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The genetic landscape of *Ceratocystis albifundus* populations in South Africa reveals a recent fungal introduction event

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ABSTRACT

Geographical range expansion or host shifts is amongst the various evolutionary forces that underlie numerous emerging diseases caused by fungal pathogens. In this regard, *Ceratocystis albifundus*, the causal agent of a serious wilt disease of *Acacia mearnsii* trees in Africa, was recently identified killing cultivated *Protea cynaroides* in the Western Cape (WC) Province of South Africa. *Protea cynaroides* is an important native plant in the area and a key component of the Cape Floristic Region. The appearance of this new disease outbreak, together with isolates of *C. albifundus* from natural ecosystems as well as plantations of nonnative trees, provided an opportunity to consider questions relating to the possible origin and movement of the pathogen in South Africa. Ten microsatellite markers were used to determine the genetic diversity, population structure, and possible gene flow in a collection of 193 *C. albifundus* isolates. All populations, other than those from the WC, showed high levels of genetic diversity. An intermediate level of gene flow was found amongst populations of the pathogen. The results suggest that a limited number of individuals have recently been introduced into the WC, resulting in a novel disease problem in the area.

Introduction

Evolutionary mechanisms, such as host jumps or host range expansions, admixture effects, and fungal introductions have been shown to contribute to novel disease outbreaks by invasive alien pests including fungal pathogens (Anderson *et al.* 2004; Slippers *et al.* 2005; Desprez-Loustau *et al.* 2007; Stukenbrock & McDonald 2008; Giraud *et al.* 2010; Wingfield *et al.* 2015). In particular, expanded geographical ranges or fungal introductions into new habitats are two of the components driving the occurrence of novel diseases caused by either an adapted or selected fungal genotype with high levels of aggressiveness to new hosts (Desprez-Loustau *et al.* 2007; Pariaud *et al.* 2009).

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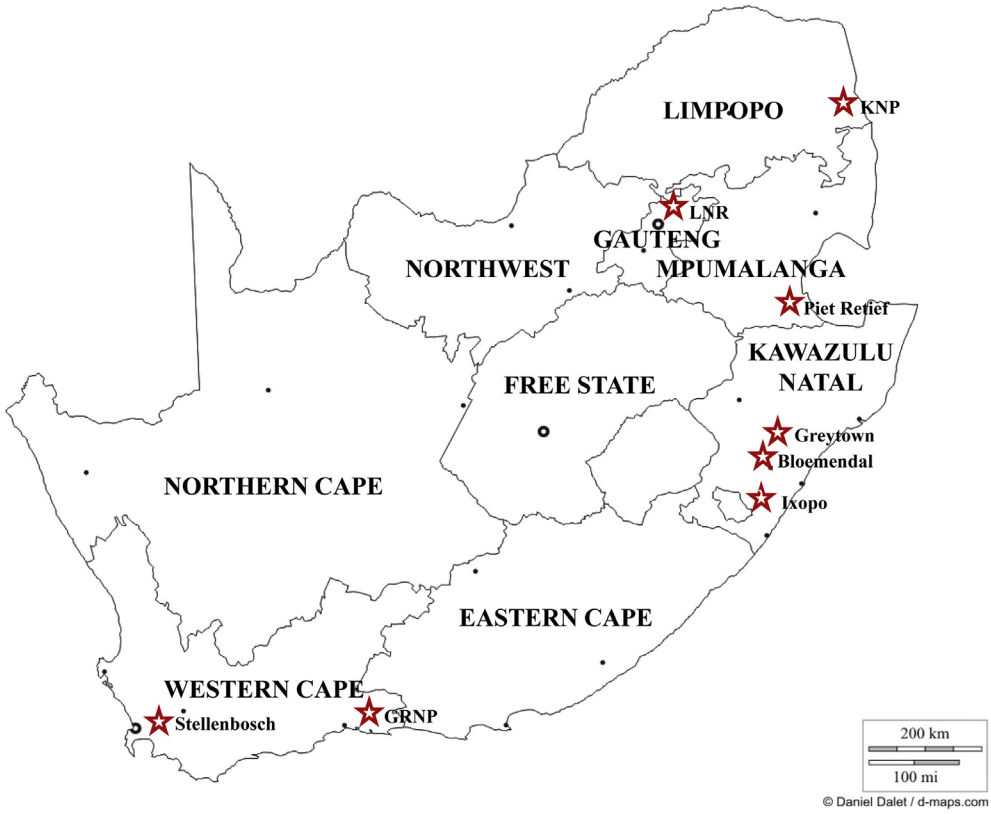

Fig 1 – Map of South Africa showing areas where *Ceratocystis albifundus* isolates were collected.

There are growing numbers of fungal or related pathogens that have been accidentally introduced into new forestry habitats, resulting in the emergence of novel fungal diseases. Some contemporary examples include laurel wilt disease caused by *Raffaelea lauricola* (Harrington *et al.* 2011), sudden oak death caused by *Phytophthora ramorum* (Grünwald *et al.* 2012), needle blight disease of pines caused by *Dothistroma septosporum* (Barnes *et al.* 2014), and wilt and die-back disease of *Acacia mangium* and serious mango decline on *Mangifera indica* caused by *Ceratocystis manginecans* (Tarigan *et al.* 2011; Al Adawi *et al.* 2014).

Species of *Ceratocystis*, as defined by de Beer *et al.* (2014), include many economically important fungal pathogens causing wilt and canker diseases on a wide variety of hosts, leading to substantial economic losses worldwide (Wingfield *et al.* 2013). One such species is *Ceratocystis albifundus* believed to be native to Africa (Roux *et al.* 2001, 2007; Barnes *et al.* 2005). This fungus was described as a novel species in the 1990's after it was identified causing a wilt and canker disease of non-native *Acacia mearnsii* trees and posing a significant threat to the sustainability of *Acacia* propagation in South Africa and neighbouring countries (Wingfield *et al.* 1996; Roux & Wingfield 2009).

Several lines of evidence exist to support the endemism of *C. albifundus* in southern Africa. It was initially isolated from a branch canker of native South African *Protea* spp. in Mpumalanga (Gorter 1977), and numerous other native South African tree genera including *Protea gaguedii* in the eastern part of the country (Roux *et al.* 2007). Generally, infections of these native hosts by *C. albifundus* are not characterized by disease (Roux *et al.* 2007), whereas it is highly virulent on non-native *A. mearnsii* in plantations. Furthermore, population genetic studies showed a relatively high level of genetic diversity of *C. albifundus* populations in southern Africa, supporting the view that it is native to southern Africa (Roux *et al.* 2001; Barnes *et al.* 2005).

In 2008, *C. albifundus* was found for the first time on *Protea cynaroides*, native to southern Africa, and one of the commercially important shrubs cultivated for cut-flowers in the Stellenbosch area of South Africa (Bezuidenhout *et al.* 2011; Crous *et al.* 2013). Further sampling in 2013 revealed severely diseased *P. cynaroides* plants from the same production site on which the disease outbreak on *P. cynaroides* caused by *C. albifundus* had previously been reported. This raised several questions pertaining to the natural distribution of the pathogen in the country because *C. albifundus* had not previously been found in that part of South Africa. This suggested that the pathogen (i) could have been introduced into the Cape Floristic Region (CFR), (ii) has recently undergone a geographic range expansion or host range expansion to *P. cynaroides* in the CFR, or (iii) that selection for the cut-flower industry has resulted in a clone or small number of clones of *P. cynaroides*

highly susceptible to *C. albifundus*. To test the first two possibilities, populations of *C. albifundus* from multiple hosts and regions including natural forests and planted trees, including the outbreak in cultivated *P. cynaroides* in the Western Cape (WC) Province of South Africa were studied using microsatellite markers.

Materials and methods

Fungal isolates

Isolates of *Ceratocystis albifundus* used in this study originated from six geographical regions of South Africa (Fig 1). These included the Kruger National Park (KNP) in the Limpopo Province, Leeuwfontein Nature Reserve (LNR) in the Gauteng Province, the Piet Retief area in the Mpumalanga Province (MP), Greytown, Ixopo, and Bloemendal farms in the Midlands of the KwaZulu-Natal (KZN) Province, Garden Route National Park (GRNP) near Knysna in the WC Province, and the Stellenbosch region of the WC. Two of these situations (KNP and LNR) represented natural woody ecosystems. Others were where trees (native in the case of *Protea cynaroides* or nonnative in the case of *Acacia* species) had been commercially propagated. Isolates from KNP, LNR, MP, and three isolates (CMW4062, CMW4063, CMW4065) from KZN were collected in previous studies and obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1, S1).

Isolations from three different orchards in WC were made from *P. cynaroides* (cultivar 'Madiba'), showing symptoms of canker and wilt and producing sexual structures (Fig 2), typical of *C. albifundus*. Cultures were made by transferring single spore masses directly from the apices of ascomata onto Petri-dishes (65 mm) containing 2 % MEA (20 g malt extract, Biolab, Midrand, South Africa; 20 g agar, Difco Laboratories, Detroit, MI) supplemented with 100 mg l^{-1} Streptomycin sulphate (SIGMA–ALDRICH, Steinheim, Germany), and incubated at 25 °C for 2 weeks in the dark. Samples from GRNP and KZN were collected from stumps of felled trees. Pieces of exposed bark were collected and placed in separate brown paper bags for each tree, and then transported to the laboratory for the further study. All samples were examined for the presence of *Ceratocystis* ascomatal structures. Where no sexual structures were found, either moisture was added to the plastic bags to induce sporulation or pieces of bark were placed between two carrot pieces (Moller & DeVay 1968) as a bait for isolation. Samples were examined daily for the presence of ascomata and isolations were made as these developed.

Identification of fungi

To ensure the correct identity of all the isolates collected in this study, they were subjected to morphological inspection

Table 1 – *Ceratocystis albifundus* isolates used in this study.

Populations	Subpopulations	Origin	Hosts
KNP (14)	KNP, Limpopo	Native hosts in natural habitat	*Terminalia sericea* (8) *Acacia grandicornuta* (2) *Combretum collinum* (1) *Acacia nigrescens* (1) *Dichrostachys cinerea* (1)
		Nonnative host in natural habitat	*Acacia mearnsii* (1)
LNR (30)	LNR, Gauteng	Native hosts in natural habitat	*Faurea saligna* (4) *Ozoroa paniculosa* (4) *Burkea africana* (2) *Combretum molle* (3) *Combretum zeyheri* (1) *Ochna pulchra* (1) *Ozoroa* sp. (1) *Nitidulid* sp. (9) *Carpophilus* sp. (2) *Acacia cafra* (1) *Combretum apiculatum* (1) *Protea gaguedi* (1)
MP (13) KZN (58)	Piet Retief, Mpumalanga Greytown, KZN (5) Ixopo, KZN (18) Bloemendal, KZN (35)	Nonnative host in commercial plantation	*Acacia mearnsii* (71)
GRNP (24)	GRNP, WC	Nonnative host in natural habitat	*Acacia melanoxylon* (3) Unidentified *Nitidulid* sp. (12) *Carpophilus hemipterus* (3) Unidentified *Staphylinid* sp. (6)
WC (54)	Stellenbosch, WC	Native host in commercial plantation	*Protea cynaroides* (54)

(): Number of isolates included in this study.

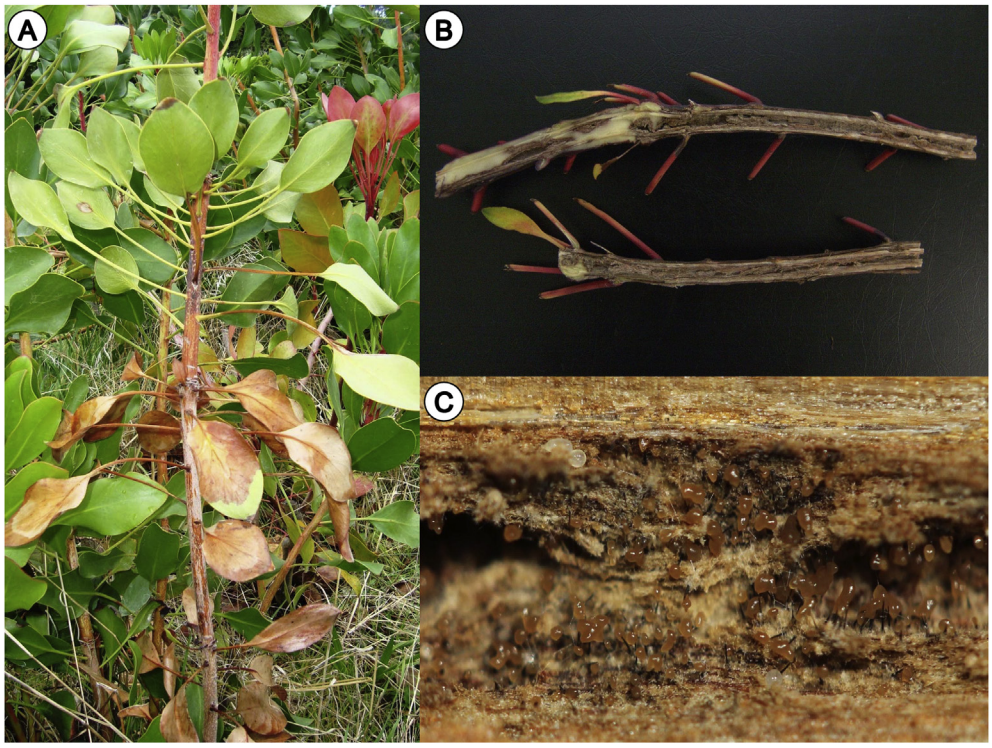

Fig 2 – Disease symptoms on *Protea cynaroides* plants infected with *Ceratocystis albifundus*. (A) Severe canker on a stem and wilted leaves. (B) Longitudinal section through a diseased *P. cynaroides* stem. (C) Sexual structures of *C. albifundus* developed in the pith of *P. cynaroides*.

using the characters described by Wingfield *et al.* (1996). In addition, two isolates from KZN and three isolates from each of the GRNP and WC, having morphological characteristics typical of *Ceratocystis albifundus* were subjected to DNA sequence comparisons based on the internal transcribed spacer regions (ITS1, ITS2) and 5.8S rDNA gene regions as described by Lee *et al.* (2015). Sequencing results were aligned in BIOEDIT ver.7.0.9.0 Sequence Alignment Editor (Hall 1999) and sequences were then used in a BLASTn analysis against the nucleotide database of NCBI [http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi] to confirm the identity of the isolates.

DNA extraction

Single ascospore-derived isolates of *Ceratocystis albifundus* were made on 2 % MEA and then incubated for 2 weeks at 25 °C. Mycelium was scraped from the agar surface of cultures using a sterilized surgical scalpel, and subsequently transferred to 1.5 ml Eppendorf tubes. Two DNA extraction methods were used. For the isolates from WC, MP, and GRNP, the CTAB based-protocol described by Möller *et al.* (1992) was used. Genomic DNA for the remainder of isolates was extracted using PrepMan™ Ultra (Applied Biosystem, Foster City, CA) following the manufacturer's instructions with slight modifications where 5 µl of RNase was added, and subsequently placed at 37 °C for 30 min. All DNA extracts were further purified with a QIAquick PCR Purification Kit (QIAGEN Co.). The concentration of DNA extracted from the samples was verified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA).

PCR for microsatellite analyses

Ten microsatellite primer sets selected from the studies of Steimel *et al.* (2004) and Barnes *et al.* (2005) were used to amplify microsatellite regions for isolates of the *Ceratocystis albifundus* populations studied (Table S2). The PCR analyses were performed in a total volume of 15 µl containing 10 ng μl^{-1} 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 $MgCl_2$ (Bioline, London, UK), 0.09 µl of MyTaq™ DNA polymerase (Bioline, London, UK). PCR reactions were conducted in a Veriti™ 96 well Thermal Cycler (Applied Biosystem, Foster City, CA) following previously described conditions (Steimel *et al.* 2004; Barnes *et al.* 2005) with slight modifications in annealing temperatures (Table S2). The amplification products stained with GelRed™ (Biotium Incorporation, USA) nucleic acid dye were analysed by electrophoresis (Mini-Sub® Cell GT, Bio-Rad, USA) 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 Doc™ EZ Imager, Bio-Rad, Richmond, CA). To confirm the size of each allele, amplicons from each

microsatellite marker were sequenced, and each confirmed product length was treated as a unique allele.

Fragment analysis

Microsatellite amplicons were analysed on an ABI PRISM™ 3500xl POP 7™ Automated DNA sequencer (Applied Biosystem, Foster City, CA). GENESCAN-600 LIZ (Applied Biosystem, Foster City, CA) was used as the internal size standard. The expected fragment lengths and allele sizes were determined using GENEMARKER ver.2.2.0 (Softgenetics, LLC, USA).

Microsatellite analyses

For the analyses, *Ceratocystis albifundus* isolates were grouped into six populations based on the host and geographical location where they had initially been collected. These included isolates from native hosts in natural habitats, resulting in two separate populations (KNP and LNR), those from a nonnative host in commercial plantations where *Acacia mearnsii* is propagated, resulting in two separate populations (MP and KZN), those from a nonnative host in indigenous forest where *Acacia melanoxylon* has been propagated in the GRNP in the WC Province and those from a native host in a commercial nursery where *Protea cynaroides* is cultivated for the cut-flower industry in the WC Province.

Population diversity estimates

A clone-corrected data set, in which genetically identical haplotypes from each of the locations were removed, was generated for each population and used for further analysis. Gene diversity (*H*; Nei 1973), the average number of alleles (Na), and the average number of effective alleles (Nef; Nielsen *et al.* 2003) were calculated in GENALEX ver.6.5 (Peakall & Smouse 2012). Allelic richness (AR) and the average number of private alleles (Npriv) were calculated using ADZE ver.1.0 (Szpiech *et al.* 2008). To account for the difference in population sample size, a rarefaction procedure was used and scaled to the size of the smallest population in this study. Genotypic diversities were computed using the R package 'poppr' using the nonclone-corrected data set (Kamvar *et al.* 2014). Four measures of genotypic diversity, the Shannon–Weiner Index (*G*; Shannon 2001), Stoddart and Taylor's Index (GD; Stoddart & Taylor 1988), unbiased genotypic diversity (Hexp; Nei 1978), and number of expected multilocus genotype (MLG) (eMLG; Hurlbert 1971) where genotype frequencies can be calculated by standardizing to sample size (Nei 1978), were employed using the R package 'poppr' (Kamvar *et al.* 2014).

Genetic structure

POPGENE ver.1.32 (Yeh *et al.* 1999) was used to estimate gene flow, calculated as Nm (gene flow) = 0.5 * (1 − Gst)/Gst (McDonald & McDermott 1993). Fst (Wright 1931) was calculated among all pairs of populations included in this study i) to determine the extent to which each population had differentiated and ii) to ascertain whether or not the defined populations could be combined, especially those from KZN, using Arlequin ver.3.5.2.1 (Excoffier *et al.* 2005; Excoffier & Lischer, 2010). The degree of genetic differentiation between all populations in all possible combinations was determined based on the values ranging from 0 (no differentiation) to 1 (significantly differentiated) between populations.

To determine whether subpopulations were present in the entire data set, the Bayesian genotype clustering method, as implemented in STRUCTURE ver.2.3.4 (Pritchard *et al.* 2000), was used. The admixture model with correlated allele frequency was used to achieve the optimum *K* value, which represents the optimal number of inferred genetic groups. The '*K*' value was set from one to ten with twenty iterations, and the program was simulated with 5 000 000 runs with the burn-in period set at 50 000 runs. The results were then subjected to analysis implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2012). Consequently, the *K* value was determined based on the Δ*K* and the median value of ln*Pr*(*K*) generated from STRUCTURE HARVESTER (Earl & vonHoldt 2012).

To cluster the estimated membership coefficients of the analysed individuals in each population and to generate the schematic representation of the inferred populations, the online software program CLUMPAK (http://clumpak.tau.ac.il/contact.html) was used (Kopelman *et al.* 2015). The spatial genetic structure of *Ceratocystis albifundus* was further analysed by distance-based methods using Principal Coordinates Analysis (PCoA) implemented in GENALEX ver.6.5 (Peakall & Smouse 2012). Nei's unbiased genetic distance (Nei 1978) was calculated among all pairs of isolates included in this study and then visualized by PCoA.

A haplotype network was constructed among clone-corrected *C. albifundus* isolates using the median-joining method with the default option implemented in NETWORK ver.4.6.1.3 (www.fluxusengineering.com). The output file (Network calculation of transversions/transitions weights) was then subjected to Postprocessing with MP calculation (Maximum parsimony option to derive all possible shortest trees) to purge all superfluous links and median vectors and to identify the network containing the shortest tree. Two haplotype networks were generated based on geographical locations and host ranges.

Analysis of molecular variance (AMOVA) was calculated to test the hypothesis of population differentiation between populations as well as between subpopulations within populations. A data set modified to hierarchical levels was prepared, and this was used to compute AMOVA using the R package 'poppr' with 999 permutations (Kamvar *et al.* 2014).

'Isolation by distance' (IBD) (Wright 1943) was tested based on Mantel tests implemented in GENALEX ver.6.5 (Peakall & Smouse 2012). Clone-corrected data sets that were used to calculate the appropriate distance matrix based on both Nei's genetic distance and Nei's unbiased genetic distance in comparison to geographic distance were generated, and then executed with 999 permutations in GENALEX ver.6.5 (Peakall & Smouse 2012).

Results

Fungal isolates and identification

All *Ceratocystis* isolates obtained from diseased *Protea cynaroides* in the WC, stumps of *Acacia melanoxylon* in GRNP, and

those from *Acacia mearnsii* in KZN were identified as *Ceratocystis albifundus*, based on morphology. These identities were then confirmed with DNA sequence comparisons where the maximum percentage identity of the isolates was almost identical (99 %) when analysed using BLAST against those of *C. albifundus* strains in NCBI. A total of 133 isolates were obtained from the WC, GRNP, and KZN. A further 60 isolates were obtained from the CMW culture collection for inclusion in the study (Table 1, S1). All ITS sequence data produced in this study were deposited in NCBI (KR559541–KR559548).

Microsatellite analysis

In total, ten microsatellite markers were optimized, resulting in two multiplex panels. The first panel consisted of amplicons from primer pairs AG7/8, AG15/16, AG17/18, CF21/22, CF23/24, and CCAG15, and the second panel comprising amplicons from primer pairs CF17/18, CCAA10, CCAA80, and CAT12X. All microsatellite markers used in this study were polymorphic. The loci had different levels of polymorphism, ranging from two alleles for CAT12X to 27 alleles for CCAG15 (Table 2). Clone-corrected data were generated for the analysis of diversity, eventually retaining 114 microsatellite haplotypes among the 193 isolates obtained (Table 2).

Population diversity estimates

The levels of *H* in the six *Ceratocystis albifundus* populations ranged from 0.167 ± 0.070 to 0.412 ± 0.083 (Table 3). Higher levels of *H* were found in the populations from native hosts in the indigenous habitats; KNP ($H = 0.396 \pm 0.092$) and LNR ($H = 0.412 \pm 0.083$). Intermediate levels of *H* were found in KZN ($H = 0.352 \pm 0.100$), MP ($H = 0.310 \pm 0.113$), and GRNP ($H = 0.321 \pm 0.079$), comprising isolates from a nonnative host propagated in commercial plantations (*Acacia mearnsii*) and those from a nonnative host in natural habitats (*Acacia melanoxylon*). However, the lowest level of *H* was observed from WC ($H = 0.167 \pm 0.070$), where isolates were all from *Protea cynaroides*, a native plant but intensively propagated clonally in an orchard situation. Higher ranges of diversity for the Nef, AR, and Npriv frequency were observed from KNP, LNR, MP, KZN, and GRNP, compared to those from WC, which were lower at 1.317 ± 0.160 for Nef, 1.364 ± 0.158 for AR, and 0.256 ± 0.197 for Npriv (Table 3). Similar ranges of genotypic diversity were obtained from results for all four tests employed in this study, G, GD, Hexp, and eMLG ranging from 1.23 to 3.62, 2.5–30.3, 0.62–1.00, and 3.68–13.00, respectively. The lowest level of genotypic diversity was observed from WC (1.23 for G, 2.5 for GD, 0.62 for Hexp, and 3.68 for eMLG) (Table 3).

Population genetic structure

The estimated gene flow between all pairs of populations of *Ceratocystis albifundus* defined in this study varied from 0.862 to 6.425. The highest level of gene flow was obtained between MP and GRNP, whilst the lowest value was obtained between Greytown (KZN) and WC (Table 4). Higher levels of gene flow (2.146), between KNP and WC, were observed despite these populations being the most geographically isolated. GRNP and WC, which are the closest to each other geographically,

Table 2 – The number of alleles found at each of ten microsatellite loci for 114 haplotypes of *Ceratocystis albifundus*.

Population	Subpopulations	Microsatellite locus									
		AG7/8	AG15/16	AG17/18	CF21/22	CF23/24	CCAG15	CF17/18	CCAA10	CCAA80	CAT12X
		Allele size range (bp)									
		308–344	271–295	302–311	246–282	155–168	318–529	261–288	134–143	277–315	341–378
KNP (10)	KNP	5	2	1	3	2	6	3	2	4	1
LNR (23)	LNR	2	4	3	3	4	8	6	2	5	2
MP (13)	Piet Retief	8	2	1	2	1	8	2	1	4	1
KZN (40)	Ixopo (15)	5	4	1	3	1	4	3	2	6	1
	Greytown (4)	2	2	1	2	1	2	2	2	1	1
	Bloemendal (21)	6	3	1	3	1	8	3	1	8	1
GRNP (16)	GRNP	4	2	2	3	2	9	3	1	4	2
WC (12)	Stellenbosch	2	2	1	1	1	4	2	1	2	1
Total number of alleles observed		12	6	3	4	4	27	8	3	11	2

(): The number of clone-corrected isolates included for the analysis.

Table 3 – Diversity indices for six populations of *Ceratocystis albifundus* in South Africa.

Population	N	Na	Nef	AR	Npriv	H	G	GD	Hexp	eMLG
KNP (14)	10	2.9 ± 0.526	2.152 ± 0.393	1.953 ± 0.247	0.519 ± 0.219	0.396 ± 0.092	2.04	6.5	0.91	8.57
LNR (30)	23	3.7 ± 0.633	2.093 ± 0.331	1.946 ± 0.208	0.671 ± 0.155	0.412 ± 0.083	3.01	17.3	0.98	11.35
MP (13)	13	3.0 ± 0.882	2.402 ± 0.707	1.812 ± 0.325	0.333 ± 0.229	0.310 ± 0.113	2.56	13.0	1.00	13.00
KZN (58)	40	3.8 ± 0.929	2.030 ± 0.367	1.807 ± 0.243	0.431 ± 0.181	0.352 ± 0.100	3.62	30.3	0.98	11.74
GRNP (24)	16	3.2 ± 0.712	2.014 ± 0.583	1.768 ± 0.228	0.551 ± 0.228	0.321 ± 0.079	2.59	10.7	0.95	10.01
WC (54)	12	1.7 ± 0.300	1.317 ± 0.160	1.364 ± 0.158	0.256 ± 0.197	0.167 ± 0.070	1.23	2.5	0.62	3.68

(): Total number of isolates included in this study.
N: Number of haplotypes after the clone-corrections.
G: Shannon–Wiener Index of MLG diversity (Shannon 2001).
GD: Stoddart and Taylor's Index of MLG diversity (Stoddart & Taylor 1988).
eMGL at the smallest sample size ≥ 2 based on rarefaction (Hurlbert 1971).

Table 4 – Estimated gene flow (below diagonal) and genetic differentiation (Fst) (above diagonal) among all pairs of *Ceratocystis albifundus* populations (P-value < 0.05).

	KNP	LNR	MP	Bloemendal	Ixopo	Greytown	GRNP	WC
KNP	–	0.010	0.011	0.013	0.016	0.013	0.011	0.042
LNR	2.569	–	0.003	0.002	0.005	0.000	0.000	0.029
MP	4.058	2.180	–	0.002	0.005	0.000	0.000	0.030
Bloemendal	2.619	2.474	2.554	–	0.004	0.003	0.002	0.031
Ixopo	3.379	2.158	2.609	6.331	–	0.011	0.005	0.035
Greytown	1.800	1.414	2.228	3.046	3.960	–	0.000	0.036
GRNP	2.641	1.941	6.425	2.267	2.300	1.640	–	0.030
WC	2.146	1.059	1.925	1.144	1.234	0.862	1.633	–
	KNP	LNR	MP	Bloemendal	Ixopo	Greytown	GRNP	WC

had intermediate levels of gene flow (1.633) (Table 4). A similar pattern was observed from the Fst test, showing that WC was most differentiated from all other populations ranging from 0.029 to 0.042. There was little population differentiation (≤0.016) between Bloemendal, Ixopo, and Greytown (KZN), suggesting that these collections could be combined as a single larger population (Table 4).

A set of genetically structured groups was revealed based on Bayesian clustering analysis in STRUCTURE ver.2.3.4 (Figs 3 and 4). Given the data sets included in this study, the optimal value for *K* based on the log-likelihood and ΔK values indicated that the most possible number of clusters is 5 (Fig 3). However, analysed individuals assigned to different genetic groups using CLUMPAK clearly corresponded to geographic locations of the isolates, resulting in the four larger clusters: KNP (green, in the web version), LNR (dark navy, in the web version), KZN (orange, in the web version), and GRNP + WC (blue, in the web version), except for the MP population being highly diverse (Fig 4). The 5th genetic group (figured in purple, in the web version, $K = 5$) is, on the other hand, composed of individuals distributed in several populations (mostly LNR and GNRP). The majority of isolates from Bloemendal, Ixopo, and Greytown (KZN) grouped into one larger cluster. Isolates from GRNP and WC were genetically distinct from all other populations and grouped in another larger cluster (Fig 4).

A distribution pattern similar to that obtained from the STRUCTURE analysis was produced from the PCoA analysis, showing that there was a consistent clustering between the WC and GRNP population. Among the six populations defined in this study, the MP population occurred at more than one location being the most scattered, throughout the South African populations and this was followed by the GRNP population (Fig 5).

The haplotype networks generated based on both the geographical locations (Fig 6) and host ranges (Fig 7) showed the same patterns where the location (KZN) or the host (*Acacia mearnsii*) was shown to represent the most dominant population. Haplotypes from MP were shown to be most scattered throughout the South African populations as seen in the result of the PCoA and STRUCTURE analyses. In addition to the results of the Bayesian clustering analysis and PCoA analysis, haplotypes from the WC were most strongly clustered with those from the GRNP, which is consistent with their geographic proximity and their genetic relatedness (Fig 6). However, there was no consistent grouping in the haplotype network constructed based only on host, except for the haplotypes from the WC, which consistently clustered with those from the GRNP (Fig 7).

An intermediate level of variance was observed between subpopulations of *C. albifundus*, but not between populations based on the hierarchical AMOVA test (Table 5). Thus, the

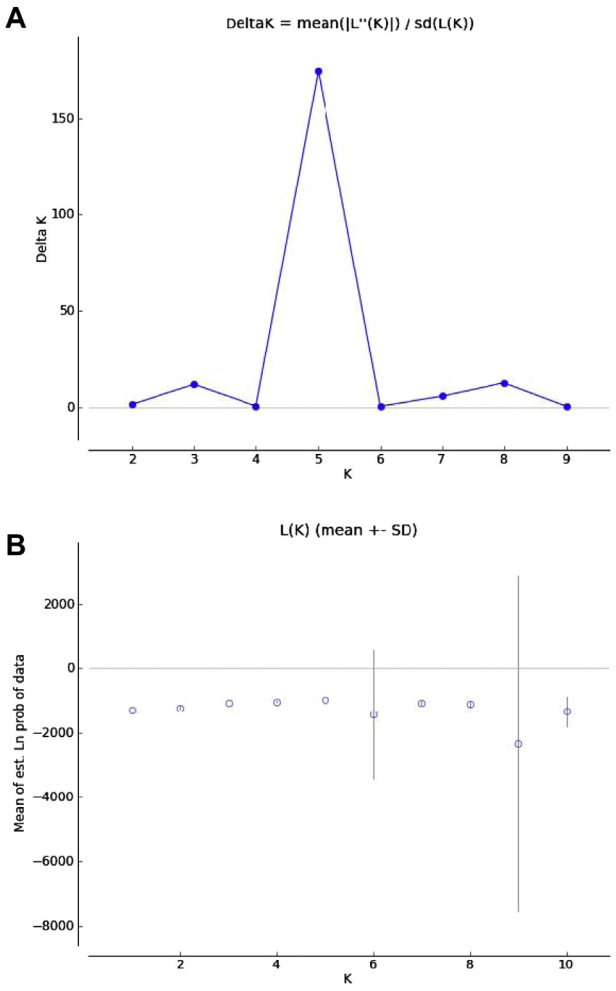

Fig 3 – Schematic representation showing the optimal number of genetic clusters based on the estimated probability of data for each *K* value. (A) Δ*K* calculated according to the method based on Evanno *et al.* (2005). (B) Graphical representation plotted based on the estimated median value and variance of probability value for each *K* value.

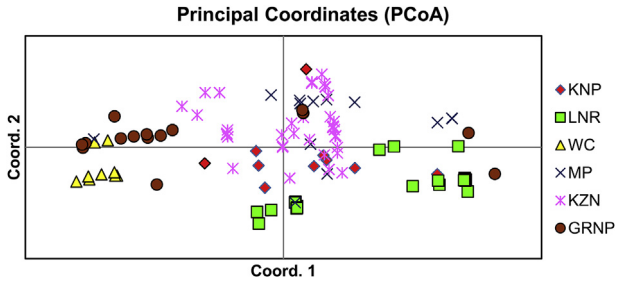

Fig 5 – PCoA among individuals from six *Ceratocystis albifundus* populations in South Africa based on Nei's genetic distance using GENALEX ver.6.5.

null hypothesis that there is no population differentiation could not be rejected (*P*-value < 0.032) (Table 5).

Mantel tests implemented in GENALEX ver.6.5 showed that there was no significant indication of IBD based on results between geographic distance and Nei's unbiased genetic distance ($R^2 = 0.011$, $P = 0.280$). No strong correlation was found when the Mantel test was performed between geographic distance and Nei's genetic distance ($R^2 = 0.018$, $P = 0.180$).

Discussion

It has previously been shown that *Ceratocystis albifundus* is most likely a native African fungus that has undergone a host shift to a nonnative host (Roux *et al.* 2001; Barnes *et al.* 2005). Previous studies, however, evaluated the genetic diversity of populations originating only from *Acacia mearnsii*. In this study, we obtained populations of *C. albifundus* from across South Africa and included not only isolates from artificially cultivated plants, but also those from native trees in native environments. Furthermore, we included a population of *C. albifundus* isolates associated with *Ceratocystis* canker on native *Protea cynaroides* being farmed for cut-flowers in an area where the pathogen was not previously known to occur. This provided the opportunity to study a native fungus on a native host but in a cultivated, monoculture environment.

A key result of this study was the low genetic diversity of isolates of *C. albifundus* associated with the disease outbreak

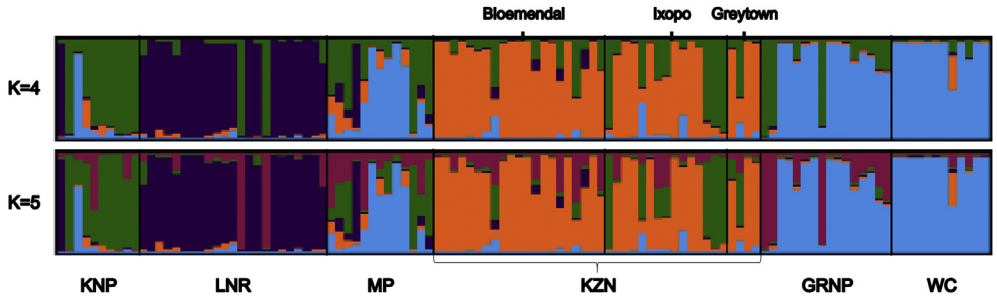

Fig 4 – Cluster analyses of *Ceratocystis albifundus* populations from South Africa inferred using STRUCTURE (*K* = 4 and 5). Each individual is represented by a bar, divided into *K* colours, where *K* is the possible number of clusters.

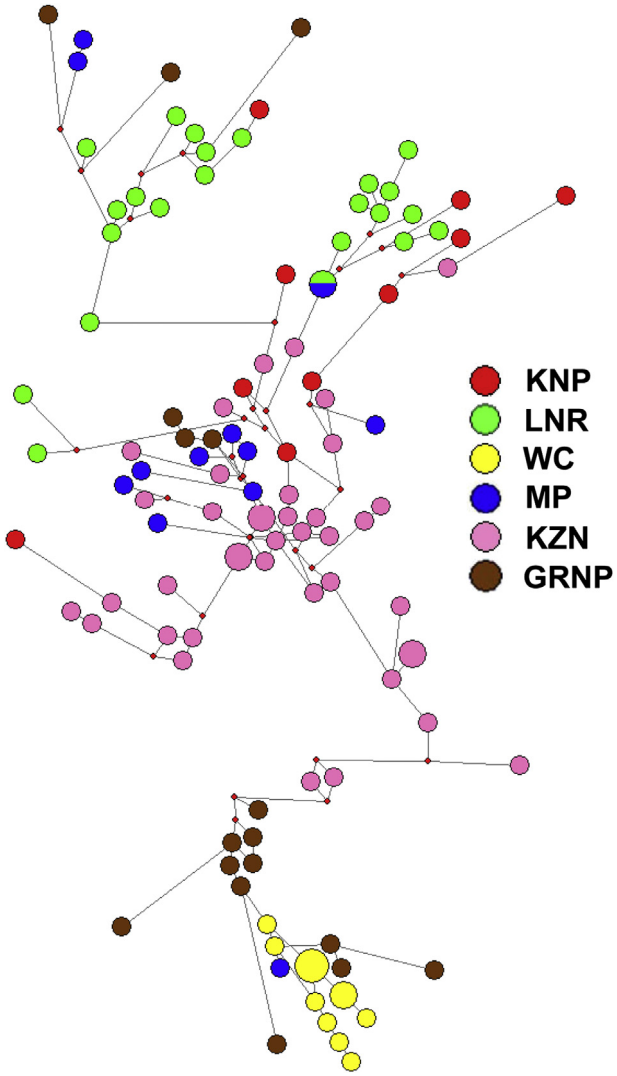

Fig 6 – Haplotype network based on geographical locations with median-joining analysis implemented in NETWORK for the South African population of *Ceratocystis albifundus*. The size of circles and the length of branches are proportional to the frequency of the haplotype found and the number of mutations, respectively. The colours of the circles reflect haplotypes from different geographical origins.

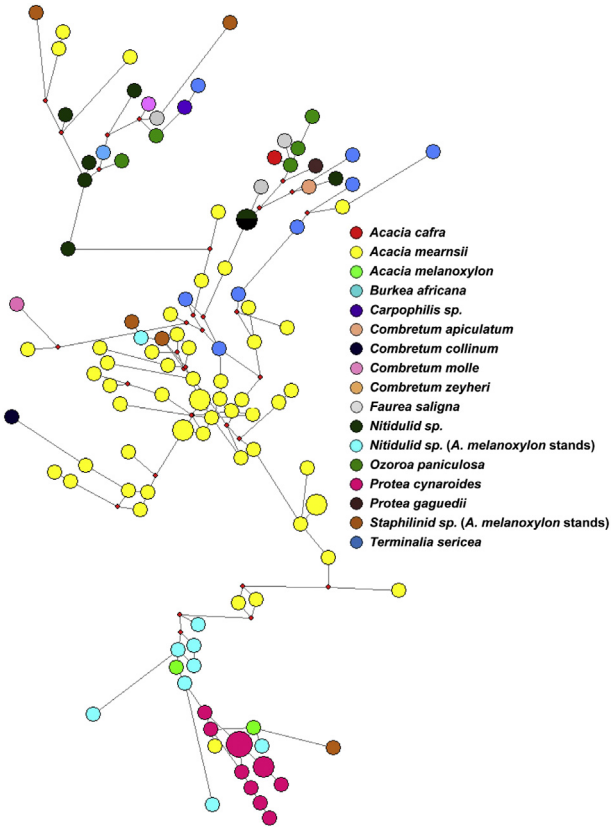

Fig 7 – Haplotype network based on host ranges with median-joining analysis implemented in NETWORK for the South African population of *Ceratocystis albifundus*. The size of circle and the length of branch are proportional to the frequency of the haplotype found and the number of mutations, respectively. Each colour in the circle represents different host.

on *P. cynaroides* in the WC. The pathogen has not been found in the region prior to 2008, and the result was indicative of an introduced pathogen. In this regard, where an organism is introduced into a new area, the population commonly experiences a genetic bottleneck, leading to a reduction in genetic diversity (Hallatschek & Nelson 2008), loss of alleles (Goodwin *et al.* 1994), and a possible change in modes of reproduction (Goodwin *et al.* 1994; Taylor *et al.* 1999). There are also several examples where a markedly reduced genetic diversity in introduced fungi or oomycetes has been found in comparison to those of the source populations (Goodwin *et al.* 1994; Milgroom *et al.* 1996; Engelbrecht *et al.* 2004; Al Adawi *et al.* 2013).

The estimated gene flow and values of Fst indicated that *C. albifundus* populations considered in this study were not highly differentiated. This was further supported based on the AMOVA, suggesting that most of the genetic variation could be attributed to variation from samples within populations but not between populations. Given the limited ability

Table 5 – Hierarchical AMOVA test for eight populations of *Ceratocystis albifundus* defined in this study.

	Df	Variations (sigma)	Percentage of variation (%)	P-value
Between populations	4	0.345	13.983	0.032
Between subpopulations within populations	3	0.334	13.558	0.001
Within samples	102	1.787	72.459	0.001
Total	109	2.467	100	

Df: Degrees of freedom.

of *Ceratocystis* species to disperse by insects over large distances (Ferreira *et al.* 2010), the spread of the pathogen could be more closely related to human-mediated movement than natural spread. In this regard, the contemporary population structure of *C. albifundus* seems to be continuous as demonstrated by the Mantel tests. This, along with the fact that haplotypes from MP and GRNP occurred at more than one location across the country, clearly show that there is a high level of movement of the pathogen, apparently driven by anthropogenic activities.

The WC and GRNP populations consistently clustered together based on the Bayesian clustering, PCoA, and haplotype network analyses. These areas are geographically closer to each other than those from which any other populations were collected. They also occur in areas more similar to each other climatically than those from the eastern part of the country, which were all from savannah environments. The distinct clustering patterns and lower levels of genetic diversity observed in the *C. albifundus* population on *P. cynaroides* in WC provide strong support for a recent introduction, possibly of a particularly virulent genotype of the pathogen. This could have been introduced into GRNP and have subsequently expanded its geographical range into WC. Since *C. albifundus* on *Protea* sp. was first found in MP (Gorter 1977), and this study showed that a MP haplotype also clustered with WC and GRNP haplotypes, it is probable that it could have been distributed from MP to WC *via* GRNP. Even though *Ceratocystis* canker of *P. cynaroides* have been reported in South Africa, this is the first report of the large scale occurrence on cultivated *P. cynaroides* cv. Madiba plants that were multiplied by vegetative propagation. This could easily have led to a genetically uniform planting stock highly susceptible to *C. albifundus*. This would be consistent with the fact that our field surveys have failed to provide evidence of *P. cynaroides* having been killed by *C. albifundus* in natural ecosystems (M.J.W. & J.R., pers. comm.).

The fact that the new disease event in the WC is associated with a native plant is not surprising. These shrubs are being propagated intensively in orchards and thus ecologically similar to other situations where the pathogen is causing disease problems. The results are also consistent with increasing numbers of studies where it has been shown that geographical expansions or introductions of pathogens into new areas underpin the emergence of new plant diseases (Anderson *et al.* 2004; Slippers *et al.* 2005; Desprez-Loustau *et al.* 2007; Stukenbrock & McDonald 2008; Giraud *et al.* 2010; Wingfield *et al.* 2015). In this regard, *C. albifundus* is an aggressive fungal pathogen, and we might expect it to be associated with new disease outbreaks on crop plants both in Africa and perhaps elsewhere in the world in the future.

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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.2016.03.001.

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