Chapter 4

Cryptic diversity and distribution of species in the *Neofusicoccum parvum / N. ribis* complex as revealed by microsatellite markers

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ABSTRACT

Delineation of cryptic species by molecular identification tools is drastically changing our view of fungal species diversity and distribution. For example, the *Neofusicoccum parvum* / *N. ribis* species complex (Botryosphaeriaceae, Ascomycetes) was thought to consist of two sister species, but genealogical concordance species recognition has led to the recent delineation of five cryptic sibling species in this complex. Their cryptic nature and the small number of isolates available in previous studies has, however, led to questions regarding the distinction, diversity and distribution of *N. cordaticola*, *N. kwambonambiense*, *N. umdonicola* and *N. parvum* on native *Syzygium cordatum* trees. Microsatellite markers were thus used to investigate inter- and intra-species genetic diversity and structure amongst 114 isolates in this complex from across the distribution of *S. cordatum* in South Africa. The microsatellite data support the fact that four distinct species exist sympatrically on this host. The distribution of these species on *S. cordatum* shows very clear structure across the *S. cordatum* distribution, with *N. parvum* isolates being dominant and most abundant in areas influenced by humans, and absent in isolated natural stands of these trees. *Neofusicoccum parvum* populations from *S. cordatum* in disturbed environments were also structured, with those from trees growing alongside stands of non-native *Eucalyptus* less genetically diverse than trees planted in urban environments. These results suggest a strong influence of human activity on the composition of *Neofusicoccum* species on *S. cordatum* and that cross infections between native and non-native plants are important in structuring the diversity of these fungi.

INTRODUCTION

The availability and improvement of molecular tools have led to the identification of numerous cryptic species in the fungal kingdom. The ability to efficiently identify such cryptic species has consequently substantially changed our understanding of species diversity and distribution (Taylor et al 2006, Bickford et al 2007). This is also true for many plant pathogenic fungi affecting economically important crops. These fungi might, however, have the ability to also infect surrounding native vegetation. To fully understand their ecological role, diversity and distribution these pathogens must not be viewed separately from those occurring on plants in the surrounding natural ecosystems.

The *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, de Wet et al 2008). These fungi have been recorded on cultivated, economically important non-native fruit and forestry trees (Slippers et al 2004b, 2007, Mohali et al 2007) and also on trees in native ecosystems (Slippers et al 2005, Burgess et al 2005, Pavlic et al 2007). They are also commonly isolated as endophytes that reside in asymptomatic 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.

Species that belong to the *Neofusicoccum parvum* / *N. ribis* complex were the most abundant species of Botryosphaeriaceae isolated from the native tree, *Syzygium cordatum* (Myrtaceae) across its native range in South Africa (Pavlic et al 2007). In that study, *N. parvum sensu lato* and *N. ribis s. l.* were identified based on ITS sequence comparison and PCR-RFLP profiles. In more recent studies, three undescribed species from this host were discovered using a Genealogical Concordance Phylogenetic Species Recognition (GCPSR) approach based on multiple locus sequence data (Pavlic et al 2009a). These were described as the phylogenetic species *N. cordaticola, N. kwambonambiense* and *N. umdonicola* (Pavlic et al 2009b). These three cryptic species are known only from *S. cordatum* in South Africa, while *N. parvum* has been found on many different hosts in the country, including native *S. cordatum* (Pavlic et al 2007), closely related to *Eucalyptus* in non-native plantations (Slippers *et al*. 2004b) and on the unrelated mango (Jacobs 2002).

A limited number of isolates was considered in GCPSR of *N. cordaticola, N. kwambonambiense, N. umdonicola* and *N. parvum* on *S. cordatum* by Pavlic et al (2009a),

which leaves a number of questions regarding the distribution and interaction of these species unanswered. The small number of fixed single nucleotide polymorphisms (SNPs) in sequenced loci in that study also demands further evidence to support distinction of the phylogenetic species. Considering the sub-clades observed in the *N. parvum* clade in combined multi-gene genealogies, the question as to whether other cryptic species also exist in this group has been raised. In order to clarify these questions, verification using an additional molecular tool is required.

Simple Sequence Repeat (SSR) or microsatellite markers are frequently applied in population genetic studies on a variety of fungal species. However, microsatellite markers have also been utilised to provide an additional molecular tool to be used in the delineation of closely related human pathogenic fungi, which had initially been recognised as phylogenetic species based on GCPSR (e.g. Fisher et al 2000, Taylor and Fisher 2003, Matute et al 2006). In the case of the Botryosphaeriaceae, Burgess et al (2001) distinguished morphotypes of *D. sapinea* with microsatellite markers. Two of these morphotypes were later shown to represent the distinct species, *Diplodia pinea* and *D. scrobiculata*, by de Wet et al (2003) using GCPSR. In these studies, the microsatellite markers were useful to type strains and species, in support of GCPSR, because they can easily be applied to large numbers of isolates, they are reproducible and they often reveal more diversity than sequence analyses alone.

Microsatellite markers have previously been developed for Botryosphaeriaceae with *Fusicoccum*-like anamorphs, which include *Neofusicoccum* species (Slippers et al 2004a). In this study, we use these markers to: (i) test the GCPSR based hypothesis of Pavlic et al (2009a) regarding the coexistence of four cryptic species, *N. parvum*, *N. cordaticola, N. kwambonambiense* and *N. umdonicola*, in the *N. parvum* / *N. ribis* complex on *S. cordatum* in South Africa, (ii) analyse their inter- and intra-species genetic structure and diversity and (iii) map their geographical distribution on *S. cordatum* trees in natural stands and in undisturbed sites or areas disturbed by human activity such as trees growing along non-native *Eucalyptus* plantations or those planted 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 *Syzygium cordatum* in different geographical locations of South Africa, between

February 2001 and March 2003 (TABLE I, II, FIG. 1). *Syzygium cordatum* trees do not grow in persistent forests, but rather in patches or as solitary trees and sampling areas were defined accordingly. Of the 114 isolates used in this study, 81 were collected from natural stands of *S. cordatum* that are isolated in natural reserves or are growing alongside *Eucalyptus* plantations (8 sites) and 33 isolates were collected from planted *S. cordatum* in urban areas (4 sites) (TABLE I, II, FIG. 1). Between 1 and 45 trees were sampled from each site. From each tree, isolations were made from dying twigs and, visually healthy twig and leaf tissues as described by Pavlic et al (2007). All isolates were identified in previous studies to belong to the *N. parvum / N. ribis* species complex including *N. parvum* (48 isolates), *N. cordaticola* (17), *N. kwambonambiense* (14) and *N. umdonicola* (35), based on DNA sequence data of at least one locus and PCR-RFLP analysis (Pavlic et al 2007, 2009a, b). All 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 1000 mL deionised 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 marker. 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 of the overlap of fragment sizes 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 Bayesian clustering algorithm in the program STRUCTURE version 2.2 (Pritchard et al 2000, Falush et al 2003) 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 genotypes indicate that they are admixed. To determine the most likely number of genetically distinct groups or clusters (*K*) in the sample, 20 independent runs of $K = 1-10$ were carried out at 100 000 Markov chain Monte Carlo (MCMC) repetitions following a burn-in of 20 000 iterations. The program was run assuming 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 taken using the highest mean log-likelihood of *K*. The analyses were run with a clone-corrected data set where only one of each of the genotypes was included in the analyses.

The program POPGENE version 1.31 (Yeh et al 1999) was used to calculate allele frequencies at each microsatellite locus and to estimate genetic diversity across all loci 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 unique alleles, number and percentage of polymorphic loci (P) and mean genetic diversity across all loci (H), which was calculated as $H = 1-\Sigma x_k^2$, where x_k is the frequency of the k^{th} allele (Nei 1973), were evaluated.

Three geographically defined populations of *N. parvum* collected in South Africa from *S. cordatum* in Kwambonambi (KWM), Pietermaritzburg (PTM) and Tzaneen (TZ) were further analysed. For each isolate, a data matrix of multistate characters was composed by assigning a different letter to each allele at each of 7 loci (e.g. ABDCEFB), and the total

number of multilocus genotypes across the dataset was determined. Each genotype was assigned a unique number and genotypic diversity (G) was calculated using the equation $G = n / n - 1 (1 - \Sigma p_i^2)$ where p_i is the observed frequency of the i^{th} genotype and n is the number of individuals sampled in the population (Stoddart and Taylor 1998). To compare genotypic diversity (G) between populations, the maximum percentage of genotypic diversity was obtained using the equation $\hat{G} = G/N \times 100$, where N is the population size (Chen et al 1994). Isolates with the same genotype were considered to be clones and only one representative of each genotype was included in the analyses.

The analysis of molecular variance (AMOVA) was carried out using software GENALEX version 6.1 (Peakall and Smouse 2006). We examine the partitioning of genetic variation among and within the three geographically defined (Kwambonambi, Pietermaritzburg and Tzaneen) populations of *N. parvum*. Analysis was performed on clone-corrected datasets, where only one representative of each genotype was included in the analysis, to prevent over-representation of alleles in frequently occurring clones.

RESULTS

Genetic structure and diversity

Six clusters were identified using STRUCTURE analyses with no prior population knowledge assumed. The identified clusters supported the species distinctions recognized by Pavlic et al (2009a, b) based on multiple gene sequence genealogies, and they distinguished added subdivision in *N. parvum*. Isolates of each of the three species, *N. cordaticola, N. kwambonambiense* and *N. umdonicola*, were grouped in three different clusters (1–3), showing no further substructure within populations of these species. The three remaining clusters (4–6) contained only *N. parvum* isolates (FIG. 2) representing subdivision of this species into three sub-populations.

For the entire dataset of 114 isolates that belong to *N. parvum* / *N. ribis* complex from native *S. cordatum,* a total of 61 alleles were observed across seven loci examined (TABLE III). There were 26 alleles detected amongst the *N. cordaticola* individuals, 16 alleles amongst the *N. kwambonambiense* individuals, 15 alleles amongst the *N. umdonicola* individuals and 38 alleles in the *N. parvum* populations (TABLE III). Only one allele was shared among all four species. Private alleles were identified in each of these groups that represent the species. Twenty private alleles were detected in *N. parvum*, eight in *N. cordaticola,* four in *N. kwambonambiense*, and two in *N. umdonicola* (TABLE III). The mean

total gene diversity across all isolates was the highest in *N. parvum* (H = 0.57). Moderate gene diversity was observed in *N. cordaticola* (H = 0.44) and low gene diversity in *N. kwambonambiense* (H = 0.27) and *N. umdonicola* populations (H = 0.21) (TABLE III).

Genotypic diversity of *N. parvum*

Twenty-three genotypes were observed among 48 isolates of *N. parvum* from *S. cordatum* in South Africa. The three geographically defined populations of *N. parvum* encompassed a total of 41 isolates represented by 22 genotypes, four of which were from the Kwambonambi (KWM) population and nine in each of the Pietermaritzburg (PTM) and the Tzaneen (TZ) populations (TABLE IV). The maximum genotypic diversity ($\hat{G}$) of the *N. parvum* populations was moderate to high for populations PTM (62.8 %) and TZ (77.5 %) and low in the KWM population (36 %) (FIG. 3, TABLE IV). The low diversity in the KWM population was due to the predominance of a single genotype (S14), which accounted for 66 % of the isolates collected in 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 the *N. parvum* dataset, the highest fraction of variability (96 %) was within populations and only 4 % among the geographic populations.

Distribution of *Neofusicoccum* spp. on *S. cordatum*

The occurrence of the four *Neofusicoccum* species on *S. cordatum* varied significantly for the twelve collection sites (FIG. 4). In ten of the twelve areas, one of the species was dominant (FIG. 4). *Neofusicoccum parvum* was the only species identified in Pietermaritzburg and it was also the dominant species in Tzaneen and Kwambonambi. In Tzaneen, *N. kwambonambiense* was found together with *N. parvum*. Kwambonambi was the only area where all four species co-exist. *Neofusicoccum cordaticola* was the dominant species in the Sabie area, and it was found together with *N. kwambonambiense*. The same composition as that in the Sabie area was found in Richards Bay, but with *N. kwambonambiense* being the dominant species. Two areas close to the Indian Ocean, Kosi Bay and Sodwana Bay had the same species compositions, with *N. umdonicola* dominant and found together with *N. cordaticola* (FIG. 4). Overall *N. parvum* was the dominant *Neofusicoccum* species on *S. cordatum* in South Africa making up 42 % of all isolates.

DISCUSSION

Application of microsatellite markers clearly supported the earlier discovery (Pavlic et al 2009a) that four sister species, *N. parvum*, *N. cordaticola, N. kwambonambiense* and *N. umdonicola*, co-exist within the *N. parvum* / *N. ribis* complex on native *S. cordatum* trees in South Africa. Intriguingly, this study also showed that there is a strong correlation between the distribution of the cryptic species in the *N. parvum* / *N. ribis* complex on *S. cordatum* and human disturbance. Thus, at sites disturbed by or in close contact with human activities, *N. parvum*, a generalist and serious pathogen with a wide geographic distribution (Slippers and Wingfield 2007, de Wet et al 2008), was dominant. This is in contrast to the three other cryptic species in the *N. parvum* / *N. ribis* complex that were dominant in natural, undisturbed stands of *S. cordatum*.

Pavlic et al (2009a) discovered four sympatric cryptic species in the *N. parvum* / N. *ribis* complex, using GCPSR based on DNA sequence data for five nuclear loci. An important outcome of the present work was the fact that the microsatellite data were in concordance with those previous findings. Private alleles were observed in each of the four *Neofusicoccum* spp., which supported their distinction in STRUCTURE. The concordant phylogenies indicated by the private microsatellite alleles, together with the fixed SNPs observed in the five sequenced loci (Pavlic et al 2009a), provide evidence for the absence of recombination amongst these alleles between the groups. These fungi not only occur sympatrically at a larger spatial scale, but in some cases inhabit the same tree or even a single leaf (Pavlic at al 2009b). A sexual state is also known in the *N. parvum* / *N. ribis* complex and the absence of recombination is, therefore, interpreted as being due to reproductive isolation over long period (Fisher et al 2000, Taylor et al 2000).

These four species identified here and by Pavlic et al (2009) also shared alleles at all loci examined. Most of the identical alleles were shared between two species and only one was shared among all four species. The occurrence of shared alleles has also been recognized in other closely related species such as in the human pathogens *Coccidioides immitis* and *C. posadasii* (Fisher et al 2002). However, the congruent phylogenies obtained using microsatellite and gene sequence data for the latter species suggest that the shared alleles between the two taxa were a result of mutational convergence or ancestral shared origin of the alleles, and not due to interbreeding (Fisher et al 2000, 2002, Taylor et al 2000). This is consistent with the interpretation of the results in the present study. The microsatellite

markers used in this study are evidently useful as a part of an integrative approach in studies on speciation and delimitation of cryptic species in the Botryosphaeriaceae.

This study on the *N. parvum* / *N. ribis* complex was focused on the single species *S. cordatum* and in a single country. More species diversity is to be expected in this group on other hosts and in other areas. For example, isolates of an undescribed *Neofusicoccum* sp., was recently identified within *N. parvum* / *N. ribis* complex from the ancient *Wollemia nobilis* and *Araucaria cunninghamii* growing in Australia and New Zealand (Slippers et al 2005). More work is needed to confirm that this is a distinct species, but patterns of variation indicated that this is most likely the case. To fully understand patterns of diversity in these fungi additional studies on native species in additional areas will be needed.

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 (Coppin et al 1997, Turgeon 1998) Although *Neofusicoccum* species produce both teleomorph and anamorph 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 and since mode of reproduction plays an important role in population structure and diversity, future studies should interrogate this question. The small population sizes did not allow us to further analyse these questions for these species.

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 *N. parvum* population associated with native *S. cordatum* in South Africa is a mixture of at least tree 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, Genetic variation identified by microsatellites and subdivision of *N. parvum* isolates into three sub-populations was consistent with significant sequence variation observed in a previous study among *N. parvum* isolates based on multiple gene sequence data (Pavlic et al 2009a). However, there was no concordance between phylogenies from multiple gene genealogies and GCPSR to further separate these groups into species. Furthermore, in this study the highest fraction of

genetic variability (96 %) was within the populations and only 4 % among the populations of *N. parvum*, which is expected for populations of the same species (Linde et al 2002, Grünig et al 2006).

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 absent from any other area. The Sabie area is at high altitude and has a climate very different to other areas sampled and with a very different floral composition surrounding the collection site (Mucina and Rutherford 2006). In contrast, *N. umdonicola* was the dominant species in the three isolated collection sites in the National Reserves in northern KwaZulu Natal including Sodwana Bay, Kosi Bay and Makuze, 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 local adaptation 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). 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 contrast, *N. parvum* was not found in the isolated natural stands of *S. cordatum*. 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 to the region on *S. cordatum* and that this species is spreading from non-native hosts to native *S. cordatum*, rather than *vice versa*.

The genotypic diversity among the isolates of *N. parvum* differed between the population from *S. cordatum* from natural stands along *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 derives from naturally grown *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). Interestingly, it is rare on *Eucalyptus* in its native range in Australia and likely originates from another host and geographic origin (Slippers et al 2004b, Burgess et al 2005). Some of the genotypes identified in the Kwambonambi population from *S. cordatum* were identical with *N. parvum* genotypes identified on *Eucalyptus* in this area (authors, unpublished). This finding strongly supports 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.

In contrast to the Kwambonambi population, two populations from planted *S. cordatum* trees in Pietermaritzburg and Tzaneen, exhibit high levels of genotypic diversity with some genotypes overlapping between them, despite the fact that these two sites are more than 600 kilometers apart. *Neofusicoccum parvum* isolates with 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 the Tokai. The *S. cordatum* trees in urban environments are surrounded by various known host of *N. parvum*, such as *Eucalyptus*, *Vitis vinifera* and *Mangifera indica* (Jacobs 2002, van Niekerk et al 2004, Slippers et al 2004b) and many ornamental plants that could harbor this species. 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*. Such movement of pathogens, in particular generalists such as *N. parvum*, could result in a population continuum over a large area, where gene flow among isolates might serve to maintain similar populations even at distant locations (McDonalds and Linde 2002).

Introduction of plants into non-native areas, changes in land use and intense 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, from isolated natural stands to environments where they have been affected by human activity provide vivid examples of this influence. This study illustrates the importance of considering surrounding native tree communities in studies that seek to understand fungal tree pathogens of importance to forestry and agriculture, and *vice versa*. Our results also

provide a foundation for future studies to characterize the biology and temporal changes of members of *N. parvum* complex in native and human disturbed environments.

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TABLE I. Isolates analysed in this study

Culture no. [1]	Other no. [2]	Identity	Geographic origin [3, 4]
CMW14056	CBS123635	*Neofusicoccum cordaticola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14035		*N. cordaticola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14041, 14042		*N. cordaticola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14054	CBS123636	*N. cordaticola*	SA, KwaZulu Natal Province, Mkuzi
CMW14122		*N. cordaticola*	SA, KwaZulu Natal Province, Richards Bay
CMW14124	CBS123638	*N. cordaticola*	SA, KwaZulu Natal Province, Richards Bay
CMW14144-14150		*N. cordaticola*	SA, Mpumalanga Province, Sabie
CMW14151	CBS123637	*N. cordaticola*	SA, Mpumalanga Province, Sabie
CMW14152		*N. cordaticola*	SA, Mpumalanga Province, Sabie
CMW13992	CBS123634	*N. cordaticola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14023	CBS123639	*N. kwambonambiense*	SA, KwaZulu Natal Province, Kwambonambi
CMW14046		*N. kwambonambiense*	SA, KwaZulu Natal Province, Kwambonambi
CMW14025	CBS123640	*N. kwambonambiense*	SA, KwaZulu Natal Province, Kwambonambi
CMW14031		*N. kwambonambiense*	SA, KwaZulu Natal Province, Kwambonambi
CMW14119-14121		*N. kwambonambiense*	SA, KwaZulu Natal Province, Richards Bay
CMW14123	CBS123643	*N. kwambonambiense*	SA, KwaZulu Natal Province, Richards Bay
CMW14153, 14154		*N. kwambonambiense*	SA, Mpumalanga Province, Sabie
CMW14155	CBS123645	*N. kwambonambiense*	SA, Mpumalanga Province, Sabie
CMW14156		*N. kwambonambiense*	SA, Mpumalanga Province, Sabie
CMW14140	CBS123641	*N. kwambonambiense*	SA, Northern Province, Tzaneen
CMW14136		*N. kwambonambiense*	SA, Northern Province, Tzaneen
CMW14079	CBS123647	*N. umdonicola*	SA, Eastern Cape Province, Gonubie
CMW14055		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14057		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14058	CBS123645	*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14098, 14099		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14059		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14060	CBS123646	*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14100, 14101		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14061, 14062		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14068		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14028		*N. umdonicola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14016		*N. umdonicola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14125, 14126		*N. umdonicola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14127	CBS123648	*N. umdonicola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14047		*N. umdonicola*	SA, KwaZulu Natal Province, Mkuze
CMW14051		*N. umdonicola*	SA, KwaZulu Natal Province, Mkuze
CMW13993-13997		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14007		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14106	CBS123644	*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14008		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14010		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW13990, 13991		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14006		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14011, 14012		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14096		*N. umdonicola*	SA, KwaZulu Natal Province, Port St Johns
CMW14080	CBS123651	*Neofusicoccum parvum*	SA, Eastern Cape Province, Gonubie
CMW14018-14022		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14024		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14027		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14032		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14036-14040		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14045		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14030		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14081, 14082		*N. parvum*	SA, KwaZulu Natal Province, Pietrmaritzburg
CMW14084		*N. parvum*	SA, KwaZulu Natal Province, Pietrmaritzburg
CMW14085	CBS123649	*N. parvum*	SA, KwaZulu Natal Province, Pietrmaritzburg
CMW14086-14095		*N. parvum*	SA, KwaZulu Natal Province, Pietrmaritzburg
CMW27901		*N. parvum*	SA, Gauteng Province, Pretoria

TABLE I. Continued

Culture no. [1]	Other no. [2]	Identity	Geographic origin [3, 4]
CMW29125		*N. parvum*	SA, Gauteng Province, Pretoria
CMW14097	CBS123650	*N. parvum*	SA, Eastern Cape Province, Port St Johns
CMW14110-14112		*N. parvum*	SA, Western Cape Province, Tokai
CMW14128		*N. parvum*	SA, Northern Province, Tzaneen
CMW14137-14139		*N. parvum*	SA, Northern Province, Tzaneen
CMW14141, 14142		*N. parvum*	SA, Northern Province, Tzaneen
CMW14143	CBS123652	*N. parvum*	SA, Northern Province, Tzaneen
CMW14129, 14130		*N. parvum*	SA, Northern Province, Tzaneen
CMW14133-14135		*N. parvum*	SA, Northern Province, Tzaneen

[1, 2] 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.

[3] SA = South Africa.

[4] All isolates were collected from *S. cordatum* by D. Pavlic.

TABLE II. Distribution of isolates of *Neofusicoccum* spp. collected from *S. cordatum* in South Africa

Collection sites [1, 2]	***N. cordaticola***	***N. kwambonambiense***	***N. umdonicola***	***N. parvum*** [3]
Tzaneen (1) **P**	-	2	-	**12**
Pretoria (2) **P**	-	-	-	2
Sabie (3) **N**	9	4	-	-
Kosi Bay (4) **N**	1	-	12	-
Mkuze (5) **N**	1	-	2	-
Sodwana Bay (6) **N**	1	-	14	-
Kwambonambi (7) **N**	3	4	5	**15**
Richards Bay (8) **N**	2	4	-	-
Pietermaritzburg (9) **P**	-	-	-	**14**
Port St Johns (10) **N**	-	-	1	1
Gonubie (11) **N**	-	-	1	1
Tokai (12) **P**	-	-	-	3
Total	17	14	35	48

[1] Numbers in brackets indicate collections sites as marked on the South African map (FIG. 1).

[2] N = Isolates were collected from *S. cordatum* trees on natural stands; P = Isolates collected from the planted trees in urban areas.

[3] Numbers in bold indicate isolates considered in population analyses.

TABLE III. Allele size (bp) and frequency at 7 loci in four *Neofusicoccum* spp. collected from *Syzygium cordatum* in South Africa

Locus	*Allele*[1]	*N. cordaticola*	*N. kwambonambiense*	*N. umdonicola*	*N. parvum*
***BotF*11**	420	-	**1.000**	-	-
	427	-	-	-	**0.104**
	428	-	-	-	**0.854**
	431	0.471	-	0.400	-
	432	-	-	0.543	0.021
	433	-	-	**0.057**	-
	435	**0.471**	-	-	-
	null	0.058	-	-	0.021
***BotF*15**	365	-	-	-	**0.187**
	374	-	-	-	**0.021**
	377	1.000	-	1.000	0.646
	378	-	-	-	**0.021**
	389	-	-	-	**0.083**
	390	-	-	-	**0.042**
	395	-	**0.857**	-	-
	396	-	**0.143**	-	-
***BotF*17**	229	-	1.000	0.971	-
	233	0.117	-	-	0.104
	234	**0.882**	-	-	-
	244	-	-	-	**0.104**
	246	-	-	-	**0.083**
	247	-	-	-	**0.021**
	249	-	-	0.029	0.479
	256	-	-	-	**0.104**
	259	-	-	-	**0.104**
***BotF*21**	203	-	0.071	0.200	-
	204	0.176	0.143	0.743	0.062
	206	-	-	-	**0.021**
	207	0.118	-	-	0.187
	208	0.118	0.143	-	0.104
	209	0.059	0.071	-	0.125
	219	0.294	0.429	-	0.500
	229	-	0.071	0.057	-
	234	**0.176**	-	-	-
	null	0.059	0.071	-	-
***BotF*23**	422	-	-	0.914	0.562
	423	-	-	-	**0.021**
	424	-	-	-	**0.042**
	425	-	-	-	**0.333**
	426	0.059	0.286	-	0.042
	427	0.059	0.714	-	-
	428	0.882	-	0.057	-
	null	-	-	**0.029**	-
***BotF*35**	222	-	-	-	**0.083**
	225	0.059	-	-	0.480
	238	-	-	0.800	0.125
	239	-	-	-	**0.083**
	244	0.529	-	-	0.187
	245	-	-	0.200	0.021
	247	**0.294**	-	-	-
	253	0.059	0.429	-	-
	255	-	**0.571**	-	-
	265	-	-	-	**0.021**
	null	**0.059**	-	-	-
***BotF*37**	303	**0.412**	-	-	-
	306	**0.412**	-	-	-
	312	-	-	1.000	0.542
	313	0.059	-	-	0.417
	314	-	-	-	**0.042**
	320	0.059	1.000	-	-
	null	**0.059**	-	-	-

TABLE III. Continued

N	**17**	**14**	**35**	**48**
n_a	**26**	**16**	**15**	**38**
No. of private alleles	**8**	**4**	**2**	**20**
Polymorphic loci	**6**	**4**	**5**	**7**
P	**85.71**	**57.14**	**71.43**	**100**
H	**0.438**	**0.271**	**0.212**	**0.572**

N = number of isolates

n_a = observed number of alleles

P = percentage of polymorphic loci

H = mean gene diversity

[1] Alleles in bold are unique for each of species

TABLE IV. *Neofusicoccum parvum* genotypes as estimated from multilocus profiles generated from the 7 microsatellite loci; Genotypes were distributed among populations collected in from *S. cordatum* in three different areas in South Africa; Kwambonambi (KWM), Pietermaritzburg (PTM) and Tzaneen (TZ)

Genotype[1]	KWM	PTM	TZ
S1			1
S2			1
S3	1		
S4		1	
S5			2
S6			
S7			1
S8			3
S9		1	1
S10		1	
S11		1	1
S12		1	1
S13	3		
S14	10	5	
S15			
S16		1	
S17		1	
S18			
S19		1	
S20			
S21			
S22		1	
S23	1		
N	**15**	**14**	**12**
N(g)	**4**	**9**	**9**
G	**0.54**	**0.88**	**0.93**
Ĝ (%)	**36**	**62.8**	**77.5**

N = number of isolates

N (g) = number of genotypes

G = Genotypic diversity (Stoddart and Taylor 1988)

Ĝ = percent maximum diversity

[1] Genotypes in bold overlap between populations

FIG. 1. Map of South Africa showing sites where the isolates were collected. Tzaneen (1U), Pretoria (2U), Sabie (3N), Kosi Bay (4NR), Mkuze (5NR), Sodwana Bay (6NR), Kwambonambi (7E), Richards Bay (8N), Pietermaritzburg (9U), Port St. Johns (10N), Gonubie (11N), Tokai (12U). U = Urban area; N = Natural stand; NR = Nature Reserve; E = Naturally regenerated *S. cordatum* amongst *Eucalyptus* stands. The timber plantations areas are highlighted in red. Source: Forestry South Africa.

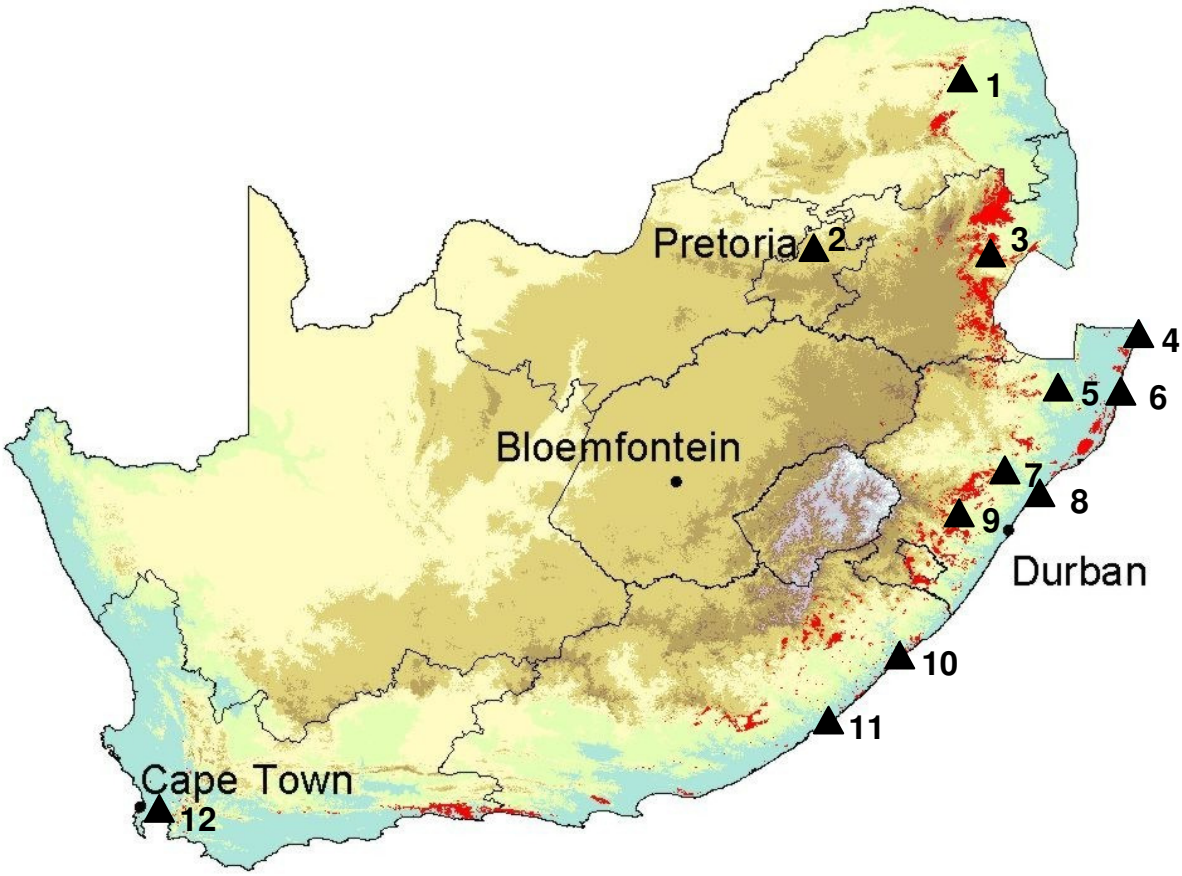

1
Pretoria
2
3
4
5
6
Bloemfontein
7
8
9
Durban
10
11
Cape Town
12

FIG. 2. The clustering outcome from STRUCTURE analyses of the clone-corrected dataset of all isolates at $K = 6$. Each color represents one cluster. Labels underneath the outcome (1–4) correspond to clusters related to each of four *Neofusicoccum* species, *N. cordaticola* (1), *N. kwambonambiense* (2), *N. umdonicola* (3) and isolates of *N. parvum* (4). Note that isolates of *N. parvum* are distributed in three clusters indicated by green, yellow and blue.

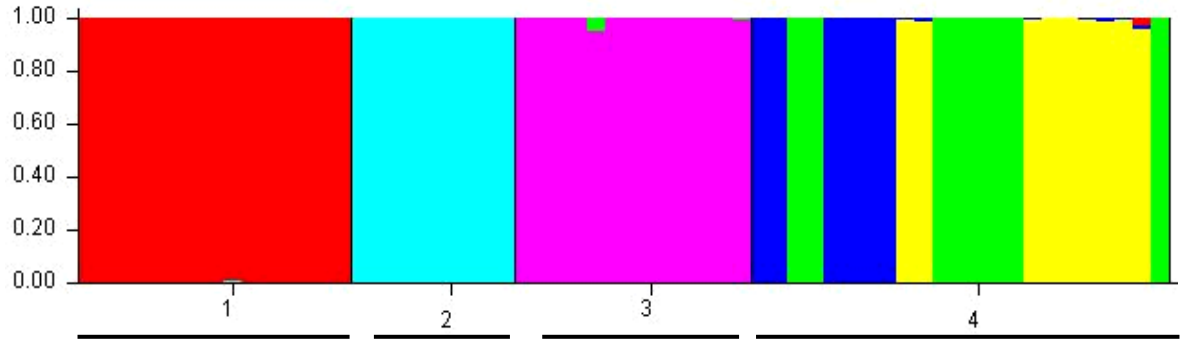
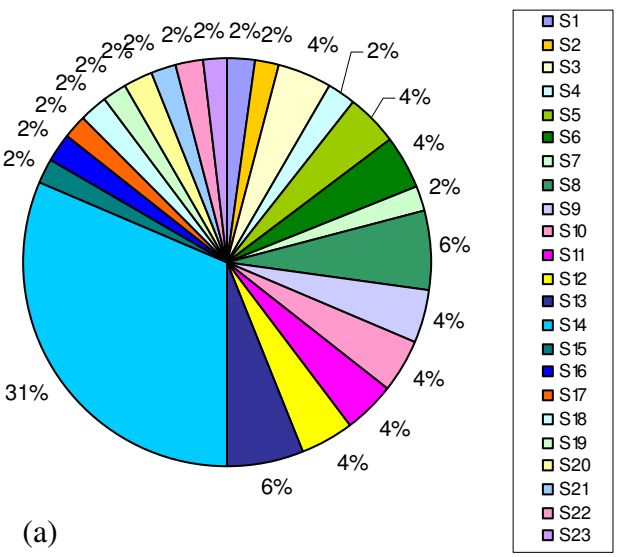

1.00
0.80
0.60
0.40
0.20
0.00
1
2
3
4

FIG. 3. Pie charts representing genotypic diversity of the *N. parvum* populations from *S. cordatum.* All South African isolates (a), the population collected from naturally regenerated trees growing amongst *Eucalyptus* plantations in the Kwambonambi (KWM) area (b), and the populations collected from planted trees in the towns of Pietermaritzburg (PTM) (c) and Tzaneen (TZ) (d). Different multilocus genotypes are indicated as S1-S23.


31%
6%
4%
4%
4%
4%
6%
2%
4%
4%
2%
2%
2%
2%
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2%
2%
2%
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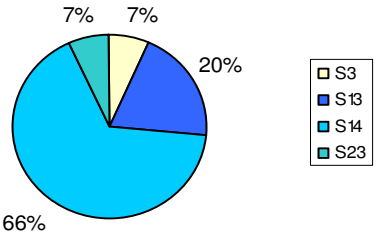
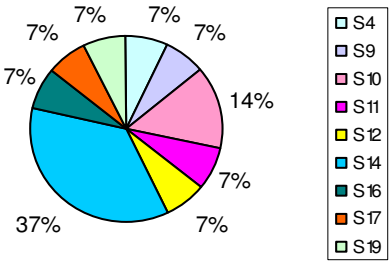
2%
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
S11
S12
S13
S14
S15
S16
S17
S18
S19
S20
S21
S22
S23

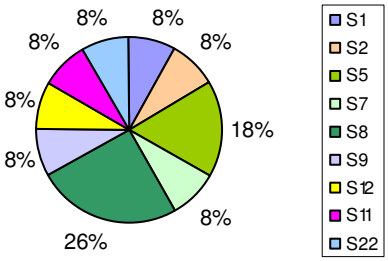
(a)


66%
20%
7%
7%
S3
S13
S14
S23

(b)

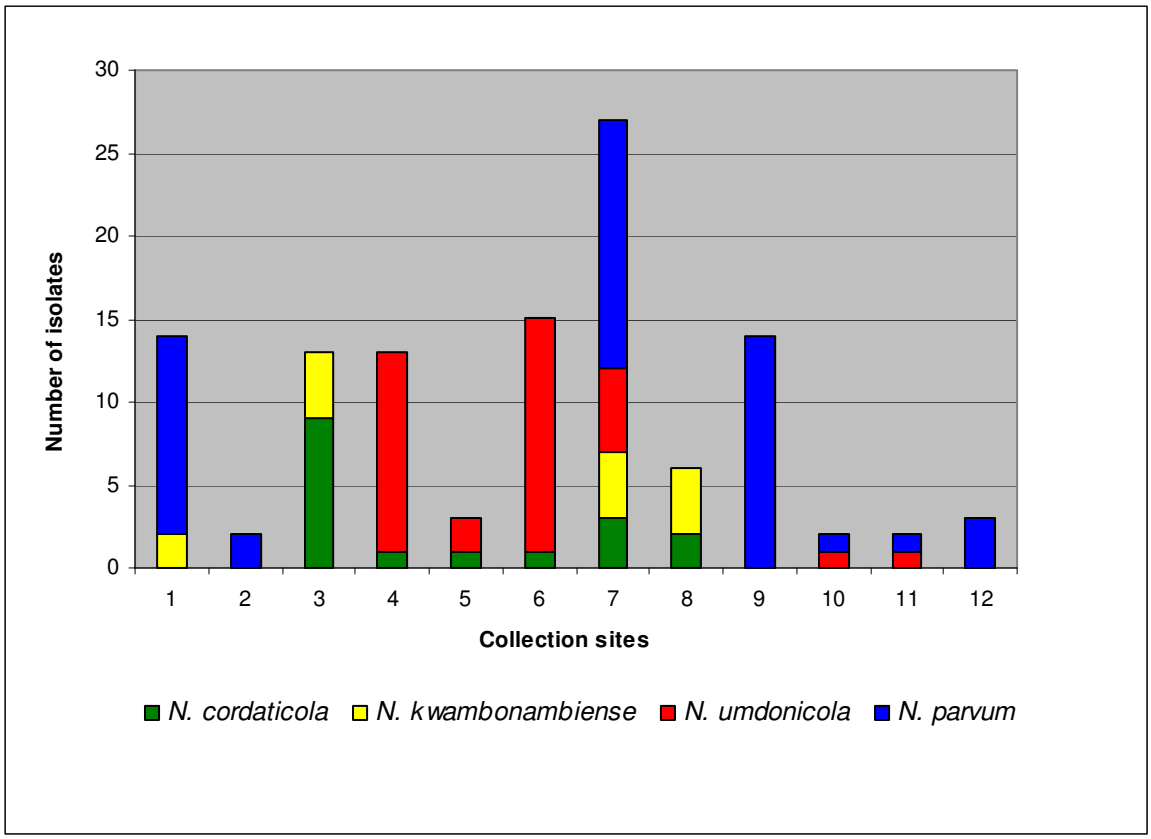

37%
14%
7%
7%
7%
7%
7%
7%
S4
S9
S10
S11
S12
S14
S16
S17
S19

(c)


26%
18%
8%
8%
8%
8%
8%
8%
8%
S1
S2
S5
S7
S8
S9
S12
S11
S22

(d)

FIG. 4. Bars representing the distribution of four species from the *Neofusicoccum parvum* / *N. ribis* complex on *Syzygium cordatum* in the twelve collection sites. Tzaneen (1U), Pretoria (2U), Sabie (3N), Kosi Bay (4NR), Mkuze (5NR), Sodwana Bay (6NR), Kwambonambi (7E), Richards Bay (8N), Pietermaritzburg (9U), Port St Johns (10N), Gonubie (11N), Tokai (12U). U = Urban area; N = Natural stand; NR = Nature Reserve; E = Naturally regenerated *S. cordatum* amongst *Eucalyptus* stands.


30
25
20
15
10
5
0
Number of isolates
1
2
3
4
5
6
7
8
9
10
11
12
Collection sites
N. cordaticola
N. kwambonambiense
N. umdonicola
N. parvum