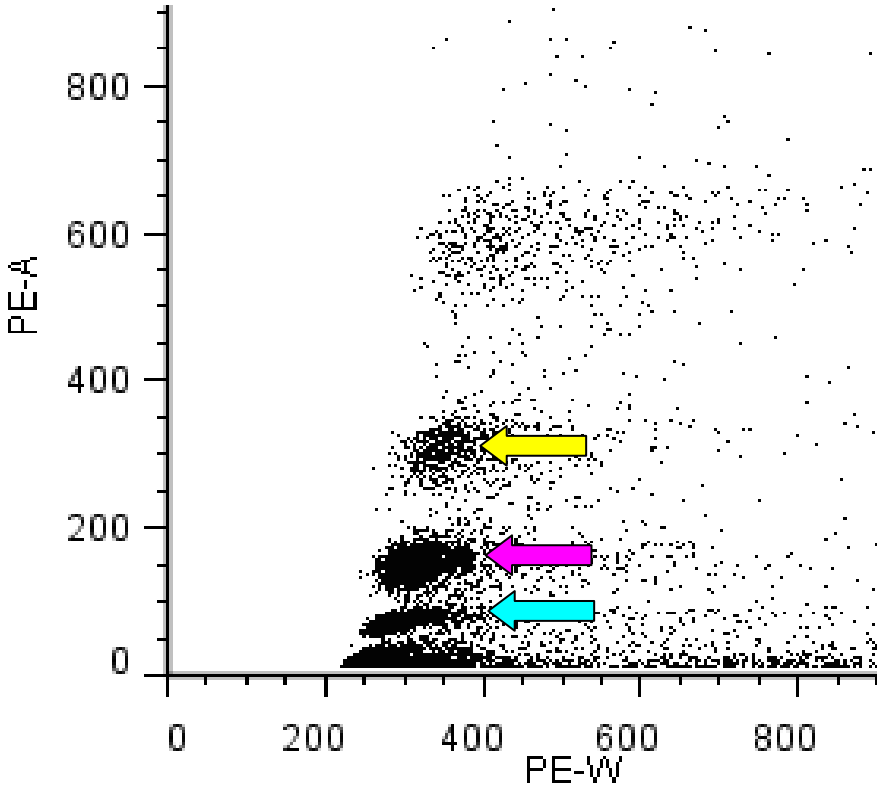
Fig. 1 Dot plot of typical female (a) and male (b) sample. Each dot represents the relative fluorescence scatter produced by a single nucleus (cell). Nuclei with the same ploidy form clusters/ populations. Populations indicated by yellow arrows are tetraploid (4N), pink arrows are diploid (2N) and blue arrows are haploid (1N).

(a)



(b)

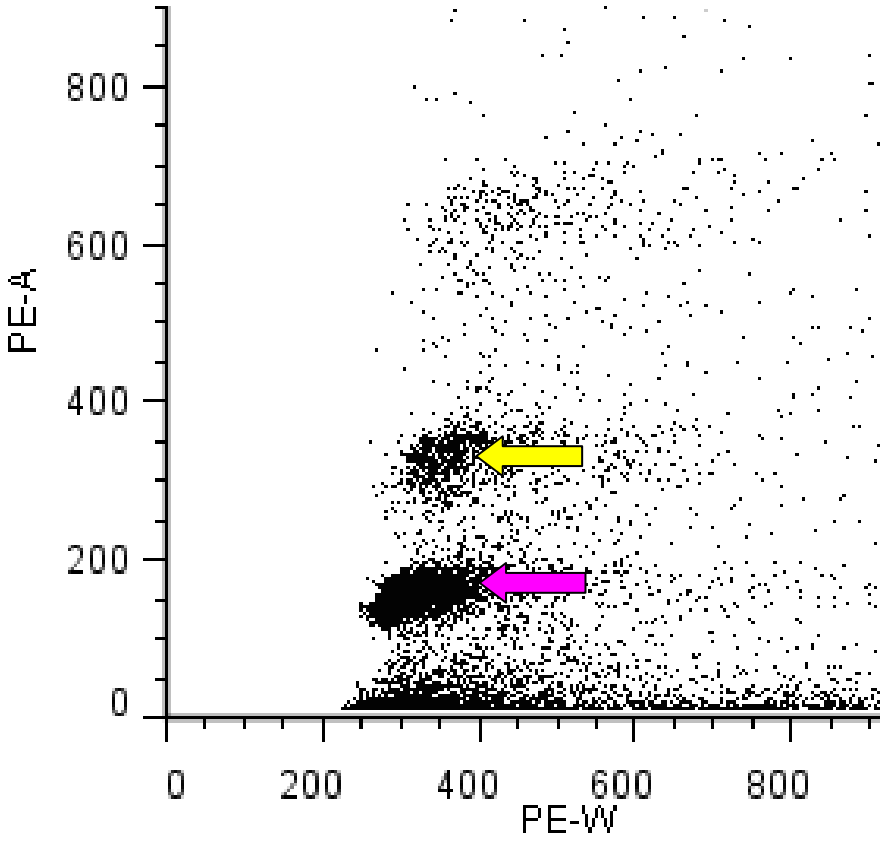


Fig. 2 Flow cytometry fluorescence histogram of (a) a female profile, (b) male profile 1 and (c) male profile 2. Fluorescence peaks are labelled according to ploidy level; 1N = haploid, 2N = diploid and 4N = tetraploid.

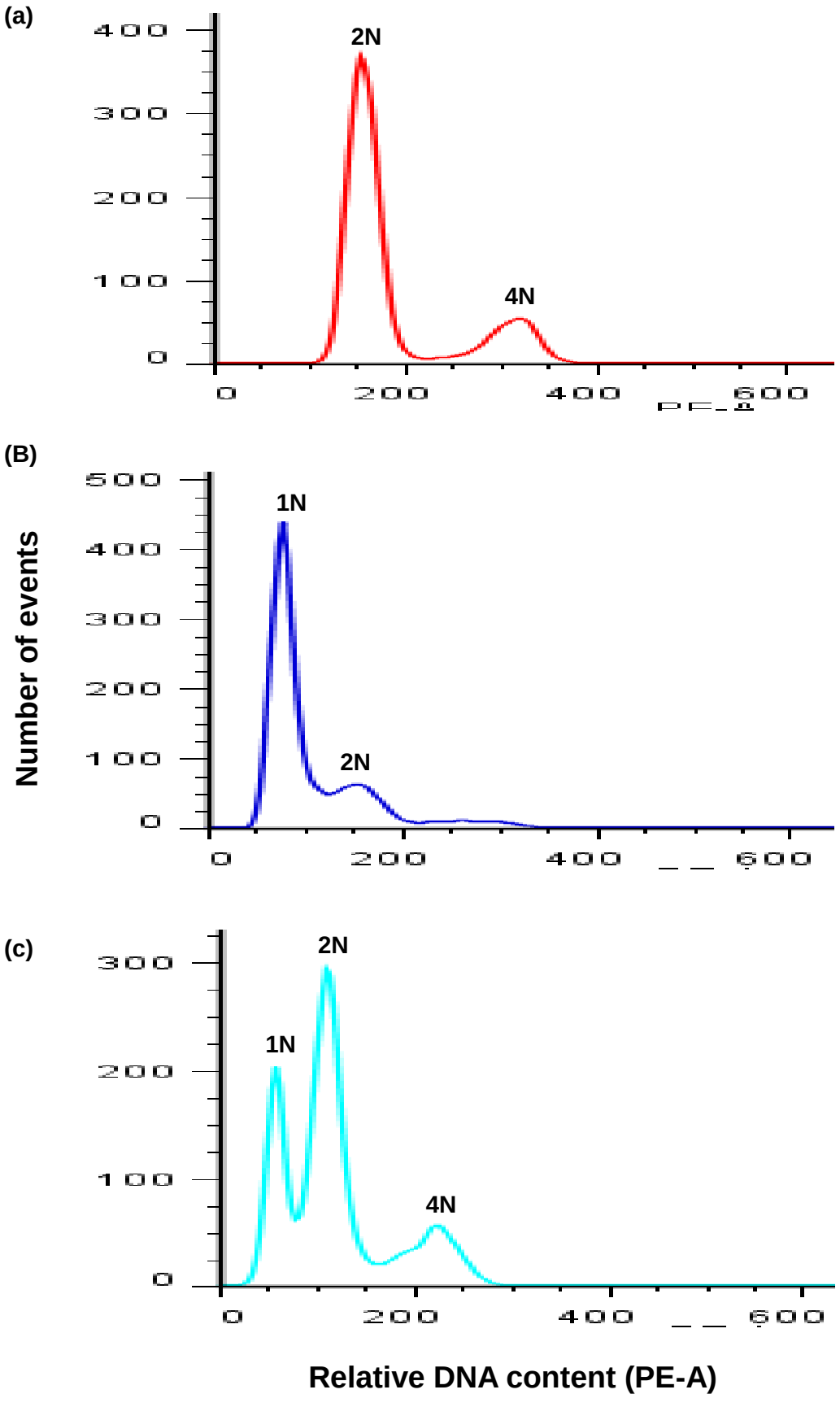
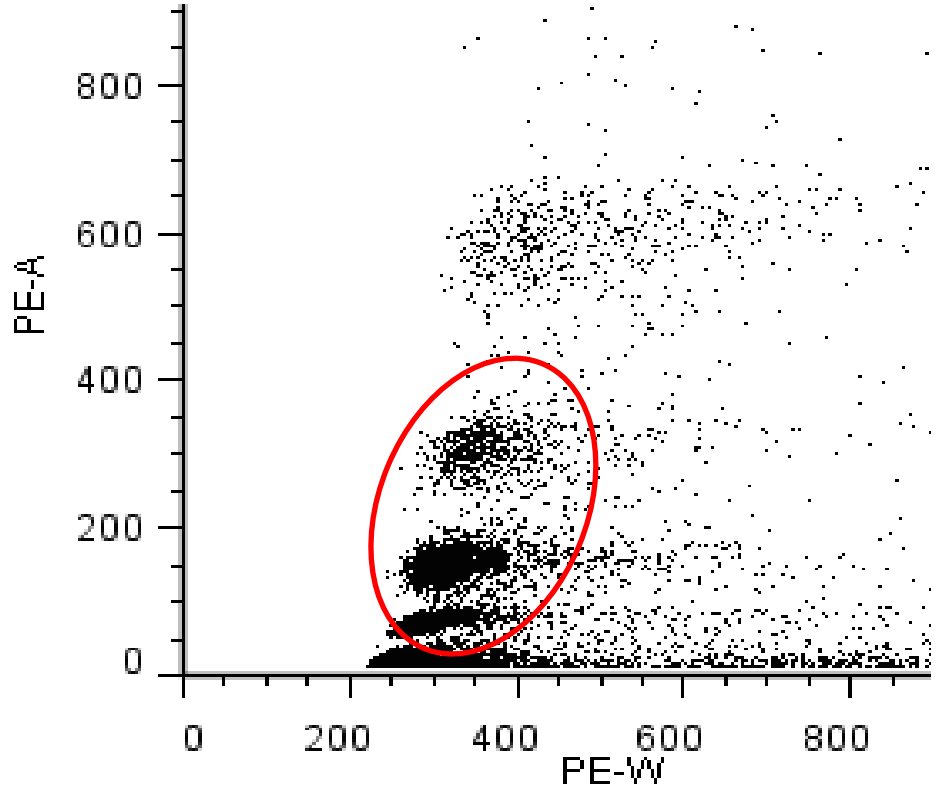


Fig.3 Determination of male profile type. (a) Dot plot of parent population gated in red. (b) Histogram showing percentages of events from the parental population in each peak; haploid (1N), diploid (2N) and tertaploid (4N).

(a)



(b)

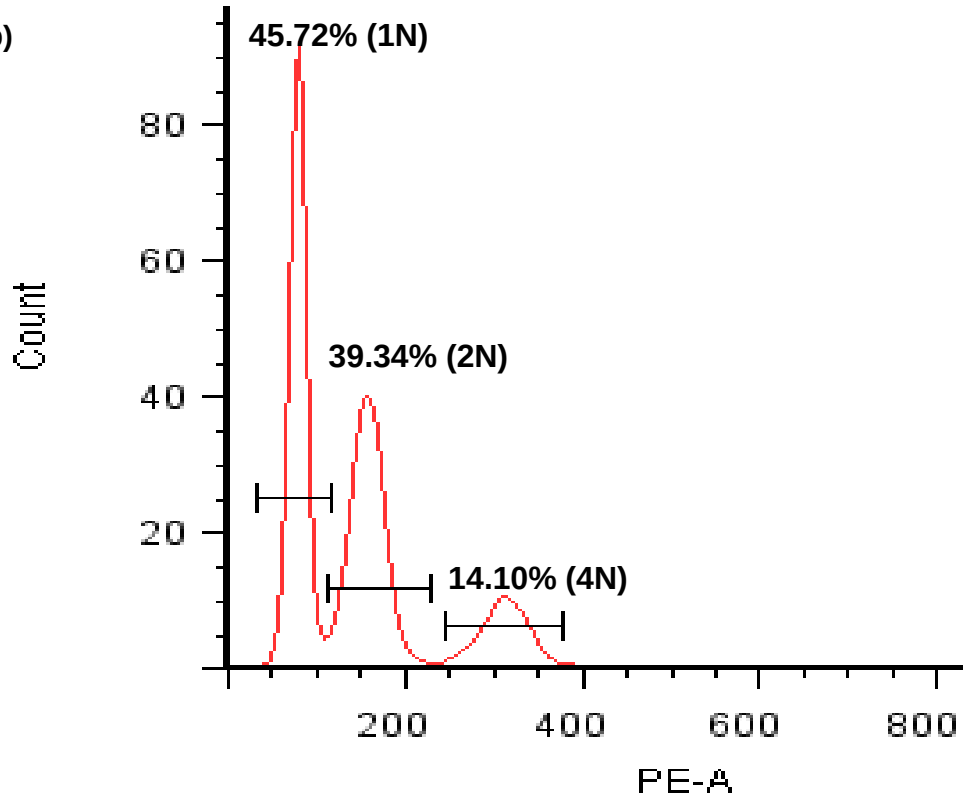
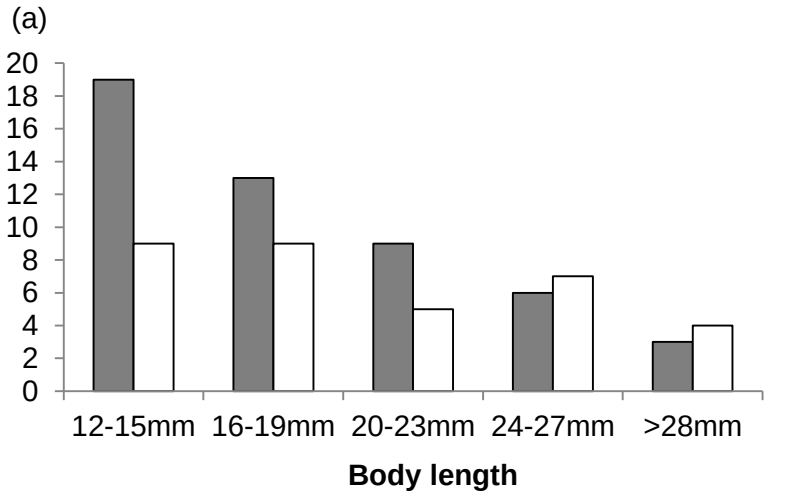
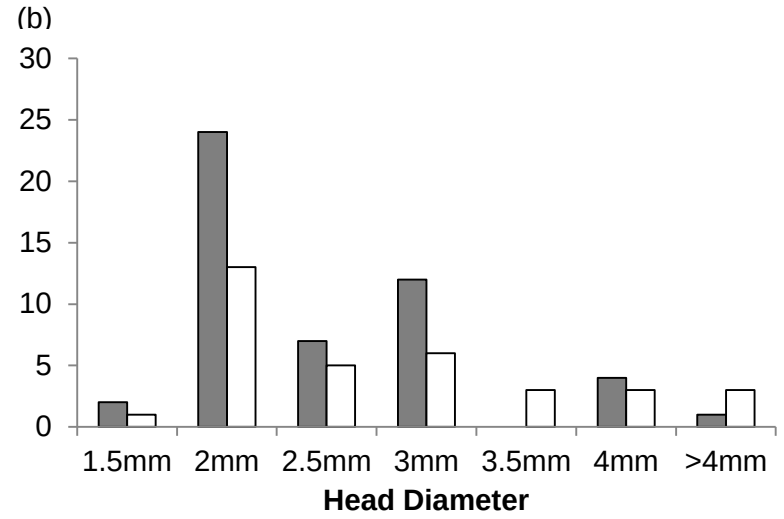
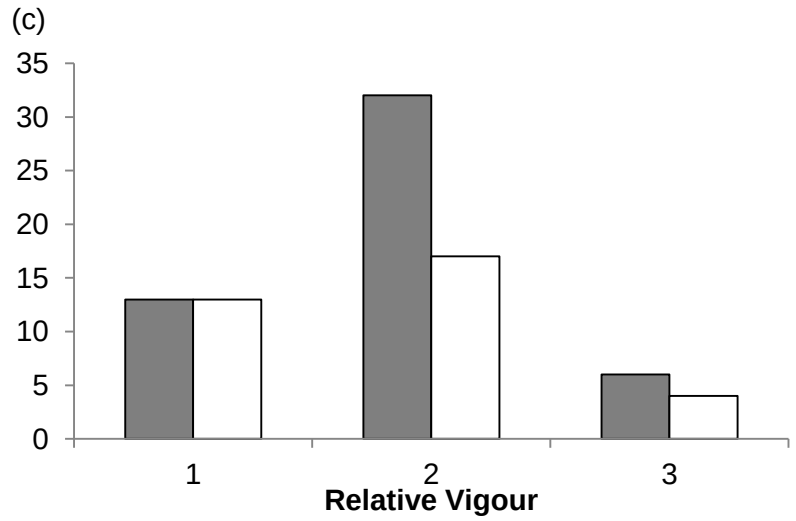


Fig. 4 Histograms of number of male wasps with DNA content histogram profile 1 (grey) and profile 2 (white) in relation to (a) body length from mandibles to the tip of the last abdominal segment, (b) head diameter at widest point, and (c) Relative vigour where 1 is very vigorous, 2 is moderately vigorous and 3 is least vigorous.







Understanding sex ratio variation in the invasive Hymenopteran *Sirex noctilio* Fabricus

Abstract

Sirex noctilio is an economically important invasive pest of commercial pine forestry in the Southern Hemisphere. Newly established populations of this woodwasp are characterised by initially highly male-biased sex ratios throughout its invasive range. These ratios subsequently revert to levels seen in the native range of *S. noctilio* in Eurasia and North Africa. This trend has not been observed in the KwaZulu-Natal population of *S. noctilio* in South Africa, which remained highly male-biased for almost a decade. The aim of this study was to determine the cause of this persistent male-bias. We found no correlation between wood moisture content, as a proxy for host quality, and sex ratio. No evidence of the sex ratio altering reproductive parasite, *Wolbachia* was found in *S. noctilio* populations in the Western Cape or in KwaZulu-Natal. We found that 73% of females in a newly established *S. noctilio* population are mated, and report a simple simulation model that indicates that female investment in daughters has a considerably greater influence on sex ratios than mating frequency or success. Microsatellite data analysis showed that KwaZulu-Natal populations of *S. noctilio* are far less genetically diverse than those in the Western Cape region of South Africa, with genotypic richness scores of 0.07 and 0.82, respectively. These data also identified diploid males at low frequencies in both the Cape (4.23%) and KwaZulu-Natal (1.28%). These results confirm the presence of a complementary sex determination mechanism in *S. noctilio*, but suggest that reduced genetic diversity is not the main driver of the male-bias observed in the KwaZulu-Natal population. Among all the factors considered, selective investment in daughters appears to have the most significant influence on male-bias in *S. noctilio* populations. Why this investment remains different in KwaZulu-Natal populations compared to other populations is not clear, but it could be influenced by mate choice based on genetic relatedness. This study lays the foundation for further investigations into mate choice and the genetic basis of sex determination in *S. noctilio*.

Introduction

The woodwasp *Sirex noctilio* has been an extremely successful invader, spreading from its native range in Europe and North Africa throughout the non-native pine plantations of the Southern Hemisphere, and into natural pine forests, as well as plantations, in North America, in less than a century (Hoebeke*, et al.* 2005; Hurley*, et al.* 2008; Tribe 1995). Invasive populations of *S. noctilio* in the Southern Hemisphere are characterised by initially highly male-biased sex ratios, with up to 32:1 males: females being recorded in Brazil, 20:1 in New Zealand, 12:1 in South Africa and 16.5:1 in Tasmania (Slippers*, et al.* 2012; Taylor 1981; Zondag & Nuttall 1977). These ratios tend to equalise with population age, and in established populations reflect ratios of ~2:1 observed in the wasps' native range (Slippers*, et al.* 2012). This trend has been observed in all areas where *S. noctilio* is now established as an invasive, except in the KwaZulu-Natal region of South Africa (Fig.1), (Spradbery & Kirk 1978; Taylor 1981; Zondag & Nuttall 1977). Unusually, sex ratios in this area have remained above 10 males: 1 female for over eight years, a longer period than in other established invasive populations. These ratios have been calculated from collections of thousands of wasps from pine plantations in KwaZulu-Natal over a number of years but preliminary data for 2012 and 2013 indicate that ratios in this area are beginning to decline (Philip Croft, personal communication).

Fisher's principle states that 'the sex ratio is in equilibrium when, in the population as a whole, the totals of effort spent producing the two sexes are equal' (Fisher 1958; Hamilton 1967). This is generally taken to mean that all populations will evolve towards a 1:1 sex ratio (Hamilton 1967). Sex ratios, however, can vary as a result of a number of factors. These include local mate competition (Hamilton 1967), host and mate quality (Bono & Herbers 2003; Craig*, et al.* 1992; Henter 2004), genetic elements such as paternal sex ratio chromosomes (Werren & Stouthamer 2003), reproductive parasites (Jiggins*, et al.* 2001), and constrained sex allocation (Godfray 1990). In *S. noctilio*, the equilibrium sex ratio

appears to remain stable between two and three males per female in both the native range and in invasive populations that have been established for longer than five years (Slippers*, et al.* 2012; Spradbery & Kirk 1978).

The persistent extreme male-bias observed in the KwaZulu-Natal *S. noctilio* populations prompted an investigation into factors which are likely to be influencing the sex ratio in these populations. We tested four hypotheses; (1) Wood moisture content of infested trees is acting as a cue for oviposition of fertilised eggs in more moist sections of trees, where wood moisture content serves as a proxy for resource quality, thus influencing sex ratios of emerging wasps; (2) The presence of the reproductive parasite *Wolbachia* is causing the production of diploid males from unfertilised, diploidised eggs, or the death of fertilised eggs due to incompatibility between *Wolbachia* strains carried in the eggs and sperm. *Wolbachia* was chosen as this parasite is predicted to infest over 65% of insect species (Hilgenboecker*, et al.* 2008); (3) Females in newly established populations of *S. noctilio* are under constrained sex allocation (experience low mating success), which causes increased production of male offspring; (4) Decreased genetic diversity in the population is acting on a complementary sex determination system, increasing levels of homozygosity at the sex determination loci and resulting in the production of diploid males, resulting in increased male-bias in these populations.

Materials and Methods

Data collection and analysis of wood moisture content and emerging wasp sex ratio

Data for the analyses of the effect of wood moisture content on sex ratio were obtained from Dr. Brett Hurley. These data reflected sex ratios of emerging wasps from billets (log sections) from trees that were felled between 28 February and 1 March 2006. Moisture

readings were taken from these billets at the time of felling, from logs cut from the bottom, middle and top sections of the trees (Hurley*, et al.* 2008). Tree sections were collected at two farms in KwaZulu-Natal, Yonderdale and Good Hope and stored in emergence drums. Each emergence drum contained tree sections, either bottom, middle or top, from three trees. Statistical analyses of the data were done in R (R Core Team 2013).

The study from which the data were sampled showed that the bottom sections of trees were significantly more moist than the middle sections, and the middle sections more moist than the top sections ($F = 1650.56$; $P < 0.0001$) (Hurley*, et al.* 2008). This study used the moisture readings taken at the closest time point to oviposition, which is a subset of the data used by Hurley *et al.* (2008). The analyses conducted in this study showed that while the bottom sections of the trees were statistically more moist than the top and middle sections, there was no significant difference between moisture content in the top and middle sections (Fig. 2). For this reason separate analyses were run, using farm, tree section and moisture as predictor variables. No significant patterns were found using moisture as the sole predictor of sex ratio, hence all further analyses were done using the interaction term of farm and tree section as the predictor variable. A 2 X 3 ANOVA was performed on sex ratio (=males/ [female+1] per log section) by site and tree section as billets of several trees were placed in single emergence drums so there was no way to reconstruct the inherent nesting of log sections within trees. Tukey HSD post hoc tests were used to examine treatment-level differences in position and position x site variables.

Sample collection for Wolbachia *screening and microsatellite analyses*

All wasps were obtained from the collections at the Forestry and Biotechnology Institute (FABI) at the University of Pretoria, South Africa. The wasp collections were from trees felled in various plantations for the purpose of screening emerging wasps for the presence of the parasitic nematode *Deladenus siricidicola*. The felled trees were sectioned and placed in

emergence drums where emerging wasps were collected and sent to FABI. Samples were taken from both the Cape and KwaZulu-Natal populations.

For *Wolbachia* screening purposes, thirty females were sampled from both the Western Cape and KwaZulu-Natal collections from 2009, as well as thirty males from the KwaZulu-Natal collections from 2009. Females were dissected to obtain eggs that were used for *Wolbachia* screening. Male reproductive organ dissection was not possible because storage in alcohol had made the samples inordinately brittle for this purpose. To obtain DNA from sperm, the last two segments of the abdomens, where the male reproductive organs are located, were removed and used a source of DNA.

Ninety four male wasps (collected during the November 2010 through February 2011 emergence period) from both the Western Cape and KwaZulu-Natal regions were sampled for microsatellite screening. Wasps were dissected to obtain thorax tissue for DNA extraction.

DNA extraction and Wolbachia *screening*

DNA extraction was performed using the PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California, USA) following the manufacturers' instructions with some modifications. Samples were freeze-dried prior to extraction. On addition of 80 µl of Prepman Ultra Sample Preparation Reagent, prior to heating, the samples were homogenised using bashing beads in a Retsch MM301 mill (VERDER Group, Netherlands).

Wasp eggs and sperm were tested for *Wolbachia* infection by amplifying *Wolbachia* specific 16S ribosomal DNA with forward primer W-Specf (5'-CATACCTATTCGAAGGGATAG-3') and reverse primer W-Specr (5'-AGCTTCGAGTGAAACCAATTC-3') (Werren & Windsor 2000). PCRs were performed in a total volume of 25 µl. Each reaction contained 1X PCR-

buffer + $MgCl_2$ (Roche Diagnostics, Randburg, South Africa), 200 µM dNTP's (Roche Diagnostics, Randburg, South Africa), 1 unit FastStart Taq polymerase (Roche Diagnostics, Randburg, South Africa), 400 nM forward primer, 400 nM reverse primer (Inqaba Biotech, South Africa) and 1 µl DNA template. A DNAEngine Peltier Thermal Cycler (Bio Rad, Hercules, California, USA) was used for all PCRs. Cycling conditions were 4 minutes at 95 ^{o}C, two cycles of 2 minutes at 95 ^{o}C, 1 minute at 60 ^{o}C and 1 minute at 72 ^{o}C, followed by 35 cycles of 30 seconds at 95 ^{o}C, 1 minute at 60^{o}C and 45 seconds at 72^{o}C. A final extension step was carried out for 5 minutes at 72 ^{o}C. PCR products were analysed by agarose gel electrophoresis on a 1% agarose gel. PCR products were loaded with 30X Gelred (BIOTIUM, Hayward, California, USA).

Proportion of females with constrained sex allocation

Constrained females are defined as those that have no sperm in their spermatheca (Godfray 1990). Wasps were collected in black panel traps from recently invaded pine plantations in the Mpumalanga province of South Africa in 2012 as part of a collaborative trial between FABI and USDA-APHIS. The wasps were collected from three trap sites over a six week emergence period. Ninety eight females were dissected and their spermatheca (sperm storage organs; (Fig. 3) were crushed on a slide using a scalpel blade and stained using bromophenol blue (Sigma-Aldrich). The slides were viewed under a Zeiss Axioskop 2 plus light microscope at 10X, 20X, 40X and 100x magnification and screened for the presence or absence of sperm, which in *S. noctilio* has a distinctive shape with 'spikes' radiating from a central 'hub' (Fig. 3). Photographs were taken of all slides that showed the presence of sperm using a Zeiss AxioCam ICc3 camera and the Axiovision SE64 Rel.4.8 software.

Female investment in diploid eggs (daughters), where sex allocation is unconstrained by mating status (i.e., in mated females), can influence sex ratio. For this reason a simulation model was run in R (R Core Team 2013) which systematically varied the frequency of

unconstrained females (mating frequency) and the proportional female investment in fertilisation (daughters) among mated females. This allowed for the examination of the effect on sex ration of both mating frequency and proportional investment in daughters.

DNA extraction and microsatellite screening

DNA was extracted using the *prep*GEM^{TM} Insect kit DNA extraction kit (ZyGEM Corporation Ltd, Hamilton, New Zealand) following the manufacturers' instructions. Samples were amplified at 13 microsatellite loci reported in Santana *et al.* (2009) using the QuantiTect® Multiplex PCR Kit (QIAGEN, Hilden, Germany) and primer stocks from Inqaba Biotech (Pretoria, South Africa)(Santana*, et al.* 2009). Amplification was performed using the manufacturers' instructions, but modified such that all reactions were scaled down to a total volume of 8.2 µl. Cycling conditions were 95 ^{o}C for 15 minutes followed by fifty cycles of 94 ^{o}C for 1 minute and 60 ^{o}C for 1.5 minutes. Amplified products were visualised using agarose gel electrophoresis on a 2 % agarose gel, PCR products were loaded with 30 X Gelred (BIOTIUM, Hayward, California, USA). Visualised PCR products were analysed by electrophoresis in an ABI PRISM 3100 automated DNA Sequencer (Applied Biosystems, Foster City, California, USA). The data collected were analysed using Genemapper software (version 3.0; Applied Biosystems, Foster City, California, USA) for genotyping of all samples at the 13 loci. All allele calls made by the software were checked manually and in cases where complete genotypes were not obtained samples were excluded from further analyses.

Analysis of genetic diversity

Genetic diversity was calculated from microsatellite data sets excluding diploid males using GenClone2.0 to calculate the number of distinct multilocus genotypes (MLGs) and genotypic richness (Arnaud-Haond & Belkhir 2007). Gene diversity and allelic richness were used as additional indices of genetic diversity and calculated using fstat v. 2.9.3.2 (Goudet 2001).

Results

Wood moisture content

Significantly fewer males were found to have emerged from the top sections of trees at both Good Hope and Yonderdale, however no significant differences were found in numbers of emerging females (Fig. 4). Similarly, no pattern was found for sex ratio when using the interaction between farm and tree section as a predictor variable because no significant differences were found in sex ratio in the different tree sections. There was, however, a significant difference in the sex ratio in the bottom section of trees at the two farms (Fig. 5).

Wolbachia *screening*

All *Wolbachia* presence/ absence PCRs were negative. This confirmed that this reproductive parasite was absent in all male and female *S. noctilio* specimens screened (Fig. 6). All positive controls amplified an expected fragment of ~430 bp.

Proportion of females with constrained sex allocation

Of 96 females dissected, 71 were found to have sperm in their spermatheca (Fig. 7). The simulation model showed that at high frequencies of constrained (unmated) females, sex ratio would be male biased regardless of the level of investment in daughters by mated females. Applying the simulation model (Fig. 8), at the frequency of constrained females observed here (~27%), if females fertilise 50% of their eggs, the sex ratio would be 2: 1.

Microsatellite scoring and analysis

Multilocus genotypes (MLGs) were obtained for 70 wasps from the Western Cape and 78 wasps from KwaZulu-Natal. Of the loci analysed, seven were polymorphic in the Cape population and three were polymorphic in the KwaZulu-Natal population. These populations had 55 and 6 distinct MLGs, respectively, with no unique MLGs identified in the KwaZulu-Natal populations. Diploid males were found in both the Cape (4.2 %) and KwaZulu-Natal (1.3 %) populations (Table 1).

Analysis of genetic diversity

Microsatellite diversity in the KwaZulu-Natal population was lower than in the Cape population. The KwaZulu-Natal population had 6 distinct MLGs, and a genotypic richness of 0.07 whereas the Cape population had 55 distinct MLGs and a genotypic richness of 0.82 (Table 1). Allelic richness and gene diversity indices were lower at all polymorphic loci in the KwaZulu-Natal population than in the Western Cape population (Table 2). Five of the 13 loci used were monomorphic in both populations. Unique alleles in were found in the Western Cape population, but no unique alleles were found in the KwaZulu-Natal population.

Discussion

In this study male-bias in *S. noctilio* populations in South Africa was shown not to be influenced by wood moisture content, tree section or the reproductive parasite *Wolbachia*. The data suggest that at the invasion front of *S. noctilio* in South Africa, sex ratios are correlated with a lack of genetic diversity, but little support was found for the proposed mechanism linking low genetic diversity to male bias (i.e., the overproduction of diploid males). Mating frequency is sufficiently high in the KwaZulu-Natal populations to produce

only moderate male bias such as is seen in the native and in older invasive populations (Slippers*, et al.* 2012). This suggests that female investment in male offspring is disproportionately high.

No *S. noctilio* males or females were found to be infected with *Wolbachia.* This reproductive parasite is normally associated with female biased insect populations (Werren*, et al.* 2008). While *Wolbachia* infection can increase male bias in Hymenopteran insects (Werren 1997), this was not the case in the *S. noctilio* populations examined in this study. *Wolbachia* is not the only reproductive parasite capable of altering population sex ratios (Werren*, et al.* 2008), and the possibility exists that other bacterial infections are present in *S. noctilio,* which would not have been detected by the *Wolbachia* specific primers used in this study.

Analyses of wood moisture data showed that at the time when moisture readings were taken, six months after the flight season of *S. noctilio*, wood moisture content was not correlated with sex ratio of emerging wasps. Significantly fewer males emerged from the top sections of trees from both Good Hope and Yonderdale. However, this did not appear to influence sex ratios in different tree sections, where no significant differences were found. This is in contrast to the observations in previous studies (Hurley*, et al.* 2008; Morgan & Stewart 1966) who noted that proportionally more females emerged from the bottom sections of trees, which were also found to be more moist.

Wood moisture content is not expected to vary at a regional scale over time as populations of *S. noctilio* become established; male-bias, however, does change over time. In KwaZulu-Natal for example, sex ratios were around 10 males: 1 female for 8 years after *S. noctilio* became established in the region. After 2010 the sex ratio, steadily dropped to ~3 males: 1 female in 2012 and 2013. Similarly, areas such as Mpumalanga, that have comparable weather conditions, similar tree species (*Pinus patula*) and similar management regimes (dense, unthinned stands managed for pulp wood) have more strongly male–biased sex

ratios. These populations are at the front of spread and establishment of *S. noctilio* in South Africa. This, as well as the data presented in this study, shows that changes in host quality (wood moisture) are unlikely to influence sex ratios in *S. noctilio* populations. Rather, the low amount of genetic diversity in the KwaZulu-Natal populations of *S. noctilio* may hinder the adaptation of oviposition behaviour, in terms of sex ratio of offspring, to local moisture conditions. This however would need to be thoroughly investigated as little is known about the interaction between oviposition and wood moisture content.

Results of this study showed that 27% of female *S. noctilio* were subject to constrained sex allocation (were unmated) at the invasion front of *S. noctilio* in Mpumalanga. This is a large proportion of constrained females when compared with other Hymenopteran insects (Hardy & Godfray 1990). This larger than expected proportion of constrained females could be an artefact of the timing of trap capture in that unmated females caught in traps may ultimately have mated. This result could also have been influenced by the fact that some of the traps used had male pheromone lures. However, even if mating frequency is underestimated here, constrained sex allocation does not adequately explain the extreme male bias in *S. noctilio* populations.

This study showed that Cape populations of *S. noctilio* were far more genetically diverse than the KwaZulu-Natal populations. The low genetic diversity observed in the KwaZulu-Natal population is not unusual because this population was at the invasion front of *S. noctilio* in South Africa at the time of sampling. The population is hypothesised to have arisen from the movement of infested wood from the Cape, because there is no continuous distribution of *Pinus* between this region and other regions where the wasp has been found (Hurley*, et al.* 2007; Tribe & Cillie 2004). For this reason the population is expected to have experienced a population bottleneck associated with a newly established population (Dlugosch & Parker 2008). The fact that no unique multilocus genotypes were found in the KwaZulu-Natal population of *S. noctilio* supports previous work suggesting the Western

Cape as the source of the KwaZulu-Natal *S. noctilio* populations (Hurley*, et al.* 2007). Similarly, the comparatively large level of genetic diversity observed in the Cape *S. noctilio* population, as well as the large number of unique multilocus genotypes identified supports recent work showing that *S. noctilio* has been introduced into South Africa more than once (Boissin*, et al.* 2012).

The existence of diploid males in *S. noctilio* populations in South Africa shows that a complementary sex determination mechanism exists in the woodwasp (van Wilgenburg*, et al.* 2006). This is not unexpected as this mechanism is thought to be ancestral in the Hymenoptera (Asplen*, et al.* 2009; de Boer*, et al.* 2012; Goulet & Huber 1993; Schmieder*, et al.* 2012). The fact that only three of thirteen microsatellite loci were polymorphic in the KwaZulu-Natal population implies that, if more diploid males had been present, they might not have been detected by microsatellite analyses, which rely on allelic differences at microsatellite loci to identify diploid males. For this reason, flow cytometry is being investigated as a tool for use in *S. noctilio* ploidy determination (Chapter 3), because it has been used in ploidy determination studies of other Hymenopteran insects (Aron*, et al.* 2003). The fact that diploid males were present at such low frequencies, suggests that there is a mechanism that prevents mating with males that are similar genetically.

The investment versus constraint model developed in this study suggests that female investment in female offspring has a greater influence on sex ratio than frequency of constrained females. At the level of mating observed (73%), females would need only to fertilize ~16% of their eggs to explain the 10 males: 1 female sex ratio that was seen in the KwaZulu–Natal population for a period of eight years. Should 95% have been mated, the percentage of fertilized eggs needed to explain a sex ratio of 10 males: 1 female would fall to 10%. Similarly, to achieve a sex ratio of 2 males: 1 female at a level of 25% constrained sex allocation (unmated), females would need to fertilize ~45% of eggs, whereas if only 5% of females were constrained, ~38% of eggs would need to be fertilised to maintain a 2 males: 1

female sex ratio. Hence, the model shows that while large changes in levels of constrained sex allocation are needed to significantly alter sex ratios, a small change in investment in female offspring can achieve the same change in sex ratio. This is an important observation given the evidence that in Hymenoptera with complementary sex determination, females will avoid mating with males that are inordinately genetically similar (Ruf*, et al.* 2013; Thiel*, et al.* 2013). Whether this is the case in *S. noctilio* has yet to be determined, although the investment versus constraint model suggests that post-mating mechanisms preventing fertilisation with genetically similar sperm, potentially by differential sperm use, would be highly effective.

The results of this study highlight the need for further exploration of sex determination in *S. noctilio*. Future studies directed at elucidating the genetic basis for sex determination *S. noctilio*, such as the identification of the sex determination locus and identifying whether one or more loci are involved in sex determination, will lead to a better understanding of the male-bias observed in newly established populations of the woodwasp. This study also highlighted the relevance of exploring preferential mating or fertilisation based on genetic similarity in *S. noctilio* populations. Such studies will allow for an improved understanding of the massive variation in sex ratios observed in invasive populations of *S. noctilio*.

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Table 1 Comparison of genetic diversity between the Western Cape (n=70) and KwaZulu-Natal (n=78) populations

	Western Cape	**KwaZulu-Natal**
Number of diploid males	3	1
Percentage diploid males	4.23%	1.28%
Number of polymorphic loci	7	3
Number of distinct MLGs	55	6
Genotypic richness (R)	0.82	0.07

Table 2 Diversity indices for Western Cape (WC, n=70) and KwaZulu-Natal (KZN, n=78) populations per locus

Locus	Gene diversity		Allelic richness*		
	WC	KZN	WC	KZN	Combined WC and KZN
Sn231	0.502	0	2	1	2
Sn576	0.498	0	2	1	2
Sn350	0	0	1	1	1
SnA2	0.555	0.461	4	2	4.693
SnB4	0	0	1	1	1
Sn104	0.03	0	2	1	2
Sn177	0	0	1	1	1
Sn185	0.369	0	2	1	2
Sn525	0	0	1	1	1
Sn641	0	0	1	1	1
Sn90	0.435	0	3	1	3
SnA7	0.412	0.1	2	2	2
SnB2	0.633	0.167	3	2	3

*Allelic richness was calculated based on the minimum sample size of n=67.

Fig. 1 Map of the Republic of South Africa showing provinces where wasp samples originated (asterisks).

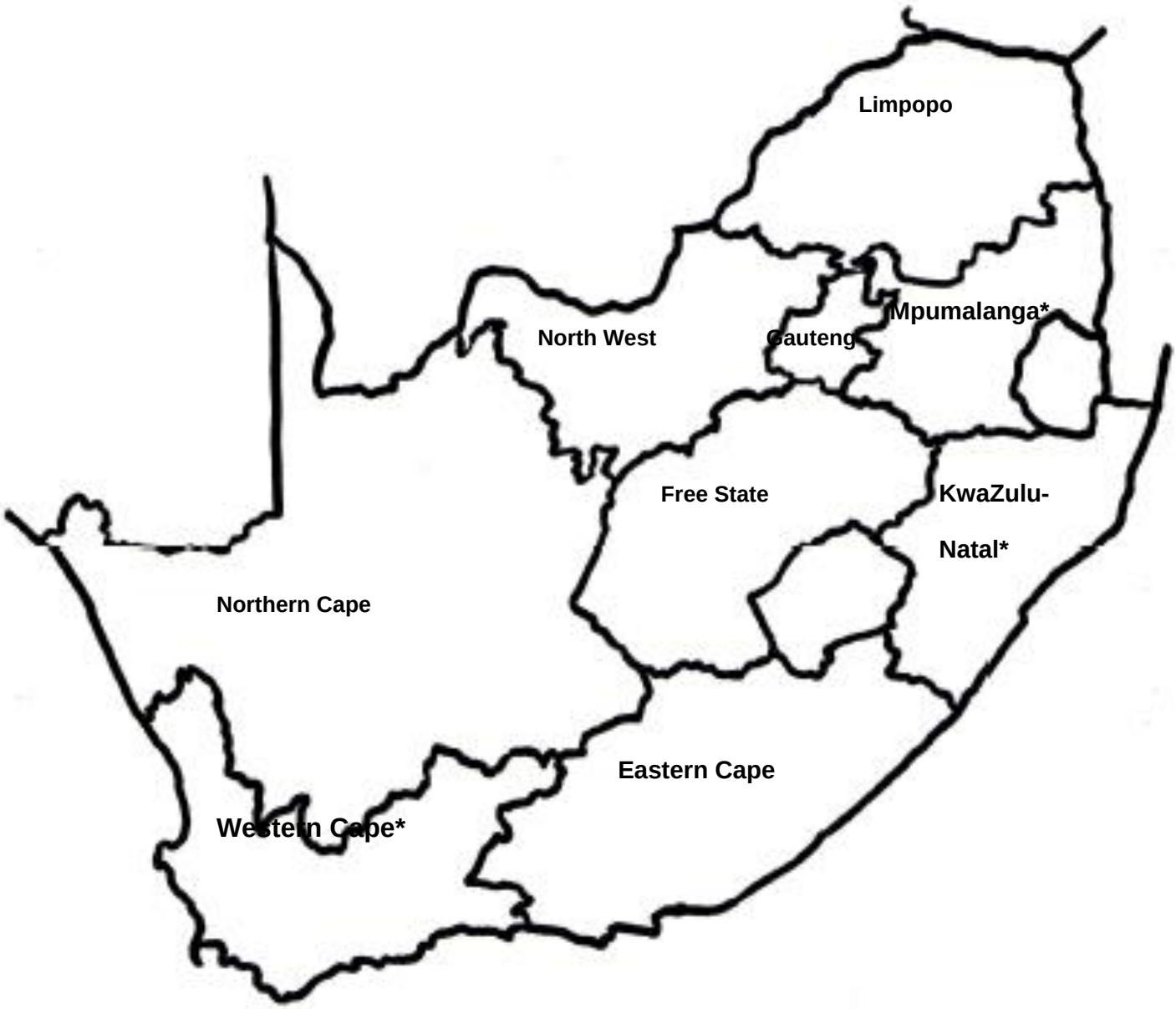


Fig 2 Box plot showing moisture content as a function of the interaction between farm and tree section. Where moisture content differs significantly among tree sections data are labelled with different letters. Outliers are indicated by open circles. At Good Hope (GH) the middle and top are significantly less moist than the bottom section. At Yonderdale (Y) there is no significant difference in moisture content between bottom and top sections of trees.

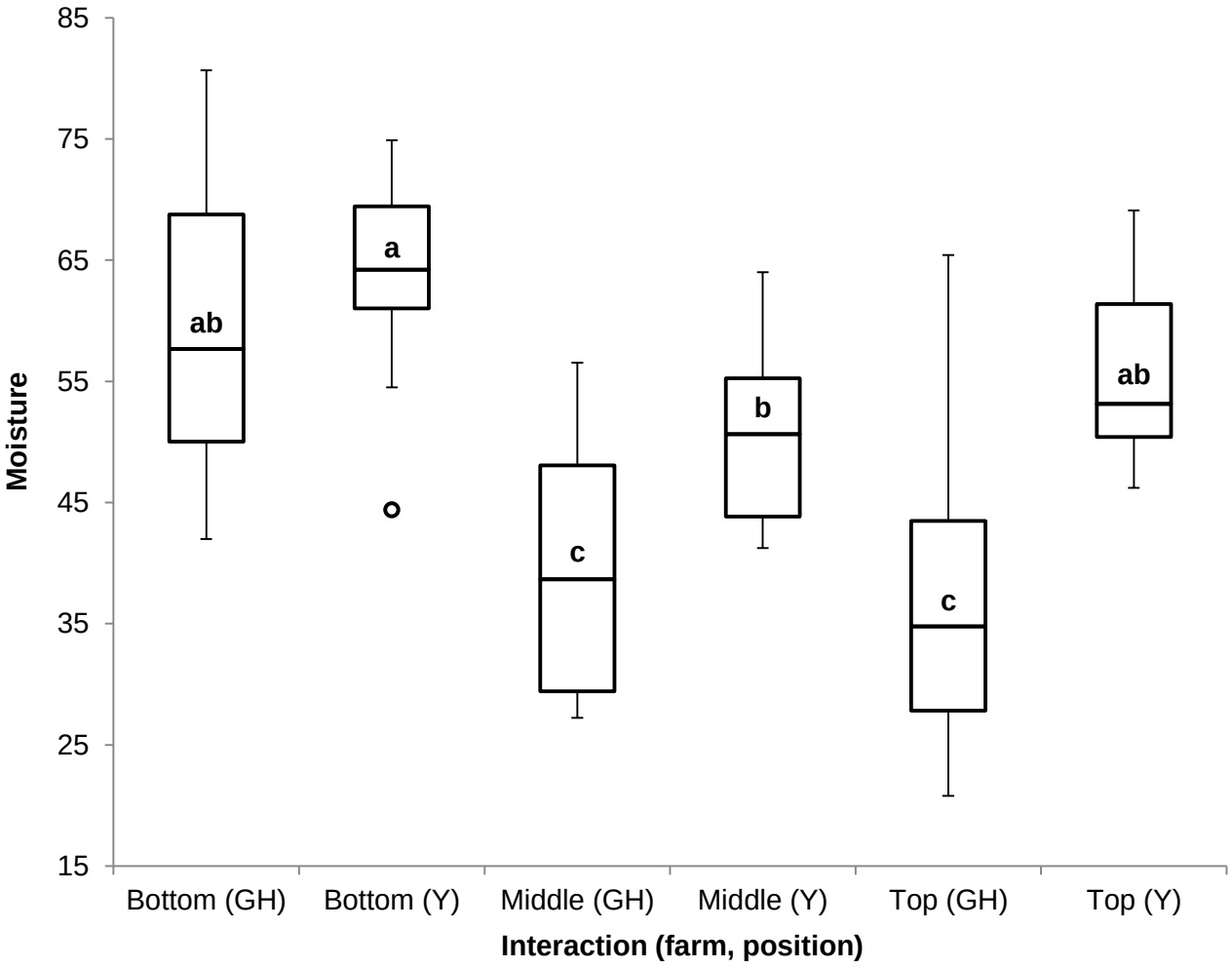


Fig. 3 Position and appearance of spermatheca in female *S. noctilio*. (a) Dorsal view highlighting the area where spermatheca is found. (b) Spermatheca (white arrow) and mycangia (asterisks) situated above the spermatheca.

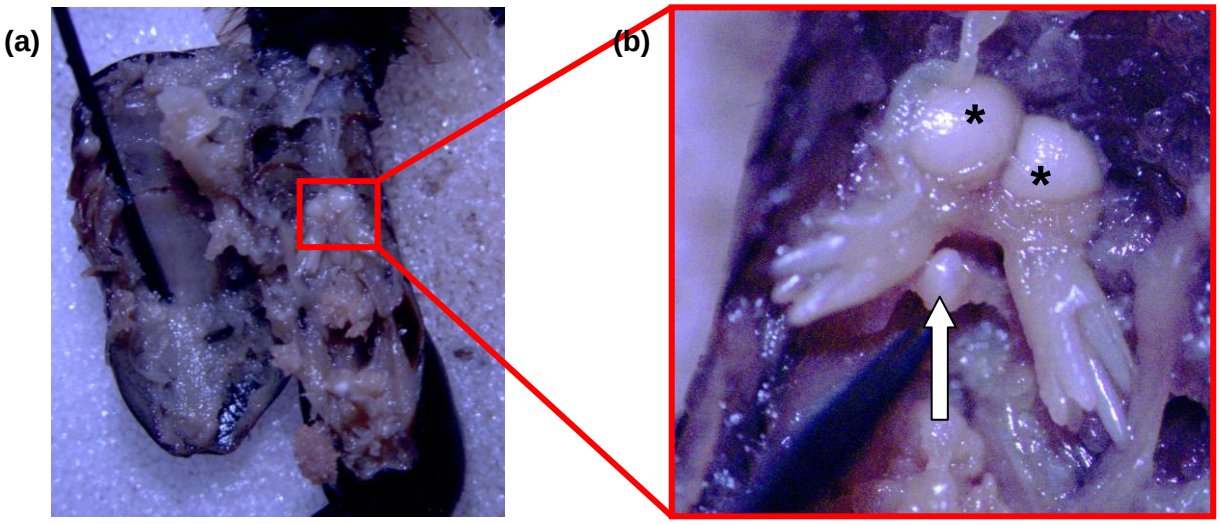
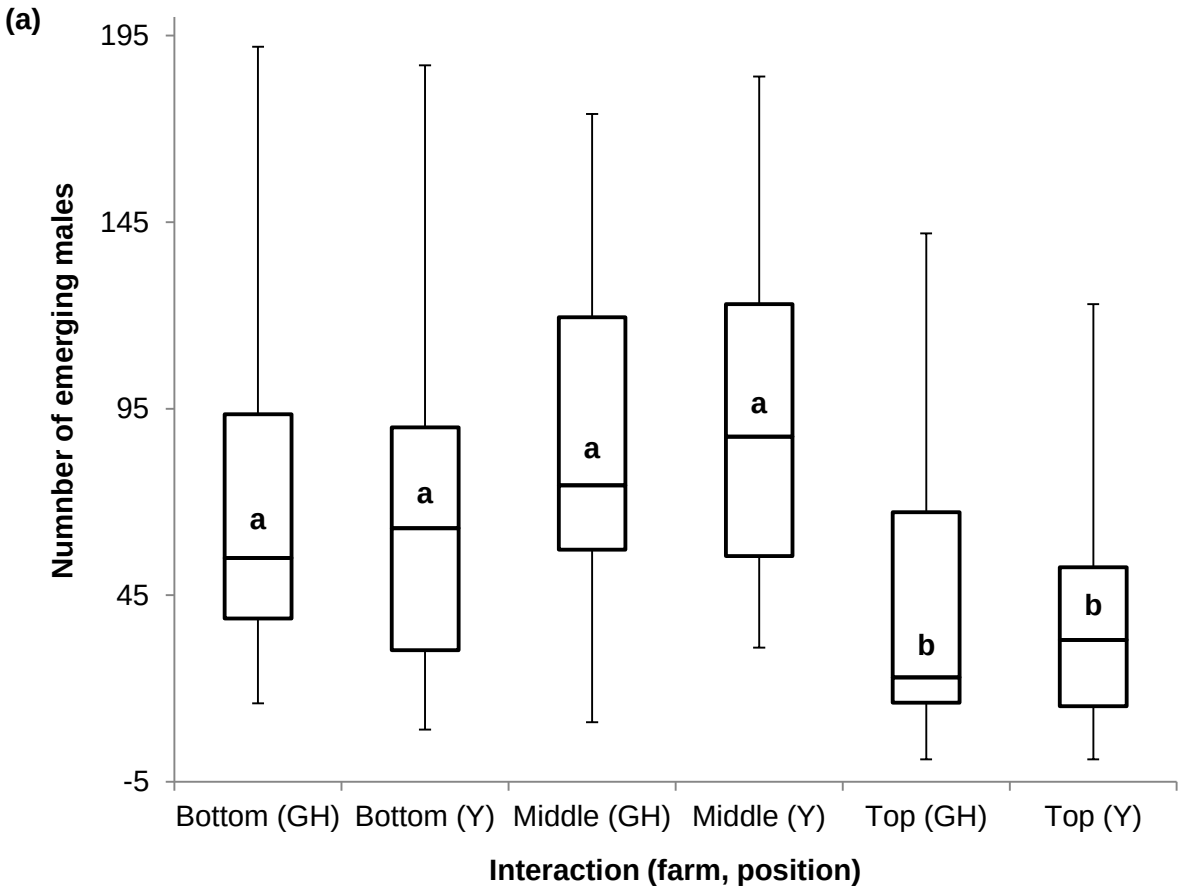


Fig. 4 Box plot of number of emerging wasps as a function of tree section. Where numbers of emerging wasps differ significantly among tree sections data are labelled with different letters. Outliers are indicated by open circles. Good Hope = GH, Yonderdale = Y (a) Significantly fewer males emerge from the top sections of trees at both GH and Y. (b) No significant differences in number of emerging females exist between tree sections at the same farm, however there is a significant difference in the number of females emerging from the bottom sections of logs at Good Hope and Yonderdale.



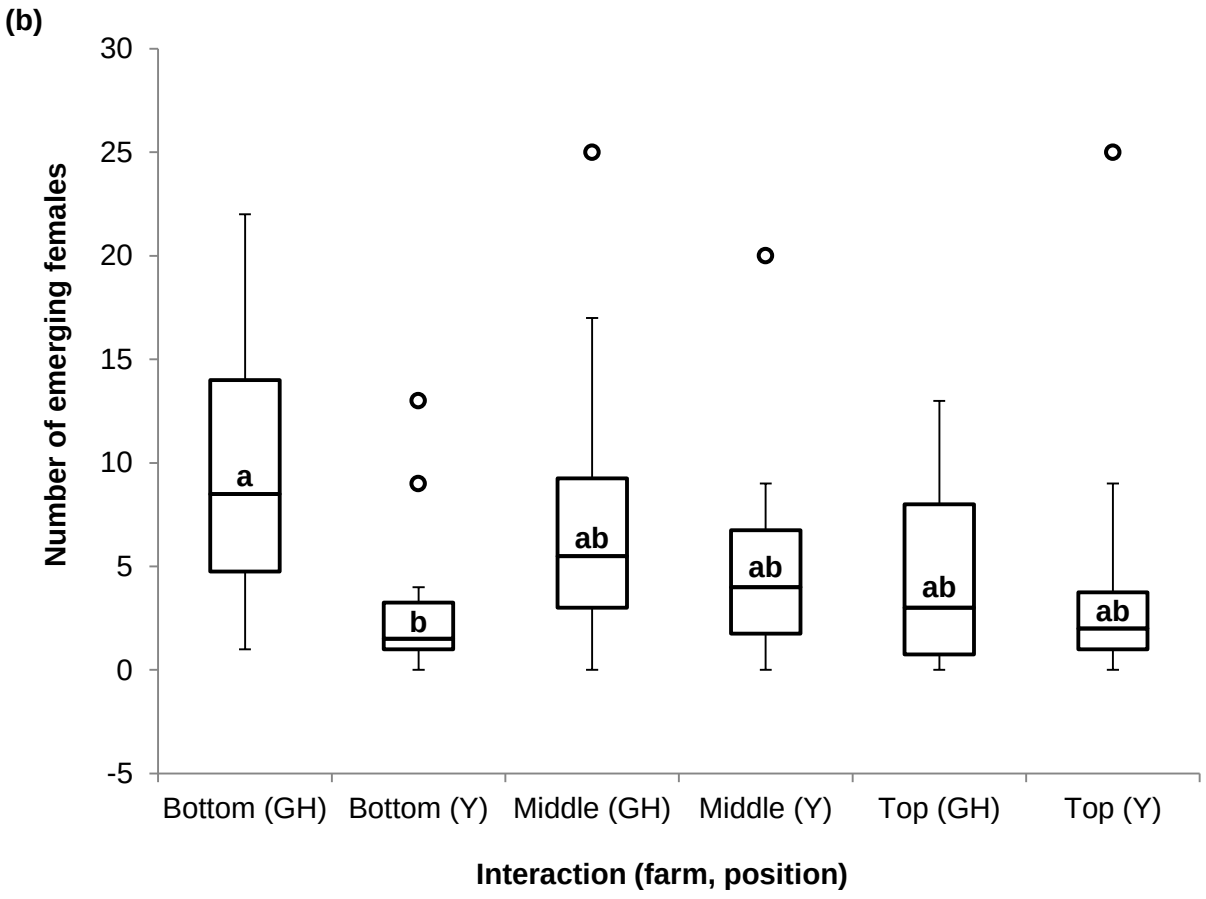


Fig. 5 Box plot of the natural log of the sex ratio of emergent wasps as a function of the interaction between farm and tree section. Where numbers of emergent wasps differ significantly among tree sections data are labelled with different letters. Outliers are indicated by open circles. Good Hope = GH, Yonderdale = Y. The natural log of sex ratio is used for visualisation purposes only; all statistical analyses were performed on a non-log-transformed data set. No significant differences in sex ratio exist between tree sections.

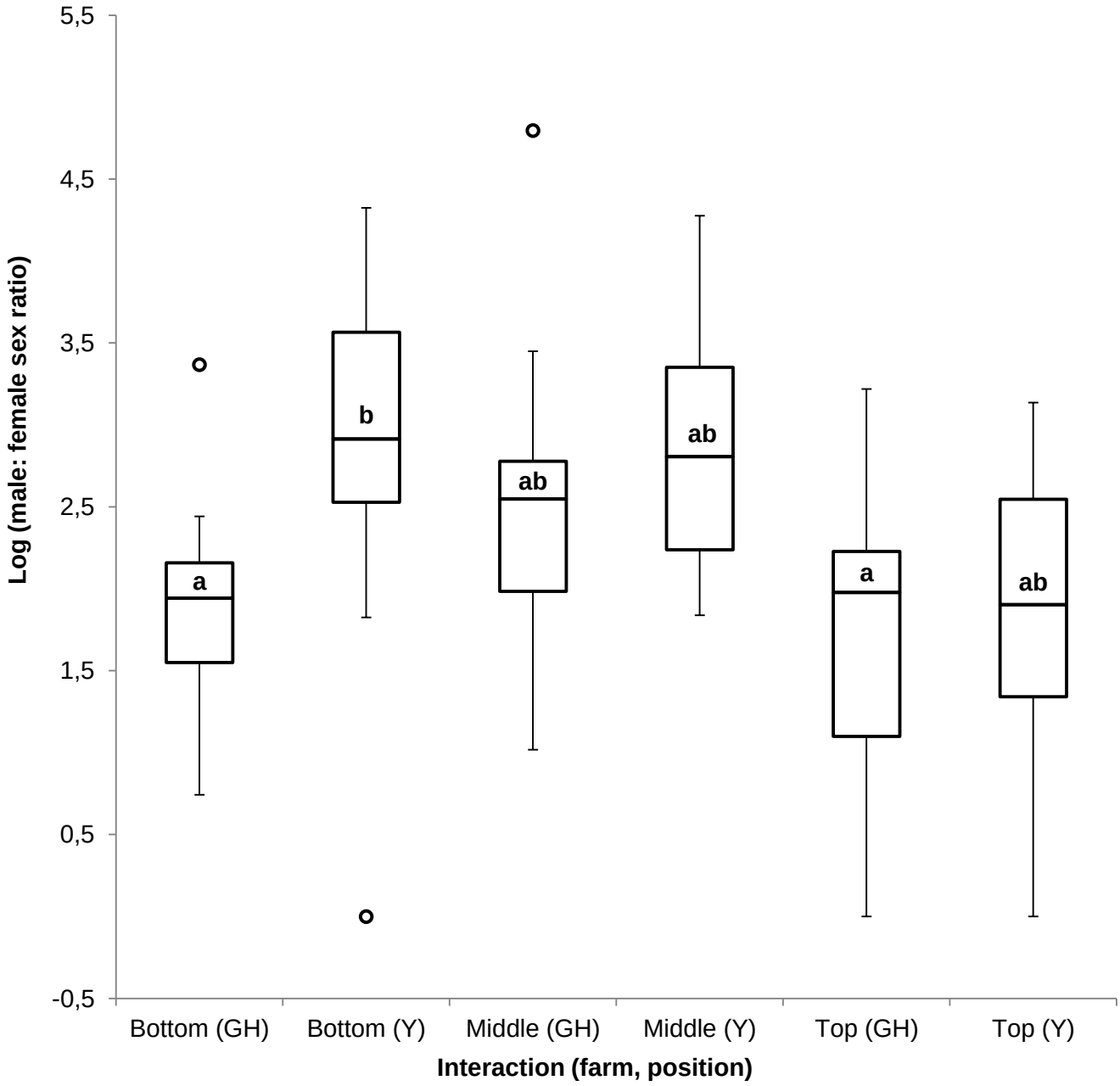


Fig. 6 Agarose electrophoresis gel of *Wolbachia* PCR. H-A are column names and 1-12 are row numbers. Lanes marked * contain 100bp ladder, lanes marked N contain negative controls, and lanes marked P contain positive controls.

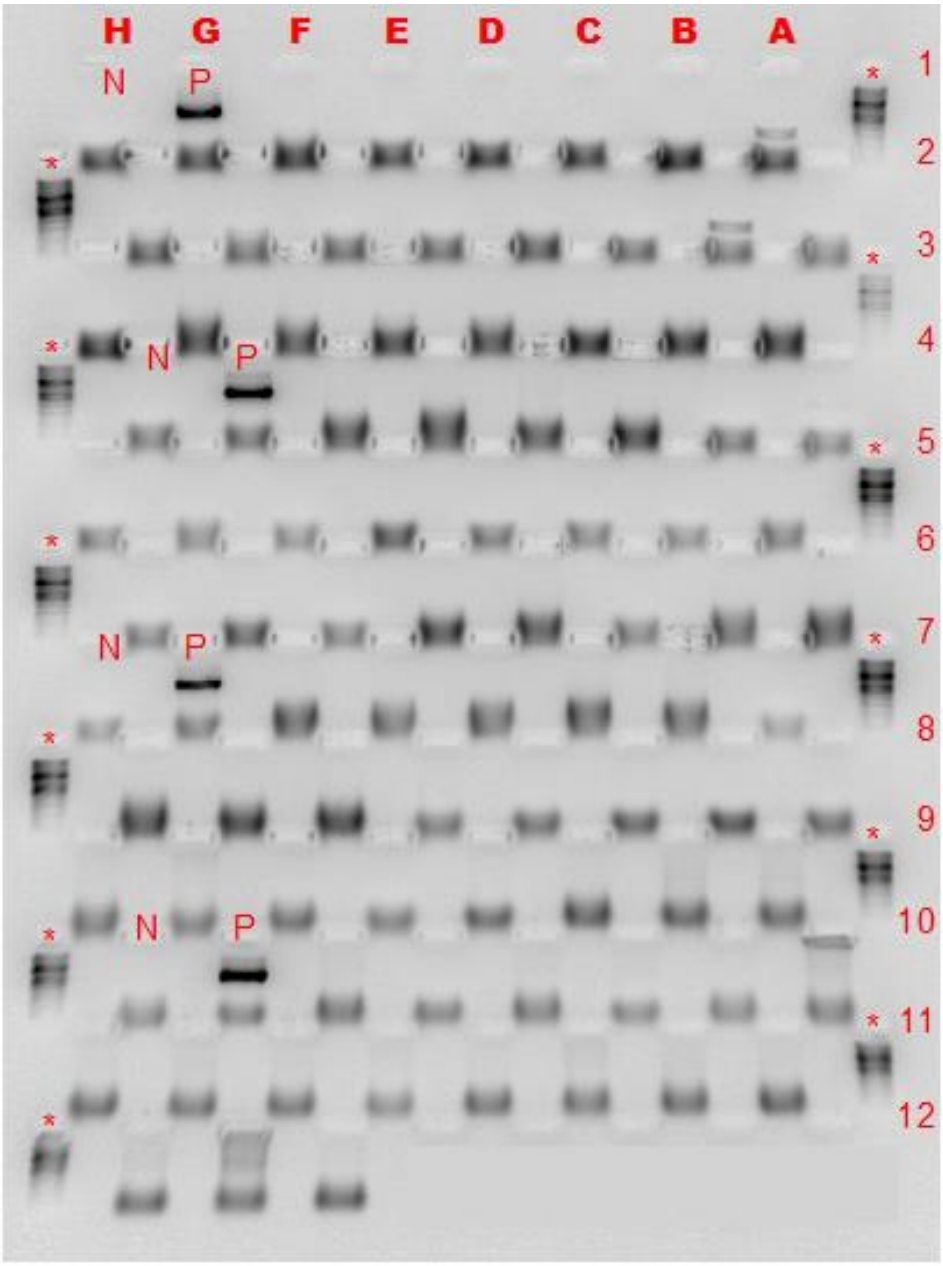


Fig. 7 *Sirex noctilio* sperm cells (indicated by white arrows) 100X magnification

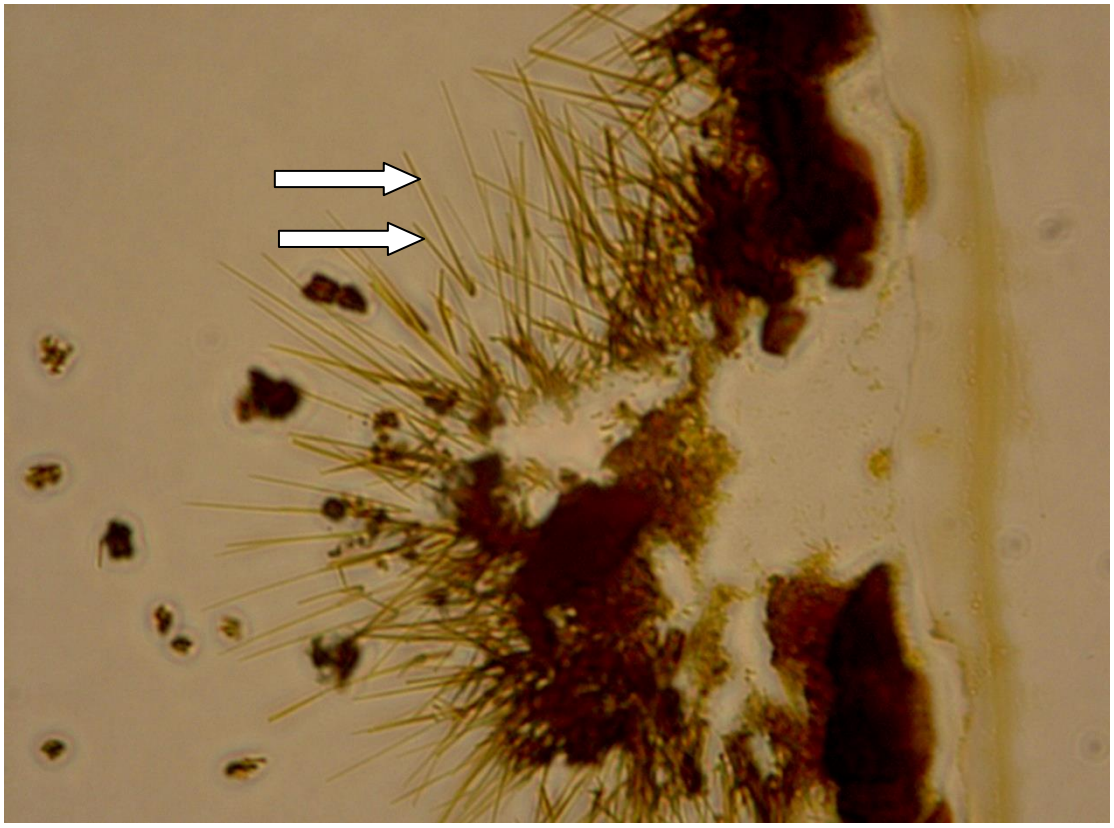
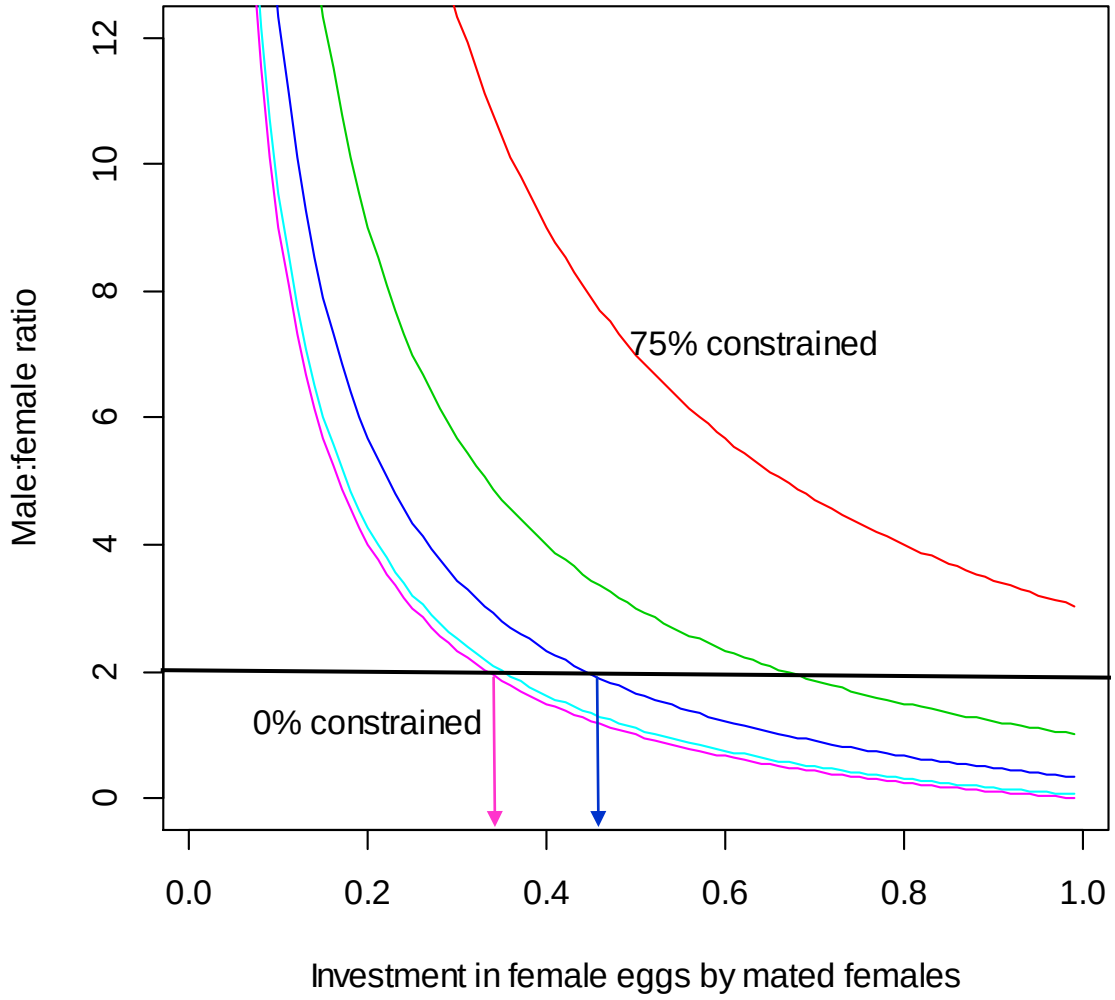


Fig. 8 Simulation model of effect of mating frequency and investment in daughters on sex ratio. Purple, light blue, dark blue, green and red lines indicate 0%, 5%, 25%, 50%, and 75% constrained females respectively. Black line indicates sex ratio in the native range of *Sirex noctilio*. Blue and purple arrows indicate proportion of female investment in daughters required to achieve a 2:1 sex ratio at 25% and 0% frequency of constrained females.



Thesis title: Sex determination and symbiont transmission in the *Sirex-Amylostereum* mutualism

Student: Amy Lorraine Wooding

Supervisors: Prof. Bernard Slippers

Prof. Michael J. Wingfield

Prof. Jaco M. Greeff

Dr. Jeffrey R. Garnas

Dr. Brett P. Hurley

Institution: Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria

Department: Department of Genetics

Degree: MSc

The obligate mutualism which exists between *Sirex* woodwasps and *Amylostereum* fungi has been thought to be highly specific; with each *Sirex* species carrying only one species of fungal symbiont. This was observed in the native range of *S. noctilio* in Europe and North Africa and in its invasive range throughout the Southern Hemisphere. *Sirex noctilio*, along with its obligate fungal symbiont *A. areolatum*, has recently become established in Eastern North America. This is the first time that *S. noctilio* has been introduced into a region where native *Sirex* and *Amylostereum* species exist. As *A. areolatum* is not known to occur in Eastern North America, this invasion provided the perfect opportunity for investigating the specificity of the mutualism between *Sirex* and *Amylostereum* species. We found that in the great lakes region of Canada 75.3% of the native *S. nigricornis* woodwasp specimens examined carried invasive *A. areolatum.* Similarly the 3.5% of the *S. noctilio* specimens examined carried the native *A. chailletii*. This shows that the observed pattern of specificity of the mutualism between *Sirex* and *Amylostereum* species is likely the result of factors such as geographic distribution and host association preventing contact between *Sirex* and *Amylostereum* species, rather than biological mechanisms preventing symbiont switching.

Newly established populations of *S. noctilio* are typically highly male biased, with sex ratios of up to 32 males: 1 female being recorded. This bias generally declines to ~2 males: 1 female; reflecting sex ratios observed in the wasps' native range in Europe and North Africa, within five years of establishment. This trend was not observed in the KwaZulu-Natal region of South Africa, where sex ratios remained above 10 males: 1 female eight years after establishment. Understanding population dynamics of invasive insects is important for control programmes, particularly against *S. noctilio* whose primary biological control agent is only spread via female wasps. The persistent skew in sex ratios in KwaZulu-Natal was investigated in order to identify the factor(s) driving the maintenance of the extreme male–bias in these populations. It was found that host quality as related to wood moisture content did not influence sex ratio. Similarly the sex ratio altering reproductive parasite *Wolbachia* was not found in the sampled *S. noctilio* specimens. The study showed that female investment in female offspring has the largest immediate effect on observed sex ratios. Microsatellite analyses showed that in KwaZulu-Natal the population genetic diversity is extremely low. They also revealed that *S. noctilio* uses a complementary sex determination system, where heterozygosity at the sex determination locus results in female development and hemizygosity or homozygosity at the locus results in male development, as evidenced by the presence of diploid males. As discussed in the final chapter, this suggests that a mechanism might exist which reduces mating with genetically similar males. This agrees with the finding that female investment in daughters is the factor with the largest influence on sex ratio.