Chapter 2

Multiple gene genealogies and phenotypic data reveal cryptic species of the Botryosphaeriaceae: A case study on the *Neofusicoccum parvum / N. ribis* complex

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ABSTRACT

Neofusicoccum parvum and *N. ribis* (Botryosphaeriaceae, Ascomycetes) are closely related, plant pathogenic fungi with a worldwide distribution on a wide range of woody hosts. Species boundaries in the *N. parvum* / *N. ribis* complex have eluded definition, despite the application of various tools for characterization. In this study, we test the hypothesis that only one species exists amongst isolates from the *N. parvum* / *N. ribis* complex, identified from *Syzygium cordatum* trees across their native distribution in South Africa. Genealogical concordance phylogenetic species recognition (GCPSR) was applied based on concordance of genealogies obtained from DNA sequence data for five nuclear loci. These data showed that the single species hypothesis must be rejected. Rather, all analyses support the existence of three previously unrecognised, cryptic species within the *N. parvum* / *N. ribis* complex from *S. cordatum*, in addition to *N. parvum* and *N. ribis*. The three lineages reflecting these cryptic taxa are sympatric across their geographical range, indicating barriers to gene flow other than geographic isolation. Phenotypic characters failed to detect all the species uncovered by the GCPSR. Sequence data of the Internal Transcribed Spacer (ITS) of the ribosomal DNA locus, which is thought to be useful for barcoding in fungi, did not distinguish all the species with confidence. RNA polymerase II subunit (RPB2) was the most informative to distinguish all the species *a posteriori* to the application of GCPSR. The results reflect the critical importance of using multiple gene genealogies and adequate sampling to identify cryptic species and to characterise the true diversity within the Botryosphaeriaceae.

INTRODUCTION

Most fungal species are identified solely based on phenotypic characters. However, morphological features used to define species might not be noticeable until well after genetic separation has occurred (Taylor et al 2006). The rapidly increasing number of taxonomic studies utilizing DNA sequence comparisons is revealing increasing numbers of cryptic fungal species and species complexes, previously identified as single morphospecies (Taylor et al 2000, Bickford et al 2006). This is especially true where the genealogical concordance phylogenetic species recognition (GCPSR), a form of phylogenetic species concept (PSC), has been applied (Taylor et al 2000). The GCPSR is based on concordance of multiple gene genealogies and has been used to study cryptic speciation in important human and plant pathogenic fungal complexes, such as *Fusarium graminearum* and *Gibberella fujikuroi* (O'Donnell et al 2000a, b, Steenkamp et al 2002), *Aspergillus flavus* and *A. fumigatus* (Geiser et al 1998, Pringle et al 2005), *Coccidioides immitis* (Koufopanou et al 1997) and others. These studies have revealed numerous previously unidentified, cryptic species.

Since molecular data have been incorporated in species separation and identification of the Botryosphaeriaceae, new sibling species have been recognized within morphologically described taxa. In some cases multiple gene sequence data, using the GCPSR (although not always explicitly stating it as such), needed to be combined with phenotypic characters to identify closely related species. For example, the GCPSR was effectively used to detect *Diplodia scrobiculata* as a sister species of *D. pinea* (de Wet et al 2003). *Neofusicoccum eucalypticola* and *N. australe,* were also identified using the GCPSR as sister species of *N. eucalyptorum* and *N. luteum*, respectively (Slippers et al 2004c, d). The cryptic species recognized in these studies were overlooked or uncertain when using morphology or single-locus sequence data alone (Denman et al 2000, Smith et al 2001, Zhou and Stanosz 2001, Pavlic et al 2007).

Neofusicoccum parvum and *N. ribis* are closely related species that belong to the Botryosphaeriaceae (Ascomycetes, Botryosphaeriales) (Crous et al 2006). *Neofusicoccum ribis* was originally described from *Ribes* spp. in New York, USA as "*Botryosphaeria*" *ribis* (Grossenbacher and Duggar 1911), while *Neofusicoccum parvum* was described from Kiwifruit and a *Populus* sp. in New Zealand as "*Botryosphaeria*" *parva* (Pennycook and Samuels 1985). Both of these species were subsequently identified as pathogens on numerous woody hosts worldwide (Punithalingam and Holliday 1973, Slippers et al 2004a, Mohali et al 2007, Pavlic et al 2007). These fungi are known to have both sexual (teleomorph) and asexual (anamorph) stages in their life cycle, but they are most commonly

encountered as anamorphs. Sexual reproduction in these species is still unexplored and little is known regarding their mating strategy. *Neofusicoccum parvum* and *N. ribis* overlap in the morphological characteristics of their teleomorphs and anamorphs that were used for their original descriptions, making all subsequent identifications difficult and unreliable (Grossenbacher and Duggar 1911, Pennycook and Samuels 1985). The uncertainty regarding their identification was seemingly resolved when *N. parvum* and *N. ribis* were characterised based on multiple gene phylogenies combined with phenotypic characters (Slippers et al 2004b). However, this study was based on a few ex-type and other isolates related to the types of each species. In subsequent phylogenetic analyses, where more isolates were included from larger numbers of hosts and locations, the distinction between these species became less clear (Farr et al 2005, Slippers et al 2005, Pavlic et al 2007). It thus appears to be inadequate to rely only on ex-type specimens of *N. parvum* and *N. ribis* to represent populations across the distribution of these species.

The difficulty in distinguishing *N. parvum* and *N. ribis* is illustrated by conflicting results in two related studies aimed at resolving their identity using multiple approaches. Slippers (2003) characterised a large number of isolates from different hosts and geographical regions using simple sequence repeat (SSR) markers and multiple gene DNA sequence data. In that study, these species were recognised as distinct and *sensu stricto* and *sensu lato* groups were identified for each. The *sensu lato* groups of *N. parvum* and *N. ribis* could be separated using a PCR-RFLP diagnostic tool, but not the further subdivisions of *sensu stricto* groups (Slippers 2003). In a similar study on populations of these species obtained from variety of hosts around the world, and using multiple gene DNA sequence data, SSR marker data, phenotypic characters and AFLP analysis, it was concluded that these two species could not be distinguished from each other (Sakalidis 2004). The separation of the type species was viewed as the end of a genetic continuum of populations. Both studies, however, suffered from sampling deficiencies, where some populations were undersampled, originating from different continents, and from both native and non-native hosts, where opportunities for mating were difficult to judge.

A recent study of Botryosphaeriaceae on *S. cordatum* across its native range in South Africa gave rise to a large number of isolates in the *N. parvum* / *N. ribis* complex (Pavlic et al 2007). Initial data indicated significant variation in conidial morphology and ITS rDNA sequences amongst these isolates, but without supporting a clear distinction of species. In this study we test the hypothesis that these isolates represent one species. For this purpose, we use GCPSR with multiple genes DNA sequence data for five nuclear loci. In addition, we

compare results obtained from the multilocus genealogies with a single locus approach in order to identify the most suitable loci for future recognition of cryptic species in this complex. Variation in conidial morphology, the traditional tool used to distinguish species in this group, was also compared to the GCPSR results to determine their value in species delineation.

MATERIALS AND METHODS

Fungal isolates

The 30 isolates used in this study were selected from a larger collection of 103 isolates collected during the course of a survey of the Botryosphaeriaceae on native *S. cordatum* in different geographical locations of South Africa (TABLE I). All the isolates were identified as *N. parvum* or *N. ribis sensu lato* based on PCR-RFLP analysis (Pavlic et al 2007). The 30 isolates were selected to represent the diversity observed previously in conidial morphology and ITS rDNA sequence data (Pavlic 2004, Pavlic et al 2007), as well as to represent the geographical area and different trees from which they were collected. Three isolates of each of *N. parvum* and *N. ribis* that included the ex-type specimen and two specimens linked to the ex-type were used for comparison (TABLE I). The single-conidial cultures were prepared as reported previously (Pavlic et al 2007), to ensure that only haploid genotypes were characterized for each representative culture. The collection of single-conidial strains used in this study is maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphometric analysis

In a previous study, all 103 isolates were induced to sporulate in culture and conidia were measured and characterized using light microscopy (Pavlic, 2004, Pavlic et al 2007). The lengths and widths of ten conidia were measured for each isolate and the data were analysed in this study. Averages of ten conidial measurements per isolate were calculated and used in the analyses.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from the single-conidial cultures following the modified phenol-chloroform DNA extraction method outlined in Smith et al (2001). Five different gene regions were selected for characterization, including the internal transcribed spacer

(ITS) regions 1 and 2 and the 5.8S gene of the ribosomal RNA (rRNA) (White et al 1990), the portion of gene encoding translation elongation factor 1 alfa (EF-1α) (Sakalidis 2004), Bt2 regions of the β-tubulin gene (Glass and Donaldson 1995), a portion of RNA polymerase II subunit (RPB2) (Sakalidis 2004) and locus *BotF15*, an unknown locus containing microsatellite repeats (Slippers et al 2004a). The primer sequences, their respective annealing temperatures and expected product size are presented in TABLE II. The selected regions were amplified using the polymerase chain reaction (PCR) from genomic DNA. The amplifications were performed using an Eppendorf Mastercycler PERSONAL (Perkin-Elmer, Germany) and the following protocol: 94 ºC for 2 min initial denaturation; 40 cycles of 94 ºC for 30 s, 55 or 62 ºC for 30 s, 72 ºC for 1 min; and 72 ºC for 7 min final extension. PCR products were cleaned using the High Pure PCR Product Purification kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Both strands were sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Warrington, U.K.), as specified by the manufacturer. Sequence reactions were run on an ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer, Warrington, U.K.).

The nucleotide sequences for both strands were examined with SEQUENCE NAVIGATOR version 1.0.1. (Perkin-Elmer Applied BioSystems, Inc., Foster City, California) software and alignments were done online using MAFFT version 5.667 (http://timpani.genome.ad.jp/~mafft/server/) (Katoh et al 2002). Aligned sequences for each gene region were analysed in DnaSP v. 4.00.6 (Rozas et al 2003) for nucleotide polymorphisms.

Phylogenetic analyses

To determine whether analyses of combined sequences can be conducted, statistical congruence was tested using a partition homogeneity test (PHT) (Farris et al 1995, Huelsenbeck et al 1996). The PHT was performed in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2000) using 1000 replicates and the heuristic standard search options.

Maximum-parsimony (MP) genealogies, for single genes and all five genes combined, were constructed in PAUP version 4.0b10 (Swofford 2000), using the heuristic search function with 1000 random addition replicates and tree bisection and reconstruction (TBR) selected as branch swapping algorithm. Gaps were treated as fifth characters and all characters were unordered and of equal weight. Insertions/deletions (indels), irrespective of

their size were each treated as one evolutionary event and weighted as one base substitution. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. To estimate branch support, maximum parsimony bootstrap values were determined using 1000 bootstrap replicates (Felsenstein 1985).

Bayesian analyses were performed using MrBayes v. 3.0b4 (Ronquist and Huelsenbeck 2003) for single gene data and for the combined data set of all five genes. The best-fitting evolutionary models were estimated for each gene region and for the combined data using MrModeltest v. 2.2 software (Nylander 2004). The Markov Chain Monte Carlo (MCMC) chains were initialised from a random tree and were run for 1000000 generations and trees were saved every 100 generations, counting 10000 trees. Burn in was set to 100000 generations. To determine the confidence of the tree topologies, values of Bayesian posterior probabilities (BPPs) (Rannala and Yang 1996) were estimated using MrBayes (Ronquist and Huelsenbeck 2003).

RESULTS

Morphometric analysis

Conidial lengths and widths varied significantly among the isolates. This variation was continuous and did not support any clear distinction of groups. Isolates used for DNA sequence comparisons were selected to represent the full range of conidial sizes and are indicated on the graph reflecting these data (FIG. 1).

DNA sequencing

The sequences obtained in this study have been deposited in GenBank with accession numbers as follows: ITS1, 5.8S, and ITS2 (EU821898-EU821927), EF-1α (EU821868-EU821897), β-tubulin (Bt-2a/b) (EU821838-EU821867), *BotF15* (EU821802-EU821837) and RPB2 (EU821928-EU821963). The sequence alignments and phylogenetic trees have been deposited in TreeBASE as SN3948. Polymorphic nucleotide positions observed in the five sequenced DNA regions are presented in TABLE IV.

Phylogenetic analyses

The phylogenies obtained from sequence data of the gene regions were first determined separately. MrModeltest v. 2.2 predicted appropriate evolutionary models for Bayesian analyses for each of the datasets as follows: K80 model for ITS, HKY model (Hasegawa et

al 1985) with a proportion of invariable sites (*I*) for β-tubulin, GTR model (Rodriguez et al 1990) for RPB2 and HKY model for the *BotF15* and EF-1α datasets. The topologies of trees representing all the gene regions were identical in the maximum-parsimony and Bayesian consensus analyses. Therefore, only unrooted maximum-parsimony trees are presented, with the parsimony bootstrap values and the posterior probabilities shown for well-supported branches (FIG. 2). Statistical data for individual trees are summarised in TABLE III. Five distinct groups were consistently observed, of which two correspond to *N. parvum* and *N. ribis*, while the other three groups represent distinct lineages referred to as R1, R2 and R3. The isolates from *S. cordatum* considered in this study grouped within the *N. parvum* clade (n = 14), and clades R1 (n = 5), R2 (n = 6) and R3 (n = 5).

The R3 and *N. ribis* groups were the most closely related. The three isolates of *N. ribis* (one of which is the ex-type isolate) formed a separate clade in four of the gene regions analysed, while the fifth locus (*BotF15*) contained no polymorphisms between *N. ribis* and R3 (FIG. 2). Bootstrap support and BPPs were generally low for the *N. ribis* clade except in the EF-1α dataset (FIG. 2), but each of the four gene regions contained unique fixed polymorphisms (FIG. 2, TABLE IV). Groups R1 and R2 were strongly supported in four of the five gene genealogies, except the EF-1α dataset, which had only one unique, fixed polymorphism distinguishing R1 and R2 (FIG. 2, TABLE IV). The *Neofusicoccum parvum* clade was recognised in four gene genealogies, with the exception of the β-tubulin dataset in which unique fixed polymorphisms were not identified for the *N. parvum* group (FIG. 2, TABLE IV). The phylogenies constructed based on RPB2 sequences showed the best resolution and highest support for the groups (FIG. 2), followed by the ITS rDNA sequences based genealogy.

Subsequent to individual analyses, the datasets were also analysed collectively. The partition homogeneity test for all the datasets combined indicated that there was no significant conflict among the datasets ($P \geq 0.05$) (Cunningham 1997). MrModeltest v2.2 predicted HKY model with a proportion of invariable sites (*I*) as the most appropriate evolutionary model for Bayesian analyses. Two most parsimonious trees of the same overall topology were obtained for the combined dataset (FIG. 3, TABLE III). In the phylogenetic reconstruction from this combined dataset, the same partitions observed in the individual gene genealogies were recognised. All of these were also strongly supported with bootstrap values close to or equal to100 % and posterior probabilities above 0.95 (FIG. 3).

DISCUSSION

Application of the GCPSR in this study led us to reject the hypothesis that a single variable species in the *N. parvum* / *N. ribis* complex occurs on native *S. cordatum* trees in South Africa. Analysis of five DNA sequence loci showed congruent phylogenies supporting five lineages and indicating a lack of recombination between loci amongst the lineages. The high number of shared single nucleotide polymorphisms (SNPs) and short branches in the phylogenetic trees suggest recent speciation events within the *N. parvum / N. ribis* complex. Nevertheless, the unique SNPs fixed for each of the five lineages, which were linked across all five gene regions, support their treatment as distinct species. What was previously referred to as the *N. parvum* / *N. ribis* clade, therefore, represents a species complex that contains at least five cryptic species, of which three are recognised here for the first time and designated as *Neofusicoccum* sp. R1, R2 and R3. Results of this study reflect the critical importance of using multiple gene genealogies and GCPSR to identify cryptic species and to characterise the true diversity within the Botryosphaeriaceae.

Neofusicoccum parvum and the three new phylogenetic species occur sympatrically across the native geographical range of *S. cordatum*. In addition, more than one species was identified from the same tree, apparently occupying the same niche. This raises the question as to how the genetic barriers that separate the taxa would have evolved. One hypothesis is that these species previously occurred in allopatry, or on different hosts and that they have expanded their geographical or host ranges. Alternatively, genetic barriers might have evolved in sympatry in response to ecological forces not currently known to us. Le Gac et al (2007), based on studies of *Microbotryum violaceum*, and Le Gac and Giraud (2008), after an extensive analysis of published data for various fungi, concluded that such genetic barriers frequently exist among Ascomycetes, to which the Botryosphaeriaceae also belong. This is even when they occur in sympatry and despite the absence of, or only weak, pre-zygotic mating barriers. The genetic barriers appear to be mostly post-zygotic in these fungi, and Le Gac and Giraud (2008) speculated that this is strongly influenced by some 'phylogeny-dependent' life history traits. *In vitro* mating with isolates of the Botryosphaeriaceae has not previously been achieved, making a test of these hypotheses difficult. This should be the focus of future studies if the process of evolution in the group is to be more completely understood.

The focus of this study was specifically to consider members of the *N. parvum* / *N. ribis* complex from a single native tree species occurring in a clearly defined geographical area. Previous studies on these species have considered limited numbers of isolates obtained

from various hosts, including native and non-native trees, and from different geographical regions of the world (Slippers 2003, Sakalidis 2004). In these studies *N. parvum* and *N. ribis* were either recognised as *sensu lato* groups with high levels of inter-specific and intra-specific variation (Slippers 2003) or treated as a single species (Sakalidis 2004). It is likely that the under representation of certain populations in those studies failed to reveal the concordant phylogenies between sequence data sets from different loci. Slippers (2003) recommended that species of Botryosphaeriaceae should be analyzed separately for each host and geographical area of origin due to the possibility for under-sampled, native species occurring sympatrically. The recognition of four cryptic phylogenetic species, occurring sympatrically on native *S. cordatum* supports this view.

None of our isolates from *S. cordatum* were found to represent *Neofusicoccum ribis*. This species has thus far only been confirmed from *Ribes* sp. in the USA using multiple gene phylogenies (Slippers et al 2004b). Although *N. ribis* has been reported from the other hosts and regions (Cunnington et al 2007, Mohali et al 2007) those isolates were characterized only based on the ITS sequences and their identity needs to be reconsidered. Phylogenetic species R3 is recognised in this study as the most closely related taxon to *N. ribis*. Differentiation between these two species was consistent across four gene regions with six fixed unique SNPs that distinguish them. Similarly, a recent study on Southern Hemisphere conifers based on multiple gene genealogies identified three isolates from native coniferous trees in Australia that were also more closely related to *N. ribis* than *N. parvum* (Slippers et al 2005). Four unique fixed SNPs across three gene regions distinguish these three isolates from *N. ribis*. Based on sequence comparison (data not shown) none of those isolates represent any of the phylogenetic species recognised in the present study. The number of cryptic species recognized in the *N. parvum* / *N. ribis* complex in the present study may thus increase in future when isolates from other hosts and areas are considered. It is especially important to better characterize the diversity in *N. ribis*, for which only three isolates has been confirmed thus far.

Evaluation of the single gene genealogies showed that the RPB2 gene region contains the highest number of parsimony informative characters. The RPB2 single-locus phylogeny consequently also provided the highest support for the clades or phylogenetic species. The RPB2 phylogeny was most congruent with ITS sequences and these two datasets combined were the most appropriate for delimitation of phylogenetic species in this study. The RPB2, encoding the second largest RNA polymerase subunit, with its single copy in Ascomycetes and relatively slow evolutionary rate (Liu 1999), has proven useful for

phylogenetic resolution of the Ascomycetes at different taxonomic levels (Liu 1999, Schoch et al 2006, Hofstetter et al 2007, Tang et al 2007). However, it has not been used extensively in the studies of the Botryosphaeriaceae at the species level. DNA sequence based characterisation of these fungi has most commonly been based on the ITS rDNA sequences combined with EF-1α (Luque at al 2005, Phillips et al 2005, Burgess et al 2006). Based on the data presented here, we propose that RPB2 and ITS sequences be used in combination for delimitation of species in the *N. parvum* / *N. ribis* complex in the future. Furthermore, we recommend that its utility for identification of other species of Botryosphaeriaceae should also be assessed.

The ITS rDNA sequence data has been most commonly used for DNA sequence based identification of fungi (Hajibabaei et al 2007). This locus has also been proposed as the DNA barcoding region for fungi (Nilsson et al 2006, www.allfungi.org/its-barcode.php). ITS rDNA sequence data, however, need to be used in combination with other data to delimit cryptic species. The support for the subclades obtained in phylogenetic analyses of ITS sequence data in this study was very low, leaving uncertainty as to their interpretation. Similar results have been obtained in other studies of fungi based on multiple gene genealogies, where ITS data did not provide sufficient resolution for separation of closely related species or varieties. Examples are found in *Neurospora* and *Gelasinospora* (Dettman et al 2001), the human pathogenic fungus *Cryptococcus neoformans* (Xu et al 2000) and many others. As have been discussed in previous studies (Will and Rubinoff 2004, Trewick 2007), the attempt to sort the complex task of species identification based on DNA sequences of one gene region is unlikely, especially when closely related species are considered. After the basis of the variation had been clarified using GCPSR in this study, SNPs could, however, be identified in ITS rDNA regions that would be useful for identification of cryptic species in the *N.* parvum / *N. ribis* complex.

Significant variation in conidial morphology was observed for isolates within the *N. parvum* / *N. ribis* complex from *S. cordatum*. Conidial measurements and the conidial morphology of many of the isolates differed from those in the original descriptions of *N. parvum* and *N. ribis*, suggesting that additional species could exist in this complex. This conidial morphological variation represented a continuum for the phylogenetic species recognised here using multiple gene genealogies and GCPSR. This indicates that genetically isolated species do not necessarily show divergence in character states such as conidial morphology, which is consistent for many other fungi that have been considered in a similar manner (Taylor et al 2000, Chaverri et al 2003, Dettman et al 2003, O'Donnell at al 2004).

In these studies, morphospecies were also recognised as species complexes comprising of a number of phylogenetic species when analysed using GCPSR. *A priori* selection of isolates to represent the full spectrum of the conidial variation (together with ITS sequences and geographic variation), however, proved to be useful in our study to sample representatives of different cryptic species. Observed morphological differences should thus not be underestimated for initial selection of isolates from a larger collection prior to molecular identification. This, together with molecular and ecological data, as well as adequate sampling, should be considered in combination when selecting isolates to test hypotheses regarding cryptic species in the Botryosphaeriaceae.

The common occurrence of *N. parvum sensu stricto* throughout the native distribution of *S. cordatum*, and the intraspecific genetic variation observed, suggests that this is a native fungal species. However, to address hypotheses relating to the origin of species in the *N. parvum* / *N. ribis* complex, population and phylogeographic studies are needed. The delimitation of species boundaries and diagnostic tools tested in this study provide a foundation for such further studies. Significant DNA sequence variation observed amongst *N. parvum* isolates raises questions about population differentiation or even speciation in this group. Sequence data or other more variable molecular tools, such as microsatellite markers, and extended collections is necessary to clarify the origin and distribution of this observed variability within *N. parvum*. Since the *N. parvu* / *N. ribis* species complex includes some of the most aggressive members of the Botryosphaeriaceae (Burgess et al 2005, Pavlic et al 2007), identification of variation in phenotypic characters such as pathogenicity and virulence for the newly recognized species must also be a key area for research in future.

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TABLE I. Isolates analysed in this study

Culture no. [1, 2, 3]	Other no. [1]	Identity	Geographic origin	Host
CMW13992	CBS123634	*Neofusicoccum* sp. R1	South Africa, Sodwana Bay	*Syzygium cordatum*
CMW14056	CBS123635	*Neofusicoccum* sp. R1	South Africa , Kosi Bay	*S. cordatum*
CMW14054	CBS123636	*Neofusicoccum* sp. R1	South Africa , Mkuze	*S. cordatum*
CMW14124	CBS123638	*Neofusicoccum* sp. R1	South Africa, Richards Bay	*S. cordatum*
CMW14151	CBS123637	*Neofusicoccum* sp. R1	South Africa , Sabie	*S. cordatum*
CMW14023	CBS123639	*Neofusicoccum* sp. R2	South Africa, Kwambonambi	*S. cordatum*
CMW14025	CBS123640	*Neofusicoccum* sp. R2	South Africa, Kwambonambi	*S. cordatum*
CMW14140	CBS123641	*Neofusicoccum* sp. R2	South Africa, Tzaneen	*S. cordatum*
CMW14155	CBS123642	*Neofusicoccum* sp. R2	South Africa, Sabie	*S. cordatum*
CMW14123	CBS123643	*Neofusicoccum* sp. R2	South Africa, Richards Bay	*S. cordatum*
CMW14106	CBS123644	*Neofusicoccum* sp. R3	South Africa, Sodwana Bay	*S. cordatum*
CMW14058	CBS123645	*Neofusicoccum* sp. R3	South Africa, Kosi Bay	*S. cordatum*
CMW14060	CBS123646	*Neofusicoccum* sp. R3	South Africa, Kosi Bay	*S. cordatum*
CMW14079	CBS123647	*Neofusicoccum* sp. R3	South Africa, Gonubie	*S. cordatum*
CMW14096		*Neofusicoccum* sp. R3	South Africa, Port St Johns	*S. cordatum*
CMW14127	CBS123648	*Neofusicoccum* sp. R3	South Africa, Kwambonambi	*S. cordatum*
CMW14029		*Neofusicoccum parvum*	South Africa, Kwambonambi	*S. cordatum*
CMW14082		*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14085	CBS123649	*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14087		*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14088		*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14089		*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14094		*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14097	CBS123650	*N. parvum*	South Africa, Port St Johns	*S. cordatum*
CMW14080	CBS123651	*N. parvum*	South Africa, Gonubie	*S. cordatum*
CMW14129		*N. parvum*	South Africa, Tzaneen	*S. cordatum*
CMW14135		*N. parvum*	South Africa, Tzaneen	*S. cordatum*
CMW14141		*N. parvum*	South Africa, Tzaneen	*S. cordatum*
CMW14143	CBS123652	*N. parvum*	South Africa, Palaborwa	*S. cordatum*
CMW27901		*N. parvum*	South Africa, Pretoria	*S. cordatum*
CMW9079	ICMP7933	*N. parvum*	New Zealand	*Actinidia deliciosa*
CMW9080	ICMP8002	*N. parvum*	New Zealand	*Populus nigra*
CMW9081	ICMP8003	*N. parvum*	New Zealand	*P. nigra*
CMW7772		*Neofusicoccum ribis*	USA. New York	*Ribes* sp.
CMW7773		*N. ribis*	USA, New York	*Ribes* sp.
CMW7054	CBS121.26	*N. ribis*	USA, New York	*Ribes rubrum*

[1]Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands; CMW = Forestry and Agricultural Biotechnology Institute, University of Pretoria South Africa; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand.

[2] Isolates in bold are ex-types.

[3] All isolates other than CMW 9079, CMW 9080, CMW 9081, CMW 7772, CMW 7773, and CMW 7054 were collected by D. Pavlic.

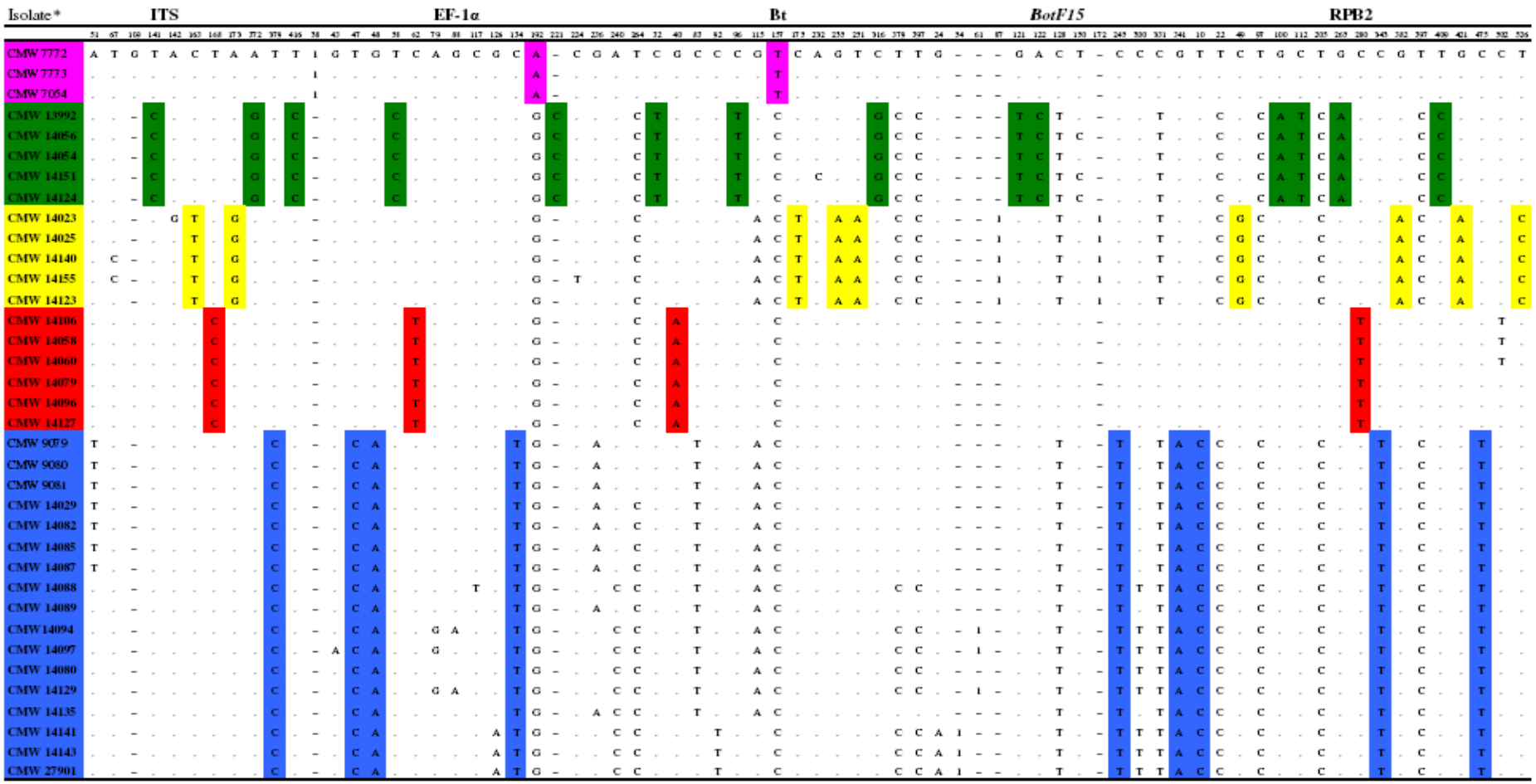
TABLE II. Primer sets used to amplify the five loci analysed in this study

Region	Oligos	Oligo Sequences	Amplicon size (bp)	AT (ºC)	Reference
ITS	ITS1	5′ TCCGTAGGTGAACCTGCGG	600	55	(White et al 1990)
	ITS4	5′ TCCTCCGCTTATTGATATGC			
EF-1α	EF-AF	5′ CATCGAGAAGTTCGAGAAGG	310	55	(Sakalidis 2004)
	EF-BR	5′ CRATGGTGATACCRCGCTC			
β-tubulin	Bt2a	5′ GGTAACCAAATCGGTGCTGCTTTC	450	55	(Glass and Donaldson 1995)
	Bt2b	5′ ACCCTCAGTGTAGTGACCCTTGGC			
RPB2	RPB2bot6F	5′ GGTAGCGACGTCACTCCC	500	55	(Sakalidis 2004)
	RPB2bot7R	5′ GGATGGATCTCGCAATGCG			
BotF15	Bot15	5′ CTGACTTGTGACGCCGGCTC	350	62	(Slippers et al 2004a)
	Bot16	5′ CAACCTGCTCAGCAAGCGAC			

TABLE III. Information on the sequence dataset and maximum parsimony (MP) trees for each locus and all five loci combined

	Locus					
	ITS	**EF-1α**	**β-tubulin**	***BotF15***	**RPB2**	**Combined all**
Total no. of alignable characters	499	286	420	376	565	2146
No. of excluded characters	0	13	0	38	0	51
Total no. of variable characters	11	17	14	13	17	72
No. of informative characters	10	14	13	13	17	67
No. of most parsimonious trees	1	1	6	1	1	2
Tree length	10	15	15	13	17	72
Consistency index (CI)	1	0.933	0.867	1	1	0.931
Retention index (RI)	1	0.989	0.979	1	1	0.989

TABLE IV. Single nucleotide polymorphisams (SNPs) from the sequence data of ITS rDNA, EF-1α, β-tubulin, *BotF15* and RPB2 loci



* Isolates in bold are obtained from *S. cordaeum*. *Neofusicoccum ribis* = pink; *Neofusicoccum* sp. R1 = green; *Neofusicoccum* sp. R2 = yellow; *Neofusicoccum* sp. R3 = red; *N. parvum* = blue. (1, –) = Insertions /deletions (indels) irrespective of their size.

FIG. 1. The averages of the lengths and widths of ten conidia measured for each of 103 isolates representing *Neofusicoccum parvum* / *N. ribis* complex from *Syzygium cordatum*. The thirty isolates used for DNA sequence comparisons in this study were selected to represent the full range of conidial sizes and are indicated on the graph as unfilled squares.

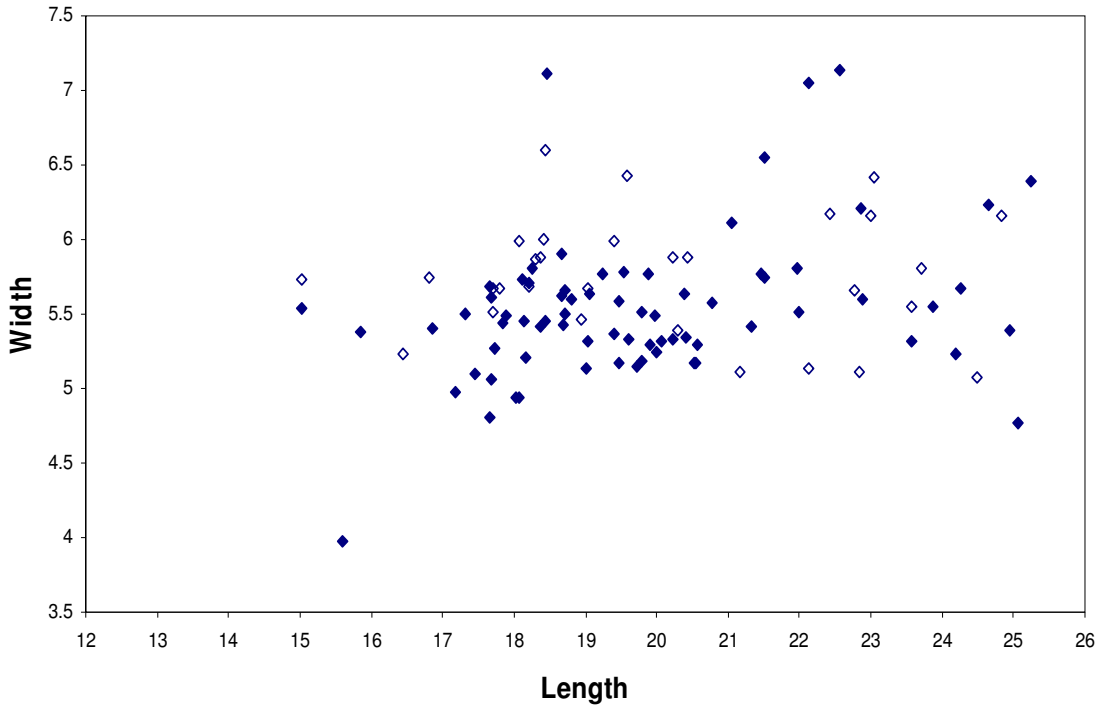
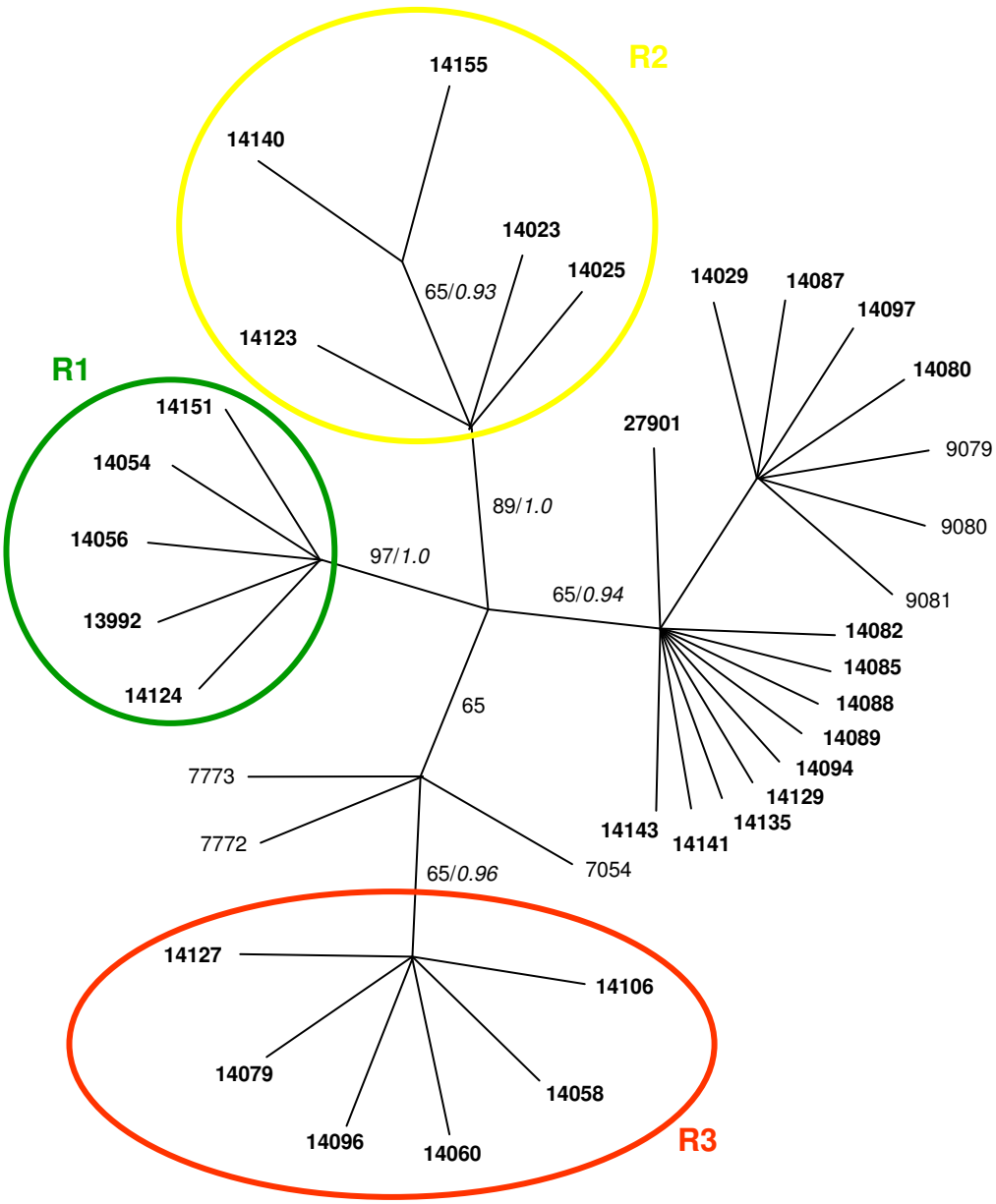
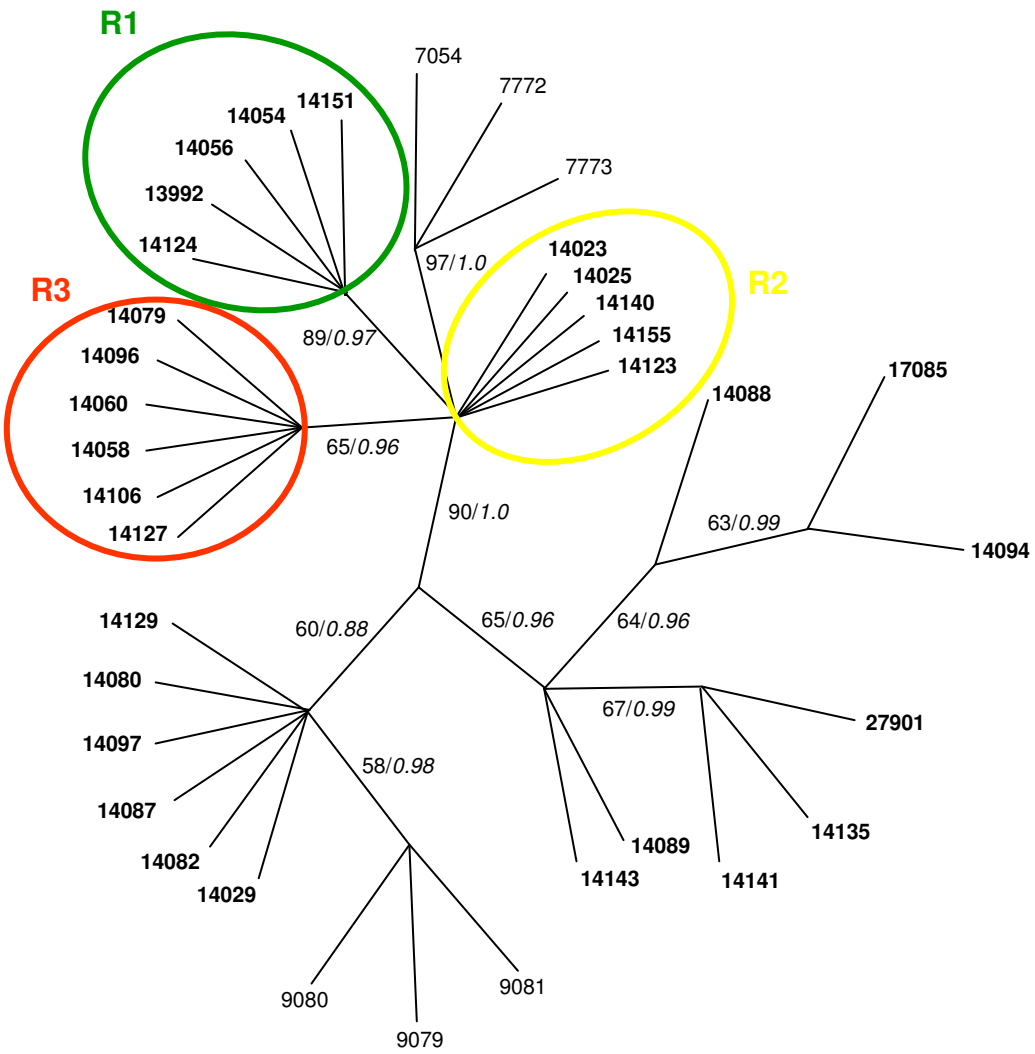

Width
Length
7.5
7
6.5
6
5.5
5
4.5
4
3.5
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26

FIG. 2. Unrooted maximum-parsimony trees resulting from the separate analysis of the sequence data of the ITS (a), EF-1α (b), Bt2 regions of the β-tubulin gene (c), locus *BotF15* (d) and RPB2 (e). Bootstrap values of maximum parsimony analyses are indicated next to the branches followed by the posterior probabilities resulting from Bayesian analysis (indicated in italics). Isolates of the *Neofusicoccum* spp. obtained from *S. cordatum* are indicated in bold.

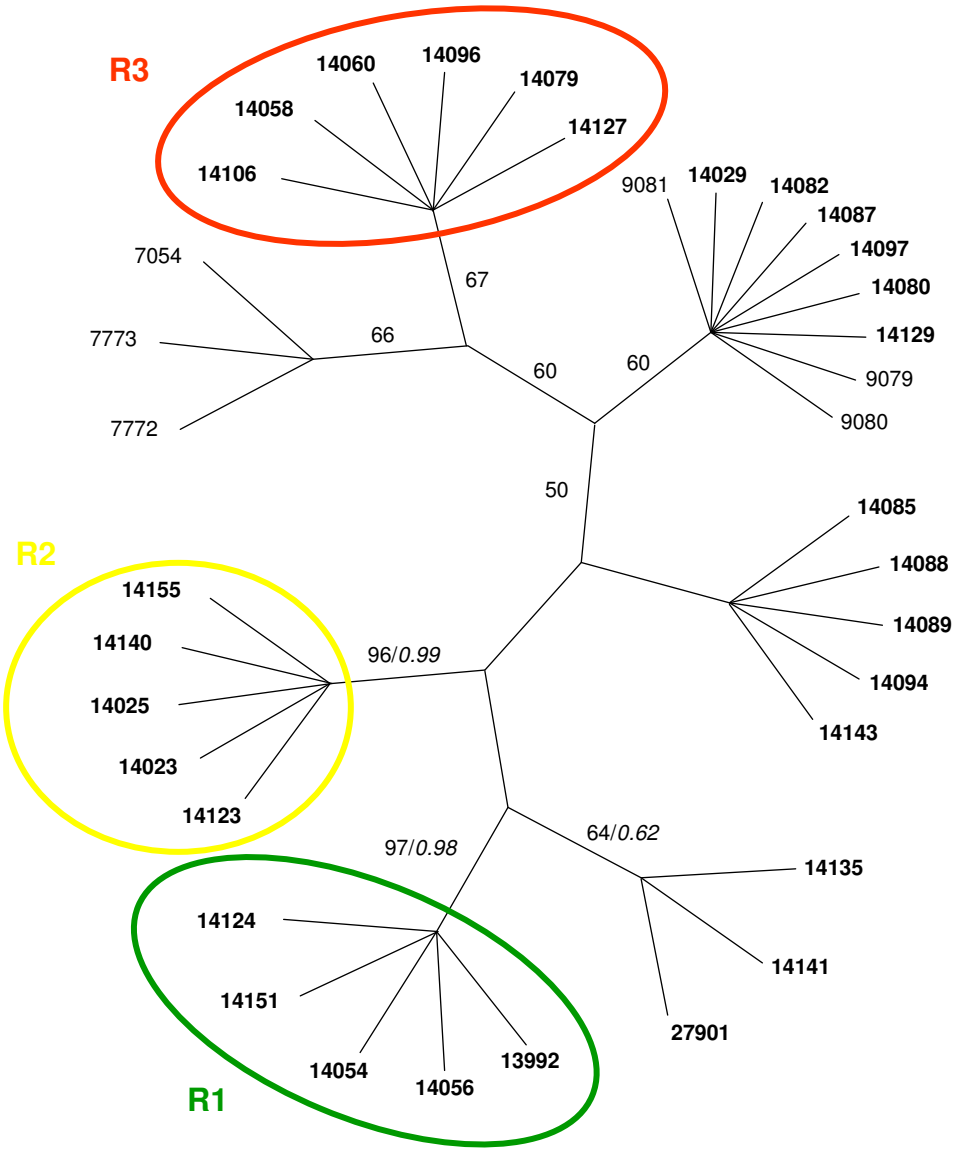
(a)


R2
14155
14140
14023
14025
65/0.93
14123
R1
14151
14054
14056
13992
14124
97/1.0
89/1.0
65/0.94
65
14029
14087
14097
14080
9079
9080
9081
27901
14082
14085
14088
14089
14094
14129
14135
14143
14141
7773
7772
7054
65/0.96
14127
14106
14079
14058
14096
14060
R3

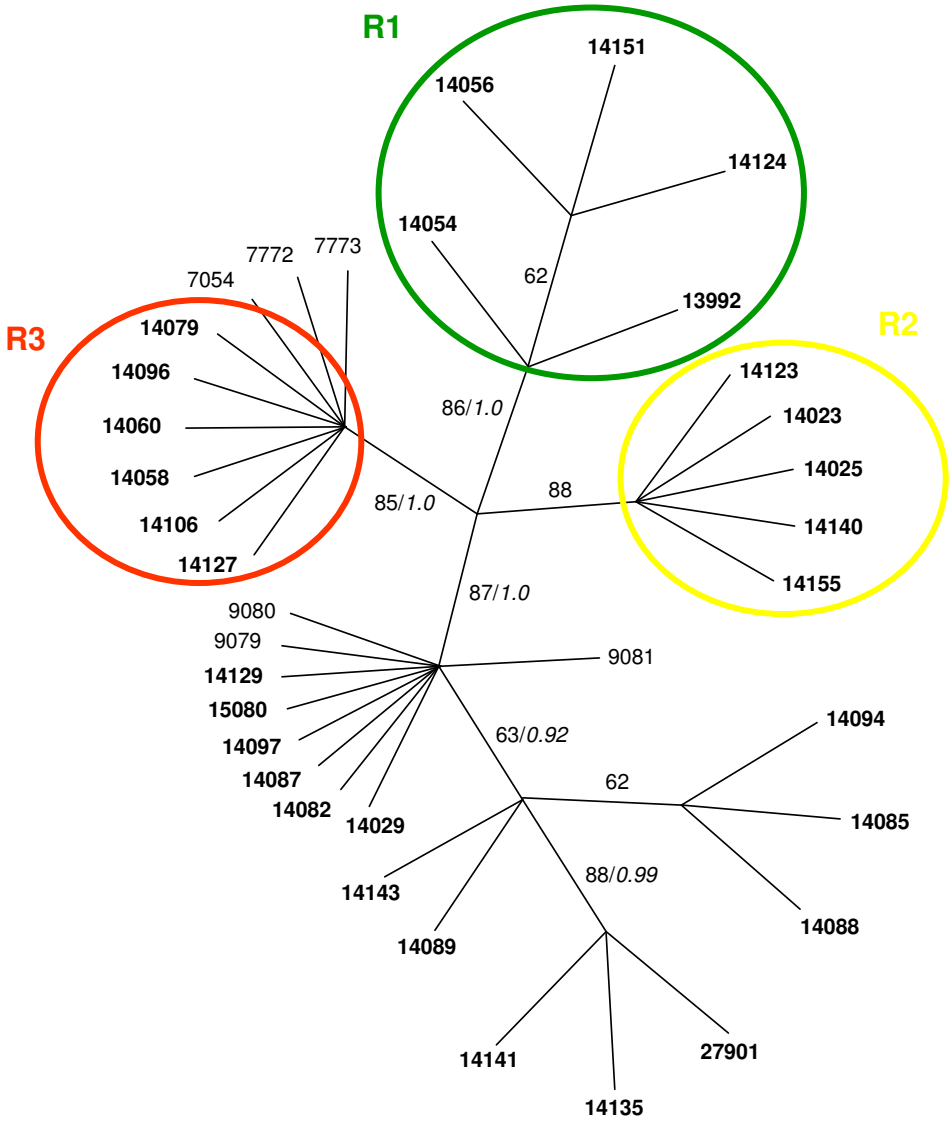
(b)


R1
R2
R3
7054
7772
7773
14151
14054
14056
13992
14124
14023
14025
14140
14155
14123
14079
14096
14060
14058
14106
14127
14088
17085
14094
14129
14080
14097
14087
14082
14029
27901
14135
14089
14143
14141
9080
9081
9079
97/1.0
89/0.97
65/0.96
90/1.0
63/0.99
60/0.88
65/0.96
64/0.96
67/0.99
58/0.98

(c)


R3
14060
14096
14079
14058
14127
14106
9081
14029
14082
14087
14097
14080
14129
9079
9080
7054
67
7773
66
60
60
7772
50
14085
14088
14089
14094
14143
R2
14155
14140
96/0.99
14025
14023
14123
97/0.98
64/0.62
14135
14124
14141
14151
27901
14054
14056
13992
R1

(d)


R1
14151
14056
14124
14054
62
13992
7772
7773
7054
R3
14079
14096
14060
14058
14106
14127
86/1.0
R2
14123
14023
14025
14140
14155
88
85/1.0
87/1.0
9080
9079
14129
15080
14097
14087
14082
14029
9081
63/0.92
14094
62
14085
14088
88/0.99
14143
14089
14141
14135
27901

(e)

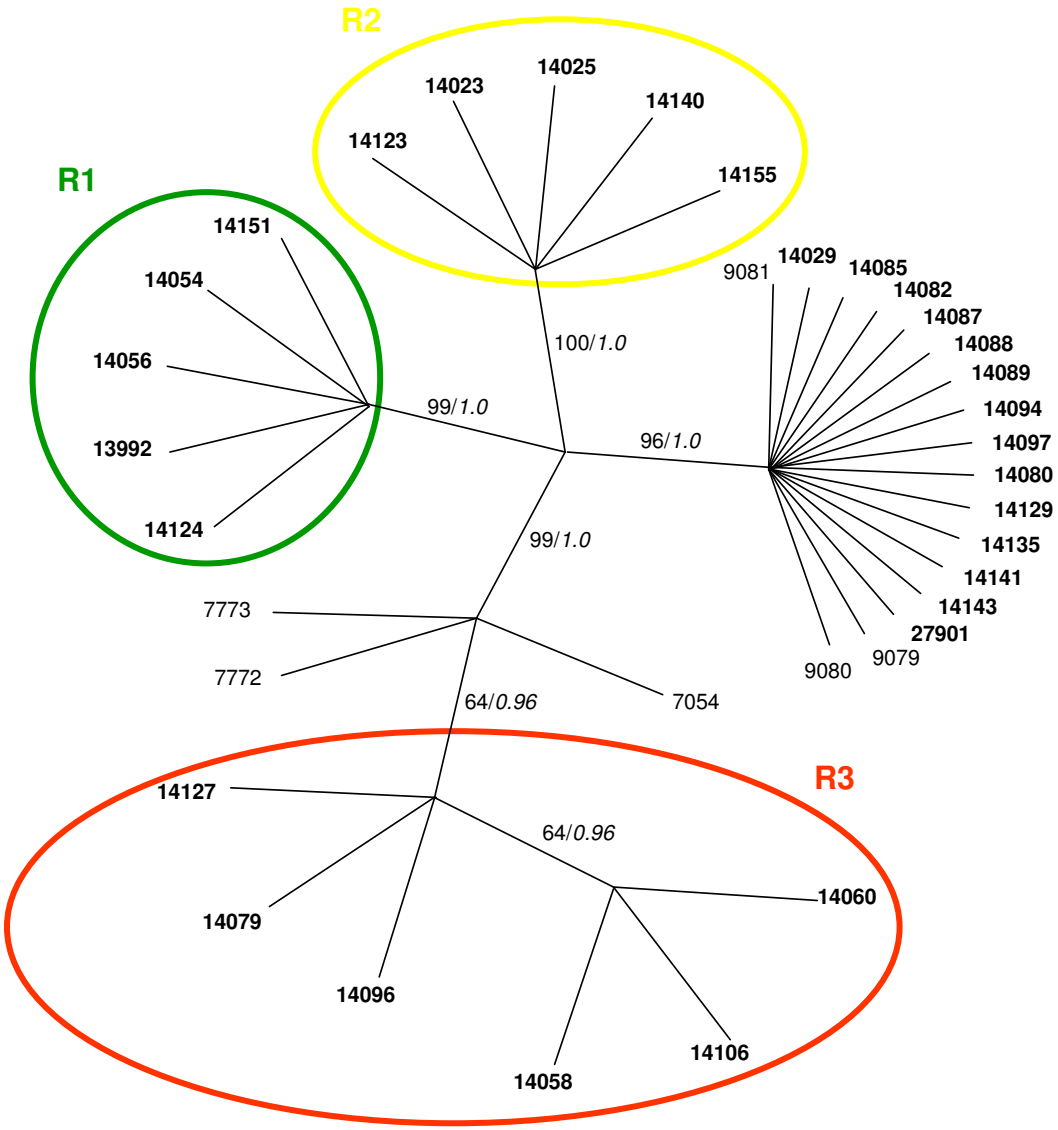
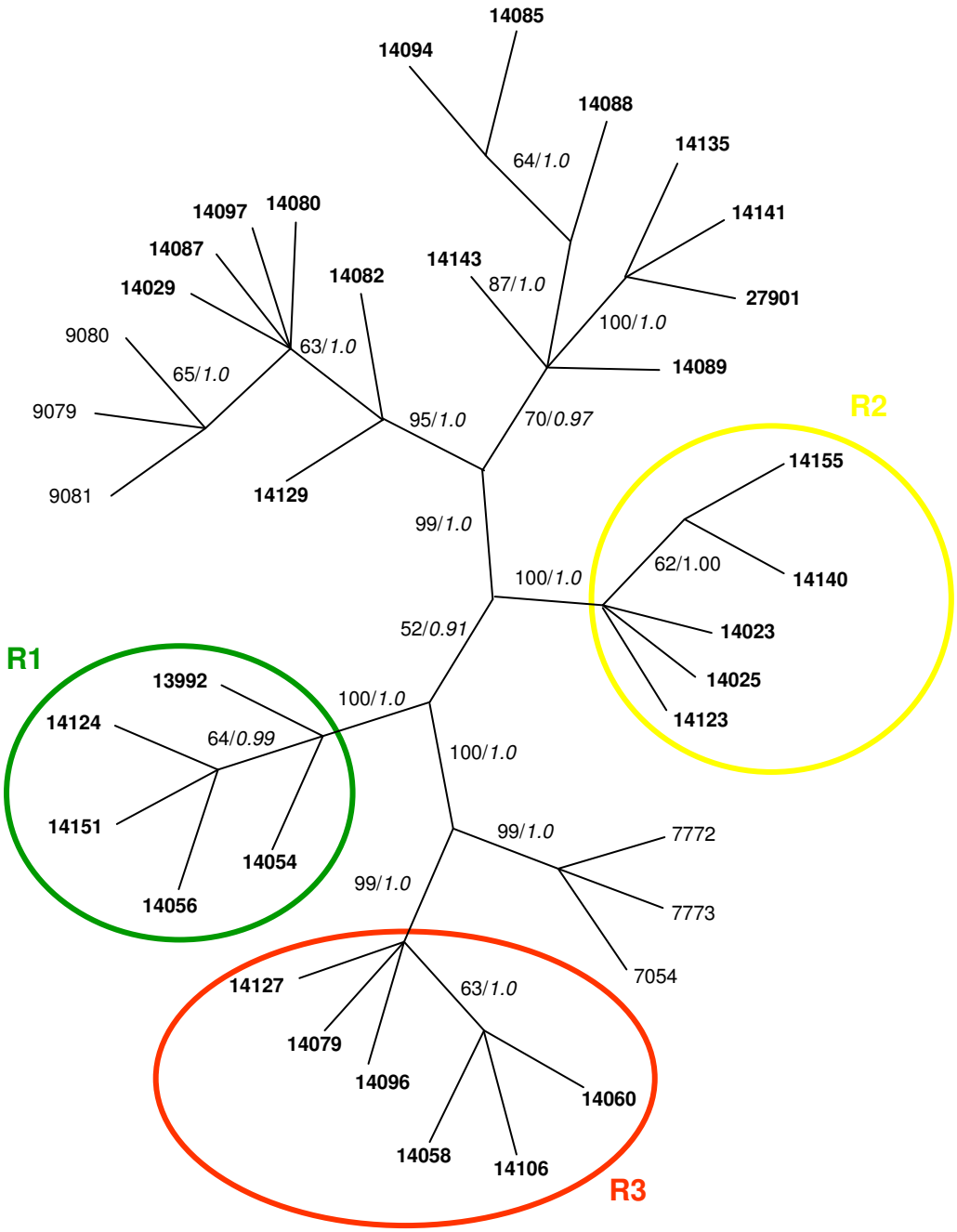

R2
14025
14023
14140
14123
14155
R1
14151
14054
14056
13992
14124
100/1.0
99/1.0
96/1.0
99/1.0
9081
14029
14085
14082
14087
14088
14089
14094
14097
14080
14129
14135
14141
14143
27901
9079
9080
7773
7772
7054
64/0.96
R3
14127
64/0.96
14060
14079
14096
14106
14058

FIG. 3. One of two unrooted maximum-parsimony trees resulting from the analysis of the combined sequence data. Bootstrap values of maximum parsimony analyses are indicated next to the branches followed by the posterior probabilities resulting from Bayesian analysis (indicated in italics). Isolates of the *Neofusicoccum* spp. obtained from *S. cordatum* are indicated in bold.


14085
14094
14088
14135
64/1.0
14141
14097
14080
14087
14143
14082
87/1.0
14029
27901
100/1.0
9080
63/1.0
14089
65/1.0
9079
95/1.0
70/0.97
R2
14155
9081
14129
99/1.0
62/1.00
14140
100/1.0
52/0.91
14023
R1
14025
13992
100/1.0
14123
14124
64/0.99
100/1.0
14151
99/1.0
7772
14054
99/1.0
7773
14056
7054
14127
63/1.0
14079
14096
14060
14058
14106
R3