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A microsatellite-based identification tool used to confirm vector association in a fungal tree pathogen

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Abstract

Many fungi live in close association with insects, and some are specifically vectored by them. One of the best examples is found in the so-called Ophiostomatoid fungi, including species of *Ceratocystis* and other genera in the Ceratocystidaceae. Our understanding of vectorship in these fungi is based predominantly on either their frequency of isolation from insects or the success with which these fungi are isolated from their insect vectors. The fact that *Ceratocystis* species mostly have casual as opposed to highly specific relationships with their insect vectors makes it difficult to prove insect vector relations. In order to provide unambiguous support for *Ceratocystis* species being vectored by insects, we interrogated whether genotypes of the tree pathogen, *Ceratocystis albifundus*, would be shared between isolates retrieved from either infected trees or nitidulid beetles. *Ceratocystis albifundus* isolates were collected from nitidulid beetles and from naturally occurring wounds on trees in the Kruger National Park (KNP) in South Africa. The genotypes of these isolates were then determined using eight microsatellite markers, and they were compared using a haploid network analyses. A high frequency of Multi-Locus Haplotypes (MLHs) derived from nitidulid beetles was found to be shared with those from wounded trees across the KNP. This provides robust support showing that nitidulid beetles play an important role in the dispersal of *C. albifundus* in the KNP.

Introduction

Many plant-colonizing insects have evolved intimate associations with a wide variety of microbial assemblages. This is also true for many fungal plant and tree pathogens such as in the case of tree-infesting bark beetles. Where fungi occur in close association with insects, for example where they are carried in specialised structures such as mycangia, it is reasonable to assume that the insects involved are the vectors of these fungi. These beetle-fungus interactions are diverse, ranging

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from antagonistic to mutualistic. However, most associations appear to be more commensal (Six 2003; Klepzig et al. 2009), with the insects playing a significant role in transporting fungal inoculum to a particular part of the plant where infection might occur.

Nitidulid beetles (Coleoptera, Nitidulidae), commonly known as sap beetles or picnic beetles, are important pests known to occur on a variety of agricultural products (Hinton 1945). They are also commonly associated with fresh tree wounds (Moller and DeVay 1968a; Juzwik and French 1983; Heath et al. 2009). For this reason, nititdulid beetles have also been associated with important tree pathogens in the Ceratocystidaceae and have been implicated as vectors of these pathogens (Moller and DeVay 1968a; Juzwik and French 1983; Heath et al. 2009).

Species of *Ceratocystis* in the Ceratocystidaceae as defined by de Beer et al. (2014) are known to be common associates of insects. *Ceratocystis albifundus* is one example of an economically important tree pathogen that causes a canker and wilt disease on non-native *Acacia mearnsii* trees in Africa (Roux and Wingfield 2013). In a recent study where the epidemiology of *C. albifundus* was considered, the pathogen was

frequently isolated from the nitidulid species, *Brachypeplus depressus*, *Carpophilus bisignatus* and Ca. *hemipterus* (Heath et al. 2009). The presence of *C. albifundus* isolated from these nitidulids, collected either in traps or on fungal mats occurring under bark flaps, suggested that they are vectors of the pathogen (Heath et al. 2009). However, the assumed vectorship is based only on association and without fulfilling Leach's rules (Juzwik and French 1983; Harrington et al. 2008; Nkuekam et al. 2012) that are usually applied to prove vectorship.

In the same manner that Koch's Postulates are required to confirm the role of microbes in causing plant disease, Leach's Rules (Leach 1940) are applied to prove that insects are the vectors of plant pathogens. In this case, a pathogenic fungus must not only be isolated from an asscociated insect, but must be shown to be naturally transferred to and then re-isolated from the host plant. Leach's Rules can be demonstrated by (i) a close association between an insect and infected plants, (ii) regular visits of the insect to wounded healthy plants, (iii) the presence of the pathogen on the insect following visitation to a diseased plant and (iv) the development of disease on plants following visits by pathogen-infested insects.

In some cases where plant pathogens are vectored by insects, it is not possible to fulfill all of the criteria of Leach's Rules. A common failure in insect/fungus interaction studies is the assumption of vectorship based on association without proof of natural transfer to a host by the insect. This is due to the fact that vector studies with insects under artificial conditions can be difficult; demanding access to adult insects infected with traceable fungal strains. In this regard, linking the pathogens to their assumed insect vectors has largely relied either on isolation success or on frequency at which fungal pathogens were successfully recovered.

PCR-based sequencing technologies have become commonly available to identify genotypes of microbial strains. This makes it possible and affordable to trace the genetic identity of fungal individuals unambiguously using DNA sequence comparisons. Amongst the most commonly applied and contemporary tools to identify genotypes of fungal strains include microsatellite markers (Udupa et al. 1998; Ivors et al. 2006). These markers provide an opportunity to confirm that a particular insect is an effective vector of a fungal plant pathogen in the absence of artificial inoculations and caged insect studies.

The aim of this study was to provide robust evidence that nitidulid beetles act as vectors of *Ceratocystis* species using microsatellite markers and to test whether this can be done without cage experiments. This would then provide an effective means to confirm a vector relationship without completing Leach's Rules of proof. The model used in this case was the important fungal tree pathogen *C. albifundus* that can be found in the Kruger National Park (KNP) of South Africa. Animals and especially elephants in the park induce wounds on trees that attract insects such as nitidulid beetles, from which *C. albifundus* can be isolated.

Materials and methods

Fungal isolates

Three sampling sites, Pretoriuskop (Coordinates: 25°.16′67.05″S, 31°.26′65.81″E), Tshokwane (Coordinates: 24°.78′60.85″S, 31°.85′86.17″E) and Lower Sabie (Coordinates: 25°.11′81.95″S, 31°.91′47.04″E) in the southern part of the KNP were selected for field investigations. A collection of *C. albifundus* isolates was made from nitidulid beetles and plant tissues with which they were associated. The tissue samples including cambium and bark flaps were from wounds on native trees damaged by animals.

Pieces of bark and cambium from wounds were inspected for the presence of sexual structures resembling *C. albifundus* using a $10 \times$ magnification hand lens. Infected plant samples were then placed in separate brown paper bags for each tree, and transported to the laboratory. Nitidulid beetles associated with bark wounds were collected only from the Pretoriuskop area, using an aspirator, and they were transported to the laboratory in 1.5 ml Eppendorf tubes stored at 4 °C.

For the isolations, the bark and cambium samples were examined with a dissection microscope for the presence of long-necked, light coloured ascomata bearing sticky ascospore masses at their apices, typical characteristic of *C. albifundus* (Wingfield et al. 1996). Individual ascospore masses were transferred to MEA plates, and their growth was assessed daily. Isolates were also obtained from white mycelial strands on the plant samples. In cases where sexual structures were absent, samples were placed in incubation chambers to induce sporulation, or pieces of infected cambium were placed between two carrot pieces to bait for *C. albifundus* (Moller and DeVay 1968b). Once cultures had sporulated, ascospore droplets were transferred to 2% malt extract agar (MEA: 20 g malt extract, Biolab, Midrand, S. Afr.; 20 g agar, Difco Laboratories, Detroit, Mich, USA) supplemented with 100 mg/l streptomycin sulphate (Sigma-Aldrich, Steinheim, Germany), and incubated at 25 °C for two weeks in the dark.

Isolations from nitidulid beetles were achieved either by plating the insects directly onto 2% MEA plates or by carrot baiting. For the direct isolations, insects were allowed to crawl over the surface of 2% water agar (WA: 20 g agar, Difco Laboratories, Detroit, Mich) and the resulting cultures were purified by sub-culturing from single hyphal tips or ascospore droplets onto 2% MEA supplemented with 100 mg/l streptomycin sulphate (Sigma-Aldrich, Steinheim, Germany), and incubated at 25 °C for two weeks in the dark. For carrot baiting, insects were squashed onto the surface of a carrot slice

(Moller and DeVay 1968b). Samples were incubated at 25 °C to induce sporulation, and isolations were made as spore masses were produced at the apices of the ascocarp necks.

Identification of fungi and insect vectors

The identity of the *Ceratocystis* isolates was confirmed using the morphological characters described by Wingfield et al. (1996). Identification based on morphology was confirmed using DNA sequence comparisons based on the internal transcribed spacer regions (ITS1, ITS2) and 5.8S rDNA gene regions (White et al. 1990; Gardes and Bruns 1993).

The CTAB based-protocol described by Möller et al. (1992) was used to extract genomic DNA from single ascospore-derived isolates of *C. albifundus* that were cultured on 2% MEA for two weeks at 25 °C. Mycelium was scraped from the agar surface using a sterilized surgical scalpel, and subsequently transferred to 1.5 ml Eppendorf tubes. Where DNA was extracted from nitidulid beetles, a prepGEM™ extraction kit (zyGEM Ltd., Hamilton, NZ) was used, following the protocols provided by the manufacturer. All DNA extracts were calibrated using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA) and adjusted to a working concentration of 15 ng/μl of genomic DNA.

Sequencing was done following the procedures described by Lee et al. (2015) and sequences were manually aligned in BIOEDIT ver.7.0.9.0 Sequence Alignment Editor (Hall 1999). Sequences were then subjected to a BLASTn analysis against the nucleotide database of NCBI [http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi].

Nitidulid beetles were first grouped based on morphotypes, and selected specimens representing each morphotype were subjected to DNA sequence comparisons. The mitochondrial cytochrome oxidase I gene region (MT-COX1) was amplified and sequenced using the universal primer sets C1-J-2183 (CAA CAT TTA TTT TGA TTT TTT GG) and TL2-N-3014 (TCC AAT GCA CTA ATC TGC CAT ATT A) (Simon et al. 1994). PCR reactions were prepared in a total volume of 25 μl, containing 15 ng of genomic DNA, 0.5 μl of the forward primer (10 pM), 0.5 μl of the reverse primer (10 pM), 5 μl of 5 × reaction buffer containing 5 mM dNTPs and 15 mM MgCl₂ (BIOLINE, London, UK), 0.09 μl of MyTaq™ DNA polymerase (BIOLINE, London, UK). The thermal cycling conditions included 5 min at 96 °C for initial denaturation, 35 cycles of 60 s at 94 °C, 60 s at 50 °C and 90 s at 72 °C, and a final extension for 10 min at 72 °C. All other steps involved in the sequencing procedure were the same as those described by Lee et al. (2015). MT-COX1 sequences were then analysed using BLASTn and the nucleotide database of NCBI [http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi].

Microsatellite amplification and fragment analysis

Microsatellite primer sets (Table 1) previously developed by Barnes et al. (2005) and Steimel et al. (2004) were used to determine the genotypes of all *C. albifundus* isolates. The PCR analyses were carried out in a total volume of 25 μl containing 15 ng of genomic DNA, 0.5 μl of the forward primer (10 pM), 0.5 μl of the reverse primer (10 pM), 5 μl of 5 × reaction buffer containing 5 mM dNTPs and 15 mM MgCl₂ (Bioline, London, UK) and 0.09 μl of MyTaq™ DNA

Table 1 Microsatellite primers used in this study

Primers	Sequence (5'-3')	Annealing temperature	References
AG15	CTT GAC CGA CCT GCC GAT TG	62°C	Barnes et al. 2005
AG16	GGA TAG CAG CGA CAA GGA CC		
AG17	GTC GGT GGT GGA GAC GGT C	62°C	
AG18	CGG CCC TGC CAA CGG ATG		
CF17	GAA ACC GAG AGT CAT CGT CC	62°C	
CF18	CGA GCC AAG ACG TTC ATT GAA G		
CF21	GCG TTG AAA GAT GTG GCG TG	62°C	
CF22	GCA CTA GCA GAA TAG AAT GCA G		
CF23	CAT GAT CGA CAA GGG CGC TG	60°C	
CF24	CAG GGA ATT CCC GAT GGC AG		
CCAA10-F	TGA CAC GCG CTT CAC TAA CAG	58°C	Steimel et al. 2004
CCAA10-R	TGC ACC ATA CCC AGG GGA CA		
CCAA80-F	ACC CGT CTC GTA TTG GCT AT	58°C	
CCAA80-R	AAT CGT TCG CAT TCA GGT GG		
CAT12X-1F	ACA AAA GAC GGC ACG CAT ACA	62°C	
CAT12X-1R	TGG GGA GAA GTC TGA GTA GAG GGA CAA		

polymerase (Bioline, London, UK). PCR reactions were conducted in a Veriti™ 96 well Thermal Cycler (Applied Biosystems, Foster City, CA) with thermocycling conditions previously described by Barnes et al. (2005) and Steimel et al. (2004) with slight modifications made in annealing temperatures (Table 1). The amplification products stained with GelRed™ (Biotium Incorporation, USA) nucleic acid dye were electrophoresed on 1.5% (w/v) agarose gels in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.0), and then visualized under UV light (Gel DocTM EZ Imager, Bio-Rad, Richmond, CA) to confirm the sizes of the amplicons.

Amplicons of expected length were analyzed on an ABI PRISM™ 3500xl POP 7™ Automated DNA sequencer (Applied Biosystem, Foster City, CA) where GENESCAN-600 LIZ (Applied Biosystem, Foster City, CA) was used as the internal size standard. GENEMARKER ver.2.2.0 (Softgenetcis, LLC, USA) was then used to determine the allele sizes of each microsatellite amplicon.

Haplotype network analysis

All Multi-Locus Haplotypes (MLHs) of *C. albifundus* were used to construct a haplotype network. This was implemented in NETWORK ver.4.6.1.3 (www.fluxusengineering.com) using the median joining method with the default option. Network calculation was first made depending on the transversion/transition weights. MP calculations based on the outfile obtained was performed using the Maximum parsimony option to derive all possible shortest trees. This purged all superfluous links and identified the network containing the shortest tree.

Results

Identification of fungal isolates and insect vectors

All *Ceratocystis* isolates having light–coloured ascomatal bases were tentatively identified as *C. albifundus*. Their identity was further confirmed based on ITS sequence data. All isolates identified as *C. albifundus* had almost identical similarity (99%) when analysed using BLASTn searches against those sequences of *C. albifundus* strains retrieved from NCBI. All isolates used in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 2). All ITS sequence data produced in this study was deposited in NCBI (KX945362 to KX945364).

A total of 114 *C. albifundus* isolates were obtained from all three sites (Pretoriuskop, Tshokwane and Lower Sabie) surveyed in the KNP. These included 83 from two native tree species (*Terminalia sericea* and *Lannea stuhlmannii*) and 31 from nitidulid beetles. The 31 beetles collected include two insect genera, representing *Brachypeplus* sp., *Brachypeplus ater*, *Carpophilus apicipennis*, Ca. dimidiatus and Ca. hemipterus (Tables 2, 3). The recovery rates of *C. albifundus* differed for the different insect taxa, ranging from 16% to 37% (Table 3).

Microsatellite analysis and fragment analysis

Of ten microsatellite primer pairs used in this study, eight primer sets produced amplicons of the expected size. After optimization for analysis, these microsatellite markers (Table 1), were divided into two multiplex panels for further fragment analysis. All eight markers were polymorphic. The

Table 2 *Ceratocystis albifundus* isolates collected from the Kruger National Park in this study

Sources	Collection areas	Hosts	CMW Number
Isolates from trees (83)	Pretoriuskop area	*Terminalia sericea* (27)	CMW41510, CMW41512-41518, CMW41521-41530, CMW42118-42121, CMW42123-42126, CMW43680
	Tshokwane area	*Terminalia sericea* (24)	CMW41531-41545, CMW41580-41588
	Lower Sabie area	*Terminalia sericea* (4)	CMW41546-41549
		Lannea stuhlmannii (28)	CMW41550-41564, CMW41566-41573, CMW41575-41579
Isolates from insects (31)	Pretoriuskop area	*Brachypeplus* sp. (1)	CMW41594
		Brachypeplus ater (10)	CMW41595-41596, CMW41601-41605, CMW41610, CMW42199, CMW42202
		Carpophilus apicipennis (7)	CMW41598-41599, CMW41608, CMW42197-42198, CMW42200-42201
		Carpophilus dimidiatus (6)	CMW41600, CMW41606-41607, CMW42193-42195
		Carpophilus hemipterus (7)	CMW41589-41593, CMW41609, CMW42196

(): Numbers in brackets indicate number of isolates obtained

Table 3 Summary of recovery rates of *Ceratocystis albifundus* isolates from insects in Kruger National Park

Collection areas	Vectors	Number of vectors collected	Number of isolates retrieved	Recovery rates
Pretoriuskop area	*Brachypeplus* sp.	3	1	33%
	Brachypeplus ater	31	10	32%
	Carpophilus apicipennis	23	7	30%
	Carpophilus dimidiatus	38	6	16%
	Carpophilus hemipterus	19	7	37%

number of alleles generated from each marker ranged from two (CF23/24, AG17/18, CCAA10-F/R and CAT12X-1F/R) to four (CF21/22 and CCAA80-F/R) (Data not shown). In total, 67 unique haplotypes were recovered, representing 38 haplotypes (Pretoriuskop: 16, Tshokwane: 14, Lower Sabie: 8) from trees and 29 from insects (*Brachypeplus* sp.: 1, *Brachypeplus ater*: 9, *Carpophilus apicipennis*: 7, Ca. *dimidiatus*: 5 and Ca. *hemipterus*: 7).

Haplotype network analysis

The haplotype network generated in NETWORK ver.4.6.12 (www.fluxusengineering.com) showed that there was a significant frequency of MLHs shared between isolates recovered from trees and insects as well as between geographic locations (Fig. 1). Of MLHs obtained in this study, 13 MLHs recovered from nitidulid beetles were found to be shared with 46 MLHs recovered from trees at Pretoriuskop (16), Lower Sabie (21) and Tshokwane (9). In total, 46 MLHs from trees accounted for 55.4% shared with those recovered from insects. MLHs obtained from nitidulid beetles at Pretoriuskop were also found on trees at Lower Sabie and Tshokwane. The Tshokwane and Lower Sabie areas are on average 75 km apart from one another and also from the Pretoriuskop area (Fig. 1).

Discussion

Results of this study provided unequivocal evidence that *C. albifundus* is vectored by nitidulid beetles. This was shown by identifying the same genotypes of the pathogen from tree wounds and nitidulid beetles collected at different locations. As far as we are aware, this study is the first to apply molecular tools to show that *Ceratocystis* species are vectored by the insects with which they are commonly associated. In addition, the results are consistent with previous observations (Cease and Juzwik 2001; Heath et al. 2009; Nkuekam et al. 2012; Soulioti et al. 2015) that nitidulid beetles transfer *Ceratocystis* to new host substrates, and that this relationship is not specific to host or beetle species.

Occurrence of the same genotypes of *C. albifundus* over relatively large distances were observed as has been found in previous studies (Lee et al. 2016). For example, the same MLH's were found at Pretoriuskop and Tshokwane as well as at Lower Sabie and Pretoriuskop, which are all at least 70 kms apart. In cases where the dispersal and flight distances of bark and nitidulid beetles have previously been considered based on mark-recapture experiments (Morris et al. 1955; Weslien and Lindelöw 1990), it was found that most tagged beetles could fly for at least ~1.6 km from the point of release. For this reason, it seems likely that *C. albifundus* would have been vectored by nitidulid beetles in a "step-wise" manner. This is
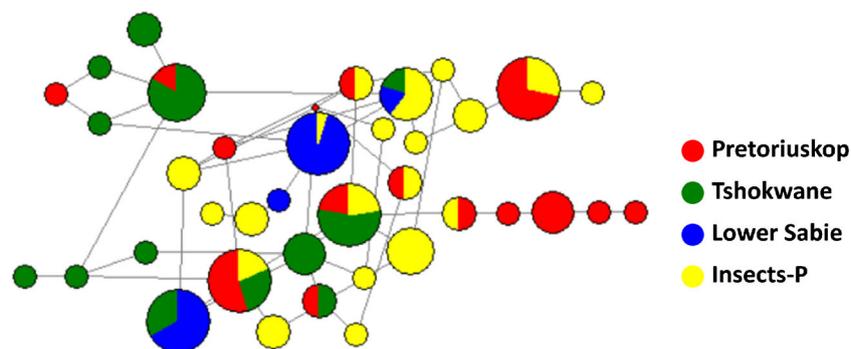
Fig. 1 Schematic representation of *Ceratocystis albifundus* MLHs recovered from native trees and nitidulid beetles in KNP. Each colour represents either isolates of *C. albifundus* from insects collected from the Pretoriuskop area in KNP (yellow in colour) or those isolated from wounds on trees in three different areas, Pretoriuskop area (red in colour), Tshokwane area (green in colour), Lower Sabie area (blue in colour), in KNP

in contrast to the pathogen being carried directly across longer distances by the same insect individuals. This would also confirm that these insects not only carry the fungus, but they successfully transfer it to fresh wounds. While nitidulid beetles are clearly vectors of *C. albifundus*, it is also possible that other vectors could be responsbile for the dispersal of this pathogen.

It is well established that insects can be the primary vectors of fungal plant pathogens (Batra 1967; Six 2003; Klepzig and Six 2004). This has mainly been shown using conventional approaches such as either isolation success or the frequency with which the fungi were isolated from insect vectors. In the present study, a high number of the same MLHs that were clustered between isolates from either insect vectors or trees was recovered. Furthermore, the fact that MLHs were shared between isolates recovered from Tshokwane, Lower Sabie and Pretoriuskop shows clearly that insect vectors are directly involved in disseminating *C. albifundus* in KNP.

Ceratocystis albifundus appears to have a non-specific relationship with its nitidulid vectors. This is at least in comparison to some insect-associated fungi that have co-evolved obligate relationships with their vectors. For the Ophiostomatoid fungi, the broad group in which *Ceratocystis* resides (Wingfield et al. 1993; de Beer et al. 2014), a truly obligate relationship exists only for species that are carried in mycangia such as the case with *Ceratocystiopsis ranaculosus* and its vector *Dendroctonus frontalis* (Bridges and Perry 1987; Moser et al. 1995). In this study, it was found that *C. albifundus* is vectored by two different genera of nitidulid beetles. Along with the fact that the isolation rate of *C. albifundus* from the nitidulid beetles was not high in this study, it is clearly also not specific to either of them. These insects are apparently able to infest wounds on many different tree species (Heath et al. 2009), and there is consequently no evidence of a tight vector specificity.

Since the first recognition of a link between wood discolouration, insect damage and fungi by Hartig (1844), there have been many studies shedding light on the various aspects of insect associations with species in *Ceratocystis* sensu stricto (Moller and DeVay 1968a; Cease and Juzwik 2001; Heath et al. 2009; Nkuekam et al. 2012; Soulioti et al. 2015). Leach's rules were established in order to determine whether disease development is directly associated with fungal pathogens carried by insects (Leach 1940) and specifically to separate the issue of mere association and that of successful transfer. A difficulty in achieving the requirements of these rules has been the need for cage experiments where the insects can be shown to transfer the fungi that they carry to host trees and to allow establishment to occur. In this study, we have applied molecular tools to effectively achieve the same goal. This approach should in future provide a relatively simple means as an alternative to fulfil the Leach's rules and to test for effective transfer without needing to engage in complex and often very difficult experiments with insects.

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