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Ecology and population structure of a tree wound-infecting fungus in a native South African forest environment

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ABSTRACT

Ceratocystis tsitsikammensis was first isolated from bark harvesting wounds on two indigenous tree species in the Afromontane forests of the Western Cape Province of South Africa. Inoculation studies indicated that it is a potential pathogen of native *Rapanea melanophloeos* trees. In this study, we investigated the distribution, ecology and biology of *C. tsitsikammensis* in the Garden Route National Park of South Africa. Isolates were obtained from wounds on *R. melanophloeos*, three non-native hosts as well as from nitidulid and staphylinid beetles visiting wounds on these trees. The genetic diversity and population biology of the fungus was examined using microsatellite markers. Its mating strategy was also determined by amplifying its mating type genes and the fungus was shown to be homothallic. Despite the homothallic nature of the fungus, high levels of random mating and absence of genetic structure was found in the investigated population, suggesting a strong effect of gene flow, probably linked to insect dispersal. The gene diversity of *C. tsitsikammensis* was similar to that of a related fungus, *Ceratocystis albifundus*, that is known to be native in Africa. This, together with the fact that *C. tsitiskamensis* is not known elsewhere, within or outside South Africa, suggests that it is native and endemic to the Cape Afromontane region.

Introduction

The genus *Ceratocystis* (Microascales, Ceratocystidaceae), as recently redefined by de Beer *et al.* (2014), includes pathogens of economically important trees and root crops worldwide (Upadhyay 1981; Wingfield *et al.* 1993; Roux & Wingfield 2009). These pathogens induce various disease symptoms, including stem cankers, wilting and root rot resulting in tree/plant death (Kile 1993; Seifert *et al.* 2013). The type species, *Ceratocystis fimbriata*, was described as a pathogen of sweet

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potato (*Ipomea batata*) in the United States of America (USA), (Halsted 1890). Since then, numerous congener species have been found associated with diseases on various plants on all continents, excluding Antarctica. Some species that have caused significant economic losses include *Ceratocystis manginecans*, the causal agent of mango sudden decline in Oman and Pakistan (van Wyk *et al.* 2007) and the wilt and die-back of *Acacia mangium* in Indonesia (Tarigan *et al.* 2011; Fourie *et al.* 2016), *Ceratocystis cacaofunesta* causing wilt, canker and death of cacao (*Theobroma cacao*) in South and Central America and the Caribbean islands (Engelbrecht & Harrington 2005) and *Ceratocystis platani*, the cause of a canker stain disease on plane trees (*Platanus* sp.) in the USA and Europe (Gibbs 1981; Panconesi 1999; Baker *et al.* 2003; Ocasio-Morales *et al.* 2007).

Ceratocystis species colonize their hosts principally through wounds, which may result from animal browsing, insects, wind and hail damage, as well as human management activities (DeVay *et al.* 1968; Roux & Wingfield 1997; Roux *et al.* 2004; Mbenoun *et al.* 2014). Infection can also occur via root grafts between adjacent plants, but this has been ascertained only for *Ceratocystis fagacearum* (Gibbs & French 1980; Blaedow & Juzwik 2010) and *C. platani* (Mutto 1986). Natural overland transmission is generally provided by insects, facilitated by adaptations in these fungi for insect dispersal (Moller & DeVay 1968; Gibbs & French 1980; Redfern *et al.* 1987; Hayslett *et al.* 2008; Heath *et al.* 2009a). These include the production of globoid ascomata with elongated necks exuding sticky ascospore masses at their apices (Ingold 1961; Upadhyay 1981; Malloch & Blackwell 1993). These sticky spores easily attach to the bodies of cohabiting insects (Malloch & Blackwell 1993) and are also adapted for endozoic transfer (Juzwik & French 1986). All species, with the exception of *Ceratocystis caryae*, produce fruity volatiles that are attractive to insects (Hanssen 1993; Kile 1993; Johnson *et al.* 2005). Nitidulid beetles (Coleoptera: Nitidulidae) are the most common vectors of *Ceratocystis* species (Hinds 1972; Appel *et al.* 1990; Heath *et al.* 2009a; Kamgan Nkuekam *et al.* 2012; Mbenoun *et al.* 2014). Other insects that appear to contribute to the dispersal of these fungi include drosophilid flies (Diptera: Drosophilidae) (Moller & DeVay 1968; Hinds 1972), staphylinid beetles (Coleoptera: Staphylinidae) (Hinds 1972; Kamgan Nkuekam *et al.* 2012) and bark beetles (Coleoptera: Scolytidae) (Juzwik *et al.* 2008).

Agricultural activities have contributed to broaden the distribution of many *Ceratocystis* species via the trade and movement of infected plant material and products (Ferreira *et al.* 2011; Wingfield *et al.* 2013). These include important pathogens such as *C. platani*, *C. manginecans*, and *C. eucalypticola*, which have been introduced to new regions where they have initiated new disease outbreaks on exotic as well as native host plants (Roux & Wingfield 2009). Emergence of new diseases caused by native *Ceratocystis* species on non-native hosts has also been reported. One vivid example is the wattle wilt disease affecting Australian *Acacia* trees (*Acacia mearnsii*) commercially grown in Africa (Morris *et al.* 1993; Wingfield *et al.* 1996; Roux *et al.* 1999; Roux & Wingfield 2009). This disease is caused by *Ceratocystis albifundus*, an indigenous African fungus commonly occurring as a saprobe on wounds on various trees in natural South African woodlands (Morris *et al.* 1993; Wingfield *et al.* 1996; Roux *et al.* 1999; Roux & Wingfield 2009).

The emergence of wattle wilt disease has prompted an interest in the diversity of *Ceratocystis* species in natural as well as human-modified ecosystems in Africa as a potential source of new pathogens of agricultural and forestry tree crops. This has resulted in the discovery of several previously unknown species, most of which were new to science (Barnes *et al.* 2003; Kamgan Nkuekam *et al.* 2008; Heath *et al.* 2009b; van Wyk *et al.* 2012; Mbenoun *et al.* 2014). There is evidence that some of these fungi found in plantation forest environments, including *Ceratocystis eucalypticola* and *Ceratocystis pirilliformis*, may have originated outside Africa (Mbenoun *et al.* 2014). This is also supported by population genetic studies suggesting their probable recent introduction into South Africa (van Wyk *et al.* 2006; Kamgan Nkuekam *et al.* 2009). Another group of species, mostly from native ecosystems have, along with *C. albifundus* been shown to form an African lineage within the genus *Ceratocystis* (Mbenoun *et al.* 2014). However, unlike *C. albifundus* for which abundant information has been generated regarding its genetic diversity (Roux *et al.* 2001; Barnes *et al.* 2005; Lee *et al.* 2016), distribution and insect associations (Heath *et al.* 2009 a,b), almost nothing is known regarding the biology or origins of its closest relatives.

Ceratocystis tsitsikammensis was described from the Garden Route National Park (GRNP), in the Western Cape Province of South Africa (Kamgan Nkuekam *et al.* 2008). It was first isolated from bark harvesting wounds on native *Rapanea melanophloeos* and *Ocotea bullata* trees (Kamgan Nkuekam *et al.* 2008). Inoculation experiments on *R. melanophloeos* resulted in significant lesions, highlighting the need to monitor this fungus as a potentially important pathogen. Until recently, *C. tsitsikammensis* was known only from GRNP and several collections undertaken in natural and plantation ecosystems across and outside South Africa have failed to recover this fungus. In 2014, *C. tsitsikammensis* was isolated from *Virgilia divaricata* and *A. mearnsii* in the GRNP (Van der Colff 2014), suggesting that it may have a wider host range and, more importantly, this host range includes commercially planted non-native trees.

The objective of this study was to increase our knowledge regarding *C. tsitsikammensis*. We determined the mating strategy of the fungus and investigated its geographic distribution, biology, ecology and possible impact on *R. melanophloeos* in the GRNP. The genetic diversity and structure of *C. tsitsikammensis* was studied using microsatellite markers, developed for congener species, to determine whether it is an indigenous species that readily disperses between native and non-native plants. In addition, the role of insects associated with tree wounds as potential dispersal agents was considered.

Materials and methods

Study area

This study was conducted in the GRNP of the Western Cape Province of South Africa. The GRNP forest covers about 35 765 ha stretching from sea level to altitudes of more than 1000 m. Its floristic composition spans eight types of

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Afromontane forests (Rutherford *et al.* 2006). Commercial plantations of *Eucalyptus* species, *Acacia melanoxylon* and *Pinus* species are common in the area. Samples were collected from three forests including the Outeniqua mountain forest comprising the Groenkop forest from which *Ceratocystis tsitsikammensis* was first collected (33°84′773″ S, 22°92′195″ E) as well as the Diepwalle forest (34° 00′436″ S, 23°19′935″ E) and the Olifantsvlakte forest (33° 93′812″ S, 23° 62′473″ E). These sampling sites span a distance of approximately 150 km (Fig 1).

Sample collection

During Sep. 2013, wounds (10 × 10 cm) were made on the trunks of twenty randomly selected *Rapanea melanophloeos* trees per site by removing the bark and exposing the vascular cambium. Flaps of bark resulting from this wounding method were retained on the tree to avoid rapid desiccation of the wounds. Samples from non-native trees were collected opportunistically at the collection sites where damaged trees were observed and included five trees of *Acacia melanoxylon* at the Outeniqua forest, fifteen *Eucalyptus* trees at Diepwalle, and two *A. melanoxylon* trees at Olifantsvlakte. Three days after wounding, sap feeding insects including nitidulid and staphylinid beetles were collected from the fresh wounds on *R. melanophloeos* using an aspirator. These were individually maintained in Eppendorf tubes containing pieces of tissue paper and stored at 4 °C in a cool box until further analyses could be made. Insects were also collected opportunistically from wounds on damaged plantation trees.

Three weeks after wounding, pieces of wood and bark were collected from the artificially induced wounds and examined for the presence of ascomata with long black necks characteristic of the sexual state of *Ceratocystis* species, as well as for the presence of mycelial growth, using a 10× magnification hand lens. When fruiting structures were absent, samples were incubated in moist chambers at room temperature to induce sporulation. Samples from open surfaces of stumps on non-native trees were collected, assessed and handled in a similar fashion.

Isolations from plant material

Collected plant material was assessed for fungal growth on three separate occasions including the day of collection, the third day after collection and on the seventh day after collection, using a dissecting microscope. Fungal isolates were obtained by lifting single ascospore masses from the tops of long-necked ascomata, or single strands of light brown to greyish fluffy mycelium, using a sterile surgical needle and transferring them to 2 % malt extract agar (MEA: 20 g malt extract, 15 g agar, Biolab, Midrand, South Africa, and 1 L deionized water) supplemented with 0.05 g L^{-1} streptomycin sulphate (Sigma–Aldrich, Steinheim, Germany). Cultures were incubated at room temperature for seven days and inspected regularly. Colonies from these primary isolations were purified by transferring single hyphal tips to fresh MEA plates and spore suspensions from these cultures were preserved in 15 % glycerol solution at −70 °C. Cultures were also freeze-dried and stored at −70 °C until further analyses could be undertaken. Representative isolates were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (Supplementary Table 1).

Isolations from insects

Isolations of *Ceratocystis* species from insects were made using carrot baiting (Moller & DeVay 1968). A maximum of three insects per tree wound were randomly selected and individually

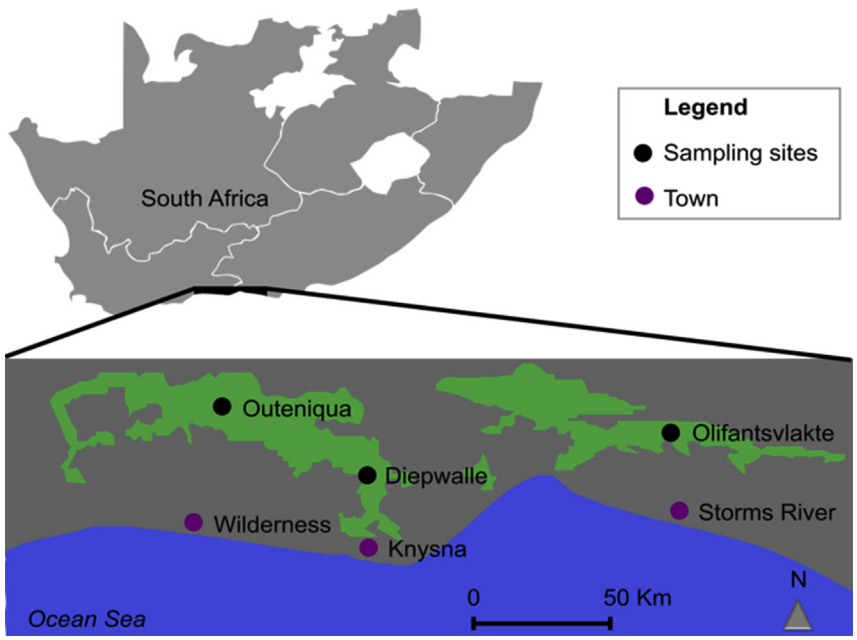

Fig 1 – Outline map of South Africa showing position of the Garden Route National Park (GRNP) in South Africa and sampling sites for *C. tsitsikammensis*.

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crushed between two carrot discs. These discs containing the crushed insects were then wrapped with Parafilm, incubated at room temperature in plastic containers sterilized with 95 % alcohol and monitored for 10–15 d. Mycelium or ascospore masses of putative *Ceratocystis* species were collected from the growth on carrot discs and transferred to 2 % MEA using sterilized surgical needles. Cultures were then purified from a single hyphal tip and preserved as described above.

DNA extraction and isolate identification

To confirm the identities of the *Ceratocystis* species obtained from trees and insects, each isolate was grown for two weeks on 2 % MEA. Mycelium was scraped from the surfaces of the MEA plates using sterilized surgical blades and transferred into 1.5 μL Eppendorf tubes. DNA was extracted using the CTAB method (Möller *et al.* 1992). Identities of isolates were confirmed using β-tubulin sequences generated with primers βt1a and βt1b (Glass & Donaldson 1995). Reactions were prepared in 25 μL total volumes including 0.5 μL (2.5 U) MyTaq™ DNA polymerase (Bioline), 5 μL 5× MyTaq reaction buffer (supplied with the enzyme), 0.5 μL of the βt1a (5′-TTCCCCCGTCTCCACTTCTTCATG-3′) and βt1b (5′-GACGAGATCGTTCATGTTGAACTC-3′) forward and reverse primers (Glass & Donaldson 1995), 1 μL DNA template (75 ηg/μL) and 17.5 μL sterile distilled water (SABAX water, Adcock Ingram, Bryanston, South Africa). Reactions were performed using a BIO-RAD (Hercules, CA, USA) thermo cycler. Thermal cycling conditions included an initial denaturation step at 96 °C for 2 min followed by 35 cycles of 30 s at 94 °C, 60 s at 52 °C and 90 s at 72 °C. A final step of 10 min at 72 °C completed the program. PCR products were stained with GelRed™ (Biotium, USA) nucleic acid dye, electrophoresed on 2 % agarose gels along with a 100 bp molecular marker (Fermentas O′ Gene Ruler™) and visualized under UV illumination to verify successful amplification. Amplified PCR products were purified by gel filtration through 6 % G-50 Sephadex®, in Sephadex® columns (Sigma, Steinheim, Germany).

For the sequencing PCR, forward and reverse sequencing reactions contained 2.5 μL sequencing buffer, 0.5 μL Big Dye (Perkin-Emmer, Warrington, UK), 1 μL of either the forward or reverse primer (10 mM), 3 μL of purified PCR product and sterile Sabax® water in 12 μL final volumes for each reaction. Thermal cycling conditions were 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C. Sequencing products were purified by filtration as described above and base calling was conducted on an ABI 3500xl sequencer (Thermo Fisher Scientific, Carsbad, USA). Forward and reverse sequences were aligned in CLC Main Workbench (CLC Bioinformatics, Denmark). Consensus sequences were aligned with sequences of described *Ceratocystis* species using the MAFFT multiple sequence alignment program version 7 (Katoh & Standley 2013) and phylogenetic analyses were conducted using maximum likelihood (ML) searches in PAUP 4.0 (Swofford 2002).

Genotyping and population genetic parameters

Three microsatellite marker sets were used, including two developed from *Ceratocystis fimbriata s.l* (Barnes *et al.* 2001; Steimel *et al.* 2004). and a set developed for *Ceratocystis manginecans* (Fourie *et al.* 2016). The three microsatellite sets comprised of 16, 11, and 10 markers respectively. The microsatellite markers were assigned to three panels based on their fluorescence and amplicon sizes (Table 1). PCR reagents and thermal cycling conditions published for each set of microsatellite markers were tested and further optimized where necessary. To verify successful amplification and approximate sizes of amplicons, PCR products were stained with GelRed™ (Biotium, USA) nucleic acid dye, electrophoresed on 2 % agarose gels along with a 100 bp molecular size marker (Fermentas O′ Gene Ruler™) and visualized under UV illumination. PCR products for each isolate were pooled based on the expected size of amplicons and the type of fluorescent label attached to the primer. From the pooled sample, 2 μL was mixed with 0.14 μL Genescan-500 Liz size standard (Applied BioSystems, Foster City, California) and 10 μL HIDI formamide. These were separated on a 36 cm capillary with POPTM4 polymer on an ABI Prism 3500XL sequencer. Allele sizes for PCR products were determined with GENEMARKER version V2.2.0 (SoftGenetics, Pennsylvania, USA). Allelic data obtained were used for further analyses. In order to minimize the effect of clonal reproduction on the estimates of the population genetic

Table 1 – Characteristics of the polymorphic markers used in this study, their arrangement into panels for fragment analysis, and number and size of observed amplicons of 116 isolates of *C. tsitsikammensis*.

Locus code	AF2[a]	AF4[a]	AG17[b]	CfAAG8[c]	CfCAA9[c]	CfCAA15[c]	CfCAA80[c]	CfCAT1[c]	CfCAT9X[c]	CfGACA650[c]
Motifs	Tri	Tri	Compound	Tri	Tri	Tri	Tri	Tri	Tri	Compound
Dye colour	VIC	PET	PET	NED	FAM	NED	FAM	VIC	NED	VIC
Panel arrangements	Panel 1	Panel 1	Panel 2	Panel 1	Panel 1	Panel 2	Panel 2	Panel 3	Panel 3	Panel 3
Observed allele sizes	195	262	404	166	220	334	288	232	266	177
	198	265	409	174	224	347	299	235	275	184
	207		414		227	350	302	242	292	
			421		237	352				
					240	355				
						358				
Number of observed alleles	3	2	4	2	5	6	3	3	3	2

a Fourie *et al.* 2016.
b Barnes *et al.* 2001.
c Steimel *et al.* 2004.

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parameters (Grünwald *et al.* 2003), repeated haplotypes from the same site or host individual (tree or insect) were removed (clone-corrected) prior to the analyses.

Genetic diversity

Genetic characterization of *Ceratocystis tsitsikammensis* included determining the gene diversity (H), allelic richness and evenness within populations, as well as the distribution of haplotypes among host groups (insects and trees) and sampling sites. Gene diversity (H) (Nei 1973) was determined using the program POPGENE version 1.3.2 (Yeh *et al.* 1999) with and without clone-correction. For the comparison of the allelic richness and evenness among populations, the unbiased allelic richness and evenness was calculated using the rarefaction approach (Hurlbert 1971; Smith & Grassle 1977; Leberg 2002; Kalinowski 2005). Allelic richness was calculated using the program FSTAT 2.9.3 (Goudet 2001) using rarefaction to the smallest sample size for clone-corrected and non-clone corrected populations. The allelic richness and evenness generated in GenAlEx 6.5 (Peakall & Smouse 2012) were visualized using Venn diagrams.

Population structure and differentiation

To assess the role of ecological factors, including geographic locations, tree species and insect associates, on the genetic clustering of the collected *Ceratocystis tsitsikammensis* populations, four separate datasets were generated based on sampling sites, hosts (trees and insects), sampling sites excluding isolates from insects, and host trees excluding isolates from insects.

All four datasets were used for separate Bayesian clustering analyses in STRUCTURE version v. 2.3.4 (Pritchard *et al.* 1999). STRUCTURE analyses use allelic frequencies to assign isolates to memberships or genetic clusters. Prior to STRUCTURE analyses, populations of *C. tsitsikammensis* were clone-corrected. Analyses were performed using 500 000 MCMC repetitions after a burn-in of 100 000 generations, where K values ranged from 1 to 10 and the number of iterations was fixed at 20. The data were analysed using the admixture ancestral model that assumes inbreeding within populations (Pritchard *et al.* 1999). This model was assigned to the correlated allele frequencies across population clusters, appropriate when weak genetic structure is expected (Pritchard *et al.* 1999).

The most likely value for K, corresponding to the optimal K value with the higher likelihood and lower standard deviation, was inferred using L(K) (Pritchard *et al.* 1999) and DeltaK (Evanno *et al.* 2005) maximum likelihood-based methods implemented in STRUCTURE HARVESTER web v.0.6.93 (Earl 2012; http://taylor0.-biology.ucla.edu/structureHarvester/). To calculate the best values of K, the analysis was re-run (assignment) five times using 1 000 000 MCMC repetitions after a burn-in period of 200 000. The results were graphically visualized in CLUMPAK (Kopelman *et al.* 2015). A 90 % identity cut-off was used to assign individual MLHs to clusters. MLHs having a membership proportion of less than 90 % in a single cluster were considered admixed.

The genetic grouping proposed by STRUCTURE was compared to that produced by Principal Coordinate Analysis (PCoA) in GenAlEx and the Median-joining networks (MJN) generated in NETWORK 4.6 (Brandelt *et al.* 1999). For PCoA, the standardized covariance method and haploid distance matrix for clustering of haplotypes was used. MJN was used to better visualize the distribution of MLHs of *C. tsitsikammensis* between sampling sites, tree hosts and insects.

To determine partitioning of molecular variation among isolates within and among the different sources of variation, including sampling sites and host species, Analysis of Molecular Variance (AMOVA) was performed using GenAlEx.

Mode of reproduction

Because most *Ceratocystis* species reproduce both sexually and asexually, evidence for random mating between individuals in the Olifantsvlakte, Diepwalle, and Outeniqua populations, as well as all three populations combined was assessed using the alternative measure of the index of association, *rBarD* (Agapow & Burt 2001). The assumption of non-random mating (linkage disequilibrium) is rejected when: (i) the observed value for *rBarD* falls within the bell curve produced by the simulation of a randomly mating population or (ii) when $P < 0.05$ (Agapow & Burt 2001).

Determination of mating system

The mating system of *Ceratocystis tsitsikammensis* was determined using two sets of mating type makers developed from the genome of an ex-type isolate of *C. tsitsikammensis* (Wilken Unpublished). Primers MAT 1-1-1-5: AACTTTGGAGGGGAGATCC and MAT 1-1-1-3: AGATATCCACGAGCTAGACA that amplify approximately 343 bp of the MAT 1-1-1 idiomorph and primers MAT 2-5: AATACCCACCAGAAATCAGC and MAT 2-3: ATTCAATGTGCCGATACCG that amplify ~449 bp of the MAT 1-2-1 idiomorph were screened on 24 isolates in two separate PCR reactions. The PCR reactions were prepared and performed as described above, with an annealing temperature set at 52 °C for both reactions. Isolates that amplified both the MAT 1-1-1 and MAT 1-2-1 idiomorph were considered homothallic and those that lacked the MAT 1-2-1 idiomorph were considered as resulting from unidirectional mating type switching.

Results

Isolates obtained

A total of 116 isolates of *Ceratocystis tsitsikammensis* were utilized in this study. *Ceratocystis tsitsikammensis* isolates were obtained from 38 (46 %) of the 82 wounded trees, 37 (54 %) of the 69 crushed nitidulid beetles and six (75 %) of the eight crushed staphylinid beetles (Table 2, Supplementary Table 1). Nitidulid and staphylinid beetles were collected from wounds of *Acacia melanoxylon*, *Eucalyptus* sp. and *Rapanea melanophloeos*. Fifty (64 %) insects were collected from *R. melanophloeos*, 16 (21 %) from *A. melanoxylon*, and 11 (14 %) from *Eucalyptus* sp. Nitidulid beetles were collected at all three sampling sites and the staphylinid beetles only at Outeniqua (Table 3).

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Table 2 – Distribution of *C. tsitsikammensis* isolates and multilocus haplotypes (MLHs) among sampling sites, host trees and insects in the GRNP.

Sampling site	Host	No. of trees/insects sampled	No. of trees/ insects with *C. tsitsikammensis*	No. of *C. tsitsikammensis* isolates	Total no. of MLHs per tree/ insect species	MLHs code (see Fig 3)
Diepwalle	*Eucalyptus* sp.	15	5	7	6	5, 41, 42, 43, 44, 49
	Rapanea melanophloeos	20	8	9	6	1, 9, 16, 31, 41, 42,
	Nitidulid beetle	10	7	10	8	20, 12, 14, 15, 16, 19, 51, 62
Olifantsvlakte	*Acacia melanoxylon*	2	1	1	1	20
	Nitidulid beetle	17	10	10	9	21, 29, 30, 32, 33, 38, 47, 66, 67
	R. melanophloeos	20	8	26	22	2, 7, 11, 12, 14, 16, 17, 19, 20, 27, 33, 36, 53, 55, 56, 57, 58, 60, 61, 63, 64, 65
Outeniqua	*A. melanoxylon*	5	4	2	2	3, 37
	Nitidulid beetle	42	20	17	11	22, 23, 24, 25, 32, 34, 35, 37, 48, 52, 59
	R. melanophloeos	20	12	31	22	4, 6, 7, 8, 10, 14, 16, 18, 19, 20, 26, 34, 38, 39, 40, 45, 46, 50, 54, 62, 64, 67
	Staphylinid beetle	8	6	3	3	4, 13, 28
Total		159	81	116		

Table 3 – Summary of the number of insects obtained from sampled trees and forests.

Sampling sites	Insects	No. of insects per tree species			Total
		A. melanoxylon	*Eucalyptus* sp.	*R. melanophloeos*	
Diepwalle	Nitidulid	0	3	7	10
Olifantsvlakte	Nitidulid	3	5	9	17
Outeniqua	Staphylinid	2	3	3	8
	Nitidulid	11	0	31	42
	Total	16	11	50	77

Microsatellite amplification and polymorphism

Of the 37 microsatellite markers tested, five from Barnes *et al.* (2001), eleven from Steimel *et al.* (2004) and all ten from Fourie *et al.* (2016), amplified successfully for *Ceratocystis tsitsikammensis*. From the fragment analyses, ten markers were found to be polymorphic with the number of alleles ranging from two to six per locus (Tables 1, 4 and 5).

Genetic diversity

The genetic diversity was analysed for both the sampling sites (Table 4) and all the hosts (Table 5). A total of 82 MLHs were obtained from both the sampling sites and hosts (Tables 1, 4 and 5). For the total population of all *Ceratocystis tsitsikammensis* isolates combined, 67 clone corrected MLHs were obtained (Table 1, Supplementary Table 2). The allelic richness values

Table 4 – Population genetic parameters of *C. tsitsikammensis* per sampling site in the GRNP of South Africa.

Population genetic parameters	Diepwalle	Olifantsvlakte	Outeniqua	Total population
No. of isolates	26	37	53	116
No. of polymorphic loci	9	9	9	10
Allelic richness[a]	2.40	3.06	2.79	3.06
Allelic richness (cc)[b]	2.40	2.99	2.78	3.01
H[c]	0.31	0.32	0.28	0.32
H (cc)[d]	0.31	0.35	0.31	0.36
MLHs[e]	17	30	35	82

a Allelic richness using non clone-corrected data and.
b Allelic richness using clone-corrected data, based on rarefaction to smallest sample size of 26.
c Nei's gene diversity using non clone-corrected data.
d Nei's gene diversity using clone-corrected data set.
e Number of multilocus haplotypes per sampling site.

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Table 5 – Population genetic parameters of *C. tsitsikammensis* per sampled host in the GRNP.

Population genetic parameters	*A. melanoxylon*	*Eucalyptus* sp.	Nitidulid beetles	*R. melanophloeos*	Staphylinid beetles	Total population
No. of isolates	3	7	37	66	3	116
No. of polymorphic loci	3	6	9	9	4	10
Allelic richness[a]	NA	1.70	2.08	2.40	NA	2.45
Allelic richness (cc)[b]	NA	1.70	2.14	2.43	NA	2.47
No. of private alleles[c]	1	0	0	3	1	4
H[d]	0.16	0.23	0.26	0.31	0.20	0.32
H (cc)[e]	0.16	0.24	0.30	0.33	0.26	0.36
MLHs[f]	3	6	27	43	3	82

a Allelic richness using non clone-corrected data with rarefaction to the smallest sample size of 7 (populations of *A. melanoxylon* and staphylinid beetles excluded due to small sample size = NA).
b Allelic richness using clone-corrected data with rarefaction of 6.
c Number of private alleles in clone-corrected data.
d Nei's gene diversity using non clone-corrected data.
e Nei's gene diversity using clone-corrected data.
f Number of multilocus haplotypes per sampled host tree/insect species.

over sampling sites varied from 2.40 to 3.06 and from 2.40 to 3.01 for non clone-corrected and clone-corrected data respectively (Table 4). The highest value of allelic richness was obtained from Olifantsvlakte. For the sampling sites, Nei's gene diversity values ranged between 0.28–0.32 and 0.31–0.35 for the non clone-corrected and clone-corrected datasets respectively (Table 4). Olifantsvlakte contained the highest gene diversity while Outeniqua contained the largest number of MLHs (Table 4).

The allelic richness values over hosts varied between 1.70 and 2.43 for both non clone-corrected and clone-corrected data with the highest allelic richness obtained for isolates from *Rapanea melanophloeos* (Table 5). Nei's gene diversity varied between 0.16–0.31 and 0.16–0.33 for non clone-corrected and clone-corrected data respectively (Table 5). The population of isolates from *R. melanophloeos* contained the highest gene diversity and contained the largest number of MLHs (Table 5).

The Venn diagram (Fig 2A), showed that 29 (88 %) of the 33 alleles were shared between sampling sites, and 28 (85 %) between hosts (Fig 2B). Private alleles were obtained from Olifantsvlakte and Outeniqua (Table 4, Fig 2A) as well as from *Acacia melanoxylon*, *R. melanophloeos*, and staphylinid beetles (Table 5, Fig 2B).

Population structure

Analyses for population structure using STRUCTURE were inconclusive as no clear population structure could be detected for any of the optimal K values tested (Supplementary Figs 1 and 2). The random distribution of MLHs of the GRNP population of *Ceratocystis tsitsikammensis* was also confirmed in the PCoA (Fig 3). The distribution of the 67 clone-corrected MLHs between sampling sites using the MJN showed that 11 (16.4 %) MLHs were shared between sampling sites (Fig 4A), 14 (21 %) between the sampled trees and insect species and 23 (34.3 %) between at least two sampled individual hosts (Fig 4B). About 13 (56.5 %) and 20 (87 %) of the shared MLHs haplotypes were nitidulid beetle and *Rapanea melanophloeos* associated respectively (Fig 4B). The distribution of MLHs between hosts within sampling sites showed that, in Diepwalle (Fig 4C), *R. melanophloeos* shared two and one MLHs with *Eucalyptus* sp. and nitidulid beetles respectively. From Olifantsvlakte (Fig 4D), *R. melanophloeos* shared one MLH with *Acacia*

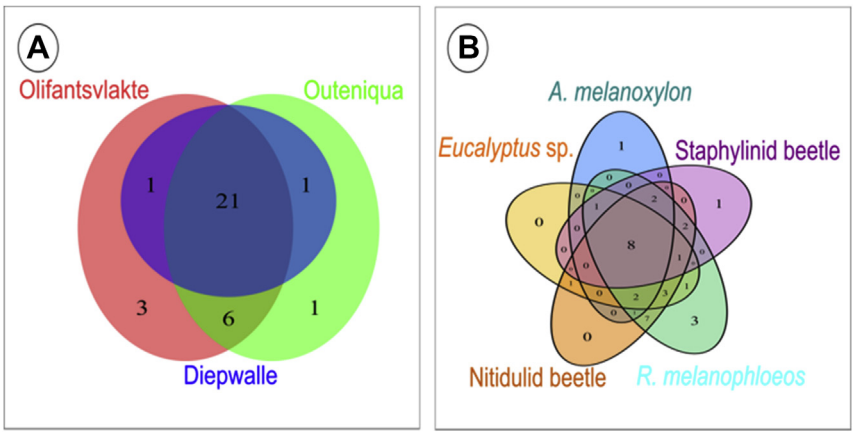

Fig 2 – Allelic richness and evenness between (A) sampling sites and (B) host trees and insects.

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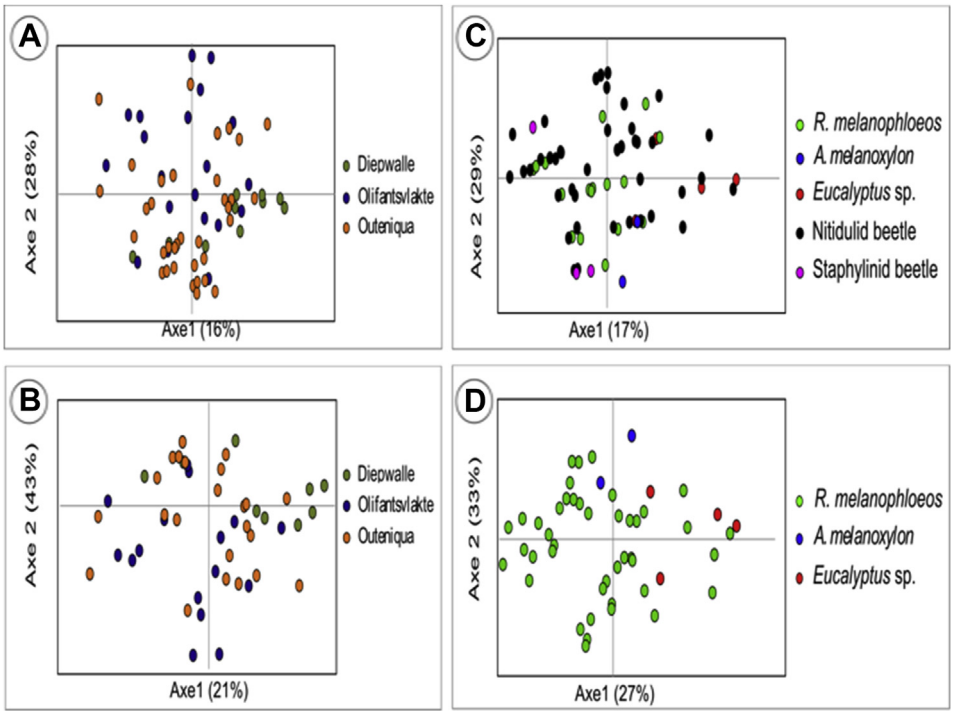

Fig 3 – PCoA analysis showing the absence of structure in *C. tsitsikammensis* haplotype distribution, (A) between sampling sites, (B) between sampling sites excluding isolates from insects, (C) between tree hosts and insects, (D) between tree hosts without isolates from insects.

melanoxylon and another MLH with nitidulid beetles. From Outeniqua (Fig 4E), *R. melanophloeos* shared one MLH with staphylinid beetles and another with nitidulid beetles. Nitidulid beetles also shared one MLH with *A. melanoxylon* in Outeniqua (Fig 4E).

Analysis of molecular variance (AMOVA) showed that most of the variation observed was within the analysed populations rather than between populations (Supplementary Fig 3). These results suggest that the GRNP population of *C. tsitsikammensis* is highly admixed and represents a single population.

Mode of reproduction

The observed *rBarD* values fell within the bell-curve of the simulated distribution of a randomly mating population for the overall GRNP population of *Ceratocystis tsitsikammensis* (Fig 5A) as well as for the Diepwalle (Fig 5B), Olifantsvlakte (Fig 5C) and Outeniqua (Fig 5D) subpopulations. The observed *rBarD* values and the *P*-values greater than 0.05 reject the hypothesis of non-random mating indicating that *C. tsitsikammensis* reproduces sexually in the GRNP.

Determination of mating system

All isolates screened for mating type had the MAT 1-1-1 gene, 16 (66 %) had both MAT 1-1-1 and MAT 1-2-1 and 8 (33 %) lacked a MAT 1-2-1 gene (Supplementary Table 3). The presence of both mating types is characteristic of homothallism, while the lack of the MAT 1-2-1 in some isolates confirms that unidirectional mating type switching takes place in *Ceratocystis tsitiskamensis*. These results are consistent with the *rBarD* results suggesting that sexual reproduction, or outcrossing, occurs in the GRNP population of the fungus.

Discussion

Although a previous greenhouse inoculation study showed that *Ceratocystis tsitsikammensis* can result in the death of *Rapanea melanophloeos* trees (Kamgan Nkuekam *et al.* 2008), no diseased *R. melanophloeos* trees were observed in this study in their natural environment. The fungus displayed a relatively high genetic diversity, low population differentiation and evidence of sexual reproduction. These characteristics as well as its restricted geographic range, suggest that *C. tsitsikammensis* is native to the GRNP of South Africa. At least in this situation, it should not be considered as a threatening pathogen for native trees.

In the present study, the overall gene diversity for *C. tsitsikammensis* (H = 0.36) was in the range of that of the native African fungus *Ceratocystis albifundus* in South Africa (H = 0.38) (Barnes *et al.* 2005), but also in the range of congener species considered non-native in South Africa, including *Ceratocystis eucalypticola* (H = 0.36) (van Wyk *et al.* 2006) and *Ceratocystis pirilliformis* (H = 0.34) (Kamgan Nkuekam *et al.* 2009). The genetic diversity of a fungal population, determined by both measures of gene and genotypic diversity (McDonald 1997), is substantially affected by the age of a population (McDonald 1997). Populations that have evolved over long periods at a specific location are expected to have greater numbers of alleles as a result of repeated mutations and genetic drift (McDonald 1997; Sakai *et al.* 2001). High levels of genetic diversity,

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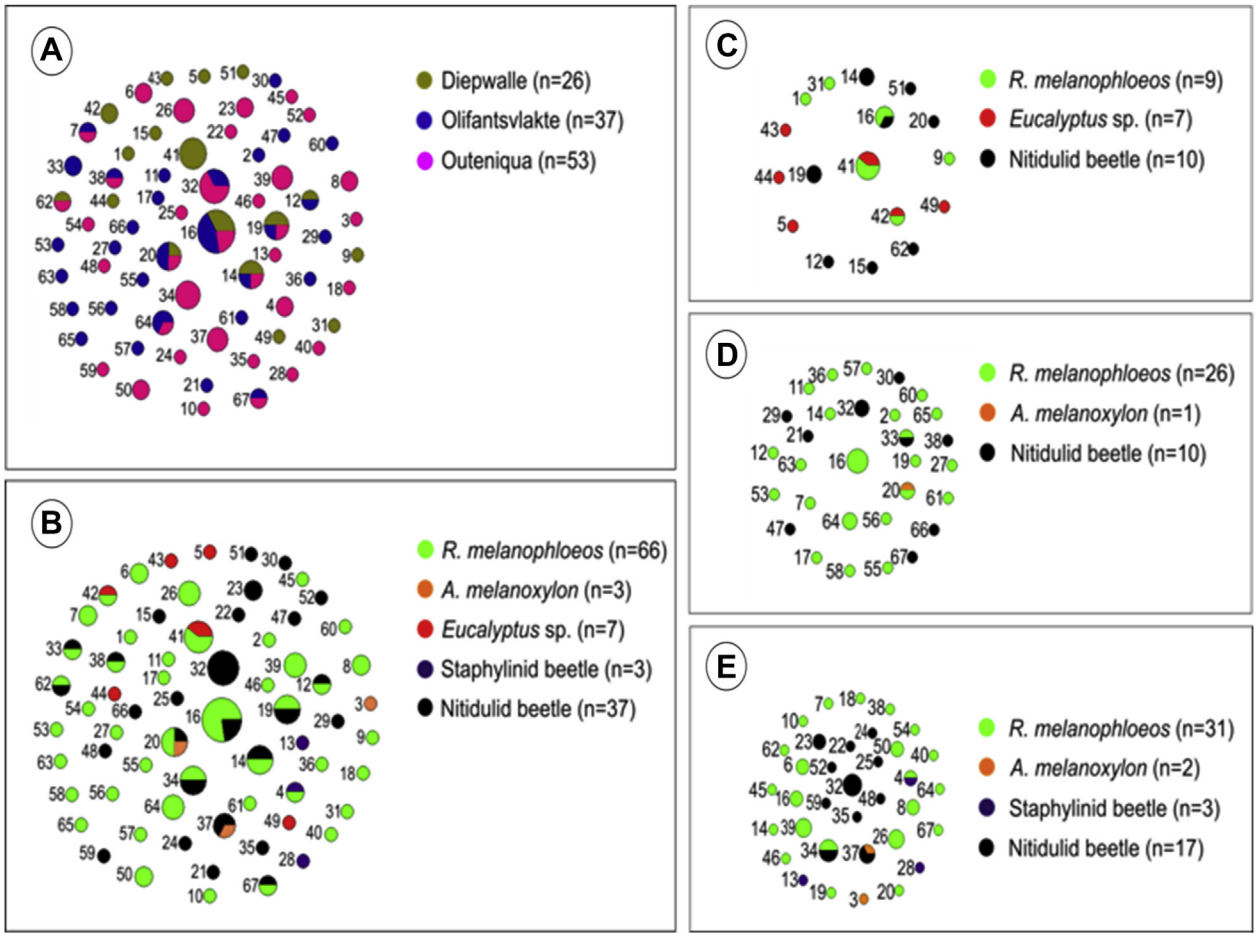

Fig 4 – Haplotype distribution and frequencies (A) between sampling sites, (B) between host plants and insects, (C) between hosts at Diepwalle, (D) between hosts at Olifantsvlakte, (E) between hosts at Outeniqua. The numbers indicate the haplotypes (see Table 2) and the size of the circles their frequencies. "N" represents the total number of isolates used. Shared haplotypes were present in all sites and on all hosts.

therefore, provide an indication of the center of origin of a species, although multiple introductions of exotic species could also generate high genetic diversity, as has for example been shown for *Diplodia sapinea* Fr., a pathogen responsible for shoot blight and die-back diseases of plantation pine trees in South Africa (Bihon *et al.* 2012).

A high number of *C. tsitsikammensis* alleles were shared between sampling sites, host trees and insects. As indirect measures of gene flow (Slatkin 1985), the allelic richness and evenness are indications of life-history traits in natural populations, such as dispersal abilities and reproductive strategies (McDonald 1997). High values such as those observed for the GRNP population of *C. tsitsikammensis*, are characteristic of a fungus that is well adapted to the environmental heterogeneity of its geographic range (Slatkin 1987; Sakai *et al.* 2001).

Mating strategies represent important life history traits in natural populations of fungi. In this regard, *Ceratocystis* species have been reported to be predominantly homothallic or self-fertile (Harrington *et al.* 1998; Wilken *et al.* 2014). Homothallic populations of *Ceratocystis* species are, however, capable of outcrossing as result of a unidirectional mating type switching system, that allows some self-fertile isolates to produce both self-fertile and self-sterile offspring (Harrington & McNew 1997; Wilken *et al.* 2014). This irreversible process, also observed for *C. tsitsikamensis* in the present study can generate high genetic diversity in natural populations of these fungi, such as that observed for the GRNP population.

The largely random distribution of haplotypes throughout the GRNP could be ascribed to the dispersal of *C. tsitsikammensis* over large distances by nitidulid and staphylinid beetles, which were associated with most of the dominant MLHs in all three sampled sites. The haplotype richness of *C. tsitsikammensis* isolated from insects in this study was high and included numerous haplotypes not found on the host plants. This result underlined the important role of insects for the dispersal of the fungus within and between locations as well as host trees. Long distance dispersal of fungal haplotypes by insects has been shown in population studies on other insect associated fungi (Marin *et al.* 2009; Ferreira *et al.* 2011). Insect dispersal could also explain the observed lack of population structure found for *C. tsitsikammensis*, as is the case for other insect-associated Ceratocystidaceae such as *Endoconidiophora polonica* Siemaszko & C. Moreau, dispersed by the bark beetle, *Ips typographus* L. (Marin *et al.* 2009).

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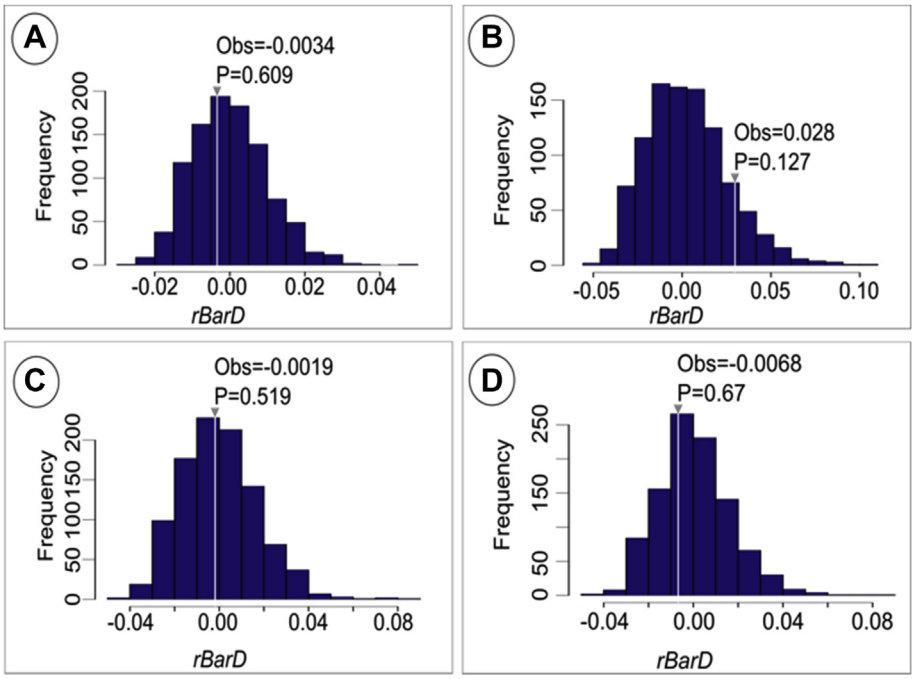

Fig 5 – Linkage disequilibrium testing (*rBarD*, *P*-values > 0.05), showing evidence for random mating of *C. tsitsikammensis*, in (A) the overall GRNP population of *C. tsitsikammensis*, (B) the Diepwalle population, (C) the Olifantsvlakte and (D) the Outeniqua population.

The dispersal of *Ceratocystis* and other species in the Microascales by insects is well-established (True *et al.* 1960; Hinds 1972; Gibbs & French 1980; Juzwik & French 1983; Appel *et al.* 1990; Health *et al.* 2009; Kamgan Nkuekam *et al.* 2012; Mbenoun *et al.* 2014). Nitidulid beetles are well-recognised vectors of the oak wilt pathogen *Ceratocystis fagacearum* (True *et al.* 1960; Juzwik & French 1983; Webber & Gibbs 1989) and several *Ceratocystis* species described from Africa (Heath *et al.* 2009b; Mbenoun *et al.* 2014). In the GRNP, we recovered *C. tsitsikammensis* from nitidulid as well as staphylinid beetles. The association of staphylinid beetles with *Ceratocystis* species is not new, but has not received as much attention as other associations. Fungi in the Ceratocystidaceae have previously, for example, been associated with staphylinid beetles on Aspen trunk wounds in Colorado in the USA (Hind 1972), as well as from wounds of *Eucalyptus* trees in Australia (Kamgan Nkuekam *et al.* 2012). This is not surprising given that four of the 32 subfamilies of Staphylinidae are mycophagous (Frank & Thomas 2013) and many taxa favour moist habitats (Frank & Thomas 2013), such as tree wounds. Considering their ubiquitous distribution, staphylinid beetles may, therefore, play an important role in the dispersal of economically important *Ceratocystis* species in other parts of the world.

Conclusions

The host range of *Ceratocystis tsitsikammensis* was expanded to include *Acacia melanoxylon* and *Eucalyptus* sp. in this study. Together with previous studies by Kamgan Nkuekam *et al.* (2008) and Van der Colff (2014) this brings the current host range of this fungus to six, of which three hosts are non-native to South Africa. Results from this study suggest that *C. tsitsikammensis* is native to the Garden Route National Park (GRNP) of South Africa. The studied population is genetically diverse and undergoing sexual reproduction. *Ceratocystis tsitsikammensis* is associated with various insects, including nitidulid and staphylinid beetles that contribute to the genetic diversity and structure of the fungus. Furthermore, no evidence of disease was found on *Rapanea melanophloeos* in the studied native forests, supporting the above assumption and it is thus not likely to represent a threat to this ecosystem.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.09.002.

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