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

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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* spp., are known to transmit various fungal pathogens. It has thus been hypothesised 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 *Bo. cinerea*, respectively. The sensitivity of these primers when fungal DNA was mixed with fungus gnat DNA was tested at various concentrations. General fungal primers were used to detect any other fungi on *B. difformis*. Neither *F. circinatum*, *Bo. 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 1:3 727 fungus to fungus gnat DNA, and the ability of C_{729+} and C_{729-} to detect *Bo. cinerea* at a minimum of 3.4pg and a ratio of 1:14 691 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.

Keywords: *Botrytis cinerea*, *Bradysia difformis*, CIRC1A, CIRC4A, C_{729+}, C_{729-}, DNA markers, fungus gnats, *Fusarium circinatum*, Sciaridae

Introduction

Bradysia difformis Frey is a nematoceran diptera belonging to the family Sciaridae. These insects are commonly referred to as fungus gnats or black fungus gnats. *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 (Hurley *et al.*, 2007), 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 (Hurley *et al.*, 2007).

Bradysia difformis is known as a pest in European nurseries. In Britain, *B. difformis* damages ornamentals (Gouge and 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; Springer, 1995a; 1995b) 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. Therefore, fungus gnats are potential carriers of fungi, including fungal pathogens, within nurseries. Pathogens transmitted by *Bradysia* spp. include *Verticillium albo-atrum* Reinke and Berthold on alfalfa plants (Kalb and 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. *radicislycopersici* Jarvis and Shoemaker on bean and tomato plants (Gillespie and Menzies, 1993). *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 and Blakeslee, 1980; Viljoen *et al.*, 1994).

Symptoms of diseased seedlings include tip die-back, damping-off, chlorotic or reddish-brown needle discolouration, and wilting (Barnard and Blakeslee, 1980; Rowan, 1982).

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

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 *Bo. 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 (Hurley *et al.*, 2007), we were unable to isolate *F. circinatum* from *B. difformis* collected at the four major pine-growing nurseries in South Africa. However, the use of *Fusarium* selective medium (Nash and 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 and French, 1993; Ouellet and Seifert, 1993; Taylor, 1993; Parry and Nicholson, 1996; Schilling *et al.*, 1996; Pryor and 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 *Bo. cinerea* in strawberry farms (Rigotti *et al.*, 2002). The potential exists to use these primers for the detection of *F. circinatum* and *Bo. 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 twofold. The first objective was to test fungus gnats for the presence of *F. circinatum* and *Bo. 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°00'34"S, 30°00'41"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) (Figure 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 sterile tweezers. Fifteen fungus gnats from each nursery were placed into 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. difformis* was based on previous studies that showed *B. difformis* to be the only fungus gnat present in the sampled nurseries (Hurley *et al.*, 2007). The presence of just one species of fungus gnat was further confirmed by molecular studies (BPH and co-authors, unpublished data).

DNA extraction

Prior to DNA extraction, the samples were centrifuged for 6min (16 000 x g) and the ethanol decanted. The remaining sample was rinsed with distilled water, centrifuged for a further 5min (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

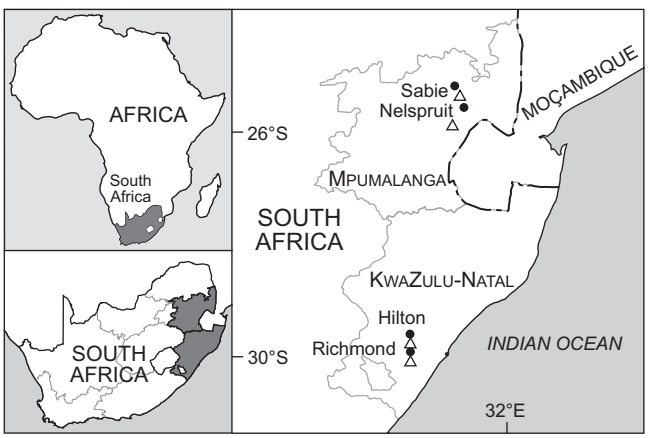

Figure 1: Location of the four pine-growing nurseries where *B. difformis* was collected. Triangles show position of nurseries, in relation to the nearest towns/cities

used for vials with individual fungus gnats and mycelium of *F. circinatum* and *Bo. cinerea*, and 200µl used for vials with many fungus gnats.

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

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

Species-specific primers were used to test for the presence of *F. circinatum* and *Bo. cinerea* on the fungus gnat samples. *F. 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 *Bo. 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 2µl of DNA extract, 1 x PCR buffer, 0.2mM of each dNTP, 2.75mM $MgCl_2$, 3.75 units *Taq* polymerase (ThermoRed DNA polymerase — Saveen and Werner AB, Malmö, Sweden) and 0.2mM of each primer, and were made up with distilled water to reach a volume of 25µl. All amplifications throughout the study were done using a GeneAmp® PCR System 2700 (Applied Biosystems) thermocycler and were programmed for an initial denaturation of the DNA at 95°C for 2min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 60°C for 45s and elongation at 72°C for 30s, and concluding with a final elongation at 72°C for 7min. All PCR products were run on 1% agarose gels, stained with ethidium bromide, and amplicons were visualised 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, isolated from infected *P. patula* seedlings in South Africa. The positive control for the *Bo. cinerea*-specific primers was DNA from the strain 98G1, isolated from infected *P. sylvestris* seedlings in Sweden (Capieau *et al.*, 2004). Both the positive controls for *F. circinatum* and *Bo. cinerea* were strains associated with disease symptoms in the nurseries.

Dilution series were done to determine the minimum amount of *F. circinatum* and *Bo. 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). Tenfold serial dilutions of *F. circinatum* and *Bo. cinerea* DNA were prepared in distilled water. *Fusarium circinatum* and *Bo. cinerea* DNA were also diluted with DNA from both individual and pooled fungus gnats. Dilutions of *F. circinatum* in DNA extract from a single fungus gnat ranged from 6:1 to 1:1 600, and from 1:3.7 to 1:37 000 in DNA extract from pooled fungus gnats. Dilutions of *Bo. cinerea* with pooled fungus gnat DNA ranged from 1:14.7 to 1:147 000. For both dilutions in water and dilutions in fungus gnat DNA, the amount of fungal DNA ranged from 13.4ng to 1.34pg for *F. circinatum* and from 3.4ng to 340fg for *Bo. cinerea*, in a 25µl PCR reaction, as described above.

General fungal primers

Originally, the primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') (Gardes and 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. Thus, the primers ITS1F and ITS4B (5' CAGGAGACTTGTACACGGTCCAG) (Gardes and Bruns, 1993) and NL1 (5' GCATATCAATAAGCGGAGGAAAAG3') and NL4 (5' GGTCCGTGTTTCAAGACGG 3') (O'Donnell, 1993) were used. The region amplified by 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 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 50µl. Amplifications were done as described for the species-specific primers, except that the initial denaturation was at 94°C for 5min, and during the following 35 cycles annealing was at 50°C for 30s and elongation at 72°C for 1min. All PCR products were separated using electrophoresis on 1% agarose gels, stained with ethidium bromide, and the amplicons visualised 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 (50µg ml^{-1}). 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 200µl of sterile distilled water. Primers M13F (5' GGAAACAGCTATGACCATGATTACGC) and M13R (5' CAGGAAACAGCTATGAC) (Vieira and Messing, 1982) were used to amplify the fungal insert. PCR reaction mixtures were the same as described for the general fungal primers, except that 0.3mM of each primer was used and 25µl of bacterial colony suspension was used as template. The PCR thermocycler program was the same as for the ITS primers, except that elongation at 72°C was for 30s during the 35 cycles. PCR products were evaluated using agarose gel (1%) electrophoresis and ethidium bromide staining and then visualised 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 Version

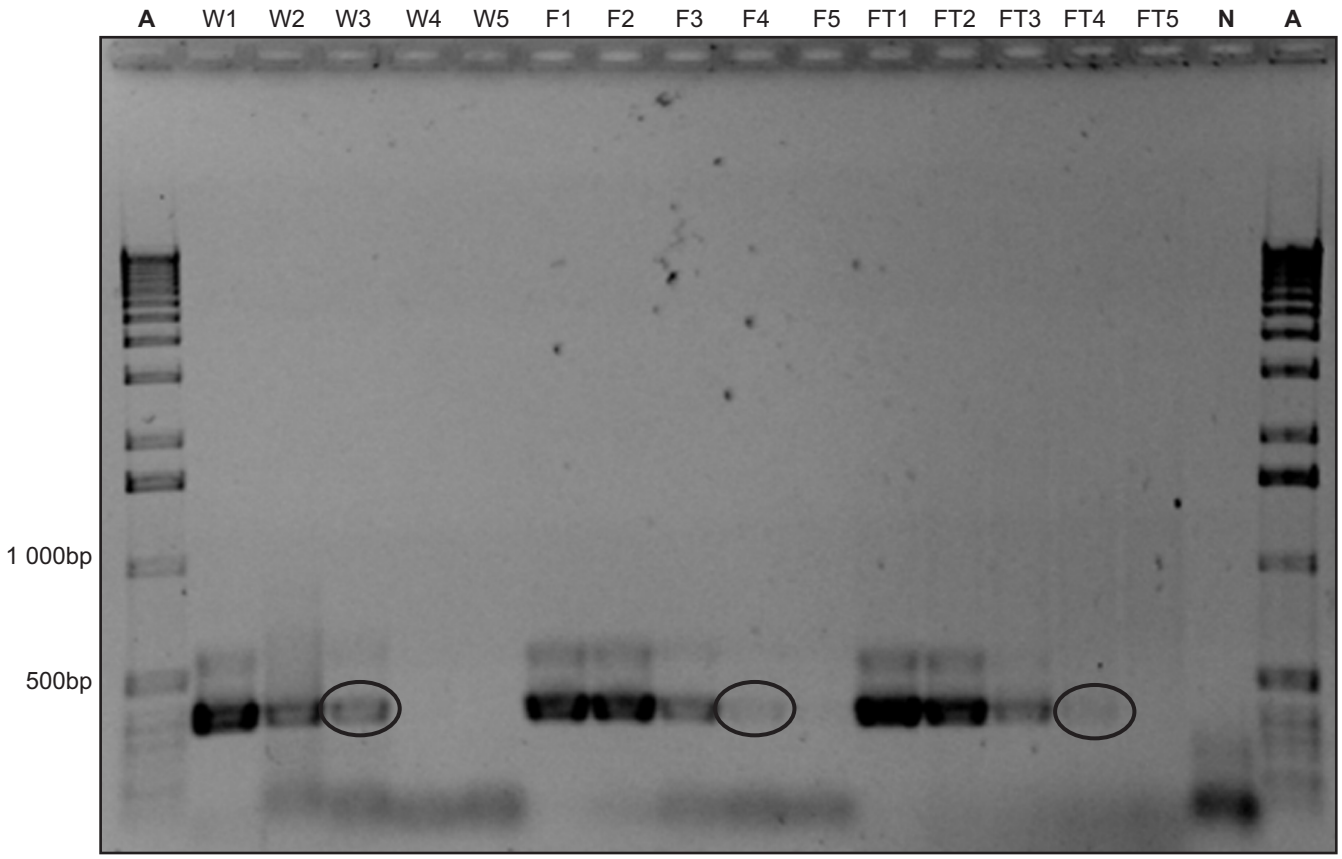

Figure 2: Agarose gel (1%) showing the PCR amplicons resulting from *F. circinatum* dilution series using *F. circinatum*-specific primers CIRC1A and CIRC4A. Lanes where bands are visible indicate that DNA was detectable using the PCR test used in this study. The circles indicate the faintest band on the gels. Lane W1 is undiluted *F. circinatum* DNA (13.4ng genomic DNA or 6.7ng μl^{-1}) and serves as a positive control. Lanes W2–W5 are 10-fold dilutions of *F. circinatum* genomic DNA with water, where the amount of DNA is 1.34ng, 134pg, 13.4pg and 1.34pg, respectively. Lanes F1–F5 are the dilutions of *F. circinatum* DNA extract (6.7ng μl^{-1}) with DNA extract from a single fungus gnat (1.1ng μl^{-1}) with the ratios of 6:1, 1:1.6, 1:16, 1:160 and 1:1 600 fungus DNA to fungus gnat DNA. Lanes FT1–FT5 are the dilutions of *F. circinatum* DNA extract (6.7ng μl^{-1}) with DNA extract from pooled fungus gnats (25ng μl^{-1}) in the ratios of 1:3.7, 1:37, 1:370, 1:3 700 and 1:37 000 fungus DNA to fungus gnat DNA. The amount of *F. circinatum* DNA in Lanes F1–F5 and FT1–FT5 is: 6.7ng, 1.34ng, 134pg, 13.4pg and 1.34pg. Lane N is the negative control and Lane A contains a 1kb ladder

2.0 (Applied Biosystems, Foster City, California, USA), using the manufacturers' specifications, and adding 3µl of cleaned PCR product and 0.16mM of either primer in a total volume of 10µl. Sequence products were analysed on an ABI 310 Genetic Analyzer (Applied Biosystems). The electropherogram obtained was analysed using Seqman (DNASTAR, Inc.). The closest sequence match was then determined using a BLAST search (Altschul *et al.*, 1997) option in GenBank (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 were tested for the presence of *F. circinatum* and *Bo. cinerea* DNA, using species-specific primers. Neither *F. circinatum* nor *Bo. cinerea* were detected from any of the fungus gnats. 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 134pg of the fungus DNA was present. When diluted with single and pooled fungus gnat DNA extract, *F. circinatum* was detected to a ratio of 1:164 and 1:3 727 of fungus to fungus gnat DNA, respectively, equivalent to 13.4pg of fungus (Figure 2). The dilution test for *Bo. cinerea* showed that *Bo. cinerea* could be detected when diluted in water at a ratio of 1:100 or when 34pg of the fungus was present. When diluted with DNA extract of pooled fungus gnats, *Bo. cinerea* was detected up to a ratio of 1:14 691 of fungus to fungus gnat DNA, equivalent to 3.4pg of fungus (Figure 3).

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

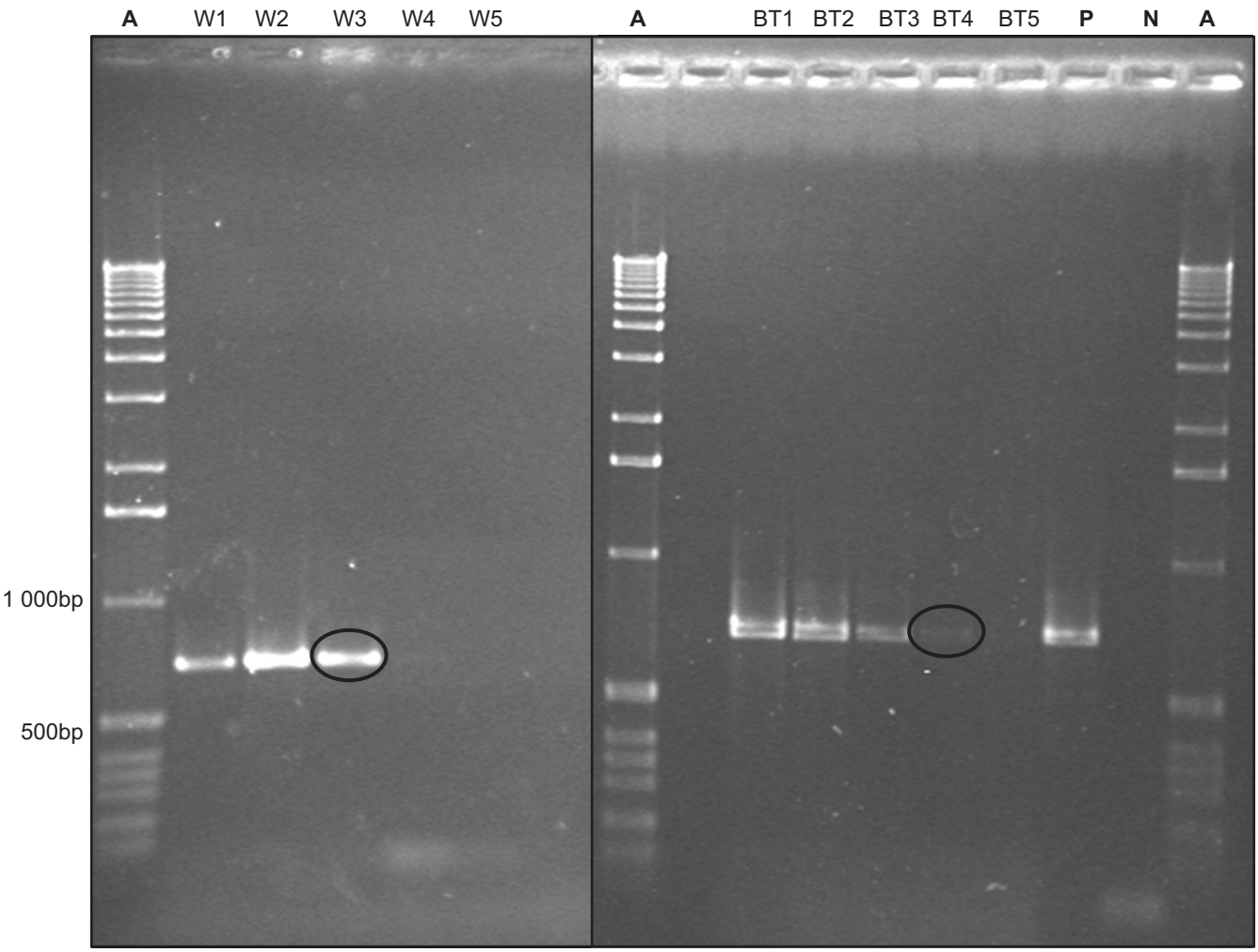

Figure 3: Agarose gel (1%) showing the PCR amplicons resulting from the *Bo. cinerea* dilution series using *Bo. cinerea*-specific primers C_{729+} and C_{729-}. Lanes where bands are visible indicate that DNA was detectable using the PCR test used in this study. The circles indicate the faintest band on the gels. Lane W1 is undiluted *Bo. cinerea* DNA (3.4ng genomic DNA or 1.7ng μl^{-1}) and serves as a positive control. Lanes W2–W5 are 10-fold dilutions of *Bo. cinerea* DNA with water, where the amount of DNA is 340pg, 34pg, 3.4pg and 340fg, respectively. Lanes BT1–BT5 are the dilutions of *Bo. cinerea* DNA extract (1.7ng μl^{-1}) with DNA extract from pooled fungus gnats (25ng μl^{-1}) in the ratios of 1:15, 1:150, 1:1 500, 1:15 000 and 1:150 000 fungus DNA to fungus gnat DNA. The amount of *Bo. cinerea* DNA in Lanes BT1–BT5 is: 1.7ng, 340pg, 34pg, 3.4pg and 340fg. Lane N is the negative control, Lane P is an additional positive control and Lane A contains a 1kb ladder

cloning multiple-band products. Sixty-seven per cent 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 that 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 ectomycorhiza, *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 characterise them.

Discussion

In this study, we attempted to detect *F. circinatum* and *Bo. cinerea* from DNA isolated from *B. difformis,* using species-specific primers. The results showed that neither of these fungi was present. These findings, together with those in Hurley *et al.* (2007), 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 *Bo. cinerea* and *Fusarium* spp. have been shown to be carried by fungus gnats in other nursery environments (Keates *et al.*, 1989; Gillespie and Menzies, 1993; James *et al.*, 1995).

Using a dilution series, we showed that DNA from *F. circinatum* and *Bo. 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 amplification using C_{729+} and C_{729-} primers allowed for the detection of *Bo. 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 *Bo. cinerea* DNA, but 2pg when mixed with other DNA. The sensitivity of the CIRC1A and CIRC4A and C_{729+} and C_{729-} primers is also comparable to that of species-specific primers developed for other pathogens. For example, the limit for detection for *Fusarium culmorum* (W.G. Sm.) Sacc., *Fusarium graminearum* Schwabe, *Alternaria radicina* Meier, Drechs., and Eddy, and *Leptosphaeria maculans* (Desm.) Ces. et de Not. is 50pg, 5pg, 0.2pg and 0.1pg, respectively (Taylor, 1993; Shilling *et al.*, 1996; Pryor and Gilbertson, 2001). Thus, the sensitivity of the primers used in this study is 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 *Bo. cinerea* on *B. difformis*.

It is probable that *B. difformis* does not have sufficient contact with *F. circinatum* and *Bo. cinerea* to be of importance in the movement of these pathogens in a nursery. Fungus gnats have been reported to carry pathogens by trans-stadial transmission (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 ingesting pathogenic fungi increases if they are feeding on or are close to infected plant tissue. However, *B. difformis* larvae very seldom inhabit the plugs of the pine seedlings (BPH pers. obs.), possibly 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 and 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. It might be expected that *B. difformis* adults might acquire *F. circinatum* and *Bo. cinerea* when moving around on infected plants. Our studies indicate that this does not occur on a frequent basis. One possible explanation is that the population of *B. difformis* is lower in South African forestry nurseries than in other nurseries where *Bradysia* species have been implicated in the movement of *Fusarium* spp. and *Bo. cinerea* (Keates *et al.*, 1989). Alternatively, nursery sanitation involving the removal of infected plants may decrease the inoculum of these pathogens to levels low enough that frequent contact between *B. difformis* adults and the pathogens does not occur.

The two main conclusions from this study are, firstly, that despite a history of association between fungus gnats and fungal pathogens, neither *F. circinatum*, *Bo. cinerea* or any other fungal pathogens were detected on *B. difformis*. The fungi that were detected were common soil inhabitants and mycorrhizal fungi. This indicates that *B. difformis* does not have a major role in the movement of pathogenic fungi in South African forestry nurseries. Other factors should be investigated to explain the movement of these fungi. Secondly, 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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