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Molecular and phenotypic characterization of three phylogenetic species discovered within the *Neofusicoccum parvum/N. ribis* complex

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Abstract: *Neofusicoccum parvum* and *N. ribis* are closely related species whose identities often have been confused. These fungal plant pathogens were identified recently as the most abundant species of Botryosphaeriaceae (Ascomycetes) isolated from native *Syzygium cordatum* trees in South Africa. In another study using multiple gene genealogies from five nuclear loci three undescribed cryptic phylogenetic species as well as *N. parvum* were identified among 30 of these isolates. The aim of this study was to clarify the identity of the remaining isolates in the *N. parvum/N. ribis* complex from *S. cordatum* in South Africa, to describe newly identified cryptic species and to test their pathogenicity. Based on the RNA polymerase II subunit (RPB2) sequence comparisons, the isolates were identified as *N. parvum* or one of three previously recognized phylogenetic species that are described here as *N. cordaticola, N. kwambonambiense* and *N. umdonicola*. These species cannot be separated a priori based on morphological characteristics, although a posteriori analysis of variance showed that the differences in conidial length and width between the species were statistically significant. The isolates of the newly described species as well as *N. parvum* and *N. ribis* were tested for pathogenicity on *S. cordatum* under greenhouse conditions. Isolates representing the three new species were significantly more aggressive than *N. parvum* and *N. ribis* with *N. kwambonambiense* being the most aggressive. This study resolved long-standing questions of identity of species within *N. parvum/N. ribis* complex and lays a foundation for further studies on this group of pathogens.

Key words: Botryosphaeriaceae, GCPSR, multiple gene genealogies, *Neofusicoccum*, phylogenetic species, speciation, *Syzygium*

INTRODUCTION

The phylogenetic species concept (PSC) (Taylor et al 2000) and genealogical concordance phylogenetic species recognition (GCPSR) have been increasingly applied in studies of species boundaries in both human- and plant-pathogenic fungi (e.g. Koufopanou et al 1997; Geiser et al 1998; O'Donnell et al 2000a, b, 2004; Steenkamp et al 2002; Pringle et al 2005). In these studies, using GCPSR based on concordance of multiple gene sequence genealogies, numerous cryptic species and species complexes were revealed in fungal taxa previously identified as one morphospecies. GCPSR also was used with good results in the detection of cryptic species within Botryosphaeriaceae, for example *Diplodia scrobiculata* as a sister species of *D. pinea* (de Wet et al 2003) and *Neofusicoccum eucalypticola* and *N. australe* as sister species of *N. eucalyptorum* and *N. luteum* respectively (Slippers et al 2004b, c). The cryptic species recognized in these studies could not have been acknowledged based on morphology or single-locus data alone, methods commonly used for identification of Botryosphaeriaceae (e.g. Jacobs and Rehner 1998, Denman et al 2000, Smith et al 2001, Zhou and Stanosz 2001, Pavlic et al 2004).

Neofusicoccum parvum and *N. ribis* are closely related cryptic species within the recently described genus *Neofusicoccum* (Botryosphaeriaceae, Ascomycetes) (Slippers et al 2004a, Crous et al 2006). Although known to develop teleomorph (sexual) structures, these fungi are commonly encountered in their anamorph (asexual) stage (Pennycook and Samuels 1985, Slippers et al 2004a, Pavlic et al 2007). The cosmopolitan distribution, sympatric occurrence on native and non-native hosts, as well as plasticity and overlap in the morphological characteristics of both

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their teleomorphs and anamorphs, make these species difficult to distinguish based on morphological, ecological and geographical criteria. Consequently these plant pathogens often have been mistaken for each other. These species also could not be separated with confidence based on ITS sequence data alone, the method most commonly used in molecular identification and phylogenetic analyses of the Botryosphaeriaceae (Smith et al 2001, Zhou and Stanosz 2001, Slippers et al 2005, Pavlic et al 2007).

Nucleotide sequence data from multiple genes were used to distinguish the identity of the type specimens of *N. parvum* and *N. ribis* (Slippers et al 2004a). However, when more isolates were included in subsequent analyses, many clustered intermediate to the type but did not clearly cluster with either of these species (Ahumada 2002, Rodas 2003, Slippers 2003, Slippers et al 2005). These isolates have been referred to as the *N. parvum/N. ribis* complex. Isolates that belong to the *N. parvum/N. ribis* complex could be separated into two groups with a PCR-RFLP fingerprinting technique. They then were referred to as *N. parvum sensu lato* and *N. ribis sensu lato* (Slippers 2003). It was not clear in those studies however whether these groups comprise more than one cryptic species or represent interspecific variation.

Neofusicoccum parvum sensu lato and *N. ribis sensu lato* were identified as the most abundant species of Botryosphaeriaceae isolated from native *Syzygium cordatum* (Myrtaceae) in South Africa (Pavlic et al 2007). In a subsequent study, using multiple gene genealogies of five nuclear loci three undescribed cryptic phylogenetic species as well as *N. parvum*, were identified among these isolates (Pavlic et al 2009). None were identified as *N. ribis*. In this study we characterize a larger collection of these isolates with genotypic data and combine this with phenotypic characteristics such as conidial morphology and pathogenicity to describe the taxa. Consequently three new phylogenetically recognized cryptic species within the *Neofusicoccum parvum/N. ribis* species complex are described here as *N. cordaticola* sp. nov., *N. umdonicola* sp. nov. and *N. kwambonambiense* sp. nov.

MATERIALS AND METHODS

Isolates.—The 103 isolates used in this study were collected during the survey of the Botryosphaeriaceae on native *S. cordatum* in South Africa 2001–2003 (TABLE I). The collection spanned the north to south natural distribution of *S. cordatum* in South Africa, from Tzaneen in the Northern Province to Gonubie in Eastern Cape Province. Isolations were made from dying twigs and asymptomatic, visually healthy twigs and leaves, as described in Pavlic et al (2007). Isolations also were made from visually healthy fruits. Fruits were washed in running tap water and surface disinfected by spraying them with 70% ethanol and left dried on filter paper. The disinfected fruits were halved and pieces from the fruit pulp (2 mm^2) were placed on 2% malt extract agar (MEA) and incubated and maintained as described in Pavlic et al (2007). All cultures have been maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and representative isolates have been deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

DNA sequence comparisons.—Thirty isolates from *S. cordatum* were selected and identified by Pavlic et al (2009) as *N. parvum* or one of the undescribed phylogenetic species termed *Neofusicoccum* sp. R1, R2 and R3. This distinction was based on multiple gene genealogies of DNA sequence data for five nuclear loci, including the internal transcribed spacer rDNA (ITS1, 5.8S and ITS2), partial translation elongation factor 1-α (EF-1α), β-tubulin-2 (βt-2a/b), a portion of the RNA polymerase II subunit (RPB2) and locus *BotF15* (an unknown locus containing a simple sequence repeat); the results were compared with a single gene sequence data. The RPB2 region was found to contain the most informative characters considering fixed single nucleotide polymorphisms (SNP) in each species. Following the same protocol as Pavlic et al (2009), a portion of the RNA polymerase II subunit (RPB2) was sequenced for the remaining 73 isolates. The type specimens and two specimens related to the types of *N. parvum* and *N. ribis* were included for comparison. The nucleotide sequences from one strand were examined with Sequence Navigator 1.0.1. software (Perkin-Elmer Applied BioSystems Inc., Foster City, California), and alignments were prepared online with MAFFT 5.667 (http://timpani.genome.ad.jp/~mafft/server/) (Katoh et al 2002) to compare it to the data from Pavlic et al (2009).

Phylogenetic analyses.—A maximum parsimony (MP) tree was constructed in PAUP 4.0b10 (Swofford 2000) with 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. Branches of zero length were collapsed, and all multiple equally parsimonious trees were saved. To estimate branch support, maximum parsimony bootstrap values were determined with 1000 bootstrap replicates (Felsenstein 1985).

Bayesian analyses were performed with MrBayes 3.0b4 (Ronquist and Huelsenbeck 2003) and the best-fitting evolutionary model was estimated with MrModeltest 2.2 software (Nylander 2004). Markov Monte Carlo (MCMC) chains were initialized from a random tree and were run 2 000 000 generations and trees were saved every 100 generations, counting 20 000 trees. Burn-in was set at 1000 generations, leaving just over 38 002 trees from which the consensus tree was calculated. To determine the confidence of the tree topologies, values of Bayesian posterior

TABLE I. Isolates considered in this study

Culture No.[1, 2, 3, 4]	Other No.[1]	Identity	Geographic origin	Host	Substratum	GenBank[5] RPB2
CMW13992[a]	CBS123634	*Neofusicoccum cordaticola*	Sodwana bay, S. Africa	*Syzygium cordatum*	twig	*EU821928*
CMW14035[c]		*N. cordaticola*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389275
CMW14041		*N. cordaticola*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389277
CMW14042		*N. cordaticola*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389276
CMW14056[d]	CBS123635	*N. cordaticola*	Kosi bay, S. Africa	*S. cordatum*	twig	*EU821933*
CMW14054	CBS123636	*N. cordaticola*	Mkuze, S. Africa	*S. cordatum*	twig	*EU821936*
CMW14144		*N. cordaticola*	Sabie, S. Africa	*S. cordatum*	twig	FJ389269
CMW14145		*N. cordaticola*	Sabie, S. Africa	*S. cordatum*	leaf	FJ389271
CMW14147		*N. cordaticola*	Sabie, S. Africa	*S. cordatum*	leaf	FJ389270
CMW14148		*N. cordaticola*	Sabie, S. Africa	*S. cordatum*	leaf	FJ389274
CMW14149		*N. cordaticola*	Sabie, S. Africa	*S. cordatum*	leaf	FJ389268
CMW14150		*N. cordaticola*	Sabie, S. Africa	*S. cordatum*	leaf	FJ389273
CMW14151	CBS123637	*N. cordaticola*	Sabie, S. Africa	*S. cordatum*	twig	*EU821952*
CMW14152		*N. cordaticola*	Sabie, S. Africa	*S. cordatum*	twig	FJ389272
CMW14124[h]	CBS123638	*N. cordaticola*	Richards bay, S. Africa	*S. cordatum*	fruit	*EU821955*
CMW14023	CBS123639	*Neofusicoccum kwambonambiense*	Kwambonambi, S. Africa	*S. cordatum*	twig	*EU821930*
CMW14025[b]	CBS123640	*N. kwambonambiense*	Kwambonambi, S. Africa	*S. cordatum*	twig	*EU821931*
CMW14031		*N. kwambonambiense*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389280
CMW14046		*N. kwambonambiense*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389282
CMW14136		*N. kwambonambiense*	Tzaneen, S. Africa	*S. cordatum*	twig	FJ389286
CMW14140[g]	CBS123641	*N. kwambonambiense*	Tzaneen, S. Africa	*S. cordatum*	twig	*EU821949*
CMW14153		*N. kwambonambiense*	Sabie, S. Africa	*S. cordatum*	twig	FJ389285
CMW14154		*N. kwambonambiense*	Sabie, S. Africa	*S. cordatum*	twig	FJ389283
CMW14155	CBS123645	*N. kwambonambiense*	Sabie, S. Africa	*S. cordatum*	fruit	*EU821953*
CMW14156		*N. kwambonambiense*	Sabie, S. Africa	*S. cordatum*	fruit	FJ389284
CMW14119		*N. kwambonambiense*	Richards bay, S. Africa	*S. cordatum*	fruit	FJ389279
CMW14120		*N. kwambonambiense*	Richards bay, S. Africa	*S. cordatum*	fruit	FJ389248
CMW14121		*N. kwambonambiense*	Richards bay, S. Africa	*S. cordatum*	fruit	FJ389281
CMW14123[h]	CBS123643	*N. kwambonambiense*	Richards bay, S. Africa	*S. cordatum*	fruit	*EU821954*
CMW13990[a]		*Neofusicoccum umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	twig	FJ389310
CMW13991		*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	twig	FJ389293
CMW13993		*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	twig	FJ389306
CMW13994		*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	twig	FJ389300
CMW13995		*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	twig	FJ389298
CMW13997		*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	twig	FJ389289
CMW14006		*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	twig	FJ389295
CMW14007		*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	twig	FJ389303
CMW14106	CBS123644	*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	leaf	*EU821929*
CMW14008		*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	leaf	FJ389287
CMW14010		*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	twig	FJ389304
CMW14012		*N. umdonicola*	Sodwana bay, S. Africa	*S. cordatum*	twig	FJ389290
CMW14016		*N. umdonicola*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389297
CMW14028		*N. umdonicola*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389294
CMW14055[d]		*N. umdonicola*	Kosi bay, S. Africa	*S. cordatum*	twig	FJ389305
CMW14057		*N. umdonicola*	Kosi bay, S. Africa	*S. cordatum*	twig	FJ389301
CMW14058	CBS123645	*N. umdonicola*	Kosi bay, S. Africa	*S. cordatum*	twig	*EU821934*
CMW14098		*N. umdonicola*	Kosi bay, S. Africa	*S. cordatum*	twig	FJ389288
CMW14099		*N. umdonicola*	Kosi bay, S. Africa	*S. cordatum*	twig	FJ389307
CMW14059		*N. umdonicola*	Kosi bay, S. Africa	*S. cordatum*	twig	FJ389291
CMW14060	CBS123646	*N. umdonicola*	Kosi bay, S. Africa	*S. cordatum*	twig	*EU821935*
CMW14100		*N. umdonicola*	Kosi bay, S. Africa	*S. cordatum*	twig	FJ389299
CMW14101		*N. umdonicola*	Kosi bay, S. Africa	*S. cordatum*	twig	FJ389311
CMW14068		*N. umdonicola*	Kosi bay, S. Africa	*S. cordatum*	twig	FJ389309
CMW14047		*N. umdonicola*	Mkuze, S. Africa	*S. cordatum*	twig	FJ389308

TABLE I. Continued

Culture No.[1, 2, 3, 4]	Other No.[1]	Identity	Geographic origin	Host	Substratum	GenBank[5] RPB2
CMW14051		*N. umdonicola*	Mkuze, S. Africa	*S. cordatum*	twig	FJ389292
CMW14096[e]		*N. umdonicola*	Port St. Johns, S. Africa	*S. cordatum*	leaf	*EU821943*
CMW14079[f]	CBS123647	*N. umdonicola*	Gonubie, S. Africa	*S. cordatum*	leaf	*EU821945*
CMW14127	CBS123648	*N. umdonicola*	Kwambonambi, S. Africa	*S. cordatum*	fruit	*EU821956*
CMW14125		*N. umdonicola*	Kwambonambi, S. Africa	*S. cordatum*	fruit	FJ389296
CMW14126		*N. umdonicola*	Kwambonambi, S. Africa	*S. cordatum*	fruit	FJ389302
CMW14018		*Neofusicoccum parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389333
CMW14019		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389317
CMW14021		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389321
CMW14022		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389322
CMW14024		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389320
CMW14027[b]		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389339
CMW14029		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	*EU821932*
CMW14030		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389319
CMW14032[c]		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389332
CMW14036		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389318
CMW14038		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389335
CMW14039		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389316
CMW14040		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389334
CMW14045		*N. parvum*	Kwambonambi, S. Africa	*S. cordatum*	twig	FJ389314
CMW14081		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	twig	FJ389338
CMW14082		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	twig	*EU821937*
CMW14085	CBS123649	*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	leaf	*EU821938*
CMW14086		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	leaf	FJ389312
CMW14087		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	twig	*EU821939*
CMW14088		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	twig	*EU821940*
CMW14089		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	leaf	*EU821941*
CMW14090		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	twig	FJ389336
CMW14091		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	leaf	FJ389337
CMW14092		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	twig	FJ389315
CMW14093		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	twig	FJ389323
CMW14094		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	twig	*EU821942*
CMW14095		*N. parvum*	Pietermaritzburg, S. Africa	*S. cordatum*	twig	FJ389329
CMW14097[e]	CBS123650	*N. parvum*	Port St. Johns, S. Africa	*S. cordatum*	leaf	*EU821944*
CMW14080[f]	CBS123651	*N. parvum*	Gonubie, S. Africa	*S. cordatum*	leaf	*EU821946*
CMW14112		*N. parvum*	Tokai, Cape Town, S. Africa	*S. cordatum*	leaf	FJ389326
CMW14128		*N. parvum*	Tzaneen, S. Africa	*S. cordatum*	twig	FJ389313
CMW14129		*N. parvum*	Tzaneen, S. Africa	*S. cordatum*	twig	*EU821947*
CMW14130		*N. parvum*	Tzaneen, S. Africa	*S. cordatum*	twig	FJ389327
CMW14133		*N. parvum*	Tzaneen, S. Africa	*S. cordatum*	twig	FJ389330
CMW14134		*N. parvum*	Tzaneen, S. Africa	*S. cordatum*	twig	FJ389328
CMW14135		*N. parvum*	Tzaneen, S. Africa	*S. cordatum*	twig	*EU821948*
CMW14137		*N. parvum*	Tzaneen, S. Africa	*S. cordatum*	twig	FJ389324
CMW14138		*N. parvum*	Tzaneen, S. Africa	*S. cordatum*	twig	FJ389325
CMW14139		*N. parvum*	Tzaneen, S. Africa	*S. cordatum*	twig	FJ389340
CMW14141[g]		*N. parvum*	Tzaneen, S. Africa	*S. cordatum*	twig	*EU821950*
CMW14142		*N. parvum*	Palaborwa, S. Africa	*S. cordatum*	twig	FJ389331
CMW14143	CBS123652	*N. parvum*	Palaborwa, S. Africa	*S. cordatum*	twig	*EU821951*
CMW27901		*N. parvum*	Pretoria, S. Africa	*S. cordatum*	twig	*EU821957*
CMW9079	ICMP7933	*N. parvum*	New Zealand	*Actinidia deliciosa*		*EU821961*
CMW9080	ICMP8002	*N. parvum*	New Zealand	*Populus nigra*		*EU821962*
CMW9081	ICMP8003	*N. parvum*	New Zealand	*Populus nigra*		*EU821963*
CMW7772		*Neofusicoccum ribis*	New York, U.S.A.	*Ribis* sp.		*EU821958*

TABLE I. Continued

Culture No.[1, 2, 3, 4]	Other No.[1]	Identity	Geographic origin	Host	Substratum	GenBank[5] RPB2
CMW7773		*N. ribis*	New York, U.S.A.	*Ribis* sp.		*EU821959*
CMW7054	CBS121.26	*N. ribis*	New York, U.S.A.	*Ribis rubrum*		*EU821960*

[1] 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, the Netherlands; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand.

[2] Isolates used in pathogenicity trials are in boldface.

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

[4] Isolates of different *Neofusicoccum* spp. collected from a single tree or from one leaf, twig or fruit are marked with the same latter.

[5] Sequence numbers in italics were obtained from the GenBank public database. All others were obtained in this study.

probabilities (BPP) (Rannala and Yang 1996) were estimated with MrBayes (Ronquist and Huelsenbeck 2003).

Morphological characteristics.—In an earlier study the 103 isolates (TABLE I) were induced to sporulate in culture as described by Pavlic et al (2007). Conidia were mounted in lactophenol on microscope slides and inspected by light microscopy. Ten measurements of conidial lengths and widths were taken for each isolate, and the ranges and averages as well as length and width ratio were calculated. Measurements were made and digital photographs taken with a HRc Axiocam digital camera and accompanying Axiovision 3.1 software (Carl Zeiss Ltd., Munich, Germany). SAS® 8.2 undmc vm/cms statistical software was used to analyze variability in conidial lengths and widths among the isolates. Single conidial cultures grown on 2% malt extract agar (MEA) at 25 C under continuous near fluorescent light were used to characterize culture morphology as described by Pavlic et al (2007).

Pathogenicity.—A total of 20 isolates representing the three new *Neofusicoccum* species and *N. parvum* identified from *S. cordatum* as well as type specimens of *N. parvum* and *N. ribis* (TABLE I) were selected for pathogenicity trials under greenhouse conditions. Isolates obtained from *S. cordatum* were selected randomly for inoculations, and all isolates were grown on 2% MEA at 25 C under continuous near fluorescent light 7 d before inoculation.

Twenty-month old *S. cordatum* saplings were grown in pots in an open plant nursery and moved into the greenhouse for acclimatization 4 wk before inoculations. The greenhouse temperature was constant (25 C) and regular day/night conditions were kept. Trees were inoculated in spring–summer (Oct–Nov 2007). Each isolate was inoculated into stems of 10 trees, and 10 trees were inoculated with sterile MEA plugs as control. The inoculations were carried out following the procedure described by Pavlic et al (2007). The inoculated trees were arranged in a randomized block design. The trial was repeated under the same conditions.

Tree diameter at the inoculation height and the length of the lesion developed 6 wk after inoculations were measured. SAS® 8.2 undmc vm/cms statistical software was used to analyze variability in lesion lengths among the isolates. We modeled lesion length as a linear function of greenhouse, fungal species and isolates nested within the species, interaction of greenhouses and fungal species and interaction of greenhouses and isolates nested within the species. This model was repeated with tree diameter as the covariable. The 95% confidence limits were determined for all means based on full model analysis of variance (ANOVA). Differences between means were considered significant at $P \leq 0.05$.

RESULTS

Phylogenetic analyses.—The sequence alignment consisted of 550 characters of which 16 were parsimony informative and were included in the analyses. The parsimony analyses resulted in one most parsimonious tree (CI = 1.0, RI = 1.0) (FIG. 1). MrModeltest 2.2 predicted K80 as an appropriate evolutionary model for Bayesian analyses. The topologies of the trees were identical in the maximum parsimony and Bayesian consensus analyses. Therefore only the consensus tree derived from Bayesian analyses is presented with the parsimony bootstrap values and the posterior probabilities shown at the branches (FIG. 1). The sequences of *N. ribis* were used as outgroup. Ingroup taxa formed four distinct clades of which one corresponded to *N. parvum*, while the other three clades 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 = 43) and clades R1 (n = 15), R2 (n = 14) and R3 (n = 31).

The sequences obtained in this study have been deposited in GenBank (TABLE I). The sequence alignment and phylogenetic trees have been deposited in TreeBASE as SN4175.

Morphological characteristics.—No differences were observed in cultural morphology among the isolates

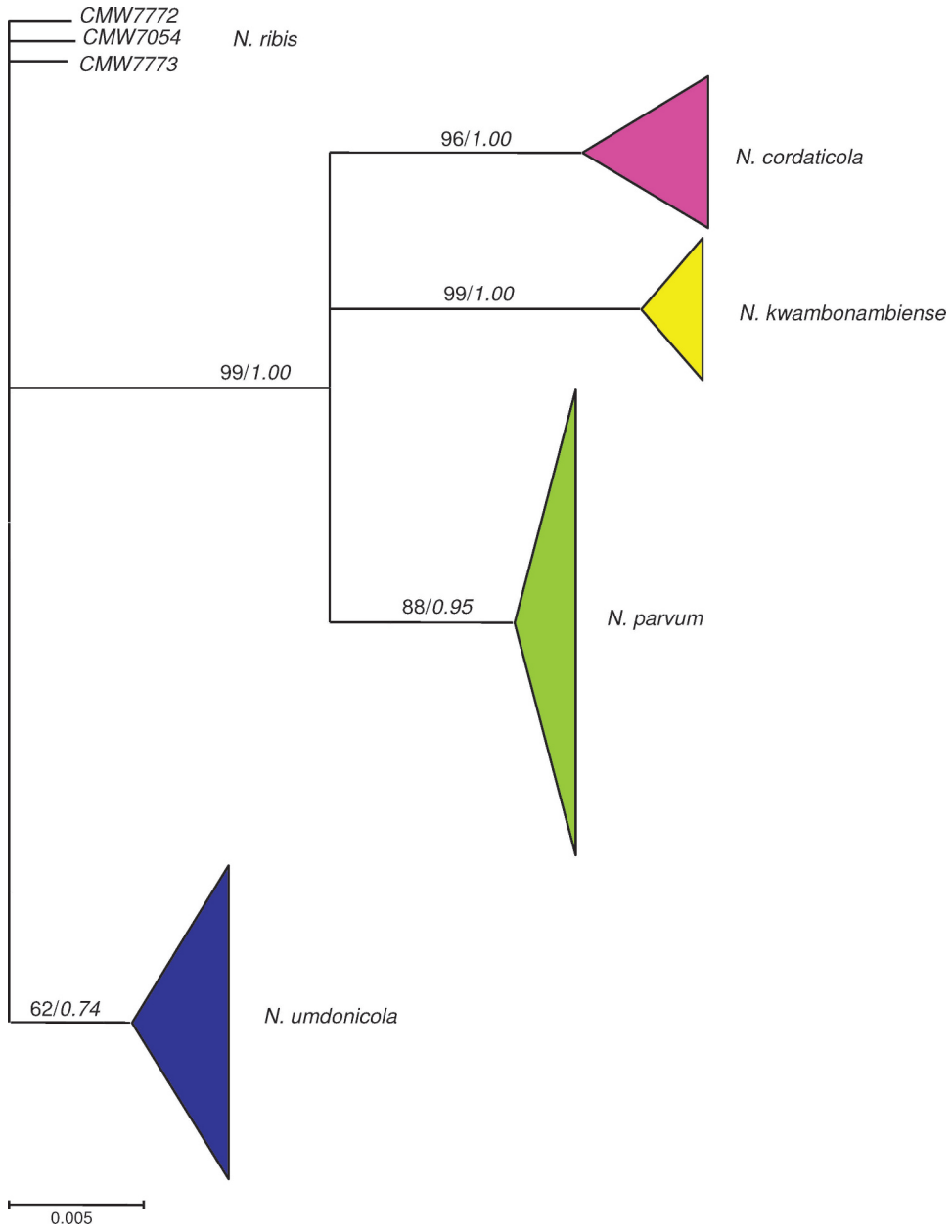

FIG. 1. Consensus phylogram of 38 002 trees resulting from Bayesian analyses of the RNA polymerase II subunit (RPB2) sequence data of the *Neofusicoccum* species in the *N. parvum/N. ribis* complex. The tree is rooted to sequences of *Neofusicoccum ribis*. Bootstrap values of maximum parsimony analyses are indicated above the branches followed by the posterior probabilities resulting from Bayesian analysis (indicated in italics).

of the different *Neofusicoccum* species analyzed in this study. Cultures were initially white with fluffy aerial mycelium, turning pale olivaceous gray from the middle of colony after 3–4 d. They formed thick aerial mycelium, occasionally with columns of the mycelium in the middle of colony reaching the lid. The margins were regular with the reverse sides of the colonies olivaceous gray to black.

Conidial dimensions (lengths and widths) of isolates that belong to the *N. parvum/N. ribis* complex from *S. cordatum* are highly variable and overlap among newly recognized species (FIG. 2). As such these characteristics cannot be used for morphological species recognition a priori. However a posteriori analysis of variance showed that the differences in conidial length and width among phylogenetically recognized species in the *N. parvum/N. ribis* complex were statistically significant ($P \leq 0.001$). Therefore conidial measurements are included in the description of newly recognized

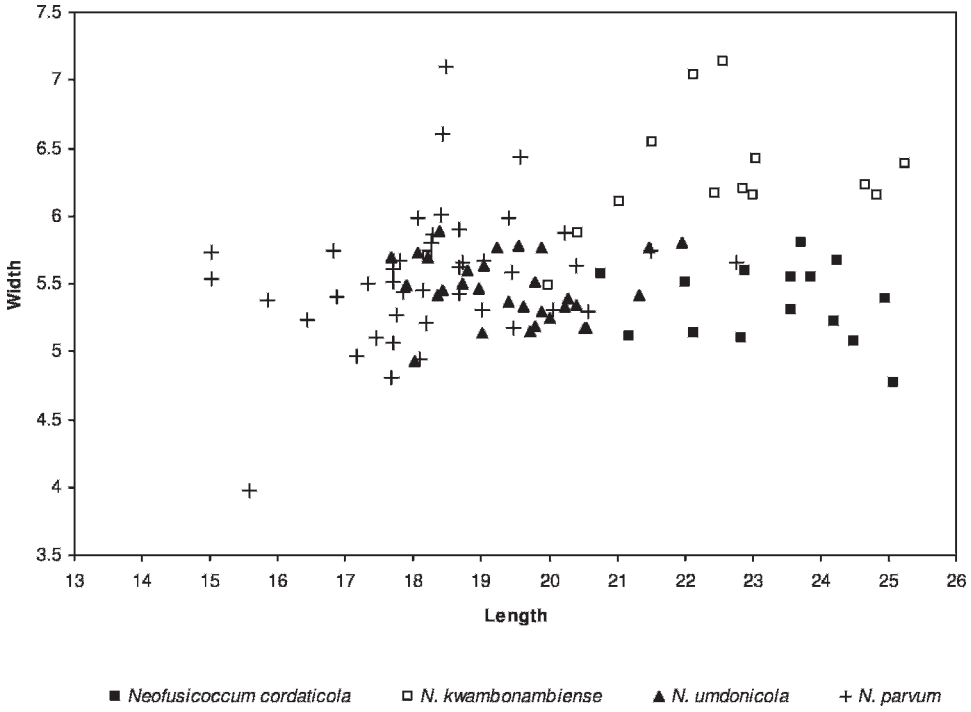

FIG. 2. The averages of the lengths and widths of 10 conidia measured for each of 103 isolates representing *Neofusicoccum parvum/N. ribis* complex from *Syzygium cordatum*.

phylogenetic species. On average conidia of *Neofusicoccum* sp. R1 and R2 are longer than those of *Neofusicoccum* sp. 3, and with rounded apices. Conidia of *Neofusicoccum* sp. R1 are on average longer and narrower with a higher length to width ratio than those of *Neofusicoccum* sp. R2, which are shorter and wider with lower length to width ratio. *Neofusicoccum* sp. R3 differ from the *Neofusicoccum* sp. R1 and R2 by conidia that are on average shorter with tapered apices, but they overlap in shape and size with those of *N. parvum* identified in this study, as well as *N. parvum* and *N. ribis* described by Slippers et al (2004a). Although the conidia of different ages (2–6 wk) were examined, as well as after discharge from pycnidia and until germination, no septate conidia were observed for any of newly recognized species or *N. parvum*.

Pathogenicity.—All isolates induced lesions on stems of *S. cordatum* saplings within 6 wk, demonstrating potential pathogenicity of all species. The respective *Neofusicoccum* species that were re-isolated from the edge of the lesions on the inoculated trees were the same as those used for inoculations. Small lesions developed on some trees inoculated with a sterile MEA plugs as controls. No species of Botryosphaeriaceae were re-isolated from controls. Therefore the lesions associated with the controls are considered reaction of trees to inoculation wounds.

Analyses of variance showed that the interactions between mean lesion lengths produced in two trials were statistically significant ($P \leq 0.05$) and therefore data from these trials could not be combined. Data for both trials are presented on the same graph (FIG. 3). Statistical analyses showed no correlation between tree diameter and lesion length. With exception of two *N. parvum* isolates (CMW14143, CMW9079), one isolate of *N. ribis* (CMW7054) and another of *Neofusicoccum* sp. R1 (CMW14151), all other isolates in trial 1 produced lesions significantly different from the controls (FIG. 3). Lesions produced by four isolates of *N. parvum* (CMW14080, CMW14143, CMW9079, CMW9080), one of *N. ribis* (CMW7054) and another of *Neofusicoccum* sp. R2 (CMW14140) in the second trial were not significantly different from the control (FIG. 3). All other isolates in the second trial produced lesions significantly different from the controls (FIG. 3). Intraspecific variation in mean lesion length was observed for all four species obtained from *S. cordatum* and at the 95% significance level for some of isolates in both trials (FIG. 3). Mean lesion lengths produced by some of the isolates (CMW 14097, 14140, 14155, 14058, 14106) differed significantly between two trials (FIG. 3). In such cases significantly smaller lesions were observed on trees that were in better condition.

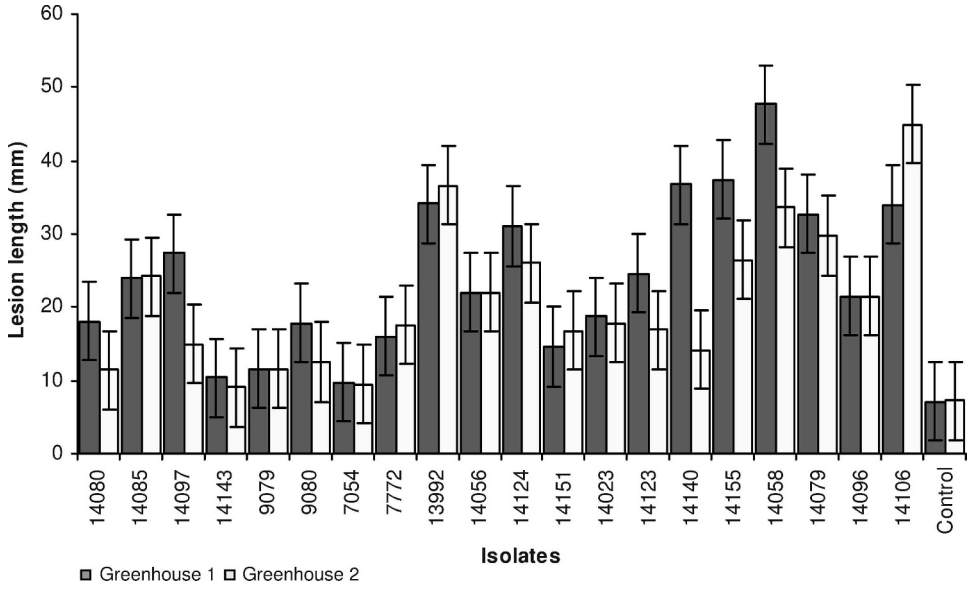

FIG. 3. Mean lesion lengths (mm) obtained for each isolate of different species of the *Neofusicoccum* 6 wk after inoculation on *Syzygium cordatum*. Bars represent 95% confidence limits for each isolate. *N. parvum* (CMW14097, 14080, 14085, 14143, 9079, 9080); *N. ribis* (CMW7772, 7054); *N. cordaticola* (CMW13992, 14056, 14151, 14124), *N. kwambonambiense* (CMW14023, 14140, 14155, 14123); *N. umdonicola* (CMW14106, 14058, 14079, 14096); C = Control.

TAXONOMY

Based on combined sequence data of five gene regions, four phylogenetic groups were recognized within the *N. parvum/N. ribis* species complex from native *S. cordatum* in South Africa. Three of these groups are closely related but clearly separated from *N. parvum* and *N. ribis* and are recognized as three undescribed phylogenetic species. These species can be consistently diagnosed based only on genotypic characters. The three new phylogenetic species are therefore described here.

Neofusicoccum cordaticola MB512498 Pavlic, Slippers, M.J. Wingfield, sp. nov.

= *Neofusicoccum* sp. R1 *sensu* Pavlic et al Mol Phylogenet Evol 51:259–268 (2009)

N. cordaticola speciebis aliis in complexo specierum *N. parvi/N. ribis* similis; conidia *N. cordaticola* hyalina unicellularia anguste fusiformia vel ovalia apicibus rotundatis 18–28 × 4.5–7 µm. *N. cordaticola* a speciebus aliis locis 5 nuclearibus differt: ITS1, 5.8S, et ITS2 sitibus 141 (C), 372 (G) et 416 (C); loco ''translation elongation factor (1-α)'' dicto sitis 58 (C) et 221 (C); loco ''β-tubulin-2'' dicto sitis 32 (T), 96 (T) et 316 (G); loco *BotF15* sitis 121 (T) et 122 (C); et loco ''RNA polymerase II subunit'' dicto sitis 100 (A), 112 (T), 265 (A) et 409 (C).

Neofusicoccum cordaticola is morphologically similar to other species in the *N. parvum/N. ribis* species complex. Conidia of *N. cordaticola* are hyaline, unicellular, narrowly fusiform to oval, apices rounded 18–28 × 4.5–7 µm (av. 150 conidia 23.3 × 5.3 µm, l/w 4.3). *N. cordaticola* differs from other species in the *N. parvum/N. ribis* complex by uniquely fixed nucleotides in five nuclear loci: internal transcribed spacer rDNA (ITS1, 5.8S, and ITS2) position 141 (C), 372 (G) and 416 (C); translation elongation factor (1-α) positions 58 (C) and 221 (C); β-tubulin-2 position 32 (T), 96 (T) and 316 (G); locus *BotF15* position 121 (T) and 122 (C); RNA polymerase II subunit positions 100 (A), 112 (T), 265 (A) and 409 (C).

Teleomorph. Not known.

Etymology. Refers to the host *Syzygium cordatum* from which isolates were collected, (*in*)*cola* = an inhabitant.

Habitat. Symptomless branches and leaves, dying branches and pulp of ripe fruits of *Syzygium cordatum*.

Known distribution. South Africa.

HOLOTYPE. SOUTH AFRICA. KWAZULU-NATAL PROVINCE: Sodwana Bay on *Syzygium cordatum*, Mar 2002, D. Pavlic, (PREM 60066, a dry culture ex CMW 13992 on pine needles; ex-type culture CMW 13992 = CBS 123634).

Additional specimens examined. TABLE I.

Neofusicoccum kwambonambiense MB512499 Pavlic, Slippers, M.J. Wingfield, sp. nov.

= *Neofusicoccum* sp. R2 *sensu* Pavlic et al Mol Phylogenet Evol 51:259–268 (2009)

N. kwambonambiense speciebis aliis in complexo specierum *N. parvi/N. ribis* similis; conidia *N. kwambonambiense* hyalina unicellularia fusiformia vel ellipsoidia apici-

bus rotundatis 16–28 × 5–8 µm. *N. kwambonambiense* a speciebus aliis locis 4 nuclearibus differt: ITS1, 5.8S, et ITS2 sitibus 163 (T) et 173 (G); loco ''β-tubulin-2'' dicto sitis 175 (T), 235 (A), et 251 (A); loco *BotF15* sitis 87 et 172; loco ''RNA polymerase II subunit'' dicto sitis 49 (G), 382 (A), 421 (A), et 526 (C).

Neofusicoccum kwambonambiense is morphologically similar to other related species in the *N. parvum/N. ribis* species complex. Conidia of *N. kwambonambiense* are hyaline, unicellular, fusiform to ellipsoid, apices rounded 16–28 × 5–8 µm (av. 140 conidia 22.3 × 6.3 µm, l/w 3.6). *N. kwambonambiense* differs from other species in the *N. parvum/N. ribis* complex by uniquely fixed nucleotides in four nuclear loci: internal transcribed spacer rDNA (ITS1, 5.8S, and ITS2) position 163 (T) and 173 (G); β-tubulin-2 position 175 (T), 235 (A) and 251 (A); locus *BotF15* position 87, and 172; RNA polymerase II subunit positions 49 (G), 382 (A), 421 (A) and 526 (C).

Teleomorph. Not known.

Etymology. Refers to the town, Kwambonambi, South Africa, from where the type isolate was collected.

Habitat. Symptomless branches and leaves, dying branches and pulp of ripe fruits of *Syzygium cordatum*.

Known distribution. South Africa.

HOLOTYPE. SOUTH AFRICA. KWAZULU-NATAL PROVINCE: Kwambonambi on *Syzygium cordatum*, Mar 2002, D. Pavlic, (PREM 60067, a dry culture ex CMW 14023 on pine needles; ex-type culture CMW 14023 = CBS 123639).

Additional specimens examined. TABLE I.

Neofusicoccum umdonicola MB512500 Pavlic, Slippers, M.J. Wingfield, sp. nov.

= *Neofusicoccum* sp. R3 *sensu* Pavlic et al Mol Phylogenet Evol 51:259–268 (2009)

N. umdonicola speciebis aliis in complexo specierum *N. parvi/N. ribis* similis; conidia *N. umdonicola* hyalina unicellularia fusiformia vel ovalia apicibus angustatis 15–23.5 × 4.5–6.5 µm. *N. umdonicola* a speciebus aliis locis 4 nuclearibus differt: ITS1, 5.8S, et ITS2) situ 168 (C); loco ''translation elongation factor (1-α)'' dicto situ 62 (T); loco ''β-tubulin-2'' dicto situ 40 (A); loco ''RNA polymerase II subunit'' dicto situ 280 (T).

Neofusicoccum umdonicola is morphologically similar to other related species in the *N. parvum/N. ribis* species complex. Conidia of *N. umdonicola* are hyaline, unicellular, fusiform to oval, apices tapered 15–23.5 × 4.5–6.5 µm (av. 310 conidia 19.4 × 5.5 µm, l/w 3.5). *N. umdonicola* differs from other species in the *N. parvum/N. ribis* complex by uniquely fixed nucleotides in four nuclear loci: internal transcribed spacer rDNA (ITS1, 5.8S and ITS2) position 168 (C); translation elongation factor (1-α) positions 62 (T); β-tubulin-2 position 40 (A); RNA polymerase II subunit position 280 (T).

Teleomorph. Not known.

Etymology. Refers to common Zulu and also KZN-English name, Umdoni for the *Syzygium cordatum*, the host from which isolates were obtained, (*in*)*cola* = an inhabitant.

Habitat. Symptomless branches and leaves, dying branches and pulp of ripe fruits of *Syzygium cordatum*.

Known distribution. South Africa.

HOLOTYPE. SOUTH AFRICA. KWAZULU-NATAL PROVINCE: Kosi Bay on *Syzygium cordatum*, Mar 2002, D. Pavlic, (PREM 60068, a dry culture ex CMW 14058 on pine needles; ex-type culture CMW 14058 = CBS 123645).

Additional specimens examined. TABLE I.

DISCUSSION

In this study we described three phylogenetic species within the *N. parvum/N. ribis* species complex from native *S. cordatum* in South Africa, namely *Neofusicoccum cordaticola*, *N. kwambonambiense* and *N. umdonicola*. These species were recognized by Pavlic et al (2009) using the genealogical concordance phylogenetic species recognition (GCPSR) as a form of phylogenetic species concept (PSC) (Taylor et al 2000), based on DNA sequence data for five nuclear loci. The phylogenetic species are characterized primarily by fixed single nucleotide polymorphisms (SNPs) (O'Donell et al 2004, Grünig et al 2008) that were identified for each of three species described in this study. Although many cryptic, phylogenetic species have been recognized recently in the fungal kingdom, there are very few descriptions of these species. This is the first description of phylogenetic species in the Botryosphaeriaceae using sequence data as defining characters.

Neofusicoccum kwambonambiense is the sister species of *N. ribis*. Despite the fact that these two species can be distinguished with DNA sequence data from multiple loci, these two species cannot be separated from each other or from *N. parvum* with conidial morphology observed in this study. Slippers et al (2004a) used septation of conidia to distinguish *N. parvum* and *N. ribis*, but such septa were not observed in this study for any of the newly described species or *N. parvum*. Because conidial septation is not a constant character in *Neofusicoccum* spp. it cannot be used as a reliable feature in separation and identification of these species.

Pathogenicity trials showed that *N. kwambonambiense* is the most aggressive to *S. cordatum* of all five species tested in this study. There is no significant difference in pathogenicity between *N. cordaticola* and *N. umdonicola* to *S. cordatum*, but they both appear to be significantly more aggressive to this host

then *N. parvum* and *N. ribis*. Barring *N. ribis*, all of these species were isolated from *S. cordatum* growing in close association with commercially grown *Eucalyptus* plantations in South Africa. In an earlier study isolates of *N. parvum*, *N. cordaticola* and *N. kawambonambiense* (the latter two species were identified then as *N. ribis sensu lato* [Pavlic et al 2007]) were recognized as more aggressive to *Eucalyptus* than to *S. cordatum* in greenhouse trials. In the field pathogenicity trials on different *Eucalyptus* clones grown commercially in Venezuela (Mohali 2005) and Colombia (Rodas 2003) isolates identified as ''*N. ribis*'' were shown to be highly aggressive to *Eucalyptus*. It is possible that some of these isolates represent cryptic species in the *N. parvum/N. ribis* complex. The trials conducted on different *Eucalyptus* clones in Venezuela showed that *N. parvum* was significantly more aggressive than *N. ribis* (Mohali 2005). Clearly most members of the *N. parvum/N. ribis* complex have potential to become important pathogens to native and commercially grown Myrtaceae.

All three new species grow endophytically on different parts of *S. cordatum* tree. These include symptomless twigs, leaves and fruits. More than one species were commonly found within a single tree and even within one leaf or one fruit. Species of Botryosphaeriaceae are known as endophytes that grow within plant tissues without exhibiting disease symptoms (Smith et al 1996, Pavlic et al 2004, Slippers and Wingfield 2007) and also were identified as seedborne, for example *N. parvum* in *Podocarpus falcatus* and *Prunus africana* seeds (Gure et al 2005). As endophytes they can be easily moved into new regions and pose an equally serious threat to native and cultivated plants alike (Burgess and Wingfield 2002, Slippers and Wingfield 2007). Occurrence of more than one species within a small piece of plant tissue or in one fruit of *S. cordatum* implies that more than one species can be easily introduced into a new area with this plant material. This is important given that these new species are more aggressive than known species *N. parvum* and *N. ribis* on *Syzygium*.

The correct identification of plant pathogenic fungi is of utmost importance for quarantine and control measures. A PCR-RFLP fingerprinting technique was developed to distinguish *sensu lato* groups of *N. parvum* and *N. ribis* (Slippers 2003). Alves et al (2007) designed MSP-PCR (microsatellite-primed polymerase chain reaction) and rep-PCR (repetitive-sequence-based polymerase chain reaction) fingerprinting methodologies for rapid identification of species of Botryosphaeriaceae, including closely related species such as *N. parvum* and *N. ribis*, or *N. luteum* and *N. australe*. Such PCR-based methodologies are quick and reliable for the identification of large numbers of isolates, and development of such methods for the identification of new *Neofusicoccum* species should be considered in future studies. The isolates recognized by Rodas (2003), Slippers (2003), Mohali (2005) and Slippers et al (2005) as *N. ribis sensu lato* group, based on PCR-RFLP profiles, should be re-evaluated because these groups can comprise cryptic species, such as those described in this study. As it was shown in Pavlic et al (2009), the RPB2 sequences are the most valuable for delimitation of these cryptic species and should be used in further identification and re-evaluation of species in the *N. parvum/N. ribis* complex.

In many studies on Botryosphaeriaceae preliminary groupings of isolates have been based on cultural and conidial morphology (e.g. Slippers et al 2004a; Burgess et al 2005; Pavlic et al 2007, 2008). In those studies groups identified based on morphological characters usually were found congruent with those recognized based on DNA sequence data and vice versa. Of interest, in our earlier study on Botryosphaeriaceae from *S. cordatum* in South Africa differences in conidial morphology were used to select isolates from *N. parvum/N. ribis* group for further ITS rDNA sequencing (Pavlic et al 2007). Groups recognized based on differences in conidial morphology were consistent with groupings observed within *N. parvum/N. ribis* clade based on ITS sequences. These observations initiated further study of this group of isolates and recognition of cryptic species based on multiple gene genealogies (Pavlic et al 2009). Despite its use in selection of isolates for further study, the variation among the larger group of isolates was continuous and overlapping between what was later identified as distinct species. The morphological characters alone thus were insufficient for confident identification of all isolates representing the species in the *N. parvum/N. ribis* complex.

The use of molecular tools and specifically DNA sequence data let us detect and discriminate numerous new species. Without these powerful tools closely related or cryptic species and species complexes would stay unrecognized. However morphological and other phenotypic characteristics such as pathogenicity cannot be underestimated because differences in these characteristics may indicate presence of cryptic species and present valuable data in their delimitation, as it is shown in this study. Thus an integrated approach should be imperative in species delineation and identification of Botryosphaeriaceae, as was suggested by Dayrat (2005) and Roe and Sperling (2007).

The species described in this study are recognized only from native *Syzygium cordatum*. These species were not recognized during intensive studies on related or other non-native hosts grown in proximity

(Jacobs 2002, Slippers et al 2004b). This indicates that more studies should focus on identification of fungal species on native trees. They are clearly a source of fungal diversity, which could serve as a source of inoculum on economically important crops. Furthermore such studies on fungi on native trees will give us an opportunity to extend our knowledge about the natural history, ecology and biogeography of fungal biodiversity that at present is poorly understood.

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