Molecular Phylogenetics and Evolution 51 (2009) 259–268

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

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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ARTICLE INFO

Article history:
Received 17 June 2008
Revised 5 December 2008
Accepted 16 December 2008
Available online 27 December 2008

Keywords:
Botryosphaeriaceae
Cryptic species
Fixed single nucleotide polymorphisms
GCPSR
Multiple gene genealogies
Syzygium cordatum

ABSTRACT

Neofusicoccum parvum and *N. ribis* (Botryosphaeriaceae, Ascomycetes) are closely related, plant pathogenic fungi with a world-wide 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 characterisation. 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.

1. 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 utilising 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 *Giberella 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 recognised 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 recognised 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).

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doi:10.1016/j.ympev.2008.12.017

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 world-wide (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 *Syzygium 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.

2. Materials and methods

2.1. 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 1). 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 1). The single-conidial cultures were prepared as reported previously (Pavlic et al., 2007), to ensure that only haploid genotypes were characterised for each representative culture. The collection of single-conidium 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.

2.2. Morphometric analysis

In a previous study, all 103 isolates were induced to sporulate in culture and conidia were measured and characterised using light microscopy (Pavlic, 2004; Pavlic et al., 2007). The lengths and widths of 10 conidia were measured for each isolate and the data were analysed in this study. Averages of 10 conidial measurements per isolate were calculated and used in the analyses.

2.3. 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 characterisation, 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 2. 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 °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 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, UK), as specified by the manufacturer. Sequence reactions were run on an ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer, Warrington, UK).

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.

Table 1
Isolates analysed in this study.

Culture no.[a,b,c]	Other no.[a]	Identity	Geographic origin	Host
CMW 13992	CBS 123634	*Neofusicoccum* sp. R1	Sodwana Bay, South Africa	*Syzygium cordatum*
CMW 14056	CBS 123635	*Neofusicoccum* sp. R1	Kosi Bay, South Africa	*S. cordatum*
CMW 14054	CBS 123636	*Neofusicoccum* sp. R1	Mkuze, South Africa	*S. cordatum*
CMW 14124	CBS 123638	*Neofusicoccum* sp. R1	Richards Bay, South Africa	*S. cordatum*
CMW 14151	CBS 123637	*Neofusicoccum* sp. R1	Sabie, South Africa	*S. cordatum*
CMW 14023	CBS 123639	*Neofusicoccum* sp. R2	Kwambonambi, South Africa	*S. cordatum*
CMW 14025	CBS 123640	*Neofusicoccum* sp. R2	Kwambonambi, South Africa	*S. cordatum*
CMW 14140	CBS 123641	*Neofusicoccum* sp. R2	Tzaneen, South Africa	*S. cordatum*
CMW 14155	CBS 123642	*Neofusicoccum* sp. R2	Sabie, South Africa	*S. cordatum*
CMW 14123	CBS 123643	*Neofusicoccum* sp. R2	Richards Bay, South Africa	*S. cordatum*
CMW 14106	CBS 123644	*Neofusicoccum* sp. R3	Sodwana Bay, South Africa	*S. cordatum*
CMW 14058	CBS 123645	*Neofusicoccum* sp. R3	Kosi Bay, South Africa	*S. cordatum*
CMW 14060	CBS 123646	*Neofusicoccum* sp. R3	Kosi Bay, South Africa	*S. cordatum*
CMW 14079	CBS 123647	*Neofusicoccum* sp. R3	Gonubie, South Africa	*S. cordatum*
CMW 14096		*Neofusicoccum* sp. R3	St. Johan's Port, South Africa	*S. cordatum*
CMW 14127	CBS 123648	*Neofusicoccum* sp. R3	Kwambonambi, South Africa	*S. cordatum*
CMW 14029		*Neofusicoccum parvum*	Kwambonambi, South Africa	*S. cordatum*
CMW 14082		*N. parvum*	Pietermaritzburg, South Africa	*S. cordatum*
CMW 14085	CBS 123649	*N. parvum*	Pietermaritzburg, South Africa	*S. cordatum*
CMW 14087		*N. parvum*	Pietermaritzburg, South Africa	*S. cordatum*
CMW 14088		*N. parvum*	Pietermaritzburg, South Africa	*S. cordatum*
CMW 14089		*N. parvum*	Pietermaritzburg, South Africa	*S. cordatum*
CMW 14094		*N. parvum*	Pietermaritzburg, South Africa	*S. cordatum*
CMW 14097	CBS 123650	*N. parvum*	St. Johan's Port, South Africa	*S. cordatum*
CMW 14080	CBS 123651	*N. parvum*	Gonubie, South Africa	*S. cordatum*
CMW 14129		*N. parvum*	Tzaneen, South Africa	*S. cordatum*
CMW 14135		*N. parvum*	Tzaneen, South Africa	*S. cordatum*
CMW 14141		*N. parvum*	Tzaneen, South Africa	*S. cordatum*
CMW 14143	CBS 123652	*N. parvum*	Palaborwa, South Africa	*S. cordatum*
CMW 27901		*N. parvum*	Pretoria, South Africa	*S. cordatum*
CMW 9079	ICMP 7933	*N. parvum*	New Zealand	*Actinidia deliciosa*
CMW 9080	ICMP 8002	*N. parvum*	New Zealand	*Populus nigra*
CMW 9081	ICMP 8003	*N. parvum*	New Zealand	*P. nigra*
CMW 7772		*Neofusicoccum ribis*	New York, USA	*Ribes* sp.
CMW 7773		*N. ribis*	New York, USA	*Ribes sp.*
CMW 7054	CBS121.26	*N. ribis*	New York, USA	*Ribes rubrum*

[a] 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.
[b] Isolates in bold are ex-types.
[c] All isolates other than CMW 9079, CMW 9080, CMW 9081, CMW 7772, CMW 7773, and CMW 7054 were collected by D. Pavlic.

Table 2
Primer sets used to amplify the five loci analysed in this study.

Region	Oligos	Oligo sequences	Amplicon size (bp)	AT (°C)	Reference
ITS	ITS1	5′ TCCGTAGGTGAACCTGCGG	600	55	(White et al., 1990)
	ITS4	5′ TCCTCCGCTTATTGATATGC			
EF-1a	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			

2.4. 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) 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 Huelsenback, 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 v2.2 software (Nylander, 2004). The Markov Chain Monte Carlo (MCMC) chains were initialised from a

random tree and were run for 1,000,000 generations and trees were saved every 100 generations, counting 10,000 trees. Burn in was set to 100,000 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 Huelsenback, 2003).

3. Results

3.1. 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).

3.2. 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 4.

3.3. Phylogenetic analysis

The phylogenies obtained from sequence data of the gene regions were first determined separately. MrModeltest v2.2 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 (Rodríguez 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 3. 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 ($n = 5$).

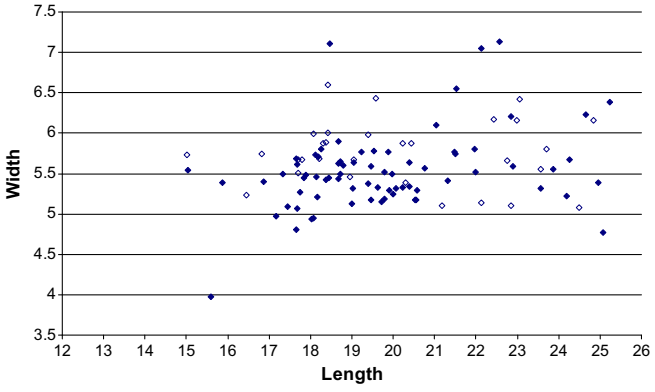

Fig. 1. The averages of the lengths and widths of ten conidia measured for each of 103 isolates representing *Neofusicoccum parvum/N. ribis* complex from *Syzygium cordatum*. The thirty isolates used for DNA sequence comparisons in this study were selected to represent the full range of conidial sizes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

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 4). 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 4). 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 4). 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 ($P \geqslant 0.05$) (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 3). 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 to 100% and posterior probabilities above 0.95 (Fig. 3).

4. Discussion

Application of the GCPSR in this study led us to reject the hypothesis that a single variable species in the *N. ribis*/*N. parvum* 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. ribis/N. parvum* 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

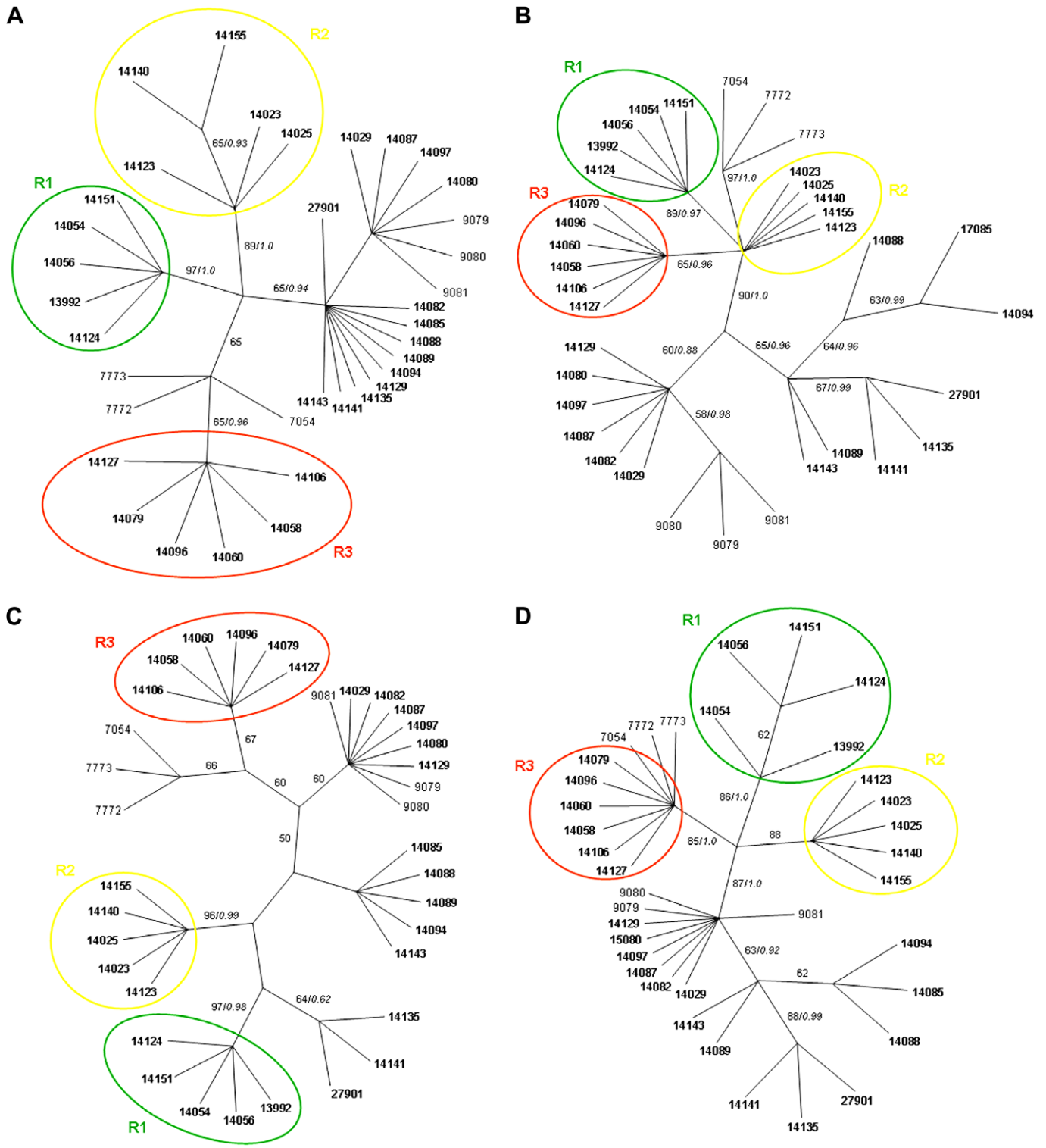

Fig. 2. Unrooted maximum-parsimony trees resulting from the separate analysis of the sequence data of the ITS (A), EF-1α (B), Bt2 regions of the β-tubulin gene (C), locus *BotF15* (D) and RPB2 (E). Bootstrap values of maximum parsimony analyses are indicated next to the branches followed by the posterior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

(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.

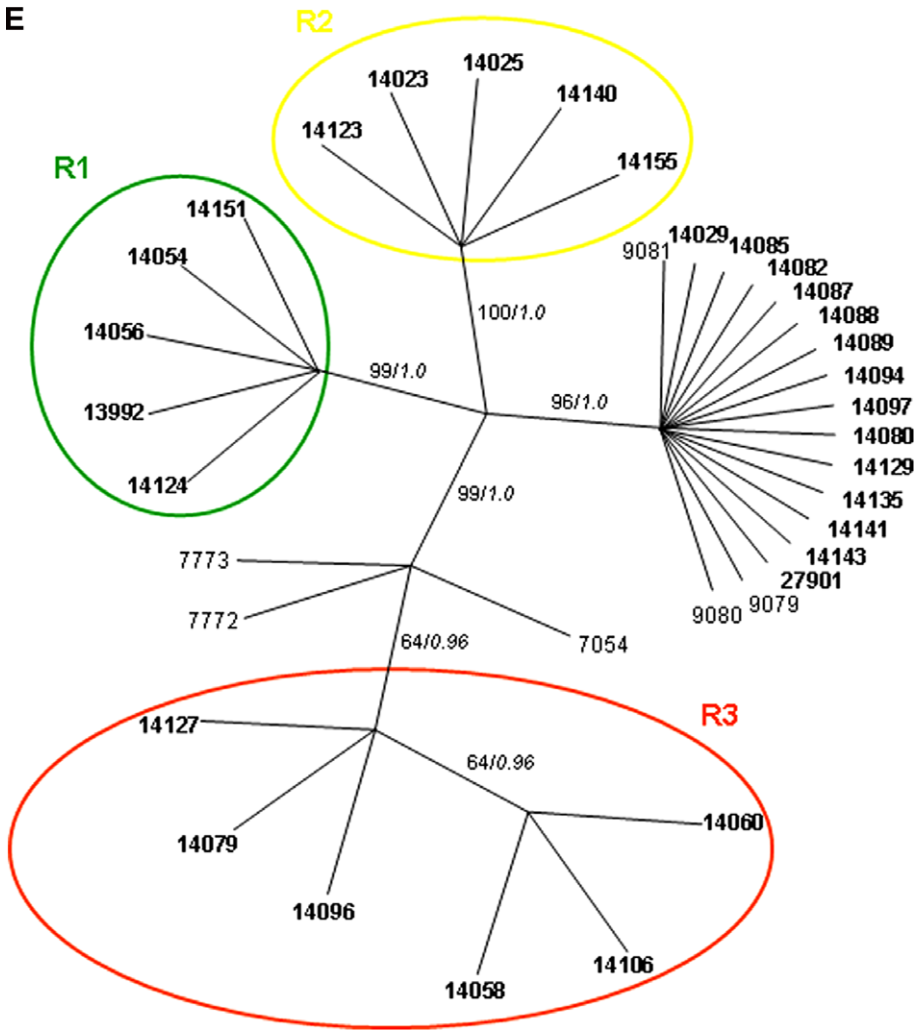

Fig. 2 (*continued*)

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 analysed 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 characterised only based on the ITS sequences

Table 3
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 4
Single nucleotide polymorphisams (SNPs) from the sequence data of ITS rDNA, EF-1α, β-tubulin, *BotF15* and RPB2 loci.

e*				ITS														EF-1																	Bt																	*BotF15*																		RPB2								
	51	67	109	141	142	163	168	173	372	379	416	38	43	47	48	58	62	79	88	117	126	134	192	221	224	236	240	264	32	40	83	92	96	115	157	175	232	235	251	316	379	397	24	54	61	87	121	122	128	150	172	245	300	331	341	10	22	49	97	100	112	205	265	280	343	382	397	409	421	475	502	526
7772	A	T	G	T	A	C	T	A	A	T	T	1	G	T	G	T	C	A	G	C	G	C	A	_	C	G	A	T	C	G	C	C	C	G	T	C	A	G	T	C	T	T	G	0	0	0	G	A	C	T	0	C	C	C	G	T	T	C	T	G	C	T	G	C	C	G	T	T	G	C	C	T
7773	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	A	_	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7054	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	A	_	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
13992	.	.	_	C	.	.	.	.	G	.	C	0	.	.	.	C	.	.	.	.	.	.	G	C	.	.	.	C	T	.	.	.	T	.	C	.	.	.	.	G	C	C	.	-	-	-	T	C	T	.	-	.	.	T	.	.	C	.	C	A	T	C	A	.	.	.	C	C	.	.	.	.
14056	.	.	_	C	.	.	.	.	G	.	C	0	.	.	.	C	.	.	.	.	.	.	G	C	.	.	.	C	T	.	.	.	T	.	C	.	.	.	.	G	C	C	.	-	-	-	T	C	T	C	-	.	.	T	.	.	C	.	C	A	T	C	A	.	.	.	C	C	.	.	.	.
14054	.	.	_	C	.	.	.	.	G	.	C	0	.	.	.	C	.	.	.	.	.	.	G	C	.	.	.	C	T	.	.	.	T	.	C	.	.	.	.	G	C	C	.	-	-	-	T	C	T	.	-	.	.	T	.	.	C	.	C	A	T	C	A	.	.	.	C	C	.	.	.	.
14151	.	.	_	C	.	.	.	.	G	.	C	0	.	.	.	C	.	.	.	.	.	.	G	C	.	.	.	C	T	.	.	.	T	.	C	.	C	.	.	G	C	C	.	-	-	-	T	C	T	C	-	.	.	T	.	.	C	.	C	A	T	C	A	.	.	.	C	C	.	.	.	.
14124	.	.	_	C	.	.	.	.	G	.	C	0	.	.	.	C	.	.	.	.	.	.	G	C	.	.	.	C	T	.	.	.	T	.	C	.	.	.	.	G	C	C	.	-	-	-	T	C	T	C	-	.	.	T	.	.	C	.	C	A	T	C	A	.	.	.	C	C	.	.	.	.
14023	.	.	_	.	G	T	.	G	.	.	.	0	.	.	.	.	.	.	.	.	.	.	G	_	.	.	.	C	.	.	.	.	.	A	C	T	.	A	A	.	C	C	.	-	-	1	.	.	T	.	1	.	.	T	.	.	C	G	C	.	.	C	.	.	.	A	C	.	A	.	.	C
14025	.	.	_	.	.	T	.	G	.	.	.	0	.	.	.	.	.	.	.	.	.	.	G	_	.	.	.	C	.	.	.	.	.	A	C	T	.	A	A	.	C	C	.	-	-	1	.	.	T	.	1	.	.	T	.	.	C	G	C	.	.	C	.	.	.	A	C	.	A	.	.	C
14140	.	C	_	.	.	T	.	G	.	.	.	0	.	.	.	.	.	.	.	.	.	.	G	_	.	.	.	C	.	.	.	.	.	A	C	T	.	A	A	.	C	C	.	-	-	1	.	.	T	.	1	.	.	T	.	.	C	G	C	.	.	C	.	.	.	A	C	.	A	.	.	C
14155	.	C	_	.	.	T	.	G	.	.	.	0	.	.	.	.	.	.	.	.	.	.	G	_	T	.	.	C	.	.	.	.	.	A	C	T	.	A	A	.	C	C	.	-	-	1	.	.	T	.	1	.	.	T	.	.	C	G	C	.	.	C	.	.	.	A	C	.	A	.	.	C
14123	.	.	_	.	.	T	.	G	.	.	.	0	.	.	.	.	.	.	.	.	.	.	G	_	.	.	.	C	.	.	.	.	.	A	C	T	.	A	A	.	C	C	.	-	-	1	.	.	T	.	1	.	.	T	.	.	C	G	C	.	.	C	.	.	.	A	C	.	A	.	.	C
14106	.	.	.	.	.	.	C	.	.	.	.	0	.	.	.	.	T	.	.	.	.	.	G	_	.	.	.	C	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.
14058	.	.	.	.	.	.	C	.	.	.	.	0	.	.	.	.	T	.	.	.	.	.	G	_	.	.	.	C	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.
14060	.	.	.	.	.	.	C	.	.	.	.	0	.	.	.	.	T	.	.	.	.	.	G	_	.	.	.	C	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.
14079	.	.	.	.	.	.	C	.	.	.	.	0	.	.	.	.	T	.	.	.	.	.	G	_	.	.	.	C	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
14096	.	.	.	.	.	.	C	.	.	.	.	0	.	.	.	.	T	.	.	.	.	.	G	_	.	.	.	C	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
14127	.	.	.	.	.	.	C	.	.	.	.	0	.	.	.	.	T	.	.	.	.	.	G	_	.	.	.	C	.	A	.	.	.	.	C	.	.	.	.	.	.	.	.	-	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
9079	T	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	.	T	G	_	.	A	.	.	.	.	T	.	.	A	C	.	.	.	.	.	.	.	.	-	-	-	.	.	T	.	-	T	.	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
9080	T	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	.	T	G	_	.	A	.	.	.	.	T	.	.	A	C	.	.	.	.	.	.	.	.	-	-	-	.	.	T	.	-	T	.	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
9081	T	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	.	T	G	_	.	A	.	.	.	.	T	.	.	A	C	.	.	.	.	.	.	.	.	-	-	-	.	.	T	.	-	T	.	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14029	T	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	.	T	G	_	.	A	.	C	.	.	T	.	.	A	C	.	.	.	.	.	.	.	.	-	-	-	.	.	T	.	-	T	.	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14082	T	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	.	T	G	_	.	A	.	C	.	.	T	.	.	A	C	.	.	.	.	.	.	.	.	-	-	-	.	.	T	.	-	T	.	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14085	T	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	.	T	G	_	.	A	.	C	.	.	T	.	.	A	C	.	.	.	.	.	.	.	.	-	-	-	.	.	T	.	-	T	.	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14087	T	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	.	T	G	_	.	A	.	C	.	.	T	.	.	A	C	.	.	.	.	.	.	.	.	-	-	-	.	.	T	.	-	T	.	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14088	.	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	T	.	T	G	_	.	.	C	C	.	.	T	.	.	A	C	.	.	.	.	.	C	C	.	-	-	-	.	.	T	.	-	T	T	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14089	.	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	.	T	G	_	.	A	.	C	.	.	T	.	.	A	C	.	.	.	.	.	.	.	.	-	-	-	.	.	T	.	-	T	.	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14094	.	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	G	A	.	.	T	G	_	.	.	C	C	.	.	T	.	.	A	C	.	.	.	.	.	C	C	.	-	1	-	.	.	T	.	-	T	T	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14097	.	.	_	.	.	.	.	.	.	C	.	0	A	C	A	.	.	G	.	.	.	T	G	_	.	.	C	C	.	.	T	.	.	A	C	.	.	.	.	.	C	C	.	-	1	-	.	.	T	.	-	T	T	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14080	.	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	.	T	G	_	.	.	C	C	.	.	T	.	.	A	C	.	.	.	.	.	C	C	.	-	-	-	.	.	T	.	-	T	T	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14129	.	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	G	A	.	.	T	G	_	.	.	C	C	.	.	T	.	.	A	C	.	.	.	.	.	C	C	.	-	1	-	.	.	T	.	-	T	T	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14135	.	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	.	T	G	_	.	A	C	C	.	.	T	.	.	A	C	.	.	.	.	.	.	.	.	-	-	-	.	.	T	.	-	T	.	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14141	.	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	A	T	G	_	.	.	C	C	.	.	.	T	.	.	C	.	.	.	.	.	C	C	A	1	-	-	.	.	T	.	-	T	T	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
14143	.	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	A	T	G	_	.	.	C	C	.	.	.	T	.	.	C	.	.	.	.	.	C	C	A	1	-	-	.	.	T	.	-	T	T	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.
27901	.	.	_	.	.	.	.	.	.	C	.	0	.	C	A	.	.	.	.	.	A	T	G	_	.	.	C	C	.	.	.	T	.	.	C	.	.	.	.	.	C	C	A	1	-	-	.	.	T	.	-	T	T	T	A	C	C	.	C	.	.	C	.	.	T	.	C	.	.	T	.	.

ates in bold are obtained from *S. cordatum*. *Neofusicoccum ribis* = pink; *Neofusicoccum* sp. R1 = green; *Neofusicoccum* sp. R2 = yellow; *Neofusicoccum* sp. R3 = red; *N. parvum* = blue.

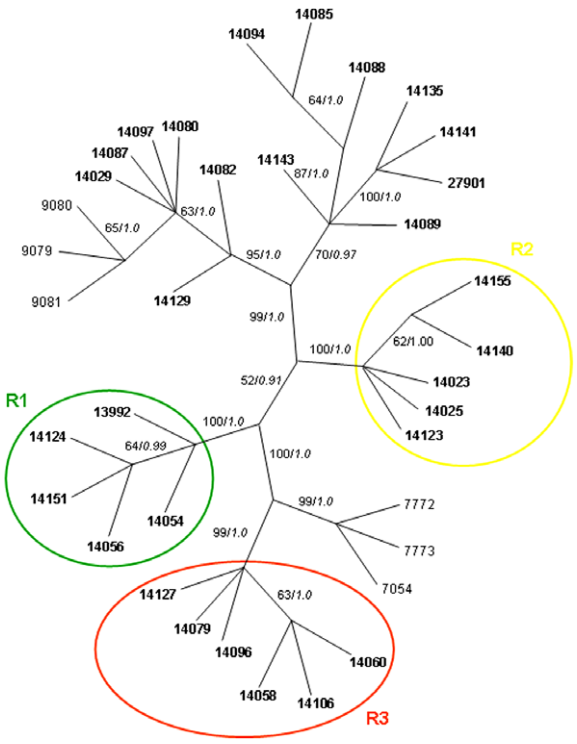

Fig. 3. Unrooted maximum-parsimony tree resulting from the analysis of the combined sequence data. Bootstrap values of maximum parsimony analyses are indicated next to the branches followed by the posterior probabilities resulting from Bayesian analysis (indicated in italics). Isolates of the *Neofusicoccum*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

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 recognised in the *N. parvum/N. ribis* complex in this study may thus increase in future when isolates from other hosts and areas are considered. It is especially important to better characterise 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 et al., 1999), has proven useful for phylogenetic resolution of the Ascomycetes at different taxonomic levels (Liu et al., 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 et 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 *Cryptoccocus 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 et 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. parvum/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 pheno-

typic characters such as pathogenicity and virulence for the newly recognised species must also be a key area for research in future.

Acknowledgments

We thank the National Research Foundation (NRF), members of Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry, and the Department of Science and Technology (DST)/NRF Centre of Excellence in Tree Health Biotechnology (CTHB), South Africa for financial support. We would also like to thank the three anonymous reviewers for their helpful comments and suggestions for improving the manuscript.

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