Fungus gnats in forestry nurseries and their possible role as vectors of *Fusarium circinatum*

by

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Declaration

I, the undersigned, hereby declare that the thesis submitted herewith for the degree *Magister Scientiae* to the University of Pretoria contains my own independent work. This work has hitherto not been submitted for any degree at any other University.

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TABLE OF CONTENTS

Acknowledgments **1**

Preface **3**

CHAPTER 1 **6**

Fungus gnats as vectors of *Fusarium circinatum* in forestry nurseries: A review

1. Introduction 7

2. Taxonomy and description of fungus gnats 9

3. Insect – fungus interactions 10

    3.1 Biology and ecology of fungus gnats 10

    3.2 Fungus gnats as vectors of fungal pathogens 13

    3.3 *Fusarium circinatum* in forestry nurseries 15

    3.4 Fungus gnats as vectors of *Fusarium circinatum* 16

4. Management of fungus gnats 17

    4.1 Cultural control and attractants 17

    4.2 Chemical control 17

    4.3 Biological control 19

    4.4 Management of fungus gnats in South Africa 26

5. Conclusions 27

6. References 27

CHAPTER 2 **42**

Fungus gnats and other diptera in South African forestry nurseries and their possible association with the pitch canker fungus

Abstract 43

Introduction 44

Materials and Methods 46

Results 48

Discussion 49

References 53

CHAPTER 3 **66**

Diversity and phylogeography of *Bradysia difformis* Frey. (Sciaridae: Diptera) in South African forestry nurseries

Abstract 67

Introduction 68

Materials and Methods 70

Results 72

Discussion 74

References 80

CHAPTER 4 **98**

Molecular detection of fungi carried by *Bradysia difformis*

Frey. (Sciaridae: Diptera) in South African forestry nurseries

Abstract 99

Introduction 100

Materials and Methods 103

Results 108

Discussion 110

References 112

Summary **127**

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PREFACE

There are many examples of associations between insects and fungi. Where the fungi involved are pathogens, such associations may be of economic importance. Insects of no economic concern alone can also become important pests because of their association with fungal pathogens. Insects may assist in the spread of pathogens by carrying them on or in their bodies. Insects may also predispose plants to infection by creating wounds during feeding, oviposition or other behavioural activities. Knowledge of associations between insects and fungal pathogens often form a crucial component in the management strategy of these pathogens.

The pitch canker fungus, *Fusarium circinatum*, causes severe disease symptoms on mature pines in the USA. Various insects have been implicated as vectors of this disease. In South Africa, *F. circinatum* is reported only to cause disease on pine seedlings, where it results in severe losses in nurseries. Various insects are present in the nursery that could possibly be associated with the spread of the fungus or the infection of its hosts. Amongst these insects, fungus gnats are the prime suspects due to their history of association with fungal pathogens in other nurseries. The presence of fungus gnats in South African pine nurseries and their possible association with *F. circinatum* and other pathogens has never been investigated critically. The objective of this study was to expand the base of knowledge of fungus gnats in South African pine nurseries, and to consider their possible association with *F. circinatum* and their population structure within and between nurseries.

The literature review provides a summary of fungus gnats in the nursery environment. This includes their description, biology and association with fungal pathogens. Information from these studies is used to evaluate the possible association between fungus gnats and *F. circinatum* in South African pine nurseries. In nurseries around the world where fungus gnats are considered pests, various control options have been used, and these are further discussed.

The first research aim of this study was to determine whether fungus gnats are present in the major pine nurseries of South Africa. Thus, in Chapter 2, surveys were undertaken in four of the major pine nurseries. All fungus gnats collected were identified to species level. Other diptera collected were identified to family level. Furthermore, all diptera collected were isolated on general and selective growing medium to examine for the presence of *F. circinatum*.

Results from Chapter 2 showed that only one species of fungus gnats was present in the nurseries and it was present in all four of the nurseries surveyed. This raised interesting questions regarding the phylogeographic structure of these populations and the diversity within and between populations. These questions are addressed in Chapter 3 using analysis of mitochondrial COI sequence data from fungus gnats collected in the four nurseries. Of particular importance was the interpretation of these results as it pertains to the movement of fungus gnats between populations, together with their associated fungi.

Using general and specific growing medium to isolate fungal pathogens from insects is not necessarily an accurate method. Pathogens may be overgrown by faster growing

fungi before they are noticed, especially if they are present only in small amounts. Chapter 4 examined the use of DNA-based methods as a tool to detect fungal pathogens on fungus gnats. Fungus gnats were collected from the same four nurseries as in Chapter 2. Species-specific primers for *F. circinatum* and *Botrytis cinerea* were used to detect these fungi. Dilution series were done to examine the sensitivity of the primers. General primers were used to detect other fungi.

This dissertation includes some of the first studies ever undertaken on fungus gnats in South African pine nurseries. Their association with the very virulent pitch canker fungus is also considered in some detail. It is my hope that these studies will form a foundation for future research on fungus gnats in South Africa.

CHAPTER 1

Fungus gnats as vectors of *Fusarium circinatum* in forestry nurseries: A review

INTRODUCTION

Many associations between insects and fungi are evident in nature. One such association involves fungi that are parasitic on insects. For example, some fungi in the Entomophthorales grow through the insect's joints and between the plates of the exoskeleton (Carlile & Watkinson 1994), obtaining nutrients from the insect's body at the insect's expense. Alternatively, other associations between insects and fungi involve the insects feeding on the fungi (Gullan & Cranston 1994), for example the fungus-eating *Drosophila* species (Drosophilidae: Diptera) (Carlile & Watkinson 1994). In many cases, such associations between insects and fungi are mutualistic, with both the insect and the fungi benefiting from the relationship (Batra & Batra 1967).

Associations between insects and fungi where plants are a food source can be detrimental to the plant. In these cases, the threat to the plant would be less or even non-existent in the presence of only one member of the insect-fungus relationship. Examples are numerous, one example being the relationship between siricid wasps and their pathogenic fungi (Parkin 1942, Slippers *et al.* 2003). This insect-fungi pair can cause high mortality to their host plants (Neumann & Marks 1990), but the insect or fungus alone pose no serious threat (Coutts 1969).

The pitch canker fungus, *Fusarium circinatum* Nirenberg *et* O'Donnell (formerly *Fusarium subglutinans* (Wollenw. et Reinking) Nelson *et al*. f. sp. *pini*) and its vectors have been investigated in California (Hover *et al*. 1996, Gordon *et al*. 2001). Pitch canker is a disease of pines caused by *F. circinatum*. *Fusarium circinatum*

infects its host via wounds, which may result from mechanical or weather-related damage, but may also be due to insects (Dwinell *et al.* 1981, Dwinell *et al.* 1985). Insects have also been recorded to carry *F. circinatum*, implicating these insects as vectors of *F. circinatum* (Fox *et al.* 1991, Storer *et al.* 1994, Hover *et al.* 1996, Storer *et al.* 1999). The insects in this relationship would benefit by having an increased food resource in the form of stressed trees and the fungus would benefit by being provided with, and often transported to wounds on pines (Storer *et al.* 1999).

In South Africa, *F. circinatum* was first reported in a forestry nursery, where it resulted in high mortality of *Pinus patula* Schlechtend & Cham. seedlings (Viljoen *et al.* 1994). Stem cankers on larger trees, such as those found in the United States (Gordon *et al.* 2001), have, thus far, not been recorded in South Africa (Viljoen *et al.* 1997). Nevertheless, the spread of the fungus to plantations is feared, as *Pissodes nemorensis* Germar., an insect associated with the spread of *F. circinatum* in the United States (Dwinell *et al.* 1981, McCain *et al.* 1987) infests pine plantations in South Africa (Kirsten *et al.* 2000, Gebeyehu & Wingfield 2003).

No vectors of *F. circinatum* on pine seedlings in South African forestry nurseries have been recorded. However, fungus gnats (Sciaridae, Mycetophilidae: Diptera) have been suggested to play a role in the transmission of *F. circinatum*. These insects are known to act as vectors of fungal pathogens in nurseries, including those producing plants for forestry (James *et al.* 1995, Landis 1996). Fungus gnats have an association with fungi, with the fungi forming a major part of the larval diet (Gardiner *et al.* 1990). Thus, due to the nature of fungus gnats in transmitting fungi and their regular contact

with fungi, these insects are suspected as being vectors of *F. circinatum* in South African forestry nurseries.

The aim of this review is to consider the association between fungus gnats and fungal pathogens. The association is considered in a worldwide context, including nurseries of a wide variety of crops. The significance of fungus gnats as possible vectors of the pitch canker pathogen, in forestry nurseries, is also discussed.

1. TAXONOMY AND DESCRIPTION OF FUNGUS GNATS

The families Sciaridae and Mycetophilidae belonging to the order Diptera, include slender, long-legged flies with elongated coxae (van Tonder 1994, Barraclough & Londt 1996, Landis 1996, Gill & Dutky 1997). These insects are known as fungus gnats. Sciaridae and Mycetophilidae are classified under the same suborder as the Culicidae (mosquitoes), namely Nematocera. In contrast to the Culicidae, the taxonomy and biology of both Sciaridae and Mycetophilidae is not well known. The small size and superficial homogeneity of insects in these families has discouraged many collectors and taxonomists from studying fungus gnats (Matile 1980, Steffan 1980).

The Afrotropical fungus gnat fauna is especially poorly known and no complete key for either family is available. Only isolated descriptions of new species, incomplete lists of species from certain areas and partial keys to genera and species have been published (Edwards 1925, Matile 1980, Steffan 1980, Väisänen 1994, Rudzinski 1997a,b,c). Taxonomic work on the Afrotropical Sciaridae and Mycetophilidae,

including a complete key to the species in these families, is greatly needed. Such a tool would be invaluable for future biological and ecological studies on these insects.

Members of the Mycetophilidae are often referred to as fungus gnats, whereas members of the Sciaridae are referred to as dark-winged fungus gnats (Barraclough & Londt 1996). The differences between the adults of these families are presented in Table 1 and Figure 1. Adults of both families are small (2 – 4 mm), have elongated coxae and long, beaded antennae and are sometimes referred to as long-horned flies (van Tonder 1994, Barraclough & Londt 1996, Gill & Dutky 1997). Larvae of both families are about 0.5 mm in length when fully grown, translucent to white in colour and have black heads (Landis 1996). In the Sciaridae, adult females are larger than adult males (Binns 1981).

The taxonomy of fungus gnats is generally based on the morphology of the adults. However, the eggshells of these insects may have external, morphological species-specific features, useful for taxonomic purposes. Carcupino & Lucchi (1995) investigated the eggshell fine structure of *Bradysia aprica* (Winnertz) (Sciaridae) and compared its morphology to that of other fly species. As research into the eggshell structure of fungus gnats and other Diptera develops, ootaxonomy may provide a viable option to identify the fungus gnats present in a nursery.

2. INSECT – FUNGUS INTERACTIONS

2.1 Biology and Ecology of Fungus Gnats

Sciarids and mycetophilids have a broad distribution and range of habitats. Fungus gnats are found in rotten wood, under the bark of fallen trees, associated with wild fungi, as well as in leaf mold and manure-piles (Binns 1981, Barraclough & Londt 1996). In a study using suction cups over cereal fields, Sciaridae and Mycetophilidae were found to be the second and fourth, respectively, most dominant family present (Binns 1981). The Sciaridae was found to be the most dominant insect family present in a study using emergence trapping in beech woods (Binns 1981).

The focus of this review is on fungus gnats occurring in nurseries. The relatively stable climate in nurseries permits the continuous reproduction and presence of fungus gnats in winter. Fungus gnat larvae, pupae and adults have been recorded in nurseries of various crops. These crops include legumes (Gillespie & Menzies 1993, Springer 1995), mushrooms (Huang *et al*. 1992, Eicker & Ludick 1993, Grewal & Richardson 1993, White 1997, White 1999, Jess & Klipatrick 2000), fuchsias (Gouge & Hague 1995b), cucumbers (Rutherford *et al.* 1985, Jarvis *et al*. 1993), alfalfa (Leath & Newton 1969, Huang & Harper 1985), tomatoes (Gillespie & Menzies 1993), forestry plants (James *et al.* 1995, Landis 1996), clovers (Springer & Carlton 1993) and other ornamentals (Harris *et al.* 1995). Adult gnats have often been observed hovering around these crops, close to the soil surface (Landis 1996).

The life cycle of fungus gnats is dependent on the species and the temperature, but generally, the entire cycle requires less than one month to complete. From Harris (1993), Harris *et al*. (1996), Landis (1996) and Gill & Dutky (1997) the life cycle is summarized as follows:

Any substrate high in organic matter is suitable for the fungus gnats to deposit their eggs. In nurseries, this includes pots, plug trays and areas under the greenhouse benches, particularly if moss, algae or weeds are present. Damp, peat – vermiculite medium with a high organic content is highly attractive to fungus gnats. One hundred to two hundred eggs are deposited on the soil surface by adult gnats. After three to six days, the translucent larvae emerge. Four larval instars are present in *Bradysia* spp. The larvae store fat, resulting in later instars becoming whiter. Pupation occurs approximately 10 – 15 days after the eggs hatch. The final instar larvae attain an upright position under the debris and produce a small chamber of glistening threads. Emergence of the adult depends on environmental conditions, but this is generally after five to six days. Adults mate soon after emergence and adult females will oviposit two days later. Adults of *Bradysia* spp. live for approximately three days.

In captivity, adult fungus gnats have fed on sucrose solutions (Binns 1981), but it is not certain what adult gnats consume in nurseries. Fungus gnat larvae are known to feed on animal excrement, decaying and living plant tissues, and fungal hyphae and fruiting bodies, including cultivated mushrooms (Anas & Reeleder 1987, Anas & Reeleder 1988, Springer 1995, Barraclough & Londt 1996). In nurseries, the larvae feed on decaying and healthy plant roots, causing a reduction in plant vigor (Kennedy 1974, Springer 1995a, Springer 1995b). Feeding wounds may provide infection sites for various pathogenic microorganisms (Springer 1995b). Leath & Newton (1969) for example showed that alfalfa plants injured by *Bradysia* spp. larvae prior to inoculation of *Fusarium* spp. had a greater mortality than uninjured plants. Similarly, feeding wounds of fungus gnat larvae on pine seedlings may increase infection of *F.*

circinatum and other pine pathogens. Experimental research is needed to support this hypothesis.

Fungi present in nurseries provide another source of food for fungus gnat larvae. Kennedy (1974) and Gardiner *et al.* (1990) stated that fungi provide an essential and even complete nutrient source for *Bradysia impatiens* (Johannsen). *Bradysia impatiens* ingests the mycelium, oospores and zoospore cysts of *Pythium* spp. (Gardiner *et al.* 1990). Larvae of *B. coprophila* (Lintner) have thus been used as a biological control agent to degrade the sclerotia of the plant pathogen, *Sclerotinia sclerotiorum* Lib. (Anas & Reeleder 1988, Gracia-Garza *et al.* 1997). Generally, however, the association that fungus gnats have with fungi and plants enhances, rather than reduces the impact of plant pathogens in nurseries.

2.2 Fungus Gnats as Vectors of Fungal Pathogens

Fungus gnats may be external and / or internal vectors of fungi. Internal vectors carry fungi inside their bodies, while external vectors carry fungi on the exterior body parts. External transmission of fungi may occur when fungus gnat larvae or adults come into contact with a fungus during feeding or while moving within the nursery (Kalb & Millar 1986, Whipps & Budge 1993). Both adult and larval stages of fungus gnats may have contact with fungi in this way and so serve as external vectors.

Certain phytophagous insects have been shown to ingest and transmit fungi within the nursery (Huang & Harper 1985). Gardiner *et al.* (1990) recorded that the larvae of *B. impatiens* ingest mycelium, oospores and zoospores of *Pythium* spp. Most oospores

and zoospores still appeared intact inside the digestive tract of the larvae, as mycelium was the main nutrient source. Therefore, viable fungal spores may occur in the faeces of these insects (Whipps & Budge 1993) and in the corpses of fungus gnat larvae, within forestry nurseries. If the substrate is suitable, these spores will germinate and establish a fungal colony. Fungus gnat larvae can thus be considered as potentially important internal vectors of fungi.

The role of adult fungus gnats as vectors of fungi is controversial (James *et al*. 1995), however, trans-stadial transmission of oospores from the larval to adult stage ensures that a small percentage of the oospores ingested by the larva can remain in the gut of the adult insects (Jarvis *et al*. 1993). The presence of mature oospores of *Pythium aphanidermatum* (Edson) Fitzp. in the gut of shore flies (Ephydridae) decreased from 97 % in first and second instar larvae, to 20 % in third instar larvae and pupae, to 10 % in adult flies (Goldberg & Stanghellini 1990). As the adult shore flies were not exposed to the fungus, the oospores in the gut remained from the larval stage. Similarly, adults of *B. impatiens* possessed 1.67 % of oospores ingested by the larval stage, in the gut (Jarvis *et al.* 1993). Although only a small percentage of oospores survive to the adult stage, it is still possible for adults to be internal vectors of fungi, without necessarily feeding on fungi.

The role of fungus gnats as vectors of fungi has been demonstrated in various studies. Kalb & Millar (1986) investigated the association between fungus gnat adults and the Verticillium wilt causing fungus, *Verticillium albo-atrum* Reinke & Berthold. Adult flies were observed to walk over infected stems and leaflets and then to move to healthy plants. Fungus gnats (*Bradysia* spp.) were introduced to plants grown in a

greenhouse free of *V. albo-atrum*. After the introduction of the fungus gnats, 31 – 41 % of the plants became infected with the pathogen. A similar study by Gillespie & Menzies (1993) showed that fungus gnat adults carry *Fusarium oxysporum* Schlecht f. sp. *radicis-lycopersici* Jarvis and Shoemaker to healthy bean and tomato plants. These studies provide good evidence of a relationship between fungus gnats and fungal pathogens.

3.3. *Fusarium circinatum* in Forestry Nurseries

Fusarium circinatum, the pitch canker fungus, was first reported in 1946 on *Pinus virginiana* Mill. in North Carolina, United States (Hepting & Roth 1946). Subsequently, *F. circinatum* has been recorded from Japan (Muramoto *et al*. 1988), Mexico (Santos & Tovar 1991), South Africa (Viljoen *et al*. 1994) and Chile (Wingfield *et al*. 2002), on a wide variety of hosts. Originally, *F. circinatum* was thought to only infect large pine trees, but this fungus also infects pine seedlings causing serious losses in nurseries (Barnard & Blakeslee 1980, Rowan 1982, Viljoen *et al*. 1994, Wingfield *et al*. 2002).

Fusarium circinatum has not been recorded on mature pine trees in South Africa, but has caused serious losses in the nurseries (Viljoen *et al*. 1994). *Fusarium circinatum* causes lesions on the root collar and the cotyledonary node region of the seedlings (Barnard & Blakeslee 1980, Dwinell *et al*. 1985, Viljoen *et al*. 1994). Symptoms of diseased seedlings include tip die-back, damping-off, chlorotic or redish brown needle discoloration and wilting (Barnard & Blakeslee 1980, Rowan 1982, Dwinell *et al*. 1985). Susceptibility trials have shown that three of the most important commercial

pine species in South Africa, *P. patula*, *P. elliottii* and *P. radiata*, are all susceptible to *F. circinatum* (Viljoen *et al*. 1995). *Pinus patula*, the most widely planted species in South Africa, is extremely susceptible to *F. circinatum* (Viljoen *et al*. 1995).

3.4. Fungus Gnats as Vectors of *Fusarium circinatum*

Fungus gnats are potential vectors of any fungi that they consume or with which they come into contact. *Fusarium* spp. have been recovered from germinating seedlings, in the root areas and in bark or soil medium associated with pine seedling propagation (Viljoen *et al.* 1994, Ocamb & Juzwik 1995). These are the same substrates where fungus gnats are found and the possibility exists for fungus gnats to be external or internal vectors of *Fusarium* spp.

The role of fungus gnats as vectors of *F. circinatum* has not been investigated. However, there is good anecdotal evidence to suggest that nurseries with severe losses due to the pitch canker fungus, also tend to have high populations of fungus gnats. Similarly, spraying with chemical insecticides has substantially lowered infection levels. Many of the fungi transmitted by fungus gnats (Table 2) are pathogenic and result in severe crop losses. Certain *Pythium* spp. are highly pathogenic to tomato plants, causing damping-off of the seedlings (Robertson 1973). *Botrytis cinerea* Pers. *ex* Nocca. & Balb. causes grey mold and is regarded as a very important pathogen of conifer seedlings (James *et al.* 1995). It is possible that *F. circinatum* is another important pathogen whose spread is assisted by fungus gnats.

3. MANAGEMENT OF FUNGUS GNATS

4.1. Cultural Control and Attractants

Fungus gnats prefer to deposit their eggs in substrates that are damp and high in organic matter (Harris 1993, Landis 1996). Therefore, nursery practices must exclude these conditions. Harris (1993), van Tonder (1994) and Landis (1996) discussed various methods to control fungus gnats in forestry nurseries. Over-watering must be prevented and adequate drainage provided. Nurseries must be cleared of weeds, moss and algae regularly. Placing stone chips or ash under the nursery tables will decrease the dampness and presence of weeds, moss and algae. The medium used in nurseries may also affect fungus gnat infestations. Lindquist *et al.* (1985) noted that the emergence of *B. coprophila* was greater from composted hardwood bark medium than from composted pine bark medium. By eliminating attractive breeding, mating and feeding sites, the incidence of fungus gnats in the nursery will decrease.

Other forms of control include the use of screening vents as barriers to the fungus gnats and the use of an attractant light trap (Harris *et al.* 1996). However, screening vents are often impractical and light traps may attract unwanted pests into the nursery.

4.2. Chemical Control

Negative environmental impacts and high costs are often associated with chemical control. However, at times, chemical control is not only feasible, but the only form of control available. A large range of chemicals is available for use against fungus gnats,

but none of these are registered for use in South African forestry nurseries (Nel *et al*. 2002).

Many of the chemicals tested against fungus gnats are in the form of IGRs (Insect Growth Regulators). IGRs target the development or metabolism of the insect (Gullan & Cranston 1996). Various IGRs have been tested to control fungus gnats. These include methoprene, diflubenzuron, cyromazine, hexaflumuron, fenoxycarb, pyriproxyfen and azadirachtin (Eicker & Ludick 1993, White 1997, White 1999, Ludwig & Oetting 2001).

Eicker & Ludick (1993) compared the efficacy of two IGRs, namely, methoprene and diflubenzuron, on the control of the sciarid *Lycoriella auripila* (Winnertz), a pest in South African mushroom farms (*Agaricus bisporus* (Lange) Imbach). Methoprene is a juvenile hormone mimic. It disrupts the process of morphogenesis in the insect, particularly during pupation, resulting in the emergence of sterile adults. Diflubenzuron is a chitin synthesis inhibitor. It prevents the formation of chitin, which is essential for the development of the cuticle. Moulting does not occur and the insects grow until the exoskeletons are inordinately tight and then they die. Both treatments provided adequate control of *L. auripila*.

Besides IGRs, other forms of chemical control have also been used against fungus gnats. These include aldicarb (oxime carbamate), bendiocarb (carbamate), chlorpyrifos (organophosphate), diazinon (organophosphate), methiocarb (carbamate), permetherin (synthetic pyrethroid), oxamyl (oxamic carbamate), mercaptothion (organophosphate), calcium oxalate and sinapic acid (Lindquist *et al*. 1985, Harris

1993, van Tonder 1994, Bartlett & Keil 1997, White 1997, Jess & Klipatrick 2000, Ludwig & Oetting 2001). Lindquist *et al.* (1985) compared aldicarb, bendiocarb, oxamyl, diazinon and methoprene for the control of *B. coprophila*. Oxamyl and aldicarb resulted in the best control. White (1997) investigated the use of calcium oxalate and sinapic acid to control *L. auripila* in a commercial mushroom farm. Both calcium oxalate and sinapic acid provided 50% control.

Chemical control may achieve a temporary solution to fungus gnat problems. However, it involves continuous financial and environmental expense. Another disadvantage of chemical control for extended periods is the acquisition of resistance to the chemical, by the insect. The short life cycle of fungus gnats enables them to rapidly develop resistance within the population to chemical treatment. There is already widespread resistance by *L. auripila* to diazinon (White 1997), while *L. mali* has evolved resistance to permethrin in the U.K (Bartlett & Keil 1997). Control measures with less maintenance, cost and impact on the environment are needed.

4.3. Biological Control

The importance of biological control for the management of pests and pathogens is increasing. Biological control provides an environmentally sound option and often requires less maintenance and cost than other control measures. However, the initial introduction of a biological control agent requires appropriate research. Biological controls should be introduced within an integrated pest management context. Scouting, accurate identification, establishing action thresholds, maintenance of records and the correct release strategy are needed (Landis 1996).

Numerous biological control agents are used against fungus gnats. Biological control agents used include entomopathogenic nematodes (Grewal & Richardson 1993, Gouge & Hague 1995a,b,c), parasitoids (Hellqvist 1994), predatory mites (Ali *et al.* 1999, Enkegaard & Brødsgaard 2000), entomopathogenic fungi (Huang *et al.* 1992) and bacteria (Osborne *et al.* 1985). These biological control agents have been used and tested in various nurseries, but no work on the control of fungus gnats in forestry nurseries of South Africa has been reported.

Nematodes

Nematodes may attack the larval and adult stage of fungus gnats. The mechanism of attack by the nematode *Steinernema feltiae* (Filipjev) (Steinernematidae) has been closely studied by Poinar (1992), Grewal & Richardson (1993), Gouge & Hague (1995a,b). This includes the following steps:

Steinernema feltiae has a mutualistic association with toxic bacterium *Xenorhabdus*. The infective third instar juvenile of *S. feltiae* enters into the host haemocoel via the mouth or anus. *Xenorhabdus* is then released from oesophageal vesicles, resulting in septicemia and death of the host insect. The nematodes then feed on the septicemic tissue, reproduce and when the food reserves in the host cadaver are depleted, new infective juveniles leave to find living hosts. Due to the small size of fungus gnats and the consequently limited food reserves, successful reproduction requires that only a few infective juveniles enter the host.

Certain species of nematodes have a greater efficacy against fungus gnats than others. Gouge & Hague (1995c) tested the efficacy of various nematodes, namely *S. feltiae,*

S. anomali, S. riobravis, S. carpocapsae, S. scapterisci, S. glaseri and two *Heterorhabditis* spp., against six sciarid species, namely, *B. amoena, B. confinis, B. tritici, B. paupera, L. auripila* and *L. solani. Steinernema feltiae* was the most effective control agent. All six sciarid species were susceptible to infestation by *S. feltiae*. Hay & Richardson (1995) compared the susceptibility of *L. mali* to various nematode species. *Steinernema feltiae* was the most virulent and *S. kraussei* the least virulent. Similarly, for the control of *Bradysia coprophila* (Lintner) on rooted poinsettias (*Euphorbia pulcherrima* Willd. Ex Klotzsch), *S. feltiae* was significantly more effective than *S. carpocapsae*, as well as *Bacillus thuringiensis* var. *israelensis* and kinoprene (Harris *et al*. 1995). *Steinernema feltiae* is also effective against Phoridae (Diptera) (Scheepmaker *et al*. 1998b).

The establishment rates of *S. feltiae* on the sciarid host *Bradysia paupera* (Tuomikoski), were investigated by Gouge & Hague (1995a). Infection by *S. feltiae* occurred three hours after invasion, adults developed after 27 hours and new infective juveniles were produced after 48 hours. The establishment rate thus varies depending on the nematode and host species.

Most of the above-mentioned work regarding the use of *S. feltiae*, involves the SN strain. Other strains of *S. feltiae* exist and have been used to control fungus gnats. Grewal *et al.* (1993) compared the SN strain, obtained from France, and the ScP strain, obtained from Poland, of *S. feltiae* against the sciarid *L. mali* in a mushroom farm. At low pest densities, no significant differences were observed and 85 – 94 % reduction of *L. mali* was obtained. At high pest densities, the SN strain resulted in 51 – 73 % reduction of *L. mali*, while the ScP strain caused 56 – 83% reduction. The ScP

strain was also more persistent in the casing material. This suggests that the ScP strain may be the most effective strain, however, the host and environmental conditions may influence which nematode species or strain is more effective in any given situation.

Another entomopathogenic nematode, *Tetradonema plicans* Hungerford showed greater efficacy in controlling sciarids than *S. carpocapsae* and *Heterorhabditis bacteriophora* (Peloquin & Platzer 1993). *Tetradonema plicans* caused 74% to 80% mortality of sciarids in four months. It is clear that *S. feltiae* and *T. plicans* are the favoured nematodes to use for the control of fungus gnats.

The optimum dosage or number of nematodes used for biological control is important for nursery practices. Grewal & Richardson (1993) compared various application rates of infective nematodes to control *L. auripila* on mushroom farms. The most reliable and cost effective rate of application tested was 3 x 10^6 nematodes per tray (5.4 x 10^6 nematodes / m^2), which achieved 93 % reduction in *L. auripila*. Scheepmaker *et al*. (1998a) reported control rates for *L. auripila* of 96 – 98 % at only 3 x 10^6 nematodes / m^2, applied to the compost. Gouge & Hague (1995b) used a concentration of 7.8 x 10^5 nematodes / m^2 to control *B. paupera* on glasshouse fuchsias and obtained 92 % reduction of the pest.

Besides the concentration used, the method by which nematodes are applied is also important. Scheepmaker *et al*. (1998a) applied nematodes to the compost, while Gouge & Hague (1995b) applied the nematodes as a suspension through a hydraulic sprayer. Both methods were effective. The method used to apply nematodes must depend on the nursery techniques being utilized.

Caution is needed when using nematodes in conjunction with fungicides. The fungicides cinnamaldehyde (cinnamate) and a hydrogen dioxide / peroxyacetic acid mixture caused 100% mortality of *S. feltiae* (Krishnayya & Grewal 2002). However, another fungicide, azoxystrobin, caused no mortality. The use of nematodes does not exclude the use of fungicides, but does restrict which fungicides may be used.

It is evident that there has been extensive research on the use of nematodes as biological control agents against fungus gnats. The biology and application of the nematode is known. Further, the difference in virulence between species and even strains of the nematodes has been investigated. This past research and the high success rate of nematodes to control fungus gnats, makes nematodes an attractive control option in nurseries.

Parasitoids

The use of parasitoids as biological control agents for fungus gnats has received little attention. However, Hellqvist (1994) conducted an extensive study on the biology of a *Synacra* spp. (Hymenoptera: Diapriidae), a parasitoid of *B. paupera* in Swedish greenhouses. *Synacra* spp. is suggested to have been introduced accidentally into Sweden.

The mechanism of parasitism by *Synacra* spp., has been described by Hellqvist (1994). When in the vicinity of the host larva beneath the surface, the parasitoid examines the surface carefully with its antennae. On locating the larva, the parasitoid bends its abdomen down at 90° to its thorax and pushes out the ovipositor to make

contact with the larva. The parasitoid then punctures the cuticle of the larva with its ovipositor and inserts an egg inside its host. One egg is inserted at a time, but if host densities are low one host may be parasitised many times. Second to fourth instars are parasitised. The host is killed shortly after it pupates.

Two factors contribute towards the suitability of *Synacra* spp. as a biological control agent against fungus gnats. Firstly, *Synacra* spp. have been recorded to have a higher potential rate of population increase than that of its host, *B. paupera*. Secondly, unlike entomopathogenic nematodes, *Synacra* spp. have the ability to disperse and so follow the migrations of fungus gnats within the nursery. The disadvantage of *Synacra* spp. as biological control agents is that, unlike entomopathogenic nematodes, they do not kill the larval stage of the fungus gnat. Damage is still caused by the larvae before the fungus gnats die in their pupal stage. (Hellqvist 1994)

Mites

Predatory mites have been used as biological control agents against fungus gnats. *Hypoaspis miles* (Berlese) (Acari: Hypoaspidae) is a commonly used mite (Ali *et al*. 1999), but *Lasioseius fimetorum* Kang (Acari: Podocinidae) (Enkegaard & Brødsgaard 2000) and *Geolaelaps* sp. nr. a*culeifer* (Canestrini) (Gillespie & Quiring 1990) have also been used. *Geolaelaps* sp. nr. *aculeifer* gave a 30% reduction in *Bradysia* spp. (Gillespie & Quiring 1990), demonstrating that mites can form an important component of an integrated pest management strategy.

The rapid and easy rearing of *H. miles* and its high tolerance to starvation, contribute to its success as a biological control agent (Wright & Chambers 1994). *Hypoaspis*

miles controlled *Bradysia* spp. on cylamen and poinsetta at 55 mites / pot (Chambers *et al*. 1993). In conjunction with two IGRs (with methoprene and diflubenzuron as active ingredients respectively) *H. miles* controlled the sciarid *L. solani*. The IGRs had no negative effect on *H. miles* (Wright & Chambers 1994).

Fungi

Entomopathogenic fungi have not been widely used as biological control agents against fungus gnats. However, their potential for future use is evident from the work of Huang *et al*. (1992). Huang *et al*. (1992) used the fungus *Erynia ithacensis* Kramer (Zygomycetes: Entomophthorales) to control fungus gnats in a mushroom farm. Infected insect cadavers were placed in pest-infested areas. Pest-infested areas were also sprayed with water to establish a non-uniform relative humidity distribution. This enhanced the performance of *E. ithacensis* and 59 % control of the gnats was obtained. Huang *et al*. (1992) thus demonstrated the ability of fungi to control fungus gnats. However, research is needed to investigate the option more thoroughly and then to decide whether levels of control can be obtained, that compete with those of the entomopathogenic nematodes and other biological control agents.

Bacteria

Bacillus thuringiensis Berliner var. *israelensis* (Goldberg) is a common biological control agent for various pests, including fungus gnats. *Bacillus thuringiensis* var. *israelensis* produces a crystalline protein toxin that kills the larvae of fungus gnats (Gill & Dutky 1997). Forty-eight hours after infection, the cuticle of the larvae loose all integrity and the larvae disintegrate (Osborne *et al*. 1985). The efficacy of *B.*

thuringiensis var. *israelensis* may vary depending on medium, moisture and other environmental variables.

Bacillus thuringiensis var. *israelensis* has proved to be a successful control agent for fungus gnats. A survival rate of only 8% was obtained when *Bradysia coprophila* was exposed to *B. thuringiensis* var. *israelensis* (Osborne *et al*. 1985). When exposed to water only, 84% survival was obtained. Similarly, Cantwell & Cantelo (1984) reported over 90% control of *L. mali* (Fitch) by *B. thuringiensis* var. *israelensis*. A dipteran strain of *B. thuringiensis* used by White (1999), proved effective for the control of *L. auripila* on cultivated mushrooms. *Bacillus thuringiensis* var. *kurstaki* was also tested for its ability to control *B. coprophila*, but its activity was minimal when compared to *B. thuringiensis* var. *israelensis* (Osborne *et al*. 1985). These high rates of control make *B. thuringiensis* var. *israelensis* comparable to nematodes for the control of fungus gnats.

4.4. Management of Fungus Gnats in South Africa

No research of fungus gnats and their association with fungal pathogens in forestry nurseries of South Africa has been undertaken. Neither research to investigate which families and species of flies are present in the nurseries and effective methods to control them. Control methods currently used are based on research or advice from other countries. These control methods are often specific to a certain crop or nursery. The target fungus gnat species and even family of insect may be different to that which occurs in the forestry nurseries of South Africa.

5. CONCLUSIONS

The species composition of diptera and especially fungus gnats (Sciaridae and Mycetophilidae: Diptera) in South African forestry nurseries is poorly known. Whether diptera contribute to the spread of *Fusarium* spp. and particularly the pitch canker fungus, *F. circinatum*, is also unknown. However, fungus gnats have been shown as vectors of fungal pathogens in various nurseries, worldwide. Fungus gnats may also increase the incidence of fungal pathogens when the larvae produce wounds in the roots of seedlings. Therefore, it seems probable that fungus gnats and / or other diptera assist the spread of *F. circinatum* within forestry nurseries.

Cultural, chemical and biological control could be used to reduce the impact of fungus gnats. Biological control, especially using nematodes and bacteria, are preferred because of their high success and low threat to the environment. Research in South Africa will be needed to identify the families and species present in local forestry nurseries and determine their possible role, as vectors of the pitch canker fungus.

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Table 1. Differences between adults of the families Sciaridae and Mycetophilidae as described by MacAlpine (1981) and Barraclough and Londt (1996).

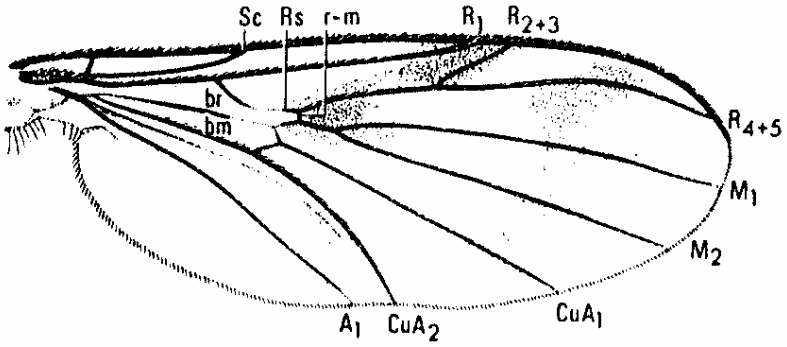
	Sciaridae	Mycetophilidae
Wings	grey to black	clear
wing venation	stem and fork of M subequal in length, fork distinctly bell-shaped	fork of M usually longer than stem, fork is lanceolate (fig. 2)
Body	grey to black	yellow to brown
Eyes	meet in a narrow eye bridge above antennae (fig. 1)	do not meet above antennae (fig. 1)
mesothoracic pleural sclerites	bare	usually at least weakly haired

Table 2. Fungi recorded to be transmitted by fungus gnats (Sciaridae and Myceophilidae: Diptera)

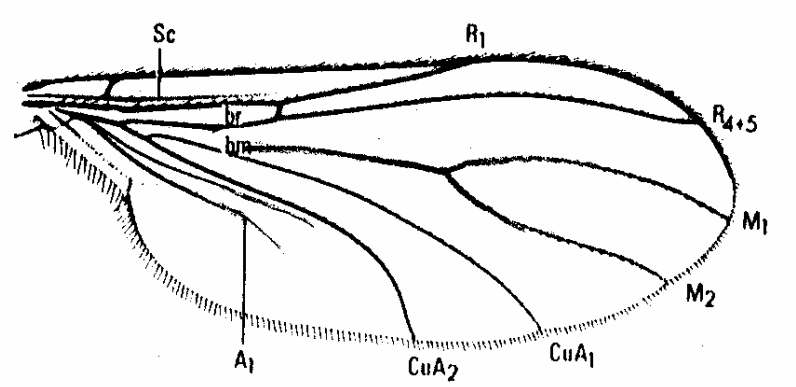
Fungus	Nursery Crop	Reference
Pythium spp.	cucumber	Gardiner *et al*. (1990)
Verticillium albo-atrum	alfalfa	Kalb (1986) Huang & Harper (1985)
Botrytis spp.	conifers	James *et al*. (1995)
Fusarium spp.	alfalfa, beans, tomatoes	Leath & Newton (1969) Gillespie & Menzies (1993)

Figure 1. Comparison of wing venation between the families Sciaridae and Mycetophilidae (Diptera) (MacAlpine 1981). Abbreviations: A_1, branch of anal vein, br and bm, basal cells, CuA_{1-2}, branches of cubitus anterior, M_{1-2}, branches of media, Rs, radial sector, R_{1-5}, branches of radius, r-m, radiomedial vein, Sc, subcosta.

a.


Sc
Rs
r-m
R1
R2+3
br
bm
R4+5
M1
M2
A1
CuA2
CuA1

b.


Sc
R1
br
bm
R4+5
M1
M2
A1
CuA2
CuA1

CHAPTER 2

Fungus gnats and other diptera in South African forestry nurseries and their possible association with the pitch canker fungus

ABSTRACT

Fusarium circinatum is the causal agent of a serious disease of seedlings in South African pine nurseries. Insects and especially fungus gnats (Diptera: Sciaridae, Mycetophlidae) are suspected of transmitting this fungus in nurseries. The aim of this study was to ascertain which species of gnats are present in South African pine nurseries, and to consider whether these and other diptera carry *F. circinatum*. Dipteran fauna were thus surveyed in four major forestry nurseries between 2000 and 2001. Fungi were isolated from these insects and resulting *Fusarium* spp. were identified. *Bradysia difformis* was the only fungus gnat species found and it occurred in all nurseries. Other Dipteran families collected included Agromyzidae, Cecidomyiidae, Chironomidae, Ephydridae, Muscidae, Simulidae and Tachinidae. This is the first report of *B. difformis* in South Africa. *F. circinatum* was not isolated from any of the Diptera collected. However, *Fusarium oxysporum* and *F. stilboides* were isolated from Chironomidae, but these fungi are not considered important pathogens in the nurseries surveyed.

INTRODUCTION

Nematoceran diptera belonging to the families Sciaridae and Mycetophilidae have a broad distribution and a wide range of habitats. These insects, known as fungus gnats, are found in rotten wood, under the bark of fallen trees, associated with wild fungi as well as in leaf mould and manure piles (Binns 1981a, Barraclough & Londt 1996). They are also found in nurseries of various crops, including legumes (Gillespie & Menzies 1993, Springer 1995a), mushrooms (Eicker & Ludick 1993), fuchsias (Gouge & Hague 1995), cucumbers (Jarvis *et al.* 1993), alfalfa (Leath & Newton 1969), tomatoes (Gillespie & Menzies 1993), forestry plants (James *et al.* 1995, Landis 1996), cloves (Springer & Carlton 1993) and other ornamentals (Harris *et al.* 1995).

Fungus gnat larvae feed on animal excrement (Barraclough & Londt 1996), decaying and living plant tissues (Springer 1995a, Barraclough & Londt 1996), and fungal structures (Anas & Reeleder 1988), including cultivated mushrooms (Binns 1981b). The diet of adult fungus gnats is not clearly known, although they have been fed on sucrose solutions in captivity (Binns 1981b). In nurseries the larvae also feed on decaying and healthy plant roots as well as fungi (Hungerford 1916, Kennedy 1974).

The feeding of fungus gnat larvae on healthy roots causes a reduction in plant vigour (Kennedy 1974, Springer1995a,b) and provides infection sites for various pathogenic fungi (Springer 1995b). Fungus gnat larvae come into contact with fungi while moving in the soil, feeding on infected plant roots or they feed directly on the fungi. Therefore, fungus gnats can directly or indirectly act as vectors of fungal pathogens.

Pathogens reported to be transmitted by fungus gnats include *Botrytis cinerea* Pers. :Nocca. & Balb on conifer seedlings (James *et al.* 1995), *Verticillium albo-atrum* Reinke & Berthold on alfalfa plants (Kalb & Millar 1986) and *Fusarium oxysporum* Schlecht f. sp. *radicis-lycopersici* Jarvis and Shoemaker on bean and tomato plants (Gillespie & Menzies 1993).

In South African forestry nurseries, the pitch canker fungus, *Fusarium circinatum* Nirenberg et O'Donnell (formerly *Fusarium subglutinans* (Wollenw. et Reinking) Nelson *et al.* f. sp. *pini*) is a serious pathogen of pine seedlings (Viljoen *et al.* 1994, Wingfield *et al.* 2002). This fungus causes lesions at the root collars and the cotyledon node regions of seedlings (Barnard & Blakeslee 1980, Viljoen *et al.* 1994). Symptoms of diseased seedlings include tip die-back, damping-off, chlorotic or reddish-brown needle discoloration and wilting (Barnard & Blakeslee 1980, Rowan 1982).

The association between fungus gnats and fungal pathogens in other crops has led to the suggestion that these insects may be vectors of *F. circinatum* in South African forestry nurseries, although no surveys for these insects has ever been undertaken. These nurseries commonly apply insecticides for the control of fungus gnats, hoping to reduce the impact of *F. circinatum*. There is, however, no experimental evidence showing an interaction between fungus gnats and *F. circinatum,* and chemical control of fungus gnats may represent an unnecessary expense and threat to non-target, possibly beneficial insects. Conversely, a greater knowledge of fungus gnat biology and control would be required if an interaction between these insects and *F. circinatum* does exist. The aim of this study was to determine whether Sciaridae or Mycetophilidae are present in the forestry nurseries of South Africa. Furthermore, we

considered whether these diptera, or any other diptera in South African forestry nurseries, might be involved in transmitting *F. circinatum*.

MATERIALS AND METHODS

Collection sites

Diptera were collected in four of the major pine growing nurseries in South Africa, over a two-year period, from 2001 to 2002. Two of the nurseries were in the Mpumalanga province, near Nelspruit (approximately 25˚34"S, 30˚41"E) and Sabie (approximately 25˚06'28"S, 30˚47'05"E). The other two nurseries were in KwaZulu-Natal, near Richmond (approximately 29˚51'54"S, 30˚15'50"E) and Hilton (approximately 29˚33'50"S, 30˚18'24"E) (www.gpswaypoints.co.za) (Fig. 1). These nurseries were selected specifically because serious losses due to *Fusarium circinatum* have been reported in them.

Collection of insects

Only adult diptera were collected for identification. This was because the keys used to identify the diptera rely primarily on the adult stages and sufficient numbers of the larvae proved difficult to collect within the nursery environment. Initially, yellow sticky traps were used to capture the adults (James *et al.* 1995). These traps consisted of yellow sheets of plastic (14.0cm x 7.5cm) covered with insect glue (Flytac). The traps were placed randomly within nurseries, amongst the pine seedlings. Some of the traps were suspended just above the seedlings, while others were placed upright on

the seedling trays, at the level of the seedlings. The traps were successful in catching the adult fungus gnats and other diptera, but due to the sticky nature of the traps, the specimens could not be removed intact for effective identification. Paraffin and similar liquids could not be used to remove the insects from the traps, as the fungi on these insects would have been killed.

As a replacement to sticky traps, aspirators were used to collect specimens from around the nurseries (Gardiner *et al.* 1990, Goldberg & Stanghellini 1990). When swarms of diptera were observed, sweep nets were used and the diptera were then collected from the net using an aspirator. Although this method was time consuming, the specimens remained in good condition for later identification.

Identification of Diptera

Dipteran families were identified using the keys of Borror & White (1970), McAlpine (1981), Barraclough (1995) and Barraclough & Londt (1996). Dr. Mervin Mansell (ARC Biosystematics, Pretoria, South Africa) assisted in confirming some of the identifications. Specimens belonging to the families of Sciaridae and Mycetophilidae were divided into morpho-species and later identified by Dr. Hans-Georg Rudzinski (Entomographisches Studio, Schwanewede, Germany).

Isolation and identification of fungi

Primary isolations of fungi from dipteran specimens were crushed and placed on *Fusarium* selective medium (Nash & Snyder 1965). To purify fungi, isolates were

transferred to 2% malt extract (15g malt extract, Merck, South Africa, plus 20g agar). *Fusarium* spp. were placed on Synthetic Low-Nutrient Agar (SNA) medium (Nirenberg & O'Donnell 1998) to promote the formation of the microconidia for species identification. Single conidial isolates were made from cultures of *Fusarium* spp. Each of these isolates was transferred to half-strength potato dextrose agar (PDA) (Difco, South Africa) and Carnation Leaf Agar (CLA). *Fusarium* spp. were identified using the keys provided in Nelson *et al.* (1983). Identifications of the *Fusarium* spp. were confirmed by Dr. W.F.O. Marasas (PROMEC, Medical Research Council, South Africa).

RESULTS

Identification of insects

Fungus gnats of the family Sciaridae were collected in this study. No Myecetophilidae were collected. These insects were collected in all four nurseries considered (Table 1). The collections also represented only the single species, *Bradysia difformis* Frey (= *Bradysia paupera* (Tuomikoski)) (Fig. 2 & 3). The overall sex ratio of *B. difformis*, from all four nurseries, was 24.3 males to one female.

Other dipteran families found in the nurseries included Agromyzidae (leaf miners), Cecidomyiidae (gall midges), Chironomidae (midges), Ephydridae (shore flies), Muscidae (muscids), Simulidae (black flies) and Tachinidae (tachinids) (Table 1). Of these, only Ephydridae were recorded in all of the nurseries (Table 1). Chironomidae were present in three of the four nurseries and were observed swarming in large

numbers. These swarms were often confused with fungus gnats by nursery employees. Muscidae and Agromyzidae were collected in two of the four nurseries. Cecidomyiidae, Simulidae and Tachinidae were collected in only one nursery (Table 1).

Isolation and identification of fungi

Fusarium circinatum was not isolated from any of the dipteran specimens. Two other *Fusarium* spp., namely *F. oxysporum* and *F. stilboides*, were isolated from chironomids. *F. oxysporum* was isolated from these insects at the Nelspruit and Pietermaritzburg nurseries. *F. stilboides* was isolated from chironomids collected only at the Nelspruit nursery. Over 100 chironomids were isolated from each nursery.

DISCUSSION

Results of this study showed that fungus gnats are common in South African forestry nurseries suffering from pitch canker fungus infections. It was intriguing that these insects belonged to only a single species, which was clearly well distributed geographically in South African *Pinus* nurseries. This is the first report of *Bradysia differmis* in Africa. This is interesting particularly as other *Bradysia* species have previously been recorded in South Africa (Rudzinski 1997a,b,c).

The genus *Bradysia* has a worldwide distribution (Dennis 1978, Bechev 2000), but *B. differmis* has been recorded only in Britain (White *et al.* 2000), Norway and Sweden (Hellqvist 1994), the USA and Brazil (Menzel *et al.* 2003) in the past. The insect

appears to have been introduced into South Africa from the northern hemisphere. It is not clear how this might have happened although imported plants, growth medium or logs would be possible sources of these insects.

Bradysia difformis is known as a pest in European nurseries. In Britain, *B. difformis* damages ornamentals (Gouge & Hague 1995) and is a minor pest of mushrooms (Binns 1981b, White *et al.* 2000). *Bradysia difformis* is the dominant sciarid in Norwegian greenhouses and possibly the most common pest in Swedish greenhouses (Hellqvist 1994). In these greenhouses, *B. difformis* larvae feed on the roots and root collar region of the plants. It is possible that *B. difformis* larvae in South Africa feed on pine seedlings, but this must still be tested experimentally.

No association between *B. difformis* and fungal pathogens has been recorded. However, various other *Bradysia* species have been found to transmit fungal pathogens, thus increasing their pest status (Kalb & Millar 1986, Keates *et al.* 1989, Gillespie & Menzies 1993). In two of these cases, *Fusarium* species were transmitted (Keates *et al.* 1989, Gillespie & Menzies 1993). It is, therefore, surprising that *B. difformis* that are clearly living in a close association with *F. circinatum* were not found to carry the fungus. It is possible that larger numbers of insects would have yielded cultures of the fungus, but these insects are clearly not important vectors of *F. circinatum.*

Although a high male to female sex ratio was observed for *B. difformis* in the study, this may not be representative of the actual sex ratio in the nurseries. Males congregate at the surface of the growing medium to mate with emerging females and

engage in a zigzag running motion when attracted by the female's sex pheromone (Binns 1981b, Harris *et al.* 1996). Females are less conspicuous or active than males, and generally remain on the underside of leaves (Harris *et al.* 1996). In this study, flies were collected from the seedlings, the seedling beds and when they were observed flying in the vicinity of seedlings. The more conspicuous flies would, therefore, have been collected more often than those under the leaves of the seedlings, resulting in a biased sex ratio.

Besides fungus gnats, ephydrids were the only other dipteran family collected in all four nurseries. Ephydrids occur in a variety of habitats, from oil pools to saline marshes and they utilise a diverse range of food resources, including algae, bacteria, plants, fungi and decaying carcasses (Foote 1995, Hesler 1995). Most species are not economically important, but certain species have been recorded as pests of crops (Foote 1995, Hesler 1995) and plants in greenhouses (Corbaz & Fischer 1994, Stanghellini *et al.* 1999). In greenhouses, the ephydrid *Scatella stagnalis* has been shown to acquire and transmit the pathogen *Thielaviopsis basicola* from infected to healthy corn-salad plants (Stanghellini *et al.* 1999) and *F. oxysporum* f. sp. *lycopersici* from infected to healthy tomato plants (Corbaz & Fischer 1994). Stanghellini *et al.* (1999) isolated *T. basicola* from the larvae, pupae, adults and faeces of *S. stagnalis*. None of the other dipteran families collected from the nurseries are reported to be associated with plant pathogenic fungi or with pine seedlings.

Isolations in this study were only from adult diptera and it is possible that the larvae carry the pathogen. Sciarid larvae feed on fungi and plant roots (Anas & Reeleder 1988, Gardiner *et al.* 1990, Springer 1995, Barraclough & Londt 1996), a habit not

shared by the adults. Thus the larvae may come into contact with fungal pathogens while moving in the soil or during feeding. It is also possible that sciarid larvae wound roots allowing infection by *F. circinatum* to occur, which may explain why nursery managers have reported a reduction in the incidence of pitch canker fungus infections, when insecticide treatments are used (Wingfield, unpublished). This role of the insects will need to be considered in the future.

Fusarium oxysporum and *F. stilboides* were the only *Fusarium* spp. isolated from the dipteran specimens. Both *F. oxysporum* and *F. stilboides* were isolated from chironomid specimens. Chironomids have been recorded to transmit Trichomycetes (Zygomycota) (Slaymaker *et al.* 1998). These fungi live obligately within the gut of their hosts and are not plant pathogens. There are no reports of chironomids transmitting plant pathogenic fungi or of having any association with such fungi (Barraclough & Londt 1996).

Neither *F. oxysporum* nor *F. stilboides* are considered important pathogens of pines in South African forestry nurseries. *F. oxysporum* has been isolated from the necrotic roots of seedlings of *Pinus patula* (Viljoen *et al.* 1994), *P. strobus* (Ocamb & Juzwik 1995) and other conifers, such as *Pseudostuga menziesii* (Douglas Fir) (Bloomberg 1971, Bloomberg 1976). However, the pathogenicity of *F. oxysporum* on *P. patula* is significantly lower than that of *F. circinatum* (Viljoen *et al.* 1994), which is considered the primary cause of mortalities in South African pine nurseries. *Fusarium stilboides* is not pathogenic to pine, but causes disease symptoms on other crops, including coffee (Phiri *et al.* 2001), passion fruits (Ismail 2000) and bamboo (Zhang 2000).

Fusarium circinatum was not isolated from adult Sciaridae or Ephydridae, or any of the other dipteran families collected in this study. This is despite the history of sciarids and epydrids transmitting fungal pathogens, including *Fusarium* spp., in nurseries of other crops and the occurrence of *F. circinatum* from all the nurseries where the specimens were collected. These results indicate that adult diptera do not play a major role in the transmission of *F. circinatum* in South African pine nurseries. *B. difformis* larvae possibly influence the incidence of *F. circinatum* in the nursery and this needs to be further investigated.

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Table 1. Diptera recorded in four of the major pine growing nurseries in South Africa during the period 2001 to 2002. (K = Klipkraal nursery, M = Mountain Home nursery, N = Ngodwana nursery, R = Richmond nursery, * = Sciaridae, the only family of fungus gnats recorded.)

Suborder	**Family**	**Nursery**			
		K	**M**	**N**	**R**
Cyclorrhapha	Agromyzidae	✓	✓		
Cyclorrhapha	Ephydridae	✓	✓	✓	✓
Cyclorrhapha	Muscidae		✓		✓
Cyclorrhapha	Tachinidae		✓		
Nematocera	Cecidomyiidae				✓
Nematocera	Chironomidae		✓	✓	✓
Nematocera	*Sciaridae	✓	✓	✓	✓
Nematocera	Simulidae		✓		

Figure 1. Location of the four major pine-growing nurseries sampled between 2001 and 2002. K = Klipkraal (GFP) nursery, Mpumalanga province, M = Mountain Home (Mondi) nursery, KwaZulu-Natal province, N = Ngodwana (Sappi) nursery, Mpumalanga province, R = Richmond (Sappi) nursery, KwaZulu-Natal province.

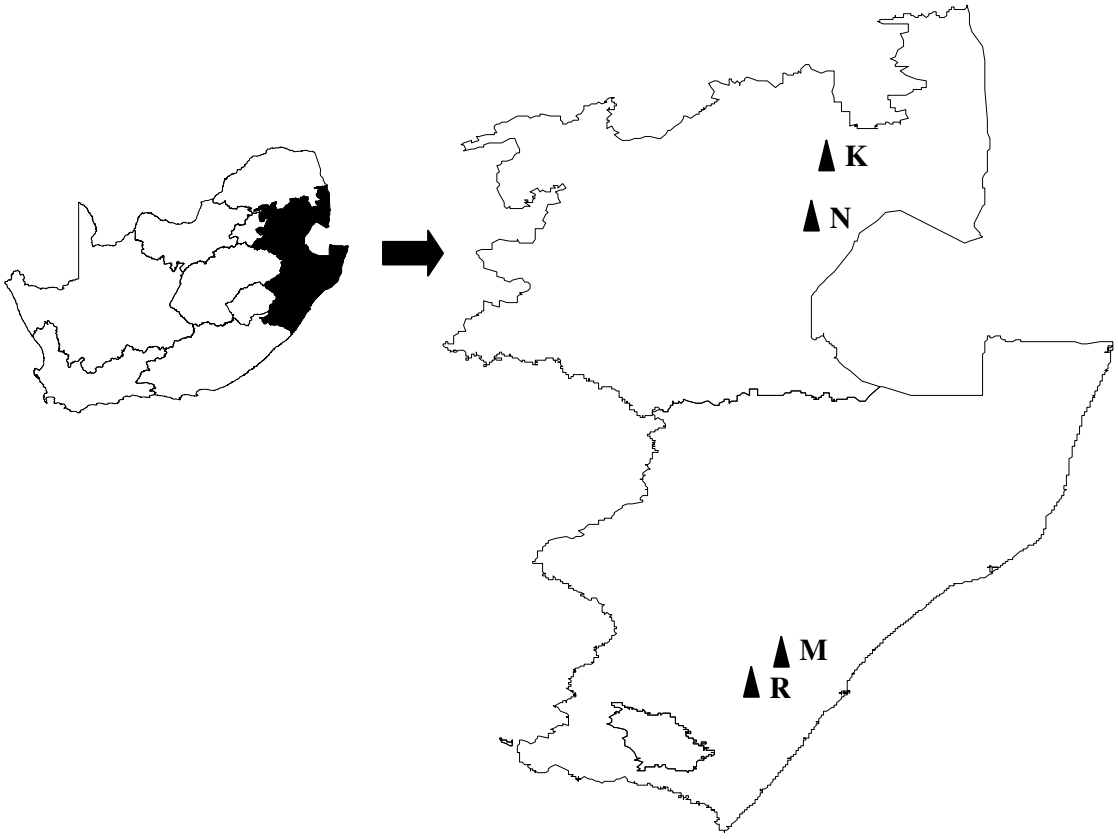
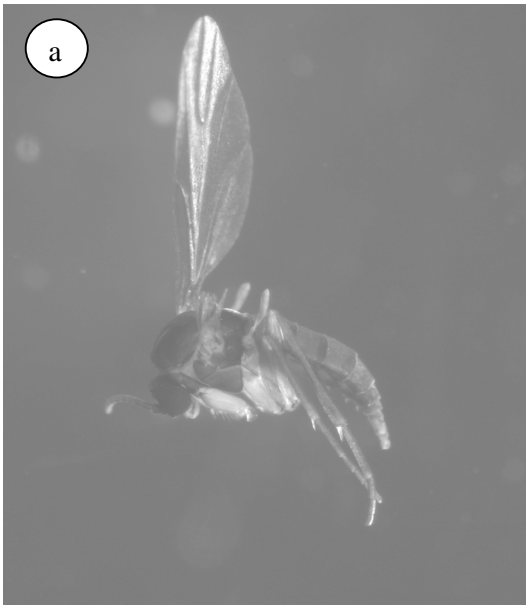

K
N
M
R

Figure 2. *Bradysia difformis.* a. female, b. male, c. wing venation, showing symmetrical fork of M starting distal to the point where R1 joins C. C = costa, CuA = cubitus anterior veins, M = media veins, R = radius veins).


a


b

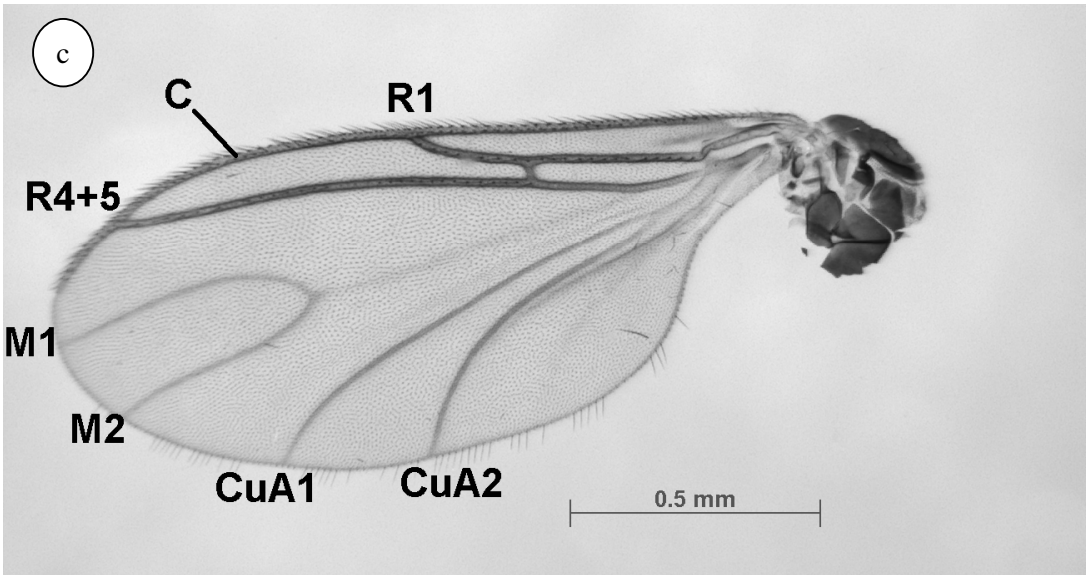
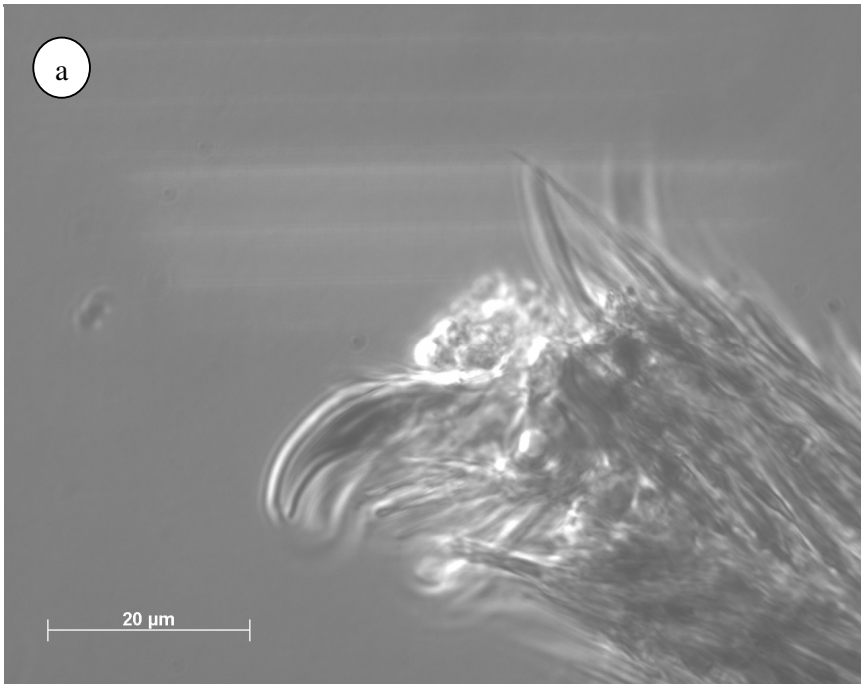
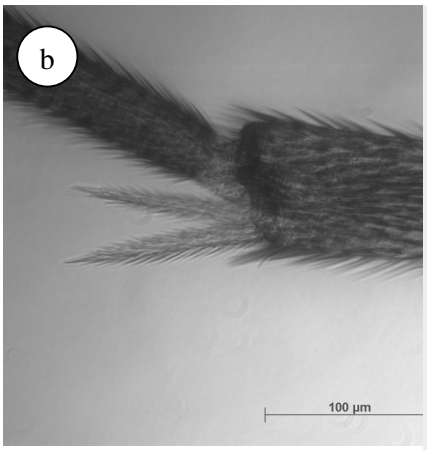
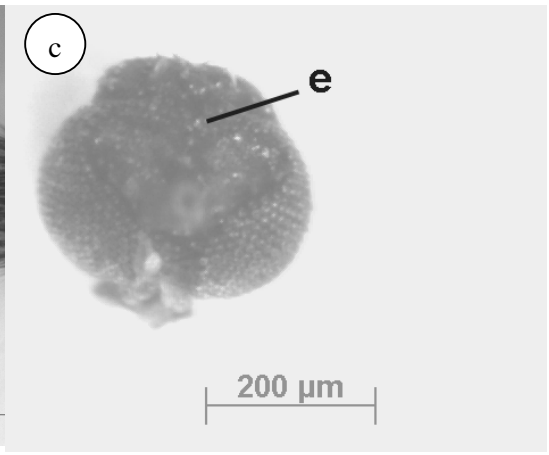

c
C
R1
R4+5
M1
M2
CuA1
CuA2
0.5 mm

Figure 3. *Bradysia difformis*. a. Tarsal claw, simple – teeth absent, b. hind tibia, showing two apical spurs, c. eye-bridge, showing complete eye-bridge above base of antennae. e = eye bridge.


a
20 µm


b
100 µm


c
e
200 µm

CHAPTER 3

Diversity and phlyogeography of *Bradysia difformis* Frey. (Sciaridae: Diptera) in South African forestry nurseries

ABSTRACT

Bradysia difformis (Sciaridae: Diptera) has recently been identified from South African forestry nurseries. This fungus gnat species is also present in Europe, Brazil and the United States, where it occurs in nurseries, fields and mushroom houses. The presence of *B. difformis* in all the major forestry nurseries in South Africa as the dominant and only sciarid species raises intriguing questions regarding its origin and population genetic structure. A portion of the mitochondrial COI gene (395bp) was thus analysed for 56 fungus gnat individuals from four populations collected in pine growing nurseries of South Africa. Sequence analysis revealed 10 polymorphic nucleotide sites, forming eight different COI haplotypes. Maximal sequence divergence was 1.5%. The majority of the variation was within populations, as opposed to between them. This was also reflected in the high estimated migration rate (13.47 – infinite) and low genetic differentiation (-0.00154 – 0.03580) between populations. The insect populations originating from collection sites most distant from each other had the highest migration rate and the populations closest to each other had the highest genetic differentiation. This suggests that natural migration is not a major determining component of the phylogeographic structure. This pattern most likely reflects multiple and / or relatively large introductions of *B. difformis* into South Africa from its origin, combined with subsequent and continued movement of plants between nurseries. These patterns are similar to those for other human-associated fly species and reflect their ease of movement. The results also provide an indication of the magnitude and threat of biological movement into and within South African forest nurseries.

INTRODUCTION

Bradysia difformis is a nematoceran dipteran residing in the family Sciaridae. These and other sciarids are commonly referred to as fungus gnats or black fungus gnats. The genus *Bradysia* has a worldwide distribution (Dennis 1978, Bechev 2000) with *B. difformis* recorded from Azerbaijan, Czech Republic, Germany, Great Britain, Finland, Italy, Japan, Latvia, Netherlands, Norway, Russia, Spain, Sweden, Switzerland and more recently USA, Brazil and South Africa (Hellqvist 1994, White *et al.* 2000, Menzel *et al.* 2003, Chapter 2).

Bradysia difformis typically occurs in glasshouses, mushroom houses, on the stems and roots of various young plants, on ornamental plants in gardens, on peat moss, and in deciduous and coniferous forests (Menzel *et al.* 2003). Sciarid larvae feed on animal excrement, decaying and living plant tissues, and fungal structures, including cultivated mushrooms (Binns 1981, Anas & Reeleder 1988, Springer 1995, Barraclough & Londt 1996). The diet of adult fungus gnats has not been established, although they have been artificially fed on sucrose solutions in captivity (Binns 1981).

The recent discovery of *B. difformis* in South African forestry nurseries (Chapter 2) represents the first report of this insect in the Afrotropical region (Rudzinski pers. comm.). It has been assumed that *B. difformis* was introduced into South Africa, although a path of entry has not been established. However, eggs or larvae could easily have been accidentally imported into the country on plant material, growth medium or logs.

In South Africa, *B. differmis* has been recorded in all the major forestry nurseries and it is apparently the only sciarid present in these facilities (Chapter 2). The insect is suspected to be involved in transmitting or predisposing pine seedlings to the pitch canker fungus, *Fusarium circinatum* Nirenberg & O'Donnell in these nurseries. This pathogen represents one of the most serious impediments to pine production in South Africa (Viljoen *et al.* 1994; Wingfield *et al.* 2002) and the role of *B. differmis* in facilitating infection is currently being studied.

Mitochondrial DNA (mtDNA) has been used extensively for population studies on insects (Sperling & Hickey 1994, Funk *et al.* 1995, Roderick 1996, Ready *et al.* 1997, Caterino *et al.* 2000, Bae *et al.* 2001, Scheffer & Grissell 2003, Hufbauer *et al.* 2004). The advantages of using mtDNA in such population studies includes its high evolutionary rate, absence of recombination and conserved areas that allow primers to be used across taxa (Roderick 1996). Because mtDNA is clonal and only maternally inherited, its effective population size (Ne) is expected to be only one fourth of that for nuclear autosomal genes. As a result, population structure due to stochastic events such as introductions that cause bottlenecks and migration, can be more effectively deduced using mitochondrial DNA variation (Rossi *et al.* 1996)

The presence of *B. differmis* in all the major forestry nurseries in South Africa and its status as the only sciarid species present in these facilities raises intriguing questions relating to its origin and movement. The aim of this study was to evaluate the genetic diversity of *B. differmis* populations in South Africa. Furthermore, we considered whether geographic structure of the insect populations is due to founder effects and genetic drift in geographically isolated populations and whether there are signs of

gene flow between the populations of *B. difformis* in South Africa. We therefore investigated the diversity and phylogeographic structure of *B. difformis* populations from four South African pine nurseries. To achieve this, a portion of the COI gene of the mtDNA was used, corresponding to that for other related organisms (Bae *et al*. 2001. This allowed direct comparison of our results with those of previous studies on introduced and native diptera.

MATERIALS AND METHODS

Collection of fungus gnats

Bradysia difformis individuals were collected from the four main pine-growing nurseries in South Africa. Two of the nurseries near Nelspruit (approximately 25˚34"S, 30˚41"E) and Sabie (approximately 25˚06'28"S, 30˚47'05"E) are situated in the Mpumalanga province. The other two nurseries near Richmond (approximately 29˚51'54"S, 30˚15'50"E) and Hilton (approximately 29˚33'50"S, 30˚18'24"E) are in the KwaZulu-Natal province.

Adult fungus gnats were collected during January 2005. Yellow plastic sheets (14.0cm x 7.5cm) covered with insect glue (Flytac) were used as traps. The traps were placed randomly within nurseries, amongst the pine seedlings. Some of the traps were placed upright on the seedling trays, but the majority of the traps were suspended below the nursery benches. Fungus gnats were removed from the traps using fine tweezers and each insect was placed into a separate vial containing 96% ethanol.

Phylogeny and population biology

DNA extraction

Prior to DNA extraction, the ethanol was removed from the samples. The samples were centrifuged for 6 minutes (16 000 x g) and rinsed with distilled water. The samples were then centrifuged for a further five minutes (16 000 x g) and the water was removed. DNA was extracted using the PrepMan™ Ultra Sample Preparation Reagent Protocol (Applied Biosystems), with 100µl of PrepMan™ Ultra Sample Preparation Reagent used per fungus gnat.

Amplification and sequencing

A portion of the COI gene, including the membrane-spanning helices M3, M4 and M5, external loops E2 and E3, and internal loop I2 (Lunt *et al.* 1996), was amplified by PCR using the primers CI-J-1751 (5' GGAGCTCCTGACATAGCATTCCC) and CI-N-2191 (5' CCCGGTAAAATTAAAATATAAACTTC) (Simon *et al.* 1994). PCR reaction mixtures contained final concentrations of: 2µl of DNA extract, 5µl of dNTPs and buffer, 1.5µl of $MgCl_2$ and *Taq* polymerase (ThermoRed DNA polymerase (Saveen & Werner AB, Malmö, Sweden)), 1µl of each primer and made up with sterile water to reach a volume of 50µl. Amplifications were done using a GeneAmp® PCR System 2700 (Applied Biosystems) thermocycler and programmed for an initial denaturation of the DNA at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 30 s, and concluding with a final elongation at 72 °C for 7 min.

All PCR products were run on 1 % agarose gels, stained with ethidium bromide, and visualized under UV light. PCR products were cleaned using VioGene (Techtum Lab, Umeå, Sweden). Cycle sequence reactions were performed with the ABI PRISMTM BigDyeTM 10x Terminator Cycle Sequencing Ready Reaction Kit v.2.0 (Applied Biosystems, Foster City, CA), using the manufacturers specifications, and analysed on a ABI 310 Genetic analyzer (Applied Biosystems).

Analysis of data

Raw sequence data were analysed using Seqman software (DNASTAR, Inc.). Both forward and reverse sequences were analysed for all isolates. Alignment files were produced in ClustalX (Thompson *et al.* 1997). All further analyses of haplotype and nucleotide diversity, genetic distance and migration rates, AMOVA, and a minimum spanning network were calculated using DnaSP v.4 (Rozas *et al.* 2003) and Arlequin v.2 (Scheider *et al.* 2000). An unrooted tree was produced in Splitstree v.4β, using the BioNJ algorithm and an uncorrected p-value (Huson 1998). A BLAST search (Altschul *et al.* 1997) was done in GenBank to determine the closest sequence match.

RESULTS

Collection of fungus gnats

A total of 56 fungus gnats were collected from the four nurseries, representing four different populations (Table 1). Of these, 15 were collected from the Sabie nursery, 14 from the Hilton nursery, 14 from the Neslpruit nursery and 13 from the Richmond

nursery. The specimens were identified as fungus gnats by morphology. From previous collections (Chapter 2), it was known that the fungus gnats collected were *B. differmis*.

Phylogeny and population biology

A 395bp portion of the COI gene was successfully extracted, amplified and sequenced from the fungus gnats (Figure 1, Table 1). The sequence data confirmed that only one species of fungus gnat had been collected. The BLAST search showed the closest sequence match to be the corresponding COI sequence of an unidentified species of Sciaridae (accession number = AY485389; identities = 392 / 395 (99%)), followed by various species of *Phytomyza* and *Anopheles*, which are both nematocerans and thus related to *B. differmis*.

Sequence analysis revealed 10 polymorphic nucleotide sites representing eight COI haplotypes. Of the 10 polymorphic nucleotide sites, seven were in the third position of the codon, one was in position two and two were in position one. The first codon mutation in position 135 of the sequenced COI gene resulted in the change of the amino acids isoleucine to phenylalanine in haplotype seven. The second codon mutation in position 364 of the sequenced COI gene resulted in the change of the amino acid alanine to valine in haplotypes six and seven (Figure 2). The other mutations did not result in amino acid changes. Sequence divergence of the 8 haplotypes ranged from 0.25 to 1.52% (Table 2, Figure 3). The largest sequence divergence was between haplotypes two and six and haplotypes six and seven. These haplotypes are separated by six mutations each (Figure 4). Three of the haplotypes

were present in all four of the populations and a further two haplotypes were present in three of the populations (Figure 4).

All (100%) of the variation was within populations, as opposed to between them (F_{ST} = -0.00093, p<0.0001) (AMOVA, Distance method: Pairwise distance). The variation greater than 100% and negative F_{ST} value can occur in the absence of genetic structure because the true value of the parameter to be estimated is zero (Schneider *et al.* 2000). Within-locality genetic diversity was high with haplotype diversity ranging from 0.75 to 0.90 and nucleotide diversity ranging from 0.0032 to 0.0072 (Table 3). Insects from the Hilton nursery had the highest genetic diversity, with seven haplotypes present and those from the Richmond nursery had the lowest genetic diversity, with only four haplotypes present.

Genetic differentiation (F_{ST}) was low and female migration rates (N_m) high between populations (Table 4). None of the populations were significantly different from each other with regards to genetic distance. Genetic distance was highest and migration rate lowest between populations of insects from the Hilton and Richmond nurseries, despite the close proximity of these sites. Genetic distance was lowest and migration rate infinite between populations of insects from Richmond and Sabie nurseries, despite the fact that these two populations were most distant from each other.

DISCUSSION

In this study, we investigated the phylogeographic population structure of the fungus gnat, *B. differmis*, in South Africa, using mitochondrial COI sequence data. The data

allowed significant resolution of geographic structure, gene flow and genetic diversity of these flies in four different pine growing nurseries. The results provide an indication of the magnitude of biological movement into and within forestry nurseries.

Bradysia difformis showed relatively high genetic diversity for an introduced population (Pi = 0.005, maximal sequence divergence = 1.52%). The maximal sequence divergence is comparable to those for many other recent studies using the COI gene, but on native populations of insects. These include populations of a parasitoid wasp (Shufran *et al.* 2004), leaf beetle (Funk *et al.* 1995) and migratory dragonfly (Artiss 2004) where sequence divergence was 1.4, 3.8 and 2.3%, respectively. However, it is not comparable to other native populations, such as the red turpentine beetle (*Dendroctonus valens*) where the sequence divergence was up to 15% (Cognato *et al.* 2005). High sequence divergence values have also been recorded for introduced populations, such as *D. valens* populations introduced to China, where the sequence divergence was 2.0% (Cognato *et al.* 2005). In this case the sequence divergence in the native range was up to 15%. The above mentioned studies were for insects of varying biology, habitat and distances between populations. Direct comparison of genetic structure between populations of these insects and that of *B. difformis* must be viewed with some circumspection. However, they do provide a basis to illustrate the range of genetic diversity within which *B. difformis* falls.

There is a striking similarity between the population structure of *B. difformis* presented in this study, and that of the scatopsid fly *Coboldia fuscipes* (Bae *et al.* 2001). Both *C. fuscipes* and *B. difformis* are nematoceran flies and both occur in a nursery-like environment. *Coboldia fuscipes* was studied in oyster mushroom farms in

Korea and *B. difformis* in pine nurseries in South Africa, where each is thought to have been introduced. Maximum sequence divergence was similar between *B. difformis* haplotypes (1.52%) and *C. fuscipes* haplotypes (1.2%). The number of haplotypes was also similar between populations, with eight haplotypes between *B. difformis* populations and ten in *C. fuscipes* populations. The maximum distance between populations was similar in both cases, being approximately 525km between *B. difformis* populations and approximately 310km between *C. fuscipes* populations. These results might reflect common patterns of variation for introduced flies associated with nurseries, greenhouses or other high intensity systems where there are high levels of human activity.

The relatively high genetic diversity found within populations of *B. difformis* suggests either multiple introductions of the fungus gnat into the nurseries and / or a large single introduction of many haplotypes. Most of the haplotypes were shared between all or most of the populations. It therefore seems unlikely that multiple, independent introductions into different nurseries, would produce a pattern of such uniformity. A more likely explanation is that there have been multiple introductions of *B. difformis* into South Africa, followed by movement of fungus gnats between the nurseries. This raises important questions concerning the origin of the insects and whether these introductions are continuing.

Haplotype five identified in *B. difformis* in this study appears to be ancestral. This haplotype is central to the network, is the most common haplotype and it occured in insects from all locations tested. When a population of insects is introduced, the most common haplotypes of the source population tend to be introduced with a higher

probability. For example, introduced populations of *Drosophila buzzatii* in Spain and Australia contained only the main haplotype from the native populations in South America (Rossi 1996). As more introductions occur, it is probable that less common haplotypes are also sampled in proportionate amounts. The fact that such a population structure is reflected for *B. differmis* in South Africa provides further support for the view that there have been multiple and / or large introductions of this insect. The relationship between the haplotypes also suggests a single source population for the South African introductions, as many of the haplotypes are shared between insects at the different locations sampled. In order to verify this view, it will be necessary to consider the genetic structure of native populations of *B. differmis*, but this was not in the scope of this study.

We believe that *B. differmis* is an introduced insect in South Africa, originating most likely in Europe or Asia, where it has been well known for extended periods of time (Binns 1981, Hellqvist 1994). In South Africa, the insect has only been confirmed from forestry nurseries, where most seedlings that are grown are pines and eucalypts, which are both non-native in the country. Although some species of sciarids have been described from South Africa (Rudzinski 1997a,b,c), no comprehensive surveys of this group of insects has been undertaken in the country. Prior to recent investigations (Chapter 2) there have also not been comprehensive surveys of the diptera inhabiting forestry nurseries. Further studies are needed to consider the genetic structure of *B. differmis* from native populations from other countries and those that seem likely to have been introduced into for example Brazil and the USA (Menzel *et al.* 2003). Genetic diversity would be expected to be higher in native populations (Hale & Singh 1987, Irvin *et al.* 1998).

One of the primary findings of this study is the very close similarity between populations of *B. differmis* in four different nurseries, which were in some cases very distant from each other. High gene flow between nurseries and little or no genetic drift affecting the population structures was also observed. Gene flow was well above the minimum level expected to prevent phylogeographic divergence of populations, i.e. $N_m = 1$ (Wright 1969). The shared haplotypes and co-occurrence of low frequency haplotypes in distant populations are also possible consequences of high gene flow (Hale & Singh 1987). The results indicate that fungus gnats migrate or are moved frequently.

Binns (1981) noted that newly fertilized sciarid females tend to move to new environments, but Hungerford (1916) reported that although sciarids can move rapidly on the soil surface, they are weak fliers. It is thus unlikely that *B. differmis* migrated to such great distances between the nurseries considered in this study. Adult *B. differmis* live for only a few days (Harris *et al.* 1996) and migration between nurseries would only occur over a considerable period of time where continuous food and habitat resources were available along the route between nurseries. Furthermore, the physical distances between the populations considered in this study do not explain the high migration and low genetic difference values between populations. When combined, these observations suggest that the exchange of genetic material between populations is strongly influenced by human movement or human-assisted movement as opposed to natural migration. The movement of seedling trays and bark medium between nurseries is known to occur in South Africa (pers. observation) and this could allow *B. differmis* to move between nurseries. Although this movement is not

frequent, it appears to be sufficient to ensure the low genetic differentiation between the populations that was observed. *Bradysia difformis* could also move by means of other plants, such as ornamentals, that are kept in the nurseries, or by the movement of wood (Menzel *et al.* 2003). Other diptera, such as *Drosophila melanogaster*, have also been recorded to be a high migration species due to human-mediated transport (Hale & Singh 1987).

The apparently high migration rate between populations of *B. difformis* could assist the movement of associated fungi between these nurseries. Fungus gnats have been associated with fungal pathogens in various nurseries (Kalb & Millar 1986, Gillespie & Menzies 1993, James *et al.* 1995). Of particular concern is the fact that *B. difformis* could be a potential vector or predisposing agent for the pitch canker fungus *Fusarium circinatum* in South African forestry nurseries. *Fusarium circinatum* is the most serious impediment to pine seedling production in South Africa (Viljoen *et al.* 1994; Wingfield *et al.* 2002) and its movement within and between pine nurseries is of major concern to the forestry industry.

The frequent movement of *B. difformis* between nurseries reflects the general biological movement into South Africa and between distinct agricultural regions within the country. It is already known from population genetic studies that fungi such as *Diplodia pinea*, *Botryospaeria eucalyptorum* and various other *Botryosphaeria* spp. on forestry and other agricultural hosts have been introduced into South Africa multiple times (Burgess *et al.* 2004, Slippers *et al.* 2004). The human associated introduction of the woodwasp *Sirex noctilio* into South Africa, and its subsequent

rapid movement throughout the country provides another important example (Carnegie *et al.* 2005).

The overall results of this study indicated that there is a significant similarity in population structure and pattern of spread of dipteran species associated with agricultural practice, especially in nurseries. Previous reports indicate that these organisms are easily introduced into new environments and that they can spread multiple times. There is clearly an urgent need for more effective measures to reduce the non-intentional spread of forestry and agricultural pests into and within South Africa.

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Table 1. Specimens of *Bradysia difformis* us

Collecting locality	Collection date	Specimen number	COI haplotype	GenBank accession number
Klipkraal nursery, Sabie	28.01.2005	K1	1	DQ060445
		K2	2	DQ060446
		K3	3	DQ060447
		K4	4	DQ060448
		K5	4	DQ060449
		K6	1	DQ060450
		K7	5	DQ060451
		K8	4	DQ060452
		K9	2	DQ060453
		K10	1	DQ060454
		K11	2	DQ060455
		K12	5	DQ060456
		K13	1	DQ060457
		K14	2	DQ060458
		K15	6	DQ060459
Mountain Home nursery, Hilton	20.01.2005	M1	3	DQ060460
		M2	5	DQ060461
		M3	1	DQ060462
		M4	5	DQ060463
		M5	3	DQ060464
		M6	2	DQ060465
		M8	5	DQ060466
		M9	6	DQ060467
		M10	6	DQ060468
		M11	2	DQ060469
		M12	4	DQ060470
		M13	3	DQ060471
		M14	7	DQ060472
		M15	6	DQ060473
Ngodwana nursery, Ngodwana	28.01.2005	N1	5	DQ060474
		N2	3	DQ060475
		N3	5	DQ060476
		N4	5	DQ060477
		N5	5	DQ060478
		N6	5	DQ060479
		N8	4	DQ060480
		N9	5	DQ060481
		N10	5	DQ060482
		N11	5	DQ060483
		N12	4	DQ060484
		N13	2	DQ060485
		N14	6	DQ060486
		N15	5	DQ060487
Richmond nursery, Richmond	19.01.2005	R1	8	DQ060488
		R2	5	DQ060489
		R3	5	DQ060490
		R4	5	DQ060491
		R5	4	DQ060492
		R6	2	DQ060493
		R8	8	DQ060494
		R9	2	DQ060495
		R10	5	DQ060496
		R11	4	DQ060497
		R12	5	DQ060498
		R14	2	DQ060499
		R15	5	DQ060500

Table 2. Sequence divergence values between the eight haplotypes of *B. differmis*.

Haplotype	1	2	3	4	5	6	7	8
1	0	0.76	0.51	0.51	0.25	0.76	0.76	0.25
2		0	0.76	0.76	0.51	1.52	1.01	1.01
3			0	0.51	0.25	1.27	0.76	0.76
4				0	0.25	1.27	0.25	0.76
5					0	1.01	0.51	0.51
6						0	1.52	1.01
7							0	1.01
8								0

Table 3. Diversity estimates of *B. differmis* within localities and of all localities combined.

Locality	N	NH	H	S	Pi
Klipkraal	15	6	0.848	8	0.0056
Mountain Home	14	7	0.890	9	0.0072
Ngodwana	14	5	0.593	8	0.0032
Richmond	13	4	0.744	5	0.0041
All localities	56	8	0.809	10	0.0050

Note: N = sample size, NH = number of haplotypes, H = haplotype diversity, S = number of polymorphic sites, Pi = nucleotide diversity.

Table 4. Genetic differentiation (F_{ST}) between populations of *B. differmis* and per-generation female migration rates (N_M) based on pairwise distances of COI sequence data haplotypes and approximate direct distances (D) between locations (km).

	Sabie	**Hilton**	**Nelspruit**
Hilton	F_{ST} = -0.01845 N_M = Infinite D = 480		
Nelspruit	F_{ST} = 0.01709 N_M = 28.76 D = 60	F_{ST} = -0.00154 N_M = Infinite D = 425	
Richmond	F_{ST} = -0.03075 N_M = Infinite D = 525	F_{ST} = 0.03580 N_M = 13.47 D = 45	F_{ST} = -0.00383 N_M = Infinite D = 475

Note: None of the p-values for F_{ST} were significant at the 95% level.

Figure 1. Example of an agarose gel showing fragments of PCR amplicons of various *B. difformis* specimens, obtained using primers CI-J-1751 and CI-N-2191. Lane N is the negative control, which did not contain *B.difformis* DNA extract. Lane A contains a 100bp size marker.

A K8 K9 K10 K11 K12 K13 K14 K15 N7 N8 N9 N10 N11 N12 N13 N14 N15 **N** **A**

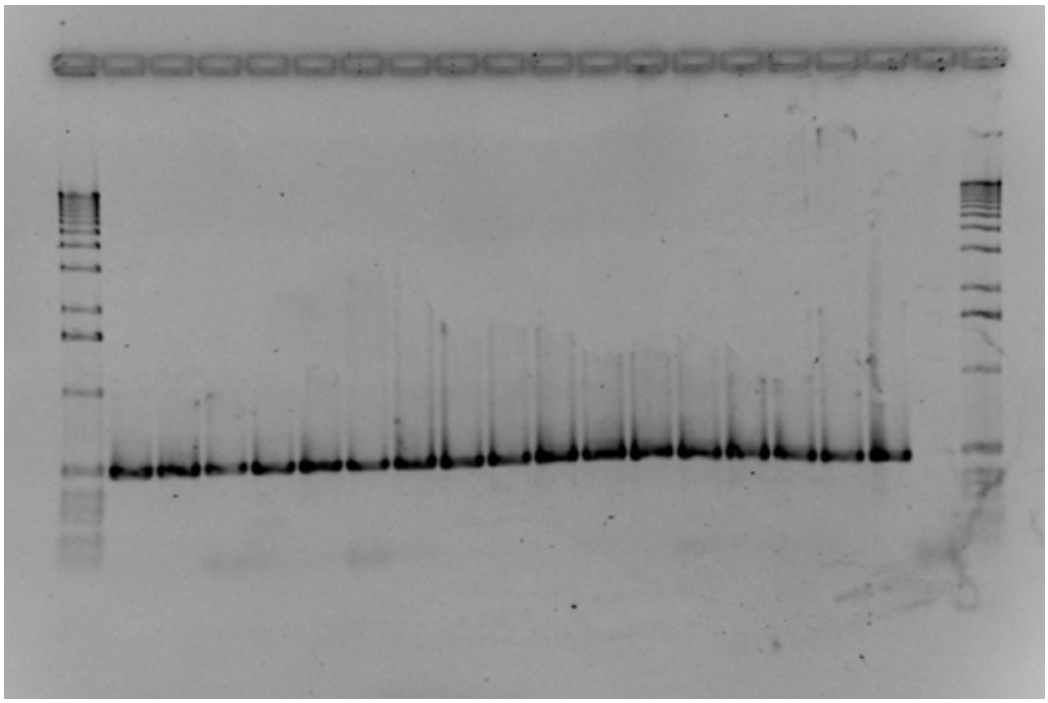


Figure 2. Sequence data of part of the mitochondrial COI gene of 56 *B. difformis* specimens, showing the polymorphic sites in the eight haplotypes obtained.

```
[              10         20         30         40         50]
[               *          *          *          *          *]
Hap_1  TTTGATTATTGCCACCCTCTTTAACACTTTTATTAACTAGAAGGTTAGTA
Hap_2  ..................................................
Hap_3  ..................................................
Hap_4  ..................................................
Hap_5  ..................................................
Hap_6  ..................................................
Hap_7  ..................................................
Hap_8  ..................................................

[              60         70         80         90        100]
[               *          *          *          *          *]
Hap_1  GAAAGAGGTACAGGTACTGGATGAACTGTTTATCCTCCATTATCGTCAAC
Hap_2  ..................................................
Hap_3  ..........................................A.......
Hap_4  ..........................................A.......
Hap_5  ..........................................A.......
Hap_6  ..........................................A.......
Hap_7  ..........................................A.......
Hap_8  ..................................................

[             110        120        130        140        150]
[               *          *          *          *          *]
Hap_1  AATTGCTCATTCAGGGGCCTCTGTTGATCTATCAATTTTTTCTCTTCATT
Hap_2  ......C...........................................
Hap_3  ..................................................
Hap_4  ..................................................
Hap_5  ..................................................
Hap_6  ..................................................
Hap_7  ..................................T...............
Hap_8  ..................................................

[             160        170        180        190        200]
[               *          *          *          *          *]
Hap_1  TAGCAGGAATTTCTTCAATTTTAGGTGCAGTAAATTTTATTTCTACAATT
Hap_2  ..................................................
Hap_3  ..................................................
Hap_4  ..................................................
Hap_5  ..................................................
Hap_6  ..................................................
Hap_7  ..................................................
Hap_8  .......G..........................................

[             210        220        230        240        250]
[               *          *          *          *          *]
Hap_1  ATTAATATACGAGCCCCAGGAATATCTTTTGATAAATTACCTTTATTTAC
Hap_2  ..................................................
Hap_3  ..................................................
Hap_4  ..................................................
Hap_5  ..................................................
Hap_6  ..................................................
Hap_7  ..................................................
Hap_8  ..................................................

[             260        270        280        290        300]
[               *          *          *          *          *]
Hap_1  TTGATCTGTTTTAATTACAGCAGTTTTATTATTATTATCTTTACCAGTAT
Hap_2  ..................................................
Hap_3  ..................................................
Hap_4  ..................................................
Hap_5  ........................C.........................
Hap_6  ..................................................
Hap_7  ..................................................
Hap_8  ..................................................

[             310        320        330        340        350]
[               *          *          *          *          *]
Hap_1  TAGCAGGAGCAATTACTATATTATTAACTGACCGAAATTTAAATACTTCA
Hap_2  ..................................................
Hap_3  ..................................................
Hap_4  ..................................................
Hap_5  ..................................................
Hap_6  ..................................................
Hap_7  ..................................................
Hap_8  ..................................................

[             360        370        380        390     ]
[               *          *          *          *     ]
Hap_1  TTTTTTGATCCAGCAGGAGGGGGAGACCCAATTTTATATCAACAT
Hap_2  ..........................................
Hap_3  ..........................................
Hap_4  ..................A.....T.................
Hap_5  ..........................................
Hap_6  ............T.............................
Hap_7  ............T.............................
Hap_8  ............G........T.....................
```

Figure 3. An unrooted tree, using the BioNJ algorithm and an uncorrected p-value, showing the relationship between haplotypes of *B. differmis*. Haplotypes are represented by H1 to H8. Correlating specimen numbers are shown in Table 1.

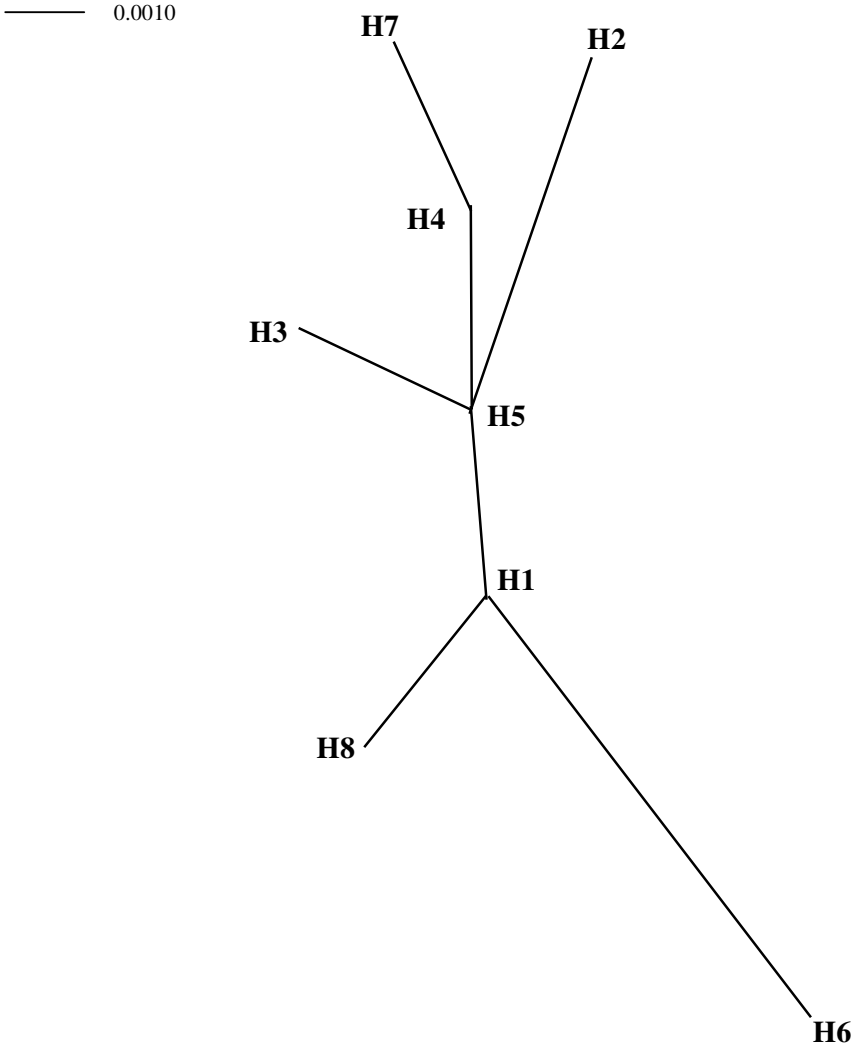
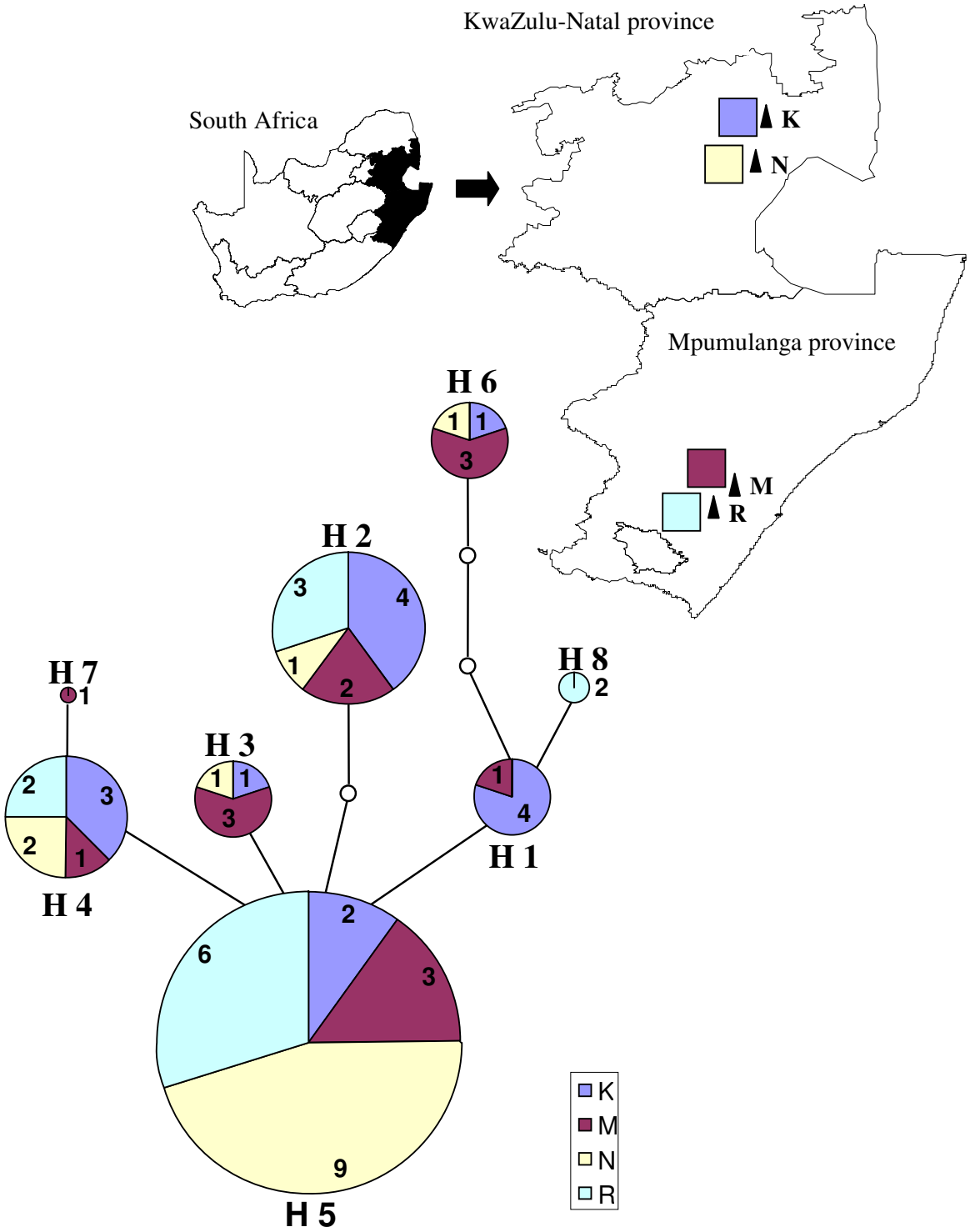

0.0010
H7
H2
H4
H3
H5
H1
H8
H6

Figure 4. Map of South Africa showing the different localities where *B. differmis* specimens were collected and an unrooted haplotype network showing the relationship between *B. differmis* haplotypes and the proportion of each haplotype found in the different localities. The branches are single nucleotide mutations between haplotypes and the empty circles are unsampled haplotypes. The number of *B. differmis* specimens that comprise each segment is indicated. K = Klipkraal nursery; N = Ngodwana nursery; M = Mountain Home nursery; R = Richmond nursery. Haplotypes are represented by H1 to H8.


KwaZulu-Natal province
South Africa
K
N
Mpumulanga province
M
R
H 6
1 1
3
H 2
3 4
1 2
H 8
2
H 7
1
H 3
1 1
3
1
4
H 1
2 3
2 1
H 4
2
6 3
9
H 5
K
M
N
R

CHAPTER 4

Molecular detection of fungi carried by *Bradysia difformis* Frey. (Sciaridae: Diptera) in South African forestry nurseries

ABSTRACT

Bradysia difformis (Sciaridae: Diptera) has recently been identified from South African forestry nurseries, and is thought to have been introduced into the country. Fungus gnats, including *Bradysia* sp. are known to transmit various fungal pathogens. It has been hypothesized that *B. difformis* might be responsible for the rapid spread of the pathogen *Fusarium circinatum* within South African forestry nurseries. Previous studies have, however, failed to confirm this assumption. In this study we attempted to determine the association between *B. difformis* and the two nursery pathogens *F. circinatum* and *Botrytis cinerea,* using sensitive DNA based markers. A total of 60 fungus gnats and four combined collections of 25-30 fungus gnats were obtained from four of the major forestry nurseries in South Africa. The species-specific primers CIRC1A and CIRC4A and C_{729+} and C_{729-} were used in an attempt to detect *F. circinatum* and *B. cinerea*, respectively. The sensitivity of these primers when fungal DNA was mixed with fungus gnat DNA was tested at various concentrations. The general fungal primers, ITS1F and ITS4B and NL1 and NL4, were used to detect any other fungi on *B. difformis*. Amplicons resulting from PCR with these general primers were sequenced, or multiple band products were cloned and sequenced. Neither *F. circinatum*, *B. cinerea* nor any other fungal pathogens were detected on *B. difformis*. This is despite the ability of CIRC1A and CIRC4A to detect *F. circinatum* at a minimum of 13.4pg and a ratio of 1:3727 grams fungus to fungus gnat DNA, and the ability of C_{729+} and C_{729-} to detect *B. cinerea* at a minimum of 3.4pg and a ratio of 1:14691 grams fungus to fungus gnat DNA. Other fungi were detected using the general fungal primers, but none of these fungi were pathogens. We conclude that *B. difformis* does not play a major role in the movement of these or other fungal pathogens in South African forestry nurseries.

INTRODUCTION

Bradysia difformis Frey (Sciaridae: Diptera) are commonly referred to as fungus gnats or black fungus gnats (Barraclough & Londt 1996). *Bradysia difformis* has been reported in Britain (White *et al.* 2000), Norway and Sweden (Hellqvist 1994), and recently also in the USA, Brazil (Menzel *et al.* 2003) and South Africa (Chapter 2) where it is thought to have been introduced. In South Africa, *B. difformis* was detected in all the major forestry nurseries, where it appears to be the only fungus gnat present (Chapter 2).

Bradysia difformis is known as a pest in European nurseries. For example, in Britain, *B. difformis* damages ornamentals (Gouge & Hague 1995) and is a minor pest of mushrooms (Binns 1981; White *et al.* 2000). In Norway and Sweden, *B. difformis* is a common greenhouse pest (Hellqvist 1994). In these greenhouses, *B. difformis* larvae feed on the roots and root collar regions of plants. The pest status of *B. difformis* in South Africa, however, is not yet known.

The feeding of fungus gnat larvae on healthy roots causes a reduction in plant vigour (Kennedy 1974; Springer1995a,b) and provides infection sites for various pathogenic fungi (Springer 1995b). Fungus gnat larvae come into contact with fungi while moving in the soil, feeding on infected plant roots or feeding directly on the fungi. Fungus gnat adults come into contact with fungi while moving on infected plants, emerging from soil after pupation or by trans-stadial transmission, where fungal material from the larval gut persists in the adult gut (Huang & Harper 1985, Gardiner *et al.* 1990, Jarvis *et al.* 1993). Therefore, fungus gnats are potential carriers of fungi,

including fungal pathogens, within nurseries. Pathogens transmitted by *Bradysia* spp. include *Verticillium albo-atrum* Reinke & Berthold on alfalfa plants (Kalb & Millar 1986), *Botrytis cinerea* Pers.:Fr., *Fusarium* spp. and *Phoma* spp. on conifer seedlings (Keates *et al.* 1989, James *et al.* 1995) and *Fusarium oxysporum* Schlecht f. sp. *radicis-lycopersici* Jarvis and Shoemaker on bean and tomato plants (Gillespie & Menzies 1993). Yet, *Bradysia difformis* has not been implicated in the transmission of fungal pathogens.

In South African forestry nurseries, the pitch canker fungus, *Fusarium circinatum* Nirenberg et O'Donnell is a serious pathogen of pine seedlings (Viljoen *et al.* 1994; Wingfield *et al.* 2002). This fungus causes lesions at the root collars and the cotyledon node regions of seedlings (Barnard & Blakeslee 1980; Viljoen *et al.* 1994). Symptoms of diseased seedlings include tip die-back, damping-off, chlorotic or reddish-brown needle discoloration and wilting (Barnard & Blakeslee 1980; Rowan 1982).

Botrytis cinerea Pers.:Fr. is also a common pathogen in South African forestry nurseries, where it infects both *Eucalyptus* and *Pinus* seedlings (Crous *et al.* 1989). *Botrytis cinerea* colonizes and sporulates on dead needles, from which it moves to healthy plant tissue. Severely infested stems may be girdled and die (Mittal *et al.* 1987).

The association between fungus gnats and fungal pathogens in other crops has led to the suggestion that *B. difformis* may transmit fungal pathogens such as *F. circinatum* and *B. cinerea* in South African forestry nurseries. *Fusarium* spp. have been isolated from *Bradysia* spp. in conifer seedling greenhouses in British Columbia, Canada,

using *Fusarium* selective media (Keates *et al.* 1989). *Botrytis cinerea* has been isolated from *Bradysia* spp. in conifer seedling greenhouses in Idaho, USA, using potato dextrose agar (PDA) (James *et al.* 1995) and in British Columbia, Canada, using acidified potato dextrose agar (APDA) and malt agar (MA) (Keates *et al.* 1989). In a previous study (Chapter 2), we were unable to isolate *F. circinatum* from *B. difformis* collected at the four major pine growing nurseries in South. However, the use of *Fusarium* selective medium (Nash & Snyder 1962) in that study may not have been sufficiently efficient to detect *F. circinatum* in small quantities or when other faster growing *Fusarium* species are present. Using *Fusarium* selective medium also excludes the detection of non-target but potentially interesting fungal species. Even when the more general nutrient agar was used, these species may not have been detected if other faster growing and / or more dominant fungi were present, or where the fungi were unable to grow in culture.

DNA-based methods offer an alternative for the detection of fungi from environmental samples, including insects. The polymerase chain reaction (PCR) has been used as a tool to detect various pathogens, using primers designed to specifically amplify a region of the pathogen DNA (Henson & French 1993, Ouellet & Seifert 1993, Taylor 1993, Parry & Nicholson 1996, Shilling *et al.* 1996, Pryor & Gilbertson 2001). Such species-specific primers have been developed and used to detect airborne conidia of *F. circinatum* in *Pinus radiata* plantations (Schweigkofler *et al.* 2004) and *Botrytis cinerea* in strawberry farms (Rigotti *et al.* 2002). The potential exists to use these primers for the detection of *F. circinatum* and *B. cinerea*, possibly being transmitted by *B. difformis* in South African forestry nurseries. Use of more general

fungal primers, combined with cloning, enables the detection of other fungi, possibly unknown, that may also be of interest.

The objectives of this study were two-fold. The first objective was to test fungus gnats for the presence of *F. circinatum* and *B. cinerea*, two fungal pathogens known to occur in South African nurseries, using species-specific primers. The second objective was to consider whether any other fungi might be transmitted by *B. difformis* using more general fungal primers.

MATERIALS AND METHODS

Collection of fungus gnats

Fungus gnats were collected in four of the major pine growing nurseries in South Africa. Two of the nurseries were in the Mpumalanga province, near Nelspruit (approximately 25˚34"S, 30˚41"E) and Sabie (approximately 25˚06'28"S, 30˚47'05"E). The other two nurseries were in KwaZulu-Natal, near Richmond (approximately 29˚51'54"S, 30˚15'50"E) and Hilton (approximately 29˚33'50"S, 30˚18'24"E) (www.gpswaypoints.co.za) (Fig. 1). These nurseries were selected due to the severity of the losses caused by *F. circinatum* in them.

Adult fungus gnats were collected in January 2005. Yellow plastic sheets (14.0cm x 7.5cm) covered with insect glue (Flytac) were used as traps. The traps were placed randomly within nurseries, amongst the pine seedlings. Some of the traps were placed upright on the seedling trays, but the majority of the traps were suspended below the

nursery benches. The traps were successful in catching the adult fungus gnats and other diptera. Fungus gnats were removed from the traps using fine tweezers. Fifteen fungus gnats from each nursery were placed in separate vials, while others where placed collectively in a single vial. The vials contained 96% denatured ethanol and were stored at 5°C. The identity of these fungus gnats as *B. differmis* was based on previous studies that showed *B. differmis* to be the only fungus gnat present in the sampled nurseries (Chapter 2). The presence of just one species of fungus gnat was further confirmed by molecular studies (Chapter 3).

DNA extraction

Prior to DNA extraction, the samples were centrifuged for 6 minutes (16 000 x g) and the ethanol decanted. The remaining sample was rinsed with distilled water, centrifuged for a further 5 minutes (16 000 x g) and then the water was removed. DNA was extracted using the PrepMan™ Ultra Sample Preparation Reagent Protocol (Applied Biosystems), with 100µl of PrepMan™ Ultra Sample Preparation Reagent used for vials with individual fungus gnats and mycelium of *F. circinatum* and *B. cinerea*, and 200µl used for vials with many fungus gnats.

DNA from single isolates of *F. circinatum* and *B. cinerea* were included as positive controls and for the dilution series test. Mycelium was taken from actively growing cultures of *F. circinatum* and *B. cinerea*. DNA was extracted as described for the fungus gnats with 100µl of PrepMan™ Ultra Sample Preparation Reagent.

Specific primers for *F. circinatum* and *B. cinerea*

Species-specific primers were used to test for the presence of *F. circinatum* and *B. cinerea* on the fungus gnat samples. *Fusarium circinatum* specific primers were CIRC1A (5' CTTGGCTCGAGAAGGG) and CIRC4A (5' ACCTACCCTACACCTCTCACT) (Schweigkofler *et al.* 2004). The primers amplify a 360bp DNA fragment in the intergenic spacer region of the nuclear ribosomal RNA operon. The *B. cinerea* specific primers were C_{729+} (5' AGCTCGAGAGAGATCTCTGA) and C_{729-} (5' CTGCAATGTTCTGCGTGGAA) (Rigotti *et al.* 2002). The primers amplify a 700bp fragment of an unknown genomic nuclear locus. PCR reaction mixtures contained final concentrations of: 2 µl of DNA extract, 1x PCR buffer, 0.2 mM of each dNTP, 2.75 mM $MgCl_2$, 3.75 units *Taq* polymerase (ThermoRed DNA polymerase (Saveen & Werner AB, Malmö, Sweden)), 0.2 mM of each primer and made up with distilled water to reach a volume of 25 µl. All amplifications throughout the study were done using a GeneAmp® PCR System 2700 (Applied Biosystems) thermocycler and programmed for an initial denaturation of the DNA at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 45 s and elongation at 72 °C for 30 s, and concluding with a final elongation at 72 °C for 7 min. All PCR products were run on 1 % agarose gels, stained with ethidium bromide, and amplicons were visualized under UV light.

Both negative and positive controls were included in the PCR reaction. The negative control was the same PCR mixture, but excluded the DNA extract. The positive control for the *F. circinatum* specific primers was DNA from the strain FCC3579. The positive control for the *B. cinerea* specific primers was DNA from the strain 98

G1. Both the positive controls for *F. circinatum* and *B. cinerea* were strains associated with disease symptoms in the nurseries.

Dilution series were done to determine the minimum amount of *F. circinatum* and *B. cinerea* DNA necessary to result in visible amplicons after PCR and gel electrophoresis, using the species-specific primers. DNA concentrations were determined by Ultrospec 2000 (Pharmacia Biotech). 10-fold serial dilutions of *F. circinatum* and *B. cinerea* DNA was prepared in distilled water. *Fusarium circinatum* and *B. cinerea* DNA were also diluted with both DNA from individual and pooled fungus gnats. Dilutions of *F. circinatum* in DNA extract from a single fungus gnat ranged from 6:1 to 1:1600 and from 1:3.7 to 1:37000 in DNA extract from pooled fungus gnats. Dilutions of *B. cinerea* with pooled fungus gnat DNA ranged from 1:14.7 to 1:147000. For both dilutions in water and dilutions in fungus gnat DNA, the amount of fungal DNA ranged from 13.4 ng to 1.34 pg for *F. circinatum* and 3.4 ng to 340 fg for *B. cinerea*, in a 25 µl PCR reaction, as described above.

General fungal primers

Originally the primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') (Gardes & Bruns 1993) and ITS4 (5' TCCTCCGGCTTATTGATATGC 3') (White *et al.* 1990) were used to examine the fungal community on the fungus gnats. However, the ITS4 primer was found to co-amplify fungus gnat DNA. Initial results also indicated a dominant presence of Basidiomycetes. Therefore, the primers ITS1F and ITS4B (5' CAGGAGACTTGTACACGGTCCAG) (Gardes & Bruns 1993) and NL1 (5' GCATATCAATAAGCGGAGGAAAAG3') and NL4 (5'

GGTCCGTGTTTCAAGACGG 3') (O'Donnell 1993) were used. The region amplified by the ITS1F and ITS4B included the 3' end of the 18S (small subunit) rDNA gene, the first internal transcribed spacer (ITS 1), the complete 5.8S rRNA gene, the second ITS (ITS 2) region and the 5' end of the 28S (large subunit) rRNA gene. The region amplified by the NL1 and NL4 included the 5' end of 28S rDNA spanning domains D1 and D2. PCR reaction mixtures were as described above, except that 7.5 units *Taq* polymerase (ThermoRed DNA polymerase) were used in a total volume of 50 µl. Amplifications were done as described for the species specific primers, except that the initial denaturation was at 94 °C for 5 min, and during the following 35 cycles annealing was at 50 °C for 30 s and elongation at 72 °C for 1 min. All PCR products were separated using electrophoresis on 1 % agarose gels, stained with ethidium bromide, and the amplicons visualized under UV light.

Where multiple bands were obtained, the PCR products of these samples were combined and cloned into the pCR® 2.1-TOPO® vector (Invitrogen), transformed into *Escherichia coli* (TOP10F' One Shot® chemically competent cells) and grown on LB medium containing ampicillin (50 µg/ml). The protocol given in the TOPO TA Cloning® manual was followed. Bacterial colonies carrying vectors with the fungal inserts were picked up with a sterile tip and suspended in 200 µl of sterile distilled water. Primers M13F (5' GGAAACAGCTATGACCATGATTACGC) and M13R (5′ CAGGAAACAGCTATGAC) (Vieira & Messing 1982) were used to amplify the fungal insert. PCR reaction mixtures were the same as described for the general fungal primers, except that 0.3 mM of each primer was used and 25 µl of bacterial colony suspension was used as a template. The PCR thermocycler program was the same as for the ITS primers, except that elongation at 72 °C was for 30 s during the

35 cycles. PCR products were evaluated using agarose gel (1%) electrophoresis, ethidium bromide staining and visualized using UV light.

Single amplicons generated using the general fungal primers and products obtained from the PCR of the cloned amplicons were purified using VioGene (Techtum Lab, Umeå, Sweden) and sequenced. Cycle sequence reactions were performed with the ABI PRISM™ BigDye™ 10x Terminator Cycle Sequencing Ready Reaction Kit v.2.0 (Applied Biosystems, Foster City, CA) using the manufacturers specifications, and adding 3 µl of cleaned PCR product and 0.16 mM of either primer and in a total volume of 10 µl. Sequence products were analysed on an ABI 310 Genetic analyzer (Applied Biosystems). The electropherogram obtained was analyzed using Seqman (DNASTAR, Inc.). The closest sequence match was then determined using a BLAST search (Altschul *et al.* 1997) option in GenBank (http://www.ncbi.nlm.nih.gov/).

RESULTS

DNA extraction and species-specific primers

For each nursery, DNA successfully extracted from 15 individual fungus gnats, and DNA extracted from the combined collection of 25-30 fungus gnats, was tested for the presence of *F. circinatum* and *B. cinerea* DNA using species-specific primers. Neither *F. circinatum* nor *B. cinerea* were detected from any of the fungus gnats (Fig. 2 & 3). Furthermore, the dilution test for *F. circinatum* showed that DNA from this fungus could be detected when diluted in water to a ratio of 1:100 or when 134 pg of the fungus was present. When diluted with single and pooled fungus gnat DNA

extract, *F. circinatum* was detected to a ratio of 1:164 and 1:3727 ng of fungus to fungus gnat DNA respectively, equivalent to 13,4 pg of fungus (Fig. 4). The dilution test for *B. cinerea* showed that *B. cinerea* could be detected when diluted in water at a ratio of 1:100 or when 34 pg of the fungus was present. When diluted with DNA extract of pooled fungus gnats, *B. cinerea* was detected up to a ratio of 1:14691 ng of fungus to fungus gnat DNA, equivalent to 3,4 pg of fungus (Fig. 5).

General fungal primers

The ITS1F and ITS4 primers were used on the four pooled fungus gnat samples and 26 single fungus gnats. Of these, 70% showed a band of about 850bp and 17% showed a band of about 600bp. Five different bands were found from cloning multiple band products. Sixty seven percent were the 850bp band, 8% were the 600bp band and the remaining bands were between 53bp and 280bp.

BLAST searches with sequence data from the 850bp band product revealed it was the co-amplified product of the ITS1 portion of Basidiomycete fungi (*Sebacina* sp.), amplified by the ITS1F primer, and the ITS2 portion of the fungus gnat DNA, amplified by the ITS4 primer. The BLAST search showed that the closest match to the 600bp band was of a *Cladosporium* sp. The smaller bands most closely matched uncultured ectomycorrhiza, *Sebacina* endomycorrhiza and *Antrodia* spp. Further amplifications were attempted using ITS1F and ITS4B and NL1 and NL4, but no additional fungi were identified. None of the fungi mentioned above are pathogenic and it was not within the scope of this study to further characterize them.

DISCUSSION

In this study, we attempted to detect *F. circinatum* and *B. cinerea* from DNA isolated from *B. difformis* using species-specific primers. The results showed that neither of these fungi were present. These findings, together with those in Chapter 2, where *F. circinatum* was not isolated from *B. difformis* using *Fusarium* specific medium, provide convincing evidence that *B. difformis* is not a major factor in the movement of these two pathogens in South African forestry nurseries. This is despite the occurrence of *B. difformis* and the two pathogens in the same nurseries and the fact that *B. cinerea* and *Fusarium* spp. have been shown to be carried by fungus gnats in other nursery environments (Keates *et al.* 1989, Gillespie & Menzies 1993, James *et al.* 1995).

Using a dilution series, we showed that DNA from *F. circinatum* and *B. cinerea* can be detected at very low concentrations using species-specific primers, even in the presence of high concentrations of *B. difformis* DNA. Using the primers CIRC1A and CIRC4A *F. circinatum* DNA could be detected when as little as 13.4pg of DNA was present when this DNA was mixed with *B. difformis* DNA. These results compare with the work of Schweigkofler *et al.* (2004), who showed that with these primers, 10pg was the limit for detection.

PCR amlification using C_{729+} and C_{729-} primers allowed for the detection of *B. cinerea* DNA down to 3.4pg when this DNA was mixed with *B. difformis* DNA. Rigotti *et al.* (2002) found the limit for detection using these primers to be approximately 0.2pg, with only *B. cinerea* DNA, but 2pg when mixed with other DNA. The sensitivity of

the CIRC1A and CIRC4A and C_{729+} and C_{729-} primers are also comparable to species-specific primers developed for other pathogens. For example, the limit for detection for *Fusarium culmorum, F. graminearum, Alternaria radicina* and *Leptosphaeria maculans* is 50pg, 5pg, 0.2pg and 0.1pg respectively (Taylor 1993, Shilling *et al.* 1996, Pryor & Gilbertson 2001). The sensitivity of the primers used in this study are comparable to those used in other studies to detect fungi and we suggest that they are sufficiently sensitive to detect the presence of *F. circinatum* and *B. cinerea* on *B. difformis*.

Fungus gnats have been reported to carry pathogens by trans-stadial transmission (Huang & Harper 1985, Gardiner *et al.* 1990, Jarvis *et al.* 1993). For such transmission, the fungus gnat larvae feed on the spores of the fungi while living in the soil. Some of the ingested fungi remain viable in the digestive tract, even after pupation, and into the adult stage. The fungus is thus spread through the faeces and carcass of the adult. The probability of fungus gnat larvae to ingest pathogenic fungi increases if they are feeding on or close to infected plant tissue. However, *B. difformis* larvae were seldom observed to inhabit the plugs of the pine seedlingsh, most likely because they do not find the bark and vermiculite medium a suitable habitat. *Bradysia difformis* is thought to oviposit under the nursery benches and in the environment surrounding the nursery, where soil is present. This behaviour would thus limit its contact in larval form with infected pine seedlings.

Fungus gnat adults may carry fungal pathogens externally on their bodies. Adults can obtain fungi when they pupate and emerge as adults from the soil, thus making contact with any pathogens in the soil, or when walking on diseased plants (Kalb &

Millar 1986). As *B. difformis* do not pupate inside the seedling plug, the probability that emerging adults come into contact with these fungi is small. *Bradysia difformis* adults might acquire *F. circinatum* and *B. cinerea* when moving around on infected plants. No evidence found in this study supported that this occurs in South African nurseries. Nursery sanitation involving the removal of infected plants may be a factor contributing to low inoculum of the fungus, and consequently less chance of contact between *B. difformis* adults and the pathogen.

Despite a history of association between fungus gnats and fungal pathogens, neither *F. circinatum*, *B. cinerea* or any other fungal pathogens were detected on *B. difformis*. This indicates that *B. difformis* does not have a major role in the movement of these fungi in South African forestry nurseries. Species-specific primers provide a useful and sensitive technique for detecting fungi on insects in nurseries and other environments. Such techniques also provide a tool to better understand insect-fungal associations, such as those involving the transmission and possible vectorship of pathogens by insects.

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Figure 1. Location of the four pine-growing nurseries where *B. diffformis* was collected. Triangles show position of nurseries. Closest towns / cities indicated by letters, where S = Sabie, Mpumalanga province; N = Nelspruit, Mpumulanga province; H = Hilton, KwaZulu-Natal province; R = Richmond, KwaZulu-Natal province.

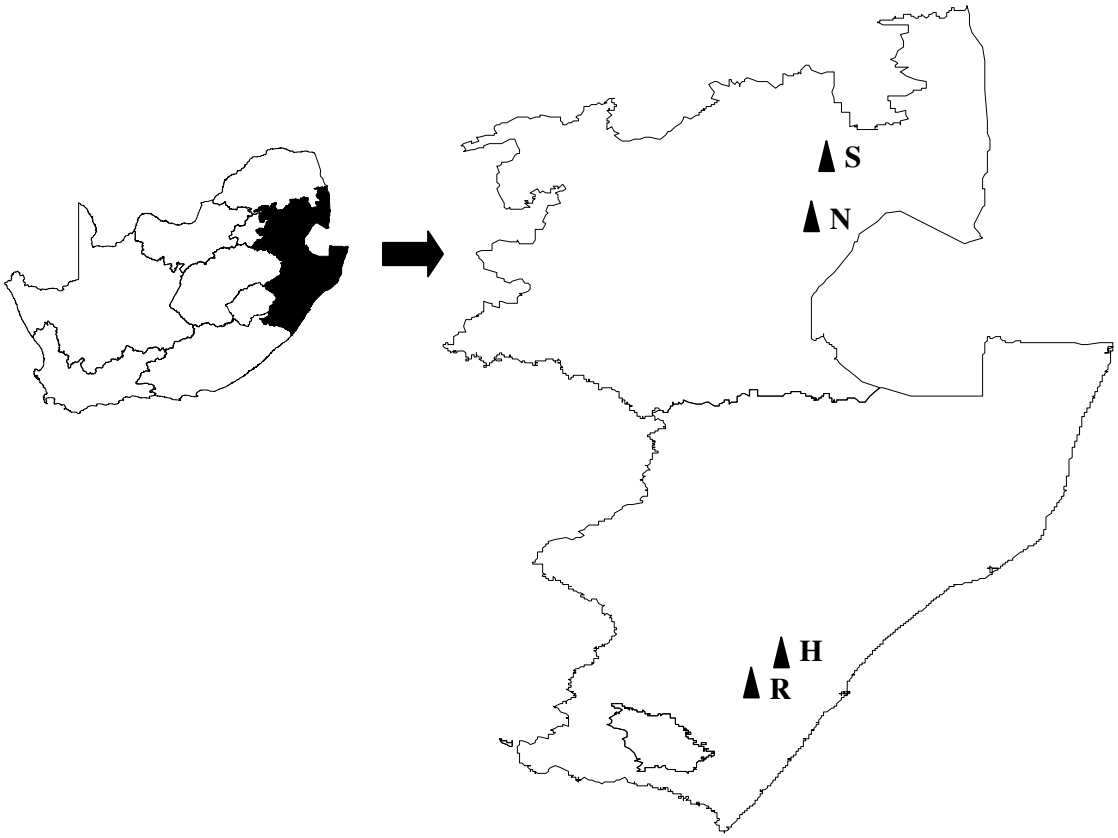

S
N
H
R

Figure 2. Example of an agarose gel showing the absence of PCR amplicons obtained from DNA isolated from *B. difformis* specimens, using *F. circinatum* specific primers CIRC1A and CIRC4A. This shows that this fungus was not detected on the fungus gnats. Lanes K9 to K15 and M4 to M15 contain PCR products from reactions containing DNA isolated from single *B. difformis* specimens. Lanes RT, NT, KT and MT represent the PCR reaction products from the PCR using the DNA from pooled fungus gnats. Lane P is the positive control, lane N is the negative control, and lane A contains a 1 kb ladder.

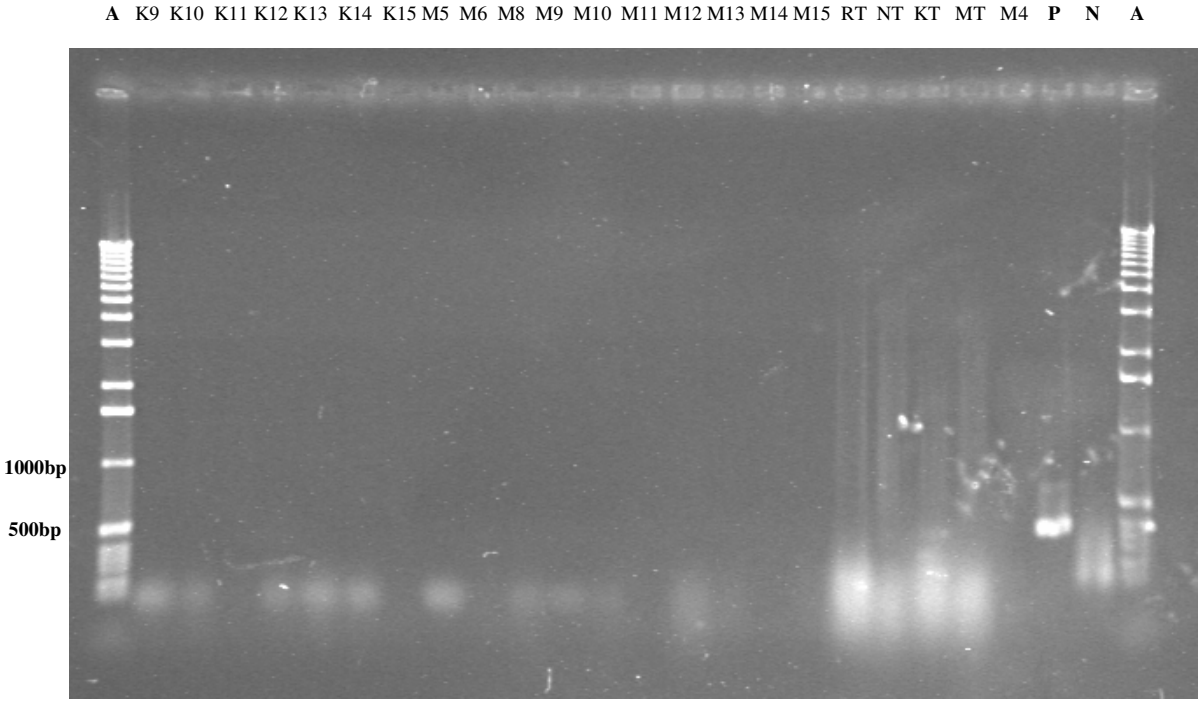

A K9 K10 K11 K12 K13 K14 K15 M5 M6 M8 M9 M10 M11 M12 M13 M14 M15 RT NT KT MT M4 P N A
1000bp
500bp

Figure 3. Example of an agarose gel showing the absence of PCR amplicons obtained from DNA isolated from *B. differmis* specimens, using *B. cinerea* specific primers C_{729+} and C_{729-}. This shows that this fungus was not detected from DNA isolated from fungus gnats. Lanes K8 to K15 and M5 to M13 contain PCR products from reactions containing DNA isolated from single *B. differmis* specimens. Lane P is the positive control, lane N is the negative control, and lane A contains a 1 kb ladder.

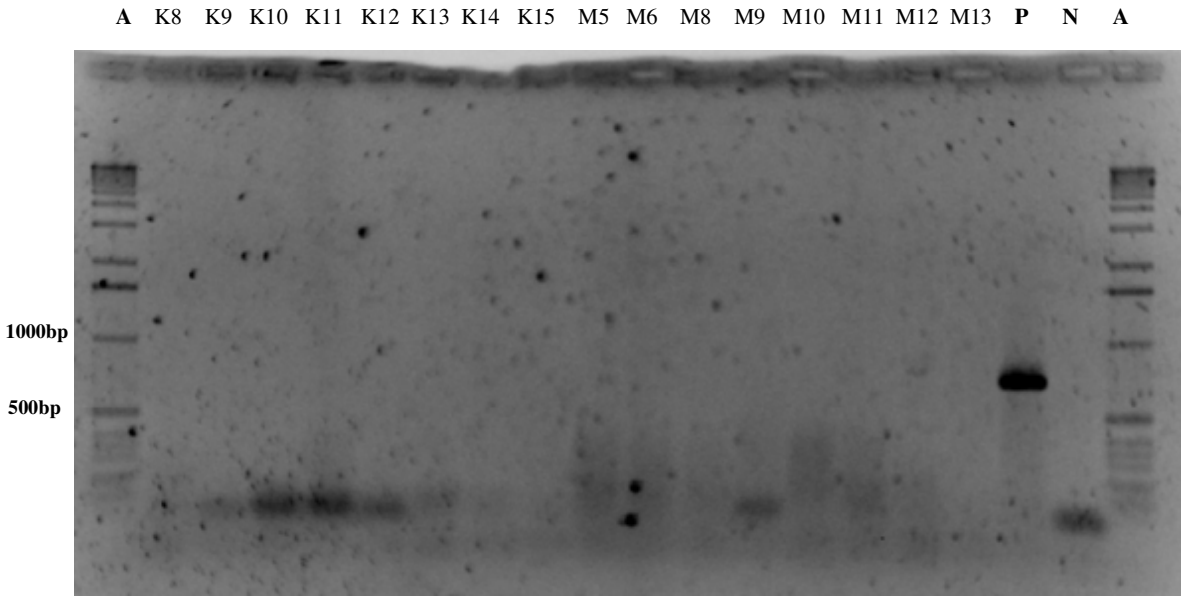

A K8 K9 K10 K11 K12 K13 K14 K15 M5 M6 M8 M9 M10 M11 M12 M13 P N A
1000bp
500bp

Figure 4. Agarose gel (1%) showing the PCR amplicons resulting from *Fusarium circinatum* dilution series using *F. circinatum* specific primers CIRC1A and CIRC4A. The circles indicate the faintest band on the gels. Lane W1 is undiluted *F. circinatum* DNA (13.4 ng genomic DNA or 6.7 ng/µl) and serves as a positive control. Lanes W2 to W5 are 10-fold dilutions of *F. circinatum* genomic DNA with water, where the amount of DNA is 1.34 ng, 134 pg, 13.4 pg and 1.34 pg, respectively. Lanes F1 to F5 are the dilutions of *F. circinatum* DNA extract (6.7 ng/µl) with DNA extract from a single fungus gnat (1.1 ng/µl) with the ratios of 6:1, 1:1.6, 1:16, 1:160 and 1:1600 fungus DNA to fungus gnat DNA. Lanes FT1 to FT5 are the dilutions of *F. circinatum* DNA extract (6.7 ng/µl) with DNA extract from pooled fungus gnats (25 ng/µl) in the ratio of 1:3.7, 1:37, 1:370, 1:3700 and 1:37000 fungus DNA to fungus gnat DNA. The amount of *F. circinatum* DNA in F1 to F5 and FT1 to FT5 are: 6.7 ng, 1.34 ng, 134 pg, 13.4 pg, 1.34 pg. Lane N is the negative control and lane A contains a 1 kb ladder.

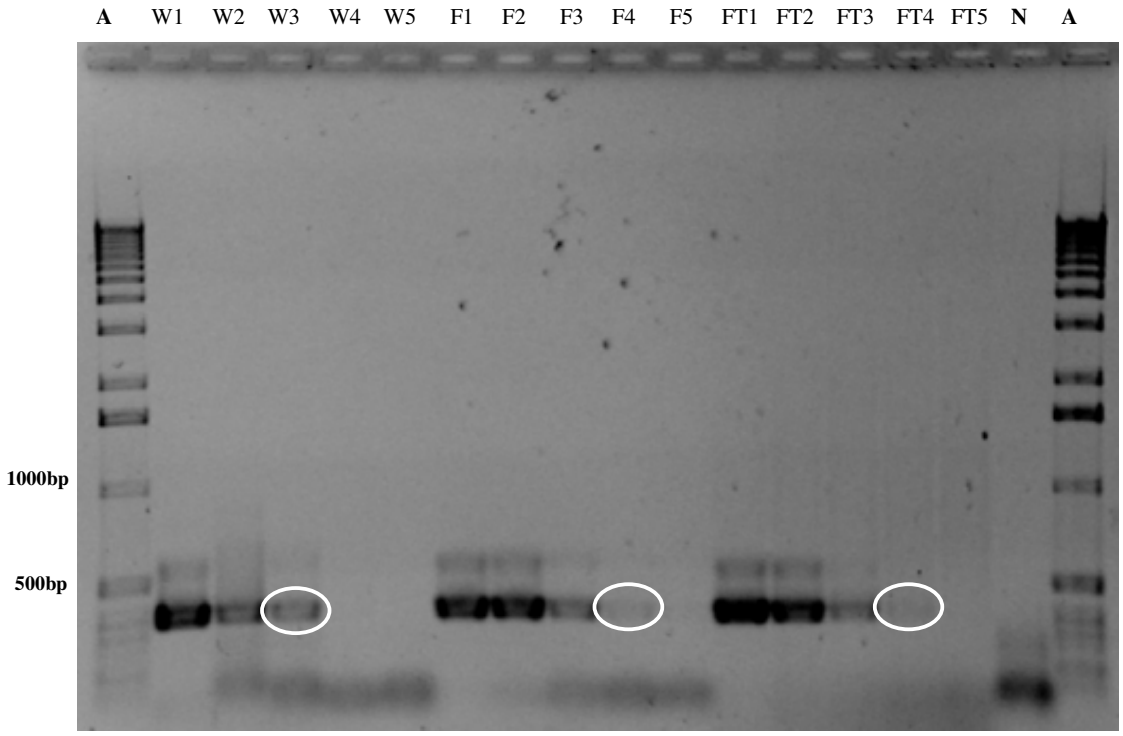
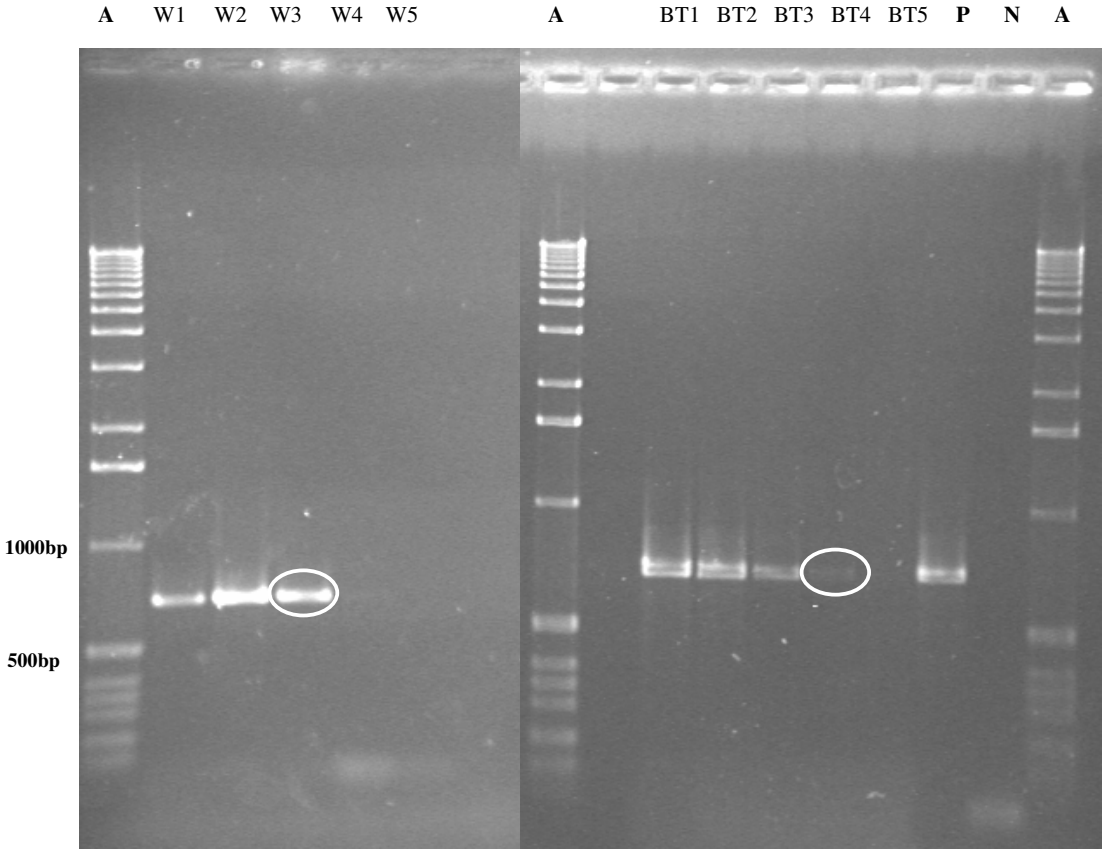

A
W1
W2
W3
W4
W5
F1
F2
F3
F4
F5
FT1
FT2
FT3
FT4
FT5
N
A
1000bp
500bp

Figure 5. Agarose gel (1%) showing the PCR amplicons resulting from the *Botrytis cinerea* dilution series using *B. cinerea* specific primers C_{729+} and C_{729-}. Lanes where bands are visible indicate that DNA was detectable using the PCR test used in this study. The circles indicate the faintest band on the gels. Lane W1 is undiluted *B. cinerea* DNA (3.4 ng genomic DNA or 1.7 ng/µl) and serves as a positive control. Lanes W2 to W5 are 10-fold dilutions of *B. cinerea* DNA with water, where the amount of DNA is 340 pg, 34 pg, 3.4 pg and 340 fg, respectively. Lanes BT1 to BT5 are the dilutions of *B. cinerea* DNA extract (1.7 ng/µl) with DNA extract from pooled fungus gnats (25 ng/µl) in the ratio of 1:15, 1:130, 1:1500, 1:15000 and 1:150000 fungus DNA to fungus gnat DNA. The amount of *B. cinerea* DNA in BT1 to BT5 is: 1.7 ng, 340 pg, 34 pg, 3.4 pg and 340 fg. Lane N is the negative control, lane P is an additional positive control and lane A contains a 1 kb ladder.


A
W1
W2
W3
W4
W5
A
BT1
BT2
BT3
BT4
BT5
P
N
A
1000bp
500bp

SUMMARY

This dissertation treats studies on fungus gnats and their association with the pathogenic fungus *Fusarium circinatum* in South African pine nurseries. In the literature review, the history of associations between fungus gnats and fungal pathogens is treated. Both fungus gnat adults and larvae have been implicated in the transmission of pathogens, although it is predominantly the larvae that facilitate the movement of these organisms. Fungus gnats in different nursery environments transmit various fungal pathogens. But, the association between fungus gnats and pathogens, particularly *Fusarium circinatum*, in South African pine nurseries had not been investigated prior to this study. In cases where fungus gnats were considered pests due to their association with fungal pathogens or from the direct damage due to larval feeding, various control measures are used. These include cultural, chemical and biological control.

Studies on the occurrence of fungus gnats in South African pine nurseries showed that *Bradysia difformis* was the only fungus gnat species present in the four nurseries surveyed, and it was present in all these nurseries. *Bradysia difformis* is most probably an alien invasive insect introduced from Europe, where it is a well-known pest in various nurseries due to the feeding habit of the larvae. *Bradysia difformis* has not specifically been implicated in the movement of pathogens, but various other *Bradysia* species are known for such associations. Our results showed that neither *B. difformis* nor any of the other diptera collected in this study carried *F. circinatum*. Among the other dipteran families collected, only Ephydridae could potentially be involved in transmitting pathogens, and this requires further study.

Analysis of mitochondrial COI sequence data from fungus gnats collected in the four nurseries provided interesting information on the population structure and diversity within as well as between populations. Of particular interest was that most of the variation was within and not between populations. The results indicated that the high diversity within populations was not due to natural migration, but most probably resulted from multiple and / or large introductions of *B. differmis* into the nurseries, followed by continued movement between the nurseries.

The species-specific primers CIR1A and CIR1B and C_{729+} and C_{729-} were used to detect *F. circinatum* and *Botryis cinerea*, respectively. Dilution series showed that these primers can detect *F. circinatum* and *B. cinerea* in minute amounts (13.4pg and 3.4pg fungus DNA) when mixed with disproportionate amounts of fungus gnat DNA. This suggests that these primers can provide an effective tool for detecting the pathogens on fungus gnats or other insects. Neither *F. ciricinatum* nor *B. cinerea* were detected on any of the fungus gnats collected. Other fungi were collected using general fungal primers, but none of these were pathogenic. This suggests that *B. differmis* is not involved in the movement of *F. circinatum*, *B. cinerea* or other fungal pathogens in these nurseries.

The results of the studies presented in this dissertation have shown that *B. differmis* does not play a major role in the movement of *F. circinatum* or other pathogenic fungi in pine nurseries of South Africa. Species-specific primers were found to be a useful tool in detecting pathogens on insects and this method should be further tested on other insects that have a potential to transmit pathogens in the nurseries. Although *B. differmis* was not found to carry any pathogens in this study, the frequent movement

of fungus gnats between nurseries shows the ease with which such insects could spread fungal pathogens not just within, but also between nurseries.