

New host range and distribution of *Ceratocystis pirilliformis* in South Africa

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Abstract

Ceratocystis pirilliformis was first described from wounds on *Eucalyptus* trees in Australia and subsequently found as a common wound inhabitant on these trees in South Africa. During a recent disease survey carried out to identify *Ceratocystis* species infecting tree wounds in South Africa, *C. pirilliformis* was isolated from various tree species, including a native *Rapanea* species and two non-native hosts, *Acacia mearnsii* and *E. grandis* growing in the Western Cape Province. These new collections from the Western Cape Province, together with isolates of *C. pirilliformis* previously collected from *Eucalyptus* species in the eastern part of the country, provided the opportunity to investigate the possible movement of the pathogen since it was first recorded in South Africa. The identity of newly collected isolates of *C. pirilliformis* was confirmed using DNA sequence comparisons using partial β -tubulin and TEF-1 α gene regions. Microsatellite markers were screened on a collection of *C. pirilliformis* isolates from the Western Cape Province. Results showed that this population has higher levels of population genetic estimates such as private allele frequency, allelic richness and gene diversity. Inoculation tests were performed to determine whether *C. pirilliformis* is a pathogen of *A. mearnsii*, which is planted as an important plantation forestry tree in the country. All isolates were shown to be highly virulent. Results of the study consequently revealed that *C. pirilliformis* has a wider host range and geographic distribution in South Africa than previously recognized and that it is a potentially important pathogen of *A. mearnsii*.

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Introduction

Species of *Ceratocystis* include numerous economically important fungal pathogens that cause diseases of a wide range of plants, especially angiosperm trees (Kile 1993; Roux and Wingfield 2013; Seifert et al. 2013). Disease symptoms include rots of various root crops, cankers on the stems of trees, streaked discolouration of the xylem tissues, wilt, and tree death (Kile 1993; Roux and Wingfield 2009). *Ceratocystis* species require wounds to infect hosts that arise from natural or accidental damage to roots or stems (DeVay et al. 1968; Roux et al. 2007). Some species have also established important symbioses with wood-infesting insects that facilitate their dispersal (Heath et al. 2009; Al Adawi et al. 2013; Ploetz et al. 2013; Soulioti et al. 2015).

Since the first report of a disease caused by a species of *Ceratocystis* on exotic plantation trees in Brazil (Ribeiro et al. 1988), there have been increasing numbers of studies reporting new *Ceratocystis* diseases on forest plantation trees globally (Roux and Wingfield 2009; Tarigan et al. 2011). These include the *Ceratocystis* wilt disease of *Acacia mearnsii* De Wild. caused by *C. albifundus* M.J. Wingf., De Beer & M.J. Morris in South Africa (Morris et al. 1993; Wingfield et al. 1996), wilt and death of *Eucalyptus* species caused by *C. eucalypticola* M. van Wyk & M.J. Wingf. (as *C. fimbriata sensu lato*) in Brazil (Ferreira et al. 1999; van Wyk et al. 2012), the Republic of Congo (Roux et al. 2000), Uganda (Roux et al. 2001) and Uruguay (Barnes et al. 2003a) and a serious canker and wilt disease of *A. mangium* Willd. in Indonesia caused by *C. manginecans* M. van Wyk, Al Adawi & M.J. Wingf. (Tarigan et al. 2011; Fourie et al. 2015).

Until recently, the genus *Ceratocystis* was used in the broad sense to include a wide variety of morphologically and ecologically similar, but in many cases very different, fungi. These have now been accommodated in a suite of different genera as suggested by Wingfield et al. (2013a) and implemented by de Beer et al. (2014). *Ceratocystis* is now restricted to species phylogenetically related to *Ceratocystis fimbriata sensu stricto*, first described for the sweet

potato pathogen by Halsted (1890). The name *Ceratocystis* used in the present study is based on the classification provided by de Beer et al. (2014).

Ceratocystis pirilliformis I. Barnes & M. J. Wingf. was first discovered as the causal agent of sap-stain on wounded *Eucalyptus nitens* (H. Deane & Maiden) Maiden near Canberra in Australia (Barnes et al. 2003b). It was subsequently found on *Eucalyptus* species in the Mpumalanga and KwaZulu-Natal Provinces of South Africa (Roux et al. 2004). Based on low levels of genetic diversity in the country, Nkuekam et al. (2009) suggested that *C. pirilliformis* was most likely introduced to South Africa from Australia. This view, along with the fact that *C. pirilliformis* was first found in Australia (Barnes et al. 2003b), is further supported by the absence of any *C. pirilliformis*-associated diseases on *E. nitens* in Australia where this tree is native (Roux et al. 2004).

During recent tree disease surveys in the Western Cape Province of South Africa, a *Ceratocystis* species with pear-shaped ascomatal bases, typical of those of *C. pirilliformis*, was consistently found colonizing artificially-induced wounds on *Acacia mearnsii* in the Stellenbosch area. It was also found on the stumps of recently felled *A. mearnsii*, *E. nitens* and *Rapanea* species in the Garden Route National Park of the Western Cape Province. This raised questions concerning the movement of *C. pirilliformis* in South Africa subsequent to its first discovery in the eastern part of the country. The aims of this study were thus i) to confirm the identification of the newly collected isolates resembling *C. pirilliformis* from the Western Cape areas, ii) to trace the movement of the pathogen in South Africa using microsatellite markers and iii) to determine whether the fungus has the capacity to infect *A. mearnsii* by conducting pathogenicity trials using selected isolates collected in this study.

Materials and Methods

Fungal isolates

Collections of *Ceratocystis* species were made from *A. mearnsii* in the Stellenbosch area (ST), and *A. mearnsii*, *Eucalyptus grandis* W. Hill ex Maid. and *Rapanea* species in the Garden Route National Park (GRNP) of the Western Cape Province (WC) (Table 1, 1S). Wounds on the stems of trees that had either been artificially induced with a machete or axe, or arose from tree harvesting, were surveyed four weeks after wounding in December 2013,

Table 1. *Ceratocystis pirilliformis* isolates from three provinces and four hosts in South Africa.

Geographic area		GPS coordinate	Host	Population	Sample size
Province	Location				
Western Cape	Stellenbosch, Knorhoek	S33°53.133' E018°52.608'	<i>Acacia mearnsii</i> (30)	ST	30
	Garden Route National Park, Knysna, Diepwalle	S33°56.909' E23°09.434'	<i>A. mearnsii</i> (3)	GRNP	15
			<i>Eucalyptus grandis</i> (8)		
Mpumalanga	Garden Route National Park, Knysna, Humansdrop	S34°09.788' E24°38.309'	<i>Rapanea</i> sp. (4)	ESA	38
	Bushbuckridge, Waterhoutboom,	S24°56.829' E030°55.213'	<i>E. grandis</i> x <i>E. camaldulensis</i> (8)		
	Sabie, Bergvliet Plantation	S25°03.242' E030°51.680'	<i>E. grandis</i> x <i>E. camaldulensis</i> (19)		
KwaZulu-Natal	Paulpietersburg, Eersteling Plantation	S27°31.843' E030°48.123'	<i>E. grandis</i> (11)		

(): Number of isolates included in this study.

and January 2014. Bark flaps surrounding the wounds were examined for sexual structures typical of *Ceratocystis* species using a 10 × magnification hand lens. Pieces of exposed bark bearing ascomata were collected separately for each tree in paper bags, and transported to a laboratory in order to make isolations. Samples were inspected regularly for the presence of *Ceratocystis* species producing ascospore masses at the tips of ascomatal necks. Cultures were made by directly transferring single drops of ascospores to separate Petri dishes (65 mm) containing 2% MEA (20 g Malt Extract; 20 g Agar, Biolad, Midland, South Africa) supplemented with 100 mg/l of the antibiotic streptomycin (SIGMA-ALDRICH, Steinheim, Germany) and incubated at 25°C for two weeks in the dark. All isolates obtained in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1S).

In addition to the isolates obtained in this study, isolates of *C. pirilliformis* from the study of Nkuekam et al. (2009) were obtained from the CMW collection in FABI and used for comparative purposes when considering the genetic diversity of populations of *C. pirilliformis* in South Africa. These isolates had been collected from Bushbuckridge (Waterhoutboom) and Sabie (Bergvliet Plantation) in the Mpumalanga Province and Paulpietersburg (Eersteling Plantation) in the Province of KwaZulu-Natal (ESA), South Africa (Table 1, 1S).

DNA extraction

Genomic DNA was extracted from all the isolates collected in the survey and those obtained from the CMW culture collection. Mycelium was scraped from the surfaces of the cultures using a sterile surgical scalpel and transferred to 1.5 ml Eppendorf tubes. Genomic DNA was extracted following the technique described by Möller et al. (1992). The concentration of the extracted DNA was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc, USA), and was diluted to a working concentration of 15 ng/μl. After quantification, DNA extracts were used in subsequent PCR reactions for species identification and for microsatellite amplification.

Identification of fungi

Isolates (Table 1, 1S) collected from ST and GRNP were identified based on morphological

characters and DNA sequence comparisons. All isolates were identified using light microscopy (C-DS, SMZ645, NIKON, Japan) following the description of Barnes et al. (2003b). In addition to the morphological identifications, two isolates (CMW42018, CMW42020) from ST and two isolates (CMW42042, CMW42336) from GRNP were randomly selected and used for species identification using DNA sequence comparisons.

DNA sequence analysis was performed following the techniques described by Lee et al. (2015) based on part of the β -tubulin 1 gene region (BT1) using primers Bt1a and Bt1b (Glass and Donaldson 1995), and the Translation Elongation Factor-1 alpha (TEF-1 α) regions with primers TEF1F and TEF2R (Jacobs et al. 2004). The PCR amplification was carried out in a total volume of 15 μ l containing 15 ng/ μ l of genomic DNA, 0.5 μ l of each forward and reverse primer (10 pM) for both BT1 and TEF-1 α gene regions, 5 μ l of 5 \times reaction buffer containing 5 mM dNTPs and 15 mM MgCl₂ (Bioline in London, UK) and 0.09 μ l of MyTaqTM DNA polymerase (Bioline in London, UK). PCR reactions were conducted in a VeritiTM 96 well Thermal Cycler (Applied BiosystemTM, Foster City, CA) using the same conditions as those described by van Wyk et al. (2006). The amplification products stained with GelRedTM (Biotium Incorporation, USA) nucleic acid dye on a 1.5% (w/v) agarose gel in 1 \times TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.0) were separated by electrophoresis (Mini-Sub[®] Cell CT, Biolad, USA), and then visualized under UV light (Gel DocTM EZ Imager, Bio-Rad, Richmond, CA).

Phylogenetic analyses

Sequences for each gene region were combined in a single data set and were aligned with published sequences of *Ceratocystis* species retrieved from GenBank (Table 2S), using the online version of MAFFT ver.7.215 (<http://mafft.cbrc.jp/alignment/server/>) (Katoh et al. 2002). Maximum likelihood (ML) analyses were performed using RAxML HPC BlackBox ver.8.1.11 (Stamatakis 2006; Stamatakis et al. 2008) using the default option with GTR substitution model implemented in the CIPRES cluster server (<https://www.phylo.org/>) at the SanDiego Supercomputing Center.

Pathogenicity tests

The ability of *Ceratocystis* species collected in this study to cause disease on *A. mearnsii* was tested on approximately three-year-old *A. mearnsii* trees following the same technique as previously described by Lee et al. (2015). Twenty trees were inoculated with each isolate (one hundred trees in total) and twenty trees inoculated with sterile agar were used as controls. Inoculations were conducted in a jungle stand near Pretoria East, Gauteng Province of South Africa in December 2014. Five isolates (CMW42042, CMW42336, CMW42018, CMW42019, CMW42020) were used in the inoculation experiment and lesion lengths were measured six weeks after the inoculation. Analysis of Variance (ANOVA) and Tukey's Honestly Significance Difference (Tukey's HSD) test were used to determine whether there were significant differences in aggressiveness between isolates used for the inoculation trial based on a P-value computed using R ver.3.1.0 (<http://www.r-project.org/>; R Core Team 2014). Re-isolations were made to ensure that the inoculated fungi were responsible for the infections observed and to fulfil the requirements of Koch's postulates.

Microsatellite amplification and scoring of alleles

To determine the genetic diversity and contemporary population structure of the isolates, 17 microsatellite primer sets (Table 3S), previously developed by Barnes et al. (2001) and Steimel et al. (2004), were screened using the same conditions described by the authors, with slight modifications in the annealing temperature (Table 3S).

The size of microsatellite amplicons was determined with an ABI PRISM™ 3500x1 POP 7™ Automated DNA sequencer (Applied Biosystems™ / Life Technologies. Carlsbad, US). GENESCAN-600 LIZ (Applied Biosystems™ / Life Technologies. Carlsbad, US) was used as the internal size standard. Determination of expected allele sizes, based on fragment length, was accomplished using GENEMARKER ver.2.2.0 (Softgenetics, LLC, USA).

Each allele was sequenced to confirm the size of PCR products amplified with each microsatellite marker. Each confirmed product length was treated as a unique allele. PCR reaction conditions and DNA sequencing of these alleles followed the same procedures described above.

Population genetic analyses and random mating

To determine the movement of *C. pirilliformis* in South Africa, isolates were grouped into three larger populations based on sampling location: ST from Stellenbosch area, GRNP from the Garden Route National Park and ESA from the KwaZulu-Natal and Mpumalanga Provinces. The confidence for being able to cluster each sampling site into these populations was based on results from Rst calculations (Slatkin 1995) implemented in ARLEQUIN ver.3.5.2.1 (Excoffier et al. 2005, 2010). A Multi-Locus Haplotype (MLH) data set, in which genetically identical haplotypes were clone-corrected from each of the locations, was generated for each population and used for further analysis. The number of different alleles (N_a), the number of effective alleles (N_e ; Nielsen et al. 2003) and gene diversity (H ; Nei 1973) were calculated using the software program GENALEX ver.6.5 (Peakall and Smouse 2012). ADZE ver.1.0 (Szpiech et al. 2008) was used to compute the private allelic richness (P) and allelic richness (AR). Given that populations had different sample sizes in this study, a rarefaction procedure using ADZE, capable of adjusting P and AR to a specific sample size, was used. The sample sizes were standardized to the smallest clone-corrected population, GRNP in this study ($N=7$). R package “poppr” (Kamvar et al. 2014) was used to compute the genotypic richness (eMLG; Hurlbert 1971) and three measures of genotypic diversity using the non clone-corrected data set. These genotypic estimates computed included the Shannon-Weiner Index (G ; Shannon 2001), Stoddart and Taylor’s Index (GD ; Stoddart and Taylor 1988) and unbiased genotypic diversity (H_{exp}) where genotype frequencies were standardized according to sample size (Nei 1978).

Evidence for random mating for each *C. pirilliformis* population was tested using the R package “poppr” with 999 randomizations (Kamvar et al. 2014). The null hypothesis of no linkage observed amongst alleles at different loci would be indicative of the population having undergone sexual reproduction based on the index of association (I_A) and multilocus linkage disequilibrium ($r-D$).

Population genetic structure

Genetic structure for the studied populations was inferred based on the Bayesian genotype clustering method implemented in STRUCTURE ver.2.3.4 (Pritchard et al. 2000). The optimum K value, referred to as the optimal number of inferred genetic groups, was

determined with the admixture model with correlated allele frequency. The 'K' value was set to test from 1 to 9 possible clusters with thirty iterations, and the program was simulated with 5,000,000 runs with the burn-in period set at 500,000 runs. Results obtained from the STRUCTURE analysis were then subjected to the analysis implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2012) to determine the K value based on the ΔK and the median value of $\ln Pr(K)$. The online software program CLUMPAK (Kopelman et al. 2015) was used to group the estimated membership coefficients for the analysed individuals in each of the clusters.

The spatial genetic structure of all the *C. pirilliformis* isolates was analyzed by the haploid network analysis based on the median joining method with the default option implemented in the software package NETWORK ver.4.6.12 (www.fluxusengineering.com). The output file obtained from the calculation for transversions/transitions weights was then subjected to Post-Processing with MP calculation (Maximum Parsimony option to derive all possible shortest trees) to purge all superfluous links and median vectors, and to identify a network containing the shortest tree.

Analysis of molecular variance (AMOVA) was calculated to test the hypothesis of population differentiation between isolates from different geographic locations. A data set based on the geographic location and one on host, from which the pathogen was initially isolated, was generated and then used to compute AMOVA using R package "poppr" with 999 permutations (Kamvar et al. 2014).

Results

Fungal isolates

A *Ceratocystis* species with pear-shaped ascomatal bases was consistently isolated from freshly made wounds on *A. mearnsii* trees from ST and stumps of *A. mearnsii*, *E. grandis* and *Rapanea* species from GRNP in the Western Cape Province, South Africa (Table 1, 1S). Based on morphology (Barnes et al. 2003b), these isolates were tentatively identified as representing *C. pirilliformis*.

Phylogenetic analyses

Maximum Likelihood (ML) analyses using the combined gene regions based on part of the BT1 and TEF-1 α regions resulted in a well-supported placement of the four isolates selected for confirmation of identity (CMW42018, CMW42020, CMW42236, CMW42042) with authenticated isolates of *C. pirilliformis* (CMW6569, CMW6579, CMW15235, CMW15236) retrieved from GenBank (Fig. 1). These results confirmed the morphological identification of the fungus as *C. pirilliformis*. All sequence data generated for isolates, CMW42042, CMW42336, CMW42018, CMW42020, were submitted to GenBank (KR349571 to KR349578).

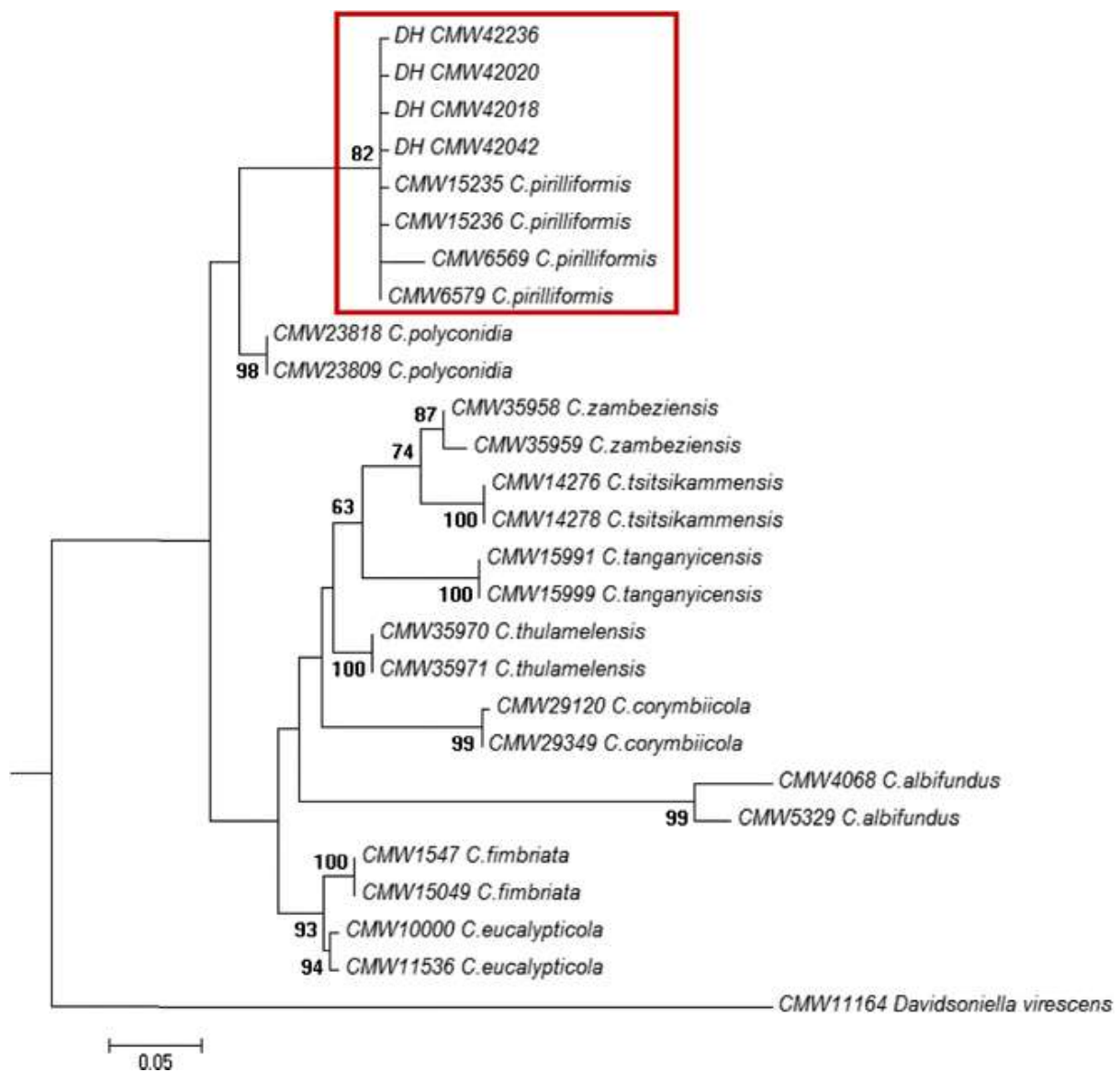


Fig. 1. RAxML phylogeny of 10 *Ceratocystis* species based on combined data sets of the BT1 and TEF-1 α gene regions. Branch support values were indicated above and below the branches, and only bootstrap values above 50% were presented from the analysis.

Pathogenicity tests

All five isolates used in the inoculation trial produced distinct lesions on the stems of *A. mearnsii* trees with blue/black streaking observed in the cambium six-weeks after the inoculations (Fig. 2). This is in contrast to the control inoculations where there were no signs of disease development (Fig. 2). There was no significant difference between lesion lengths for the five inoculated isolates, but these were all significantly different from the controls (P-value = 1.049e-09, at the 95% confidence level) (Fig. 3). Re-isolations from the lesions resulted in *C. pirilliformis* isolates morphologically indistinguishable from those inoculated onto the trees.

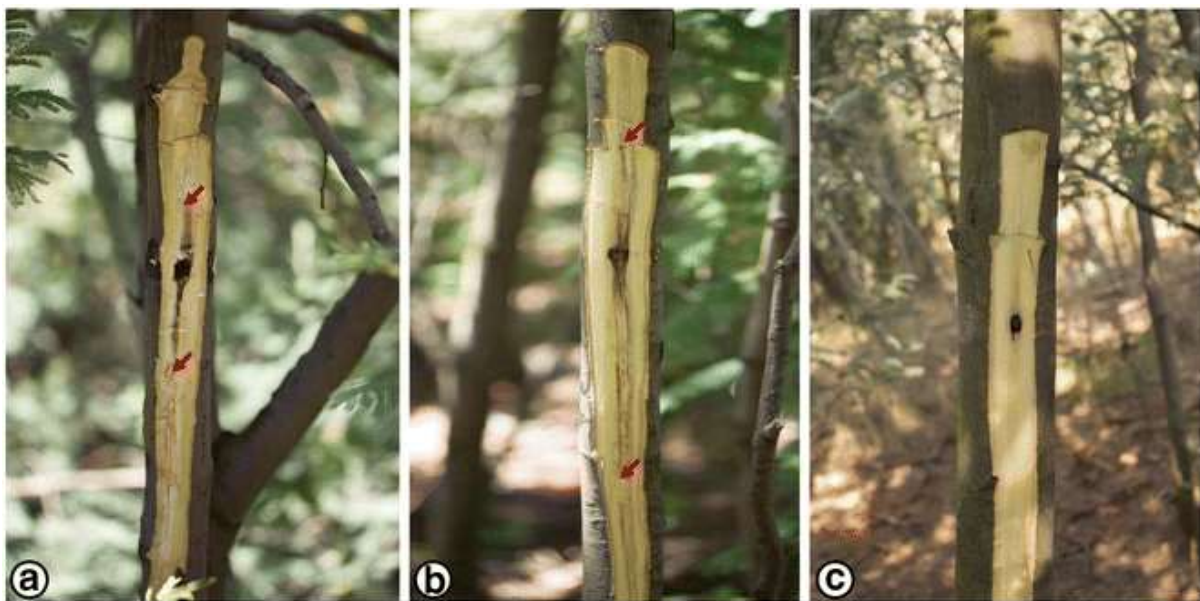


Fig. 2. Disease symptoms on the stems of *Acacia mearnsii* trees resulting from artificial inoculations with *Ceratocystis pirilliformis*. (a): Lesion caused by *C. pirilliformis*, (b): streaked vascular discolouration caused by *C. pirilliformis*, (c): control (sterile malt extract agar). Arrows indicate the limits of the lesions developed after infection by *C. pirilliformis*.

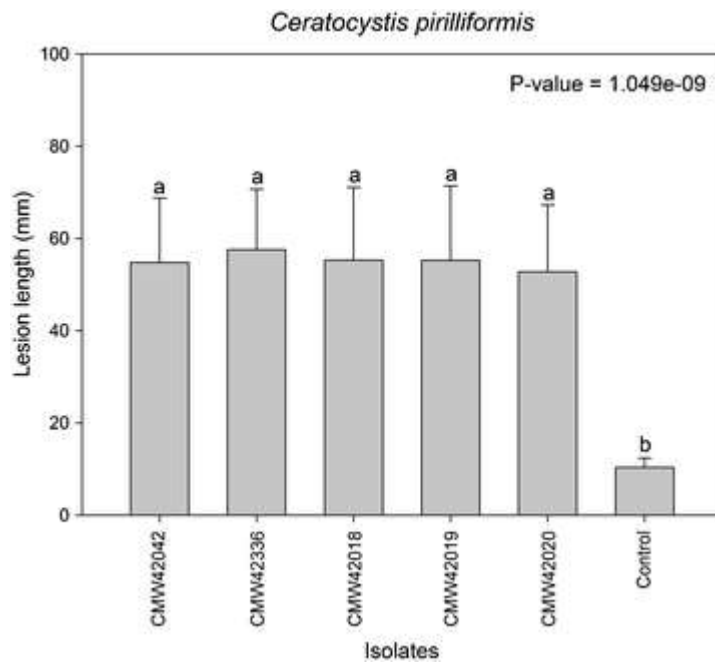


Fig. 3. Mean lesion lengths on *Acacia mearnsii* trees six weeks after inoculation with *Ceratocystis pirilliformis*. The sterile agar was used in control inoculations. Standard error bars annotated with the same letters are not significantly different at $P=1.049e-09$.

Microsatellite amplification and scoring of alleles

Seventeen microsatellite markers were optimized (Table 3S), resulting in two multiplex panels. Of the 17 microsatellite markers, nine markers (CF17/18, CF21/22, CF23/24, CCAA80-F/CCAA81R, GACA6K-1F/GACA6X-2R, AAG8-1F/AAG8-1R, AAG9-1F/AAG9-1R, CCAA9-F/CCAA9-R, CAG900-1F/CAG900-2R) were monomorphic in all the isolates screened. The remaining eight polymorphic markers (AG7/8, CF11/12, CF13/14, CF15/16, CfCAG15F/R, DBVCAT1-1F/WCAT1-1R, CAGDL2-5-1F/R, CCAG15-F/R) produced between 2 and 4 alleles per marker (data not shown).

Population genetic analyses and random mating

Estimated genetic differentiation based on *R*_{st} indicated that there was no population differentiation between isolates collected from Diepwalle and Humansdrop from GRNP (equal to zero) and similarly not between Bushbuckridge, Sabie and Paulpietersburg from ESA (equal to zero) (Table 2). These isolates were, therefore, combined resulting in three larger populations (ST, GRNP and ESA) based on areas from which they had been collected

Table 2. Estimated genetic differentiation (Rst) between *Ceratocystis pirilliformis* populations from different sampling locations (P-value < 0.05).

	Knorhoek (ST)	Diepwalle (GRNP)	Humansdrop (GRNP)	Bushbuckridge (ESA)	Sabie (ESA)	Paulpietersburg (ESA)
Knorhoek (ST)	-					
Diepwalle (GRNP)	0.015	-				
Humansdrop (GRNP)	0.016	0.000	-			
Bushbuckridge (ESA)	0.014	0.000	0.000	-		
Sabie (ESA)	0.013	0.000	0.000	0.000	-	
Paulpietersburg (ESA)	0.013	0.000	0.000	0.000	0.000	-

Table 3. Population diversity indices of *Ceratocystis pirilliformis* populations.

	N ^a	Na ^b	Nef ^c	P ^d	AR ^e	H ^f	G ^g	GD ^h	Hexp ⁱ	eMLG ^j
ST	16	1.765±0.250	1.389±0.165	0.185±0.085	1.187±0.062	0.176±0.058	2.69	14.22	0.992	9.62
GRNP	7	1.353±0.147	1.241±0.100	0.133±0.080	1.151±0.059	0.130±0.051	1.35	3.77	0.857	4.00
ESA	23	1.471±0.286	1.294±0.174	0.237±0.106	1.110±0.061	0.105±0.058	2.61	12.30	0.960	8.36

^aN: Number of haplotypes after the clone-correction^bNa: No. of different alleles^cNef: No. of effective alleles (Nielsen et al. 2003)^dP: Private allele frequency^eAR: Allelic richness^fH: Gene diversity (Nei 1973)^gG: Shannon-Wiener Index of MLG diversity (Shannon 2001)^hGD: Stoddart and Taylor's Index of MLG diversity (Stoddart and Taylor 1988)ⁱHexp: Unbiased genotypic diversity (Nei 1978)^jeMGL: Number of expected MLG at the smallest sample size ≥ 3 based on rarefaction (Hurlbert 1971)

(Table 1).

Gene diversity (H) values for the *C. pirilliformis* populations observed in this study ranged from 0.105 ± 0.058 to 0.176 ± 0.058 (Table 3). The highest level of gene diversity ($H=0.176\pm 0.058$) was obtained for the ST population, followed by GRNP ($H=0.130\pm 0.051$). ESA had the lowest gene diversity ($H=0.105\pm 0.058$). For computed population statistics encompassing the number of different alleles (Na), the number of effective alleles (Nef) and allelic richness (AR), ST showed the highest level of population estimates (Table 3). The highest allelic richness (P), on the other hand, was obtained from ESA ($P=0.237\pm 0.106$) (Table 3). A similar pattern regarding genotypic diversity was observed in which the ST population had the highest ($G=2.69$, $GD=14.22$, $Hexp=0.992$, $eMLG=9.62$), while isolates from the GRNP had the lowest level of genotypic diversity ($G=1.35$, $GD=3.77$, $Hexp=0.857$, $eMLG=4.00$) (Table 3).

The null-hypothesis that alleles observed at different loci are randomly associated and the population is freely undergoing recombination was supported for the populations from ST and ESA with $P=0.32$ and $P=0.76$ respectively (Table 4). Multilocus linkage disequilibrium ($I_A=1.152$, $r-D=0.288$) was, however, observed in the isolates from the GRNP ($P=0.004$) (Table 4).

Table 4. Test for linkage disequilibrium of *Ceratocystis pirilliformis* (P-value < 0.01).

	I_A^a	$r-D^b$	P-value ($r-D$)
ST	0.074	0.011	0.320
GRNP	1.152	0.288	0.004
ESA	-0.050	-0.025	0.760

^a I_A : index of association

^b $r-D$: multilocus linkage disequilibrium (rBarD)

Population Structure

There was congruency from both the *delta K* (ΔK) and the log-likelihood [$\ln Pr(X|X)$], yielding $K=2$ as the possible number of population clusters given the data set included in this study (Fig. 4). In the CLUMPAK analysis, the single mode of clustering was obtained from $K=2$ (Fig. 5). Bayesian clustering results obtained from STRUCTURE indicated that the isolates in this study could be grouped into two major clusters. All the isolates from ST and

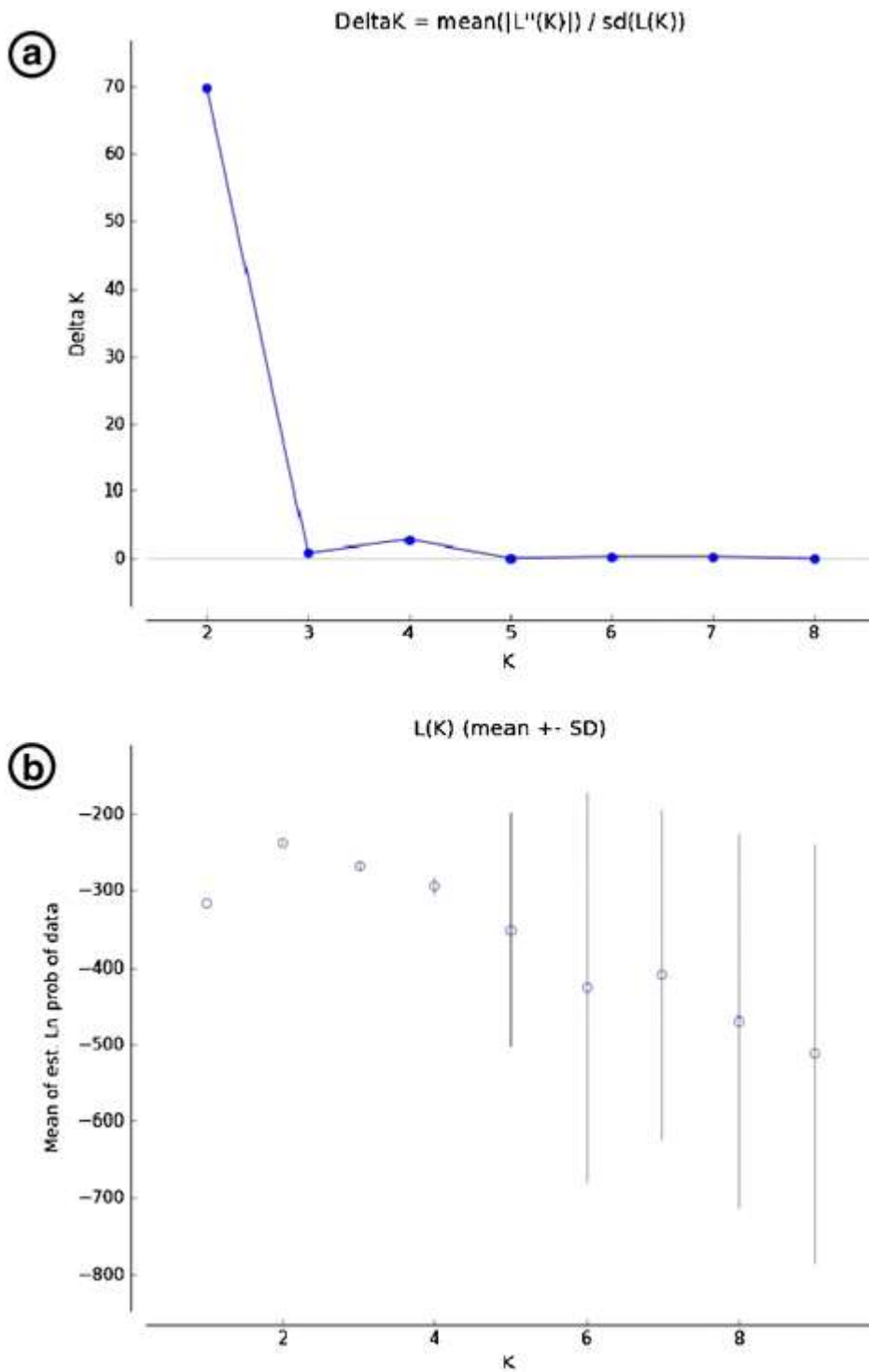


Fig. 4. Schematic representation showing the possible number of clusters based on the estimated probability of data for each K value (parameters used for the analysis: admixture model and allele frequencies correlated with three replicates for each K value), (a): ΔK calculated according to the method based on Evanno et al. (2005), (b): graphical representation plotted based on the estimated median value and variance of probability value for each K value.

GRNP in the Western Cape region clustered into one group, whereas all the isolates from ESA (Bushbuckridge, Sabie and Paulpietersburg) made up the second cluster. These

groupings clearly reflect the geographical proximity of the areas from which the isolates had been collected (Fig. 5).

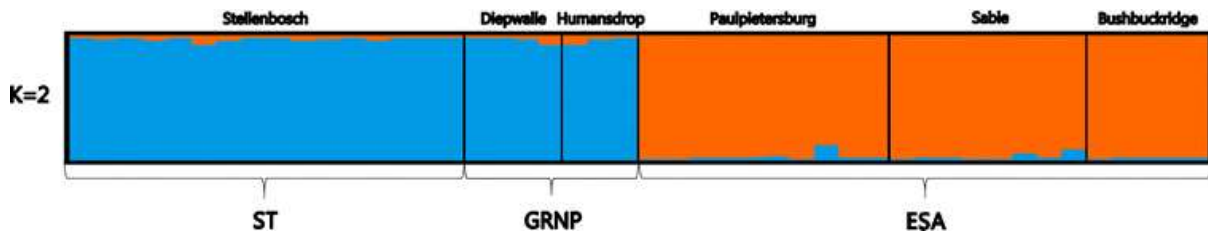


Fig. 5. Population structure of *Ceratocystis pirilliformis* isolates from different collection sites in South Africa. Two genetically distinct homogenous groups were defined based on the analysis implemented in STRUCTURE ($K=2$). Isolates from the Western Cape (ST and GRNP) grouped together, while isolates from MP and KZN (ESA) grouped together.

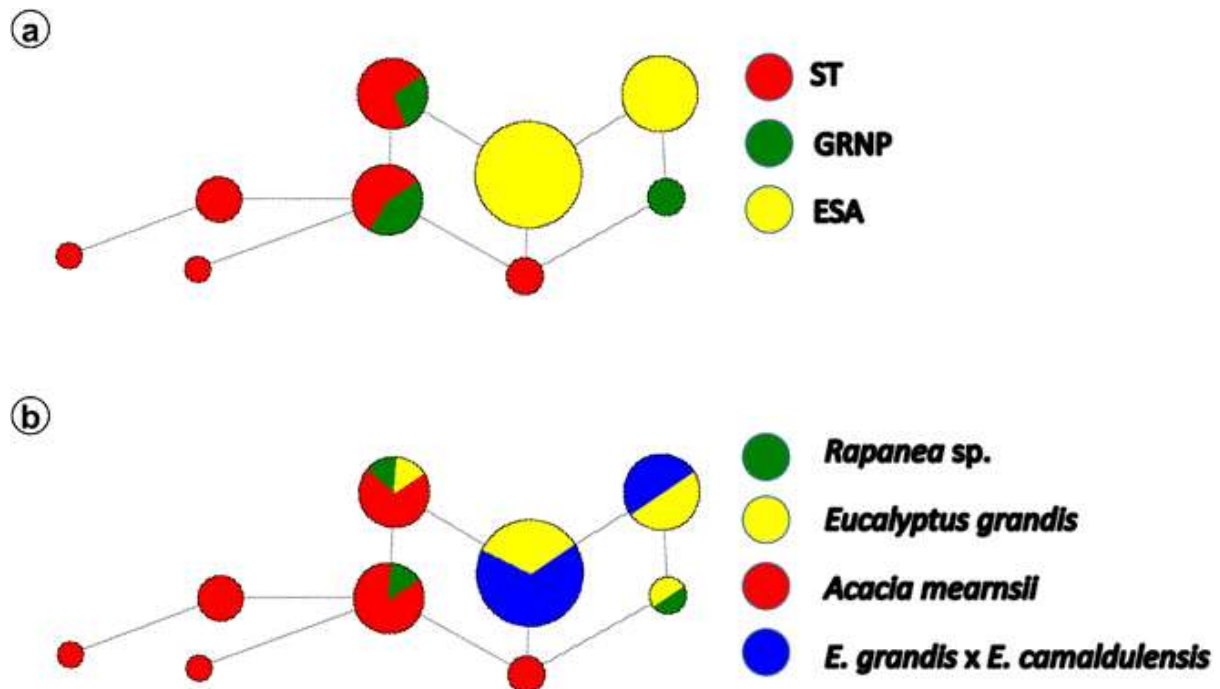


Fig. 6. Haplotype network generated using the median-joining analysis based on geographic location (a) and host (b) implemented in NETWORK ver.4.6.12 for the *Ceratocystis pirilliformis* populations. The size of circle is proportional to the number of the haplotype used. Each colour in the circle represents the different geographical origin and host from which *C. pirilliformis* was initially isolated. The biggest circle consists of 15 closely related Multi-Locus Haplotypes.

The haplotype network generated for the *C. pirilliformis* populations included in this study provided results similar to those obtained from STRUCTURE, reflecting the geographic location of the isolates (Fig. 6). Overall, two events in which closely related haplotypes (nine haplotypes from ST and five haplotypes from GRNP) were shared between ST and GRNP

were observed, showing direct movement of individuals between these two geographic locations. With regards to host, isolates with the same MLH were found on *A. mearnsii*, *E. grandis* and *Rapanea* species, providing added support of movement of pathogen between hosts from GRNP and ESA (Fig. 6).

Hierarchical AMOVA showed that most of the variance was distributed within the population. 65% of the variation observed was attributed to within population variation and only 35% between populations (P-value = 0.001, Table 5).

Table 5. Hierarchical AMOVA test for *Ceratocystis pirilliformis* grouped based on geographical proximity and host range.

	Df*	Variations	Percentage of variation (%)	P-value
Between pops (ST, GRNP, ESA)	5	0.71	35.29	0.001
Within samples	39	1.30	64.71	0.001
Total	44	2.01	100	

*Df: Degrees of freedom

Discussion

New collections of *Ceratocystis pirilliformis* arising from various hosts including a native *Rapanea* species and two non-native hosts (*A. mearnsii* and *E. grandis*) growing in the Western Cape Province enabled us to consider the movement of this pathogen in South Africa. Given the population diversity estimates obtained, the results suggest that the pathogen has been in the Western Cape Province for longer than in the eastern regions of the country. Along with new host records and an expanded geographic distribution for *C. pirilliformis* in the country, we further showed that the isolates from the Western Cape Province (ST) were able to infect *A. mearnsii* trees, resulting in distinct lesions on the stems of the inoculated trees. This suggests that *C. pirilliformis* is a putative fungal pathogen of this non-native Australian *Acacia* species in South Africa.

The fact that the highest level of genetic diversity was observed from the Western Cape population of *C. pirilliformis* raised an interesting view regarding the putative origin of the pathogen in South Africa, subsequent to its first arrival from Australia. Low levels of genetic diversity (Hallatschek and Nelson 2008), loss of alleles (Goodwin et al. 1994) and changes in reproduction modes (Goodwin et al. 1994; Taylor et al. 1999) are some of the factors that can

be applied to determine whether a pathogen has been introduced into a new environment. In this regard, it can be argued that *C. pirilliformis* was most likely introduced to the Western Cape Province, and that it subsequently expanded its geographical range into the eastern part of South Africa given the population diversity estimates (H, Na, Nef and AR) obtained in this study.

The haplotype network analysis and Bayesian clustering results showed that isolates from ST and GRNP where *A. mearnsii* grows as an invasive alien tree, are closely related. These areas are also geographically closest to each other. In addition, the haplotype network analysis generated based on host clearly showed that haplotypes from *A. mearnsii*, *Eucalyptus* species and *Rapanea* species growing in GRNP are closely related to those from ESA. The fact that successive introductions into a new environment could arise from an invasive population that had successfully established in non-native areas is generally referred to as ‘beachhead’ effect (Lombaert et al. 2010). In this sense, some particularly virulent haplotypes of *C. pirilliformis* introduced to ST, possibly from Australia, might have gradually moved to GRNP, eventually leading to the expansion of host and geographical ranges with the successful establishment in ESA. This view can be supported by the fact that haplotypes shared between ST and GRNP were observed, indicating there has been direct movement of this pathogen, between these areas, most likely driven by anthropogenic activities (Santini et al. 2013; Wingfield et al. 2015).

Higher levels of genotypic diversity in comparison to relatively low levels of gene diversity observed between populations is indicative of either multiple introductions or frequent outcrossing events (Bengtsson et al. 2012; Gross et al. 2012). The impact that mode of reproduction has on population diversity is significant. In this regard, measures of genotypic diversity can be meaningful in determining the degree to which sexual reproduction occurs in a given population since the frequency of genotypes in the population is affected by mode of reproduction (Anderson et al. 1992; McDonald 1997). Relatively higher levels of genotypic diversity obtained in ESA and the fact that similar ranges of Na and Nef were observed in relation to GRNP suggest that a significant level of sexual reproduction is occurring, and this was further supported by significant linkage equilibrium based on I_A and $r-D$ obtained from this population.

Results of this study have shown that *C. pirilliformis* is able to infect *A. mearnsii* trees,

resulting in distinct lesions in the stems. The link between emerging infectious disease caused by either adapted or selected fungal genotypes with particular aggressiveness to new hosts and evolutionary mechanisms encompassing host range expansions, admixture effects and fungal introductions has been treated in numerous recent studies (Anderson et al. 2004; Slippers et al. 2005; Desprez-Loustau et al. 2007; Stukenbrock and McDonald 2008; Pariaud et al. 2009; Giraud et al. 2010; Robert et al. 2012; Barnes et al. 2014; Rius and Darling 2014; Wingfield et al. 2010, 2015). Although *C. pirilliformis* has never been found associated with naturally dying trees, it remains possible that it could emerge as a threat to *A. mearnsii* in South Africa in the future.

The significant increase in newly emerging diseases of trees is most likely attributed to large numbers of pests and pathogens being moved globally through agricultural trade and on wood packaging material especially in the forestry environment (Haack 2006; Santini et al. 2013; Wingfield et al. 2013b). The fact that haplotypes of *C. pirilliformis* from GRNP occur in more than one geographic location suggests that anthropogenic activities are closely linked to the movement of the pathogen in South Africa. This is especially when one considers that this pathogen is unlikely to be dispersed so widely by insects over such great distances (Heath et al. 2009; Ferreira et al. 2010). There are numerous examples of *Ceratocystis* species that have been accidentally introduced into new areas where they have caused significant economic losses in yield or threaten natural ecosystems (Panconesi 1999; Santini and Capretti 2000; Engelbrecht et al. 2004; Tarigan et al. 2011; Al Adawi et al. 2014). Thus, the importance of emerging new infectious disease as a consequences of fungal introductions such as the one that has been illustrated in this study should not be under-estimated.

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