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Botryosphaeriaceae species overlap on four unrelated, native South African hosts

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ABSTRACT

Botryosphaeriaceae represents an important and diverse family of latent fungal pathogens of woody plants. We address the question of host range of these fungi by sampling leaves and branches of four native South African trees, including *Acacia karroo* (*Fabaceae*), *Celtis africana* (*Cannabaceae*), *Searsia lancea* (*Anacardiaceae*), and *Gymnosporia buxifolia* (*Celastraceae*). Two new species of the *Botryosphaeriaceae*, namely *Tiarosporella africana* sp. nov. and *Aplosporella javeedii* sp. nov. were identified, together with five known species, including *Neofusicoccum parvum*, *Neofusicoccum kwambonambiense*, *Spencermartinsia viticola*, *Diplodia pseudoseriata*, and *Botryosphaeria dothidea*. Most *Botryosphaeriaceae* occurred on more than one host. With the exception of *S. lancea*, which was infected by *A. javeedii* all the hosts were infected by more than one *Botryosphaeriaceae* species. Collectively, the results suggest that some intrinsic host factors, possibly combined with local environmental conditions, affect the distribution and co-infectivity of various hosts by the *Botryosphaeriaceae*. This would counteract the general ability of a species in the *Botryosphaeriaceae* to infect a broad range of plants. The combination of host and environmental factors might also explain why some *Botryosphaeriaceae* with apparently broad host ranges, are found on different suites of hosts in different areas of the world.
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Introduction

Fungi residing in the *Botryosphaeriaceae* (*Ascomycota*: *Botryosphaeriales*) have been characterised from a wide variety of trees. They commonly occur as endophytes in asymptomatic plant tissues (Smith *et al.* 1996b), but some species are also important pathogens. The shift in habit from endophyte to being virulent pathogens typically occurs when trees are subjected to stress (Slippers & Wingfield 2007). Some *Botryosphaeriaceae* infect several different hosts, which may or may not be related to each other. Other species are known from only a single host. While there appear to be some distinct patters of host association for those species that infect conifers as opposed to angiosperms (De Wet *et al.* 2008), relatively little is known regarding the epidemiology and host ranges of these intriguing fungi.

Species of *Botryosphaeriaceae* occur widely in South Africa and they have been found on virtually every tree species that has been sampled for them. Hosts include native trees such as *Terminalia catappa* (*Myrtales*: *Combretaceae*) (Begoude *et al.* 2010), *Pterocarpus angolensis* (*Fabales*: *Fabaceae*) (Mehl *et al.* 2011), *Syzygium cordatum* (*Myrtales*: *Myrtaceae*) (Pavlic *et al.* 2007), *Acacia mellifera* (*Fabales*: *Fabaceae*) (Slippers *et al.* 2013),

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Acacia karroo (Jami *et al.* 2012), and woody species of *Leucadendron*, *Leucospermum*, and *Protea* (*Proteales*: *Proteaceae*) (Denman *et al.* 2003). Nonnative hosts of the *Botryosphaeriaceae* in South Africa include *Pinus* spp. (*Pinales*: *Pinaceae*), *Eucalyptus* spp. (*Myrtales*: *Myrtaceae*), *Prunus* spp. (*Rosales*: *Rosaceae*), and *Vitis vinifera* (*Vitales*: *Vitaceae*) (Damm *et al.* 2007a; Smith *et al.* 1996a; Van Niekerk *et al.* 2004). Despite relatively intensive sampling over many years, most native woody plants in South Africa have not been sampled for the presence of *Botryosphaeriaceae*.

Some species of *Botryosphaeriaceae* have broad host ranges, occurring on both native and nonnative hosts in a sampled area. For example, *Neofusicoccum vitifusiforme* (Van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips, *N. australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum kwambonambiense* Pavlic, Slippers & M.J. Wingf., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Diplodia seriata* De Not., *Spencermartinsia viticola* (A.J.L. Phillips & J. Luque) A.J.L. Phillips, A. Alves & Crous and *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not., have been found on various native and nonnative trees in South Africa (Damm *et al.* 2007a; Denman *et al.* 2003; Pavlic *et al.* 2007, 2009a; Pillay *et al.* 2013; Slippers *et al.* 2007; Smith *et al.* 1996a; Van Niekerk *et al.* 2004). Some *Botryosphaeriaceae* can also infect a variety of native hosts and examples include *Dothiorella dulcispinae* Jami, Gryzenh., Slippers & M.J. Wingf., *Sphaeropsis variabilis* F.J.J. van der Walt, Slippers & G.J. Marais, and *Spencermartinsia rosulata* F.J.J. van der Walt, Slippers & G.J. Marais, that infect different *Acacia* species (Jami *et al.* 2012; Slippers *et al.* 2013), *Lasiodiplodia pseudotheobromae* A.J.L. Phillips, A. Alves & Crous from *P. angolensis*, *T. catappa*, and *S. cordatum* (Begoude *et al.* 2010; Mehl *et al.* 2011; Pillay *et al.* 2013), and *Neofusicoccum protearum* (Denman & Crous) Crous, Slippers & A.J.L. Phillips that infects *Leucadendron laureolum*×*Leucadendron salignum* and *Protea* spp. (Denman *et al.* 2003). In contrast, some species have thus far been found only on a single host plant, for example *Tiarosporella urbis-rosarum* Jami, Gryzenh., Slippers & M.J. Wingf., *Diplodia allocellula* Jami, Gryzenh., Slippers & M.J. Wingf., *Dothiorella brevicollis* Jami, Gryzenh., Slippers & M.J. Wingf., *Dothiorella oblonga* F.J.J. van der Walt, Slippers & G.J. Marais, *Spencermartinsia pretoriensis* Jami, Gryzenh., Slippers & M.J. Wingf., *Spencermartinsia capri-amissi*, *Neofusicoccum viticlavatum* (Van Niekerk & Crous) Crous, Slippers & A.J.L. Phillips, and *Lasiodiplodia pyriformis* F.J.J. van der Walt, Slippers & G.J. Marais (Jami *et al.* 2013; Slippers *et al.* 2013; Van Niekerk *et al.* 2004). This pattern of association could be attributed to a sampling effect. For example, sampling has not been particularly intensive for most tree species and sampling has also tended to focus on particular areas. It is thus not clear whether species known from a limited number of hosts are host specific, or if they simply have not been sampled from other hosts.

Acacia karroo has been subjected to intensive surveys for *Botryosphaeriaceae* across various geographical areas in southern Africa (Jami *et al.* 2012, 2013; Slippers *et al.* 2013). A large diversity of *Botryosphaeriaceae* has been found during these studies, including *T. urbis-rosarum*, *D. allocellula*, *S. variabilis*, *Do. brevicollis*, *Do. dulcispinae*, *N. vitifusiforme*, *S. viticola*, *S. pretoriensis*, *S. rosulata*, *N. australe*, *N. parvum*, *N. kwambonambiense*, *B. dothidea*, and *L. theobromae*. Some of these species are known from hosts other than *A. karroo*, while others have been reported only from this tree. As in other systems, the question arises as to whether this reflects the level of host specificity or if it is due to a sampling bias.

The aim of this study was to determine patterns of overlap of the *Botryosphaeriaceae* occurring on *A. karroo* and three unrelated and commonly occurring tree species that grow in areas surrounding it. These trees included *Celtis africana* (*Rosales*: *Cannabaceae*), *Searsia lancea* (*Sapindales*: *Anacardiaceae*), and *Gymnosporia buxifolia* (Celastrales: *Celastraceae*). Sampling was made at a particular point in time and at a single location to exclude the effect of temporal and geographical diversity. We also considered the level of diversity of *Botryosphaeriaceae* in different tissues on these hosts. It was thus anticipated that the results would provide a rudimentary estimation of the patters of diversity for *Botryosphaeriaceae* in South Africa that might be expected across different hosts.

Materials and methods

Collection of samples and isolations

Healthy plant material from *Acacia karroo* and three commonly occurring and surrounding tree species, namely *Celtis africana*, *Searsia Lancea*, and *Gymnosporia buxifolia* were collected in October 2011 (spring). Ten healthy and cooccurring trees of each species were randomly chosen for sampling. Three healthy branches including leaves were collected from each tree, placed in paper bags, and transferred to the laboratory to be processed for isolations. Samples were obtained from a nature reserve area in Pretoria, South Africa.

For each sample, 12 pieces (0.5 cm in length) of tissue were taken from each branch and 12 pieces were cut from the simple leaves. The samples were surface disinfested in 10 % hydrogen peroxide for 2 min, rinsed three times in sterile distilled water and cultured on 2 % malt extract agar (MEA) (Biolab, South Africa). Single hyphal-tips of isolates displaying a cultural morphology typical of the *Botryosphaeriaceae*, such as rapid growth and white to black mycelium with aerial hyphae, were transferred to fresh plates until pure cultures had been obtained. Single hyphal-tip cultures of these isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and duplicate isolates of the new species were deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), The Netherlands.

DNA sequence analyses

Isolates utilised in this study were grouped based on culture morphology. DNA was extracted (Lee & Taylor 1990) from fungal mycelium of 5-day-old single hyphal-tip cultures of three to five representatives for each morphological group. Four gene regions were used for comparison based phylogenetic analyses to determine the identities of the unknown isolates. These included the internal transcribed spacer region of the ribosomal RNA (rRNA) operon amplified with primers ITS-1F (Gardes & Bruns 1993) and ITS-4 (White *et al.* 1990), the

translation elongation factor 1-α (EF1-α) gene amplified with primers EF1-728F and EF1-986R (Carbone & Kohn 1999), the β-tubulin gene using primers Bt2a and Bt2b (Glass & Donaldson 1995) and the large subunit rDNA (LSU) gene region using primers LR0 and LR5 (Vilgalys & Hester 1990).

The conditions and procedures for PCR, sequencing and phylogenetic analyses were the same as those described in Jami *et al.* (2012). The phylogenetic analyses for all the datasets were performed using Maximum Likelihood (ML) and Bayesian analyses. For ML analyses, the best nucleotide substitution models for each dataset were found separately with Modeltest 3.7 (Posada & Buckley 2004). The model for GTR + G ($G = 0.2390$, $I = 0.0$) was chosen for the combined datasets of ITS, LSU, TEF-1α, β-tubulin. The ML analyses were performed in PAUP 4.0b10 and confidence levels were determined with 1000 bootstrap replications. Bayesian analyses using the Markov Chain Monte Carlo (MCMC) method were performed to ascertain the topology of trees obtained with ML. The MCMC analyses, with four chains, started from random tree topology and lasted 3 000 000 generations. Trees were saved every 100th generation. The burn-in number was graphically estimated (3000) from the likelihood scores and trees outside this point were discarded in the analyses. The consensus trees were constructed in MEGA version 4 and posterior probabilities were assigned to branches after a 60 % majority rule.

Morphological characteristics

To induce sporulation, cultures were inoculated onto sterilized twigs of *Acacia karroo* placed on the surface of 2 % MEA (Biolab), and these were incubated at 25 °C under near-UV light (Jami *et al.* 2012). Fifty released conidia, and 30 pycnidia and conidiogenous cells were measured for the isolates chosen to represent holotypes for each putative new species, and the ranges and averages were computed. Measurements and digital images were made with an HRc Axiocam digital camera and accompanying Axiovision 3.1 software (Carl Zeiss, Munich, Germany). Dried cultures representing type specimens were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Colony morphology and colour were determined for cultures grown on MEA at 5–35 °C, at 5 °C intervals, in the dark. For these, 6 mm diam. mycelial plugs were taken from the edges of actively growing 4-day-old single conidial cultures, and transferred to the centres of 90 mm diam. Petri dishes containing MEA. Three replicate plates were used for each isolate per temperature. Two measurements perpendicular to each other were taken of the colony diameter daily until the mycelium of the fastest growing isolates had covered the plates and averages were computed. Colony colours were assigned using the designations of Rayner (1970).

Statistical analyses of species diversity

To determine the variability and overlap of the *Botryosphaeriaceae* species from the four hosts, data generated from the isolations were subjected to statistical analyses to determine whether the variation was significant or not. In addition, the variability and overlap in diversity and species between tissue types (branches and leaves) for each host and in total were determined. A one-way ANOVA with the general linear model procedure was used with JMP (version 10, SAS Institute Inc. 2012).

Results

Collection of samples and isolates

A total of 191 isolates were obtained from the four host trees, with 119 from branches and 72 from leaves. These included 82 isolates from *Acacia karroo* (50 % of sampled trees), 72 from *Celtis africana* (40 % of sampled trees), three from *Searsia lancea* (10 % of sampled trees) and 34 isolates from *Gymnosporia buxifolia* (50 % of sampled trees). Isolates from *A. karroo* included 42.9 % of total isolates, while those from *S. lancea* included only 1.5 % of the total collection.

DNA sequence analyses

The sequence datasets for the ITS, TEF-1α, β-tubulin, and LSU rDNA regions were analysed individually and in combination. The ITS sequence dataset contained 552 characters (excluding 366 and including 186 characters) with RI = 0.972, RC = 0.809, HI = 0.167 and TL = 301.8. The TEF-1α dataset contained 287 characters (excluding 60 and including 227 characters) with RI = 0.891, RC = 0.550, HI = 0.383 and TL = 523. The β-tubulin dataset contained 366 characters (excluding 239 and including 127 characters) with RI = 0.965, RC = 0.825, HI = 0.302, and TL = 185. The LSU dataset contained 848 characters (excluding 460 and including 484 characters) with RI = 0.983, RC = 0.906, HI = 0.078 and TL = 549. The tree statistics for the combined dataset were RI = 0.854, RC = 0.416, HI = 0.513, TL = 2148 (TreeBase Accession No. S12358), and the partition homogeneity test (PHT) on the datasets gave a *P*-value of 0.01.

The topology of the trees emerging from the ML, MP, and MrBayes analyses were similar for the individual gene regions, as well as in the combined analyses, with regards to the clades representing species isolated in this study. Seven clades were identified in all the analyses and these represented *Spencermartinsia viticola*, *Botryosphaeria dothidea*, *Neofusicoccum parvum*, *Neofusicoccum kwambonambiense*, *Diplodia pseudoseriata* and two unidentified groups within the clades accommodating *Aplosporella* and *Tiarosporella*, respectively (Fig 1). The distinct groupings of two new species in *Aplosporella* and *Tiarosporella* were based on fixed sequence variants linked to the two groups and identified in the datasets (Tables 2 and 3).

From *Acacia karroo*, three species were identified, namely *B. dothidea* (CMW38114, CMW38115, CMW38116), *D. pseudoseriata* (CMW38137, CMW38138) and *S. viticola* (CMW38079). Four species, namely *S. viticola* (CMW38082), *N. kwambonambiense* (CMW38426), *Tiarosporella* sp. nov. (CMW38423, CMW38424, CMW38425, CMW38428), and *Aplosporella* sp. nov. (CMW38165, CMW38166, CMW38167) were isolated from *C. africana*. This is in contrast to *S. viticola* (CMW38080) and *N. parvum* (CMW38161) that were obtained from *Gymnosporia buxifolia*. Only the *Aplosporella* sp. nov. (CMW38168 CMW38169, CMW38170) was identified from *S. lancea*. *Spencermartinsia viticola* was common among *A. karroo*, *C. africana*, and

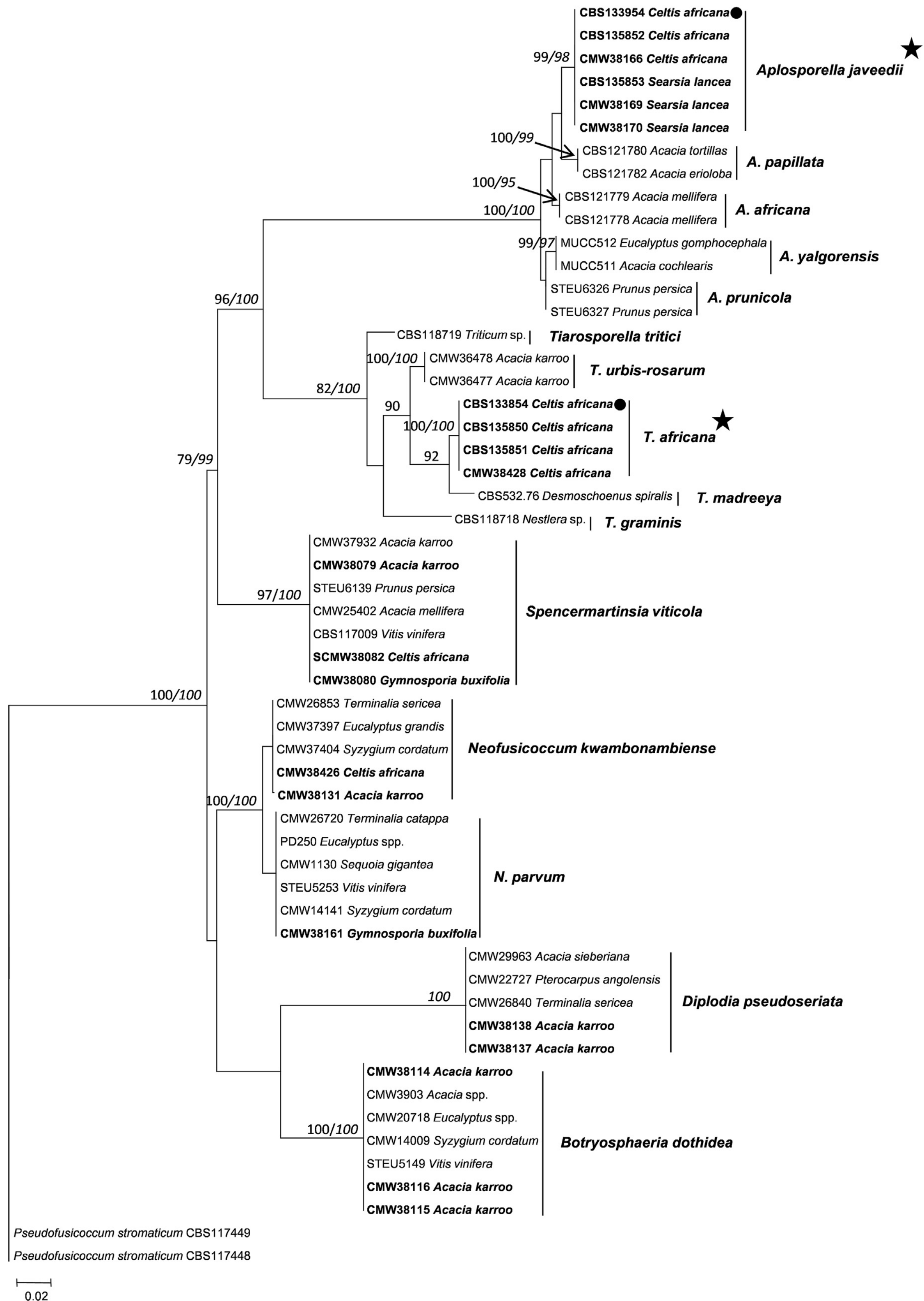

Fig 1 – Maximum Likelihood (ML) tree of the combined dataset of ITS ribosomal DNA, TEF-1α, β-tubulin, and LSU gene region sequences. Bootstrap values for ML (Piano *et al.* 2005) and MrBaysen (italic) above 60 % are given at the nodes. The tree was rooted to *Pseudofusicoccum stromaticum* (CBS117448 and CBS117449). Isolates of this study are indicated as bold. *Newly described species in this study. •Indicates for ex-type isolates.

Table 1 – Representative isolates of this study used in the phylogenetic analyses.

Isolate no.	Identity	Host	Tissue	Location	Collector	GenBank			
						ITS	EF1-α	β-Tubulin	LSU
CMW38165 CBS133954	***Aplosporella javeedii****	*Celtis africana*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769938	KC769846	KC769903	KC769979
CMW38166	*A. javeedii**	*Celtis africana*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769939	KC769847	KC769904	KC769980
CMW38167 CBS135852	*A. javeedii**	*Celtis africana*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769940	KC769848	KC769905	KC769981
CMW38168 CBS135853	*A. javeedii**	*Searsia lancea*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769941	KC769849	KC769906	KC769982
CMW38169	*A. javeedii**	*Searsia lancea*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769942	KC769850	KC769907	KC769983
CMW38170	*A. javeedii**	*Searsia lancea*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769943	KC769851	KC769908	KC769984
CMW38114	*Botryosphaeria dothidea*	*Acacia karroo*	Leaves	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769944	KC769856	KC769898	–
CMW38115	*B. dothidea*	*Acacia karroo*	Leaves	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769945	KC769857	KC769899	–
CMW38116	*B. dothidea*	*Acacia karroo*	Leaves	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769946	KC769858	KC769900	–
CMW38137	*Diplodia pseudoseriata*	*Acacia karroo*	Leaves	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769954	KC769863	KC769896	–
CMW38138	*D. pseudoseriata*	*Acacia karroo*	Leaves	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769955	KC769864	KC769897	–
CMW38131	*Neofusicoccum kwambonambiense*	*Acacia karroo*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769949	KC769862	KC769902	KC769988
CMW38426	*N. kwambonambiense*	*Celtis africana*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769948	KC769861	KF512019	KC769989
CMW38161	*N. parvum*	*Gymnosporia buxifolia*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769947	KC769859	KC769901	–
CMW38079	*Spencermartinsia viticola*	*Acacia karroo*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769952	KC769866	KC769895	KC769987
CMW38081	*S. viticola*	*Gymnosporia buxifolia*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769951	KC769865	KC769894	KC769986
CMW38082	*S. viticola*	*Celtis africana*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769950	KC769867	KC769893	KC769985
CMW38423 CBS133854	***Tiarosporella africana****	*Celtis africana*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769956	KC769852	KC769909	KC76999
CMW38424 CBS135850	*T. africana**	*Celtis africana*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769957	KC769853	KC769910	KC76999
CMW38425 CBS135851	*T. africana**	*Celtis africana*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769958	KC769854	KC769911	KC76999
CMW38428	*T. africana**	*Celtis africana*	Branches	Pretoria, South Africa	F. Jami & M. Gryzenhout	KC769959	KC769855	KC769912	KC76999

Culture collections: CMW – FABI, University of Pretoria, South Africa; CBS – Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. Isolate accession numbers in bold signify holotype cultures. Isolates for new described species are indicated with an asterisk (*) and ex-type isolates are indicated in bold type.

Table 2 – Polymorphic nucleotides from sequence data of the ITS, TEF-1α, and LSU showing the relationships between *Aplosporella papillata* and *Aplosporella javeedii*. Polymorphisms unique to *A. javeedii* are highlighted.

Identity	Isolate no.	ITS														
		111	162	166	169	168	169	176	177	181	441	447	473	482	495	553
Aplosporella papillata	CBS121780	G	T	C	C	C	G	G	A	G	G	A	G	C	G	C
A. papillata	CBS121782	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. javeedii	CBS133954	A	G	T	G	T	T	A	G	A	A	G	A	T	C	T
A. javeedii	CMW38166	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. javeedii	CBS135852	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. javeedii	CBS135853	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. javeedii	CMW38169	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. javeedii	CMW38170	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Identity	Isolate no.	TEF-1α						LSU	
		89	142	143	148	171	303	352	373
Aplosporella papillata	CBS121780	A	G	C	A	C	C	C	T
A. papillata	CBS121782	.	.	.	.	.	.	.	.
A. javeedii	CBS133954	G	C	G	C	G	G	T	C
A. javeedii	CMW38166	.	.	.	.	.	.	.	.
A. javeedii	CBS135852	.	.	.	.	.	.	.	.
A. javeedii	CBS135853	.	.	.	.	.	.	.	.
A. javeedii	CMW38169	.	.	.	.	.	.	.	.
A. javeedii	CMW38170	.	.	.	.	.	.	.	.

G. buxifolia, but was not found on *S. lancea*. The *Aplosporella* sp. nov. overlapped on *S. lancea* and *C. africana* (Table 1, Fig 6).

Morphological characteristics

The isolates in the group corresponding to *Tiarosporella* in the DNA sequence comparisons were fast-growing with white, raised aerial mycelium around the edges of the culture, with grey centres viewed from the top and bottom of the plate. These cultures produced large hyaline conidia with appendages of different sizes. Isolates in the other six groups had dark grey or olivaceous colonies with aerial hyphae and dematiaceous conidia. *Aplosporella* isolates were slow growing, and had grey-olivaceous mycelium with light, irregular edges and mostly aseptate conidia that were narrow at the centres. Other known *Aplosporella* spp. have ellipsoidal to sub-cylindrical conidia. The substantial overlap in these morphological characters allowed only limited comparisons with characteristics published for the species.

Statistical analyses of species diversity

There were no statistically significant differences between species composition (not considering frequency of individual species) on the different hosts ($P > 0.05$). Among the fungal species, *S. viticola* was the only one that had a host association that was significantly different from the other species ($P < 0.05$) in terms of frequency of occurrence. It was dominant on three of the hosts with 79.3 % of isolates from *A. karroo*, 55.6 % of isolates from *C. africana*, and 88.2 % of the isolates from *G. buxifolia*. There was no significant difference between the diversity of species found from leaves and branches ($P > 0.05$). There was also no significant difference between the frequency of species found on leaves and branches ($P > 0.05$) (Fig 4). Of the seven isolated species, *S. viticola* was the most commonly isolated from both leaves and branches. *N. parvum*, *N. kwambonambiense*, *Aplosporella* sp. nov., and *Tiarosporella* sp. nov. were found only on branches, while *D. pseudoseriata* and *B. dothidea* were exclusively isolated from leaves (Fig 5).

Table 3 – Polymorphic nucleotides from sequence data of the LSU showing the relationships between *Tiarosporella madreeya* and *Tiarosporella africana*. Polymorphisms unique to *T. africana* are highlighted.

Identity	Isolate no.	LSU										
		137	412	425	435	441	442	466	468	493	630	633
Tiarosporella madreeya	CBS532.76	T	C	C	A	T	C	T	G	T	G	A
T. africana	CBS133854	C	T	T	G	C	T	C	T	A	T	C
T. africana	CBS135850	.	.	.	.	.	.	.	.	.	.	.
T. africana	CBS135851	.	.	.	.	.	.	.	.	.	.	.
T. africana	CMW38428	.	.	.	.	.	.	.	.	.	.	.

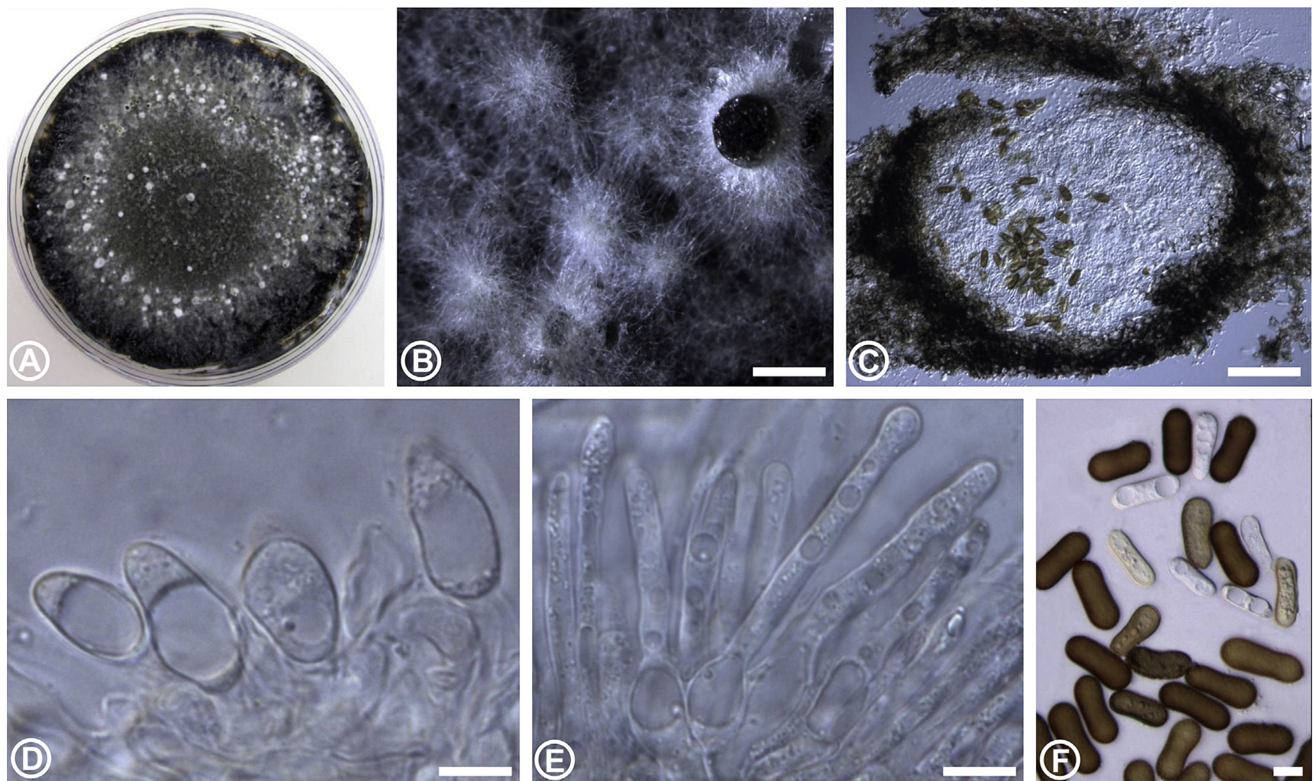

Fig 2 – Micrographs of *Aplosporella javeedii*. (A) Culture morphology on MEA in 25 °C. (B) Pycnidia (scale bar = 1000 μm). (C). Longitudinal section through pycnidium (scale bar = 100 μm). (D) Conidiogenous cells (scale bar = 5 μm). (E) Paraphyses (scale bar = 10 μm). (F) Conidia (scale bar = 5 μm).

Taxonomy

The phylogenetic analyses revealed two new taxa and these taxa were supported by morphological studies. These species are described below.

Aplosporella javeedii Jami, Gryzenh., Slippers & M.J. Wingf. **sp. nov.**

(Fig 2)

MycoBank No.: MB803637

Etymology: The name is derived from the Persian name 'Javeed Jami', meaning 'long lived'.

No *teleomorph* observed.

Pycnidia formed on MEA in 2 weeks, solitary, globose, grey-olivaceous (23''''i), unilocular, immersed to semi-immersed, average 850 × 820 μm, wall 6–10 cell layers thick, outer layers composed of dark-brown textura angularis, becoming thin-walled and hyaline towards the inner region. ***Conidiogenous cells*** formed from the cells lining the inner walls of the pycnidia, holoblastic, determinate, simple, ellipsoidal, and slightly tapered towards the apex, hyaline. ***Conidia*** aseptate, initially hyaline, becoming dark brown, smooth-walled, broadly ellipsoidal to sub-cylindrical, with rounded ends, (18.3–)21.2–24.6(–26.7) × (6.9–)8.1–9.6(–10.1) μm.

Culture characteristics: On MEA after 5 d in the dark, olivaceous to grey-olivaceous (23''''i), similar in reverse; aerial mycelium appressed, floccose, white to smoke-grey. Colonies flat with undulate edge. Growth at 5–35 °C. Growth rate 10 mm per day at an optimal temperature of 25 °C; covering the agar surface in a 90 mm diam. Petri dish after 9 d in the dark.

Specimens examined: South Africa, Gauteng Province, Pretoria, November 2011, F. Jami & M. Gryzenhout, from healthy wood section of *Celtis africana*, holotype PREM60865, ex-type culture CMW38165 = CBS133954.

Additional specimens: South Africa, Gauteng Province, Pretoria, November 2011, F. Jami & M. Gryzenhout, from healthy branch of *Celtis africana*, paratype (living cultures CMW38166, CMW38167 = CBS135852 = PREM60880) and *Searsia lancea*, paratype (living cultures CMW38168 = CBS135853 = PREM60881, CMW38169, CMW38170).

Tiarosporella africana Jami, Gryzenh., Slippers & M.J. Wingf. **sp. nov.**

(Fig 3)

MycoBank No.: MB803638

Etymology: The name refers to the Africa and the continent from which this species was collected.

No *teleomorph* observed.

Pycnidia formed on *Acacia karroo* twigs on MEA in 2–3 weeks under ultraviolet (UV), solitary, globose, dark black (29'''''m), unilocular, immersed, average 1100 × 300 μm, wall 5–7 cell layers thick, outer layers composed of dark-brown textura angularis, becoming thin-walled and hyaline towards the inner region. ***Conidiogenous cells*** formed from the cells lining the inner walls of the pycnidia, holoblastic, determinate, simple, ellipsoidal,

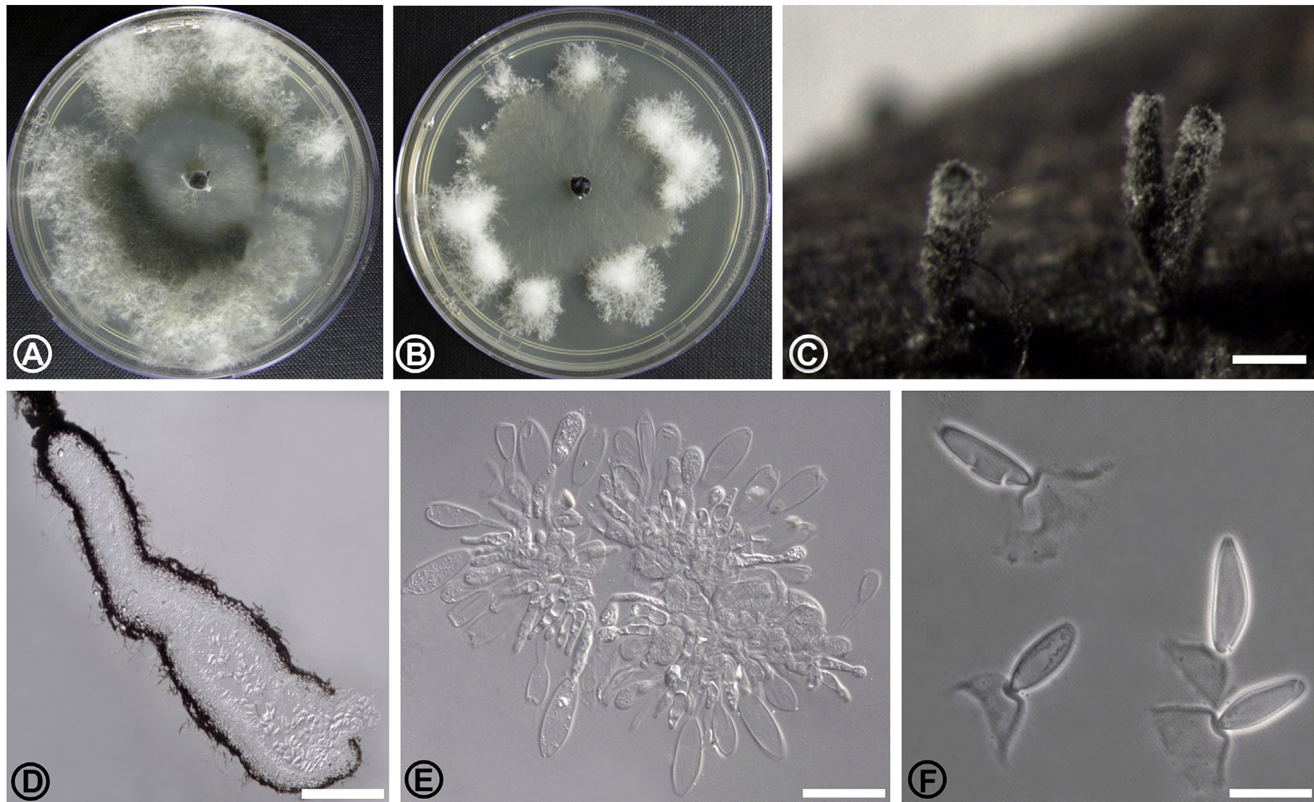

Fig 3 – Micrographs of *Tiarosporella africana*. (A) Four days culture morphology on MEA in 30 °C. (B) Four days culture morphology on MEA in 25 °C. (C) Pycnidia (scale bar = 500 μm). (D) Longitudinal section through pycnidium (scale bar = 500 μm). (E) Conidiogenous cells and young conidia (scale bar = 20 μm). (F) Conidia (scale bar = 20 μm).

and slightly tapered towards the apex, hyaline. ***Conidia*** aerogenous, solitary, hyaline, smooth, thin-walled, straight, fusiform with truncate base and obtuse apex, (15.6–) 19.5–31.8(–35.5) × (7.4–)8.6–11.6(–12.2) μm. During development, conidia are in a gelatinous sheath which may remain as an apical, hyaline, cone-like appendage that are (23.8–) 24.5–45.4(–49.9) × (11.5–)12.8–22.2(–25.11) μm.

Culture characteristics: on MEA with appressed mycelial mats, pycnidia emerging after 2–3 weeks under near-ultraviolet light on *A. karroo* twigs. Mycelium grey, becoming dark grey

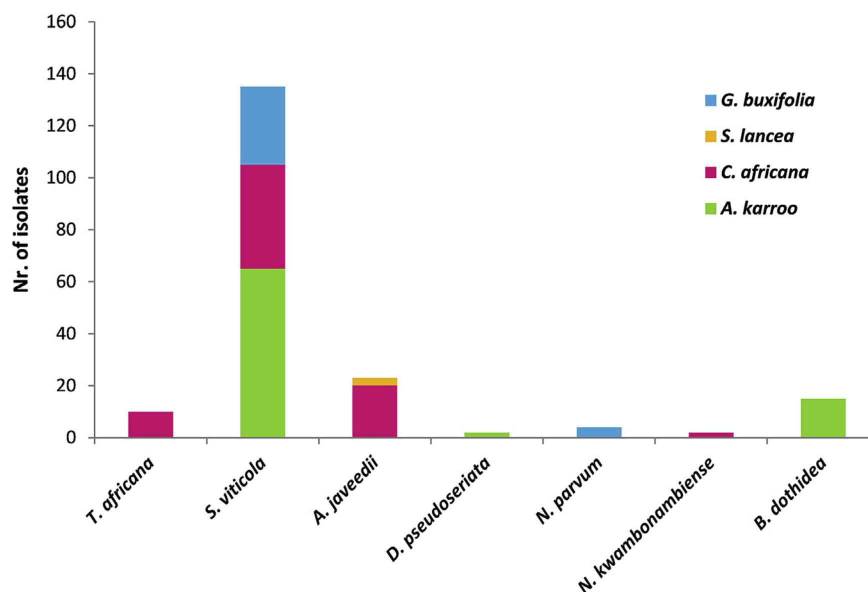

Fig 4 – Diversity of *Botryosphaeriaceae* species on four hosts, namely *Acacia karroo*, *Celtis africana*, *Searsia lancea*, and *Gymnosporium buxifolia*.

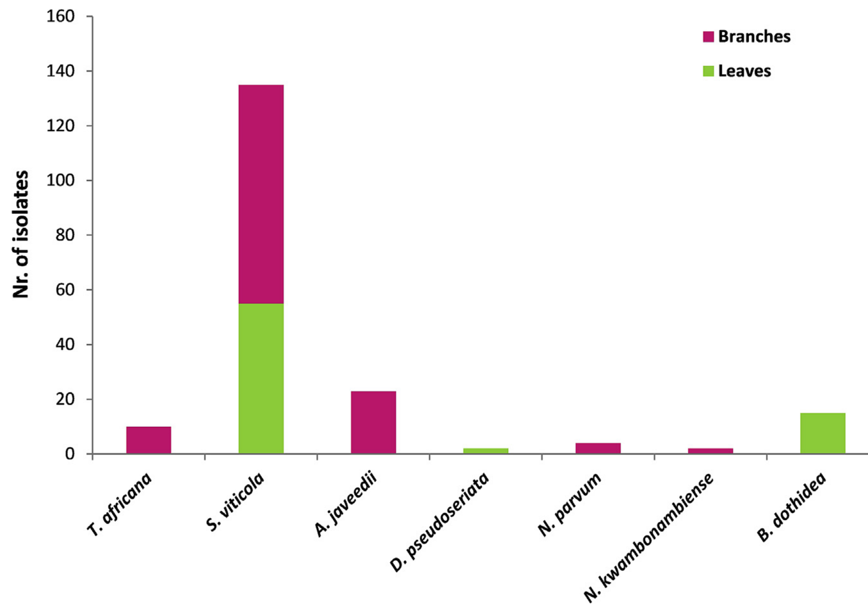

Fig 5 – Diversity of *Botryosphaeriaceae* species on leaves and branches of *Acacia karroo*, *Celtis africana*, *Searsia lancea*, and *Gymnosporium buxifolia*.

from the centre, white, and fluffy at the edges, reverse dark grey to black. Growth at 5–35 °C. Growth rate 22.5 mm per day at an optimal temperature of 30 °C; covering the agar surface in a 90 mm diam. Petri dish after 4 d in the dark.

Specimens examined: South Africa, Gauteng Province, Pretoria, November 2011, F. Jami & M. Gryzenhout, from healthy wood section of *Celtis africana*, holotype PREM60866 resulting from inoculations of living isolate to *A. karroo* twigs, living ex-type cultures CMW38423 = CBS133854.

Additional specimens: South Africa, Gauteng Province, Pretoria, November 2011, F. Jami & M. Gryzenhout, from healthy branch of *Celtis africana*, paratype (living cultures CMW38424 = CBS135850 = PREM60882, CMW38425 = CBS135851 = PREM60882, CMW38428).

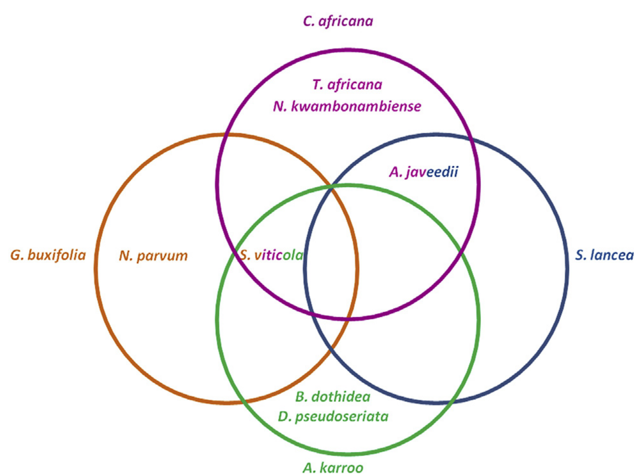

Fig 6 – The pattern of overlapping *Botryosphaeriaceae* species among *Acacia karroo*, *Celtis africana*, *Searsia lancea*, and *Gymnosporium buxifolia*.

Discussion

Seven *Botryosphaeriaceae* species were identified from the four tree species growing in close proximity to each other. These fungi included species known in South Africa (*N. parvum*, *N. kwambonambiense*, *S. viticola*, *D. pseudoseriata*, *B. dothidea*) and the two new taxa *Tiarosporella africana* and *Aplosporella javeedii*. Five of these species occurred on only a single host, but *A. javeedii* was found on two and *S. viticola* occurred on three of the tree species sampled. Results of this study, based on the single location with only four hosts sampled, represent high levels of biodiversity for the *Botryosphaeriaceae*.

Botryosphaeria dothidea, *N. parvum*, *N. kwambonambiense*, *T. africana*, and *D. pseudoseriata* were found only on one host in this study. This could be interpreted as host specificity, as has been postulated for other endophytes (Cohen 2004, 2006; Porras-Alfaro & Bayman 2011; Zhou & Hyde 2001). Some *Botryosphaeriaceae* species are also thought to have some level of host preference, such as *D. pinea*, *D. scrobiculata*, and *D. cupressi* that are found predominantly on certain conifers (Alves *et al.* 2006; De Wet *et al.* 2008). However, we do not expect that this pattern reflects host specificity in these cases, because all the fungi are known from previous studies to have broad host ranges. In particular, *B. dothidea*, *L. theobromae*, and *N. parvum* are known to have extremely broad host ranges (Punithalingam 1976; Sakalidis *et al.* 2013; Slippers & Wingfield 2007). In South Africa, *B. dothidea*, has been reported previously from *Acacia* spp., *Eucalyptus* spp., *Podocarpus* spp., *Syzygium* spp., and *Heteropyxis natalensis* (Pavlic *et al.* 2007; Slippers *et al.* 2013; Smith *et al.* 2001). Likewise, *N. parvum* has been found on *S. cordatum*, *Eucalyptus* spp., and *T. catappa* (Begoude *et al.* 2010; Pavlic *et al.* 2007; Slippers *et al.* 2004). Also, *L. theobromae* has been identified from *V. vinifera*, *S. cordatum*, *T. catappa*, and *P. angolensis* in South Africa (Begoude *et al.*

2010; Mehl *et al.* 2011; Pavlic *et al.* 2007; Van Niekerk *et al.* 2004). Given that sampling was relatively intensive at this single location, the data suggest that the occurrence of species in this study might reflect factors influencing distribution other than host specificity, such as environmental factors, and sampling effect. To determine true host ranges of these fungi, considerably more intensive and wider sampling will need to be done.

Host specificity has not previously been found for the *Botryosphaeriaceae* and this was also true for the present study. In this study, *S. viticola* was isolated from three different families of trees *Fabaceae*, *Cannabaceae*, and *Celastraceae* but not from the *Anacardiaceae*. Although some previous studies have considered larger numbers of potential host plants and were conducted over larger areas (Sakalidis *et al.* 2011a; Taylor *et al.* 2009), patterns of host association were not clear. For example, Sakalidis *et al.* (2011a) showed that at one site, *Pseudofusicoccum kimberleyense* overlapped on hosts residing in three families (*Fabaceae*, *Myrtaceae*, and *Moraceae*), but similar levels of overlap were not observed at other sites. Taylor *et al.* (2009) showed similar results with *Aplosporella yalgorensis* that were found on two tree species *E. gomphocephala* (*Myrtaceae*) and *Acacia cochlearis* (*Fabaceae*) at one site but it was not found on these trees at another sampling location. Apart from two species, *D. moneti* and *D. santali*, that were restricted to *A. rostellifera* and *Santalum acuminatum* (*Santalaceae*) respectively, the remaining species did not show any pattern of host association (Taylor *et al.* 2009). Several factors could affect these patterns of endophyte infection on a particular plant host, including biotic (e.g. plant defences, competition, etc.) and abiotic factors (e.g. local climate affecting growth, sporulation, etc.). None of these factors have, however, been studied in detail for the *Botryosphaeriaceae* on tree hosts.

The number of *Botryosphaeriaceae* species infecting the different tree hosts varied considerably in this study. Most of the trees sampled were infected by multiple (up to four) species of *Botryosphaeriaceae*. For example, *C. africana* had the most diverse assemblage of these fungi while *S. lancea* had the lowest level of diversity. These results are similar to the study of Sakalidis *et al.* (2011a) where 11 *Botryosphaeriaceae* species were found on both *Adansonia gregorii* and native surrounding trees at three sites in Australia. In that study, each host showed a different *Botryosphaeriaceae* species diversity. For example, *A. gregorii* showed the greatest species diversity (Lang *et al.* 2011) while *Melaleuca* sp. and *Calytrix* sp. were infected only by one species. Furthermore, the overlapping seven species was inconsistently found on hosts at the various sites (Sakalidis *et al.* 2011a). Taylor *et al.* (2009) showed similar results where some native Australian trees were hosts to numerous *Botryosphaeriaceae* while other native trees were host to only a single species.

In terms of understanding host defences, *S. lancea* could offer an interesting opportunity for further studies. The abundance of *Botryosphaeriaceae* found on the other hosts compared to this host (only 1.5 % of the total number of isolates) might suggest some characteristic of *S. lancea* that makes it less favourable for infection by these fungi. Future studies should consider the *Botryosphaeriaceae* on this tree in other areas of South Africa and also biochemical characteristics of this tree that might explain the low number of *Botryosphaeriaceae* in this tree as compared to, for instance, *A. karroo*.

This study revealed a number of new hosts for some of the *Botryosphaeriaceae*. For example, we isolated *S. viticola* on two new native hosts, namely *C. africana* and *G. buxifolia*. This fungus was previously known from *Prunus* spp., *V. vinifera*, *A. karroo*, and *A. mellifera* in South Africa (Damm *et al.* 2007a; Jami *et al.* 2013; Slippers *et al.* 2013; Van Niekerk *et al.* 2004). *Spencermartinsia viticola* was originally found on grapevine in Spain (Luque *et al.* 2005), but has since been reported from other areas on this host (Úrbez-Torres *et al.* 2007) and from the other hosts such as *Populus cathayana* (Zhang *et al.* 2009) and citrus (Adesemoye & Eskalen 2011). There is a clear association of this fungus with *V. vinifera* although this is clearly not fixed. The question thus arises as to where the fungus might be native and whether it has moved from commercially propagated to native plants or vice versa.

Neofusicoccum kwambonambiense represents another example of a species in the *Botryosphaeriaceae* that was isolated from *C. africana* for the first time in this study. This fungus was previously reported from *S. cordatum*, *Eucalyptus grandis*, and *A. karroo* in South Africa (Pavlic *et al.* 2009a; Pillay *et al.* 2013), from *E. dunnii* and *Corymbia torelliana* in Australia (Sakalidis *et al.* 2011b), and also from *V. vinifera* in Uruguay (Abreo *et al.* 2013). Such expansion of the known host range following expanded sampling appears to be a common pattern of recent studies on the *Botryosphaeriaceae*, and these are changing perceptions of host association drastically. For example, *N. eucalyptorum* was initially thought to be specific to *Eucalyptus* spp. in South Africa and Australia (Slippers *et al.* 2004), but was later found on other hosts in Uruguay (Pérez *et al.* 2009). These findings suggest that very extensive and global sampling will be necessary to fully understand the host associations and distribution of the *Botryosphaeriaceae*. For the present, caution would be advisable when drawing conclusions regarding host association and distribution of these fungi.

Some endophytes are known to be tissue specific (de Abreu *et al.* 2010; Fisher *et al.* 1993; Ganley & Newcombe 2006). However, results of this study provided no evidence that the *Botryosphaeriaceae* sampled are specific to either leaves or woody tissue, although the frequency of occurrence of some species such as *S. viticola* varied on tissue types. In the present study, *N. kwambonambiense* was found only on branch tissue of *C. africana*, and it has been isolated on branches of the other trees, including *S. cordatum*, *E. dunnii*, and *C. torelliana* (Pavlic *et al.* 2009b; Sakalidis *et al.* 2011b). In those studies, the samples were taken only from branches. Therefore, we cannot say that *N. kwambonambiense* is exclusive to branches. Similar to our study, Wunderlich *et al.* (2011) found no indication of tissue specificity for *Botryosphaeriaceae* species on *V. vinifera*. To fully explore the issue of variation in relative infection frequency of different species in different tissues, a metagenetics approach using either multispecies primers for the specific detection of botryosphaeriaceous species (Ridgway *et al.* 2011) or next generation sequencing might be needed to overcome potential sampling bias.

A new species of *Tiarosporella* was described in this study from a native South African tree. Several *Tiarosporella* spp. have been reported from different hosts in the U.K, U.S.A, India, Yugoslavia, and South Africa (Karadzic 2003; Sutton & Marasas 1976), but those were identified based only on morphology. Sequence data of only four species, namely *T. tritici*,

T. *graminis* var. *karroo*, T. *madreeya* (Crous *et al.* 2006), and T. *urbis-rosarum* (Jami *et al.* 2012) are available in GenBank, all of which have been isolated from different hosts in South Africa (from *Poaceae*, *Zygophyllaceae*, *Asteraceae*, and *Fabaceae*) (Jami *et al.* 2012; Sutton & Marasas 1976). It is not clear whether this current restriction of sequences for the genus exclusively from southern African isolates is due to a lack of sampling in some other regions of the world. While some areas have been fairly well sampled, this group could also have been overlooked during isolation work, because of its atypical culture morphology for *Botryosphaeriaceae*. For example, hyphae of *Tiarosporella* typically grow faster than the other *Botryosphaeriaceae*, but take longer to become grey after isolation. These atypical morphological characteristics and the fact that DNA sequence comparisons have not been conducted for species recorded outside South Africa might suggest problems regarding the identification of some collections of these fungi.

Recent studies have identified a number of unique *Aplosporella* spp. from different hosts and areas in South Africa. Of the four recently identified *Aplosporella* species, only *A. yalgorensis* was identified outside Africa from *A. cochlearis* and *E. gomphocephala* in Australia (Taylor *et al.* 2009). The other three species have all been described from southern Africa, with *A. prunicola* identified from *Prunus* in South Africa (Damm *et al.* 2007b), *A. africana* from *A. mellifera* in Namibia and *A. papillata* from *A. tortillas* and *A. erioloba* in South Africa (Slippers *et al.* 2013). The present study adds a fourth species, *A. javeedii*, and two new host records namely *C. africana* and *S. lancea*. Given fairly extensive sampling in other regions of the world, it would appear that southern Africa represents a centre of diversity for this group in the *Botryosphaeriaceae*.

The results of this study revealed the diversity of *Botryosphaeriaceae* on three previously unsampled plant families. They confirm the view that these fungi occur on most, if not all, woody plants. The data emerging from this and previous studies also suggest that many of these *Botryosphaeriaceae* are not host specific over the range of their distribution. Yet, the discovery of two new *Botryosphaeriaceae* species from a region that was previously intensively sampled for other hosts, suggest that host diversity does contribute to the diversity of *Botryosphaeriaceae* in an area. Thus, despite not being host specific, their host ranges might be limited to or more common on a certain suite of hosts in a particular area. The data, in particular from *S. lancea*, suggest that host factors could play a role in determining the diversity of *Botryosphaeriaceae* infection, even in the presence of species that have a general ability to infect many different hosts. Unravelling the limits of the host ranges of these different species, most representing plant pathogens, and how local environments influence them, remains one of the intriguing questions for this group of fungi.

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