Non-Mendelian segregation influences the infection biology and genetic structure of the African tree pathogen *Ceratocystis albifundus*

Running title: selfing versus outcrossing

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Abstract

The African fungal tree pathogen, *Ceratocystis albifundus*, undergoes uni-directional mating type switching, giving rise to either self-fertile or self-sterile progeny. Self-sterile isolates lack the *MAT1-2-1* gene and have reduced fitness such as slower growth and reduced pathogenicity, relative to self-fertile isolates. While it has been hypothesized that there is a 1:1 ratio of self-fertile to self-sterile ascospore progeny in relatives of *C. albifundus*, some studies have reported a significant bias in this ratio. This could be due to the fact that either fewer self-sterile ascospores are produced or that self-sterile ascospores have low viability. We quantified the percentage of self-sterile and self-fertile ascospores from ascospore masses in *C. albifundus* using real-time PCR. Primers were designed to distinguish between spores that contained the *MAT1-2-1* gene and those where this gene had been deleted. A significant bias towards the self-fertile mating type was observed in all single ascospore masses taken from sexual structures produced in haploid-selfed cultures. The same result was observed from a disease outbreak situation in an intensively managed field of cultivated native trees, and this was coupled with very low population diversity in the pathogen. This was in contrast to the results obtained from ascospore masses taken from the crosses performed under laboratory conditions or ascomata on native trees in a non-disease situation, where either self-fertile or self-sterile ascospores were dominant. The results suggest that reproductive strategies play a significant role in the infection biology and genetic structure of *C. albifundus* populations.

Keywords: Ceratocystidaceae, mating type, microsatellite, outcrossing, population diversity, selfing

1. Introduction

Filamentous Ascomycete fungi encompass a diverse range of reproduction strategies (Billiard et al. 2011; Heitman et al. 2013; Taylor et al. 1999), which contribute to their successful colonization of highly diverse ecological environments (McDonald & McDermott 1993). Mating in ascomycetes is determined by two allelic or idiomorphic forms, *MAT1-1* and *MAT1-2*, which represent complementary alleles that differ in length, sequences and structure (Coppin et al. 1997; Turgeon et al. 1993). *Ceratocystis albifundus* has all three *MAT* genes (*MAT1-1-1*, *MAT1-1-2* and *MAT1-2-1*) and can produce sexual offspring without requiring a partner of opposite mating type, via selfing events. Furthermore, unidirectional mating type switching can occur during meiosis, giving rise to deletion of the *MAT1-2-1* gene from the genome. This generates self-sterile mating type strains carrying only *MAT1-1-1* and *MAT1-1-2* genes. These isolates are thus unable to revert back to their self-fertile status (Harrington & McNew 1997; Perkins 1987; Wilken et al. 2014; Witthuhn et al. 2000).

Ceratocystis species, as defined by de Beer et al. (2014), include important pathogens of angiosperm trees (Roux & Wingfield, 2013; Seifert et al. 2013). Of these, *Ceratocystis albifundus* causes a wilt disease of black wattle (*Acacia mearnsii*) in South Africa and other African countries, resulting in significant economic losses (Roux et al. 2001, 2005; Wingfield et al. 2011). The pathogen has been known in South Africa for more than two decades (Morris et al. 1993) and was first reported associated with tree mortality arising from wounds made during wood gathering. Various studies have shown that *C. albifundus* is native to Africa (Barnes et al. 2005; Lee et al. 2016; Roux et al. 2001, 2007). It has recently also been reported to cause a serious wilt disease of native *Protea cynaroides* where these plants are cultivated for the cut-flower industry (Lee et al. 2016).

The reproductive modes on which fungal pathogens rely have a profound impact on the genetic structure of populations (McDonald & McDermott 1993). Sexual reproduction promotes recombination or gene exchange (Billiard et al. 2011; Lin & Heitman 2007) that can result in more effective adaptation to rapidly changing environments. This occurs by facilitating the formation and spread of new genetic combinations in a given population (Gandon et al. 1996; McDonald & Linde 2002). In contrast, asexual reproduction (selfing or clonality) ensures multiplication and transmission of advantageous allelic combinations that

are locally adapted without the re-assortment of these alleles or the introduction of new alleles (Giraud et al. 2008. 2010).

A question among the various long-standing conundrums concerning the fungal mating system is why sexual reproduction has been retained. This is given the fact that it occurs at substantial cost e.g., the time investment needed to locate a mating partner (Heitman et al. 2013) and the energy invested to produce sexual structures (Elliott 1994). In this regard, fungi with bipolar mating systems, as seen in the self-sterile form of *C. albifundus*, can be disadvantaged because sexual reproduction occurs only when strains having different mating type alleles are present. The most plausible explanation for the retention of the self-sterile form of *C. albifundus* would be to fully exploit the benefits of sexual recombination. These would include the production of novel recombinant genotypes that could facilitate a successful occupation of new habitats (Heitman 2010; Lee et al. 2015; McGuire et al. 2004; Wilken et al. 2014). Thus, while sexual reproduction has a greater cost than asexual reproduction, it potentially provides a selective advantage to these fungi.

Isolates of *C. albifundus* that are self-fertile exhibit superior fitness-related traits (Lee et al. 2015). For example, they grow more rapidly in culture and are more aggressive than self-sterile isolates in pathogenicity tests (Lee et al. 2015). A significant bias towards self-fertile isolates has also been observed among single ascospore cultures derived from ascospore masses taken from ascomata produced in culture (Lee et al. 2015). There are two possible reasons why this situation might arise. Either fewer self-sterile mating type strain ascospores originate in an ascus or, alternatively, ascospores that would give rise to self-sterile strains have lower germination rates than those that give rise to self-fertile strains (Lee et al. 2015).

In some relatives of *Ceratocystis* and members of the Ceratocystidaceae, such as species of *Endoconidiophora*, specifically *Endoconidiophora coerulescens* (de Beer et al. 2014), the segregation ratio between self-fertile and self-sterile strains isolated from ascomata was observed to be approximately 1:1 (Harrington & McNew 1997). However, this was not the case in *C. albifundus* (Lee et al. 2015), where unequal ratios of self-sterile to self-fertile isolates were found. This is similar to the situation observed in heterothallic fungi where the mating type ratio is predominantly biased towards one of the mating types (Kwon-Chung et al. 1974, 1978; Wickes et al. 1996). This distorted mating type ratio can be correlated with increased virulence (Alvarez-Perez et al. 2010; Kwon-Chung et al. 1974, 1978; Wickes et al.

1996). This is also the case in *C. albifundus* where mating type ratios were shown to be skewed strongly towards the self-fertile mating type, which had higher levels of aggressiveness (Lee et al. 2015).

The aim of this study was to compare mating type ratios in ascospore masses taken from ascomata of *C. albifundus*. This would make it possible to determine whether fewer self-sterile mating type spores were produced. We also considered whether the germination rate of the self-sterile spores might be lower than those of the self-fertile spores, eventually resulting in the observed unequal ratio. To determine the ratios of self-fertile to self-sterile ascospores, quantification was made using real-time PCR with primers designed to take advantage of the fact that the self-sterile spores are devoid of the *MAT1-2-1* gene.

2. Materials and methods

2.1. Isolates and identification of self-fertile strains

Eleven cultures of *C. albifundus* were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). Nine of these isolates were used to produce self-fertile and self-sterile mating type isolates, and the remaining two were used for crosses under laboratory conditions (Table 1, 2). Single ascospore cultures were made to produce self-fertile sand self-sterile mating type isolates as described by Lee et al. (2015). These strains were recognised as self-sterile or self-fertile based on the presence of ascomata. In cases where sexual structures were not present, mating type was confirmed using specific primer sets designed to amplify each of the mating types (Lee et al. 2015).

Table 1. Isolates of *Ceratocystis albifundus* used in this study.

Origin	Host	Purpose of use
Tanzania	Unknown native species (CMW40156)	Quantification for mating type ratio *in vitro*
Mpumalanga	*Acacia mearnsii* (CMW38512, CMW38514, CMW39133)	
Goudveld, GRNP[a]	*Acacia melanoxylon* (CMW42100, CMW42102, CMW42104)	
Stellenbosch, WC[b]	*Protea cynaroides* (CMW40624–5)	
KNP[c]	*Terminalia sericea* (CMW48857)	
	Balanites maughamii (CMW48858)	
Pretoriuskop, KNP	*Terminalia sericea* (CMW41508–12, CMW41531–5, CMW41546–50)	Quantification for mating type ratio *in vivo*
Lower Sabie, KNP		
Tshokwane, KNP		
Stellenbosch, WC	*Protea cynaroides* (CMW42408–22)	

*(): the number of isolates included in this study

[a]GRNP: Garden Route National Park

[b]WC: Western Cape Province of South Africa

[c]KNP: Kruger National Park

2.2. Fungal isolates collected from the field and identification of isolates

In addition to the ascospore masses produced on ascomata originating from single ascospores in laboratory cultures (Table 1, 2), ascospore masses were obtained from ascomata collected from naturally inflicted wounds on trees in 2013 and 2014 (Table 1, 3). These were obtained from native trees in the Kruger National Park (KNP) in the Mpumalanga Province, and from a disease outbreak on cultivated *Protea cynaroides* in Stellenbosch, Western Cape Province (WC) of South Africa. To ensure their correct identity, cultures were initially recognized as those of *C. albifundus* based on their light coloured ascomatal bases (Wingfield et al. 1996). Randomly selected isolates were then subjected to DNA sequence analysis following the methods described by Lee et al. (2015). Sequencing results were aligned in Bioedit

ver.7.0.9.0 Sequence Alignment Editor (Hall 1999) and were used in a BLASTn analysis against the nucleotide database of NCBI (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi). All the cultures used in this study were deposited in the Culture Collection (CMW) of FABI, University of Pretoria, South Africa (Table 1, 3).

2.3. Crossing experiments

To determine whether outcrossing events give rise to greater numbers of self-sterile mating type spores in a single spore mass, crosses were made using two different individuals (CMW48857 and CMW48858) of *C. albifundus*, isolated respectively from *Terminalia sericea* and *Balanites maughamii* in the KNP where outcrossing events frequently occur. For these crosses, two different cultures, representing recipient and spermatizing cultures, were prepared. Recipent cultures were produced by making single hyphal tip cultures of isolate CMW48858. These were subsequently sub-cultured until ascomata ceased to form. The presence of the *MAT1-2-1* gene in this strain was confirmed using specific primer sets designed to amplify the *MAT1-2-1* gene (Lee et al. 2015). Isolate CMW48857 was used as the spermatizing culture. The spermatizing culture was flooded with sterilized water, and the surfaces were then gently scrapped using an aseptic needle to loosen conidia into the suspension. This spore suspension was poured onto plates on which the recipient culture had been grown and gently swirled to facilitate inoculum dispersal. Cultures were incubated at room temperature and inspected periodically for the production of ascomata and ascospores.

2.4. DNA Extraction

DNA was extracted from single ascospore masses taken from the apices of *C. albifundus* ascomata using 10% Chelex 100 (Bio Rad Laboratories, Hercules, California, USA) as described by Walsh et al. (1991), with slight modifications, and subsequently subjected to quantification using real-time PCR. Because ascospore aggregate tightly in spore drops (Whitney & Blauel 1972), they were first dispersed in 30 µl of the isoparaffin solvent, Soltrol® 130 (Chemfit, Gauteng, South Africa). This solution was then aliquoted into a 10% Chelex solution.

Table 2. Results of real-time PCR-based quantification for lab isolates. The number of copies for each of the mating types were quantified from a single ascospore drop developed at the apices of the ascocarps of *Ceratocystis albifundus*.

Isolate (Origin)	Number of copies each of the mating types observed		Mating type ratio		X^2 test
	SF[a]	SS[b]	SF	SS	
CMW40156 (Tanzania)	117949	8581	13.7	1	9.45E+04
	68563	2142	32	1	6.24E+04
CMW38512 (Mpumalanga)	94701	6746	14	1	7.63E+04
	36834	4149	8.9	1	2.61E+04
	8343	783	10.7	1	6.26E+03
CMW38514 (Mpumalanga)	12318	132	93.5	1	1.19E+04
	8012	483	16.6	1	6.67E+03
	11121	295	37.6	1	1.03E+04
CMW39133 (Mpumalanga)	8637	193	44.8	1	8.07E+03
	25768	2280	11	1	1.97E+04
	8908	453	19.7	1	7.64E+03
CMW42100 (GRNP[c])	7715	134	57.5	1	7.32E+03
	4124	392	10.5	1	3.08E+03
	8028	86	93.3	1	7.77E+03
CMW42104 (GRNP)	3975	142	28	1	3.57E+03
	1208	14	87.5	1	1.17E+03
	5542	347	16	1	4.58E+03
CMW42102 (GRNP)	5962	904	6.6	1	3.73E+03
	6447	1391	4.6	1	3.26E+03
	13556	121	112.2	1	1.32E+04
CMW40624 (WC[d])	3704	64	58.1	1	3.52E+03
	8524	361	23.6	1	7.50E+03
	15145	1880	8.1	1	1.03E+04
CMW40625 (WC)	4202	1590	2.6	1	1.18E+03
	2503	1034	2.4	1	6.10E+02
	12835	1929	6.7	1	8.06E+03
CMW48857 X 48858 (KNP[e])	19673	2425	8.1	1	1.35E+04
	37	60	1	1.6	5.45E+00
	31	421	1	13.5	3.37E+16
	45	145	1	3.2	5.26E+01

*0.05 probability level (3.841), df = 1 ; [a]SF: Self-fertile ascospore in *C. albifundus* ascomata; [b]SS: Self-sterile ascospore in *C. albifundus* ascomata; [c]GRNP: Garden Route National Park; [d]WC: Western Cape Province of South Africa; [e]KNP: Kruger National Park

Table 3. The results of real-time PCR-based quantification for field isolates of *Ceratocystis albifundus* obtained from diseased *Protea cynaroides* in the Western Cape Province and the native tree, *Terminalia sericea* from the Kruger National Park in the absence of diseases.

Host (Origin)	Identity of spore used for quantifications	Mating type		Mating type ratio		X^2 test	Microsatellite profile		
		SF(MAT2)	SS(MAT1)	SF(MAT2)	SS(MAT1)		AG7/8	AG15/16	AG17/18
Protea cynaroides (Stellenbosch, the Western Cape Province, South Africa)	P1	4793	652	7.4	1	3.15E+03	-[a]	-	308
	P2	10346	861	12	1	8.03E+03	322	286	308
	P3[b]	10958	156	70.2	1	1.05E+04			
	P4	4456	1326	3.4	1	1.69E+03	322	-	308
	P5	2102	332	6.3	1	1.29E+03	322	286	308
	P6	2025	666	3	1	6.86E+02			
	P7	12469	882	14.1	1	1.01E+04	322	286	308
	P8	18089	695	26	1	1.61E+04	322	271	308
	P9	5122	3663	1.4	1	2.42E+02			
	P10	13109	159	82.4	1	1.26E+04	322	-	308
	P11	3309	3011	1.1	1	1.41E+01	322	-	308
	P12	6370	1423	4.5	1	3.14E+03			
	P13	6212	695	8.9	1	4.41E+03	322	-	308
	P14	7066	923	7.7	1	4.72E+03	322	-	308
	P15	5691	2337	2.4	1	1.40E+03			
	P16	8307	3778	2.2	1	1.70E+03	-	286	308
	P17	1496	2916	1	1.9	4.57E+02	322	-	296/308
	P18	7559	2227	3.4	1	2.91E+03			
	P19	5094	616	8.3	1	3.51E+03			
	P20	16772	2002	8.4	1	1.16E+04			
Terminalia sericea, (Kruger National Park, South Africa)	KNP1	18	341	1	18.9	2.91E+02	-	-	305/308
	KNP2[c]	301	259	1.2	1	3.15E+00	-	-	308
	KNP3	175	766	1	4.4	3.71E+02	322	-	308
	KNP6	1037	183	5.7	1	5.98E+02	-	271	-
	KNP7	295	1643	1	5.6	9.38E+02	-	-	308
	KNP8	2198	432	5.1	1	1.19E+03	321	286	308

	KNP11	543	243	2.2	1	1.15E+02			
	KNP12	843	6142	1	7.3	4.02E+03	-	271/286	-
	KNP13	609	1299	1	2.1	2.50E+02	305	-	305/308
	KNP21	20	751	1	37.6	6.93E+02			
	KNP26	1653	537	3.1	1	5.69E+02	305	-	305
	KNP61	217	858	1	4	3.82E+02			
	KNP71	3904	1357	2.9	1	1.23E+03	321	-	308
	KNP121	144	1145	1	8	7.77E+02	-	271/286	305
	KNP131	1666	984	2	1	1.76E+02			

*0.05 probability level (3.841), df = 1

[a]'-': locus that was not amplified

[b]The letters in the red colour indicated ones that were not selected for the microsatellite analysis

[c]KNP2: there is no significant difference in mating type segregation between self-fertile and self-sterile of *C. albifundus*

*Quantifications that are highlighted with grey in colour indicates that there is a significant bias observed towards self-sterile mating types in mating type segregation between self-fertile and self-sterile of *C. albifundus*

For DNA extractions, thirty ascospore masses were used, of which twenty-seven were from nine isolates (three masses from each) originating from single spore isolations induced under laboratory conditions, and three were randomly selected ascopore masses generated from a cross between two different isolates of *C. albifundus* (CMW48857 and CMW48858) (Table 2). In addition, thirty-five ascospore masses generated under field conditions (twenty from WC and fifteen from KNP) were detached from the apices of the ascomata and subsequently subjected to DNA extraction (Table 3).

2.5. Quantitative real-time PCR assay

2.5.1. Primer design

In order to obtain markers specific for each mating type locus in *C. albifundus,* two sets of primers were designed using the CLC Main Workbench ver.6.6.2 software package (CLC bio, Aarhus, Denmark). Primer set Albi_MAT1-1-2F and Albi_MAT1-1-2R was designed to amplify the *MAT1-1-2* locus, thus producing amplicons from both self-fertile and self-sterile mating type strains (Table S1, Fig 1). Primer set Albi_MAT1-2-1F and Albi_MAT1-2-1R was designed to produce amplicons only from switched strains (self-sterile mating type) (Table S1, Fig 1). This was consistent with the fact that *C. albifundus* undergoes uni-directional mating type switching, leading to the deletion of the *MAT1-2-1* gene (Fig 1).

To ascertain that PCR errors had not arisen from primer mismatches or poor RT-PCR efficiency that would lead to a bias in the quantification of self-fertile and self-sterile mating type spores, an additional primer set (Albi-121F and 121-2) was designed to amplify the *MAT1-2-1* locus (Table S1, Fig 1). This was tested using five randomly selected isolates to ensure that the quantification made from Albi_MAT1-1-2F / R was in an equal ratio to the sum of quantifications made from Albi-121F / 121-2 and Albi_MAT1-2-1F / R. The specificity of each of these primer sets was determined using conventional PCR before they were used in the real-time PCR analyses.

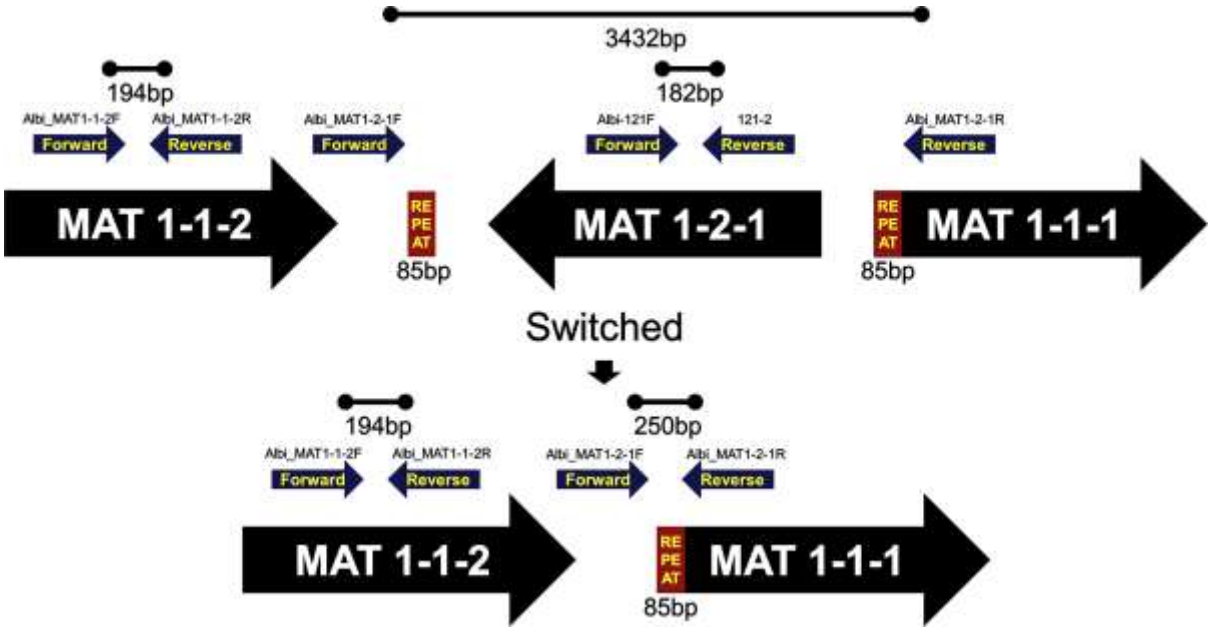

Fig 1. Schematic representation on the binding sites of primer sets designed in this study. Primer set, Albi_MAT1-1-2F and Albi_MAT1-1-2R was designed to amplify the *MAT1-1-2* locus, thus producing amplicons from both self-fertile and self-sterile mating type strains of *Ceratocystis albifundus*. Primer set, Albi_MAT1-2-1F and Albi_MAT1-2-1R was specifically designed to produce amplicons only from switched strains (self-sterile mating type). Consequently, the number of copies for the self-fertile mating type can be determined from the differences between the total number of copies quantified using the primer sets, Albi_MAT1-1-2F and Albi_MAT1-1-2R and those from the primer sets, Albi_MAT1-2-1F and Albi_MAT1-2-1R. Primer set, Albi-121F and 121-2, was further designed to check possible bias in the quantification of self-fertile and self-sterile mating type spores.

2.5.2. Real-time PCR amplification

To generate a standard curve for real-time PCR-based quantification, cultures representing either self-fertile or self-sterile strains of *C. albifundus* were incubated at room temperature for two weeks to induce ascocarp production (in the case of self-fertile strains) or mycelial growth (in the case of self-sterile strains). Ascospore masses produeced at the apices of the ascocarps or mycelium was scraped from the surfaces of cultures with a sterilized surgical scalpel, and transferred into 2 ml microcentrifuge tubes, where it was ground to a fine powder using a Ball-mill (MM301, Retsch, Haan, Germany). Genomic DNA was extracted as described by Möller et al. (1992). The quantity and quality of DNA extracted was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) that calibrates concentration and purity of DNA as PCR templates.

Real-time quantitative PCR was carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using a KAPA SYBR® FAST qPCR Kit Master Mix (2×) Universal (Kapa Biosystems, Boston, MA, USA). A 20 µl of reaction mixture was prepared in Hard-Shell® PCR Plates 96-Well WHT/CLR consisting of 10 µl of KAPA SYBR® FAST qPCR Master Mix (2×) Universal, 0.4 µl of each of the primers (5 pM), 7.4 µl of sterile water and 15 ng/µl of template DNA. Quantitative real-time PCR amplification was performed following the manufacturer's instructions: 95 °C for 3 min, followed by 40 cycles at 95 °C for 3 sec and 60 °C for 20 sec. After the amplification stage had been completed, melting curve analyses were performed from the target products based on a standard protocol suggested by the manufacturers.

The copy number for ascospores from single ascomata, representing each of the two mating types, was determined based on real time PCR-based quantification. Quantification for the self-sterile mating type spores in a spore drop was then made by interpolating the result obtained from the primer set, Albi_MAT1-2-1F and Albi_MAT1-2-1R, amplifying only the mating locus of the self-sterile mating type to the constructed standard curve. Subsequently, the copy number of all the spores was quantified based on the result obtained for the primer set, Albi_MAT1-1-2F and Albi_MAT1-1-2R, amplifying both the self-fertile and self-sterile mating type locus. Consequently, the number of copies for the self-sterile mating type was determined from the differences between the total number of spores and the number of spores lacking the *MAT1-2-1* gene. All real-time PCR reactions were conducted in duplicate to ensure reproducibility and were repeated twice.

2.5.3. Mating type quantification in a single ascospore mass

In addition to the mating type ratios obtained from ten isolates (nine isolates from the CMW culture collection in FABI and one from the cross) induced under laboratory conditions, ascomata collected from naturally infected trees in the field were also included. These could have been induced by either haploid selfing or outcrossing. In total, thirty-five single ascospore drops (twenty from WC and fifteen from KNP) were collected from the apices of ascocarps produced on freshly infected wounds on *Terminalia sericea* (native tree under natural conditions) and *Protea cynaroides* (cut flower orchard shrubs) and were used for quantification using real-time PCR.

2.5.4. Statistical analyses

A chi-square analysis (95% confidence level) was used to determine the possible differences in segregation for each mating type emerging from the single ascospore isolations. The R Stats Package using R ver.3.1.0 (http://www.r-project.org/, R Core Team, 2014) was used to analyse these data. Here, the null hypothesis was that no significant differences would be observed in the production of self-fertile and self-sterile mating type progeny from single ascospore drops.

2.6. PCR for microsatellite analyses

Microsatellite primer sets developed by Barnes et al. (2005) were used to determine the extent to which outcrossing had occurred in the given populations of *C. albifundus* in South Africa (Table S2). The PCR analyses were performed in a total volume of 15 µl containing 15 ng/µl of genomic DNA, 0.3 µl of the forward primer (10 pM), 0.3 µl of the reverse primer (10 pM), 3 µl of 5 × reaction buffer containing 5mM dNTPs and 15mM $MgCl_2$ (Bioline in London, UK), 0.09 µl of MyTaq™ DNA polymerase (Bioline in London, UK). PCR reactions were conducted in a Veriti™ 96 well Thermal Cycler (Applied Biosystem, Foster City, CA) using the same conditions described by Barnes et al. (2005), with slight modifications in the annealing temperature. This was where the microsatellite primer sets did not amplify the desired size of the PCR product, or where they produced multiple bands. After confirming that three primer sets (AG7/8, AG15/16 and AG17/18) would amplify the desired PCR fragments resulting in single amplicons, these were used in the analyses for the remaining set of isolates included in this study (Table S2). The amplification products were pre-stained with 1 µl of GelRed™ (Biotium Incorporation, USA) nucleic acid dye on a 1.5 % (w/v) agarose gel in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.0) and were analysed by electrophoresis (Mini-Sub® Cell CT, Biolad, USA), and then visualized under UV light (Gel Doc™ EZ Imager, Bio-Rad, Richmond, CA).

2.7. Genescan analyses and allele scoring

The sizes of the microsatellite amplicons were determined by electrophoresis on an ABI PRISM™ 3500xl POP 7™ Automated DNA sequencer (Applied Biosystem, Foster City, CA) and compared against a GeneScan™-600LIZ (Applied Biosystem, Foster City, CA) used as

an internal size standard. GeneMarker ver. 2.2.0 (SoftGenetics, State College, PA) was used to determine the expected allele sizes. It was assumed that where an ascoma was the result of a selfing event, all the ascospores would be genetically identical and thus would represent a single genotype. In contrast, where an ascoma was the result of a cross between two different individuals, the ascospores from a single ascoma would be the result of a recombination of the two parents and would not be identical. That is, *C. albifundus* from each of the populations that outcrossed with the genetically different mating partner would have the possibility of generating a "diploid" microsatellite profile. This is given the fact that *C. albifundus* is haploid, and single individuals would have only one allele at any given locus. In cases where two different size peaks were observed at a specific locus, cultures were scored as resulting from an outcrossing event. In contrast, where only one peak was identified, the culture was scored as not having resulted from outcrossing. By using more than one microsatellite marker, it was possible to increase the chance of observing polymorphisms.

3. Results

3.1. Isolates and identification of self-fertile strains

The self-fertile strains derived from the nine *C. albifundus* strains taken from the culture collection (Table 2) were identified by confirming the presence of sexual structures giving rise to spore drops at the apices of the ascocarps. Pairing between two different individuals of *C. albifundus* (CMW48857 and CMW48858) produced abundant ascomata within two weeks. Cultures were further incubated at room temperature until ascomata matured, which resulted in ascospore masses being large, viscous and opaque. Single ascospore masses were then randomly selected and were used for the real-time PCR-based quantification.

3.2. Quantitative real-time PCR assay

3.2.1. Primer design for real-time PCR amplification

Primer sets designed to amplify each mating type locus, *MAT1-2-1* and *MAT1-1-2*, and those amplifying only the mating locus of the self-sterile mating type lacking the *MAT1-2-1* locus, consistently generated the expected size of PCR products. The size of amplicons for the primer pairs (Albi-121F / 121_2, Albi_MAT1-1-2F / R and Albi_MAT1-2-1F / R) were

182bp, 194bp and 250bp, respectively (Table S1, Fig 1).

Primer sets, Albi-121F and 121-2, designed to determine possible bias in the quantification of self-fertile and self-sterile mating type spores and that could arise from PCR errors due to primer mismatches or poor RT-PCR efficiency, showed clearly that no such bias had occurred. This was evident from the applied chi-square analysis (Table 4).

Table 4. Results of quantifications to determine whether there is a bias in quantifying between self-fertile and self-sterile mating type spores of *Ceratocystis albifundus*.

Identity of spore used for quantifications	Q1[a]	Q2[b]	Q1+Q2	Q3[c]	X^2 test
CA1	51	17	68	86	2.10
CA2	34	60	94	86	0.36
CA3	28	98	126	114	0.60
CA4	28	30	58	46	1.38
CA5	13	7	20	19	0.03

*0.05 probability level (3.841), df = 1

[a]Quantifications using Albi-121F/121_2, amplifying only self-fertile isolates

[b]Quantifications using Albi_MAT1-2-1F/R, amplifying only self-sterile isolates

[c]Quantifications using Albi_MAT1-1-2F/R, amplifying both self-sterile and self-fertile isolates

3.2.2. Real-time PCR efficiency and specificity

A standard curve based on known DNA concentrations ranging from 6.12E+6 to 6.12E+4 was successfully constructed. This showed a strong linear relationship between the standard curve of Ct values and 10-fold serially diluted DNA. It was thus possible to quantify the actual number of spores representing each mating type in a single spore-drop (Fig S1, S2, S3). In addition to the PCR efficiency, the PCR specificity was further verified by obtaining single dissociation peaks in the melting curve analysis (Fig S4, S5, S6).

3.3. Mating type ratios in vitro

3.3.1. Mating type quantification in a single ascospore mass

Three single ascospore masses generated from each of the nine isolates produced under laboratory conditions were used to quantify the ratios of self-fertile to self-sterile ascospores

of *C. albifundus* based on real-time PCR. The results of quantitative real-time PCR consistently showed that there was a significant bias towards self-fertile ascospores in single ascospore masses (Table 2). The ratio of self-fertile to self-sterile spores obtained from ascomata generated under laboratory conditions ranged from 112.2:1 to 2.4:1 (Table 2). The biased segregation for each of the mating types quantified in this study was supported statistically based on chi-square analysis at a 95% confidence level. Thus, the null hypothesis that the segregation ratio of self-fertile to self-sterile is close to 1:1 can be rejected.

For the progeny of the laboratory crosses between isolates CMW48857 and CMW48858, there was a significant bias towards spores of the self-sterile mating type in single ascospore masses. These results were further supported statistically based on chi-square analysis at a 95% confidence level (Table 2).

3.3.2. Genescan analyses and allele scoring

A microsatellite primer pair (AG7/8) was used to determine whether crossing was successful in generating a diploid microsatellite profile. This would show that outcrossing had occurred. In cases where ascospore masses from single ascospore cultures were used for the genescan analyses, two different single alleles were observed; 330bp (CMW48857) and 321bp (CMW48858), generating a haploid microsatellite profile. However, when ascospores from the cross between individuals of *C. albifundus* (CMW48857 and CMW48858) were analysed, the diploid microsatellite profile was observed. This showed that outcrossing had occurred between the two different individuals.

3.4. Mating type ratios in vivo

3.4.1. Fungal isolates collected from the field and identification of isolates

Twenty and fifteen single spore masses were obtained from ascomata from each of the *T. sericea* trees growing naturally in the KNP and from *P. cynaroides* in the intensively managed orchard, respectively. All isolates produced sexual structures on MEA in culture and these all had morphological features typical of *C. albifundus*, including light coloured ascomatal bases (Wingfield et al. 1996). The ITS sequence data for four randomly selected isolates showed a 99% similarity when compared with sequences for other *C. albifundus* isolates in NCBI. The

sequence data obtained were submitted to NCBI (KR349567, KR349568, KR349569, KR349570).

3.4.2. Mating type quantification in a single ascospore mass

The results of quantitative real-time PCR consistently showed that there was a significant bias towards the self-fertile ascospores in single ascospore masses collected from the *P. cynaroides* in the intensively managed cut-flower orchard (Table 3). This was in contrast to the results obtained from *T. sericea* growing naturally in the KNP (Table 3). Among fifteen field samples from the KNP used in the quantification based on real-time PCR, a bias towards the self-fertile mating type spores was observed for seven samples, ranging from 5.7:1 to 1.2:1 (self-fertile : self-sterile) (Table 3). For the remaining eight samples, greater numbers of self-sterile mating spores were found in the ascospore masses and in these cases, the ratio ranged from 1:37.6 to 1:2.1 (self-fertile : self-sterile) (Table 3). The biased segregation for each of the mating types quantified in this study was supported statistically based on chi-square analysis at a 95% confidence level. This was other than for one quantification of isolate (KNP2) collected in the KNP showing 1.2:1 ratio between self-fertile and self-sterile (X^2 test, 3.15) (Table 3).

3.4.3. Genescan analyses and allele scoring

A significant correlation was found between results from the microsatellite analyses and those from the real-time PCR-based quantification. This indicated that outcrossing had occurred, on average, in greater numbers of self-sterile spores, whereas haploid selfing gave rise to greater numbers of self-fertile mating type spores (Table 3).

Only two microsatellite primer sets (AG15/16 and AG17/18) used for samples collected from the field consistently generated a diploid microsatellite profile, indicative of outcrossing having occurred (Fig 2). From both microsatellite markers, AG15/16 and AG17/18, five DNA samples used in the real-time PCR-based quantifications indicated that outcrossing had occurred. This further supported the results of the real-time PCR-based quantification showing a biased mating type ratio towards self-sterile isolates (Table 3). This is in contrast to the case where greater numbers of self-fertile isolates were found from the single spore isolates generated under laboratory conditions. Here, the haploid microsatellite profile was

obtained, indicating that haploid selfing had occurred (Table 3). However, in the case of ascospore masses, KNP3 and KNP7, obtained from naturally infected trees, greater numbers of self-sterile ascospores were produced, indicative of outcrossing events having occurred. This could be due to the fact that the parents with the identical microsatellite genotypes were involved in the cross, showing a single allele at both loci.

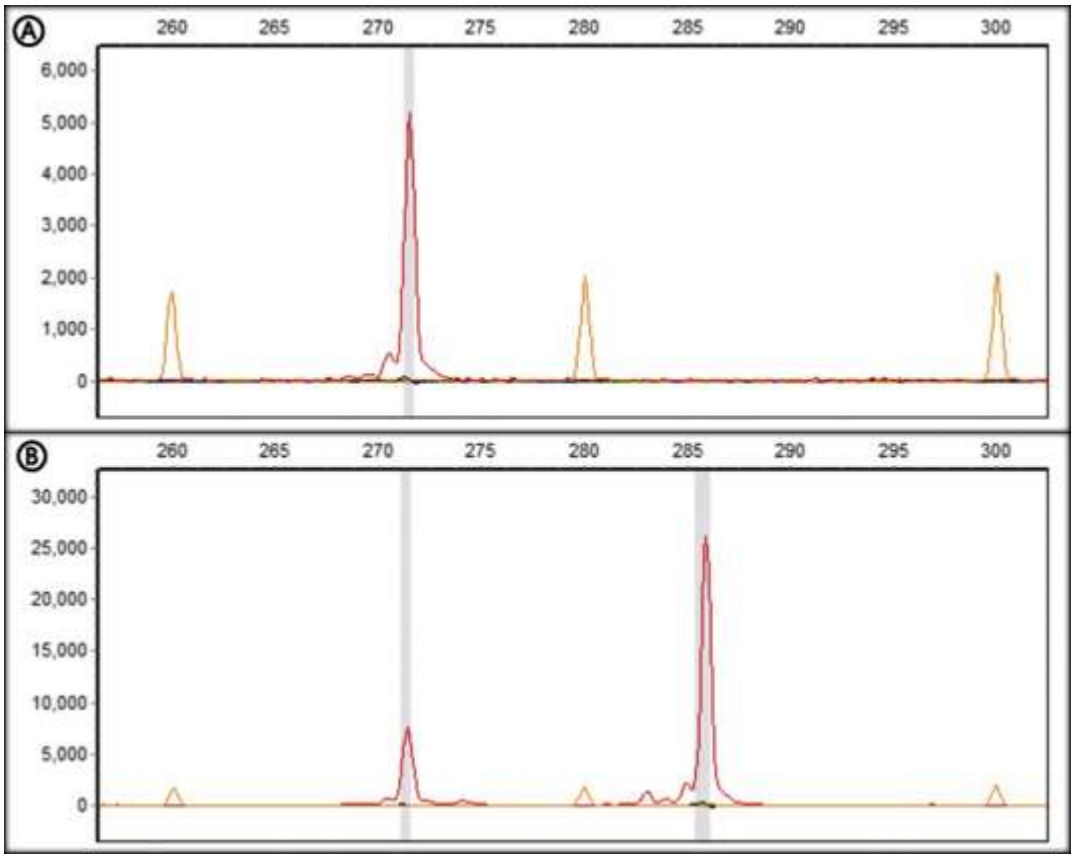

Fig 2. The results of allele scoring in GeneMarker ver.2.2.0 (SoftGenetics, State College, PA). (A): AG15/16 microsatellite primer sets with no evidence of outcrossing [a single allele; haploid microsatellite profile], (B): AG15/16 microsatellite primer sets with evidence of outcrossing [two different sizes of alleles; diploid microsatellite profile].

4. Discussion

A substantial level of variation was revealed in the ratios of self-sterile to self-fertile spores in ascospore masses from single ascomata in the African tree pathogen *C. albifundus.* This is different to the situation observed in *Endoconidiophora coerulescens*, a fungus related to *C. albifundus* and also displaying unidirectional mating switching, where a 1:1 ratio of self-fertile to self-sterile mating type strains was found (Harrington & McNew 1997). What was

particularly interesting was that the ratio of self-sterile and self-fertile spores is apparently influenced by the genotype of the parents in the cross.

A significant bias towards self-fertile ascospores was observed from the single spore isolates producing ascomata under laboratory conditions. The results are consistent with those of a previous study (Lee et al. 2015) where the mating type ratio was significantly biased to the self-fertile mating type. The present study shows that this bias is due to the fact that fewer self-sterile spores are produced and not because a higher percentage of self-sterile spores fail to germinate. Although the mechanism underlying the biased segregation ratio remains unclear, plausible hypotheses to explain this bias include the association of either of the two mating types with a lethal mutation (Kwon-Chung et al. 1978) or vulnerability of either of the two mating types to unidentified environmental stress (Kwon-Chung et al. 1978).

Quantification of ascospores collected directly from diseased *P. cynaroides* trees intensively cultivated in a cut-flower orchard showed a significantly skewed ratio towards the self-fertile mating type. This bias to self-fertile spores was also found where the ascospores were produced from haploid selfing under laboratory conditions. Thus, haploid selfing consistently gave rise to a skewed ratio towards the self-fertile mating type. In the case of the samples collected from naturally occurring trees in the KNP, seven of fifteen samples were biased towards self-fertile mating type, and the remaining samples were skewed to self-sterile mating type. This bias towards the self-sterile mating type was further observed in crosses using two different individuals of *C. albifundus* where greater numbers of self-sterile isolates were consistently produced. This was in contrast to the situation where single spore isolates were made under laboratory conditions and where haploid selfing occurs. This difference in ratios of mating type for ascomata from different environments was unexpected and has not previously been reported. It is however speculated that a bias in the mating type of *C. albifundus* isolates could relate to environmental factors as has been shown by Kwon-Chung et al. (1978) in *Cryptococcus neoformans*.

Fungi are capable of outcrossing, while retaining the ability to self under natural conditions (Brasier & Gibbs 1975; Marra & Milgroom 2001; Milgroom et al. 1993; Puhalla & Anagnostakis 1971). Although *C. albifundus* is a homothallic fungus, the extent to which outcrossing occurs in nature was unknown prior to this study. Using a real-time PCR-based quantification approach, specifically developed in this study, we were able to show that

outcrossing does occur in this fungus. The technique used ascospore masses directly and is substantially less labour intensive than methods requiring the production of single ascospore cultures. DNA from each ascospore mass was then tested for the presence of one or more microsatellite alleles at three different loci. This technique is, however, likely to under-estimate the level of outcrossing due to the difficulty in differentiating crossing events that have occurred between individuals with identical microsatellite genotypes. This is true given cases where a greater number of self-sterile ascospores were produced, as seen in the quantifications from a native and naturally infected host trees in the KNP. Nonetheless, we were able to clearly demonstrate that a high frequency of outcrossing occurs based on three microsatellite loci that were shown to be relatively variable.

Once it had been shown that outcrossing was occurring in crossing experiments as well as under field conditions, it was possible to determine whether there was a correlation between the ratios of the *MAT* loci and reproduction modes. Lower levels of genetic diversity have previously been observed in a population of *C. albifundus* isolates collected from a disease outbreak in an intensively managed *P. cynaroides* cut-flower orchard (Lee et al. 2016). In that situation, apparent haploid selfing (similar to that under laboratory conditions) could give rise to greater numbers of self-fertile spores. Conversely, greater numbers of self-sterile mating type isolates were produced from ascospore masses arising from outcrossing events such as those found in the KNP collections, where the genetic diversity of the fungus is high (Lee et al. 2016). This observation indicates that the bias in mating type ratio of *C. albifundus* is heavily dependent on whether ascomata are produced by haploid selfing or outcrossing.

It is clear that in *C. albifundus* "assortative mating" (Giraud et al. 2010) can be achieved by haploid selfing, thus preventing locally adapted allele combinations from breaking down by recombination. It might thus be expected that selfing is favoured due to the assurance of sexual reproduction when the density of mating partners is low. This would be especially true during colonization and where losing locally adapted combinations of alleles from sexual recombination would need to be avoided (Giraud et al. 2008; Gladieux et al. 2015; Jarne & Charlesworth 1993). We hypothesize that this is what has occurred in the *P. cynaroides* population typified by a serious disease outbreak and low genetic diversity (Lee et al. 2016), consequently resulting in the successful colonization of a new environment. However, in the case of the population from the KNP, there is a greater genetic diversity (Lee et al. 2016), and thus outcrossing is common.

It is intriguing that there was significant variation in the ratio of the self-sterile and self-fertile ascospores in *C. albifundus* ascomata. Some of this variation is a consequence of whether the ascomata were produced as the result of a haploid-selfing or an outcrossing event. However, the mechanism underpinning this difference is unknown. While it could be speculated that this might be the result of inbreeding depression, little is known regarding this condition in fungi. This is particularly the case in ascomycetes where the majority of the life-cycle is haploid and recessive deleterious alleles (the basis of much inbreeding depression) would be eliminated.

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Supplementary materials

Table S1. Primer sets designed for the quantitative real-time PCR in this study.

Primer	Sequence (5' - 3')	Size
Albi_MAT1-2-1F	CCA AGA TCT TTT CCA TCC TA	250bp
Albi_MAT1-2-1R	TAC GAT ATA ATG GCC CAG	
Albi_MAT1-1-2F	GTA GCT GCG TTG ATT ATA G	194bp
Albi_MAT1-1-2R	GAC GAA CCA GCT CTC AAT	
Albi-121F	TAC TAC TGC CCA TCT TGT C	182bp
121_2	CCT TCT TTT TCG GAG CCT GC	

Table S2. Microsatellite primers used in this study.

Primers	Sequences (5'-3')	Annealing temperature
AG7	CGA GAC AGC AAC ACA AGC CC	62˚C
AG8	GGG GCG GTG GTG CAA TTG TC	
AG15	CTT GAC CGA CCT GCC GAT TG	62˚C
AG16	GGA TAG CAG CGA CAA GGA CC	
AG17	GTC GGT GGT GGA GAC GGT C	62˚C
AG18	CGG CCC TGC CAA CGG ATG	

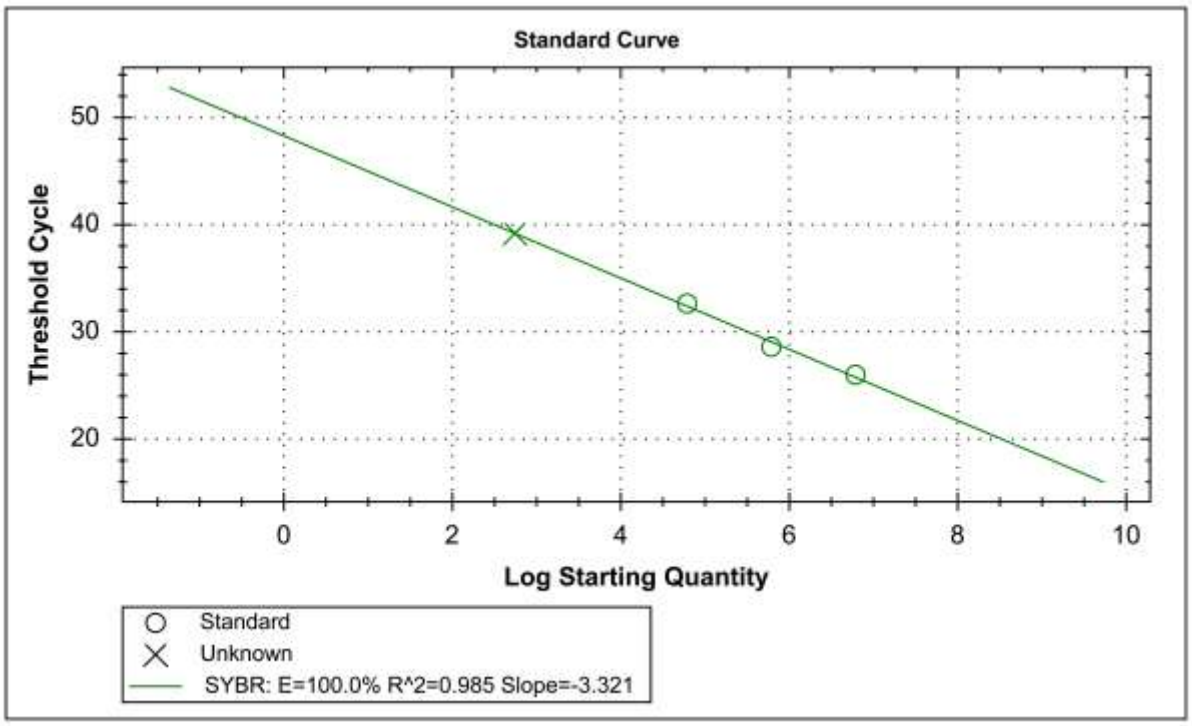

Fig S1. Standard curve generated by plotting threshold cycle (Ct) against serially diluted genomic DNA extracted for quantifications of both self-fertile and self-sterile isolate of *Ceratocystis albifundus*.

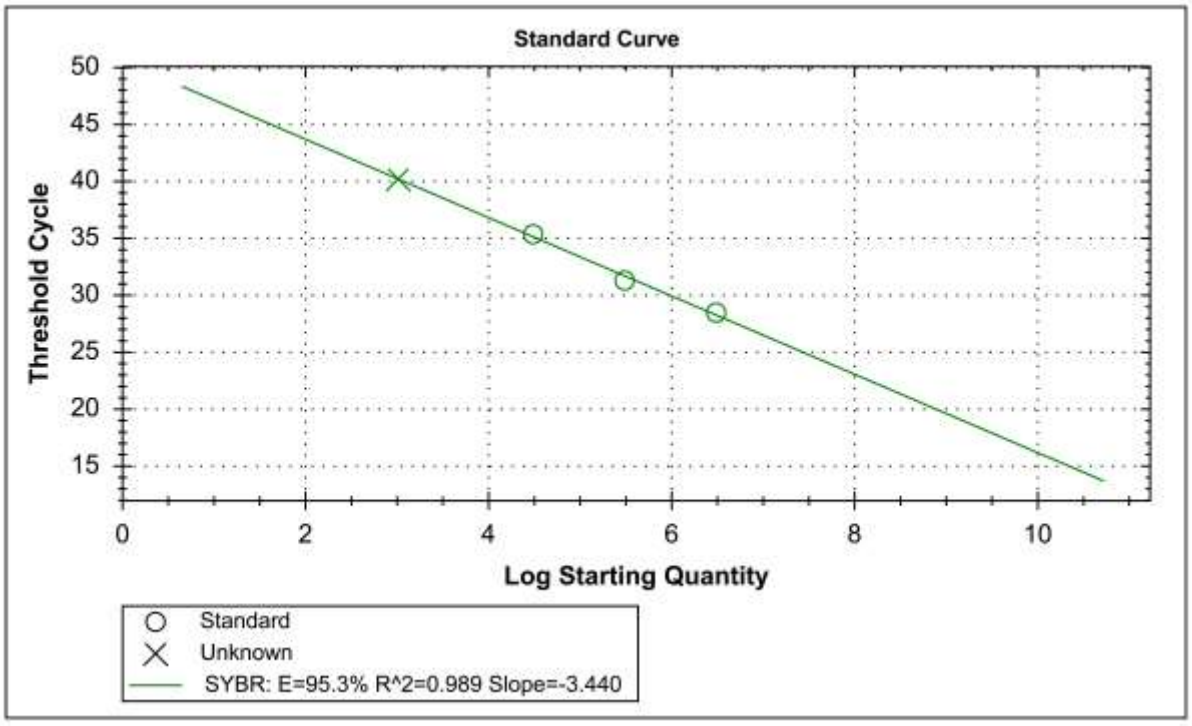

Fig S2. Standard curve generated by plotting threshold cycle (Ct) against serially diluted genomic DNA extracted from self-sterile isolate of *Ceratocystis albifundus*.

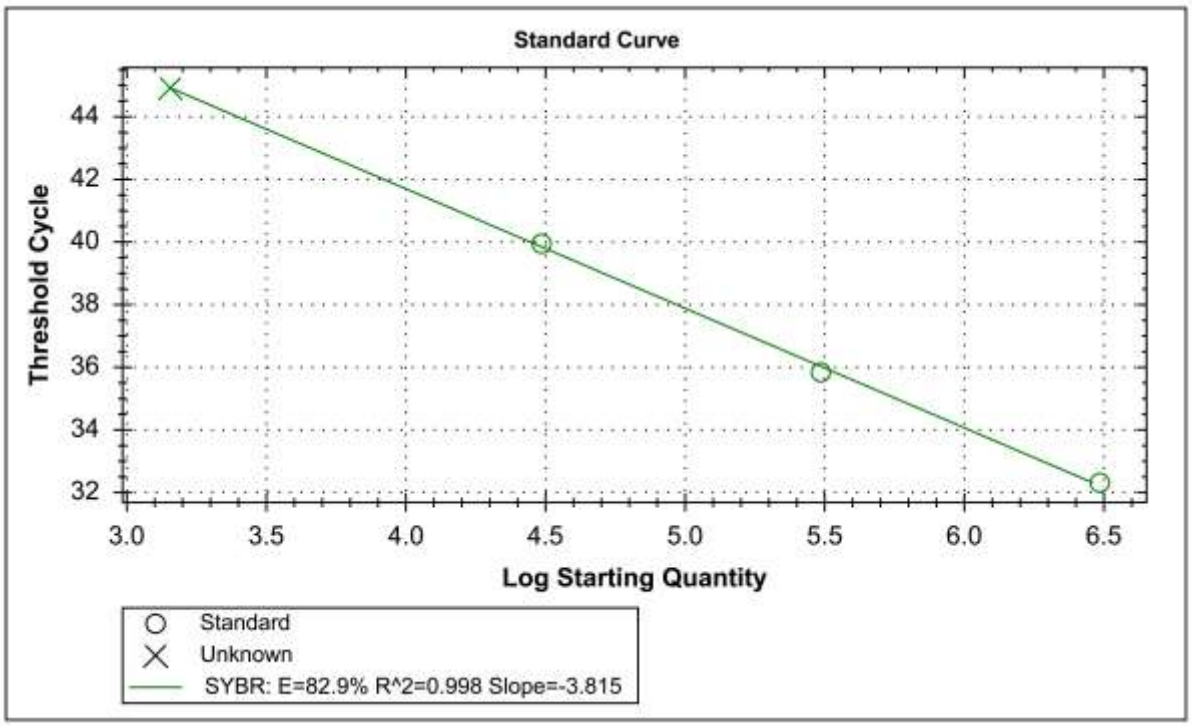

Fig S3. Standard curve generated by plotting threshold cycle (Ct) against serially diluted genomic DNA extracted for quantifications of self-fertile isolate of *Ceratocystis albifundus*.

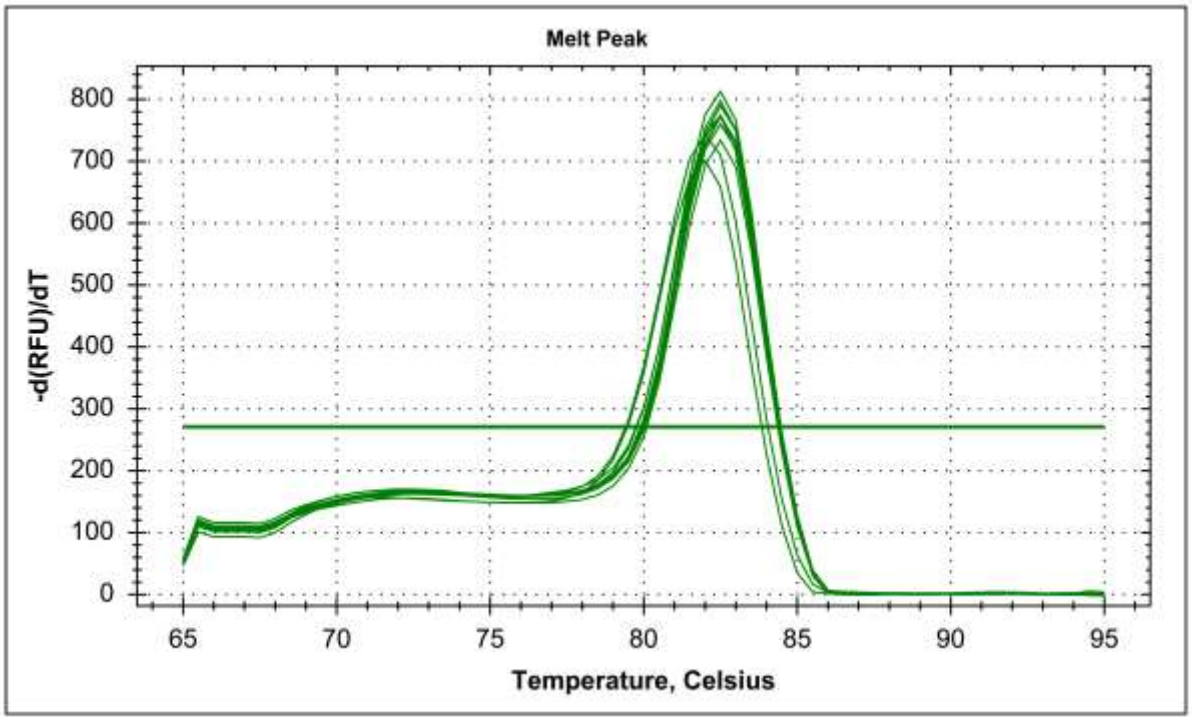

Fig S4. Profile on melting curve analysis for both self-fertile and self-sterile mating types obtained by quantitative real-time PCR reaction.

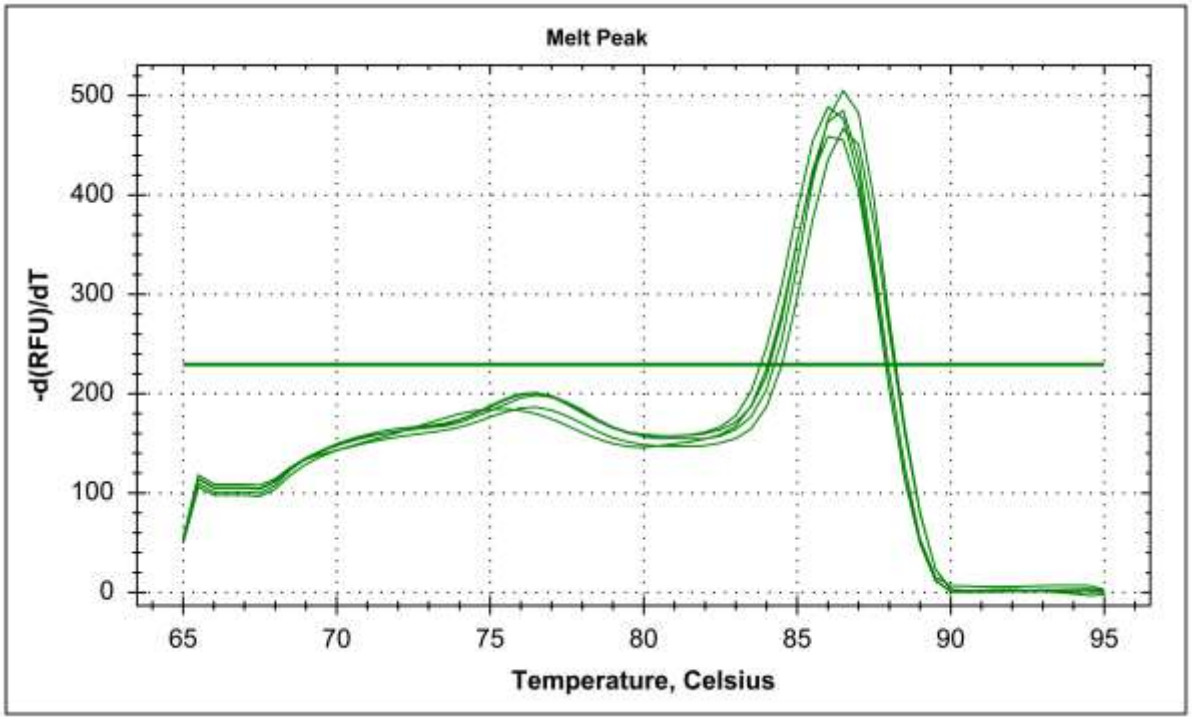

Fig S5. Profile on melting curve analysis for self-sterile mating type obtained by quantitative real-time PCR reaction.

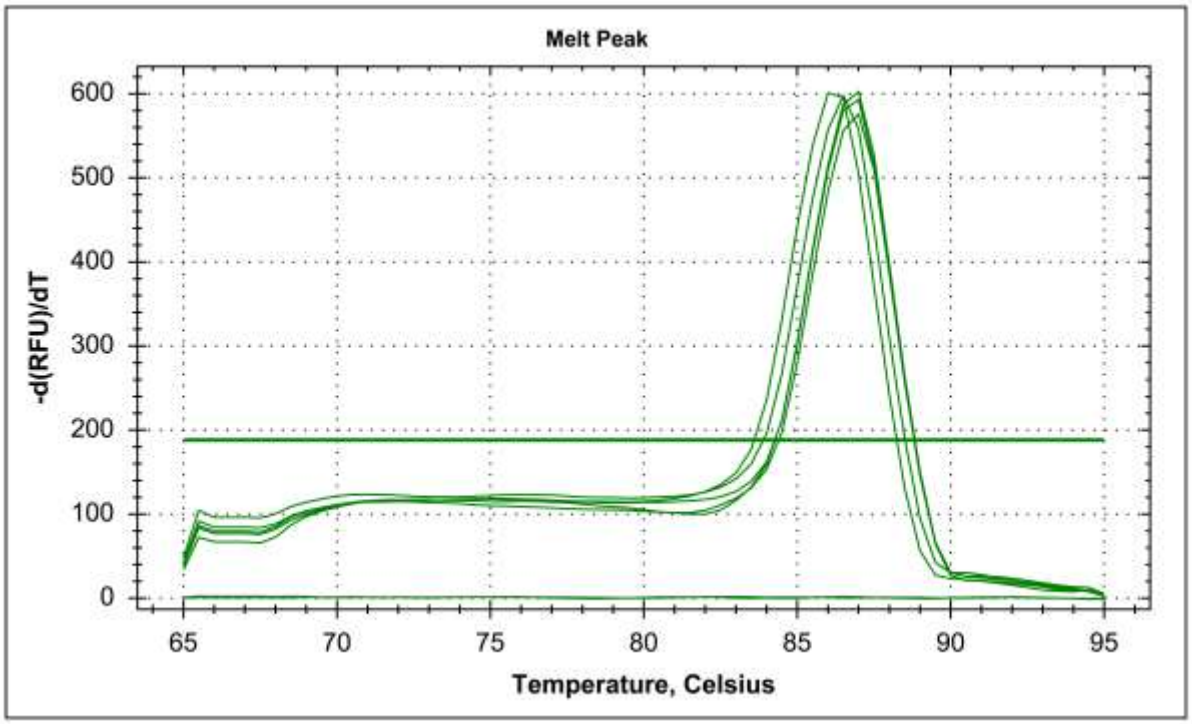

Fig S6. Profile on melting curve analysis for self-fertile mating type obtained by quantitative real-time PCR reaction.