UNIVERSITEIT VAN PRETORIA
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Ecology and genetic diversity of *Coryphodema tristis* on *Eucalyptus nitens* in South Africa

by

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A thesis submitted in partial fulfillment of the degree

Philosophiae Doctor

in the

Faculty of Natural and Agricultural Sciences

Department of Genetics

Forestry and Agricultural Biotechnology Institute

University of Pretoria

South Africa

February 2014

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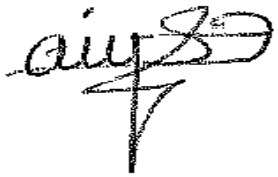
DECLARATION

I, Dawit Tesfaye Degefu declare that the thesis, which I hereby submit for the degree **Philosophiae Doctor** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.



Dawit Tesfaye Degefu

February 2014

DEDICATIONS

This work is dedicated to my father Tesfaye Degefu Kassaye who passed away when I was in South Africa for my PhD study.

It is also dedicated to all my family, my wife Frehiwot Aklilu and my lovely kids Henok Dawit and Bethelhem Dawit.

ACKNOWLEDGEMENTS

First of all I would like to thank my God for giving me the patience and endurance for completing this thesis. And I would like to thank Him for all His help in my life. Without His help this thesis would not have been possible.

I would like to express my deepest appreciation to my advisers Prof. Bernard Slippers, Dr. Brett Hurley, Dr. Jeff Garnas and Prof. Mike Wingfield for their support, guidance, encouragement and friendship throughout my post-graduate program. The lessons that I have learnt from them during this process of academic growth, especially in relation to my research, have fundamental value for my professional career. A sincere thank you also to my lab mates Alvaro Duran, Gudrun Dittrich-Schröder, Irene Barnes, Alisa Postma, Arista Fourie and Katrin Fitza for their friendship and making the Hearne-lab such a wonderful working environment.

The financial support provided by the members of the Tree Protection Co-operative Programme (TPCP), the Department of Science and Technology (DST)/ National Research Foundation (NRF) Center of Excellence in Tree Health Biotechnology and the University of Pretoria are also gratefully acknowledged.

Finally, I would like to thank my wife Frehiwot Aklilu, especially for her patience and understanding during my doctoral research years and for her immense contribution to the success of this process. Without her support and understanding it would have been immensely more difficult to commence and complete this post-graduate program.

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PREFACE

Plantations worldwide are dominated by species in the genera *Eucalyptus*, *Pinus*, *Acacia* and *Populus* that are planted in areas where they are not native. The choice of these genera is due to their desirable wood quality and growing characteristics. Their success and productivity in non-native areas is also due to their separation from natural enemies that typically decrease their productivity. These genera form the basis of rapid and extensive expansions in plantations in many parts of the world. Unfortunately the absence of natural enemies in these plantations is not permanent. A continuously growing number of emerging insect pests and microbial pathogens threaten the health of these tree species and the productivity of the plantations they make up. Understanding the origin and factors influencing the pattern of emerging pests and pathogens is thus of great significance.

Moths in the family Cossidae often infest exotic trees planted in its native range, sometimes leading to significant economic damage. The growing threat of the Cossidae to forestry calls for more thorough understanding of the diversity, biology and ecology of these insects around the world. Hence, **chapter 1** of this thesis examines Cossidae as pests both in native and non-native forests, and considers possible factors contributing to success of Cossidae in forestry. We also consider the classification, biology and major control strategies used to manage members of the Cossidae that are pests in forestry.

One source of emerging insect pests in non-native plantation forestry is native insects that eventually adapt to feed on non-native hosts. Two recent examples of this are the South African cossid moth, *Coryphodema tristis*, and the Chilean carpenterworm, *Chilecomadia valdiviana*, that started to infest non-native *Eucalyptus* spp. in plantations in these countries. In **Chapter 2** we explore the origin of the parallel host range expansion of these two insect pests on two

different continents using sequence data from the cytochrome oxidase subunit I mitochondrial DNA (COI). We test the hypothesis that the broad overlap in host range of these two geographically distant species and their apparent specificity on *Eucalyptus nitens* as an acquired host might reflect phylogenetic relatedness and cryptic invasion into one or both of these countries.

Understanding the origin of emerging pests in commercial forestry plantations is important to predict future expansion and to understand factors that might influence this process. Population genetics can help to unravel aspects of this process by helping to identify the origin and pattern of a newly emerging population. The recent jump of *C. tristis* from its broad geographic distribution and host range in South Africa to one non-native *Eucalyptus* species, *E. nitens* (the first association of the moth with the family Myrtaceae) raises many questions. In **chapter 3** we address some of these questions, namely whether this host jump constitutes a continuous (ongoing) colonization of *E. nitens* from native populations, or whether it constitutes a rare host switching event followed by isolation from the rest of the native population. To do this, we analyze patterns of diversity and structure in both nuclear and mitochondrial population genetics data, both from historical and contemporary *C. tristis* populations and from a range of hosts and areas. These hosts and areas include native hosts from across South Africa and *Vitis vinifera* from the Cape province.

Insects that feed on woody plant tissues often associate with microbes for nutrient acquisition or supplementation, in addition to many other roles microbes can play in these systems. These associations often constitute co-evolved symbioses. The interactions of microbes with insects can also influence novel host plant invasions by opening new ecological opportunities. The cossid

moths have been suggested to have an association with yeasts, and this is very noticeable in infested material by the fermenting odour that characterizes these infestations. This is also true for *C. tristis*. It was thus natural to ask what the diversity of the yeast community is within this environment, potentially to serve as a basis for future work aimed at characterizing the role of dominant species in the ecology of the insect, including its infestation of novel hosts such as *E. nitens*. **Chapter 4** addresses this question by characterizing and comparing yeast communities among different regions and hosts, and between tunnels and the insect gut of *C. tristis*. The phylogenetic relationship is also characterized and examined between isolated yeasts using DNA sequence data.

Since its discovery in 2004, *C. tristis* has spread throughout the planted range of *E. nitens* in Mpumalanga in South Africa. Notably, despite high population and close proximity the moth has not colonized the other cold tolerant *Eucalyptus* grown commercially in this region. In a preliminary study aimed at starting to unravel possible factors influencing the narrow host range on *Eucalyptus*, despite an otherwise broad host range, we explore the suitability of wood from different *Eucalyptus* spp. on the development of *C. tristis* larvae in **Chapter 5**. Experiments are done both in the lab on artificial media containing pulverized xylem tissue from different *Eucalyptus* spp. and in the field by testing the survival of *C. tristis* larvae placed on stems of different *Eucalyptus* spp.

CHAPTER 1

The threat of the cossidae to forest health with special reference to *Coryphodema tristis* and *Chilecomadia valdiviana*

ABSTRACT

Forests are complex ecosystems that provide a variety of valuable products, such as timber, fuel wood, fiber and non-wood forest products and they also contribute to socio economic development. Pest problems have a considerable influence on the health of planted and natural woodlands of this industry. Members of the family Cossidae are often of serious concern to forestry and horticulture due to their aggressive, often gregarious wood-boring behaviour. Some species of this family (e.g *Coryphodema tristis* and *Chilecomadia valdiviana*) is known to be highly polyphagous and members occupy diverse ecological zones. *Coryphodema tristis* and *Chilecomadia valdiviana* are examples of cossid species that have recently expanded their host range to include non-native species. *Coryphodema tristis* was first reported infesting the non-native plantation species, *Eucalyptus nitens*, in South Africa in 2004, and *C. valdiviana* was first recorded infesting the same species in Chile in 1992. These insects have been reported to cause substantial damage to *E. nitens*. Both species are associated with a number of native trees in their respective countries, as well as non-native fruit trees and ornamentals. Despite what appears to be extreme polyphagy in *C. tristis*, many trees, including many *Eucalyptus* species and even hybrids of *E. nitens* and *E. grandis* planted in the province of Mpumalanga, are apparently free of any cossid moth infestation. These novel host range expansions raise interesting questions regarding the factors that influence new host associations. This review examines Cossidae as pests in native and non-native forest systems. Possible factors contributing to success of Cossidae in forestry and control strategies employed to manage the group are discussed.

INTRODUCTION

Forests, both natural and planted, play a critical role in ecology, aesthetics and socio-economic development. Forest resources are amongst the most important economic assets for many countries, including temperate and tropical countries as well as developed and developing economies (Krieger 2001). Forests account for almost 30 % of the earth's total land area, nearly 3.9 billion hectares, but many of these forests are under pressure from harvesting and conversion to agriculture (Freer-Smith & Carnus 2008; Fearnside 2000; Laurance 2000; Justice *et al*. 2001; Semazzi & Yi 2001; Zhang *et al*. 2001). Managed plantations can play an important role in reducing the pressure placed on natural forests, as highly productive stands can better accommodate timber and fiber demand (Brown 1998; Jaakko Poyry 1999). Plantations worldwide are dominated by a small number of species in the genera *Eucalyptus*, *Pinus*, *Acacia*, *Populus*, *Cunninghamia*, *Larix*, *Picea*, *Tectona*, *Castanea* and *Quercus* (Kanninen 2010).

Native and introduced pests and diseases pose a very significant threat to both natural and plantation forests (Figure 1) (Wingfield *et al*. 2008; Holmes *et al*. 2009; Tubby & Webber 2010; Paine *et al*. 2011). For example, the introduction of Chestnut blight severely reduced the natural stands of chestnut in USA and Europe (Anagnostakis 1987). Similarly, the introduction of white pine blister rust (WPBR) both in Europe and then the USA caused major losses of white pine (*Pinus strobus*), western white pine (*P. monticola*) and sugar pine (*P. lambertiana*) (Maloy 2001). Forest insects have also resulted in devastating losses to forestry, such as the gypsy moth (*Lymantria dispar*) (Liebhold *et al*. 1992), pine shoot beetle (*Tomicus piniperda*) (Czokajlo *et al*. 1997), Sirex woodwasp (*Sirex noctilio*) (Hurley *et al*. 2007), and the recent expansive outbreaks of the mountain pine beetle (*Dendroctonus ponderosae*) in western North America (Wulder *et al*. 2006).

According to a report by the Food and Agricultural Organization (FAO) of the United Nations, Lepidopera are the most prevalent pests of forestry worldwide (FAO 2009; Figure, 2, 3). Within the Lepidoptera, the families Pyralidae, Tortricidae, Cossidae, Geometridae, Hyblaeidae, Arctiidae, Lymantriidae, Sesiidae and Lasiocambidae are considered the most economically important (Nair 2001; FAO 2009). Host trees of these insects include representatives of the natural forest, native and non-native plantation species throughout the world (Figure 3). As an example, some of these species include those in the families Mimosoaceae, Myrtaceae, Verbenaceae, Euphorbiaceae, Pinaceae, Oleaceae, Casuarinaceae, Lamiaceae, Rubiaceae, Dipterocarpaceae and Meliaceae, (Nair 2001; FAO 2009). The larval stages of Lepidoptera are the most destructive. They occupy various feeding guilds, mainly as defoliators, leaf miners, shoot and wood borers, or bark feeders (Powell 1980). Most of the recorded Lepidopteran pests are defoliators (FAO 2009), but wood and shoot-boring Lepidoptera in the families Cossidae, Hepialidae, Pyralidae, Phycitidae, Sesiidae and Tortricidae are also serious pests in forestry (Table 1). Of these, species in the family Cossidae are the most common major wood-boring pests in forestry (Solomon 1995).

Wood-boring moths in the family Cossidae are widespread, diverse, and are commonly cryptic and understudied. Members of the Cossidae are often of serious concern to forestry and horticulture due to their aggressive, often gregarious wood-boring behaviour, and their association with a wide range of trees and shrubs (Solomon 1995). The Cossidae are typically problematic on exotic trees planted in the native range of the moths. Some species in this family such as *C. tristis* and *C. valdiviana* are highly polyphagous, attacking a number of plant families, while others (e.g *Endoxyla cinereous*) attack members of species in same genus. Hence, the polyphagous nature of the family differs according to subfamily and the definition of polyphagy

(across genera in the same family or across families). The ability of polyphagous insects to develop on novel host trees is related to their tolerance for a wide range of diets and the capacity to withstand various tree defenses (Bertheau *et al*. 2010). Polyphagous insects are over-represented among insects that are successful colonizers of exotic trees (Parker & Hay 2005; Parker *et al*. 2006). Several reports have noted that the majority of native insect species that colonized exotic trees were polyphagous (Fraser & Lawton 1994; Novotny *et al*. 2003; Roques *et al*. 2006; Bertheau *et al*. 2010). The growing threat of the Cossidae to forestry calls for a more thorough understanding of their systematics, ecological range, possible pathways and potential distribution patterns.

This review examines Cossidae as pests in native and non-native forest systems. We discuss possible factors contributing to the success of Cossidae in forestry, the classification, biology and known host associations of the Cossidae. We also examine the major control strategies employed to manage this group of insects.

SYSTEMATICS AND CLASSIFICATION OF THE COSSIDAE

The family Cossidae consists of six sub-families, namely, Cossinae, Cossulinae, Hypoptinae, Metarbelinae, Ratardinae and Zeuzerinae. The family includes more than 110 genera and approximately 700 species worldwide (Davis *et al*. 2008). According to Edwards *et al*. (1999) the sub-family Zeuzerinae comprises the largest number of species (i.e 190 species in 30 genera) followed by Hypoptinae (170 species in 20 genera), Metarbelinae (150 species in 20 genera), Cossinae (100 species in 35 genera), Cossulinae (58 species in 6 genera) and Ratardinae (10 species in 3 genera).

The systematics and taxonomy of the family Cossidae are poorly studied, to the extent that most of the species do not yet have sub-family designation. In South Africa, the family Cossidae is represented by over 100 species (Pinhey 1975; Scholtz & Holm 1985; Picker *et al*. 2004), of which the genus *Coryphodema* is among the best-known due to its role as a pest of pear, apple, quince, loquats, olives, vines, avocado, bush willow, oak, elm, hawthorn and eucalypts (Petty 1917; Hoppner 1994; Gebeyehu *et al*. 2005). However, despite its economic importance, confusion exists regarding the taxonomy of this genus and there remains confusion regarding the sub-family in which it should reside.

BIOLOGY AND LIFE-CYCLE OF THE COSSIDAE

The Cossidae are moths of mostly medium to large in size having a wingspan from 2.5 - 24 cm (Solomon 1995). Adult moths have unspecialized mouthparts and do not feed (Solomon 1995). Most species aside from the day-flying Ratardinae (Davis *et al*. 2008) are nocturnal (Abang and Karim 2005). The life-cycle has four stages (Figure 4). Emergence of the moths is most common in the summer months and this can span a three to five month period.

Eggs are normally laid singly or in clusters on woody stems, especially on cracks and openings in the bark. The number and patterns of the eggs laid by the females vary greatly among species. *Prionoxystus robiniae* (Lepidoptera: Cossidae) (Peck) lays 200 - 1000 eggs, *P. macmurtrei* (Guerin) lays 50 - 275 eggs within a week, and *Coryphodema tristis* (Lepidoptera: Cossidae) lays 104 - 318 eggs per female (Solomon 1995; Gebeyehu *et al*. 2005). The eggs of cossids hatch after 10 - 68 days, depending on the temperature (Solomon 1995; Gebeyehu *et al*. 2005). Newly hatched larvae either construct loose silken webs and bark coverings or begin boring in to the host plant. Young larvae feed initially in the phloem and cambium but later instars bore into the xylem, forming a complex of tunnels (Figure 5).

The larvae invade the shoots, stems or roots of infested trees. In most cases the last larval instars pupate inside the tunnels of their host trees. While in the case of the seabuckthorn carpenter moth, *Holcocerus hippophaecolus* (Lepidoptera: Cossidae), the mature larvae pupate in soil around the base of the stem at a depth of approximately 10 cm (Zong *et al*. 2008). The larvae can occur solitarily such as *Chilecomadia valdiviana* (Lepidoptera: Cossidae) (Kliejunas *et al*. 2001), or gregariously within its host plant such as *C. tristis* and *H. hippophaecolus* (Gebeyehu *et al*. 2005; Zong *et al*. 2008). Emergence holes are made before pupation. Especially those larvae in *C. tristis* pupating within xylem tunnels do so in woven silk and sawdust cocoons. Pupae have rows of spines present on their abdomen, which enable them to move forward towards the emergence holes. The spines/horns present at the front of the head assist the pupae to cut through the cocoons.

The life-cycle of the Cossidae varies between 1- 4 years (Petty 1917; Solomon 1995; Gebeyehu *et al*. 2005) depending on temperature. For example, *P. robiniae* completes its life-cycle in 1 - 2 years in the southern USA, but takes 2 - 4 years in the north. *Coryphodema tristis* is reported to have a two-year life-cycle in the Western Cape, but the duration of the life-cycle in the summer rainfall area is not yet known. The life-cycle of *C. valdiviana* is also not well understood but is believed to require more than one year to complete. Overlapping generations for these insects are also suspected to occur because there are several months during the year when all four life stages can be found on the same trees (Cerda 1996; Gebeyehu *et al*. 2005).

Infestation by *C. tristis* is characterized by a number of symptoms. Their feeding produces a sap flow on the bark (Figure 6), the infestation results in round holes that penetrate deep into the sapwood, and extensive tunneling of larvae is found in the sapwood and heartwood (Figure 7). Frass and sawdust can also be found in bark crevices accompanied by holes in the bark (Figure

8) and heavily attacked trunks and branches of infested trees turn black (Figure 9). Stains and decay appear to be a symptom of more advanced infestation (Figure 10). A further symptom is that pupal casings may be found protruding from emergence holes or on the forest floor.

HOST ASSOCIATIONS OF THE COSSIDAE

Cossidae are often of serious concern to forestry and horticulture because they bore within the branches and trunks of a wide range of trees and shrubs (Nair 2001; Nair 2007). The family is known for its highly polyphagous nature and for occupying diverse ecological zones. More than 20 % of species in the family are reported to feed on multiple hosts, and many others have been recorded from only one or two hosts but are thought to also be generalists (Powell *et al*. 1999). Examples of cossid species recorded as polyphagous are *Endoxyla* spp. (Lepidoptera: Cossidae), *Xyleutes ceramicus* (Lepidoptera: Cossidae), *Zeuzera coffeae* (Lepidoptera: Cossidae), *Z. pyrina, Cossus cadambae* (Lepidoptera: Cossidae), *C. cossus, C. insularis, H. hippophaecolus and Duomitus ceramicus* (Lepidoptera: Cossidae) (Solomon 1995; Nair 2001; Kutinkova *et al*. 2006; FAO 2009). In many cases, the host ranges of these insects include native plant species as well as non-native species.

Coryphodema tristis and *C. valdiviana* are examples of cossid species that have recently expanded their host range to include non-native species. *Coryphodema tristis* was first reported infesting the non-native plantation species, *Eucalyptus nitens,* in South Africa in 2004, and *C. valdiviana* was first recorded infesting the same species in Chile in 1992 (Cerda 1995; Gebeyehu *et al*. 2005). These insects have been reported to cause substantial damage to *E. nitens*. Infestations by *C. tristis* have ranged from less than 1 % to nearly 77 % of stands (Boreham 2006). Similarly the infestation of *C. valdiviana* could be up to 5 % of infested stands (Kliejunas

et al. 2001). Both species are associated with a number of native trees in their respective countries of occurrence, as well as on non-native fruit trees and ornamentals. This includes recorded hosts such as *Acacia*, cherry, apple, quince, loquat, avocado, willow, *Nothofagus*, oak and elm for *C. valdiviana* (see Cerda 1995; Gonzalez 1989; Angulo & Olivares 1991; Kliejunas *et al*. 2001), and pear, apple, quince, loquats, olives, vines, avocado, bush willow, oak, elm and hawthorn for *C. tristis* (Petty 1917; Hoppner 1994). The appearance of *C. tristis* and *C. valdiviana* on *Eucalyptus* was the first record of these species on Myrtaceae in South Africa and Chile, respectively.

Phytophagous insects depend on their behavioral responses to plant features including phylogenetic, chemical similarity and/or pre-adaptation with the ancestral host (Ehrlich & Raven 1964; Becerra 1997; Opitz & Muller 2009). Whether these could be important factors in the relationship between *C. tristis* and *C. valdiviana* with their novel host *E. nitens* is not known. But it is possible because, as is true for of other insects, the choice of *E. nitens* by these insects may have been determined by chemical similarity and/or pre-adaptation with the ancestral host (Becerra 1997; Opitz & Muller 2009). The abundance and dominance of exotic trees in their introduced range is likely to increase the probability of colonization by native generalists (Neuvonen and Niemela, 1981). This could also be partly associated with the time elapsed since establishment in the introduced range (Kennedy & Southwood 1984; Frenzel *et al*. 2000; Brandle *et al*. 2008).

Microbial organisms are known to play a role in insect-plant interactions through environmental buffering. For example they provide various nutritional supplements (Chararas *et al.* 1983; Dowd 1989; Dowd 1991; Vega & Dowd 2005), prevent the colonization of pathogenic microbes (Six

2003; Ferrari *et al*. 2004; Loker *et al*. 2004; Scarborough *et al*. 2005), increase fitness in extreme abiotic environments (Chen *et al*. 2000; Montllor *et al*. 2002; Dunbar *et al*. 2007), assist in overwhelming plant defenses (Paine *et al*. 1997) and can provide protection from predators and parasitoids (Weis 1982; Oliver *et al*. 2003, 2005, 2006). It has been shown for *C. tristis* that various fungi and bacteria are associated with infestations (Degefu, this thesis), and it is possible that these microbes assist the insect in acquiring new host associations.

Despite what appears to be extreme polyphagy in *C. tristis*, many trees, including many *Eucalyptus* species and even hybrids of *E. nitens* and *E. grandis* planted in the province of Mpumalanga, are apparently free of any cossid moth infestation. In addition, artificial infestation of some non-host *Eucalyptus* spp. has been unsuccessful (Degefu, this thesis). Similarly, *Chilecomadia valdiviana* is also mostly found infesting *E. nitens*, although there have been a few reports of infestations on other species of *Eucalyptus* (Cerda 1996). These novel host range expansions raise interesting questions on the factors that influence new host associations.

FACTORS CONTRIBUTING TO THE SUCCESS OF HOST PLANT SELECTION

Pre-adaptation on closely related hosts

In most countries with plantations of non-native trees, shifts of native insect pests to feed on these trees are common (Strauss 2001; Fabre *et al*. 2004; see Paine 2011). For example, *Eucalyptus* plantations, which are increasing in importance around the world, are under serious threat from insect pests, including native insects that have acquired the ability to feed on these trees (Turnbull 2003; Wingfield *et al*. 2008; Paine 2011). A number of host shifts of native insects onto *Eucalyptus* have been reported to be due to pre-adaptation of the insect with native Myrtaceae plants (Kliejunas *et al*. 2001; Wingfield *et al*. 2008). In Australia, where *Eucalyptus* is

indigenous, there are a number of records of native insects shifting to *Eucalyptus* trees from other native Myrtaceae due to the pre-adaptation of those insects (Loch & Floyd 2001; Strauss 2001). For example, the larvae of the autumn gum moth, *Mnesampela privata* and some species of chrysomelids such as *Paropsis charybdis*, *P. delittlei* and *P. porosa* are possible examples of endemic species that have adapted to feed on species of *Eucalyptus* in addition to their original hosts (Bashford 1993; Lukacs 1999; Nahrung 2006). A similar situation was reported by de Little (1989) in Tasmania, where plantations of the non-local shining gum, *E. nitens*, are affected by insect pests that have originated from native eucalypt forests. There are likewise reports from Brazil where there is a high diversity of native Myrtaceae, and where, for example, the geometrid moth *Thyrinteina arnobia* and *Sarsina violascens*, known on several native Myrtaceae have shifted to feed on *Eucalyptus* (Grosman *et al*. 2005; Speight & Wylie 2001). However, this is not be the case for *C. tristis* and *C. valdiviana* where there are no records on these insects occurring on any plant in the family Myrtaceae. However, most plant species have characteristic chemical components and these could be shared among plants that are not closely related (Ehrlich & Raven 1964; Becerra 1997; Opitz & Muller 2009). This would provide the potential for host-plant selection by phytophagous insects and this might have positively influenced the novel interaction by *C. tristis* and *C. valdiviana*.

Plant secondary compounds

Secondary compounds play a decisive role in insect-plant interactions and in the evolution of host associations of herbivorous insects (Becerra 1997; Swigonova & Kjer 2004; Kergoat *et al*. 2005). There is a very diverse group of plant secondary compounds that are used by plants for defensive compounds including alkaloids, cardinolides, cyanogenic glycosides, flavonoids and terpenes (Rosenthal & Janzen 1979; Blum 1981; Cheeke 1989). For example, tannins in wood

and leaves have digestibility-reducing and protein binding properties as well as causing acutely toxic effects on insect herbivores (Swain 1979), while lignin in wood can limit digestibility (Stamopoulos 1988; Wainhouse *et al*. 1990).

Insects have developed several strategies to break down plant defense barriers, allowing them to feed, grow and reproduce on their host plants (Nishida 2002; Opitz & Muller 2009). Some herbivorous insects utilize poisonous and toxic plant secondary compounds for their own pheromone system and defense from their natural enemies (Nishida 2002). For example, plant secondary compounds such as terpenoids, phenolics and many other nitrogen containing compounds consumed by Lepidoptera are sequestered and used as to make themselves toxic or unpalatable to predators (Nishida 2002). Little is known regarding this important factor in insect plant interactions in the case of polyphagous and typically wood-boring cossid moths. Whether this could be an important factor influencing the diverse host range of cossid species has not been studied, but pre-adaptation to diverse chemical sources may at least in part explain the polyphagous nature of these insects and their novel host associations (Fraser & Lawton 1994; Novotny *et al*. 2003; Bertheau *et al*. 2010).

Escape from natural enemies

Ecological factors such as predation and parasitism can lead to selection for traits that promote or maintain novel host plant associations in phytophagous insects. Phytophagous insects select plants or plant parts not only based on their nutritional content, but also on the intensity of predation and parasitism (Price 1997). Feder (1995) suggested that oviposition by *Rhagoletis pomonella* (Tephritidae) resulted in lower parasitism rates in its derived host (apple) compared to the ancestral host (hawthorn). More recently, Murphy (2004) demonstrated that heavy larval

predation on ancestral host plants underlies a fundamental host shift from one plant family to another in the Alaskan swallowtail butterfly *Papilio machaon* aliaska (Papilionidae). Similarly, Mira & Bernays (2002) showed preference for a less suitable host plant species by the sphingid moth *Manduca sexta* reduces mortality of eggs 15-20 % due to natural enemies. Further research is needed to more thoroughly identify and understand the natural enemy community of the Cossidae, including the biology, ecological fitness and association of natural enemies with their hosts (Nicoli 1997; Hoddle 2004).

The role of symbiotic microbes

Another possible mechanism facilitating broad feeding niches is co-digestion or detoxification of food by microbial associates (Geib *et al*. 2009). The gut or other body parts of insects harbor a large diversity of beneficial microbes (Dillon & Dillon, 2004). Microbial symbionts are well recognized as providers of nutrients for their insect hosts (Gibson & Hunter 2010). Bacteria, fungi including yeasts and protozoa are the most diverse groups of organisms found associated with several insect species (Breznak 1982; Bourtzis & Miller 2003; Six 2003; Dillon & Dillon 2004). Insect-microbe interactions are not well documented in the Lepidoptera, although there are some reports of such associations (Mondy *et al*. 1998; Mondy & Corio-Costet 2000; Broderick *et al*. 2003; Genta *et al*. 2006).

Digestive symbiosis appears most common among insects that feed on wood or other highly lignified plant materials (Schloss *et al*. 2006). Cossidae are known for their associated microbes in the wood tunnels formed by their larvae, which result in staining and rotting of the wood tissue (Solomon 1995). In addition, fungi that colonize larval tunnels can be pathogens (see Hulcr & Dunn 2011) of the host trees. According to Hoppner & Ferreira (1990) fungal

discoloration of up to 13.5 % damage of the grapevine trunk was reported in association with *C. tristis*. Similarly, Gebeyehu *et al*. (2005) and Boreham (2006) reported discoloration of the tunnels made by the larvae of *C. tristis* on *E. nitens*.

Microorganisms that inhabit insect guts can play important roles in the host nutrition, development, detoxification of toxic compounds and resistance to pathogens and reproduction (Douglas 2007; Douglas 2009; Klepzig *et al*. 2009; Gibson & Hunter 2010). Therefore, understanding the taxonomy, distribution and abundance of the symbionts represents an important component of understanding their ecology and evolutionary roles. Microbial symbionts remain unexplored for most insects, including the Cossidae (Schloss *et al*. 2006).

MANAGEMENT OF COSSIDAE

Wood-boring insects that feed on xylem of living trees pose unique management challenges because their immature stages live in concealed habitats within host trees. Hence, the efficacy of spraying synthetic chemical insecticides is limited. Injection of systemic insecticides is plausible but expensive and labor-intensive (Ali & Garcia 1988; Grosman *et al*. 2009). After *C. tristis* was reported as a pest of grapevine in South Africa, insecticide trials were conducted using injection of chemicals into tunnels, fumigation and aerial spraying (Petty 1917), but this treatment over a large area was concluded to be impractical. Another option is aerial spraying of insecticides during moth emergence. This would, however, require repeated spraying due to the extended emergence period. Besides the negative economic and environmental consequences of such repeated spraying, it could also negatively affect natural enemies and lead to the development of resistance in the pest populations (Ruberson *et al*. 1998).

Pheromones are volatile chemicals used for communication in insects and many other organisms. Natural and synthetic sex pheromones are non-toxic and biodegradable and could potentially be used effectively for cossid pests (Solomon *et al*. 1978; Dix & Doolittle 1984; Dix *et al*. 1984; Dix *et al*. 1987; Facial *et al*. 1993; Trimble *et al*. 2004; Hegazi *et al*. 2009). For example, the use of synthetic sex pheromone in combination with light traps is reported to be more effective against the Leopard Moth *Z. pyrina* as compared with pheromones, light traps or insecticides alone (Hegazi *et al*. 2009). The efficacy of sex pheromones as a management tool is largely dependent on the release rate and ratio of sex pheromone components in the attractant, the preservatives added to the attractant, the release device and the number and type of traps used (Hirooka & Suwanai 1976; Haynes *et al*. 1983; Gronning *et al*. 2000; Trimble *et al*. 2004). In this regard, Solomon *et al*. (1978) reported that the use of the preservative UOP-688 with (E.E)-3,5-tetradecadien-1-0l acetate in the ratio of 10:1 resulted in the capture of significant numbers of carpenter moths. Trap density needs to balance cost constraints with trapping efficiency. For instance, Facial *et al*. (1993) showed that the use of 10 traps per hectare was sufficient to trap *Cossus cossus*, unless there were heavy infestations of the insect. Trimble *et al*. (2004) also reported that the rate of emission of pheromone from dispensers affects the efficacy of mating disruption systems. This was previously reported by Dix *et al*. (1984 and 1987) who showed that hollow fibers and septum dispensers were more effective in attracting the male moth of the carpenterworm, *Prionoxystus robiniae*. Similarly, Dix & Doolittle (1984) reported on the efficiency of different traps baited with rubber sheath dispensers in mixed hardwood forests against *P. robiniae* and *Acossus centerensis*. They reported that the diamond-carton trap design for *P. robiniae* and Pherocon1C trap for *A. centerensis* were most effective for the monitoring and management of these insects. Hence pheromones have some potential for the management of

C. tristis, particularly where they might be used in combination with other pest management strategies (Hegazi *et al*. 2009).

There are various other alternative pest management strategies for the Cossidae. The use of natural enemies is a strategy used for numerous insect pest problems (Hajek & Bauer 2007; Van Driesche *et al*. 2008). The use of natural enemies could possibly provide economic, social and ecological gains (see Menz *et al*. 1984; Tisdell 1987), while effectively controlling the pest. Different studies conducted under laboratory and field conditions revealed the potential role of natural enemies against cossid species. For instance, nematodes such as *Steinernema feltiae* (Filipjev) and *Steinernema bibionis* (Bovien) used against the cossid moth *P. robiniae* both under laboratory and field trials on infested oak trees resulted in significant mortality (Forschler & Nordin 1988a; Forschler & Nordin 1988b). Similarly, in China field trials the entomopathogenic nematode *S. carpocapsae* has been used against *H. insularis* and Z. *multistrigata* where it resulted in 93 % and 90 % mortality, respectively (Yang *et al*. 1993). However, application of parasitic nematodes could be influenced by depth, structure and moisture level within tunnels as well as densities and ages of larvae. This would influence the ability of the nematodes to disperse and infest the boring larvae inside the tunnels. Relatively little work has been done with entomopathogenic fungi against wood-boring insect pests but the results obtained are encouraging. For example, the application of *B. bassiana* conidia mixed with waste molasses and sweet potato starch into excretion holds in casuarinas trees resulted in larval mortality of 93.6 - 96.8 % against the cossids *Z. multistrigata* and *Squamura discipuncta* (=*Arbela discipuncta*) (Hajek & Bauer 2007).

Little is known regarding the natural enemies of *C. tristis* or *C. valdiviana* and how they might be used to manage these pests. *Coryphodema tristis*, which is thought to be native in South

Africa, was first reported as a pest of apple and quince in 1917 (Petty 1917) and found associated with a number of other plants representing different families. Similarly, *C. valdiviana* is a native pest species in Chile found associated with large number of host plants, both native and exotic. Thus, it is expected that natural enemies will be found associated with natural populations of *C. tristis* and *C. valdiviana* in both these countries. Hence, identifying and examining the efficacy of natural enemies of these and other cossid pests could play an important role in developing management strategies.

The use of insect-resistant plant varieties is an important component of integrated pest management in agriculture (Kennedy 2008). The importance of tree resistance in insect pest management and its role in the ecology of forest insect pests has also long been accepted (Stark 1965). The presence of variation in traits within many natural plant populations, including trees and their effects on herbivorous insect pests has been well documented (Marquis 1992; Leather 1996; Osier *et al*. 2000). For instance, Faizuddin & Dalmacio (1992) found great variability to pest resistance among the different clones of *Acacia mangium*. Similar suggestions have been made by Cobbinah & Wagner (1995) who showed highly significant variation among 21 half-sib families of *Milicia* species in growth and resistance to the gall forming psyllid, *Phytolyma lata*. Other results also reported a considerable variation in resistance to the leucaena psyllid both within and among species of *Leucaena* (Austin *et al*. 1995).

Limiting the movement of insects into new environments by closing pathways of distribution represents the most efficient strategy for the management of biological invasions (e.g. Leung *et al*. 2002; Simberloff 2005; Kenis *et al*. 2007; Garcia-Llorente *et al*. 2008; Waage & Mumford 2008). Given the threat of Cossidae to world forests, it is worth considering the pathways by which they might be introduced. Some of the most important pathways such as the trade of forest

products and domestic exchanges are the most likely routes for the movement of organisms to new environments (Kliejunas *et al*. 2001; Withers 2001; Roques *et al*. 2003; Kimoto & Duthie-Holt 2006; Haack *et al*. 2010). Transport of wood products and packaging poses a particular risk for wood-boring insects such as the cossidae, where their larval stages are completed inside the wood. Hence, knowledge of potential pathways and the management thereof is an important component of a management strategy.

Conclusions

A small number of Cossidae species are important pests in forests and plantations worldwide. Their wide host ranges and apparent adaptability have caused them to become serious pests on certain trees that are planted as exotics for agriculture and forestry industries worldwide. These moths are normally native to the areas in which they are pests, but their large host range and different geographical areas make these insects potentially serious invasive pests. As an example, the adaptability of *Z. pyrina* in the USA outside its native range provides a sobering example of the potential pest status of these insects (Kliejunas *et al*. 2001).

The integrated efforts of countries towards developing strategies for the management of insect pests must provide the necessary information to restrict the further distribution of the current potential threats that Cossidae pose to forestry worldwide. The new host associations of this insect family in different countries (Lanfranco & Dungey 2001; Gebeyehu *et al*. 2005; Boreham 2006) have resulted in severe losses to forest products. By thorough investigation and monitoring, as well as implementing effective management strategies, the spread and impact of Cossidae as forestry pests can be limited.

The studies presented in this thesis include some of the first intensive investigations of the biology of *C. tristis*. They include considerations of the origin and host range of the pest and how it has moved in South Africa. Furthermore, the feeding biology of *C. tristis* and its association with various microbes is considered. It is hoped that these studies will provide a foundation, not only to establish management strategies for the pest, but also to understand the impact that the Cossidae might have on global forestry in the future.

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TABLE

Table 1. List of the main Lepidopteran wood borers in forestry.

No	Scientific Name	Family	Host Family	Country/Region
1	*Prionoxystus robiniae*	Cossidae	Fagaceae, Oleaceae, Ulmaceae	USA
2.	*Zeuzera pyrina*	Cossidae	Oleaceae, Fagaceae, Rosaceae	USA, Europe, North Africa, Bulgaria, Italy and Japan
3	*Cossus insularis*	Cossidae	Salicaceae, Rosaceae	Japan, USA
4	*Cossus cossus*	Cossidae	Fagaceae, Oleaceae, Ulmaceae, Betulaceae, Rutaceae, Vitaceae	USA, Europe, North Africa and Asia
5	*Cossus cadambae*	Cossidae	Ebenaceae	India
6	*Phassus signifer*	Hepialidae	Verbenaceae	Thailand, Mynamer
7	*Sahyadrassus malabaricus*	Hepialidae	Verbenaceae	India
8	*Synanthedon scitula, Synanthedon novaroensis, Paranthrene robiniae*	Sesiidae	Cornaceae, Fagaceae, Salicaceae, Pinaceae	USA
9	*Endoxyla cinerea*	Cossidae	Myrtaceae and other families	Australia
10	*Zeuzera coffeae*	Cossidae	Myrtaceae, Meliaceae, Verbenaceae, Fabaceae	India, Thailand
11	*Xyleutes ceramicus*	Cossidae	Meliaceae, Verbenaceae	Thailand, Mynamer, Malaysia
12	*Alcterogystia cadambae*	Cossidae	Meliaceae, Verbenaceae	India
13	*Chilecomadia valdiviana*	Cossidae	Myrtaceae, Fagaceae, Salicaceae, Cunoniaceae, Rhamnaceae	Chile
14	*Coryphodema tristis*	Cossidae	Myrtaceae, Ulmaceae, Vitaceae, Rosaceae, Scrophulariaceae, Myoporaceae, Malvaceae and Combretaceae	South Africa
16	*Salagena discata*	Cossidae	Mangrove species	Kenya

Source: Nair, (2001), Nair, (2007), FAO, (2009), Solomon, 1995

FIGURES

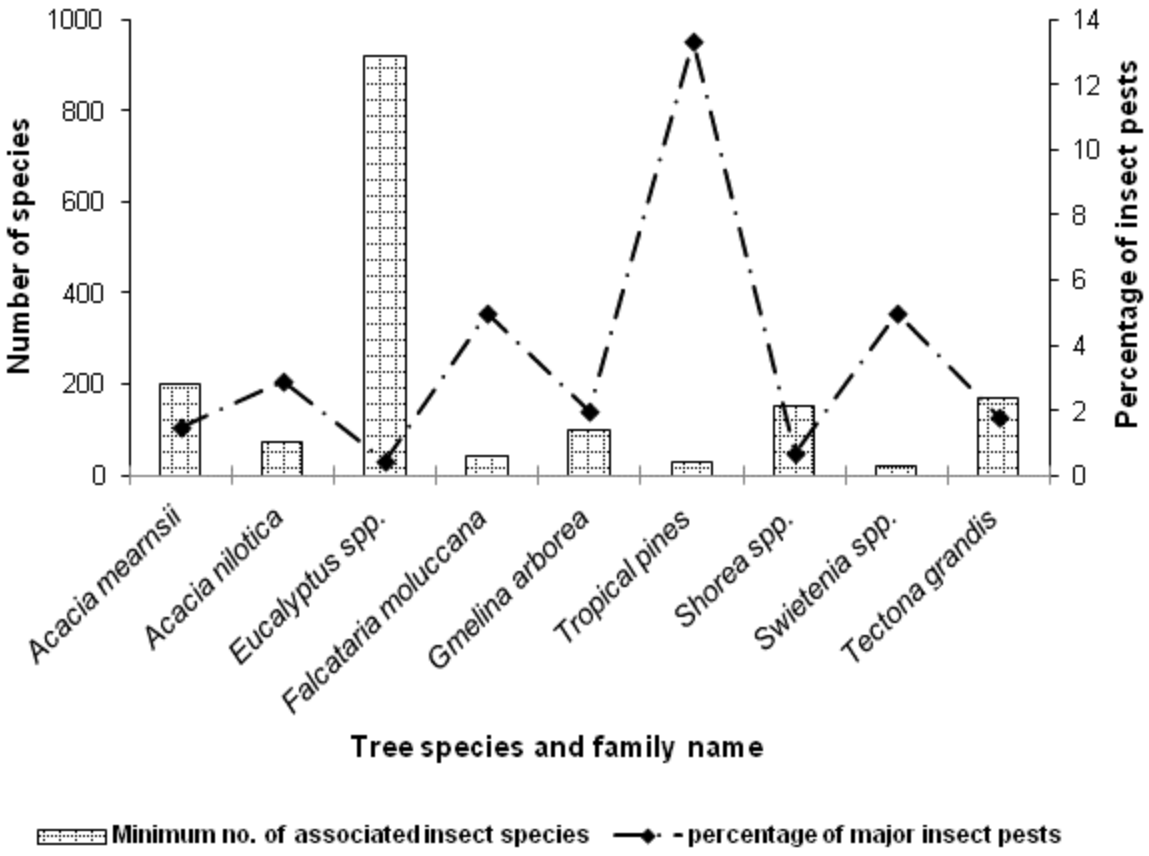

Figure 1. Some of the common tropical tree species and the number of their associated insects and major insect pests (modified from Nair, 2007).

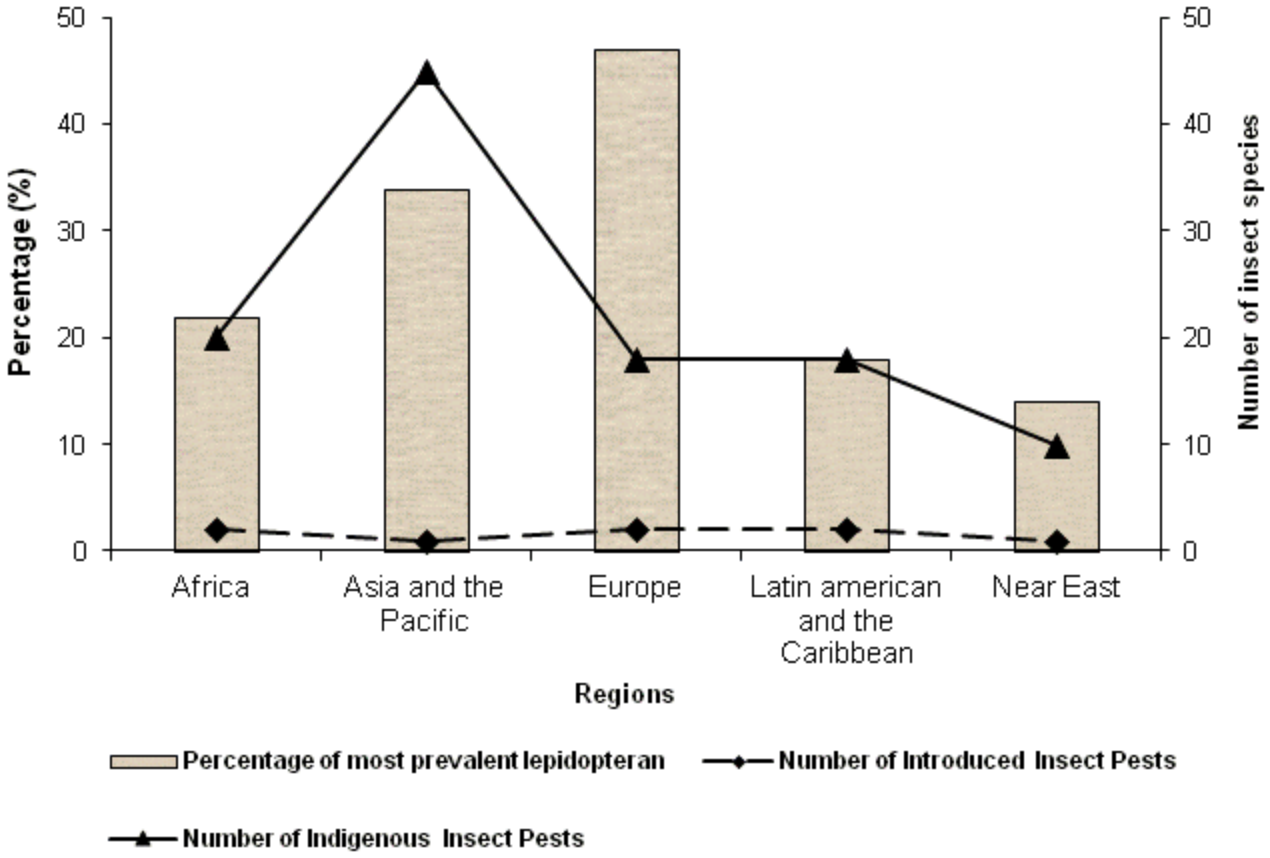

Figure 2. The distribution of most prevalent, indigenous and introduced Lepidopteran insect pests from different regions. (included countries in the region: seven countries from Africa, five countries from Asia and the Pacific, three countries from Europe, eight countries from Latin American and the Caribean and two countries from Near East). Modified from FAO, 2009.

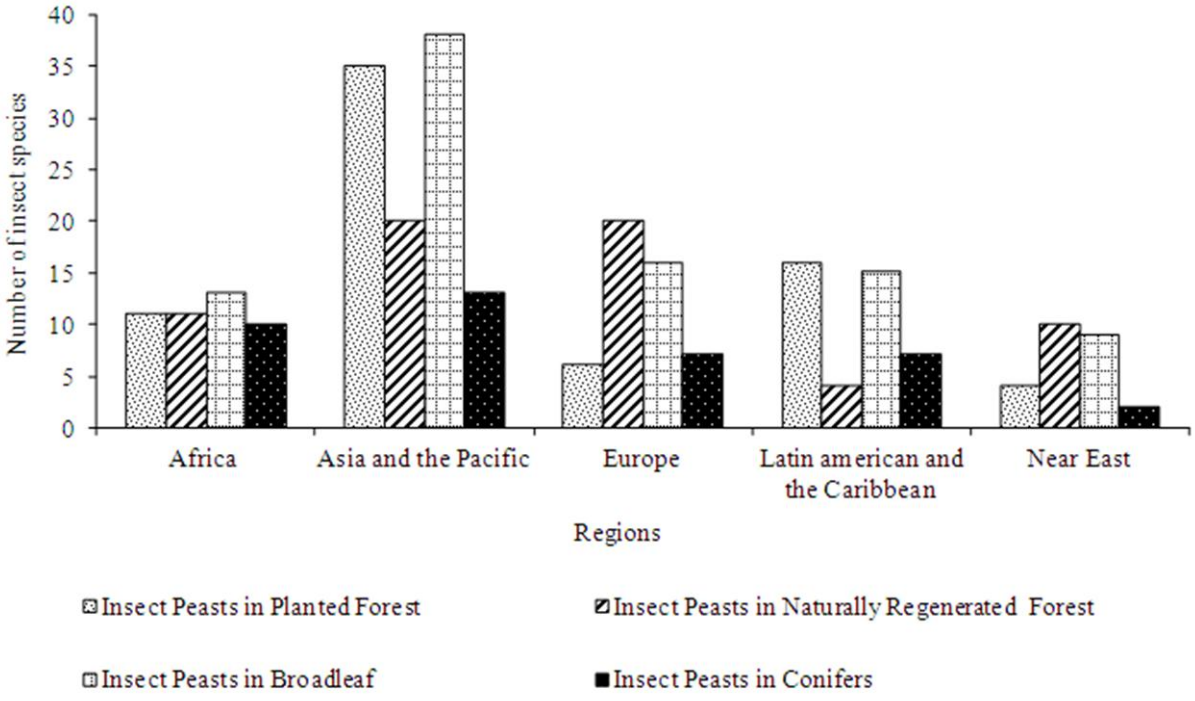

Figure 3. The distribution of Lepidopteran insect pests on different forest types (included countries in the region: seven countries from Africa, five countries from Asia and the Pacific, three countries from Europe, eight countries from Latin America and the Caribean and two countries from Near East). Modified from FAO, 2009.

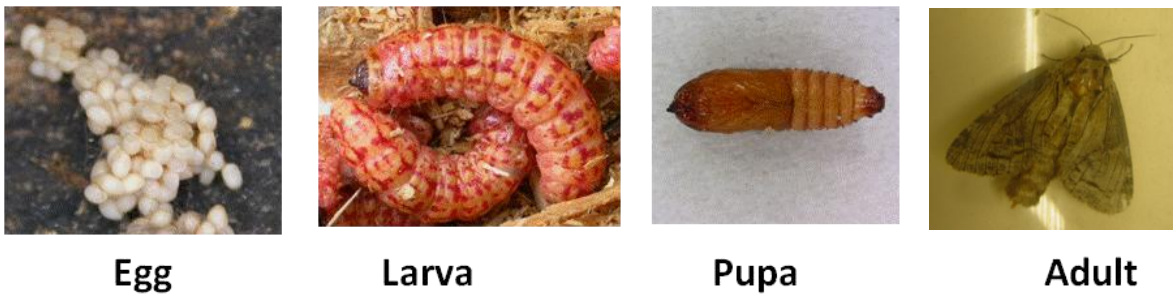

Figure 4. Life stages of *Coryphodema tristis* (Lepidoptera: Cossidae).

Figure 5. *Coryphodema tristis* early infestation and later forming tunnels inside the wood of *E. nitens*.

Figure 6. Feeding of *Coryphodema tristis* larvae produce sap flow on the bark of *Eucalyptus nitens*.

Figure 7. Larvae of *Coryphodema tristis* emergence hole extends deep into the sapwood and heartwood of *Eucalyptus nitens*.

Figure 8. Frass and sawdust are characteristic symptoms of *Coryphodema tristis* on *Eucalyptus nitens*.

Figure 9. Symptoms of old and heavily infested stands of *Eucalyptus nitens* by *Coryphodema tristis*.

Figure 10. *Coryphodema tristis* cause stains and decay inside *Eucalyptus nitens* wood.

CHAPTER 2

Parallel host range expansion in two unrelated cossid moths infesting *Eucalyptus nitens* on two continents

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Degefu D.T., Hurley B.P., Garnas J., Wingfield M.J., Ahumada R. & Slippers B. (2013). Parallel host range expansion in two unrelated cossid moths infesting *Eucalyptus nitens* on two continents. *Ecological Entomology*, 38, 112-116.

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ABSTRACT

Two cossid moths, *Coryphodema tristis* and *Chilecomadia valdiviana*, have recently become pests on *Eucalyptus nitens* in South Africa and Chile, respectively. Both *C. tristis* and *C. valdiviana* have large host ranges and high levels of similarity in their host distributions. Their infestations of *E. nitens* are the first records of these moths on Myrtaceae. The contemporaneous adoption of *E. nitens* as a novel host, despite widespread availability of native and introduced Myrtaceae, suggests a non-random pattern of invasion. Phylogenetic relatedness among the two species linked to cryptic invasion of one or both moths at some time in the recent past provides a possible explanation for this pattern. To test this hypothesis, variation in mtDNA sequences for the COI gene of *C. tristis* and *C. valdiviana* were analyzed. The COI mtDNA sequence data showed that *C. tristis* and *C. valdiviana* are highly divergent genetically, indicating that both are native on their respective continents with independent evolutionary trajectories. The parallel host range expansions to *E. nitens* on different continents appear to be unrelated events, likely driven by characteristics of the biology and / or ecology of the host.

Key words: *Coryphodema tristis*, *Chilecomadia valdiviana*, COI gene, host association

INTRODUCTION

Wood-boring moths in the family Cossidae are widespread, diverse, and are commonly cryptic and understudied. The family comprises of at least 110 genera and approximately 700 species (Davis *et al*. 2008). Members of the Cossidae are often of serious concern to forestry and horticulture due to their aggressive, often gregarious wood-boring behaviour, and association with a wide range of trees and shrubs (Nair 2001; FAO 2009). Approximately 20 % of the species are known to be polyphagous, with host ranges spanning up to seventeen families for a single species (Powell 1980). There is widespread speculation that even those species recorded from only one or a few hosts are also likely to be generalists (Powell 1980; Powell *et al*. 1999).

During the course of the past 20 years, two cossid moths, *Coryphodema tristis* and *Chilecomadia valdiviana*, have become an important pest on *Eucalyptus nitens* in South Africa and Chile, where they were recorded in 2004 and 1992 respectively (Cerda 1995; Gebeyehu *et al*. 2005). Both insects are of growing importance to plantation forestry in these two countries because there are few commercial alternatives to *E. nitens* that can be planted in areas prone to low temperatures, including frost and snow.

The roughly contemporaneous colonization of *E. nitens* by *C. tristis* and *C. valdiviana* is intriguing as neither insect had previously been recorded on a myrtaceous host. This is despite the fact that native and introduced members of this family, including *Eucalyptus*, have long been present within their ranges. Recorded hosts include *Acacia*, cherry, apple, quince, loquats, avocado, willow and elm for *C. valdiviana* (see Cerda 1996; Gonzalez 1989; Angulo & Olivares 1991), and pear, apple, quince, loquats, olives, vines, avocado, bush willow, oak, elm and hawthorn for *C. tristis* (Petty 1917; Hoppner 1994). The apparent specificity on *E. nitens* within the Myrtaceae, together with the broad overlap in host range of these two geographically distant

species, has led to speculation that the recent new host association represents a non-random pattern that could help reveal elements of the biology and/or ecology of these species (Janz *et al*. 2001; Weingartner *et al*. 2006).

Several plausible and non-exclusive hypotheses exist that might help to explain the two independent shifts of related cossid moths to *E. nitens* in the Southern Hemisphere. First, overlapping abiotic requirements in the two cossid species could result in preferential exposure to *E. nitens*, which is planted under roughly similar ecological conditions on the two continents. Further, long term exposure to a similar suite of cultivated hosts (eg. apple, quince, loquats, avocado, bush willow, and elm (Pettey 1917; Gonzalez 1989; Angulo & Olivares 1991; Hoppner 1994) could lead to convergent evolution and non-random overlap in the preference for or suitability of novel hosts (Mardulyn *et al*. 1997). Finally, phylogenetic relatedness among the two species linked to cryptic invasion of one or both moths at some time in the recent past could drive overlap in the responses of herbivorous insects to host plant cues and/or defenses (Janz & Nylin 1998; Heidel-Fischer *et al*. 2009).

In this study we report on relatedness between *C. tristis* and *C. valdiviana* using sequence similarity in the cytochrome oxidase subunit I mitochondrial DNA (COI), placing them in a phylogenetic context within the Cossidae using available data from NCBI. Membership in the same or closely related clade could in part explain the curious similarity in host use and shed light on the hypothesis of a more recent common origin. This study also reports the first published molecular identification of *C. tristis* and *C. valdiviana*.

MATERIALS AND METHODS

Sample collection and molecular procedures

One hundred and thirty two specimens of *Coryphodema tristis* and *Chilecomadia valdiviana* were collected from South Africa and Chile (Table 1). The total genomic DNA was extracted from the thorax tissues of the larvae using the protocol described by Goodwin *et al*. (1992). PCR amplification was optimized using 1µl of total genomic DNA template (50-100 ng/µl) for all samples. PCRs were run in 25 µl reactions containing 10x PCR buffer, 3 mM $MgCl_2$, 1 mM dNTP's, 1.0 U of Taq polymerase (Roche Applied Science, Mannheim, Germany), 16.75 µl of Sabax water and 0.4 µM of each primer set of LCO1490 and HCO2198 (Folmer *et al*. 1994), and C1-J-2183 (Jerry) and TL2-N-3014 (Pat) (Simon *et al*. 1994). Sequencing was performed bidirectionally using the ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems) to produce an overlap of 657 bp for the LCO1490 and HCO2198 primer combination and 743 bp for the C1-J-2183 (Jerry) and TL2-N-3014 (Pat) primer combination of the COI gene region for all samples.

Molecular data analyses

Sequence data for the forward and reverse strands were edited manually using CLC bio workbench v. 6 (www.clcbio.com) and aligned with MAFFT v. 6 (Katoh *et al*. 2002). Pairwise nucleotide diversity was calculated using combined COI sequence data in MEGA 5.0 (Tamura *et al*. 2011). For phylogenetic analysis, sequence data generated from the primer set of LCO1490 and HCO2198 were combined with 11 sequences for Cossidae obtained from GenBank. For phylogenetic comparison a Bayesian analysis was performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Selection of a nucleotide substitution model was done based on the Akaike information criterion (Akaike 1973) using the program jModel-Test (Guindon & Gascuel 2003). Nodal support was assessed from two independent runs each with four chains of 1 000 000

generations in the Markov chain Monte Carlo procedure (the first 500 000 generations were discarded as ‘‘burn-in’’).

RESULTS AND DISCUSSION

The sequence data of the COI mtDNA revealed the presence of 5 haplotypes of *C. valdiviana*, V1 – V5, and two unique haplotypes of *C. tristis*, E1 and E2, on *E. nitens*. The other two haplotypes, G1 and G2, were identified from *C. tristis* found associated with *V. vinifera*. Sequence divergence between *C. tristis* and *C. valdiviana* was high, ranging from 16.3 - 17.7 % using combined COI data (Table 2). Such levels of molecular dissimilarity strongly suggest that the two cossid species have been separated evolutionarily for considerable time (Sobti *et al*. 2007). We therefore reject the hypothesis of parallel cryptic invasion, or that host use overlap arises from close phylogenetic relatedness. A total of five COI haplotypes present in ten individuals of *C. valdiviana* collected from a single *E. nitens* site showed sequence divergence ranging between 0.1 - 3.6 % (Table 2). This diversity was partitioned into two well-supported clades (Fig 1). Sequence divergence within *C. tristis* on *E. nitens* was considerably lower (0.0 - 0.1 %). Combined divergence values for *C. tristis* was marginally higher (0 - 1.9 %) when including the samples from grapevine (*Vitis vinifera*; Figure 1, Table 2), despite geographic separation of ~1400 km between populations.

Placing *C. tristis* and *C. valdiviana* within a broader systematic context proved difficult based on mtDNA sequence data. Very little mtDNA sequence data are available on GenBank for the Cossidae - only 30 sequences from nine species were present on GenBank as of April 2012 (Mutanen *et al*. 2010). Based on the COI data, *C. tristis* and *C. valdiviana* formed distinct clades, with the latter falling outside the Cossinae (Figure 1), which contradicts the species' current

designation (see Edwards *et al.* 1999). In contrast, the clade including *C. tristis*, which has no subfamily designation, includes only members of the Zeuzerinae (Figure 1). Thus, either the subfamily is polyphyletic or *C. valdiviana* is not a true member of this group. While our sampling across the Cossidae was minimal and limits inference regarding evolutionary relationships, the family is clearly in need of taxonomic revision.

Genetic diversity for the *C. valdiviana* samples was greater than expected. The sequence divergence ranging from 0.1 - 3.6 % were surprising given the limited spatiotemporal scope of our sampling, and the fact that the insect has become established on a novel, introduced host on which it has likely only been present for a little over 20 years. This diversity was partitioned into two well-supported clades, which suggests that colonization of *E. nitens* by *C. valdiviana* occurred at least twice, and may in fact occur as regular spillover from various source populations. Low sequence divergence within *C. tristis* on *E. nitens* is more congruous with one or a few recent colonization events.

As the areas of commercial cultivation of *Eucalyptus* and other plantation tree species are expanding, these non-native trees are exposed to a broader range of native insect and pathogen species with the potential to colonize them (Paine *et al*. 2011). Thus, understanding patterns and mechanisms driving host range expansion of native insects is of critical concern. Insect pests and pathogens typically interact intimately with the environment and their hosts. In the case of *C. tristis* and *C. valdiviana* on *Eucalyptus*, we hypothesized that parallel host use was driven by specific traits of the insects in question. Specifically, we tested the idea that a high degree of relatedness might be driving similarities in host use, namely the recent shift to *E. nitens*. We reject this hypothesis based on conclusive evidence that the two insect species share only a

distant evolutionary relationship. Several alternative explanations remain. Host use overlap may simply stem from shared ecological requirements between *C. tristis* and *C. valdiviana*, may reflect convergent suite of traits in artificially selected, cultivated potential hosts, or may stem from the increasing homogenization of potential food sources linked to changing land use patterns. While more work will need to be done to further elucidate patterns of host use in cossids, the current case represents an interesting example where molecular tools aid in the understanding of evolutionary relationships with a bearing on crops of economic importance.

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TABLES

Table 1. Samples of *Coryphodema tristis* collected from *E. nitens* and *V. vinifera* in South Africa and *Chilecomadia valdiviana* from *E. nitens* in Chile.

No.	Sample site	Latitude	Longitude	Elevation (m)	Sample size	Host plant
1	Rooihoogte1	S26.05655	E30.32772	1645	10	*E. nitens*
2	Rooihoogte2	S26.06038	E30.32549	1691	10	*E. nitens*
3	Bonnie Braes	S26.16803	E30.74489	1701	10	*E. nitens*
4	Isabelladale	S26.21773	E30.63133	1718	10	*E. nitens*
5	Lothair	S26.31445	E30.62239	1666	10	*E. nitens*
6	Meadowland	S26.28522	E30.69000	1552	10	*E. nitens*
7	Elandsport1	S26.11923	E30.74505	1552	10	*E. nitens*
8	Elandsport2	S26.11616	E30.74162	1529	10	*E. nitens*
9	Ndubazi	S25.55425	E30.29552	1592	10	*E. nitens*
10	Helvetia	S25.56159	E30.29077	1622	10	*E. nitens*
11	Bambi Hotel	S25.51008	E30.37118	1677	10	*E. nitens*
12	Vredandal	S31.66435	E18.50594	-	12	*V. vinifera*
13	Chile	-	-	-	10	*E. nitens*

Table. 2. Nucleotide divergence in pairwise comparisons based on combined COI mtDNA sequence data of *Coryphodema tristis* and *Chilecomadia valdiviana*.

Haplotype Name	*Coryphodema tristis*				*Chilecomadia valdiviana*				
	Eucalyptus		Grapevine		*Eucalyptus*				
	E1	E2	G1	G2	V1	V2	V3	V4	V5
E1	0.000								
E2	0.001	0.000							
G1	0.019	0.019	0.000						
G2	0.018	0.017	0.001	0.000					
V1	0.165	0.164	0.167	0.166	0.000				
V2	0.164	0.163	0.166	0.165	0.001	0.000			
V3	0.165	0.164	0.167	0.166	0.001	0.001	0.000		
V4	0.177	0.176	0.175	0.174	0.034	0.035	0.036	0.000	
V5	0.176	0.176	0.174	0.174	0.033	0.034	0.035	0.001	0.000

FIGURE

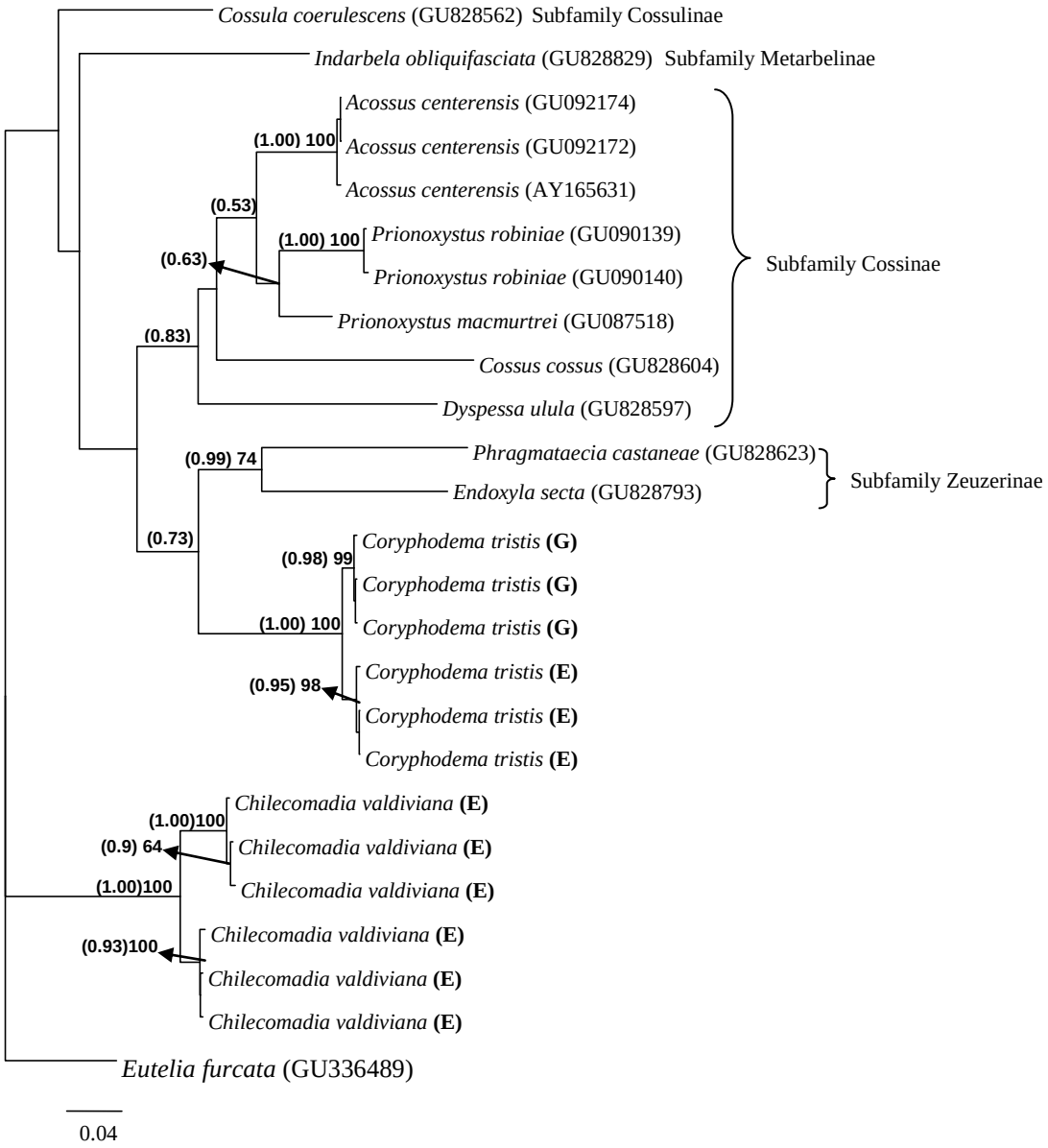

Figure 1. Bayesian phylogenetic comparison of *Coryphodema tristis* and *Chilecomadia valdiviana* from this study, and other Cossidae represented in GenBank, produced from nucleotide sequences from 657 bp mtDNA COI produced with the primer set of LCO1490 and HCO2198. Bayesian posterior probabilities are shown in brackets and bootstrap values outside brackets at the nodes. *Eutelia furcata* was used as an outgroup. Family designations are from Edwards *et al*. (1999). For *Chilecomadia valdiviana* and *Coryphodema tristis*, the letter "E" and "G" represents their host plants *E. nitens* and *V. vinifera*, respectively.

CHAPTER 3

Population genetics and origin of *Coryphodema tristis* recently colonizing non-native *Eucalyptus nitens* in South Africa

ABSTRACT

Emerging pests resulting from new associations between native pests and non-native, commercially propagated trees represent a growing threat to plantation-based industries. Knowledge of the population genetics of such pests provides opportunities to better understand the patterns and processes linked to host use and range expansion. We consider the source and extent of the *Coryphodema tristis* (Cossidae: Coleoptera) infestation of *Eucalyptus nitens* using mitochondrial cytochrome oxidase I (COI) sequence and Amplified Fragment Length Polymorphism (AFLP) data. Individuals of *C. tristis* collected from *E. nitens* plantations in Mpumalanga and *Vitis vinifera* in the Western Cape were used in these analyses together with museum specimens (COI only) from five provinces in South Africa. Fourteen COI haplotypes were recovered from the 135 sequenced individuals. Two haplotypes were recovered from contemporary *E. nitens* populations. Both these haplotypes showed high sequence similarity (1 and 2 bp difference) to a dominant haplotype collected throughout South Africa, including museum specimens from the same Highveld region collected in 1975 and 1986. AFLP analyses revealed minimal genetic diversity on *E. nitens* with very little structure between seven sampled sub-populations spanning 90 km. The populations from *V. vinifera* was clearly distinct from that on *E. nitens*, based on AFLP markers and COI sequence data and could represent a distinct species of the moth.

Key words: *Eucalyptus nitens*, population genetic differentiation, dried preserved specimens, host shift, *Vitis vinifera*

INTRODUCTION

Understanding the origin of emerging pests of commercial plantation trees represents an important step towards predicting future colonization and the factors that might influence this process. One source of emerging pests is where native insects have expanded their host range to infest non-native plantation-grown trees (Paine *et al*. 2011). In South Africa, the native cossid moth *Coryphodema tristis* (Lepidoptera: Cossidae) represents an example of such an emerging pest of plantation-grown non-native *Eucalyptus nitens* (Gebeyehu *et al*. 2005; Degefu *et al*. 2013). *Coryphodema tristis* has been reported from numerous native hosts, but its origin is unknown. It is also not known whether there have been single or multiple, independent new host associations on *E. nitens.*

Coryphodema tristis was first described by Drury in 1872 from specimens originating in the Cape Province of South Africa (Petty 1917). The insect has been described from a number of native hosts in the country and is not known to occur elsewhere in the world. The first record of *C. tristis* causing damage to crops in South Africa dates back to 1917 when it was found in apple and pear orchards as well as in vineyards (Petty 1917). Interestingly, six decades passed before this pest was reported again, causing serious damage to vineyards in the Cape Province (De Klerk 1977). The insect has also been reported from several other native and exotic trees, including species in the families Ulmaceae, Vitaceae, Rosaceae, Scrophulariaceae, Myoporaceae, Malvaceae and Combretaceae (Petty 1917; Meyer 1965; Hoppner 1991). The recent report of *C. tristis* on *Eucalyptus* is the first time that it has been associated with any host in the Myrtaceae (Gebeyehu *et al*. 2005). Interestingly, despite its wide host range and the fact that other *Eucalyptus* species are commonly found in the same area where it is infesting *E. nitens*, *C. tristis* appears to be restricted to this host.

Cold tolerant *Eucalyptus* such as *E. fastigata*, *E. macarthurii*, *E. elata* and *E. nitens* have been widely planted in the high altitude areas of the Mpumalanga and Gauteng provinces of South Africa since the early 20th century (Poynton 1979). These cold tolerant *Eucalyptus* spp. are commonly planted on high altitude sites for the production of mining timber. However, over the last 20 years this production has been increasingly focused on supplying material for pulp and paper production. This has resulted in a change in species to those with shorter rotations and that are more suitable for pulp and paper production (Swain & Gardner 2000; Dyer 2007). Stands of *E. nitens* have expanded substantially in recent years due to its snow and frost tolerance and its desirable pulping properties, with approximately 25 000 ha now planted in the Highveld region of Mpumalanga (Boreham 2006).

Damage caused by the cossid moth on *E. nitens* was first noticed in 2004, and this appeared to represent a new host association (Gebeyehu *et al*. 2005). Subsequent to its first appearance on *E. nitens* close to the town of Lothair (Mpumalanga), *C. tristis* has spread to all other plantations in the region where *E. nitens* is planted. Interestingly, this is despite the fact that some of these plantations are isolated and separated by large areas of *Pinus*. This suggests a highly developed host finding ability, as well as specificity for *E. nitens*.

Very little is known regarding inter- and intra-specific genetic diversity of the Cossidae. A recent study has, however, compared *C. tristis* with other Cossidae for which molecular data were available (Degefu *et al*. 2013). That study included *Chilecomadia valdiviana* from Chile, which has interestingly acquired a similar new host association with *E. nitens* (Lanfranco & Dungey 2001). Degefu *et al*. (2013) showed that the two cossid moth genera are highly distinct and likely

belong to different sub-families, confirming that the novel host associations represent independent events.

Nothing is known regarding the genetic diversity of the populations of *C. tristis* in South Africa. Such information could aid in detecting the source populations of *C. tristis* on *E. nitens*. In this regard, the infestation of *C. tristis* on *E. nitens* provides an ideal opportunity to test different hypotheses related to the processes by which *C. tristis* might have switched to non-native *E. nitens* trees from its base of native hosts.

The objective of this study was to investigate the historical and contemporary genetic diversity of *C. tristis* in order to better understand the source of the recent infestation on *E. nitens*. For this purpose we used mitochondrial cytochrome oxidase I (COI) sequence data and Amplified Fragment Length Polymorphism (AFLP) markers to characterize and compare the populations of *C. tristis* from different regions, hosts and times (for COI sequence data) in South Africa. We specifically considered three hypotheses, namely i) one genotype of the moth, has infested both the non-native *E. nitens* and *V. vinifera*, and consequently *E. nitens* and *V. vinifera* would share diversity with each other; ii) spillover or host range expansion of *C. tristis* on *E. nitens* originates from a substantial sub-set of the native diversity of the insect; and iii) there has been a rare expansion event that has most likely isolated a sub-set of the natural diversity of *C. tristis* (Figure 1).

MATERIALS AND METHODS

Sample collection

Coryphodema tristis larvae were collected from seven plantations in the Mpumalanga province (Figure 2). In each plantation, the larvae were collected from two infested *E. nitens* trees. Two infestation sites on each tree were selected, one towards the bottom of the tree and the other towards the top of the tree, with at least 2 - 3 meters between the infestation sites. For each infestation site, two 30 cm sections from the main stem (herein, bolts) were cut and two larvae were collected per bolt. In total, 108 *C. tristis* larvae from *E. nitens* were used in this study. In addition, 15 museum specimens were obtained from the Ditsong National Museum of Natural History, Pretoria, South Africa. Specimens were selected to represent collections from different provinces and from a range of collection dates (Table 1). Furthermore, 12 larvae were collected from infested *Vitis vinifera* (herein, grapevine) growing in Vredendal, Western Cape Province. For comparative purposes, 11 larvae of *C. valdiviana* were obtained from *E. nitens* in Chile, South America.

Cytochrome oxidase I sequence characterization

DNA was extracted from larval thoracic segments after the fat bodies had been removed and after washing with absolute ethanol and rinsing with sterile distilled water. Total genomic DNA was extracted from the thoracic tissues of caterpillars using the protocol described by Goodwin *et al*. (1992), after being freeze-dried and ground to a fine powder.

The universal primer combinations C1-J-2183 (Jerry) 5’-CAACATTTATTTTGATTTTTTGG-3’ forward and TL2-J-3014 (Pat) 5’-CCAATGCACTAATCTGCCATATTA-3’ reverse (Simon *et al*. 1994) were used to amplify a 810 bp segment of the COI sequence data. PCR amplification

was optimized using 1 µl of total genomic DNA as template (50-100 ng/µl) for fresh freeze-dried samples. PCRs were run in 25 µl reactions and included in 10x PCR buffer, 3 mM $MgCl_2$, 1 mM dNTP's, 0.4 µM of each primer, 1.0 U of *Taq* polymerase and 16 µl Sabax water. The thermal cycling conditions for fresh samples were as follows: 5 min at 96 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C and a final extension 10 min at 72 °C. PCR products were visualized on a 2 % agarose gel.

Cleaned PCR products were subjected to a sequencing PCR in 12 µl reactions. These included 3 µl cleaned PCR product, 2 µl BigDye Terminator Sequencing kit, 0.4 µM of primers and 5x sequencing buffer, with 25 cycles at 95 °C for 10s, at 48 °C for 5s, at 60 °C for 4 minutes. Sephadex-cleaned and vacuum-dried PCR products were sequenced using the ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems). Sequencing was performed bidirectionally to produce an overlap of 703 bp of the COI region. The sequenced fragments were assembled using CLC Bio Workbench version 6 (http://www.clcbio.com).

For the museum samples, one leg per pinned dry specimen was used for DNA extraction. This specimen was macerated within a PCR tube using a flame-solidified pipette tip and DNA was extracted using *prep*GEM™ Insect following the manufacturer's instructions (ZyGEM Corp Ltd, New Zealand).

Primers for the amplification of DNA from museum specimens were designed based on an alignment of sequences obtained from *C. tristis* larvae from *Eucalyptus* using CLC Bio Workbench version 6 (http://www.clcbio.com). Highly conserved regions of the sequences were used to design candidate primers to amplify shorter fragment more easily recoverable in older specimens. One set of outer and two sets of internal primers were designed, resulting in

overlapping fragments to span 702 bp of the COI gene (Figure 3). The first segment was amplified using a primer combination of CTF-5 5'-TCTCCCATATTATCTCCC-3' and CTR-296 5'-TTACTCCTGTTAGTCCTC-3' (length = 390 bp); the second segment with a primer combination of CTF-294 5'-AGGAGGACTAACAGGAGTAA-3' and CTR-573 5'-GAAATGTATGCGTCAGGG-3' (length = 279 bp) and the third segment with a primer combination of CTF-572 5'-ACCCTGACGCATACATTT-3' and CTR-728 5'-CGGGGGGTGTATTTTGAT-3' (length = 176 bp). These primer pairs were optimized on DNA extracted from freshly collected samples of *C. tristis* from *E. nitens* prior to being used to amplify DNA from museum samples.

PCR mixtures used for museum specimens were the same as those for fresh specimens, except that more DNA was added; 2-3 µl (50-100 ng/µl) of DNA template. The thermal cycling conditions were altered to optimize for the shorter amplicon sizes and different primers. For primers CTF-294 and CTR-573, the cycle consisted of 3 min at 96 °C followed by 35 cycles of 30s at 94 °C, 30s at 58 °C and 1.5 min at 72 °C and a final extension 10 min at 72 °C. For primers CTF-5 and CTR-296, and CTF-572 and CTR-728 the cycle consisted of 3 min at 96 °C followed by 35 cycles of 30s at 94 °C, 30s at 55 °C and 1.5 min at 72 °C and a final extension 10 min at 72 °C. PCR products were visualized on a 2 % agarose gel. For the museum specimens, the first PCR product was used as a template for the second PCR following cleaning using high pure PCR product purification kit (Roche diagnostics GmbH, Germany). Sequencing was performed as described for DNA from fresh specimens. The sequences from the three fragments were amplified and each of these fragments was sequenced in both directions from independent PCR reactions. The sequenced fragments were assembled using CLC bio workbench version 6 (http://www.clcbio.com) to span the target size of 702 bp COI region.

Data analysis for COI mtDNA

The COI sequence data for the forward and reverse strands were edited manually using CLC bio workbench version 6 (http://www.clcbio.com) and aligned with MAFFT version 6 (Katoh *et al*. 2002). Median joining networks (Bandelt *et al*. 1999) were calculated to examine intraspecific variation among 135 samples of *C. tristis* species using default settings as implemented in Network 4.1 (Fluxus Technology). Pairwise nucleotide diversity was calculated in MEGA 5.0 (Tamura *et al*. 2011) and using the Kimura two-parameter model of DNA evolution. The robustness of each node was tested by bootstrap analysis with 1000 replications for the Neighbor Joining tree. Analysis of molecular variance (AMOVA) for mtDNA sequence data was calculated using GenAlex v.6 (Peakall & Smouse 2005) to further show the patterns of genetic differentiation among different populations.

Amplified Fragment Length Polymorphism marker characterization

A modified AFLP (Vos *et al*. 1995) protocol was used to study the genetic variability of *C. tristis* populations collected on *E. nitens* and grapevine in Mpumalanga and the Western Cape Province respectively. The AFLP procedures included three basic steps, namely DNA template preparation, DNA template pre-amplification and AFLP-selective amplification. Approximately 150 ng of genomic DNA in 5 µl was incubated for digestion for 3 hours at 37 °C in a total volume of 30 µl which contain 6 µl of 10X R/L Buffer, 0.2 µl of 10 U/µl *Eco*RI enzyme (2u/reaction), 0.2 µl of 10 U/µl *Mse*I enzyme (2 u/reaction) and 18.6 µl nanopure water. After incubation both adaptors *Eco*RI and *Mse*I were ligated by adding 10 µl of ligation master mix containing 2 µl of 5X R/L Buffer, 4 µl of 10 mM ATP (1 mM/reaction), 0.2 µl of pmol/µl *Eco*RI adaptor (1 pmol/reaction), 0.2 µl of 50 pmol/µl *Mse*I adaptor (10 pmol/reaction), 1 µl of 1 blunt U/µl T4 DNA ligase and 2.6 µl nano-pure water to each incubated sample.

The ligation reaction was followed by pre-amplification of 5 µl DNA fragments using primers *Eco*RI and *Mse*I with no base pair extension and each containing 0.9 µl of 10 µM/µl (0.3 µM/reaction), 3 µl of 10X PCR buffer (+1.5 mM Mg) (1 X/reaction), 2.4 µl of 2.5 mM of each dNTPs (0.2 mM/reaction), 1 µl of 5 U/µl Taq polymerase (0.6 U/reaction) and 16.8 µl nanopure water to make up 30 µl total volume. PCR amplifications were then carried out using one cycle of 30 s at 72 °C, 25 cycles of 30 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C (increasing the annealing temperature by 1 s) and then one cycle of 2 min at 72 °C. The success of pre-amplification was verified using agarose gel electrophoresis of 5 µl of pre-amplification product. The remaining pre-amplification product was diluted 1:100 with nanopure water for use in the next step of selective amplification.

After screening 10 primer combinations, six were chosen for selective amplification. A reaction volume of 20 µl containing 2 µl of 10X PCR buffer (+1.5 mM Mg) (1 X/reaction), 0.4 µl of 25 mM $MgCl_2$ (0.5 mM/reaction), 1.6 µl of 2.5 mM of each dNTPs (0.2 mM/reaction), 0.8 µl of 1.0 µM IRD-700 labeled *Eco*RI primer (0.04 µM/reaction), 0.5 µl of 10 µM *Mse*I (0.25 µM/reaction), 1 µl of 5 U/µl Taq polymerase (1.2 U/reaction) and 8.7 µl nanopure water was prepared. The selective amplification was carried out using the following cycling parameters: 13 cycles of 10 s at 94 °C, 30 s at 65 °C in which the annealing temperature decreased by 0.7 s/cycle, 1 min at 72 °C, followed by 23 cycles of 10 s at 94 °C, 30 s at 56 °C, 1 min at 72 °C in which the annealing temperature increased by 1 s/cycle, with a final cycle of 1 min at 72 °C after which the reaction was maintained at 4 °C.

After selective amplification, the reaction was stopped prior to loading the selective amplification product on a polyacrylamide gel by adding 10 µl blue stop solution (LI-COR) to each reaction tube. Samples were denatured at 90 °C for 3 min and placed on ice immediately

before loading the polyacrylamide gel for electrophoresis. One µl of each of the 50 samples was run per gel with a IRD-labeled 50-700 bp size marker (LI-COR) run in two lanes as controls. The 50th sample was loaded twice on each gel to test repeatability. The AFLP products were separated in 8 % denaturing polyacrylamide gels visualized in a Li-Cor Gene Read IR 4200 DNA sequencer (LI-COR, Inc.) The electrophoresis conditions were 1500 volts, 40 watts, 40 mA of current, at 45 °C for 2.5 h.

Data analysis for AFLP markers

Popgene v. 1.32 (Yeh & Yang 1997) was used to assess the degree of polymorphism both within and between populations of *C. tristis* from the AFLP data. Nei's genetic diversity (h; Nei 1987) for dominant data was calculated such that $h = 1 - \sum x_i^2$, where x_i is the population frequency of each allele at locus i and adjusted for variation in sample sizes by multiplication with 2n/(2n-1), where n represents the sample size. G_{st} (Nei 1973) was calculated as an estimate of population differentiation and values were used to estimate gene flow using the formula $N_m = 0.5 \times (1-G_{st}) /G_{st}$ (McDermott & McDonald 1993).

Relationships between populations were determined using distance metrics that summarize the amount of genetic divergence between pairs of populations. Nei's genetic identity (S) and genetic distance (D; Nei 1978) were estimated in Popgene, such that $S = J_{xy}/(J_{xx} \times J_{yy})^{0.5}$ and D is the negative natural logarithm of S and where J_{xy} is the arithmetic mean of the probabilities that an allele drawn from populations x and y are identical and J_{xx} and J_{yy} are the arithmetic means of the probabilities that two random alleles are identical. Genetic distances were used to visualize the relationships between populations using the unweighted pair group method with arithmetic analysis to construct a dendrogram (Tamura *et al*. 2011).

Analysis of molecular variance (AMOVA) for AFLP fingerprint data was conducted. Principal coordinate analysis (PCA) was calculated to examine the genetic isolation between sampled populations visually. A Mantel test (Mantel 1967) was also used to consider the correlation between genetic and geographic distances among populations based on 1000 permutations. All analyses were performed using GenAlex v.6 (Peakall & Smouse 2005).

RESULTS

Cytochrome Oxidase I sequence analysis

A 702 bp fragment was successfully amplified from 108 larvae from *E. nitens* and 12 from *V. vinifera*. Three fragments of 390, 279 and 176 bp, which overlapped with the segment amplified for the freshly collected larva, were also successfully amplified for each of 15 museum samples. Ultimately, a corresponding segment of 702 bp of COI mtDNA gene region was compared for all 135 *C. tristis* samples. From these sequences, 14 different haplotypes were identified. The median joining phylogenetic network connected 13 of these haplotypes differing by between 1 and 25 base pairs (Figure 4). One of these haplotypes (haplotype A) was shared among populations from the Western Cape, KwaZulu-Natal, Mpumalanga, and Gauteng provinces, while all the other haplotypes were restricted to a single province. Haplotype N differed by 14-15 base pairs and was not directly connected to the other haplotypes on *E. nitens*. This haplotype was found only in samples from the Limpopo province. Haplotypes L and M were from the *C. tristis* sampled from *V. vinifera* in Vredendal, Western Cape. These haplotypes were clearly distinct in the network, with 10 bp connecting them to the closest haplotype from samples collected from other hosts.

The divergence between haplotypes ranged from 0.14 % to 3.56 %. Haplotypes from specimens recently collected from *E. nitens* (haplotypes E and F) in Mpumalanga differed by 1 and 2 base pairs, respectively, from the dominant haplotype (haplotype A) collected from the same province from unknown hosts in 1975 and 1986 (Figure 4).

Neighbour-joining analysis of the COI mtDNA haplotypes using the Kimura Two Parameters Distance Model (Figure 5) confirmed the differentiation of haplotypes L and M from other

haplotypes, with a bootstrap support value of 99 %. Furthermore, the Limpopo haplotype N also grouped as distinct from all other haplotypes. Haplotypes E and F, from the recently collected populations on *E. nitens,* grouped most closely with the museum specimens obtained from the Cape, KwaZulu-Natal, Mpumalanga and Gauteng provinces and from host trees such as oak, grapevine and other unknown sources, with a bootstrap support value of 82 %.

AMOVA performed on the COI sequence data showed that the within-population heterogeneity among the individuals of *C. tristis* accounted for 92 % of the variation. This was significantly larger than the remaining 8 % accounted for by between-population heterogeneity (Table 2). The genetic divergence F_{st} value was 0.083, indicating the absence of spatial genetic structure. This low level of population differentiation with a corresponding N_m value of 5.51 supports the possibility of a high level gene flow across the range of *C. tristis* populations.

Amplified fragment length polymorphism (AFLP) analysis

In total, 135 polymorphic AFLP fragments were identified using six *Eco*RI and *Mse*I primer combinations (E + ACT/ M + CAG; E + ACA/M + CAG; E + ACT/M + CAC; E + ACA/M + CTC; E + ACA/M + CTG and E + ACT/M + CGA). The number of polymorphic fragments scored varied among the different primer combinations, ranging from 13 - 31, with an average of 22.5 amplified fragments per primer combination. The percentage of polymorphic fragments across the 8 populations ranged from 31.1 % - 58.5 %. The percentage of polymorphic fragments was lower in the Lothair population (31.1 %) than in the Vredendal (57.8 %) and Elandspoort (58.5 %) populations (Table 3).

Genetic diversity across the 8 populations ranged from 0.124 to 0.218, with higher values for the Vredendal (0.2184) and Elandsport (0.2037) populations (Table 3) and the lowest value (0.124)

for the Lothair population. The population differentiation estimate (G_{st}) among all sampled populations was 0.334 with a corresponding gene flow value of 0.995.

The unbiased metrics of genetic identity (S) ranged between 0.97 (D = 0.03) and 0.78 (D = 0.25) for all sampled populations of *C. tristis* from *E. nitens* and *V. vinifera* (Table 4). A genetic identity matrix value of 0.97 was found for the populations collected in Rooihoogte, Meadowland, and Lothair, indicating that they are very similar. The lowest level of genetic identity (78 and 79 %) was found between populations of Ndubazi, Isabelladale and Vredandal (Table 4).

The AMOVA analysis (Table 5) showed that 30 % of the genetic variability was found among the grapevine and *E. nitens* populations. The majority of genetic variability (67 %) was within populations. When the population collected from grapevine was excluded, the AMOVA analysis showed that 96 % genetic variability was within populations of *C. tristis* and only 4 % between different *C. tristis* populations from *Eucalyptus*. This high genetic variability within populations indicates that there is low level of population differentiation, which suggests high gene flow among the populations of *C. tristis* on *E. nitens*.

Populations of *C. tristis* on grapevine and *E. nitens* clearly grouped into two major clusters (Figure 6). Geographically distinct populations from *E. nitens* all clustered together with no pattern of differentiation (Figure 7). There was no significant correlation (Mantel R = 1E-02, P = 0.365) between genetic and geographic distances among the populations collected from *E. nitens* (Figure 8).

The dendrogram drawn using the 135 polymorphic AFLP loci contained two major clades, one from Mpumalanga associated with *E. nitens* and the other from Vredendal associated with

grapevine (Figure 9). The clade represented by *C. tristis* from *E. nitens*, however, had weak (59 %) bootstrap support. The clade representing the population from grapevine in the Western Cape had a strong (100 %) bootstrap support, with one minor and weakly supported (51 % bootstrap) sub-clade.

DISCUSSION

In this study, COI mitochondrial sequence and AFLP markers were used to study the populations of a moth that has recently emerged as a serious new pest of non-native *E. nitens* in South Africa. The emerging data were used to test three possible hypotheses regarding the process that underlies this new host association. We discounted the hypotheses that i) one genotype of the moth, *C. tristis,* which is particularly invasive on new hosts, has infested the two non-native hosts, namely *E. nitens* and *V. vinifera*, and ii) a continuous spillover or host range expansion of *C. tristis* resulted in a substantial sub-set of native diversity of the insect infesting *E. nitens*. The data supported our third hypothesis that there has most likely been a rare expansion event that has isolated a sub-set of the natural diversity of *C. tristis,* which now infests *E. nitens*. This would either have been through adaptation to a new host association or via some other isolation mechanism.

One possible explanation that we tested for the recent emergence of *C. tristis* on *E. nitens* was that one genotype with low host fidelity is spreading on non-native hosts (Wood *et al*. 1999). Host fidelity in the initial stages of a host shift is considered to be a potential facilitating factor that allows divergent selection on host-associated performance traits (Wood *et al*. 1999; Diehl & Bush 1989; Bush 1994). This hypothesis was tested by comparing *C. tristis* populations on *E. nitens* and *V. vinifera*. If a particularly invasive genotype on new hosts had evolved among the populations of *C. tristis* on *E. nitens* or *V. vinifera*, it would be expected that these populations would share diversity. However, both the mitochondrial COI sequence data and the AFLP markers very clearly supported a significant divergence between these populations. This hypothesis could thus not be accepted, at least for the populations considered in this study.

A possible hypothesis to explain why the current infestation of *C. tristis* has spread so rapidly (less than a decade) throughout the *E. nitens* plantations, is that a continuous spillover of the insect from source populations could be the cause of the expansion. In this case, it might be expected that a random subset of the native diversity of *C. tristis* would be found in populations infesting *E. nitens* trees in plantations. Thus, a fairly high level of diversity would be expected in *C. tristis* populations on *E. nitens*, and possibly structured across geographic areas. This would be the typical situation for established native populations (Puillandre *et al*. 2008). There are examples of this situation for organisms other than insects where several discrete colonization events on a new host from several native populations has resulted in a high level of diversity in the newly colonized habitat (Kolbe *et al*. 2004; Genton *et al*. 2005). Similar cases have also been reported for insects, where for example there was no loss of genetic diversity reported in invasive populations of the coffee berry borer *Hypothenemus hamper* in South America (Benavides *et al*. 2005), the mosquito *Aedes albopictus* in Italy and North America (Fonseca *et al*. 2001), or for the mosquito *Aedes japonicas* in North America (Urbanelli *et al*. 2000). Similar trends have been reported by Azeredo-Espin *et al*. (1996) and Johnson & Starks (2004) for high level of diversity for an invasive population of Colorado potato beetle in Texas and an invasive wasp *Polistes dominulus* in the Northeastern United States, respectively. However, this does not appear to be the case for *C. tristis* on *E. nitens*, where a relatively narrow and unstructured amount of genetic diversity was found.

Results of this study support the fact that the emergence of *C. tristis* as a major pest on non-native *E. nitens* has occurred via a rare expansion event. This is commonly referred to as a rare event expansion, arising from single introduction events from a single native population (see Lee 2002). This explanation was supported by the fact that the COI sequence data from populations

of *C. tristis* on *E. nitens* in the Mpumalanga province formed only two mitochondrial haplotypes. Furthermore, a significant loss of genetic variability was detected, as would be expected for a small founder population undergoing a population bottleneck (Eckert *et al*. 2008; Jousson *et al*. 2000). Other insect species that also have narrow ecological requirements and/or poor colonization ability have generally been typified by reduced genetic diversity following range expansion (Stone & Sunnucks 1993; Assmann *et al*. 1994). Hence, the high level of homogeneity between the sub-populations of *C. tristis* in the Mpumalanga province most likely reflects a recent expansion of the insect. The low diversity of *C. tristis* on *E. nitens* could be the result of the recent fixation of a selectively advantageous genotype (Grapputo *et al*. 2005), but to support this view would require further evidence.

The data from this study support the fact that *C. tristis* is native in South Africa as has previously been suggested by Degefu *et al*. (2013). Museum specimens from across South Africa and collected from various hosts all belong to a common clade with less than 2.2 % variation, suggesting that no cryptic forms exist. For example, Sperling & Hickey (1994) found that the intraspecific COI mtDNA variation in the spruce budworm *Choristoneura fumifeana* ranged between 0.1 - 2.9 %, while the variation was 5.2 - 5.6 % sequence divergence with members of the sister taxon *C. fumzjkrana*. Similar ranges were also noted by Roe & Sperling (2007) for several species of various Lepidoptera and this is also true for various other insect groups (Cognato 2006).

The populations of *C. tristis* on *E. nitens* and *V. vinifera* considered in this study are approximately ~1400 km apart and are in very different parts (northeast, summer rainfall and south-west, winter rainfall) of South Africa. Apart from the fact that these populations clearly did

not originate from the same source, little if any gene flow appears to have occurred between them. This is not surprising given their geographical separation by extensive dry areas with very little tree cover. Yet, such barriers have been overcome in the past by other insects via human-assisted movement. Hurley *et al.* (2010) for example showed much less structure between nursery populations of fungus gnats across these regions; albeit clear that transport of soil, root and seedlings associated small insects such as fungus gnats will be much more likely to be moved than more conspicuous insects such as cossid moths. The extent of the divergence between the populations on *E. nitens* and *V. vinifera* (1.9 - 2.3 %), however, reached the upper limits for within species divergence (Zimmermann *et al*. 2000; Wahlberg *et al*. 2003; Cognato 2006; Roe & Sperling 2007). Future work, including additional specimens from a greater variety of hosts would contribute to our understanding of diversity in this poorly studied group of insects, and confirm the possible existence of cryptic species.

Analysis of the AFLP marker data showed that there was little structure in the diversity amongst *C. tristis* populations on *E. nitens*. The vast majority (94 %) of the diversity was contained within populations. This suggests high levels of gene flow between these populations, which expanded to infest the 30 km^2 area (Boreham 2006) where *E. nitens* has been planted for fewer than 8 years. Field observations of *C. tristis* have shown that the females have a limited ability to disperse compared with the males (authors unpublished). This is apparently associated with the fact that the emerging females have larger and heavier bodies, which would limit long distance migration.

Population genetic studies provide a powerful means characterize the differentiation between insect populations associated with different host species (Roderick 1996; Brown *et al*. 1997).

The population genetic comparison of the emerging cossid moth pest on *E. nitens*, compared to museum specimens from other hosts and geographic regions made it possible for us to better understand the source of infestation and level of diversity on *E. nitens* in the Mpumalanga province of South Africa. The relatively narrow range of diversity of *C. tristis* on *E. nitens* is consistent with a founder effect as *C. tristis* colonised *E. nitens*. This underscores the complexity and unpredictability of these processes, because this host and insect have co-occurred for many years before the colonization. It would also suggest that colonization of other *Eucalyptus* species should not be expected to be imminent, but neither should it be discounted. The extensive populations of *C. tristis* in *Eucalyptus* growing areas would increase its chances of further adaptation to *Eucalyptus* as a host.

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TABLES

Table 1. List of *Coryphodema tristis* samples obtained from the Ditsong National Museum of Natural History, Pretoria, South Africa.

Date of Sampling	Province	Location	Host plant
15/ 1992	Western Cape	Cape Town	Grapevine
22/ 1992	Western Cape	Cape Town	Grapevine
09/ 1978	Western Cape	Cape Town	no record
19/ 1967	Western Cape	Worcester	no record
04/ 1966	Western Cape	Cape Town	Grapevine
16/ 1919	KwaZulu Natal	Durban	no record
27/ 1979	KwaZulu Natal	Richards Bay	no record
29/ 1984	KwaZulu Natal	Balgowan	*Quercus palustns* (Oak)
07/ 1984	KwaZulu Natal	Balgowan	*Quercus palustns* (Oak)
28/ 1984	KwaZulu Natal	Balgowan	*Quercus palustns* (Oak)
02/ 1986	Mpumalanga	Nelshoogte	uv light trap
24/ 1975	Mpumalanga	Blyde River	no record
17/ 1975	Gauteng	Bronkhorstspruit	no record
18/ 1958	Gauteng	Bronkhorstspruit	no record
04/ 1981	Limpopo	Mariepskop	no record

Table 2. Analysis of Molecular Variance (AMOVA) for COI mtDNA sequence data of *Coryphodema tristis*.

Source	Df	SS	MS	Est. Var.	%	Nm	Fst
Among Pops	3	5.009	1.670	0.103	8%		
Within Pops	18	20.442	1.136	1.136	92%		
Total	21	25.451		1.239	100%	5.510	0.083

Table 3. Genetic variability of *Coryphodema tristis* populations measured by the observed number of polymorphic fragments and Nei's genetic diversity.

Population	n	% polymorphic fragments	Nei's genetic diversity	Number of Fragments
Rooihoogte	10	43.7%	0.1562	59
Elandsport	9	58.5%	0.2037	79
Ndubazi	4	39.6%	0.1404	53
Meadowland	6	37.8%	0.1562	51
Lothair	5	31.1%	0.1243	42
Bonnie Braes	5	35.6%	0.1435	48
Isabelladale	4	48.9%	0.1894	66
Vredendal	7	57.8%	0.2184	78

Table 4. Nei's unbiased genetic identity (above diagonal) and genetic distance (below diagonal).

Population	P1	P2	P3	P4	P5	P6	P7	P8
Rooihoogte (P1)		0.94	0.84	0.97	0.97	0.95	0.89	0.92
Elandsport (P2)	0.06		0.95	0.92	0.93	0.92	0.87	0.89
Ndubazi (P3)	0.18	0.05		0.83	0.82	0.82	0.78	0.79
Meadowland (P4)	0.03	0.08	0.19		0.97	0.96	0.89	0.91
Lothair (P5)	0.03	0.07	0.20	0.03		0.96	0.87	0.91
Bonnie Braes (P6)	0.05	0.08	0.20	0.04	0.04		0.93	0.92
Isabelladale (P7)	0.12	0.14	0.25	0.11	0.14	0.07		0.95
Vredendal (P8)	0.08	0.12	0.24	0.09	0.10	0.08	0.05	

Table 5. Hierarchical analysis of molecular variance (AMOVA) of *Coryphodema tristis* populations from *Eucalyptus nitens* and grapevine based on AFLP markers.

Source	df	SS	MS	Est. Var.	%
E. nitens & *V. vinifera*					
(From different province)					
Between *E. nitens* vs *V. vinifera*	1	16.423	16.423	1.101	30
Among Pops	6	18.399	3.067	0.107	3
Within Populations	42	101.855	2.425	2.425	67
Total	49	136.677		3.632	100
Only for *E. nitens*					
Among Pops	6	18.399	3.067	0.097	4
Within Pops	36	89.457	2.485	2.485	96
Total	42	107.857		2.582	100

FIGURES

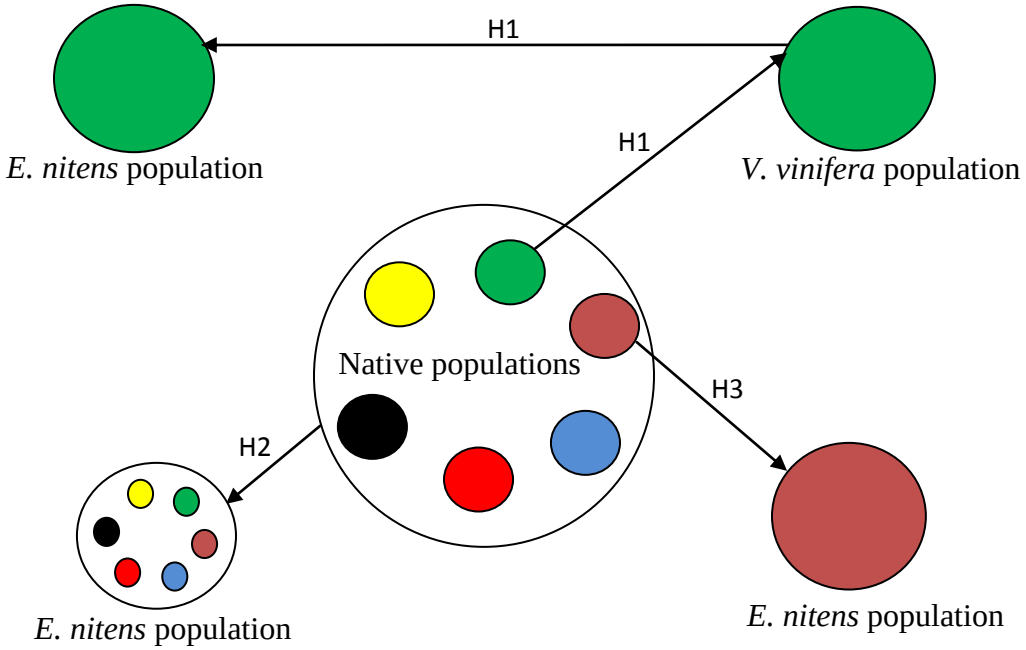

Figure 1. Diagrammatical representation of the three possible hypotheses: Hypothesis 1, *Coryphodema tristis* on *E. nitens* and *V. vinifera* would share diversity with each other and be distinct from native populations; Hypothesis 2, expansion of substantial subset of native diversity of *C. tristis* to *E. nitens*; and Hypothesis 3, Rare event expansion/single introduction events from a single native population.

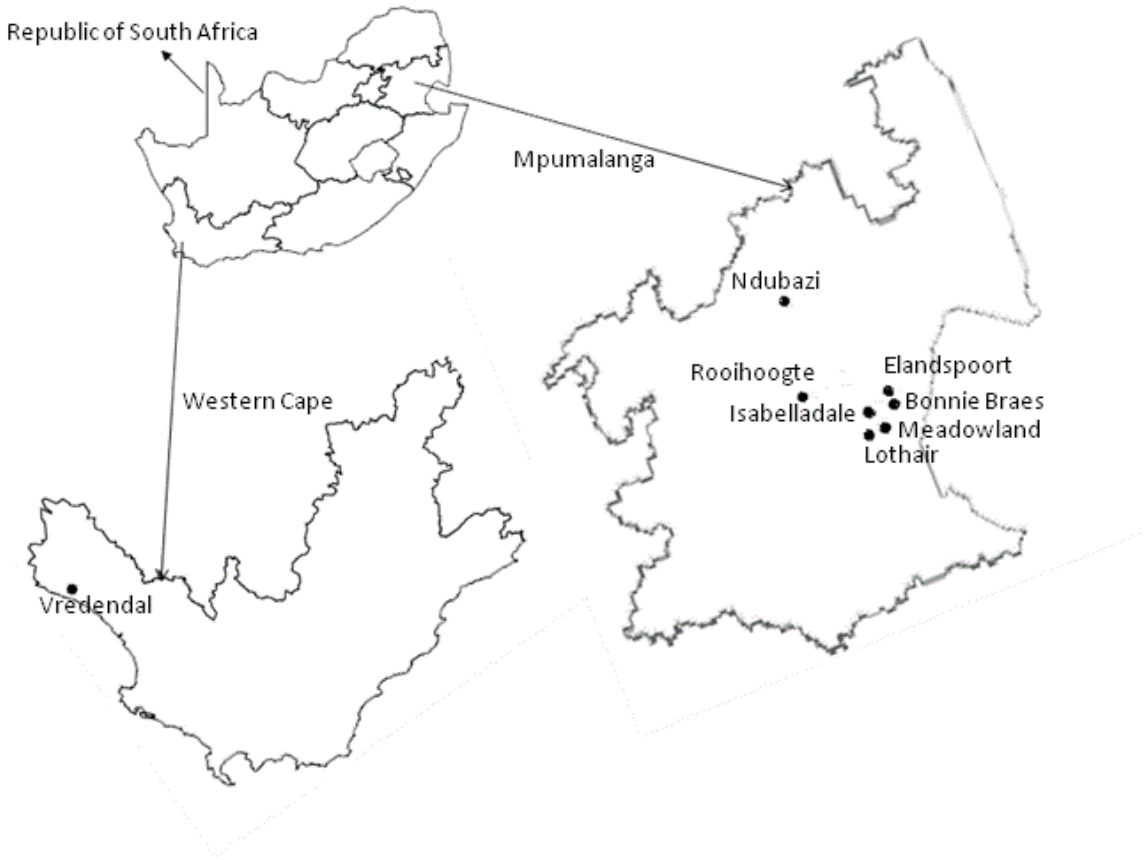

Figure 2. Sample sites of *Coryphodema tristis* from *Eucalyptus nitens* in the Mpumalanga province and *Vitis vinifera* in Western Cape Province.

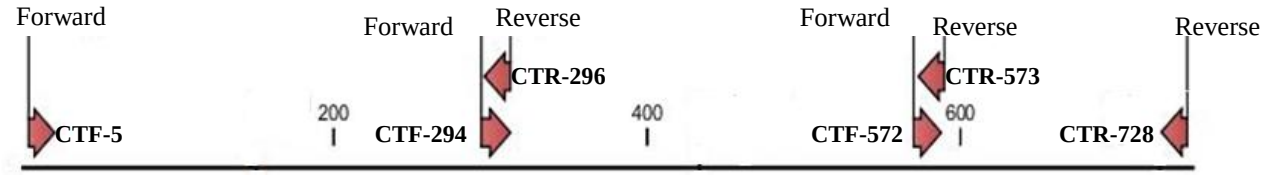

Figure 3. A diagrammatic representation of the COI barcoding region used for *Coryphodema tristis* (Cossidae) with the relative positions of the primers (arrows) indicated for amplifying the samples.

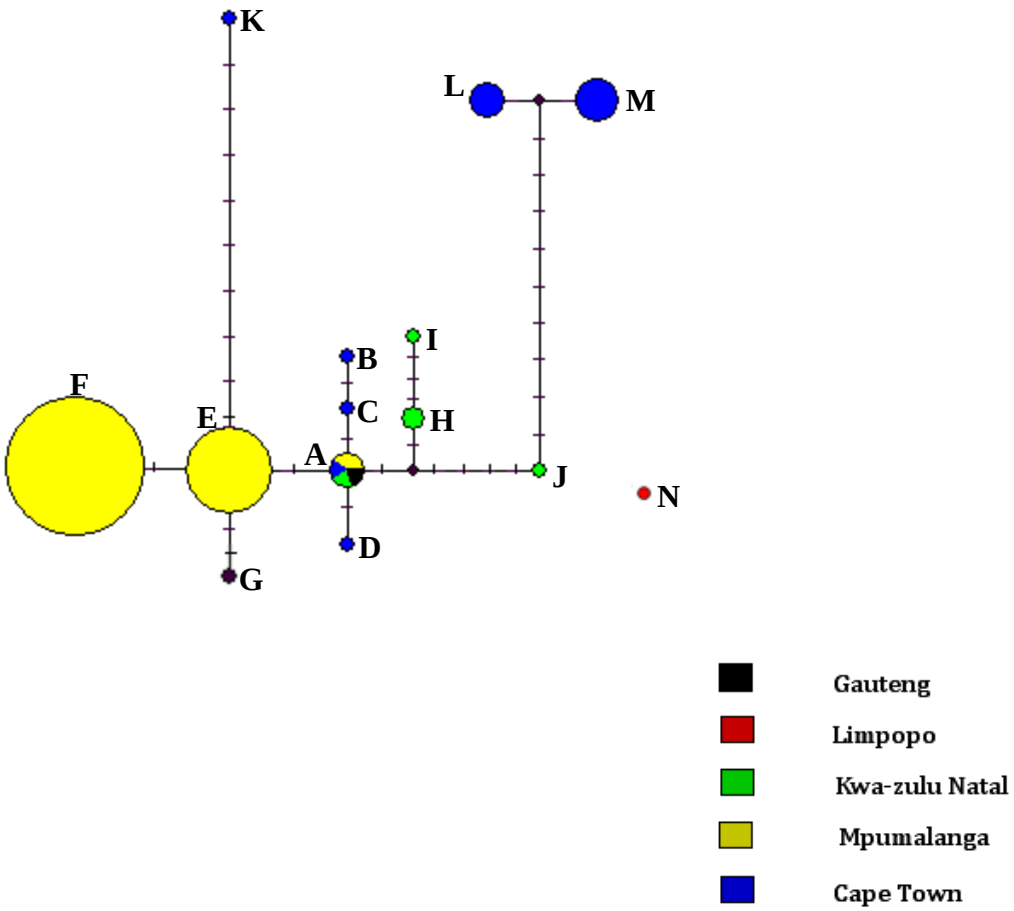

Figure 4. Median-joining network of *Coryphodema tristis* COI haplotypes. Numbers of observed haplotypes indicated by size of circles. The strikes (-) represent number of base pair differences.

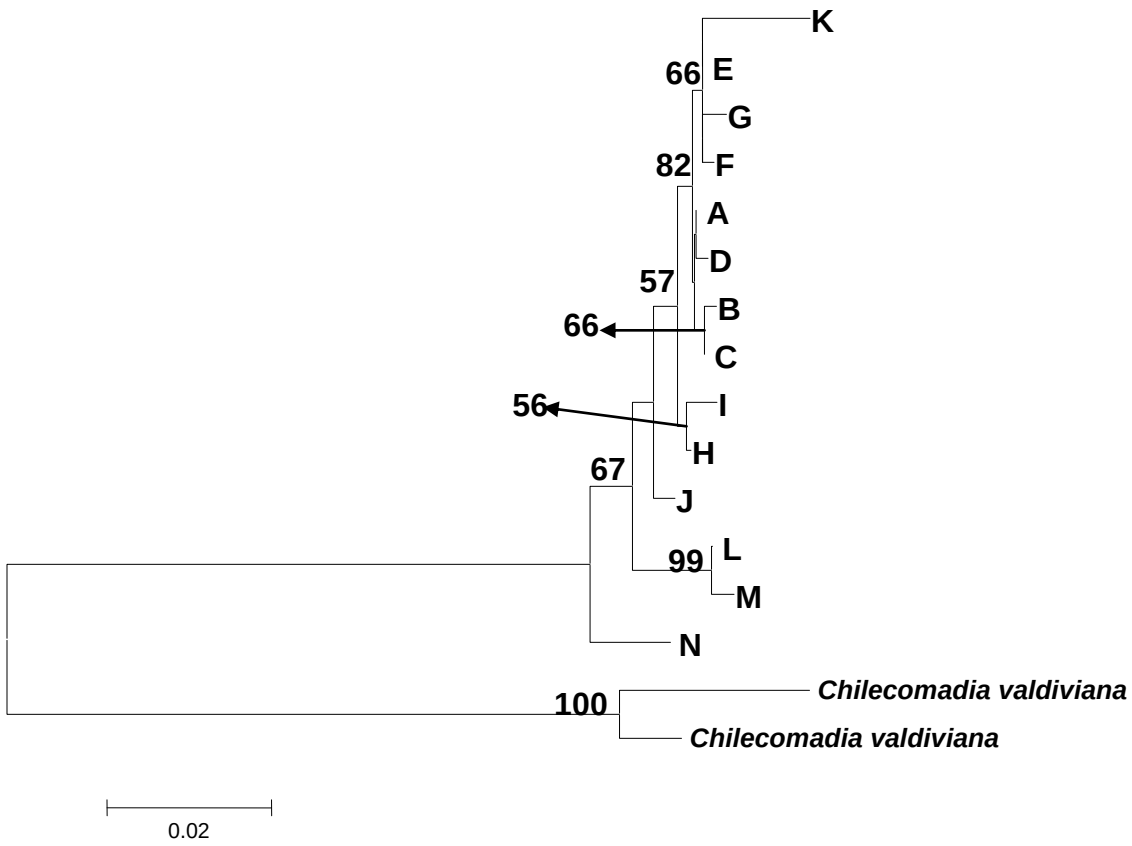

Figure 5. A neighbour joining tree constructed using the Kimura 2-parameter distance model, between 16 mtDNA COI sequences representing 14 *Coryphodema tristis* and 2 *Chilecomadia valdiviana* samples. Bootstrap values are presented at the nodes.

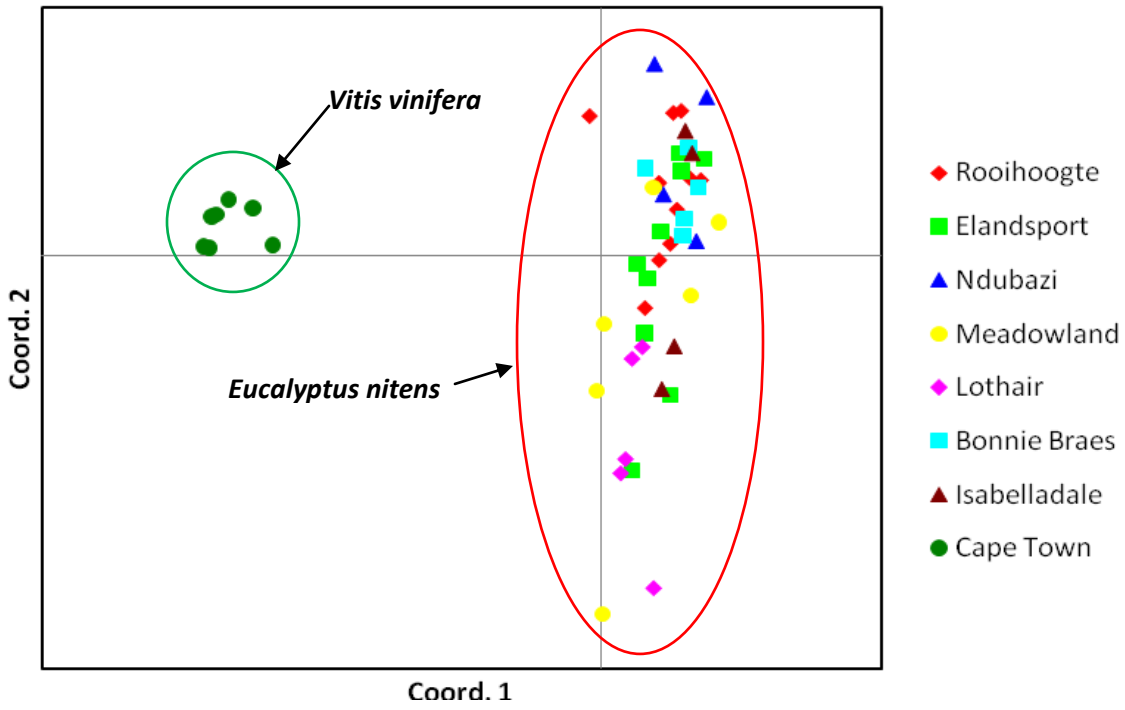

Figure 6. Principal Component Analysis (PCA) of *Coryphodema tristis* populations from *Eucalyptus nitens* and grapevine (*Vitis vinifera)*. Two distinct clusters were evident indicating some genetic isolation in between clusters.

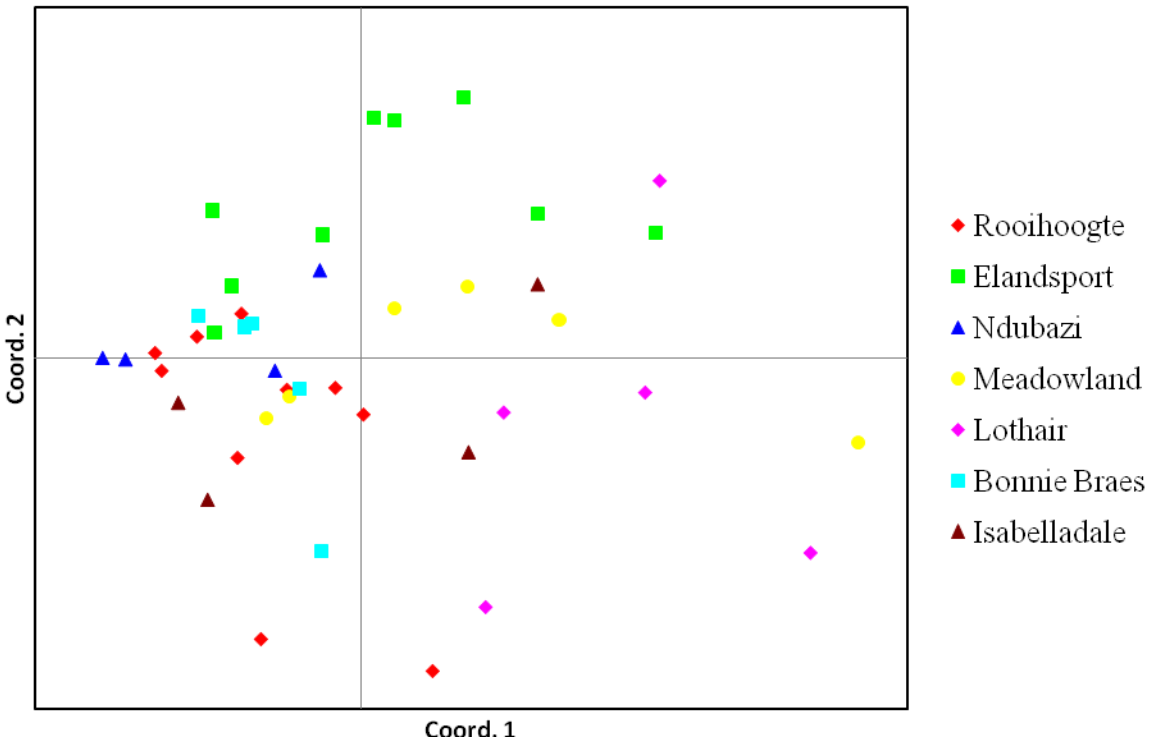

Figure 7. Principal Component Analysis (PCA) *Coryphodema tristis* populations from *Eucalyptus nitens*. The randomly scattered distribution indicating that there is no clear genetic differentiation between populations.

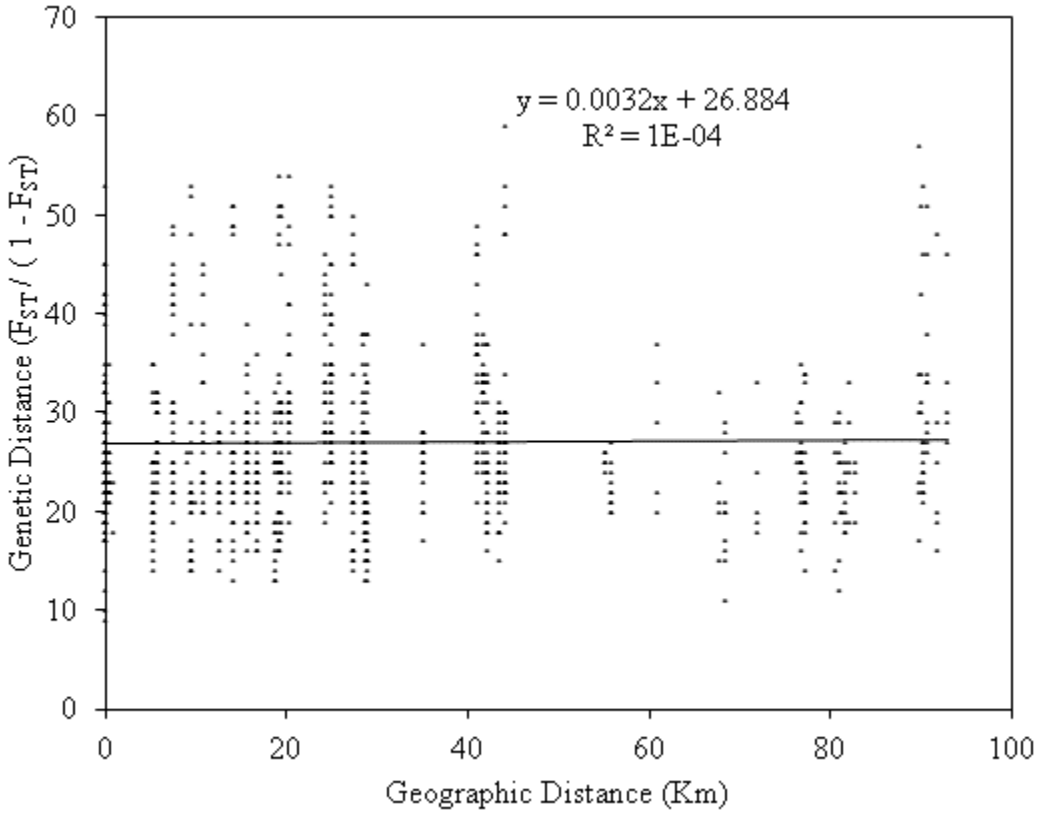

Figure 8. Regression analysis of pairwise genetic distance, F_{ST} / (*1* - F_{ST}) (AFLP markers) versus pairwise geographic distance of *Coryphodema tristis* among *E. nitens* populations.

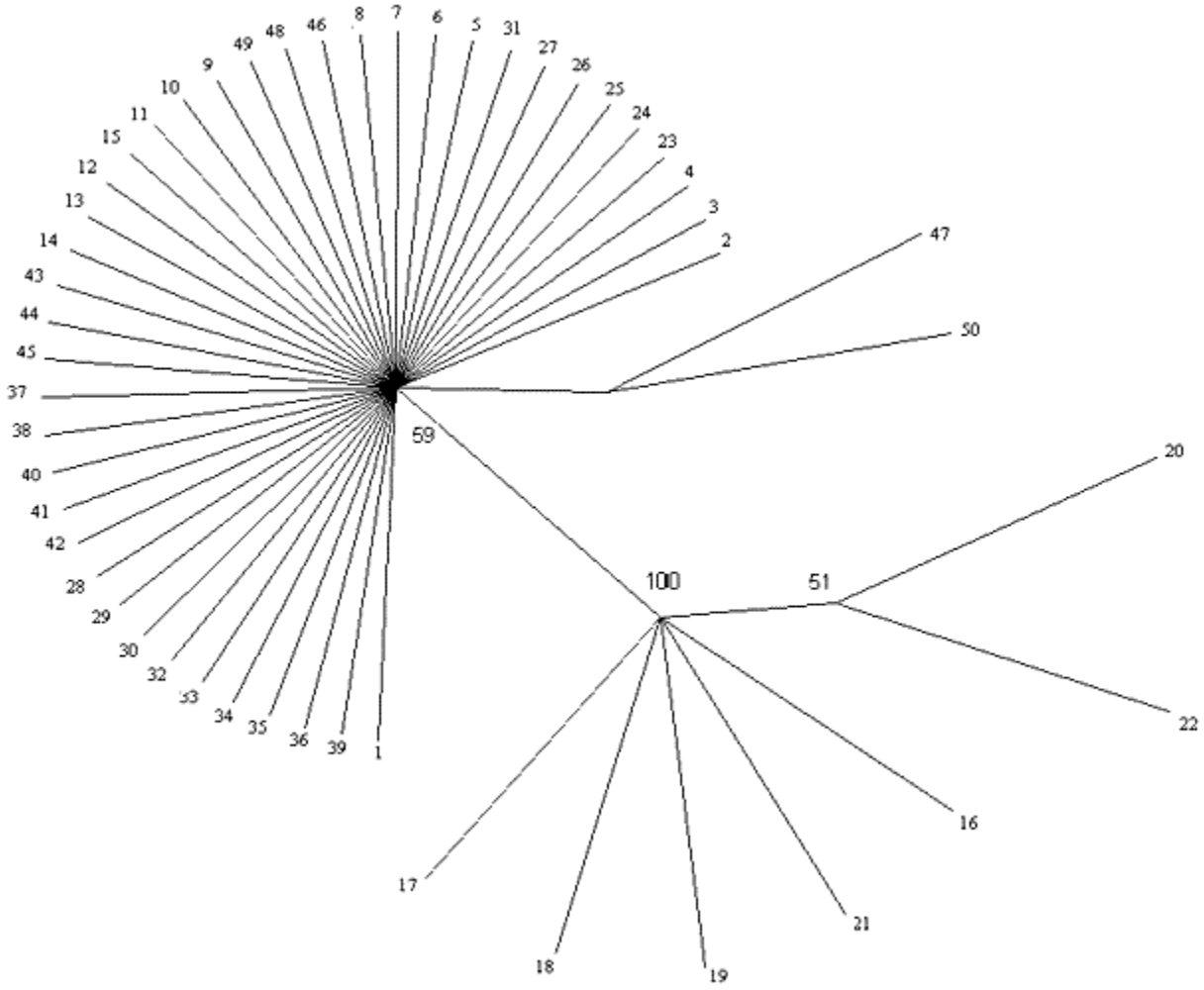

Figure 9. Dendrogram of the simple matching distance of the AFLP profiles representing genetic relationships among *Coryphodema tristis* individuals from *Eucalyptus nitens* and *Vitis vinifera*. Numbers labeled on the tree ranged from 16 - 22 represent samples of *C. tristis* individual from *Vitis vinifera*. The rest represent samples from *Eucalyptus nitens*.

CHAPTER 4

Yeast communities associated with *Coryphodema tristis* larvae and xylem tunnels in *Eucalyptus nitens* and *Vitis vinifera*

ABSTRACT

The cossid moth *Coryphodema tristis* is well known for a characteristic fermenting smell associated with the xylem tunnels of its larvae, which typically extend well into the heartwood of the infested trees. It is expected that microbes are involved in the digestion of the woody material consumed by these larvae, as is found in some other wood-boring insects. In this study, Ascomycetes yeasts that were are expected to be prominent members of the microbial community associated with these insects, were isolated from the larval digestive tracts of *C. tristis* and their tunnels in *Eucalyptus nitens* and *Vitis vinifera*. Based on comparisons of the rDNA sequence data, nine yeast species were identified including *Saccharomyces paradoxes*, *S. cerevisiae*, *Hanseniaspora valbyensis*, *Pichia manshurica*, *Kregervanrija delftensis*, *K. fluxuum*, *Wickerhamomyces myanmarensis*, *Candida salmanticensis* as well as a novel species. The analysis of dominance (P) of these yeasts showed that *P. manshurica*, a novel yeast species from larvae in *E. nitens* and *W. myanmarensis* from larvae in *V. vinifera* were the dominant and likely most important yeasts associated with *C. tristis*. The results also indicated a clear distinction between the yeast communities associated with *C. tristis* feeding on *E. nitens* and on *V. vinifera*. characterization of the yeast community of *C. tristis* represents a first step towards understanding their interactions, as well as their ecological roles in facilitating this plant-insect interaction.

Key words: Ascomycetes yeast, *Coryphodema tristis*, insect-microbial symbiosis, *Eucalyptus* pest, *Vitis* pest

INTRODUCTION

Microbes are associated with many insects and they can strongly influence the feeding behaviour and ecology of their vectors or associates. Improving the understanding of the complex interaction among insects and their internal and digestive symbionts can shed light on various aspects of the ecology of the insects involved (Klepzig *et al*. 2009). Furthermore, the microorganisms can help to explain the increased fitness of the host for example by providing the insect protection against its natural enemies (Weis 1982; Oliver *et al*. 2003, 2005, 2006). These associations could be important in order to understand the evolutionary diversification through adaptive radiation and species interactions (Schluter 2000; Funk *et al*. 2002; Rundle & Nosil 2005; Bronstein *et al*. 2006).

Apart from providing nutrition and protection to the host, insect-microbe interactions are also known to be important in insect-plant interactions, especially for those insects feeding on recalcitrant plant tissues (Hansen & Moran 2013). Evidence suggests that microbial symbionts contribute to the ability of insects to infect the host plant and potentially also to host plant specialization (Hosokawa *et al*. 2007). Microbes can contribute by enabling insects to overcome plant defenses (Paine *et al*. 1997) or by modifying or detoxifying plant allelochemicals (Douglas 1992; Dillon & Dillon 2004). Various studies have also suggested that microbe-insect interactions affect novel host plant invasions by exposing ecological opportunities through environmental buffering for their insect hosts (Simpson 1953; Schulter 2000).

In wood and phloem-feeding insects, interactions with microbes are particularly common and it is believed that they can play an important role in the digestion and the detoxification of the secondary metabolites of the plant (Dowd 1992). In aggressive tree-killing bark beetles such as

Dendroctonus frontalis, *D. ponderosae* and *Ips typographus*, their fungal symbionts have long been considered to aid in hastening the death of the trees (Krokene & Solheim 1998), although this has recently been called into question (Six & Wingfield 2011). Other examples of fungal symbionts of wood-boring insects include Ambrosia beetles, Cerambycids, Passalid beetles and the Sirex woodwasp (Dowd 1992; Sue *et al*. 2003; Nguyen *et al*. 2006). Similarly, the occurrence of yeasts in a variety of beetles and other insects has also been noted (Suh *et al*. 2003, 2004, 2006; Nguyen *et al*. 2007).

Among insect associated microbes, yeast symbionts have been found in association with a broad range of insects, including lacewings, wasps, bees and beetles (Gibson & Hunter 2005, 2009; Rosa *et al*. 2003; Torto *et al*. 2007; Benda *et al*. 2008). In these associations, yeasts can directly provide essential nutrients to the insects (e.g. amino acids, vitamins and lipids) or produce enzymes that assist in the digestion and detoxification of the diet ingested by their hosts (Chararas *et al.* 1983; Dowd 1989; Dowd 1991; Vega & Dowd 2005). Yeasts have also been shown to contribute to essential pheromone production (Dowd 1992; Vega & Dowd 2005) and they can have an important role in driving some behavioral traits of the insects, including oviposition site selection and host seeking behavior (Borden 1982; Phelan & Lin 1991; Paine *et al*. 1997; Reeves 2004).

The cossid moths (Cossidae: Lepidoptera) are a group of wood-boring insects that have been suggested to have an association with yeasts due to the fermenting odors that characterize their infestations (Vega & Dowd 2005), Certain cossid species, such as *Coryphodema tristis*, have recently become serious pests of plantation forestry species (Gebeyehu *et al*. 2005; Boreham 2006), elevating the need to understand the role of microbes in their ecology. The recent host

range expansion of *C. tristis* to *Eucalyptus nitens* in South Africa is the first record of its association with the family Myrtaceae (Boreham 2006; Gebeyehu *et al*. 2005; Degefu *et al*. 2013). Despite the wide host range of *C. tristis* on other native and non-native trees, it has not infested any other surrounding *Eucalyptus* species besides *E. nitens* (Gebeyehu *et al*. 2005). The reason for the sudden and unexpected host shift of *C. tristis* to *E. nitens* and its specificity within this genus is not clear.

The primary aim of this study was to identify the community of yeasts associated with *C. tristis* on *E. nitens* in Mpumalanga, South Africa and in *Vitis vinifera* in the Western Cape, South Africa. We considered whether the yeast communities differed in diversity or composition among different regions and hosts, and between tunnels and the insect gut. Furthermore, the phylogenetic relationships between isolated yeasts were characterized based on rDNA sequence data.

MATERIALS AND METHODS

Sample collection

Yeasts were isolated from larvae of *C. tristis* and associated tunnels in *E. nitens* from seven sites in the Mpumalanga province of northeastern South Africa and from a single *V. vinifera* (grapevine) orchard in Vredendal, Western Cape province in 2010 (Table 1). On *E. nitens*, larvae were collected from three randomly selected trees per site. Three logs (mean length ~35 cm) were collected from the top and bottom of each tree. Infestations present in the tops and bottoms of the trees were separated by 3-4 m and represented independent oviposition events, whether by the same or different females. Thus, a total of 9 logs were collected per site. Five larvae plus five associated tunnels were sampled per log. For the grapevine population, six stems were sampled from the one *C. tristis* infested *V. vinifera* orchard mentioned above.

Isolation of yeasts from tunnels and larvae

Yeasts were isolated from the *C. tristis* tunnels on *Eucalyptus* and grapevine by plating small (1-2 mm) pieces of discolored wood on YME agar (plus 100 ppm streptomycin), after removing the exposed surface of the tunnels with a sterile blade. Yeasts were also isolated from the guts of 315 and 30 larvae of *C. tristis* both from *E. nitens* and *V. vinifera*, respectively. No yeasts were found to grow on YME agar after the surface disinfested larvae were placed on YME agar for two minutes, confirming the efficacy of the treatment. Larvae were surface-disinfested by submerging them in a solution of 0.25 % sodium hypochlorite followed by 70 % ethanol for one minute. Larvae were then rinsed three times in sterile distilled water and placed on sterile filter paper to dry. Sterilized larvae were dissected in a laminar flow cabinet, where the gut sections were moved aseptically and transferred to tubes containing phosphate buffered saline (PBS) solution and this mixture was vortexed for 30 seconds to release the gut contents. Gut contents

were streaked on YME agar plates and incubated for three days after which the yeast colonies were transferred to fresh agar plates. Single colonies were transferred to new YME agar plates to purify the cultures for DNA extraction.

DNA extraction, PCR and sequencing

Yeast cells were harvested from YME agar plates and the DNA was extracted using a PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, California USA) kit and following the manufacturer's recommendations. The primer pair ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' (White *et al*. 1990) was used to amplifies the ITS (internal transcribed spacer) regions (ITS 1, ITS 2 and the intervening 5S rRNA gene), whereas LROR 5'-ACCCGCTGAACTTAAGC-3' and LR5 5'-TCCTGAGGGAAACTTCG-3' (Vilgalys & Hester 1990; Rehner & Samuels 1994) were used to amplify the D1/D2 domain of the LSU (large-subunit) rRNA gene.

The reactions for PCR amplification were performed in a final volume of 25 μl, containing 16 μl ddH_2O, 3 μl 10× Taq Buffer + $MgCl_2$, 2 μl 5mM dNTP's , 0.75 μl of each PCR primer (10 *m*M), 0.5 μl Taq DNA polymerase (5U μl^{-1}), and 2 μl of the DNA template (80 ng). Thermocycling conditions for both the ITS and LSU loci were 96 °C for 3 min, 35 cycles (94 °C for 30s, 55 °C for 30s, and 72 °C for 2 min), followed by a final extension step of 72 °C for 10 min. Amplification success was evaluated by electrophoresis of a fraction of total amplification products in 1.5 % (wt/vol) agarose gels, run for 30 min at 100 V in Tris-borate buffer. The amplified bands of the target gene regions were cleaned using Sephadex columns. The sequencing PCRs were carried out using 3 µl PCR product, 2 µl BigDye Terminator Sequencing kit (Applied Biosystems, USA), 0.4 µM of each of the forward and reverse primers and 5x

sequencing buffer. The sequencing reactions were done as follows: 25 cycles at 95 °C for 10s, at 48 °C for 5s, at 60 °C for 4 min. All Sephadex cleaned and vacuum dried sequencing PCR products were sequenced using the ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems).

Phylogenetic relationships and nucleotide divergence

DNA sequencing data were assembled and edited manually using CLC Bio Workbench v. 6.x. (www.clcbio.com). The complete sequences of the ITS and that of the D1/D2 region were compared to sequences available in GenBank using the NCBI BLASTn search program (Johnson *et al*. 2008) as well as the freely available CBS yeast sequence database (www.cbs.knaw.nl). Selected DNA sequences were aligned with MAFFT v.6 (Katoh *et al*. 2002) and adjusted visually in Bioedit (Hall 1999). Pairwise nucleotide diversity was calculated in MEGA 5.0 (Tamura *et al*. 2011). GenBank accession numbers obtained from the DNA sequences in this study were used to refer to samples.

Phylogenetic relationships were calculated using maximum-parsimony in PAUP 4.0 (Swofford 2002). Heuristic tree searches were executed using the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. Confidence limits for phylogenetic trees were estimated from bootstrap analyses (1000 replicates). *Schizosaccharomyces pombe* was the designated an outgroup species in all analyses. Base pair differences in the ITS and D1/D2 regions were calculated using the manually aligned sequence database.

Analysis of relative abundance

The dominance of yeasts isolated from the gut of *C. tristis* larvae and infected tunnels in *E. nitens* and *V. vinifera* were calculated using the following equation (Ho *et al*. 2001).

Where P_i is the percentage abundance of the i species, n_i is the number of samples with i species, and S is the number of species found in all samples and locations.

$$P_i = \frac{n_i}{\sum_{i=1}^{S} n_i} \; X\,100,\; i = 1, 2, 3 \ldots S$$

RESULTS

Phylogenetic relationships based on the LSU

The D1/D2 sequence data set was comprised of 571 characters of which 214 were parsimony informative. Phylogenetic analysis of the D1/D2 regions revealed the presence of nine yeast species (Figure 1).

Based on the D1/D2 sequences, the isolated yeast strains GM09 and GI07 had 100 % sequence similarity with that of the type strain of *S. paradoxus* (CBS 432; BR000309 and AY048155). Strain GV5 03 had 100 % sequence similarity with that of the type strain of *S. cerevisiae* (CBS 1171; AY048154) and differed by 0.4 % nucleotide (two substitutions) diversity from another *S. cerevisiae* strain (J01355). Low nucleotide diversity was observed among the yeast strains GI01 and GE2 03 that grouped with the reference strain of *H. valbyensis* (CBS 479; U73596) (0.4 %, one substitution and one deletion and 0.6 %, three substitutions), respectively, while isolate GI08 has 100 % sequence similarity with this type strain. Isolate B06 grouped with the reference strain of *K. fluxuum* (CBS 2287; EF550268) and L04 grouped with the reference strain of *K. delftensis* (CBS 2614; U75721, EF550266) 0.2 % (one substitution) nucleotide diversity (Figure 1). The isolated yeast strains M07, GV1 05 and GE1 06 had 100 % sequence similarity with that of the type strain of *P. manshurica* (CBS 209; EF550223). A second clade included isolate N006, which had 100 % sequence similarity with a *Pichia* sp. (EF550229), and differed by 0.2 % (one insertion) from that of *Pichia* sp. CBS 2150. The isolated yeast strain GH2 02 differed by 0.2 % (one substitution) from sequences of the type strain of *C. salmanticensis* (CBS 5121; (U62308). Strain GV4 05 has 100 % sequence similarity with the type strain of *W. myanmarensis* (CBS 9786; AB126678).

Phylogenetic relationships based on the ITS rDNA region

The length of the amplified fragments for the ITS region were highly variable among the taxa isolated in this study. The complete sequence of the ITS for the taxa studied ranged from 373 to 753 bp. The ITS1 and ITS2 regions showed high variability in length ranging from 75 to 362 bp and 92 to 233 bp, respectively (Table 2). Given the extent of this variation, analyses were done within genera to reduce the number of gaps and ambiguously aligned sequences.

A total of nine species were identified using the ITS sequence data, which was consistent with the D1/D2 data sets. The isolated yeast strain GH2 02 differed by 0.3 % (one substitution) from the type strain of *C. salmanticensis* (CBS 5121) (Figure 2). Strains GE2 03 and GI08 differed by 0.89 % (one substitution, one insertion and four deletions) and 2.03 % (three substitutions and four insertion) from the type strain of *H. valbyensis* (CBS 479; AY046197), respectively (Figure 3). The isolated yeast strain B06 differed by 0.99 % (three substitutions and one insertion) from the type strain of *K. fluxuum* (CBS 2287; AY923249) (Figure 4), while isolated yeast L04 has 100 % sequence similarity with the type strain of *K. delftensis* (CBS 2614; AY923246). Strain R1 07 differed by 2.3 % (eight substitutions and one deletion) from the closest type strain of *P. manshurica* (TCJ105; HM044862), while strains GE1 06 and M07 have 100 % sequence similarity with the type strain of *P. manshurica* in the CBS database (CBS 11610 and CBS 11625; database not public) (Figure 5). The strain GV1 05 differed by 5 % from the reference strains for P. *manshurica* (CBS 209; DQ104714). A second clade included isolate N006, which had 100 % sequence similarity with two *Pichia* sp. strains (CBS 2150 and CBS 2151) for which the sequence data have not been made public.

The remaining yeast strains GM09 and GI07 differed by 0.13 % (one deletion) and 0.3 % (three substitutions) from the type strain of *S. paradoxus* (CBS 432; D89891 and D89890), respectively. Strain GV5 03 differed by 0.13 % (one substitution) from the type strain of *S. cerevisiae* (CBS 1171; AM262830) (Figure 6). Strain GV4 05 had 100 % sequence similarity with the type strain of *W. myanmarensis* (CBS 9786; JX679222) (Figure 7).

Host, location and larval association

The communities of yeasts isolated from the guts of *C. tristis* larvae differed between the two host plants (*E. nitens* and *V. vinifera)* sampled in two different regions of South Africa (Table 3). The *W. myanmarensis* isolate (GV4 05) was isolated exclusively from the guts of *C. tristis* larvae infesting *V. vinifera* and from the tunnels associated with this insect (Table 3). Similarly, isolates of *K. delftensis* (L04), *K. fluxuum* (B06) and the novel yeast species (N006) were isolated only from the guts of *C. tristis* larvae from *E. nitens*, while isolates identified as *H. valbyensis* (GE2 03 and GI08) were isolated only from the larval tunnels in *E. nitens.*

Pichia manshurica (R1 07, GV1 05, GE1 06 and M07), *C. salmanticensis* (GH02), *S. paradoxus* (GM09 and GI07) and *S. cerevisiae* (GV5 03) isolates were shared between the guts of *C. tristis* from *E. nitens* and the associated tunnels. Similarly *P. manshurica*, *H. valbyensis*, *W. myanmarensis* and *S. cerevisiae* isolates were shared between the guts of *C. tristis* larvae from *V. vinifera* and their associated tunnels (Table 3).

Dominance of yeast communities

Pichia manshurica was the most commonly encountered isolated yeast species isolated from the gut of *C. tristis* infesting both *E. nitens* and *V. vinifera* and their associated tunnels. It was the dominant yeast species found in the guts of *C. tristis* feeding on *E. nitens* (44.5 %) and the

associated tunnels in the infested wood (48.19 %) (Table 4). Similarly, *P. manshurica* was the dominant species (40 %) from the guts of *C. tristis* feeding on *V. vinifera* (Table 5). *Wickerhamomyces myanmarensis* accounted for 33.3 % and 38.5 % of the isolates from *C. tristis* feeding on *V. vinifera* and its associated tunnels, respectively (Table 5).

DISCUSSION

Nine yeast species were identified from the guts of *C. tristis* larvae and their tunnels in *E. nitens* and *V. vinifera*. Eight of these species could be linked to sequences of type strains of described yeast species, while one group of isolates was similar to strains of a currently undescribed species (CBS 2150 and CBS 2151). The minimal sequence differences between the isolates and that of the associated type strains (less than 0.5 %) suggests that the yeast emerging from this study are conspecific with the species having sequences most similar to them (Kurtzman & Robnett 1997, 1998). The variation in the LSU sequences between species in some genera (e.g. *Wickerhamomyces*) were very low and additional sequence data for the ITS region were necessary to confirm their identity.

The ITS sequence data generated in this study were consistent with the identifications based on the LSU sequence data. The ITS data were generally more variable compared to the corresponding LSU data. As for LSU data, it has been suggested that isolates that are conspecific share a sequence homology of ≥ 99 % (Sugita *et al*. 1999). While it is unlikely that such a general rule could be applied indiscriminately, all the clades emerging from this study and purportedly representing a single species, showed less than 1 % variation among the isolates, including sequences for the type strains. The only exception was found in the *P. manshurica* clade, which had 96 - 98 % variation and it is possible that this clade includes isolates of more than one species.

The results of this study provide further support for the existence of an undescribed species represented by the strains CBS 2150 and CBS 2151 that are available in the CBS yeast collection (www.cbs.knaw.nl) and isolated from tanning fluid in France. This species, which is being

described elsewhere, represented the second most dominant species found in this study and only from the guts of *C. tristis* infesting *E. nitens*.

One of the isolates (GV1 05) from the guts of *C. tristis* and associated tunnels in *V. vinifera* could represent an undescribed yeast species. This strain was identified based on LSU sequences as *P. manshurica* with 100 % sequence similarity to the type strain in the CBS database (CBS 209), but differed by 3.5 % (10 substitutions, 2 deletions and 2 insertions) from the type strain CBS 209 based on the ITS region sequence comparisons. According to Sugita *et al*. (1999) the ITS nucleotide sequence similarity between identical species should be greater than 99 % suggesting that this is a discrete taxon in the *Pichia* clade.

Candida salmanticensis was isolated from both the guts of *C. tristis* and the associated tunnels of the insect in *E. nitens* and *V. vinifera*. *Candida* is a very large genus of the anamorphic Saccharomycetales, cosmopolitan and species can be found in many ecological niches including the surface of fruits and other plant organs, rotting wood, the soil, or associations with insects (especially bees) (Meyer *et al*. 1998; Webster & Weber 2007). It is thus not surprising to find a *Candida* species was found associated with *C. tristis*. However, it was interesting to find *C. salmanticensis* given that the type strain (CBS 5121) of this species was isolated from alpechin, a residue in the preparation of olive oil, in Spain, (Meyer *et al*. 1998). This species has ever been reported associated with insects. The isolation of *C. salmanticensis* isolates both from the guts of *C. tristis* and its associated *E. nitens* tunnels suggests that it could be closely involved with the host plant interaction of *C. tristis*.

Pichia manshurica was the most dominant species isolated in this study. This species was first isolated from sorghum spirits used to produce alcoholic drinks in Manchuria in 1914 (Saito

1914). *Pichia manshurica* has also been isolated from a diversity of other sources and areas, such as soil (IFO 1790), feces (CBS 7324) and partially decayed leaf tissue (IFO 1789) in Japan, exudates of black oak (*Quercus kelloggii*) (CBS 2284) in the USA, Bili wine (CBS 240) in West Africa and Palm wine toddy (CBS 4043) in Pakistan (Japan society for culture collection: www.nbrc.nite.go.jp/jscc). Even though *P. manshurica* has never been isolated from insects, a large number of different other *Pichia* species are commonly associated with insects and particularly wood-boring beetles and their frass (Kurtzman 1998).

The two *Saccharomyces* species identified in this study, *S. paradoxus* and *S. cerevisiae*, were previously isolated from a variety of niches around the world. For example, *S. paradoxus* has been isolated from *Drosophila*, soil and tree exudates, while *S. cerevisiae* has been isolated from vineyard, wine and grape must (Vaughan-Martini & Martini 1998; Naumov *et al*. 1998). *Saccharomyces paradoxus* is the most extensively studied species in the wild, and appears to be present on most continents (Naumov *et al*. 1993; Naumov *et al*. 1998; Naumov 1999). *Saccharomyces cerevisiae* is more commonly studied associated with humans and fermentation, and is more rarely isolated from nature or plants (Davenport 1974; Rosini *et al*. 1982; Sniegowski *et al*. 2002), although it has been isolated from soil beneath *Quercus* spp., flux from *Quercus alba* and Bark of *Q. velutina* and *Q. rubra* (Slavikova & Vadkertiova 1997). This species has previously been isolated from different vineyards in the Western Cape, South Africa (van der Westhuizen *et al*. 2000) and its occurrence on grape vines is perhaps not surprising. Both *S. paradoxus* and *S. cerevisiae* are also known to co-occur, as was also shown in this study (Redzepovic *et al.* 2002; Sniegowski *et al.* 2002).

Kregervanrija fluxuum and *K. delftensis* occurred in low numbers from gut of *C. tristis* associated with *E. nitens* in this study. This recently described genus was proposed by Kurtzman from a species previously described as *Pichia fluxuum* (Kurtzman 2006). The isolation of *K. fluxuum* (= *P. fluxuum*) was not surprising, as it is clearly widespread and has been found in habitats similar to those considered in this study. Previously isolates have been obtained from slime, flux, sap and black oak (*Quercus kelloggii*) (CBS 2287) from USA and partially decayed leaf (IFO 1784, IFO 1786, IFO 1787) and bark (IFO 1785) from Japan. But the collection of *K. delftensis* was surprising given that this species has previously been found only in cider and wine in the UK (CBS 2614, IFO10715) (Kurtzman 2006; Japan society for culture collection: www.nbrc.nite.go.jp/jscc).

Isolates of *H. valbyensis* were consistently found both in the guts and the associated tunnels of *C. tristis* on grapevine. This species has previously been isolated from the guts and surfaces of the dried-fruit beetle, *Carpophilus hemipterus* and *Drosophila* in California (Miller & Mrak 1953; Phaff *et al*. 1956). The species has also been identified from plant material such as partially decayed leaf (IFO 1759) and flower tissue (IFO 1758), as well as from soil in Denmark (Japan society for culture collection: www.nbrc.nite.go.jp/jscc) and it has also been reported associated with soil in Germany (CBS 479), sap of trees and tomato (CBS 281 and CBS 6618) in Japan (Cadez *et al*. 2002). Interestingly, *H. valbyensis* has also been reported from grapes collected from vineyards in the Stellenbosch-Paarl area of South Africa (van der Walt & van Kerken, 1960). The occurrence of this species from grapevine canes infested by *C. tristis* in an area where the yeast has also previously been found is thus not surprising. *Wickerhamomyces myanmarensis* was the other yeast species isolated from the guts and tunnels of *C. tristis* in grapevine stems. This species was first collected from Palm sugar (CBS 9786, IFO 11090 and NBRC 11090) from

Myanmar. Species of *Wickerhamomyces* are known from many natural substrates including forest soil, frass from insect tunnels associated with various trees species and insects, exudates and gum of various tree species, wine vats and insects such as ambrosia beetles, nitidulid beetles and larvae of *Anastrepha mucronata* (Limtong *et al*. 2009; Rosa *et al*. 2009; de Garcia *et al*. 2010; Groenewald *et al*. 2011; Kurtzman *et al*. 2011; Nakase *et al*. 2012; Ninomiya *et al*. 2013). It was thus not surprising to find a *Wickerhamomyces* species associated with *C. tristis* and its tunnels in *V. vinifera*.

Eight of the nine yeast species identified in this study were isolated from both *C. tristis* larval guts and their associated tunnels in *E. nitens* and *V. vinifera*. The only exception was *H. valbyensis* that was isolated only once from the tunnels in *E. nitens*. Of these eight species, five were present in the larval guts and the tunnels associated with *E. nitens* and three were from *V. vinifera*. Whether these yeasts have any active role in the guts of *C. tristis* larvae is not known, but given that some have biological roles in other systems, this could also be the case for *C. tristis*. For example, yeasts present in the gut of *Drosophila* such as *Candida ingens*, *Candida sonorensis*, *Pichia cactophila* and *Cryptococcus cereanus* have a nutritional role (Sang 1978; Starmer *et al*. 1982; Starmer *et al*. 1986; Ebbert *et al*. 2003). The diversity of yeasts in the guts of these insects is also not surprising, and could be beneficial. Some of the studies on the benefits of yeasts to the larvae of *Drosophila* suggest that mixed communities of yeasts improve larval survival, growth and development, albeit the mixed culture effect is to some degree host specific (Moreteau *et al*. 1992 Starmer & Barker 1986; Starmer & Fogelman 1986). This is not only true in *Drosophila*, but several other studies have also shown that insect survival, growth and reproduction are improved by gut yeast associates (see detail review by Vega & Dowd 2005). Furthermore, although little is known about yeast transmission, localization or fate within the

digestive tract, the abundance of yeasts in the gut of *C. tristis* suggests that they are likely to pass through the gut alive, and thus be spread by the larvae throughout the tunnels in the wood as is found for other insects (Ganter 2006; Rivera *et al*. 2009; Stamps *et al*. 2012). More importantly, they would have a chance to also be found associated with the adult moths, which would facilitate dispersal between trees, as has been reported for other insects (Gilbert 1980; Starmer & Fogleman 1986; Morais *et al*. 1994; Stamps *et al*. 2012).

Characterization of the yeast species associated with *C. tristis* plays an important foundation for further biological studies on this invasive pest. Further important questions that would build on the results of this study include the spatial and temporal structure of the yeast diversity associated with *C. tristis*, the specificity of the association, and the possible transmission between generations of the insect. Importantly, the results of studies of these yeasts in culture will now facilitate studies on the role that they might have in the nutrition of *C. tristis* larvae.

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TABLES

Table 1. Sample sites of *Coryphodema tristis* larvae from xylem tunnels of *E. nitens* and *V. vinifera* in South Africa.

No.	Sample site	Latitude	Longitude	Elevation (m)	Host plant
1	Rooihoogte	S26.05655	E30.32772	1645	*E. nitens*
2	Bonnie Braes	S26.16803	E30.74489	1701	*E. nitens*
3	Isabelladale	S26.21773	E30.63133	1718	*E. nitens*
4	Lothair	S26.31445	E30.62239	1666	*E. nitens*
5	Meadowland	S26.28522	E30.69000	1552	*E. nitens*
6	Elandsport	S26.11923	E30.74505	1552	*E. nitens*
7	Ndubazi	S25.55425	E30.29552	1592	*E. nitens*
8	Vredandal	S31.66435	E18.50594	-	*V. vinifera*

Table 2. Length variation of the full ITS gene region among the isolated yeast species and some reference sequences from GenBank.

Taxa	ITS1	ITS 5.8s	ITS2	Full length
Pichia manshurica (FM199959)	86	157	131	374
GV1 05	85	157	131	373
R107	86	157	135	378
M07	86	157	132	375
GE1 06	86	157	132	375
Pichia sp. nov				
N006	75	157	112	344
Kregervanrija fluxuum (DQ104734)	101	158	129	388
B06	101	158	130	389
Candida salmanticensis (EU343849)	85	158	92	335
GH2 02	85	158	92	335
Wickerhamomyces edaphicus (AB436773)	181	158	191	530
GV4 05	182	158	195	535
Saccharomyces paradoxus (AM262828)	362	158	233	753
GM09	362	158	232	752
GI07	362	158	233	753
Saccharomyces cerevisiae (AM262828)	361	158	233	752
GV5 03	361	158	233	752
Hanseniaspora valbyensis (AJ512434)	286	158	216	660
GE2 03	286	158	214	658
GI08	287	158	218	663
GI01	287	158	218	663

Table 3. Isolated yeast species identified from larval gut of *C. tristis* eating *E. nitens* and *V. vinifera* and the associated tunnels.

Species	Isolates	*E. nitens*		*V. vinifera*	
		Gut	Gallery	Gut	Gallery
Pichia manshurica	M07, R1 07, GV1 05, GE1 06	√	√	√	√
Pichia sp. nov.	N006	√	*	*	*
Kregervanrija fluxuum	B06	√	*	*	*
Kregervanrija delftensis	L04	√	*	*	*
Candida salmanticensis	GH2 02	√	√	*	*
Saccharomyces paradoxus	GM09, GI07	√	√	*	*
Saccharomyces cerevisiae	GV5 03	√	√	√	√
Hanseniaspora valbyensis	GI01, GI08, GE2 03	*	√	√	√
Wickerhamomyces myanmarensis	GV4 05	*	*	√	√

*Samples not isolated from that particular substrate.

Table 4. The distribution and abundance of yeast species identified from larval gut of *C. tristis* feeding on *E. nitens* and its associated gallery among sample populations.

	% Abundance per subpopulation							
Yeast species	Rooihoogte	Elandsport	Ndubazi	Meadowland	Bonnie Braes	Lothair	Isabelladale	% Total/all Isolate
Isolates from gut								
Pichia manshurica	45.45	41.67	47.06	33.33	38.10	52.94	52.38	44.53
Pichia sp.	18.18	16.67	17.65	27.78	23.81	11.76	14.29	18.75
Kregervanrija fluxuum	18.18	25.00	5.88	16.67	9.52	23.53	14.29	15.63
Kregervanrija delftensis	9.09	8.33	11.76	22.22	14.29	11.76	9.52	12.50
Candida salmanticensis	4.55	8.33	5.88	0.00	9.52	0.00	4.76	4.69
Saccharomyces sp.	4.55	0.00	11.76	0.00	4.76	0.00	4.76	3.91
Isolates from gallery								
Pichia manshurica	57.14	40.00	45.45	54.55	54.55	41.67	42.86	48.19
Pichia sp.	28.57	20.00	27.27	18.18	27.27	25.00	28.57	25.30
Hanseniaspora valbyensis	0.00	10.00	18.18	18.18	9.09	8.33	0.00	8.43
Candida salmanticensis	7.14	10.00	0.00	9.09	9.09	16.67	7.14	8.43
Saccharomyces sp.	7.14	20.00	9.09	0.00	0.00	8.33	21.43	9.64

Table 5. The diversity and abundance of yeast species identified from larval gut of *C. tristis* eating *V. vinifera* and its associated tunnels.

	Abundance (%)	
	Gut	Gallery
Pichia manshurica	40.00	30.77
Hanseniaspora valbyensis	13.33	7.69
Wickerhamomyces myanmarensis	33.33	38.46
Saccharomyces cerevisiae	13.33	23.08

FIGURES

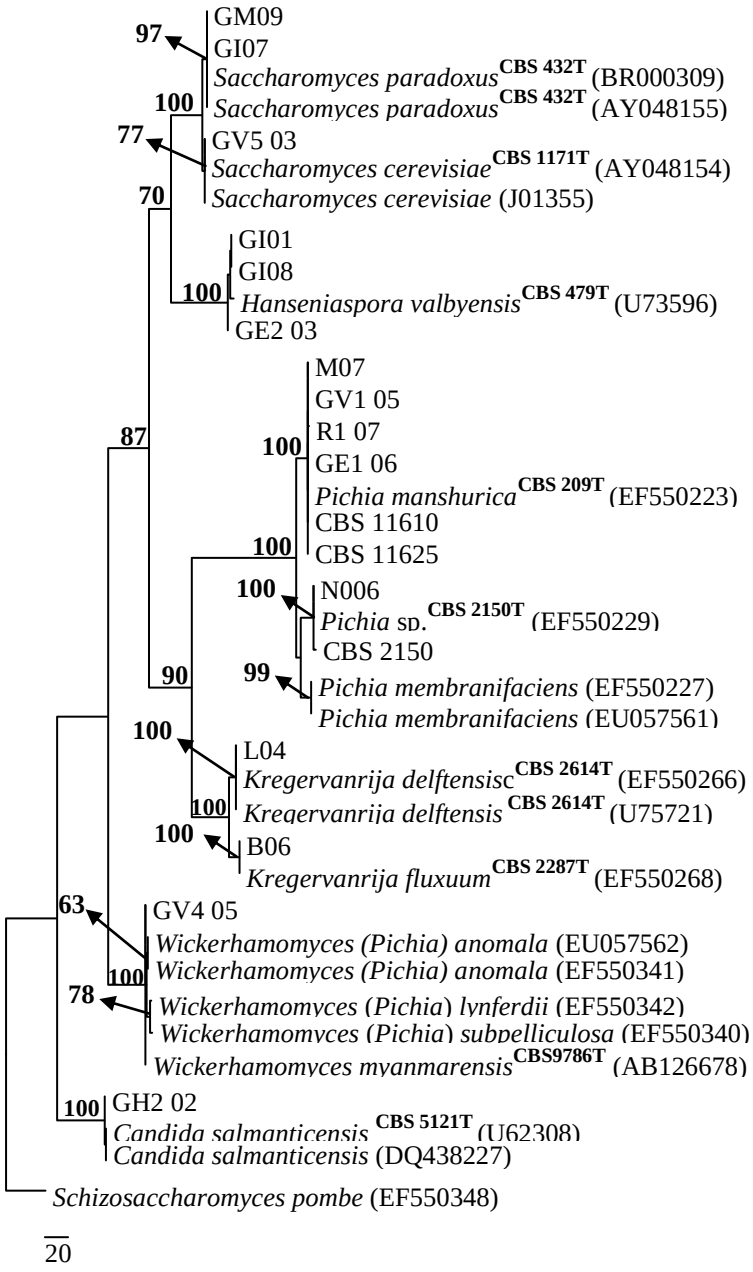

Figure 1. Phylogenetic relationships of yeast species determined from maximum parsimony analysis of gene sequences from LSU rDNA. Consistency index (CI) = 0.680; homoplasy index (HI) = 0.320; retention index (RI) = 0.935; rescaled consistency index = 0.636.Bootstrap values (>50%) calculated from 1000 replications are given in the branch node. *Schizosaccharomyces pombe* was used as an outgroup.

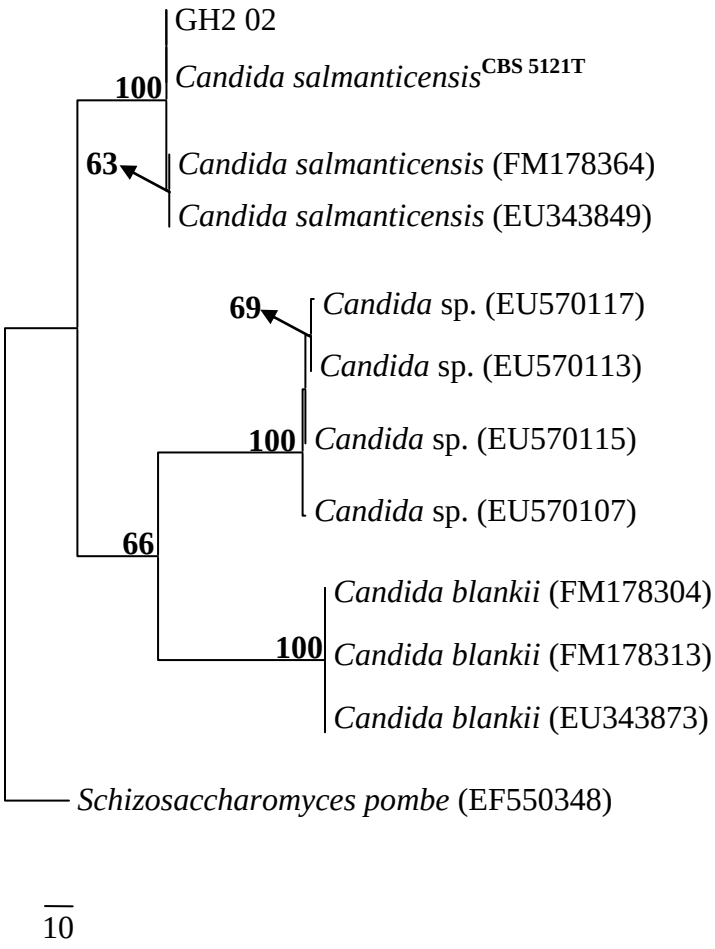

Figure 2. Phylogenetic relationships of *Candida* species determined from maximum parsimony analysis of gene sequences from ITS domains. Consistency index (CI) = 0.908; homoplasy index (HI) = 0.092; retention index (RI) = 0.957; rescaled consistency index = 0.869. Bootstrap values (>50%) calculated from 1000 replications are given in the branch node. *Schizosaccharomyces pombe* was used as an outgroup.

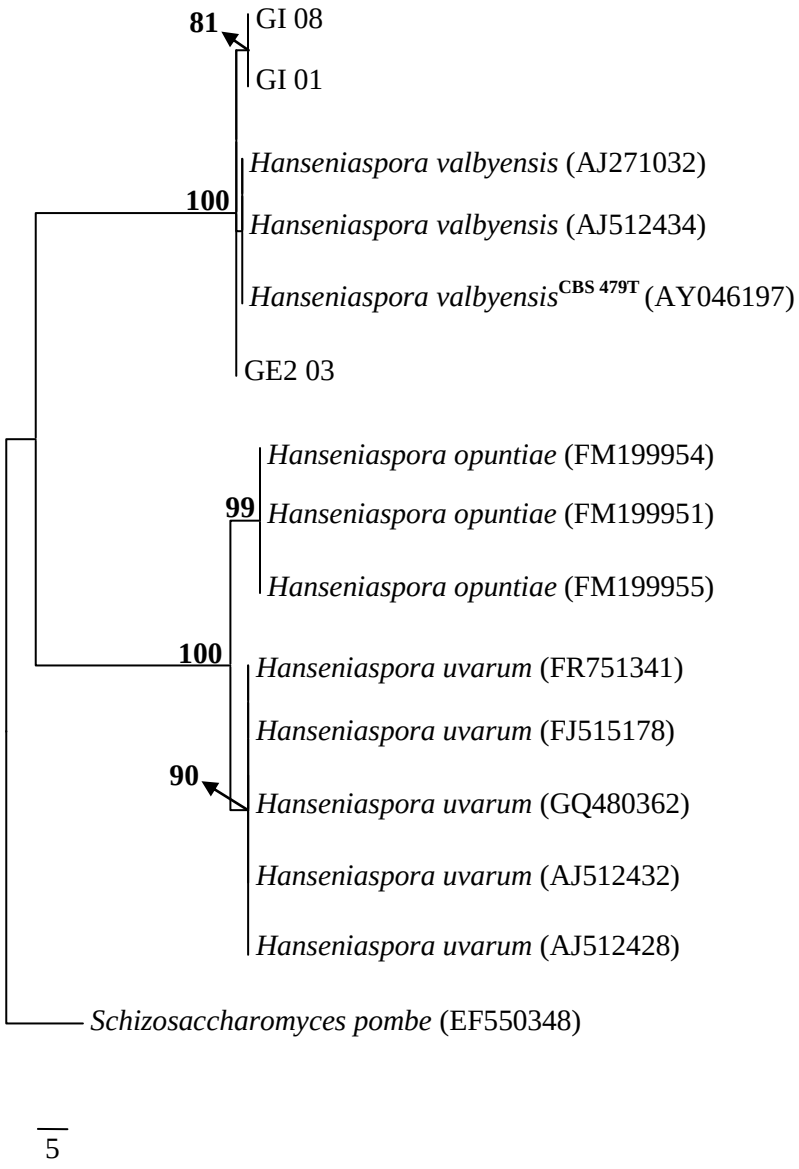

Figure 3. Phylogenetic relationships of *Hanseniaspora* species determined from maximum parsimony analysis of gene sequences from ITS domains. Consistency index (CI) = 0.969; homoplasy index (HI) = 0.032; retention index (RI) = 0.992; rescaled consistency index = 0.961. Bootstrap values (>50%) calculated from 1000 replications are given in the branch node. *Schizosaccharomyces pombe* was used as an outgroup.

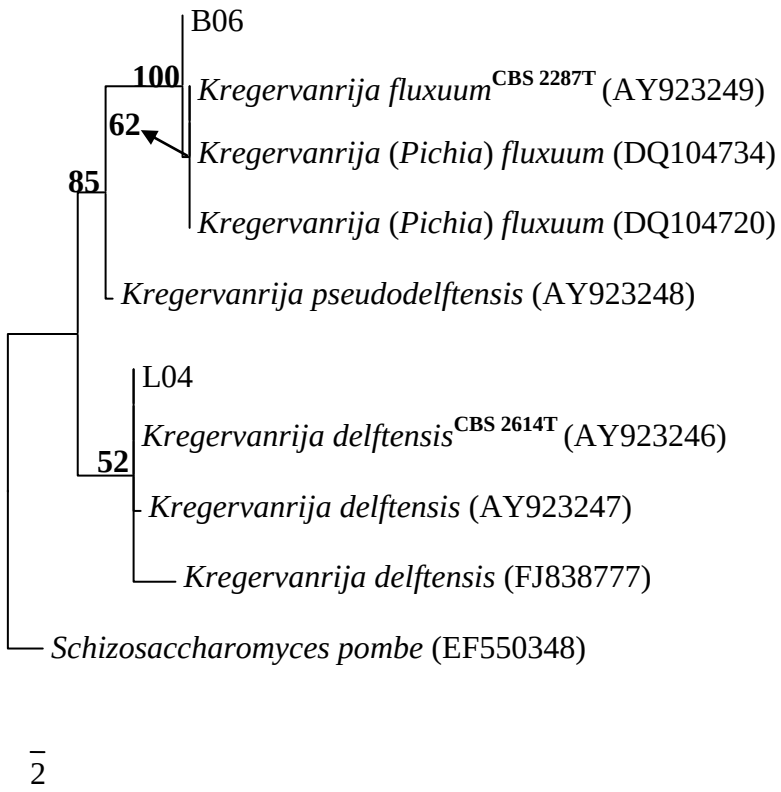

Figure 4. Phylogenetic relationships of *Kregervanrija* species determined from maximum parsimony analysis of gene sequences from ITS domains. Consistency index (CI) = 0.809; homoplasy index (HI) = 0.191; retention index (RI) = 0.885; rescaled consistency index = 0.716. Bootstrap values (>50%) calculated from 1000 replications are given in the branch node. *Schizosaccharomyces pombe* was used as an outgroup.

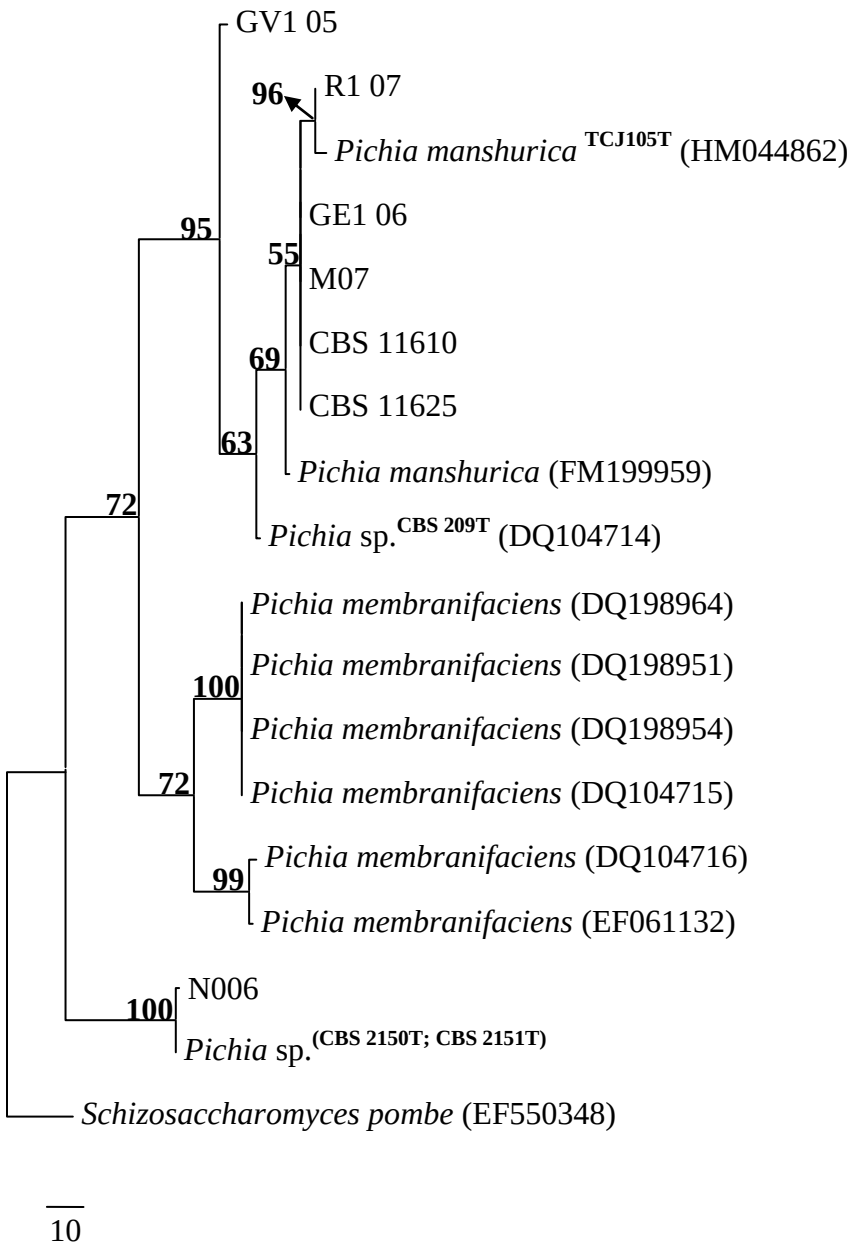

Figure 5. Phylogenetic relationships of *Pichia* species determined from maximum parsimony analysis of gene sequences from ITS domains. Consistency index (CI) = 0.849; homoplasy index (HI) = 0.151; retention index (RI) = 0.916; rescaled consistency index = 0.778. Bootstrap values (>50%) calculated from 1000 replications are given in the branch node. *Schizosaccharomyces pombe* was used as an outgroup.

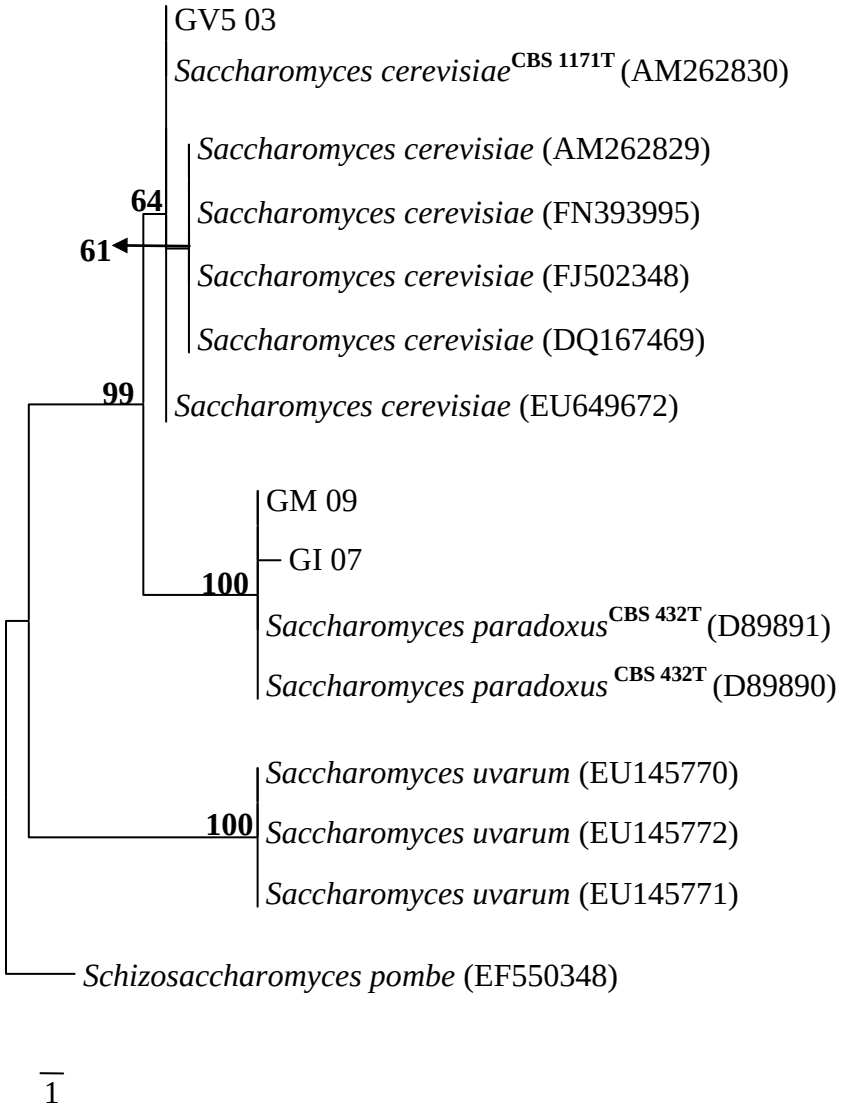

Figure 6. Phylogenetic relationships of *Saccharomyces* species determined from maximum parsimony analysis of gene sequences from ITS domains. Consistency index (CI) = 1.000; homoplasy index (HI) = 0.000; retention index (RI) = 1.000; rescaled consistency index = 1.000. Bootstrap values (>50%) calculated from 1000 replications are given in the branch node. *Schizosaccharomyces pombe* was used as an outgroup.

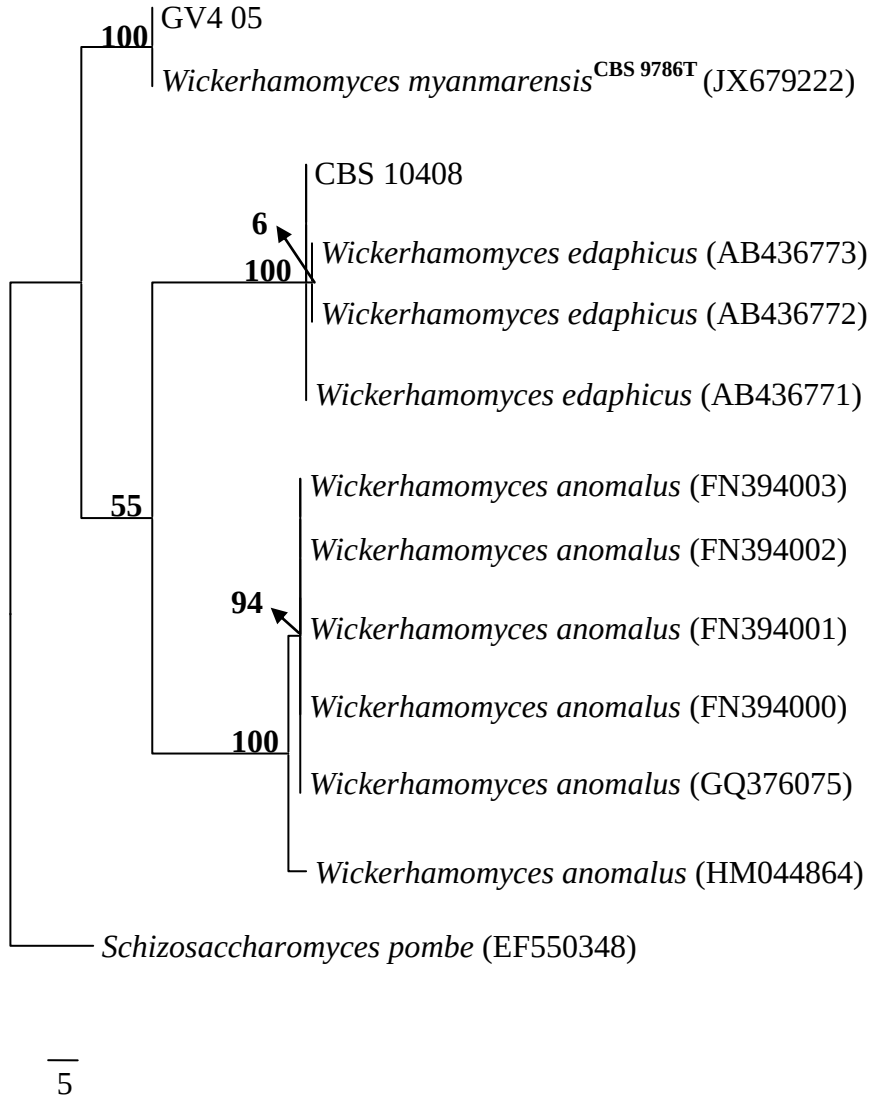

Figure 7. Phylogenetic relationships of *Wickerhamomyces* species determined from maximum parsimony analysis of gene sequences from ITS domains. Consistency index (CI) = 0.905; homoplasy index (HI) = 0.095; retention index (RI) = 0.957; rescaled consistency index = 0.866. Bootstrap values (>50%) calculated from 1000 replications are given in the branch node. *Schizosaccharomyces pombe* was used as an outgroup.

CHAPTER 5

Artificial diets and field assays suggest potential *Eucalyptus* host range of *Coryphodema tristis* (Leptidoptera: Cossidae)

ABSTRACT

Coryphodema tristis is a native wood-boring moth, found in South Africa associated with a wide range of hosts residing in at least eight plant families. In 2004, it was discovered on *Eucalyptus nitens* and became a potential threat to the South Africa forestry industry. Extensive surveys suggested that populations are currently limited to *E. nitens* (Myrtaceae), despite the distributional overlap with other closely related *Eucalyptus* spp. We used laboratory experiments to consider whether xylem from different *Eucalyptus* spp. as an ingredient in artificial media would reflect host suitability. In addition, field experiments were undertaken to assess whether *Eucalyptus* spp. other than *E. nitens* could be successfully colonized when larvae of *C. tristis* were placed on the bark surface. Laboratory experiments showed that the performance of the larvae fed on artificial diets prepared from *E. nitens* was better than on other diets prepared using wood of *E. dunnii*, *E. smithii*, and the hybrid of *E. nitens* x *E. grandis*. In the field, larvae could establish, feed and develop only when placed on bark of *E. nitens*, and not on *E. dunnii*, *E. smithii*, and the hybrid of *E. nitens* x *E. grandis*. The latter species all produced copious amounts of kino when larvae began to feed, which was not the case for feeding, *E. nitens*. These results suggest that the restricted host range on *Eucalyptus* is not due to major physiological constraints, and that tree defenses play at least some role in restricting *C. tristis* to *E. nitens*.

Key words: *Coryphodema tristis,* defense mechanism, *Eucalyptus,* host suitability, kino

INTRODUCTION

Phytophagous insects encounter host resources that vary in nutritional quality, as well as the presence, quantity and quality of physical and chemical defenses. Many classes of herbivorous insects are remarkably conservative in their selection of host plant diet (Strong *et al*. 1984; Bernays & Chapman 1994; Futuyma 1991; Bernays & Chapman 1994). Less than 10 % of herbivorous insects are thought to be truly polyphagous i.e. feeding across plant families or even orders (Chapman 1982; Price 1983; Bernays 1984). Host utilization is driven by female oviposition choice and larval survival (and in some cases larval choice). Considerable research during the course of several decades has clearly shown that plant chemistry and physical defenses, nutritional quality of consumed tissues, and plant mediated risk of natural enemy attack play an important role in both choices and outcomes related to host use (Feder 1995; Becerra 1997; Mira & Bernays 2002; Murphy 2004; Swigonova & Kjer 2004; Kergoat *et al*. 2005). In generalist insects where female host choice is not nearly as strict, relative to monophagous or oligophagous relatives, larval responses to plant traits may take on proportionally greater importance with respect to determining host range (Fraser & Lawton 1994; Novotny *et al*. 2003; Bertheau *et al*. 2010).

Amongst the insects, wood-feeding or xylophagous taxa are well represented in the Orders Coleoptera and Lepidoptera (Solomon 1995). The larvae in some species of these orders are known to feed during most of their life-cycle within woody tissues and may take several years to complete larval development (Hay & Morris 1970). Typically for lepidopteran larvae, host plant choice is almost entirely the purview of ovipositing females, especially where larval stages have limited mobility (Scheirs *et al*. 2000; Agosta & Klemens 2009; Garcia-Robledo *et al*. 2010). This may be especially acute for species that live and feed internally in plant tissues, such as leaf

miners and xylophagous insects, where larvae must cope with whatever host tissue they encounter.

Natural food plants of phytophagous insects contain nutrients, proteins and carbohydrates necessary for insect growth, together with a diverse array of metabolites often selected for defense against herbivory. Plants use different strategies to defend themselves against herbivorous insects (Gatehouse 2002; Eyles *et al*. 2010). Defensive chemistry influences diet quality as well as host selection, which are important factors underpinning host range (Franceschi *et al*. 2005). Where detailed studies have been performed, phytophagous insects ability to colonize a novel host has shown to be linked to the evolution of mechanisms to overcome plant defenses (Schluter 2000; Singer & Stireman 2005). Such behavioral or biochemical adaptations by phytophagous insects to avoid these chemicals (Dussourd 1993), or their efficient detoxification systems (Brattsten 1992), are costly and have been considered to be the main reason for the widespread specificity in plant use among herbivorous insects (Berenbaum & Zangerl 1992).

The cossid moth *Coryphodema tristis* is native in South Africa (Degefu *et al*. 2012) and has been known to infest at least eight non-native woody plants including quince, vine and apple in the Cape Province for more than a century (Petty 1917; Hoppner 1994). The host range of this insect is reported to include a number of plant families, including Rosaceae, Combretaceae, Malvaceae, Myoporaceae, Scorphulariaceae, Ulmaceae and Vitaceae (Kroon 199). It was reported for the first time from the Myrtaceae when it was discovered infesting *Eucalyptus nitens* in the Mpumalanga province in 2004 (Gebeyehu *et al*. 2005; Boreham 2006). Since then, the insect has spread throughout the planted range of *E. nitens* in that region. Noticeably, many of the other cold-tolerant *Eucalyptus* grown commercially in this region for longer periods than *E. nitens* (see

Poynton 1979) have not been infested by *C. tristis*. The sudden appearance and unusual host specificity of *C. tristis* restricted to a single species of *Eucalyptus*, despite its otherwise broad host range, is intriguing and remains to be understood.

A host range expansion similar to that of *C. tristis* has been reported for the Chilean cossid moth, *Chilecomadia valdiviana*. Interestingly, this insect inter also primarily infests *E. nitens* (Cerda 1996; Degefu *et al*. 2013), but it has also been observed on *E. dunnii* and *E. camaldulensis* (see Lanfranco & Dungey 2001). These occasional attacks on other *Eucalyptus* species raise the question whether, given sufficient time, *C. tristis* might also infest other *Eucalyptus* species in South Africa. An expansion of the host range to other *Eucalyptus* species, some more widely planted than *E. nitens*, would pose a significant threat to the sustainability of the forestry industry in this country.

The aim of this study was to investigate aspects of host suitability of *Eucalyptus* species for *C. tristis*, which could possibly have influenced the sudden appearance and restricted host range of this pest. Specifically, we considered the effect of variation in the source of wood from different *Eucalyptus* species on nutrient assimilation and growth of *C. tristis* larvae in culture. In addition, using artificial exposure in the field, we also examined the ability of *C. tristis* larvae to develop within living tissue of various *Eucalyptus* spp.

MATERIALS AND METHODS

Diet preparation

An artificial diet for *C. tristis* was modified based on those prepared for *Capnodis* spp. (Gindin *et al*. 2009) and *Spodoptera exigua* (Elvira *et al.* 2010). The diet was prepared by grinding small wood blocks from different species of *Eucalyptus*, including *E. nitens*, *E. dunnii*, *E. smithii* and a hybrid of *E. nitens* x *E. grandis* to powder. This powder (30 g) was sterilized for about 30 minutes in 400 ml of distilled water. The larger woody particles were separated from the rest of the homogenate using a course sieve (0.5 x 0.5 mm in size) after sterilization. The other diet ingredients, including sucrose, Wesson's salt mix, dry yeast/torula, methyl paraben and agar were mixed with the sterilized woody powder, and the volume adjusted to 400 ml (Part A of the diet in Table 1). The contents were thoroughly mixed before being autoclaved. A second part (part B) of the diet, including water, casein, Sorbic acid, Choline chloride and Vanderzant vitamin mix, was prepared without sterilization on a magnetic stirrer, and added to part A of the medium after it was autoclaved. The two portions of the diet (A and B) were thoroughly mixed at 60 °C. Cellulose (termed part C of the diet) was then added and the completed diets were blended using a household blender (Salton). The diet was poured into either 55 or 90 mm diameter Petri dishes, depending on the experiment for which it was required, to form a layer 3-4 mm deep.

Larval feeding performance on different species of Eucalyptus

Two sets of experiments were undertaken to assess larval feeding performance on different *Eucalyptus* spp. The first set of experiments was aimed at assessing efficiency of conversion and approximate digestibility of the different *Eucalyptus* species. These experiments were run over

short periods of 96 hrs. A second set of experiments aimed at assessing the long-term effect of feeding on xylem of non-host *Eucalyptus* spp. were run over six weeks.

Efficiency of conversion and digestibility of *Eucalyptus* spp.

For each treatment, one larva was placed in each of nine 55 mm Petri dishes containing the diets described above and made up using wood of the four *Eucalyptus* spp. The larvae used weighed approximately 88.3 ± 45.5 mg and were randomly separated with respect to size amongst the different treatments. Larvae were starved for three hours to clear their digestive tracts, after which they were weighed before being placed on the different diets. The larvae used in the treatments were allowed to feed on the diets for 96 hours. To estimate pre-treatment dry mass of larvae, a larva of similar size and wet mass was dried and weighed for each replicate.

Larvae were placed on the respective artificial diets in Petri dishes that were held in a plastic containers (26 cm width x 26 cm length x 13 cm height) covered with aluminum foil to create a dark feeding environment, and kept at 25 ± 1 °C. The feces produced by the larvae were separated from the diet every 24 hours. At the end of the experimental period (96 hours) the dry mass of the feces, as well as the remaining artificial diet was weighed. The larvae were separated from the diet for 2 - 3 hours to clear their digestive tract of feces, after which they were dried.

To calculate nutritional indices (Waldbauer 1964; Slansky & Scriber 1985) such as mass gain, food consumption and fecal production by the *C. tristis* larvae, all samples were oven dried for 48 hours at 60 °C. Nutritional indices were calculated for food ingested, food digested, relative growth rate, relative consumption rate, approximate digestibility, ingested food conversion efficiency and digested food conversion efficiency. These indices were calculated as follows:

Food ingested = Dry weight of the food offered - Dry weight of left over food

Food digested = Dry weight of food ingested - Dry weight of excreta/feces

Relative growth rate (RGR) = Dry weight gain of larva during feeding period/Duration of feeding period (Days) × Mean dry weight of larva during feeding period

Relative consumption rate (RCR) = Dry weight of food ingested/Duration of feeding period (days) x mean dry weight of the larva during the feeding period

Approximate digestibility (AD) = (Mass of food digested/Mass of food ingested) × 100

Ingested food conversion efficiency (ECI) = (Dry mass gained by larva/Dry mass of food ingested) x100

Digested food conversion efficiency (ECD) = (Dry mass gained by larva/Dry mass of food digested) × 100 and dry weight of excreta/feces of the test larvae on diets prepared with wood from different species of *Eucalyptus*.

Long-term effects of non-host diets on larval development

The treatments consisted of diets listed above, with the diet using *E. nitens* serving as positive control. The larvae for the experiment were collected from naturally infested *E. nitens* in the field. The larvae used weighed approximately 93.3 ± 50.5 mg and were randomly separated with respect to size in the different treatments. For each treatment, five larvae were placed in 90 mm Petri dishes, each containing the various diets. Each treatment was replicated four times, thus with a total of twenty larvae per treatment. The Petri dishes were kept under the same conditions as described above, in the dark in a plastic container at 25 ± 1 °C. The experiment was conducted over six weeks. The diet was replaced once a week over the six week period. Mean measurements of mass for all the larvae were taken weekly and no larvae died during the experiment.

Since we were only able to collect larvae from *E. nitens*, larval pre-conditioning on this diet was a concern. To understand the potential influence of pre-conditioning, all larvae were moved to a diet containing *E. nitens* at week six of the experiment and measured for an additional four weeks. This approach was based on the logic that if pre-conditioning played a role, then the effects of feeding on an alternative diet for six weeks should also influence larval performance when switched back to *E. nitens*. Absence of an effect when switching back to *E. nitens* after feeding on other diets, would suggest a lower chance that pre-conditioning on *E. nitens* influenced the main experiment.

Feeding response under field conditions

A field trial was conducted at Lothair plantation (S26.31445, E30.62239) at an elevation of 1666 m.a.s.l. in the Mpumalanga province of South Africa. The trial was conducted on five selected *Eucalyptus* species (treatments) grown in this region, namely *E. macarthurii*, *E. nitens*, *E. dunnii*, *E. smithii* and the hybrid of *E. nitens* x *E. grandis*. *Eucalyptus nitens* was used as a positive control. For each treatment, three trees separated by approximately three to five meters were selected.

Larvae were collected from *E. nitens* in infested stands. Thirty larvae weighing approximately 87.6 ± 32.3 mg were placed on each tree, with three groups of 10 larvae each placed at different positions on each of the three trees of different species. Each group of 10 larvae was covered with a plastic bottle having small holes for air circulation and which was attached to the stems to prevent escape. Thus, a total of 90 larvae were used per treatment. Careful examination of the potential feeding sites was made after one week, 45 days and six months after the commencement of the treatment.

Data Analyses

Data relating to the feeding indices and trends of larval feeding performance over weeks were analyzed using SAS (2001). The various digestion parameters and feeding performance among the different treatments were analyzed using one-way analysis of variance (ANOVA) by the general linear models (GLM) procedure. Tukey's Studentized Range (HSD) test was used to determine significant differences among treatment groups. The results were presented as means ± standard error. Means were not significantly different at $P>0.05$, Tukey's Studentized Range (HSD) test.

RESULTS

Efficiency of conversion and digestibility of Eucalyptus spp.

The weight of food ingested and the relative consumption rate of *C. tristis* larvae did not differ significantly between different *Eucalyptus* (Figure 1, 2). However, the relative growth rate (RGR) of the larvae differed significantly between the different diets (F=24.53; df=3,35; p<0.0001; R^2=0.70) (Figure 3). Digestibility is one of the processes involved in food utilization, which directly affects efficiency of conversion of ingested food to biomass. Approximate digestibility (AD) was significantly affected by the diet (F=34.26; df=3,35; p<0.0001; R^2=0.76; Figure 4). Approximate digestibility of diet by larvae fed on diet made with *E. nitens* tissue was significantly higher (Tukey's HSD; Figure 4) than those of larvae fed on other diets. For *E. nitens*, AD was 80.3 % for *E. dunnii* (54 %), hybrid of *E. nitens* x *E. grandis* (40.9 %) and *E. smithii* (26.2 %) (Figure 4). Similarly, efficiency of conversion of ingested food (ECI) and digested food (ECD) were significantly affected by the diet (F=42.33; df=3,35; p<0.0001; R^2= 0.80 and F=6.40; df=3,35; p<0.0016; R^2=0.36, respectively). The value of these parameters varied from 14.31 - 59.32 % for ECI (Figure 5) and 52.16 - 74.29 % for ECD (Figure 6). Furthermore, excreta/feces from larvae fed on diets prepared from non-host *Eucalyptus* tissue was significantly affected by the diet (F=12.64; df=3,35; P<0.0001; R^2=0.54; Figure 7). The larvae fed on the diet made using *E. nitens* produced less feces than those of larvae fed on other diets (Figure 7).

Long-term effects of non-host diets on larval development

All the field-collected larvae transferred to artificial diets prepared from tissue of *E. nitens* gained weight from the inception of the trials (Figure 8). Although the average weight gain was positive for all the treatments, some larvae fed on diets made up of non-host tissue, such as *E.*

dunnii, *E. smithii*, and hybrid of *E. nitens* x *E. grandis* lost weight. There was a significant difference in weight gain of larvae in all of the feeding weeks when they were fed on artificial diets prepared from host tissues of *E. nitens* as compared with diets prepared using wood of the other *Eucalyptus* species.

In the first and second week of feeding, larval mass gained was significantly affected by diet (F=50.98; df=3,15; p<0.0001; R^2=0.93 and F=17.38; df=3,15; p<0.0001; R^2=0.81, respectively; Figure 8). In the first week, larvae fed on diets made up of *E. nitens* tissue gained significantly greater mass compared with all the other diets (Figure 8). During the second week, larvae fed on diets prepared from *E. nitens*, *E. dunnii*, and *E. smithii* gained significantly greater mass than those feeding on *E. nitens* x *E. grandis*. Similarly, in the last week, mass gain was significantly affected by diet (F=34.56; df=3,15; p<0.0001; R^2=0.90). In this week there was no significant difference in mass gained by larvae feeding on diets of *E. nitens* and *E. dunnii*, while they gained significantly greater mass than larvae on diets containing *E. smithii* and hybrid of *E. nitens* x *E. grandis* (Figure 8). Diets including the hybrid of *E. nitens* x *E. grandis* sustained the poorest larval mass gain.

Feeding response under field conditions

In all three *E. nitens* trees treated with *C. tristis* larvae, the larvae had bored through the bark, penetrating deeply into the xylem tissue. Frass from this boring could seen on the surface of the feeding holes in all three *E. nitens* trees. Furthermore, there was a distinct staining of the wood beneath the bark, which is another characteristic feature of *C. tristis* infestation. There was no resin exudation associated with *C. tristis* larval infestation of the *E. nitens* trees, while this

symptom was commonly observed in all the other *Eucalyptus* spp. used in this study. Living larvae were recovered only from the three *E. nitens* trees.

None of the *Eucalyptus* spp. other than *E. nitens* treated with *C. tristis* larvae showed any evidence of a successful infestation after six months. However, in all treated trees, there was evidence of feeding damage and frass. Resin production was limited to *E. macarthurii* where it was observed at 45 days and resin exudation was observed after six months on all but the *E. nitens* trees when the wounds were opened to inspect the trees for surviving larvae.

DISCUSSION

The laboratory study using artificial diets prepared from xylem tissue of different *Eucalyptus* species clearly revealed that the xylem of different species of *Eucalyptus* has a significant effect on *C. tristis* growth and nutrition. Specifically, the feeding indices considered in this study showed very distinct differences in assimilation efficiency for diets prepared from wood tissues of *E. nitens*, *E. dunnii*, *E. smithii*, and a hybrid of *E. nitens* x *E. grandis*. Furthermore, the success of rearing wild-caught larvae under laboratory conditions was shown to have potential to understand nutrient relations and conditions necessary for the growth of *C. tristis*. Field treatments with early instar larvae on the same species of *Eucalyptus* from which diets were prepared, but also including the additional species *E. macarthurii*, showed that *C. tristis* larvae survived and developed only on *E. nitens*. These results help to explain the sudden appearance and restricted host range of *C. tristis* in South Africa, and they contribute to the knowledge on host traits that influence herbivore efficiency and fitness.

Larvae fed on diets containing pulverized *E. nitens* xylem tissue gained greater mass than those fed on other diets. This difference was attributable to the higher conversion efficiencies of ingested food (ECI) and digested food (ECD). Whether this is due to inherent differences in xylem nutritional content or digestibility or to pre-conditioning of larvae (collected from *E. nitens*) is unknown. Preference for or performance on different hosts is very often influenced by rearing conditions, and natal and/or maternal host plant effects can play an important role in these processes (Deboer & Hanson 1984; Karowe 1989; Bernays & Weiss 1996). Controlling this aspect in experiments such as those performed in the present study is difficult with *C. tristis*, This is because source individuals are readily collected only on *E. nitens*, and rearing from eggs would likely not be possible due to the long life-cycles of these insects.

Larvae fed and grew on diets including all species of *Eucalyptus* tested. This suggests that there is no inherent barrier to the colonization of additional novel hosts within the genus, at least from a nutritional perspective. However, growth rates were lower on all species tested as compared to *E. nitens*. Relative consumption rates (RCR) were similar across diets and we, therefore, found no evidence of food avoidance or alternatively, for compensatory feeding. The reduced growth of larvae on "non-host" tissue is primarily attributable to reduced digestibility (AD) and post-digestive effects (ECD). This suggests that a smaller proportion of consumed diet from non-host *Eucalyptus* wood was converted to larval biomass than from the *E. nitens* diet. This is because the indigestible material ingested by the insects could influence post-ingestive physiological process of the insect with the effect that the approximate digestibility is affected (Camara 1997). Furthermore, the high faecal levels across diets with tissues of the non-host *Eucalyptus* species strongly suggest that a high proportion of the food ingested was excreted (Zanotto *et al*. 1993).

It is possible that pre-conditioning of *C. tristis* larvae on *E. nitens* might have influenced subsequent suitability of diets made with other species of *Eucalyptus*. In order to examine the effects of pre-conditioning, we moved larvae from all diet treatments back to *E. nitens*. The assumption (supported by the increased growth rates on all novel diets at week six of the experiment) was that larvae would have adapted to the treatment diets and that these effects should carry over to influence growth on the original host, if this were an important effect. The result was that larvae all grew equally well on *E. nitens* irrespective of prior feeding treatments. While this does not rule out pre-conditioning or maternal effects, it suggests that at the very least, these are not the only or dominant factors affecting host tissue assimilation.

The suitability and acceptability of the different diets by *C. tristis* larvae varied over six weeks in the longer feeding experiment. The first week of feeding revealed that *C. tristis* larvae respond negatively to artificial diets prepared from non-host tissues as compared with the diets from the host plant, *E. nitens*. This was clear from the assimilation efficiency of the larvae that was higher on *E. nitens* than on non-host diets. This finding is supported by other studies where larvae that switch host plants show a reduction in growth rate and food utilization (Jermy *et al*. 1968; Scriber 1979, 1982; Deboer & Hanson 1984; Karowe 1989; Bernays & Weiss 1996). However, the performance of *C. tristis* on the non-host diets improved later in the experiment, suggesting that the rate or efficiency of feeding on host tissue by phytophagous insects should be measured over the entire lifetime of the herbivore, because performance may increase or decrease at different stages of the life-cycle (e.g. Scheirs *et al*. 2000). The adaptation over time may have been due to the ability of *C. tristis* larvae to increase utilization and conversion efficiency of the different diets, as has been shown for other insects (Papaj 1986; Vet & Dicke 1992).

The field experiment conducted to evaluate the ability of early-instar larvae to successfully colonize trees of five different *Eucalyptus* species provided support for the view that barriers may exist to further host switching by this insect. None of the larvae successfully colonized species other than *E. nitens*. The exact cause for this is unknown and could reflect resource quality and/or plant defences, among other possibilities (e.g., unfavorable bark microstructure, difficulties in penetrating the bark or wood, or a higher incidence of natural enemies). Many of the trees in the trial were clearly damaged by the larvae and produced comparatively large volumes of kino when compared to that seen on *E. nitens*. Kino exudation, whether induced or constitutive, is a general defense mechanism against pests and pathogens that penetrate the bark surface in many tree species (Wagner *et al*. 2002).

Systematic observations of potential infestation sites showed very little evidence of kino production on *E. nitens*, while kino exudates were recorded on all the other species of *Eucalyptus* used for this study. In a separate, similar experiment using cut logs rather than living trees, larvae formed infestations on all host species tested (authors unpublished). This suggests that defenses, such as kino, in the living trees play a role in host susceptibility. Kino production is highly variable in *Eucalyptus* (Paine *et al*. 1995) as is the quantity and quality of toxins present in this resin (Wagner *et al*. 2002; Franceschi *et al*. 2005; Keeling & Bohlmann 2006). For example, infestation of eucalypts in Australia by the wood moth *Aenetus virescens* is often aborted due to heavy gum flows (Alma 1977; Withers 2001). Similarly, native borers such as *Platypus* spp. (Milligan 1979) that infest eucalypts grown near native forests are also usually abortive (Withers 2001).

This study revealed that *C. tristis* performs significantly better when larvae are fed on diets made of *E. nitens* rather than other non-host *Eucalyptus* spp., probably because of higher nutritional value obtained from *E. nitens*. This preliminary study represents a first step towards understanding host use in this relatively poorly studied insect. Future studies including native hosts of *C. tristis* with a more comprehensive assessment of physical and chemical properties, both putative defenses and of wood tissue, will provide further knowledge on the factors influencing this recent host range expansion.

From a management perspective, it is difficult to say whether eucalypt growers should anticipate finding *C. tristis* on a wider range of planted *Eucalyptus* species. On the one hand, there appear to be no fundamental physiological constraints that preclude *C. tristis* from feeding on other species. This is what would be expected based on its generalist nature. But the process of colonization is somewhat impeded, perhaps in part due to tree defenses. It is not difficult to

imagine that successful colonization could occur, at which point the selection for adaptations to additional novel hosts could be rapid (Janz & Nylin 2008). Selecting for traits of lower nutritional value and higher resistance could be a valuable approach for the management of *C. tristis*, to the degree that such traits do not trade off with growth, yield and fiber quality.

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TABLE

Table 1. Artificial diet prepared including woody tissues of different species of *Eucalyptus*.

Steps	Ingredients	Amount of diet component
Part A	Water	400 ml
	Sucrose	16 g
	Wesson's salt mix	3.75 g
	Dry yeast/torula	30 g
	Methyl paraben	0.60 g
	Agar	9.8 g
	Host plant powder	30 g
Part B		
	Water	100 ml
	Casein	17 g
	Sorbic acid	1.2 g
	Choline chloride	0.47 g
	Vanderzant vitamin mix	2.8 g
Part C	Cellulose	140 g

FIGURES

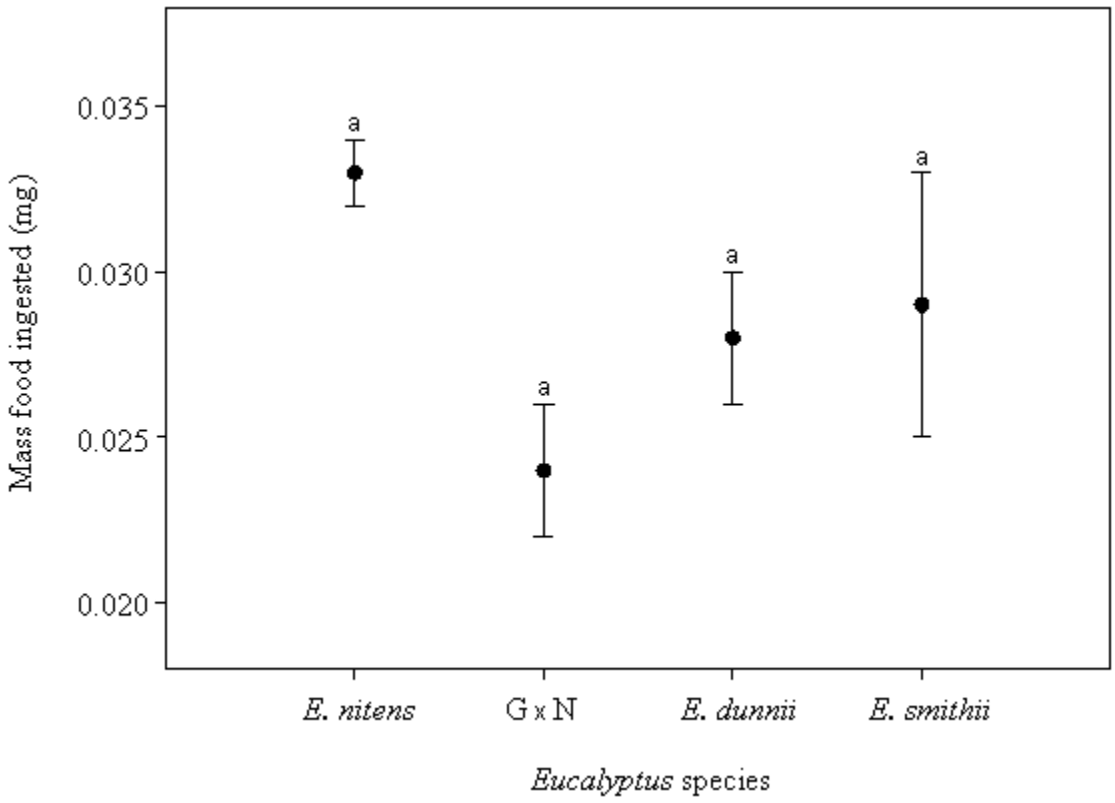


Figure 1. Mass of food ingested by the larvae of *C. tristis* feed on diets prepared from pulverized xylem tissues of different *Eucalyptus* species. Means with different letters are significantly different at $p<0.01$, at Tukey's Studentized range (HSD) test. GN: means hybrid of *E. grandis* x *E. nitens*.

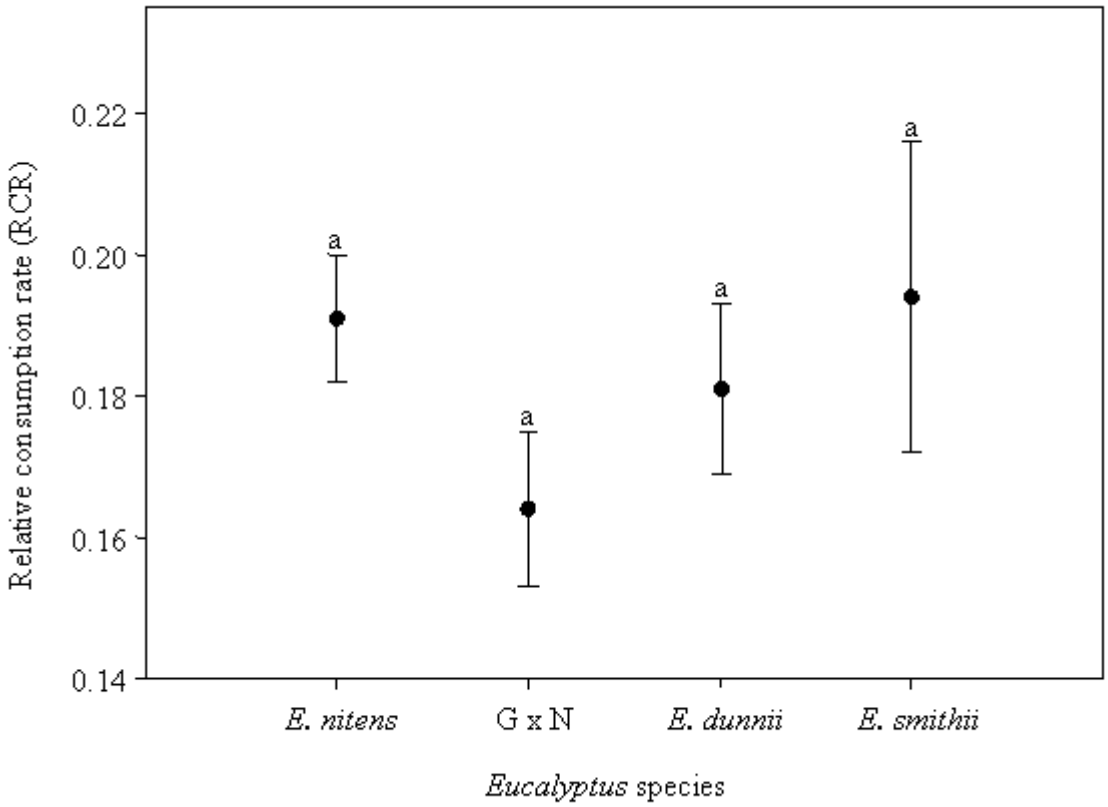


Figure 2. Relative consumption rate (RCR) by the larvae of *C. tristis* feed on diets prepared from pulverized xylem tissues of different *Eucalyptus* species. Means with different letters are significantly different at $p<0.01$, at Tukey's Studentized range (HSD) test. G x N: means hybrid of *E. grandis* x *E. nitens*.

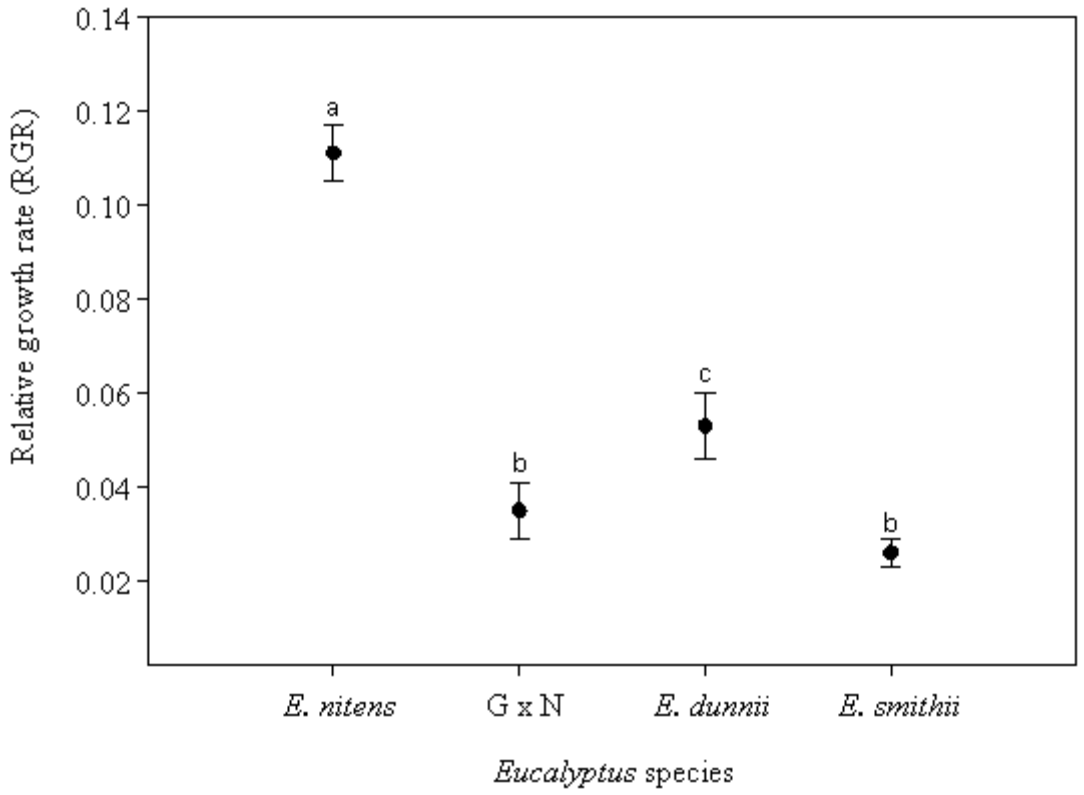


Figure 3. Relative growth rate (RGR) by the larvae of *C. tristis* feed on diets prepared from pulverized xylem tissues of different *Eucalyptus* species. Means with different letters are significantly different at $p<0.01$, at Tukey's Studentized range (HSD) test. G x N: means hybrid of *E. grandis* x *E. nitens*.

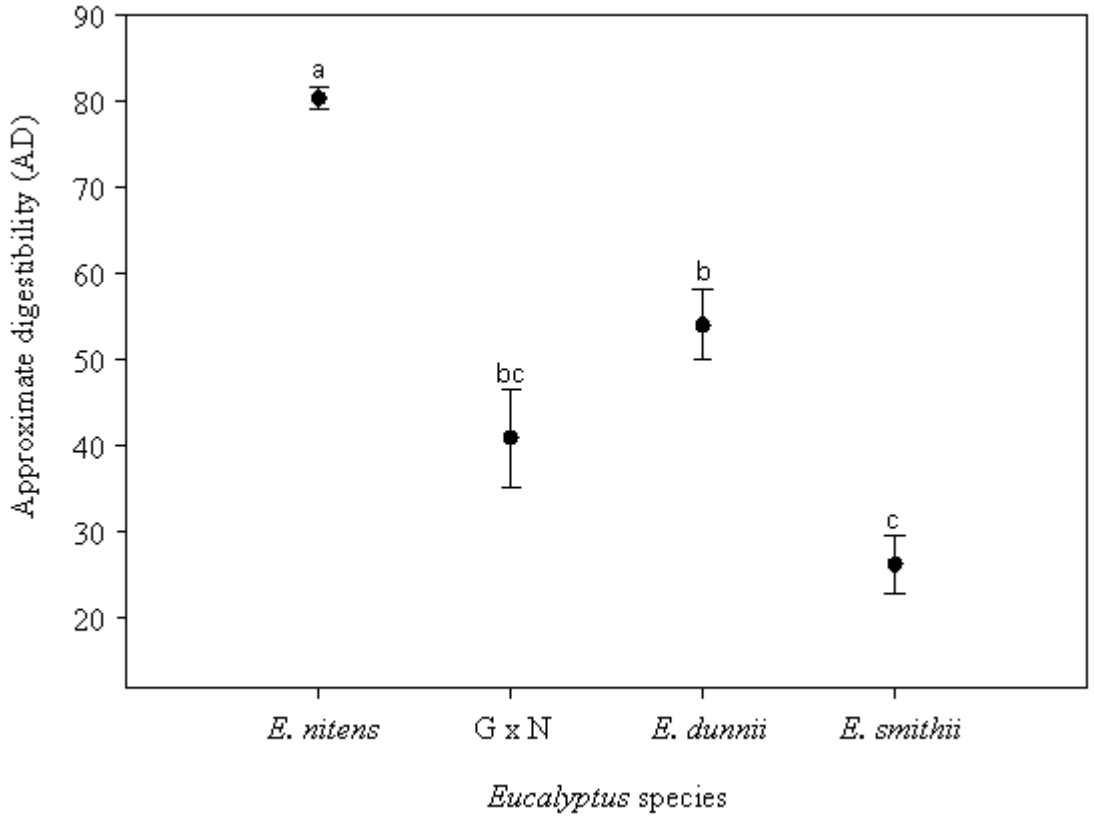


Figure 4. Approximate digestibility (AD) by the larvae of *C. tristis* feed on diets prepared from pulverized xylem tissues of different *Eucalyptus* species. Means with different letters are significantly different at $p<0.01$, at Tukey's Studentized range (HSD) test. G x N: means hybrid of *E. grandis* x *E. nitens*.

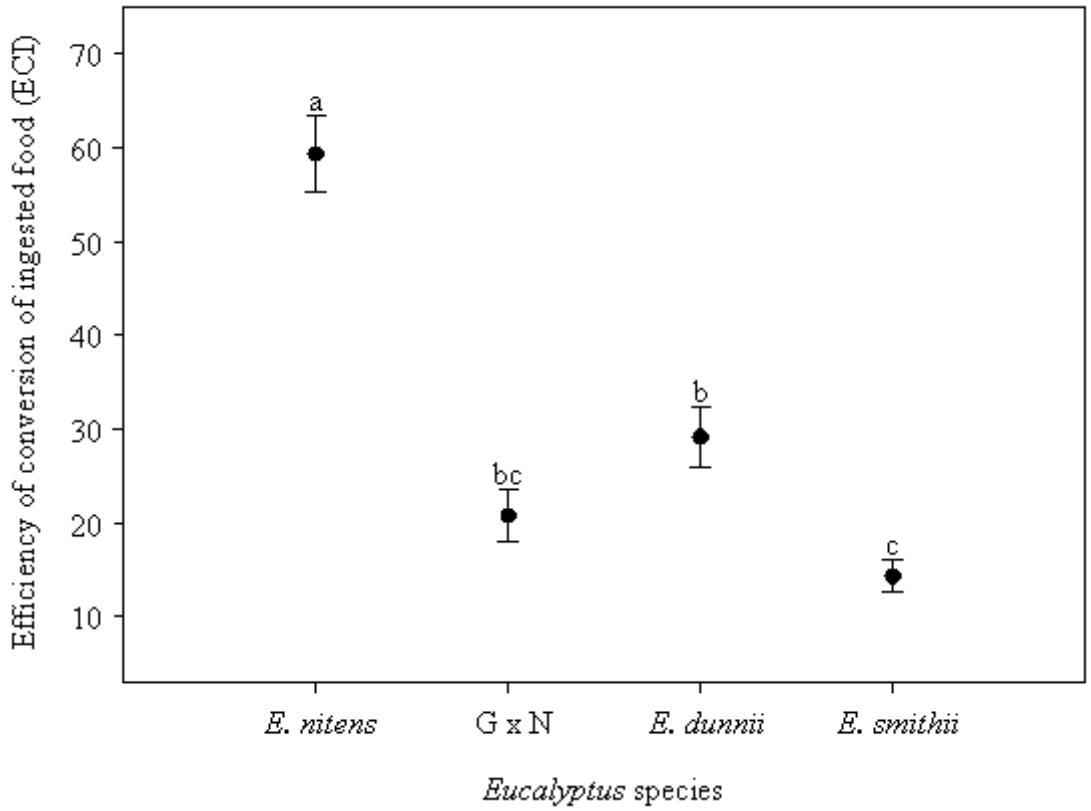


Figure 5. Efficiency of conversion of ingested food (ECI) by the larvae of *C. tristis* feed on diets prepared from pulverized xylem tissues of different *Eucalyptus* species. Means with different letters are significantly different at $p<0.01$, at Tukey's Studentized range (HSD) test. G x N: means hybrid of *E.* x *E. nitens*.

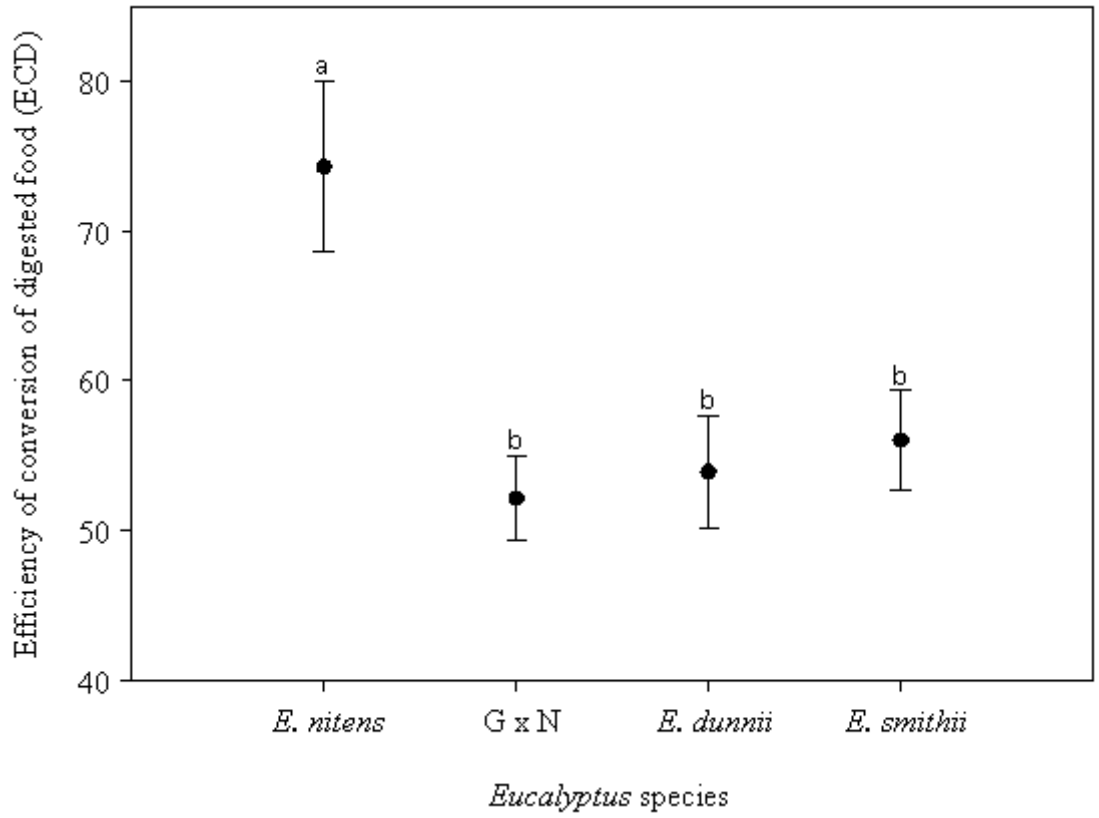


Figure 6. Efficiency of conversion of digested food (ECD) by the larvae of *C. tristis* fed on diets prepared from pulverized xylem tissues of different *Eucalyptus* species. Means with different letters are significantly different at $p<0.01$, at Tukey's Studentized range (HSD) test. G x N: means hybrid of *E. grandis* x *E. nitens*.

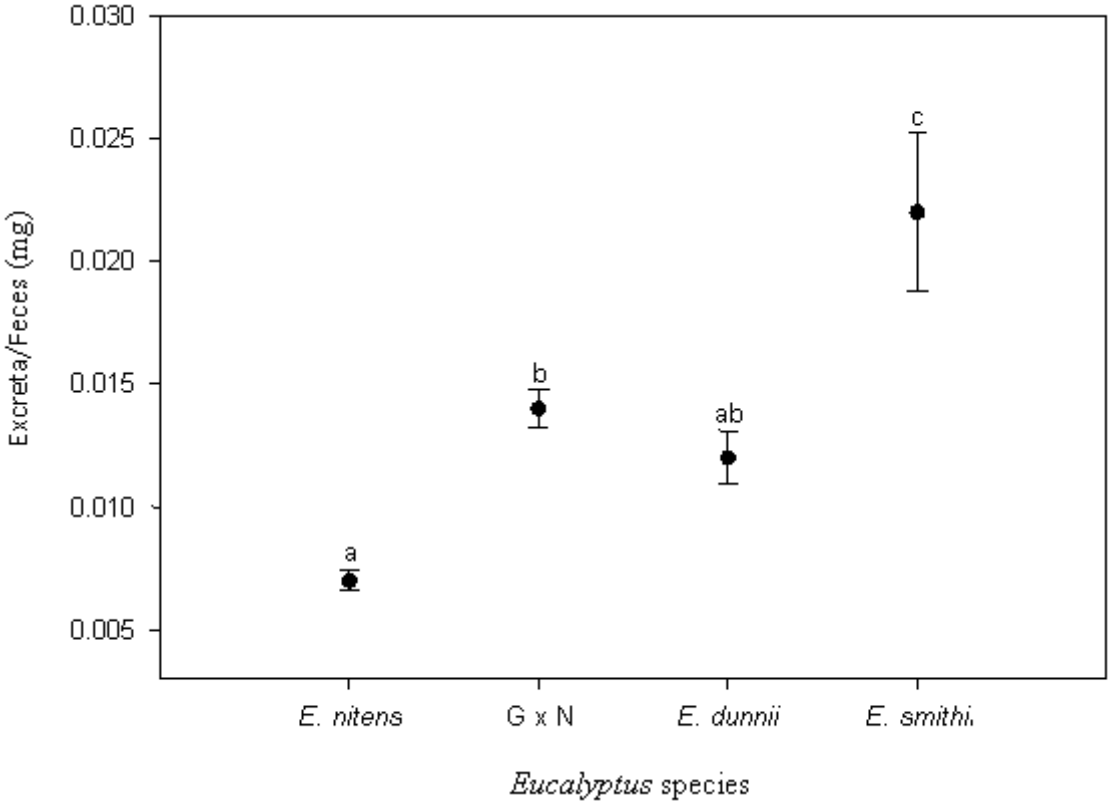


Figure 7. Dry mass of excreta/feces by the larvae of *C. tristis* fed on diets prepared from pulverized xylem tissues of different *Eucalyptus* species. Means with different letters are significantly different at $p<0.01$, at Tukey's Studentized range (HSD) test. G x N: means hybrid of *E. grandis* x *E. nitens*.

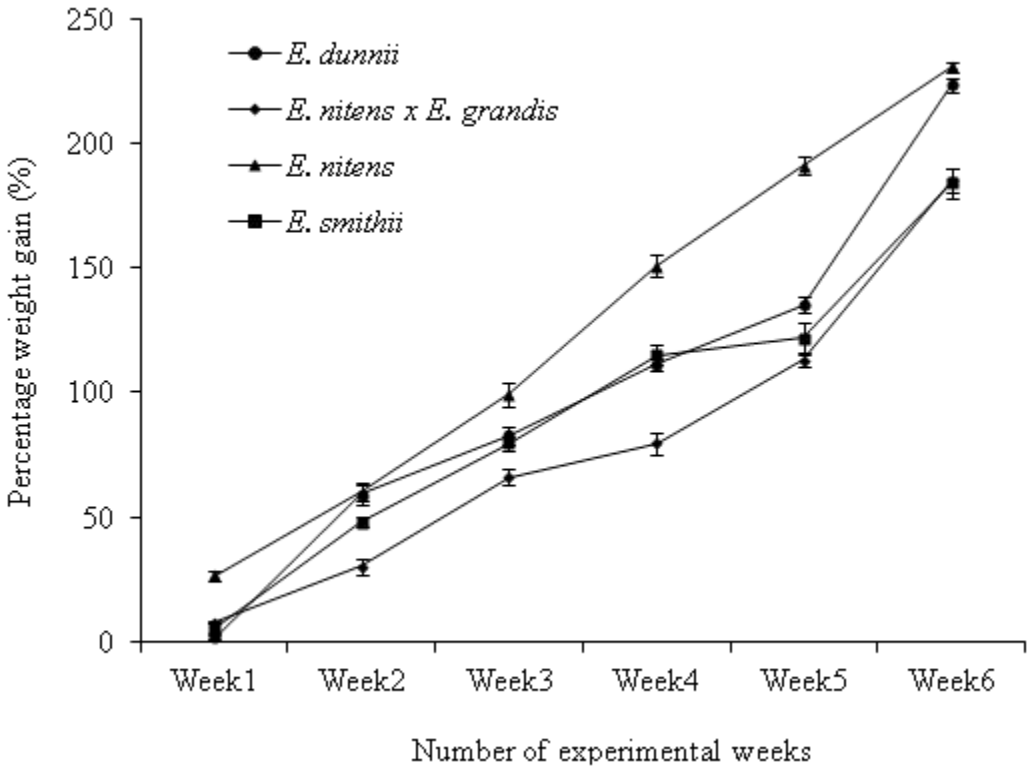
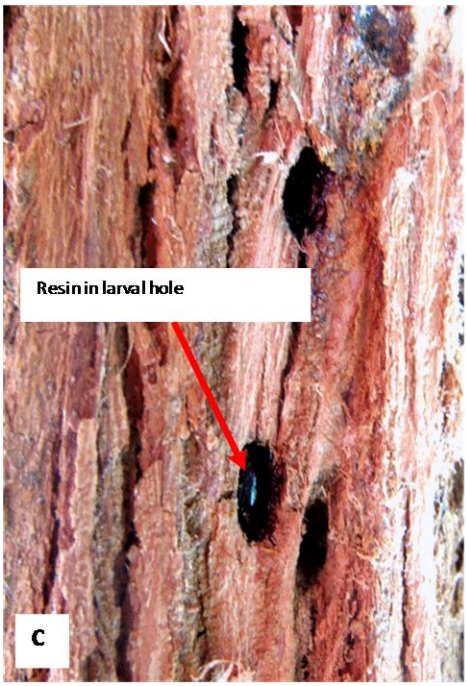
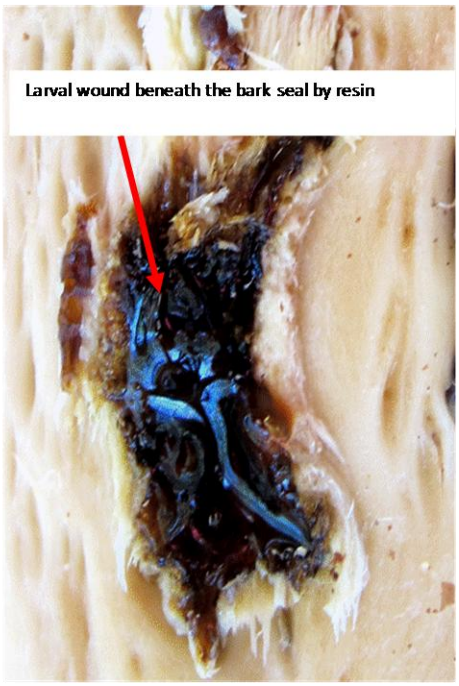
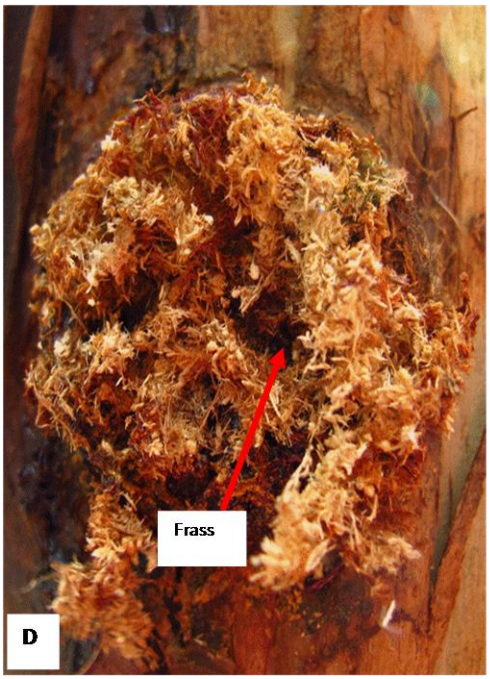
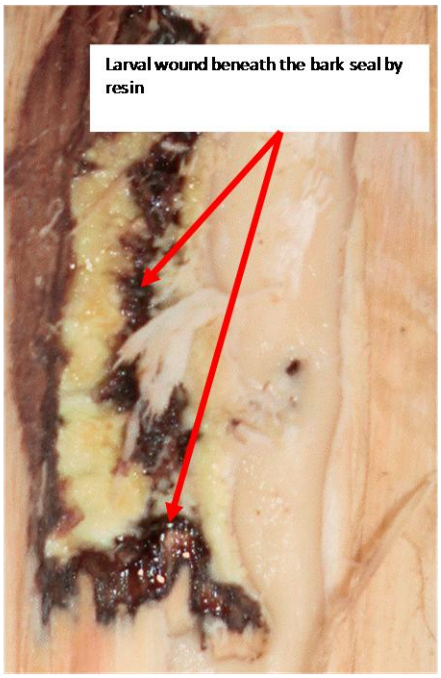


Figure 8. Influence of *Eucalyptus* spp. on percentage weight gain of *Coryphodema tristis* larvae. Average mass in milligram per five larvae per replication was measured for each treatment and mass gain was determined relative to pretreatment mass of the larvae in all the replication and treatment.









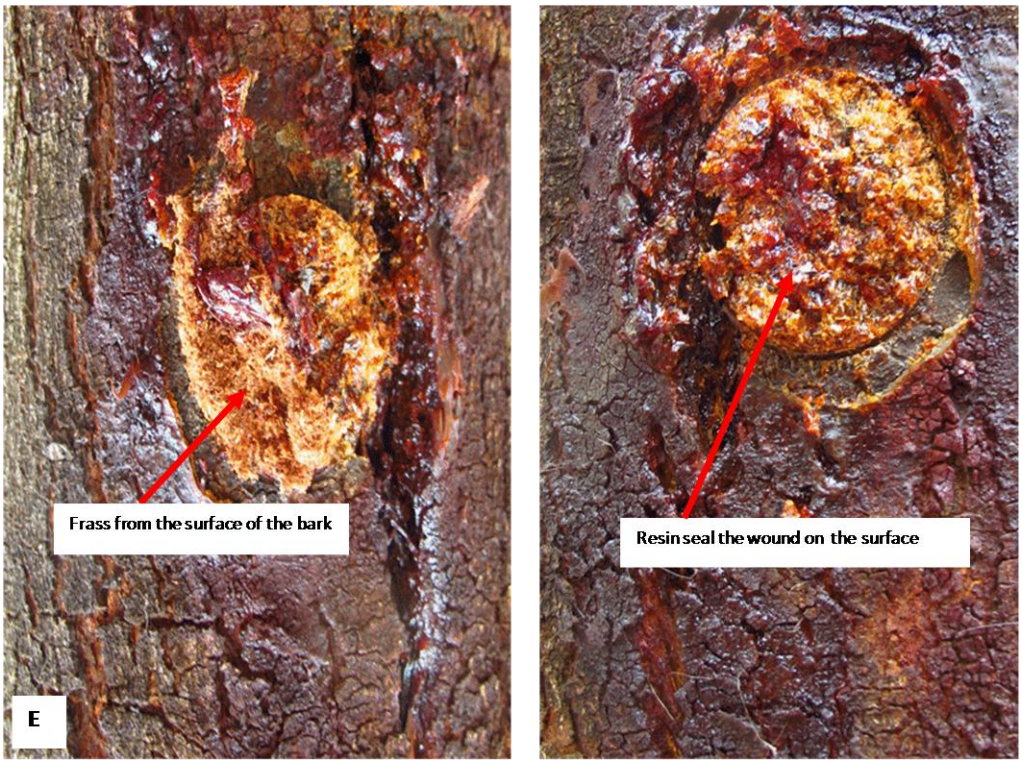


Figure 9. Defense responses of different *Eucalyptus* spp. artificially exposed to larvae of *Coryphodema tristis* in a plantation **A.** *E. nitens*, **B.** *E. dunnii*, **C.** *E. macarthurii*, **D.** *E. nitens* x *E. grandis* and **E.** *E. smithii*.

SUMMARY

Coryphodema tristis and *Chilecomadia valdiviana* are both members of the Cossidae that have recently expanded their host range to include new non-native tree species in South Africa and Chile, respectively. Both moth species are associated with a number of native trees in their respective countries, as well as non-native fruit trees and ornamentals. The appearance of *C. tristis* and *C. valdiviana* on *Eucalyptus* is the first record of these species on Myrtaceae in both countries. The parallel host range expansion by these two geographically distant cossid species raises a question about their phylogenetic relatedness and the possibility of cryptic invasion. The COI mtDNA sequence data produced and analysed in this thesis, however, showed that *C. tristis* and *C. valdiviana* are highly divergent genetically, supporting the hypothesis that both are native on their respective continents with independent evolutionary trajectories. Hence, the parallel host range expansions to *E. nitens* on different continents appear to be unrelated events, likely driven by characteristics of the biology and / or ecology of the host. Furthermore, data from mitochondrial and nuclear genomes indicate that *C. tristis* infesting *E. nitens* in the Mpumalanga province of South Africa have experienced a significant founder effect and persist with greatly reduced genetic variation relative to insects found in other provinces and different host plants in South Africa. DNA sequence analysis of museum samples of *C. tristis* from Mpumalanga and the contemporary samples from *E. nitens* confirmed that the latter populations originated from the resident populations in the province, either from native or non-native hosts.

It is well understood that symbiotic associations shape the evolution of every living organism and all levels of biological organization. Hence, it has been speculated that yeasts, which are commonly found associated with Cossidae, might be important players in host interaction of this moth. Based on comparison of LSU rDNA and ITS sequence data, nine yeast species were

identified in tunnels and guts of *C. tristis*, including *Saccharomyces paradoxes*, *S. cerevisiae*, *Hanseniaspora valbyensis*, *Pichia mandshurica*, *Pichia* sp. nov., *Kregervanrija delftensis*, *K. fluxuum*, *Wickerhamomyces myanmarensis* and *Candida salmanticensis*. The comparatively abundant genera of *Pichia* isolated from all samples in this study, as well as *Kregervanrija* from *C. tristis* on *E. nitens* and *Wickerhamomyces* from *C. tristis* on *V. vinifera*, seem to represent a major part of *C. tristis* intestinal microbial community.

The host suitability experiment conducted in this study, both under laboratory and field conditions, revealed distinctly different responses of *C. tristis* to different *Eucalyptus* tree species. While wood from all hosts was suitable for *C. tristis* larval development from a nutritional point of view, not all were optimal. Field testing, however, showed that colonies failed completely to establish on some hosts, despite the suitability of its wood in culture. This is possibly due to early defence responses of the host, with all hosts, except *E. nitens*, responding by copious production of resin soon after infestation. While preliminary, this work lays a foundation for further exploring how specific host factors might influence the strange pattern of new host association of *C. tristis* on *E. nitens*.

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Thesis title: Ecology and genetics diversity of *Coryphodema tristis* on *Eucalyptus nitens* in South Africa

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Degree: PhD