The distribution of genetic diversity in the *Neofusicoccum parvum/N. ribis* complex suggests structure correlated with level of disturbance

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Abstract

Plants and animals adapted to colonize disturbed sites might also be better invaders, but this phenomenon has not been widely considered in fungi. We investigated genetic diversity and structure amongst isolates of *Neofusicoccum parvum*, *N. cordaticola*, *N. kwam-bonambiense* and *N. umdonicola* that coexist sympatrically on a native tree, *Syzygium cor-datum*, across its distribution in South Africa. Species composition varied among stands, with dominance of *N. parvum* in disturbed stands, and absence in undisturbed stands, where the other species dominated. *N. parvum* populations from trees planted in urban environments were more genetically diverse than populations from human disturbed stands of *S. cordatum*. Bayesian analysis clustered *N. parvum* isolates in three sub-populations, suggesting three sources of origin. These results support the hypothesis that as a generalist *N. parvum* will dominate human disturbed sites and trees in urban areas, indicating strong potential for invasion, and its spreading from non-native hosts to native *S. cordatum*, rather than *vice versa*.

Keywords:
Anthropogenic disturbance
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Disturbed habitats
Diversity
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Introduction

Organisms with broad niche breadths are often prominent colonizers of disturbed environments in their home range but invaders in new regions (Lee and Gelembiuk, 2008). This is also true for some invading fungi (Fisher et al., 2012; Santini et al., 2013), although the phenomenon has not been widely studied in this group of organisms. Slippers and Wingfield (2007) noticed that a number of the prominent tree pathogenic fungi in the Botryosphaeriaceae are generalists in terms of host associations. Many of these species also appear to be widely distributed, most likely invasive in many regions, and occur in human associated, disturbed environments. In this study, we thus question whether some of these generalist species might act as "weeds", colonizing disturbed environments more effectively than native environments that are presumably more ecologically diverse and stable.

The fungal *Neofusicoccum parvum/N. ribis* species complex (Botryosphaeriaceae, Ascomycetes) (Crous et al., 2006) was thought to include two sister species that are fungal plant pathogens on a variety of woody hosts, most frequently reported from the Southern Hemisphere (Slippers and Wingfield, 2007). Recent studies of this complex on a native South African tree, *Syzygium cordatum*, resulted in the identification of three additional cryptic species using a Genealogical Concordance Phylogenetic Species Recognition (GCPSR) approach based on concordance of multiple locus sequence data (Pavlic et al., 2009a,b). These were described as the phylogenetic, cryptic species *N. cordaticola, N. kwambonambiense* and *N. umdonicola* (Pavlic et al., 2009b). Subsequently, additional cryptic species were identified in the *N. parvum/N. ribis* complex, including *N. batangarum* (Begoude et al., 2010), *N. occulatum* and *Neofusicoccum* sp. *karanda* (Sakalidis et al., 2011).

The reproductive cycle of the haploid *Neofusicoccum* species in the *N. parvum/N. ribis* complex is underexplored, and mating types of these species are not known. *Neofusicoccum parvum* and *N. ribis* are known to produce both asexual and sexual structures under field conditions, but are most commonly encountered in their asexual spore (conidial) stage (Pennycook and Samuels, 1985; Slippers et al., 2004a; Pavlic et al., 2007). Other species in the complex are known only from their asexual stage.

The global distribution and host association of members of the *N. parvum/N. ribis* complex has recently been characterized (Sakalidis et al., 2013). That study confirmed the widespread and generalist pathogen status of *N. parvum*, which was shown to occur on 90 hosts across six continents. On the contrary, *N. ribis* was identified only from a single host species, *Ribes* sp. in the USA, from which it was originally described. The other cryptic species in this complex were more widespread than *N. ribis*, but they had more restricted geographical distributions and host associations than *N. parvum* (Sakalidis et al., 2013). The cryptic species in the *N. parvum/N. ribis* complex have been recorded as pathogens on cultivated, economically important non-native fruit and forestry trees (Slippers et al., 2004b, 2007; Mohali et al., 2007) and also on forest trees in native ecosystems (Slippers et al., 2005; Burgess et al., 2005; Pavlic et al., 2007). They are commonly isolated as endophytes that reside, asymptomatically, within plant tissues of numerous woody hosts (Slippers and Wingfield, 2007). This makes them ideal candidates for undetected, long distance dispersal by humans, together with plant germplasm traded for agriculture and forestry.

Syzygium cordatum is an evergreen broad-leafed tree that belongs to the Southern Hemisphere family Myrtaceae (Palgrave, 1977), and is the most widely distributed *Syzygium* species in South Africa. Naturally seeded *S. cordatum* trees occur widely along the eastern part of the country as single trees or patches of trees remaining within human disturbed areas, e.g. amongst stands of non-native *Eucalypts* plantations, or in undisturbed stands (e.g. Nature Reserves). *Syzygium cordatum* is also planted as ornamental in urban environments across the country. The distribution of *S. cordatum* across these three different disturbance types (undisturbed, human disturbed, and in cities), and infection of four cryptic species from *N. parvum/N. ribis* complex, make this an interesting model to better understand the influence of disturbance on the diversity and distribution of these fungi.

In this study, we used previously designed SSR markers (Slippers et al., 2004a) to: (i) assess genetic diversity and structure of four species, *N. parvum*, *N. cordaticola*, *N. kwambonambiense* and *N. umdonicola* that coexist sympatrically on *S. cordatum* trees in South Africa, and (ii) analyze the distribution of the species and population diversity across three habitat types: undisturbed stands of *S. cordatum* (e.g. Nature Reserve), human disturbed stands (e.g. naturally regenerated *S. cordatum* trees remaining within non-native *Eucalyptus* plantations) and planted *S. cordatum* as ornamentals in urban areas.

Materials and methods

Fungal isolates

The isolates used in this study were collected during a survey of the Botryosphaeriaceae on native *S. cordatum* trees in different geographical locations of South Africa, between Feb. 2001 and Mar. 2003 (Supplementary Table 1, Fig 1). *Syzygium cordatum* trees do not grow in persistent forests, but rather in patches or as solitary trees and thus all available trees in 13 sampling sites were examined. Between 1 and 45 trees were examined from each site. The isolates were obtained from 1 to 17 trees per site and 1–5 isolates were collected per tree (Supplementary Table 1). From each tree, isolations were made from twigs showing die-back symptoms, and asymptomatic twig, leaf and fruit as described by Pavlic et al. (2007, 2009a,b). Of the 114 isolates used in this study, 50 were collected from undisturbed habitats of *S. cordatum* (five sites), 31 were collected from human-disturbed habitats (three sites) and 33 isolates were collected from planted *S. cordatum* trees in urban areas (five sites) (Supplementary Table 1, Table 1; Fig 1). One hundred and two isolates were identified in previous studies, and additional 12 (isolated from asymptomatic twigs) in this study, as species in the *N. parvum/N. ribis* complex including *N. parvum* (48 isolates), *N. cordaticola* (17), *N. kwambonambiense* (14) and *N. umdonicola* (35), based on DNA sequence data for at least one locus and PCR-RFLP analysis

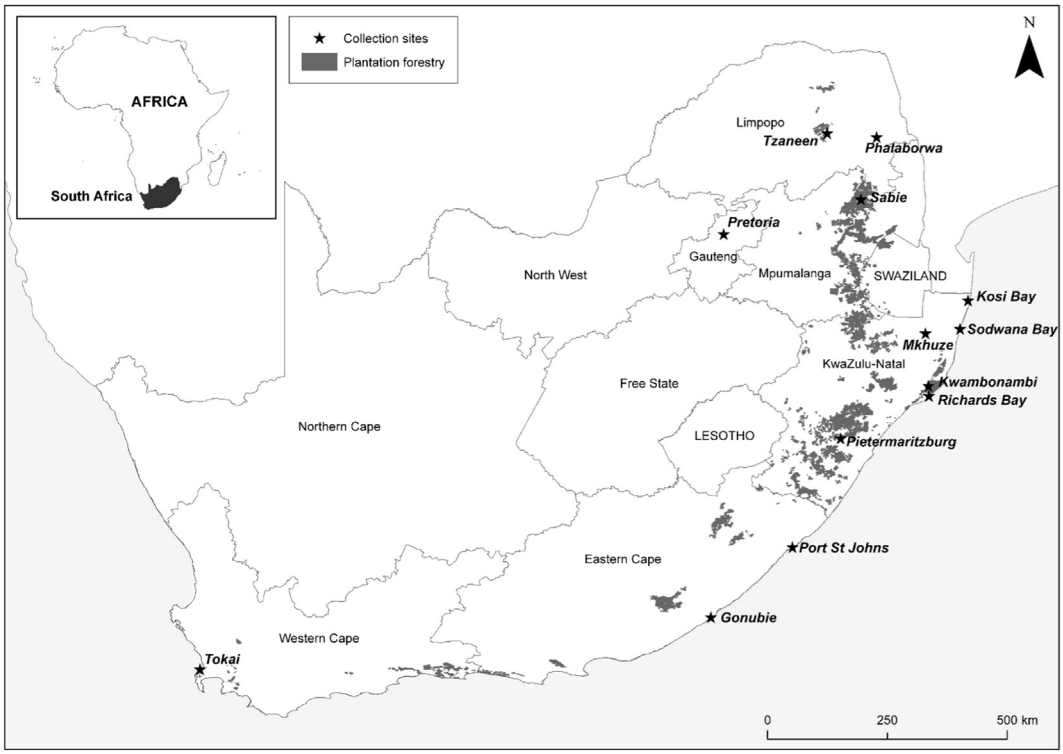

Fig 1 – Map of South Africa showing collection sites and plantation forestry. Tzaneen (P), Palaborwa (P), Pretoria (P), Sabie (UD), Kosi Bay (UD), Mkhuze (UD), Sodwana Bay (UD), Kwambonambi (HD), Richards Bay (UD), Pietermaritzburg (P), Port St. Johns (HD), Gonubie (HD), Tokai (P). P = Isolates collected from planted trees in urban areas; DU = Isolates collected from trees in undisturbed stands; HD = Isolates collected from trees human disturbed stands.

(Pavlic et al., 2007, 2009a,b) (Supplementary Table 1). Isolates of all four species were collected from twigs showing die-back symptoms and asymptomatic, visually healthy tissues. All haploid, single conidial cultures used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and representative isolates have been deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA extraction, microsatellite-PCR amplification and genotyping

The haploid, single conidial cultures were grown on 2 % malt extract agar (MEA) (20 g malt extract, 15 g agar; Biolab, Midrand, Johannesburg, S.A. and 1 000 mL deionized water) for 7 days at 25 °C in the dark. DNA was extracted from the mycelium following the modified phenol-chloroform DNA extraction method described in Pavlic et al. (2007). DNA was separated by electrophoresis on 1.5 % agarose gels, stained with ethidium bromide and visualized under ultraviolet light. DNA concentrations were estimated against λ standard size markers. Seven loci that contained microsatellite sequences were amplified for all isolates, using fluorescently-labeled primer pairs designed for species of Botryosphaeriaceae with *Fusicoccum*-like anamorphs (Slippers et al., 2004a). PCR reactions were performed using an Eppendorf Mastercycler PERSONAL (Perkin–Elmer, Germany) and the following protocol: 94 °C for 2 min initial denaturation; 40 cycles of 94 °C for 30 s, 55 or 62 °C for 30 s, 72 °C for 1 min; and 72 °C for 7 min final extension. PCR products were separated in 2 % agarose gels stained with ethidium bromide and visualized under UV light. Sizes of PCR products were estimated by comparison with a 100 bp molecular weight marker (Fermentas Life Sciences). Allele sizes of labeled microsatellite-PCR products were determined on an ABI PRISM 3100™ automated DNA sequencer (Perkin–Elmer, Warrington, U.K.) and compared against a GENESCAN–500 LIZ (Perkin–Elmer Applied Biosystems, Warrington, U.K.) internal size standard. Because fragment sizes overlapped for some of the amplicons, two separate gels were run for each sample. Allele sizes were analyzed with GENESCAN 3.7 and GENOTYPER 3 software (Perkin–Elmer Applied Biosystems, Foster City, CA, USA).

Microsatellite analyses

The program POPGENE version 1.31 (Yeh et al., 1999) was used to calculate allele frequencies at each microsatellite locus for each of four populations representing four species identified in the *N. parvum/N. ribis* complex from *S. cordatum*. For each population, the observed number of alleles (n_a), number of private alleles, number and percentage of polymorphic loci (P) were evaluated. The genetic diversity of each sample was estimated by calculating the unbiased expected heterozygosity (Hnb, Nei, 1978) in GENCLONE (Arnaud-Haond and Belkhir, 2007). Additionally, in GENCLONE, a rarefaction

Table 1 – Distribution of isolates of four *Neofusicoccum* spp. collected from *S. cordatum* trees in South Africa

Collection sites[a,b,c]	*N. cordaticola*	*N. kwambonambiense*	*N. umdonicola*	*N. parvum*[d]
Tzaneen (1) **P**	–	2	–	**10**
Phalaborwa (2) **P**	–	–	–	2
Pretoria (3) **P**	–	–	–	2
Sabie (4) **UD**	9	4	–	–
Kosi Bay (5) **UD**	1	–	12	–
Mkhuze (6) **UD**	1	–	2	–
Sodwana Bay (7) **UD**	1	–	14	–
Kwambonambi (8) **HD**	3	4	5	**15**
Richards Bay (9) **UD**	2	4	–	–
Pietermaritzburg (10) **P**	–	–	–	**14**
Port St. Johns (11) **HD**	–	–	1	1
Gonubie (12) **HD**	–	–	1	1
Tokai (13) **P**	–	–	–	3
Total	17	14	35	48

a Numbers in brackets indicate collections sites as marked on Fig 4.
b P = Isolates collected from the planted trees in urban areas; UD = Isolates collected from trees in undisturbed stands; HD = Isolates collected from trees in human disturbed stands.
c Kosi Bay, Mkhuze and Sodwana Bay are protected areas of Nature Reserves. The isolates from Kwambonambi were collected from the trees naturally growing within *Eucalyptus* plantations.
d Numbers in bold indicate isolates considered in population analyses.

method allows for comparison of heterozygosity among samples with different sample sizes. The unbiased expected heterozygosity was compared for a standard size of 10 isolates corresponding to the sample with the lowest number of isolates (population two of *N. parvum*). Allelic richness and Private allelic richness were computed using ADZE (Szpiech et al., 2008). The software also implements a rarefaction approach allowing for a comparison of the values for a standard size of 10 (corresponding to the smallest sample).

Linkage Disequilibrium between pairs of loci was investigated for each species using the Black and Krafsur (1985) algorithm available in Genetix v4.05 (Belkhir et al., 2004). The Isolation-by-Distance (IbD) hypothesis was tested using the $F_{ST}/(1 - F_{ST})$ genetic distance (Rousset, 1997) and the log geographic distances between locations. The analysis was conducted on the species represented by the largest number of samples: *N. umdonicola* and *N. parvum*, as well as population one of *N. parvum* alone using the Mantel test implemented in Genetix.

The genetic relationship of populations and species was assessed using two different methods: (i) a principal coordinate analyses (PCoA) (in Genalex v6.5, Peakall and Smouse, 2006, 2012) and (ii) Bayesian clustering (in Structure v2.3.4, Pritchard et al., 2000). The multivariate analysis (PCoA) is based on distances between multilocus genotypes of individuals and does not make any assumption on the underlying population genetic model. The PCoA was performed using genetic distances and the co-variance standardized method. Structure, on the other hand, attempts to minimize the Hardy–Weinberg Disequilibrium and Linkage Disequilibrium within clusters in order to find the most likely number of clusters in a data set.

This Bayesian clustering was used to determine whether isolates in the *N. parvum/N. ribis* complex could be subdivided into *K* genetically distinct groups. Structure assumes a model in which there are *K* populations (where *K* may be unknown), each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are probabilistically assigned to a single population, or jointly to two or more populations if their haplotypes indicate that they are admixed. To determine the most likely number of genetically distinct groups or clusters (*K*) in the sample, 20 independent runs for each $K = 1$ to 10 were carried out at 100 000 Markov chain Monte Carlo (MCMC) repetitions following a burn-in of 50 000 iterations. The program was run assuming both admixture and no admixture among the populations and additional parameters assumed were: different values of F_{ST} for different subpopulations, prior mean of F_{ST} 0.01, standard deviation (SD) of F_{ST} 0.05 and constant lambda value at 1. The most probable number of clusters was obtained using the Evanno's approach (Evanno et al., 2005) implemented online in Structure harvester (Earl and VonHoldt, 2012). The analyses were run both on the full dataset (114 isolates) and on a clone-corrected data set (61 isolates) where only one of each of the haplotypes was included in the analyses.

Analysis of molecular variance (AMOVA) was carried out using Genalex. We examined the partitioning of genetic variation among and within the geographically defined populations and regions of *N. umdonicola*, *N. parvum* and sub-population one of *N. parvum*. The other species and sub-populations two and three of *N. parvum* were not included in this analysis because of small sample size. The analysis of molecular variance was performed on the full datasets.

Results

Genetic structure and diversity

For the entire dataset of 114 isolates that belong to *N. parvum/N. ribis* complex from *S. cordatum*, a total of 61 alleles were observed across seven loci examined (Table 2). There were 26 alleles detected amongst the 17 *N. cordaticola* individuals, 16

Table 2 – Allele size (bp) and frequency at seven loci in four *Neofusicoccum* spp., *N. cordaticola* (N. c), *N. kwambonambiense* (N. k), *N. umdonicola* (N. u) and *N. parvum* collected from *Syzygium cordatum* in South Africa

Locus	Allele[a]	N. c	N. k	N. u	*N. parvum*[b]			
					Pop 1	Pop 2	Pop 3	All
BotF11	420	–	1.000	–	–	–	–	–
	427	–	–	–	0.111	0.200	–	**0.104**
	428	–	–	–	0.852	0.800	0.909	**0.854**
	431	0.471	–	0.400	–	–	0.091	–
	432	–	–	0.543	–	–	–	0.021
	433	–	–	**0.057**	–	–	–	–
	435	**0.471**	–	–	–	–	–	–
	null	0.058	–	–	0.037	–	–	0.021
BotF15	365	–	–	–	–	**0.900**	–	**0.187**
	374	–	–	–	–	**0.100**	–	**0.021**
	377	1.000	–	1.000	0.963	–	0.454	0.646
	378	–	–	–	**0.037**	–	–	**0.021**
	389	–	–	–	–	–	**0.364**	**0.083**
	390	–	–	–	–	–	**0.182**	**0.042**
	395	–	**0.857**	–	–	–	–	–
	396	–	**0.143**	–	–	–	–	–
BotF17	229	–	1.000	0.971	–	–	–	–
	233	0.117	–	–	–	–	0.455	0.104
	234	**0.882**	–	–	–	–	–	–
	244	–	–	–	–	–	**0.455**	**0.104**
	246	–	–	–	**0.148**	–	–	**0.083**
	247	–	–	–	–	–	**0.090**	**0.021**
	249	–	–	0.029	0.852	–	–	0.479
	256	–	–	–	–	**0.500**	–	**0.104**
	259	–	–	–	–	**0.500**	–	**0.104**
BotF21	203	–	0.071	0.200	–	–	–	–
	204	0.176	0.143	0.743	0.111	–	–	0.062
	206	–	–	–	–	–	**0.090**	**0.021**
	207	0.118	–	–	0.074	0.400	0.273	0.187
	208	0.118	0.143	–	–	–	0.455	0.104
	209	0.059	0.071	–	–	0.600	–	0.125
	219	0.294	0.429	–	0.815	–	0.182	0.500
	229	–	0.071	0.057	–	–	–	–
	234	**0.176**	–	–	–	–	–	–
	null	0.059	0.071	–	–	–	–	–
BotF23	422	–	–	0.914	1.000	–	–	0.562
	423	–	–	–	–	–	**0.090**	**0.021**
	424	–	–	–	–	–	**0.182**	**0.042**
	425	–	–	–	–	1.000	0.546	**0.333**
	426	0.059	0.286	–	–	–	0.182	0.042
	427	0.059	0.714	–	–	–	–	–
	428	0.882	–	0.057	–	–	–	–
	null	–	–	**0.029**	–	–	–	–
BotF35	222	–	–	–	**0.148**	–	–	**0.083**
	225	0.059	–	–	0.852	–	–	0.480
	238	–	–	0.800	–	–	0.546	0.125
	239	–	–	–	–	–	**0.364**	**0.083**
	244	0.529	–	–	–	0.900	–	0.187
	245	–	–	0.200	–	0.100	–	0.021
	247	**0.294**	–	–	–	–	–	–
	253	0.059	0.429	–	–	–	–	–
	255	–	**0.571**	–	–	–	–	–
	265	–	–	–	–	–	**0.090**	**0.021**
	null	**0.059**	–	–	–	–	–	–
BotF37	303	**0.412**	–	–	–	–	–	–
	306	**0.412**	–	–	–	–	–	–
	312	–	–	1.000	0.963	–	–	0.542
	313	0.059	–	–	0.037	1.000	0.818	0.417
	314	–	–	–	–	–	**0.182**	**0.042**
	320	0.059	1.000	–	–	–	–	–
	null	**0.059**	–	–	–	–	–	–

Table 2

Locus	Allele[a]	N. c	N. k	N. u	N. *parvum*[b]			
					Pop 1	Pop 2	Pop 3	All
N		17	14	35	27	10	11	48
n_a		26	16	15	15	13	21	38
Allelic richness		3.12 (0.63)	2.14 (0.67)	1.99 (0.31)	2.13 (0.26)	1.71 (0.18)	3.00 (0.31)	5.40 (0.71)
Allelic richness standardized for 10 isolates		2.69 (0.53)	2.02 (0.56)	1.66 (0.24)	1.70 (0.18)	1.71 (0.18)	2.93 (0.30)	3.40 (0.40)
No. of private alleles		8	4	2	3	4	10	20
Private allelic richness standardized for 10 isolates		0.99 (0.24)	0.84 (0.25)	0.37 (0.20)	0.49 (0.28)	0.62 (0.36)	1.40 (0.30)	1.78 (0.43)

N = number of isolates.
n_a = observed number of alleles.
a Alleles in bold are unique for each species.
b Populations one, two and three as defined in STRUCTURE, and all isolates of *N. parvum*.

alleles amongst the 14 *N. kwambonambiense* individuals, 15 alleles amongst the 35 *N. umdonicola* individuals and 38 alleles in the *N. parvum* populations (Table 2). Only one allele was shared among all four species. Private alleles were identified in each of these groups: twenty private alleles in *N. parvum*, eight in *N. cordaticola*, four in *N. kwambonambiense*, and two in *N. umdonicola* (Table 2). Population three of *N. parvum* displayed the highest private allelic richness (1.40) and *N. umdonicola* the lowest (0.37).

The mean total gene diversity across all isolates was the highest in *N. parvum* (Hnb = 0.579). Moderate gene diversity was observed in *N. cordaticola* (Hnb = 0.451) and low gene diversity in *N. kwambonambiense* (Hnb = 0.281) and *N. umdonicola* populations (Hnb = 0.214). The trends were similar when a correction for sample size was applied. All four species displayed significant linkage disequilibrium for all the pairwise comparisons (data not shown).

Six clusters were identified using STRUCTURE analyses with no prior population knowledge assumed (Fig 2A, B). Results were similar when using the entire dataset or the clone-corrected dataset, and using a model allowing admixture or not. Fig 2 presents the clustering on the clone-corrected dataset using an admixture model. The identified clusters supported the species distinctions recognized by Pavlic et al. (2009a,b) based on multiple gene sequence genealogies, as shown by the peak at $K = 4$ (Fig 2B), and they distinguished added subdivision in *N. parvum*, as shown by the peak at $K = 6$ (Fig 2B). Isolates of each of the three species, *N. cordaticola*, *N. kwambonambiense* and *N. umdonicola*, were grouped in three different clusters, showing no further substructure within populations of these species. The three remaining clusters contained only *N. parvum* isolates (Fig 2A) representing a subdivision of this species into three sub-populations. The PCoA analyses showed the same clustering that separated the four species (Fig 3A, B). Further separation of *N. parvum* isolates into three subgroups was also supported in the PCoA analyses (Fig 3C).

Genotypic diversity of N. parvum, *N. parvum sub-population one and* N. umdonicola

Twenty-three genotypes were observed among 48 isolates of *N. parvum* from *S. cordatum* in South Africa (Supplementary Fig 1A). The three geographically defined populations of *N. parvum* encompassed a total of 41 isolates represented by 17 genotypes, four of which were from the Kwambonambi (KWM), nine from the Pietermaritzburg (PTM) and eight from the Tzaneen (TZ) populations (Supplementary Fig 1B–D). These three geographically defined populations do not correspond to the three genetically distinct groups (sub-populations) identified in the Bayesian analyses. The genotypic diversity of the *N. parvum* populations was higher for populations PTM (Hnb = 0.532) and TZ (Hnb = 0.517) than KWM (Hnb = 0.066). The low diversity in the KWM population was due to the predominance of a single genotype (S14), which accounted for 67 % of the isolates collected in the Kwambonambi area (10 of 15 isolates). Genotype S14 was the only genotype shared between populations of *N. parvum* from Kwambonambi and Pietermaritzburg, and three genotypes (S9, S11 and S12) were shared between the PTM and TZ populations. There were no genotypes shared between KWM and TZ populations.

From the AMOVA analysis that was applied to *N. parvum* dataset, the highest fraction of genetic variability (96 %) was within the populations and only 4 % among the populations. Similarly, in the *N. parvum* sub-population one dataset, the highest fraction of variability was within populations (69 %) and 31 % ($p = 0.011$) of variability was among the geographic populations. When clustering specimens from Kwambonambi and Pietermaritzburg together in a region and against two other regions made up of specimens from (i) Tzaneen and (ii) Port Saint John's and Gonubie, the 'among regions' variance was 43 % and significant ($p = 0.022$), the 'among populations within regions' variance was 5 % (not significant, 'ns') and the 'within populations' variance was 52 % ($p = 0.015$).

AMOVA analysis of *N. umdonicola* populations showed that the 'among populations' variance was 9 % (ns) and the 'within populations' variance was 91 %. When grouping Kosi Bay, Mkhuze and Sodwana Bay in a region and against two other regions made up of specimens from (i) Kwambonambi and (ii) Port Saint John's and Gonubie, the 'among regions' variance was 32 %, 'among populations' variance was 0 % and the 'within populations' variance was 68 %, although none of these were statistically significant. The IbD tests were not significant for *N. umdonicola*, *N. parvum* and sub-population one of *N. parvum* ($p = 0.27$, $p = 0.45$, $p = 0.10$ respectively).

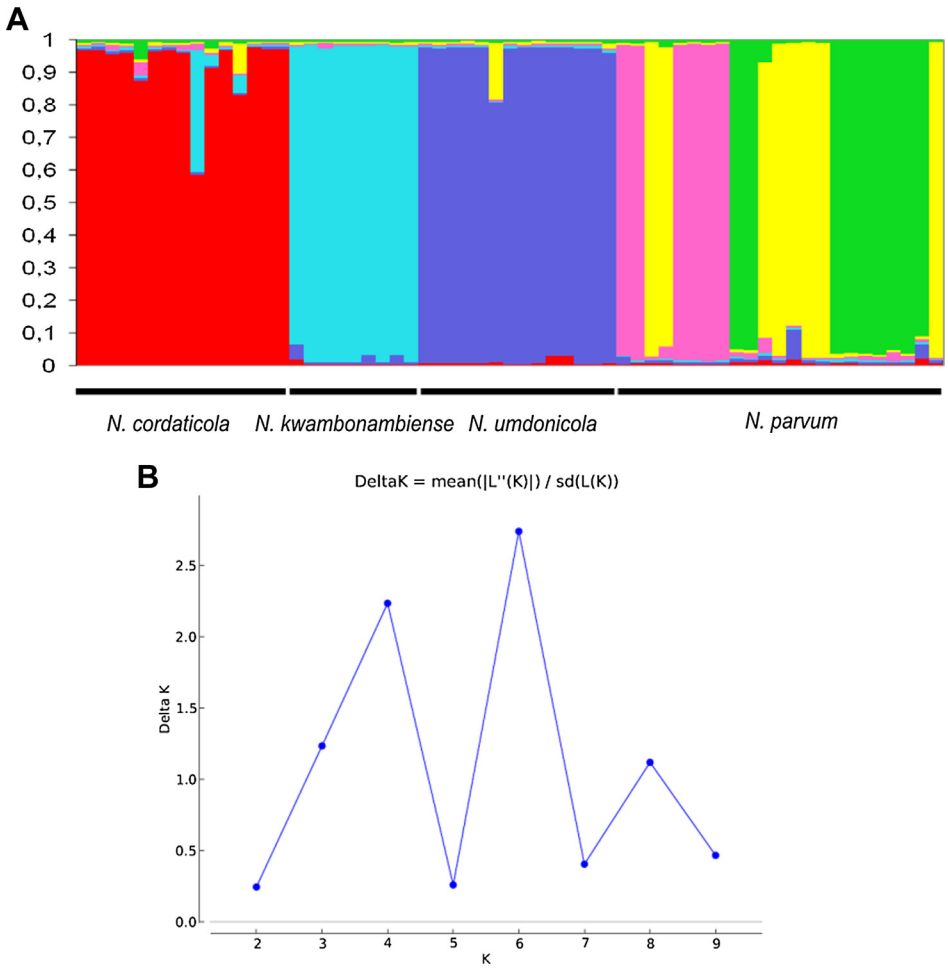

Fig 2 – The clustering outcome from STRUCTURE analyses of the clone-corrected dataset of all isolates at $K = 6$ (A). Clusters are related to each of four *Neofusicoccum* species, *N. cordaticola*, *N. kwambonambiense*, *N. umdonicola* and *N. parvum* as indicated underneath the outcome. Note that isolates of *N. parvum* are distributed in three clusters indicated by pink, yellow and green. These clusters do not correspond to geographically defined groups of *N. parvum*; Evanno's graph representing the most likely number of clusters (*K*) (B).

Distribution of Neofusicoccum *spp. among habitat types*

The distribution of the four *Neofusicoccum* species on *S. cordatum* varied for the thirteen collection sites (Fig 4). *Neofusicoccum parvum* was isolated from trees in urban areas and human-disturbed sites, and was not found in undisturbed areas. Overall, *N. parvum* was the dominant *Neofusicoccum* species on *S. cordatum* in South Africa, making up 42 % of all isolates.

Discussion

The species distribution pattern emerging from this study shows *N. parvum* as dominant and most abundant in habitats disturbed by or in close contact with human activities. This is in contrast to the three other cryptic species in the *N. parvum/N. ribis* complex that were dominant in undisturbed stands, where *N. parvum* was not found. Furthermore, *N. parvum* populations from trees planted in urban environments were more genetically diverse than populations from human disturbed but naturally regenerated stands of *S. cordatum*, indicating structure correlated with level of disturbance. It signifies a possible influence of human activity on the composition of fungal pathogens on a native host such as *S. cordatum*, and supports the hypothesis that, as with other organisms, generalist species are better invaders in human-disturbed environments.

Isolates of *N. parvum* were assigned to three sub-populations by the STRUCTURE analysis, based on allele frequencies across the loci, with very low or no admixture among the groups. This indicates that the *N. parvum* population associated with native *S. cordatum* in South Africa is a mixture of at least three independent sources, with different ancestral origins. The origin of the three groups is, however, unclear, because they could not be assigned to specific geographic regions or hosts in this study. Furthermore, *N. parvum* populations from trees planted in urban environments were more genetically diverse than populations from disturbed stands of *S. cordatum* among plantations of non-native *Eucalyptus*, indicating biological invasion with multiple introductions as a possible scenario.

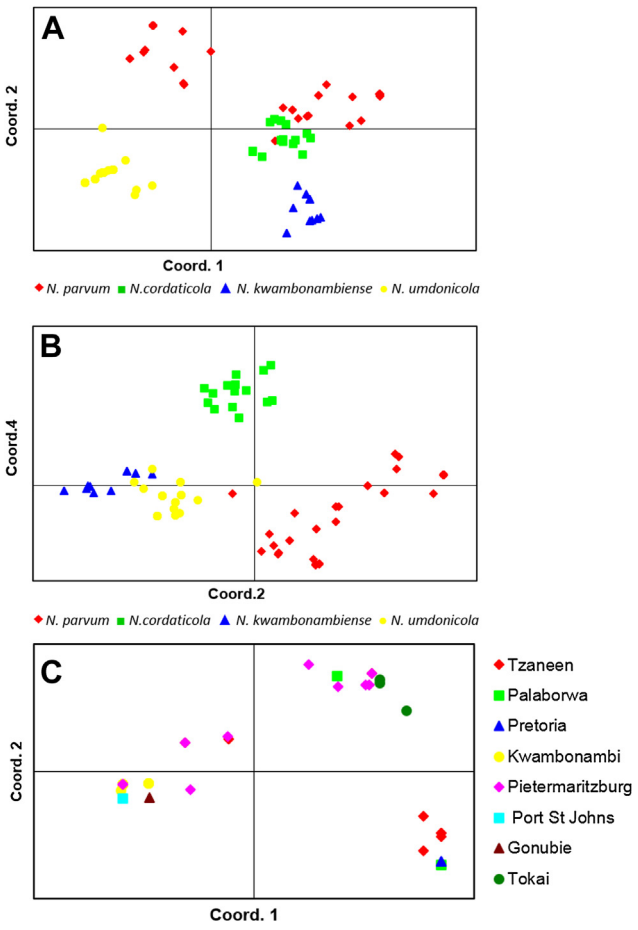

Fig 3 – Principal coordinate analyses (PCoA) of multilocus genotypes diversity for the 61 genotypes representing four different species, *N. cordaticola*, *N. kwambonambiense*, *N. umdonicola* and *N. parvum*, analyzed with seven microsatellite markers (A, B) and the twenty-three genotypes of *N. parvum* from *S. cordatum* in South Africa representing 48 isolates of *N. parvum* (C). The grouping of isolates is in accordance with clusters identified by STRUCTURE analyses (Fig 2A), indicating four species and three sub-populations within *N. parvum*.

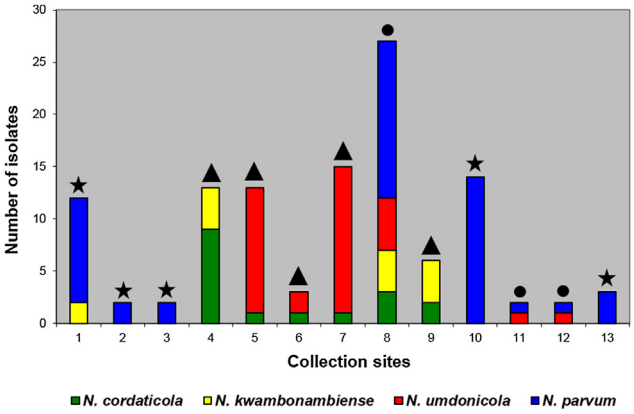

Fig 4 – Bars representing the distribution of four species from the *Neofusicoccum parvum/ribis* complex on *Syzygium cordatum* in the thirteen collection sites. Tzaneen (1P), Palaborwa (2P), Pretoria (3P), Sabie (4UD), Kosi Bay (5NU), Mkhuze (6UD), Sodwana Bay (7UD), Kwambonambi (8HD), Richards Bay (9UD), Pietermaritzburg (10P), Port St. Johns (11HD), Gonubie (12HD), Tokai (13P). P (●) = Isolates collected from planted trees in urban areas; UD (▲) = Isolates collected from trees in natural, undisturbed stands; HD (★) = Isolates collected from trees in natural, human disturbed stands.

The existence of numerous isolates with identical multilocus haplotypes, in all the different species and in particular in different populations of *N. parvum*, suggests that either asexual reproduction or a homothallic sexual cycle plays an important role in structuring these populations (Taylor et al., 1999; McDonald and Linde, 2002). Although *Neofusicoccum* species produce both sexual and asexual structures in their life cycle, these species are most commonly encountered in the asexual state, which might suggest that asexual reproduction is the main cause of the identical haplotypes. Sexual structures are known for *N. parvum*, but they have not been identified for *N. cordaticola*, *N. kwambonambiense* and *N. umdonicola*. There have been no studies considering whether they are exclusively outcrossing or alternatively, whether they can also reproduce homothallically. Since mode of reproduction plays an important role in population structure and diversity, future studies should interrogate this question. The relatively small population sizes did not allow us to further analyze such questions for these species. However, all four species analyzed in this study displayed significant linkage disequilibrium for all the pairwise comparisons. This suggests that asexual reproduction might be dominant in these fungi, as was recently suggested for Australian and South African populations of *N. parvum* from a variety of hosts (Sakalidis et al., 2013).

Distribution of the four species in the *N. parvum/N. ribis* complex across *S. cordatum* displayed clear differences on trees in environments affected by human activity or those in isolated natural stands. In most areas studied, at least two *Neofusicoccum* species coexisted on *S. cordatum*, with one of the species typically dominant. For example, *N. cordaticola* was the dominant species in the Sabie area of the Mpumalanga Province. In contrast, this species was present only in low numbers in the KwaZulu-Natal Province, co-existing either with *N. kwambonambiense* or *N. umdonicola*, and it was not present in any other area. The Sabie area is at high altitude and has a climate very different to other areas sampled, with a very different floral composition surrounding the collection site (Mucina and Rutherford, 2006). In contrast, *N. umdonicola* was the dominant species at the three isolated collection sites in the National Reserves in northern KwaZulu Natal, including Sodwana Bay, Kosi Bay and Mkhuze, which are at low altitude with a subtropical climate and surrounded by undisturbed native flora. The dominance of one species in a particular niche might be related to ecological specialization (Devictor et al., 2010) of each species to defined environmental conditions.

Neofusicoccum parvum is a known generalist that infests various woody hosts around the world (Pennycook and Samuels, 1985; Slippers and Wingfield, 2007; De Wet et al., 2008; Sakalidis et al., 2013). This was also the dominant

species in the *N. parvum/N. ribis* complex isolated from *S. cordatum* in the present study. With the exception of two isolates of *N. kwambonambiense*, only *N. parvum* was ever found on planted *S. cordatum* in urban areas. In contrast, *N. parvum* was not found in the isolated natural stands of *S. cordatum*, even though the number of sampled trees from which isolates of the four *Neofusicoccum* spp. were recovered was much higher in the natural stands than in urban areas. This effect was despite the close geographical proximity and climatic similarity of regions where other *Neofusicoccum* spp. are common. The results suggest that *N. parvum* is not native on *S. cordatum* in this region and that it is spreading from some non-native hosts to native *S. cordatum*, rather than *vice versa*. The other three species in the *N. parvum/N. ribis* complex isolated in this study were recently recorded from other hosts and countries (Sakalidis et al., 2013). However, in South Africa *S. cordatum* is the only known host of *N. cordaticola* and *N. umdonicola*, while *N. kwambonambiense* also occurs on the native tree species *Terminalia sericea* (Sakalidis et al., 2013). The area of origin of these fungi thus remains difficult to determine.

The genotypic diversity among the isolates of *N. parvum* differed between the populations from *S. cordatum* from natural stands within *Eucalyptus* plantations in the Kwambonambi area and two populations from planted *S. cordatum* in urban areas (Pietermaritzburg and Tzaneen). A low level of genotypic diversity and dominance of one multilocus genotype was observed in the *N. parvum* population from the Kwambonambi area. This population was from naturally growing *S. cordatum* that remained amongst non-native *Eucalyptus* plantations, also residing in the Myrtaceae that dominate this area. *Neofusicoccum parvum* is the most common species of Botryosphaeriaceae found in non-native *Eucalyptus* plantations in South Africa (Slippers et al., 2004b; Maleme, 2008). Some of the genotypes identified in the Kwambonambi population from *S. cordatum* were identical to *N. parvum* genotypes identified on *Eucalyptus* in this area (Sakalidis et al., 2013). These results strongly support the hypothesis that this pathogen spreads between these two hosts and that proximity to non-native *Eucalyptus* shaped the population structure of *N. parvum* on *S. cordatum* in this area. Interestingly *N. parvum* is rare on *Eucalyptus* in the native range of the tree in Australia, and the fungus is most likely native on some other host and geographic origin, which has yet to be determined (Slippers et al., 2004b; Burgess et al., 2005; Sakalidis et al., 2013).

In contrast to the *N. parvum* sub-population one that comprises Kwambonambi population, two populations from planted *S. cordatum* trees in Pietermaritzburg and Tzaneen (sub-clusters two and three, respectively) exhibited high levels of genotypic diversity with some genotypes overlapping between them. This was despite the fact that these two sites are more than 600 km apart. *Neofusicoccum parvum* isolates having different genotypes, but with the same ancestral origin (as defined by STRUCTURE), were also found on planted *S. cordatum* in other areas distant from each other, such as Pretoria and Tokai. The *S. cordatum* trees in urban environments are surrounded by various known hosts of *N. parvum*, such as *Eucalyptus*, *Vitis vinifera* and *Mangifera indica* and many ornamental plants (Jacobs, 2002; Van Niekerk et al., 2004; Slippers et al., 2004b; Sakalidis et al., 2013), which could have provided a source of this fungus. Thus, multiple introductions through human activities and movement between hosts would be common in these areas and could have influenced the genetic diversity and population structure of *N. parvum* on the planted *S. cordatum* in urban areas. Such movement of pathogens, in particular generalists such as *N. parvum*, could result in a population continuum over large areas, where gene flow among isolates might serve to maintain similar populations even at distant locations (McDonald and Linde, 2002).

Introduction of plants into non-native areas, changes in land use and intensive forestation are some of the human activities that directly influence plant pathogen movement as well as interactions with their hosts. The patterns of distribution of *N. parvum*, *N. cordaticola*, *N. kwambonambiense* and *N. umdonicola* on *S. cordatum* in different areas, ranging from isolated natural stands to environments where they have been affected by human activity provides vivid examples of this influence. This study shows that focusing on a fungal plant pathogen in only one habitat, natural forest, plantation or an urban planting, could overlook the dynamic relationship involved in invasive migration. It also highlights the importance of characterizing cryptic and population diversity of plant pathogens where one seeks to understand these patterns. More importantly, it also suggests that some (cryptic) fungal species are better adapted to colonization of disturbed environments than others. As with other organisms, this might also make them better invaders (Desprez-Loustau et al., 2007; Lee and Gelembiuk, 2008) and more important pathogens on a variety of hosts over larger areas (Slippers and Wingfield, 2007). A more complete understanding of such phenomena would inform quarantine and management options for phytopathogenic fungi.

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SUPPLEMENTARY SECTION

Table 1 Isolates analysed in this study

Culture no. [1, 2]	Other no. [3]	Tree no. [4]	Identity	Geographic origin [5]	Haplotype locations [6]	Haplotype host [7]
CMW 14128		1	*Neofusicoccum parvum*	Tzaneen, Northern Province	TZ1	S1
CMW 14129		2	*N. parvum*	Tzaneen, Northern Province	TZ2	S2
CMW 14130		3	*N. parvum*	Tzaneen, Northern Province	TZ3	S3
CMW 14133		4	*N. parvum*	Tzaneen, Northern Province	TZ4	S4
CMW 14134		5	*N. parvum*	Tzaneen, Northern Province	TZ5	S5
CMW 14135		6	*N. parvum*	Tzaneen, Northern Province	TZ6	S6
CMW 14136		7	*N. kwambonamniense*	Tzaneen, Northern Province	TZ7	S7
CMW 14137		8	*N. parvum*	Tzaneen, Northern Province	TZ8	S8
CMW 14138		9	*N. parvum*	Tzaneen, Northern Province	TZ3	S3
CMW 14139		10	*N. parvum*	Tzaneen, Northern Province	TZ9	S9
CMW 14140	CBS 123641	11	*N. kwambonamniense*	Tzaneen, Northern Province	TZ10	S10
CMW 14141		11	*N. parvum*	Tzaneen, Northern Province	TZ5	S5
CMW 14142		1	*N. parvum*	Phalaborwa, Northern Province	PB1	S3
CMW 14143		1	*N. parvum*	Phalaborwa, Northern Province	PB2	S11
CMW 14144		1	*N. cordaticola*	Sabie, Mpumalanga Province	K1	S12
CMW 14145		1	*N. cordaticola*	Sabie, Mpumalanga Province	K2	S13
CMW 14146		1	*N. cordaticola*	Sabie, Mpumalanga Province	K3	S14
CMW 14147		1	*N. cordaticola*	Sabie, Mpumalanga Province	K2	S13
CMW 14148		2	*N. cordaticola*	Sabie, Mpumalanga Province	K4	S15
CMW 14149		2	*N. cordaticola*	Sabie, Mpumalanga Province	K5	S16
CMW 14150		2	*N. cordaticola*	Sabie, Mpumalanga Province	K2	S13
CMW 14151	CBS 123637	3	*N. cordaticola*	Sabie, Mpumalanga Province	K6	S17
CMW 14152		3	*N. cordaticola*	Sabie, Mpumalanga Province	K7	S18
CMW 14153		4	*N. kwambonamniense*	Sabie, Mpumalanga Province	K8	S10
CMW 14154		4	*N. kwambonamniense*	Sabie, Mpumalanga Province	K9	S19
CMW 14155	CBS 123642	5	*N. kwambonamniense*	Sabie, Mpumalanga Province	K10	S7
CMW 14156		5	*N. kwambonamniense*	Sabie, Mpumalanga Province	K10	S7
CMW 27901		1	*N. parvum*	Pretoria, Gauteng Province	P1	S20
CMW 29125		1	*N. parvum*	Pretoria, Gauteng Province	P1	S20
CMW 14055		1	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M1	S21
CMW 14056	CBS 123635	1	*N. cordaticola*	Kosi Bay, KwaZulu Natal Province	M2	S22
CMW 14057		2	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M3	S23
CMW 14058	CBS 123645	3	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M4	S24
CMW 14059		4	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M5	S25
CMW 14060	CBS123646	5	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M6	S26
CMW 14061		6	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M7	S27
CMW 14062		7	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M3	S23
CMW 14068		8	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M5	S25
CMW 14098		9	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M8	S28
CMW 14099		10	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M9	S29
CMW 14100		11	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M10	S30
CMW 14101		12	*N. umdonicola*	Kosi Bay, KwaZulu Natal Province	M3	S23
CMW 14047		1	*N. umdonicola*	Mkuze, KwaZulu Natal Province	CF1	S31
CMW 14051		2	*N. umdonicola*	Mkuze, KwaZulu Natal Province	CF2	S27
CMW 14054	CBS 123636	3	*N. cordaticola*	Mkuze, KwaZulu Natal Province	CF3	S32
CMW 13990		1	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW1	S30
CMW 13991		1	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW1	S30
CMW 13992	CBS 123634	1	*N. cordaticola*	Sodwana Bay, KwaZulu Natal Province	SW2	S33
CMW 13993		2	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW1	S30
CMW 13994		2	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW3	S25
CMW 13995		2	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW3	S25
CMW 13996		2	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW4	S27
CMW 13997		2	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW5	S34
CMW 14006		3	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW4	S27
CMW 14007		3	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW6	S35
CMW 14008		4	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW7	S28
CMW 14106	CBS 123644	4	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW8	S36
CMW 14010		5	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW7	S28
CMW 14011		5	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW9	S26
CMW 14012		6	*N. umdonicola*	Sodwana Bay, KwaZulu Natal Province	SW4	S27

Table 1 Continued

Culture no. [1, 2]	Other no. [3]	Tree no. [4]	Identity	Geographic origin [5]	Haplotype locations [6]	Haplotype host [7]
CMW 14016		1	*N. umdonicola*	Kwambonambi, KwaZulu Natal Province	KWM1	S25
CMW 14018		2	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM2	S37
CMW 14019		2	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM3	S38
CMW 14020		2	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM4	S39
CMW 14021		3	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM2	S37
CMW 14022		4	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM2	S37
CMW 14023	CBS 123639	5	*N. kwambonamniense*	Kwambonambi, KwaZulu Natal Province	KWM5	S40
CMW 14024	CBS 123638	6	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM2	S37
CMW 14025	CBS 123640	7	*N. kwambonamniense*	Kwambonambi, KwaZulu Natal Province	KWM6	S41
CMW 14027		7	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM2	S37
CMW 14028		8	*N. umdonicola*	Kwambonambi, KwaZulu Natal Province	KWM7	S27
CMW 14030		9	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM8	S42
CMW 14031		10	*N. kwambonamniense*	Kwambonambi, KwaZulu Natal Province	KWM9	S43
CMW 14032		11	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM2	S37
CMW 14035		11	*N. cordaticola*	Kwambonambi, KwaZulu Natal Province	KWM10	S44
CMW 14036		12	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM2	S37
CMW 14037		12	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM3	S38
CMW 14038		12	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM3	S38
CMW 14039		12	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM2	S37
CMW 14040		13	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM2	S37
CMW 14041		14	*N. cordaticola*	Kwambonambi, KwaZulu Natal Province	KWM11	S45
CMW 14042		14	*N. cordaticola*	Kwambonambi, KwaZulu Natal Province	KWM12	S46
CMW 14045		15	*N. parvum*	Kwambonambi, KwaZulu Natal Province	KWM2	S37
CMW 14046		16	*N. kwambonamniense*	Kwambonambi, KwaZulu Natal Province	KWM5	S40
CMW 14125		17	*N. umdonicola*	Kwambonambi, KwaZulu Natal Province	KWM7	S27
CMW 14126		17	*N. umdonicola*	Kwambonambi, KwaZulu Natal Province	KWM7	S27
CMW 14127	CBS 123648	17	*N. umdonicola*	Kwambonambi, KwaZulu Natal Province	KWM13	S47
CMW 14119		1	*N. kwambonamniense*	Richards Bay, KwaZulu Natal Province	R 1	S48
CMW 14120		1	*N. kwambonamniense*	Richards Bay, KwaZulu Natal Province	R 1	S48
CMW 14121		1	*N. kwambonamniense*	Richards Bay, KwaZulu Natal Province	R 2	S49
CMW 14122		2	*N. cordaticola*	Richards Bay, KwaZulu Natal Province	R 3	S50
CMW 14123	CBS 123643	3	*N. kwambonamniense*	Richards Bay, KwaZulu Natal Province	R 4	S51
CMW 14124		3	*N. cordaticola*	Richards Bay, KwaZulu Natal Province	R 5	S52
CMW 14081		1	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM1	S37
CMW 14082		1	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM2	S9
CMW 14084		2	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM1	S37
CMW 14085		3	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM3	S53
CMW 14086		3	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM4	S2
CMW 14087		3	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM5	S54
CMW 14088		3	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM6	S55
CMW 14089		4	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM7	S56
CMW 14090		4	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM1	S37
CMW 14091		4	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM8	S1
CMW 14092		5	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM1	S37
CMW 14093		6	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM7	S56
CMW 14094		6	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM9	S57
CMW 14095		7	*N. parvum*	Pietermaritzburg, KwaZulu Natal Pravince	PM1	S37
CMW 14096		1	*N. umdonicola*	Port St Johns, Eastern Cape Province	SJP1	S27
CMW 14097		1	*N. parvum*	Port St Johns, Eastern Cape Province	SJP2	S39
CMW 14079	CBS 123647	1	*N. umdonicola*	Gonubie, Eastern Cape Province	G1	S35
CMW 14080	CBS123651	1	*N. parvum*	Gonubie, Eastern Cape Province	G2	S58
CMW 14110		1	*N. parvum*	Tokai, Western Cape Province	T1	S59
CMW 14111		1	*N. parvum*	Tokai, Western Cape Province	T2	S60
CMW 14112		1	*N. parvum*	Tokai, Western Cape Province	T3	S61

[1, 3] Abbreviations of culture collections: CMW = Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands

[2] Isolates identified in this study are in boldface.

[4] Tree numbers from which isolates were collected at each of sites

[5] All isolates were collected from *S. cordatum* trees in different provinces of South Africa by D. Pavlic-Zupanc

[6] Different haplotypes identified in each location

[7] Different haplotypes (S1-S61) identified among all 114 isolates from *S. cordatum* trees