*Mycologia*, 95(2), 2003, pp. 294–307.


# Circumscription of *Botryosphaeria* species associated with Proteaceae based on morphology and DNA sequence data

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***Abstract:*** *Botryosphaeria* spp. occur on and cause diseases of Proteaceae, but accurate identification has been problematic due to the lack of clear species circumscriptions of members of this genus. In this study, 46 isolates of *Botryosphaeria* from proteaceous hosts growing in various parts of the world were studied, using morphology, cultural characters and sequence data from the ITS region of the rDNA operon. Five *Botryosphaeria* spp. were found to be associated with Proteaceae. *Botryosphaeria lutea* was isolated from *Banksia* and *Buckinghamia* spp. in Australia, and a single isolate was obtained from *Protea cynaroides* in South Africa. *Botryosphaeria proteae* was associated only with South African Proteaceae, but occurred in many parts of the world. Another *Botryosphaeria* sp. that occurred exclusively on South African Proteaceae represents a new taxon that is described as *B. protearum*. This pathogen was found on South African Proteaceae cultivated in Australia; Hawaii; Portugal, including the Madeira Islands; and South Africa. *Botryosphaeria ribis* was associated with both South African and Australian Proteaceae and was isolated from material collected in Australia, Hawaii and Zimbabwe. A single occurrence of *B. obtusa* as an endophyte was recorded from *P. magnifica* in South Africa. In addition to providing a taxonomic overview of *Botryosphaeria* spp. associated with Proteaceae, this paper clarifies for the first time the global distribution of these species. A key also is provided to facilitate their identification. A large number of new host and distribution records are made and a new species of *Botryosphaeria* from Proteaceae is described.

***Key words:*** *Botryosphaeria protearum*, *Fusicoccum protearum*, ITS, key, systematics

## INTRODUCTION

Members of the plant family Proteaceae are indigenous to Australia, Central America, South Africa, South America, Southeast Asia and southwestern Pacific islands (Rebelo 1995). Proteaceae as cut flowers are valued in international markets. Consequently, certain species increasingly are being cultivated because global trade in fresh-cut flower proteas, as well as germplasm (in the form of seed and rooted cuttings), is growing. Many South African Proteaceae (e.g., *Leucadendron*, *Leucospermum* and *Protea*) are cultivated in Australia, Chile, Israel, New Zealand, Portugal (on the mainland and Azores and Madeira islands), Spain (on the mainland and Canary Islands), USA (California, Hawaii), and Zimbabwe. Similarly, some Australian Proteaceae (e.g., *Banksia* and *Telopea* spp.), are cultivated in countries other than Australia (Crous et al 2000b).

One of the factors limiting commercial production of Proteaceae is damage caused by pests and diseases (Knox-Davies 1981, Wright and Saunderson 1995). Some pathogens cause significant losses in the field and in nurseries. Others damage the appearance of blooms, and, although they are not debilitating pathogens, they are considered important for aesthetic reasons. Many pathogens associated with Proteaceae are regarded as actionable quarantine organisms, and the presence of these organisms in export shipments can result in rejection of consignments at the point of entry due to contravention of phytosanitary regulations (Crous et al 2000c, Taylor 2001).

Among the most important fungal pathogens of Proteaceae are *Botryosphaeria* spp., causing leaf spot and necrosis, shoot dieback, stem cankers and plant death (Knox-Davies 1981, Knox-Davies et al 1986). Recently it has been demonstrated that some *Botryosphaeria* spp. have an endophytic or a latent phase in their life cycles (Smith et al 1996, Swart et al 2000). This could facilitate the inadvertent introduction of pathogens into new areas,

Accepted for publication August 31, 2002.
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which also might threaten agriculture and indigenous vegetation in these regions. A number of fungal pathogens that occur on Proteaceae already have been introduced into other countries in this way (Crous et al 2000c).

Diseases caused by *Botryosphaeria* spp. have been recorded in most areas where Proteaceae are cultivated (Olivier 1951, van Wyk 1973, Benic and Knox-Davies 1983, von Broembsen 1986, Orffer and Knox-Davies 1989, Serfontein and Knox-Davies 1990, Forsberg 1993, Moura and Rodrigues 2001, Taylor et al 2001a, b). However, the species involved are often unidentified or subject to controversy largely due to the lack of clear species circumscription of members of this genus (Shoemaker 1964, Laundon 1973, Morgan-Jones and White 1987, Jacobs and Rehner 1998, Denman et al 2000). With the re-evaluation of morphological features of *Botryosphaeria* spp. (Crous and Palm 1999) and recent advances in molecular taxonomy, many of these problems now can be resolved (Jacobs and Rehner 1998, Denman et al 2000, Zhou et al 2001).

Correct identification of *Botryosphaeria* spp. associated with Proteaceae, cultivated in both Northern and Southern hemispheres, would make it possible to monitor global movement of these pathogens. It also would contribute to appropriate application of quarantine decisions. Moreover, accurate species identities are required to develop appropriate disease management strategies, because species of *Botryosphaeria* differ in their interactions with different hosts and environmental conditions (Britton and Hendrix 1982, 1986).

The aim of this study was to establish the identity of the *Botryosphaeria* spp. isolated from Proteaceae growing in different parts of the world. A table listing the different *Botryosphaeria* spp. and their proteaceous hosts were compiled (TABLE I), and a key to *Botryosphaeria* spp. associated with Proteaceae provided.

## MATERIALS AND METHODS

*Isolates.*—Isolates were obtained by making single-spore isolations from mature fruiting bodies in diseased material (Denman et al 1999) and by isolating the pathogen from stem cankers and leaves with necrosis (TABLE II). Isolates obtained from asymptomatic protea leaves (Denman 2002) also were included (endophytes, TABLE II). The plant material used for isolations represented a wide range of genera and species of Proteaceae collected in many countries (TABLE II). In total, 46 isolates of *Botryosphaeria* were obtained from Proteaceae. Thirteen isolates originated from material collected in Australia, nine isolates were from Hawaii, two isolates each originated from Madeira Islands and Portugal, 17 isolates were from South Africa, and three isolates were from material sampled in Zimbabwe (TABLE II). Twenty-one of the isolates were obtained from stem cankers, and four isolates came from plants with stem-tip dieback. Eight of the isolates originated from plants with leaf necrosis, five isolates were from leaf -spot symptoms. Eight isolates, obtained as endophytes from asymptomatic tissue in an earlier study (Denman 2002), also were included (TABLE II). Plant tissue was disinfested on the surface by placing it in 70% ethanol for 30 s, 1% NaOCl for 1 min, 30 s in 70% ethanol and rinsing in sterile water for 1 min. Pieces of tissue were cut from margins between necrotic and apparently healthy tissue and plated on 2% potato dextrose agar (PDA, Biolab, Midrand, South Africa). Hyphae growing out of the tissue were subcultured onto divided plates, containing PDA in half of the dish and carnation leaf agar (Fisher et al 1982) in the other. Divided plates were incubated 3–6 wk at 25 C under near-ultraviolet and cool-white fluorescent light with a 12 h light cycle. *Botryosphaeria* cultures subsequently were identified, based on colony morphology as evident on PDA, or, if the fungus had sporulated, on conidial or ascospore morphology. Cultures are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U) (TABLE II), and were deposited in the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands.

*Cultural studies.*—Ten isolates of the newly described species, derived from different hosts and localities, were selected for cultural growth studies on PDA. Mycelial disks 5 mm diam were cut from the periphery of growing cultures and placed at the center of PDA plates, with three plates per isolate at each temperature (5–40 C at 5 C intervals). Linear growth and colony color (Rayner 1970) were determined after 4 d. Two perpendicular readings were taken for each colony, using a digital caliper. The mean growth rates for three replicates of 10 isolates were plotted for each temperature tested.

*Sequence comparisons.*—Forty-six isolates (TABLE II) were sequenced. Methods for DNA extraction described by Crous et al (2000a) were followed. The primers ITS1 (5′TTTCCGTAGGTGAACCTGC3′) and ITS4 (5′TCCTCCGCTTATTGATATGC′) (White et al 1990) were used to amplify part of the nuclear rRNA operon, using polymerase chain reaction (PCR). The amplified region included the 3′ end of the 16S (small subunit) rDNA gene, the first internal transcribed spacer (ITS1), the 5.8S rDNA gene, the second ITS (ITS2) region and the 5′ end of the 26S (large subunit) of the rDNA gene. PCR products were purified according to the manufacturer's instructions with a commercial kit (Nucleospin Extract 2 in 1 Purification Kit, Machery-Nagel GmbH & Co., Germany). Sequencing reactions were carried out with ABI PRISM Big Dye Terminator Cycle v3.0 Sequencing Ready Reaction Kit (PE Biosystems, Foster City, California, USA) according to the manufacturer's recommendations. The reaction was done on an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut, USA).

Raw sequence data were analyzed with EditView 1.0.1 (http://www.appliedbiosystems.com) and manually aligned by inserting gaps. Phylogenetic analyses were undertaken

TABLE I. Hosts and localities of *Botryosphaeria* spp. isolated from Proteaceae

| Species | Locality | Host | Reference |
|---|---|---|---|
| *B. dothidea* | Hawaii | *Grevillea wilsonii, Leucadendron* spp., *Leucospermum* spp., *Protea compacta, P. cynaroides, Protea* cvs Lady Di, Pink Ice[a], *Telopea* sp. | Taylor et al 2001b |
| | South Africa | *Leucospermum* spp. | von Broembsen 1986 |
| *B. gaube* | Australia | *Grevillea victoriae* | Petrak 1967 |
| *B. lutea* | Australia | *Banksia* sp., *Buckinghamia* sp. | Present study |
| | South Africa | *P. cynaroides* | Present study |
| *B. obtusa* | South Africa | *P. magnifica* | Present study |
| *B. proteae* | Australia | *P. eximia, P. magnifica, P. obtusifolia* | Crous et al 2000b |
| | California | *Protea* spp. | Taylor et al 2001a |
| | Hawaii | *P. compacta, P. cynaroides, P. laurifolia, P. neriifolia, Protea* cvs Mink, Pink Ice[a] | Taylor et al 2001b |
| | South Africa | *P. eximia, P. grandiceps, P. magnifica, Protea* cvs Lady Di, Venus[a] | Denman et al 1999 |
| | | *P. cynaroides, Leucospermum cordifolium* | Swart et al 2000 |
| *B. protearum* | Australia | *Leucadendron* sp., *P. magnifica* | Present study |
| | Hawaii | *P. cynaroides* | Present study |
| | Madeira | *Leucadendron tinctum* | Present study |
| | Portugal | *Leucadendron* sp. | Present study |
| | South Africa | *Leucadendron* cv. Silvan Red[b], *P. cynaroides, P. magnifica, P. neriifolia, P. repens* | Present study |
| *B. rhodina* | Australia | *Protea* spp. | Forsberg 1993 |
| | Hawaii | *Banksia dryandroides, Leucospermum* sp., *P. cynaroides, Telopea* sp. | Taylor et al 2001b |
| *B. ribis* | Australia | *Buckinghamia* sp. | Present study |
| | | *Banksia coccinea* | Shearer et al 1995 |
| | Hawaii | *Grevillea* sp. | Taylor 2001 |
| | | *Leucospermum* sp., *P. cynaroides, Telopea* sp. | Present study |
| | South Africa | *Leucadendron argenteum, P. cynaroides* | Olivier 1951 |
| | | *Macadamia tetraphylla* | Herbert and Grech 1985 |
| | Zimbabwe | *P. cynaroides* | Present study |
| *Dothiorella banksiae* | Australia | *Banksia integrifolia* | Hansford 1954 |
| *Fusicoccum* spp. | Hawaii | *Leucospermum* spp., *P. compacta, P. cynaroides, P. laurifolia, P. neriifolia, Protea* cvs Lady Di, Pink Ice[a], *Telopea* sp. | Taylor et al 2001b |

[a] *Protea* cvs: Lady Di = *P. magnifica* × *P. compacta*, Mink = *P. neriifolia*, Pink Ice = *P. compacta* × *P. susannae*, Venus = *P. aristata* × *P. repens*.

[b] *Leucadendron* cv.: Silvan Red = *Ldn. laureolum* × *Ldn. salignum*.

with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 2000). Gaps were treated as a fifth character and all characters were unordered and of equal weight. The data matrix consisted of two outgroup taxa and 61 ingroup taxa, each sequence containing 528 characters (including gaps). Heuristic searches were carried out with stepwise simple addition and tree bisection and reconstruction (TBR) as the branch-swapping algorithm to find maximum-parsimony trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Branch support was determined with 1000 bootstrap replicates (Felsenstein 1985).

Representative *Botryosphaeria* sequences from the preliminary clades were used to obtain sequences from GenBank with a standard nucleotide-nucleotide BLAST search (Altschul et al 1997). The representative sequences from GenBank [AF27741 (*B. ribis*), AF293480 and AF27745 (*B. lutea*), AF27759 (*B. obtusa*), AF241175 (*B. dothidea*) TABLE II] were included in the analyses. The sequences of 10 *Botryosphaeria* isolates from Proteaceae in a previous study (Denman et al 2000) also were included in the analyses (TABLE II). Trees were rooted to *Mycosphaerella africana* Crous & M.J. Wingf. (AF 283690) and *Guignardia bidwellii* (Ellis) Viala & Ravaz (AF

TABLE II. Collection data and GenBank accession numbers of *Botryosphaeria* isolates studied

| Accession No. | GenBank No. | Species | Host[a] | Locality | Symptom | Collector | Reference |
|---|---|---|---|---|---|---|---|
| 97-94 | AF241175 | *B. dothidea* | *Populus nigra* | New Zealand | — | G.J. Samuels | Zhou et al 2001 |
| KJ 93.52 | AF027745 | *B. lutea* | *A. deliciosa* | New Zealand | — | G.J. Samuels | Zhou and Stanosz 2001 |
| STE-U 4402 | AF452551 | *B. lutea* | *Banksia* sp. | Australia | Stem canker | M.J. Wingfield | Present study |
| STE-U 4401 | AF452552 | *B. lutea* | *Banksia* sp. | Australia | Stem canker | M.J. Wingfield | Present study |
| STE-U 4403 | AF452553 | *B. lutea* | *Banksia* sp. | Australia | Stem canker | M.J. Wingfield | Present study |
| STE-U 3085 | AF452554 | *B. lutea* | *Buckinghamia* sp. | Australia | Leaf necrosis | P.W. Crous | Present study |
| STE-U 3088 | AF452555 | *B. lutea* | *Buckinghamia* sp. | Australia | Leaf necrosis | P.W. Crous | Present study |
| STE-U 3091 | AF452549 | *B. lutea* | *Buckinghamia sp.* | Australia | Leaf necrosis | P.W. Crous | Present study |
| STE-U 2953 | AF452550 | *B. lutea* | *Buckinghamia* sp. | Australia | Leaf necrosis | P.W. Crous | Present study |
| ATCC 56125 | AF293480 | *B. lutea* | *Eucalyptus* sp. | Australia | Stem canker | E.M. Davidson | Zhou et al 2001 |
| STE-U 4393 | AF452548 | *B. lutea* | *Protea cynaroides* | South Africa | Endophyte | L. Swart | Present study |
| KJ 93.56 | AF027759 | *B. obtusa* | Harwood shrub | New York, USA | — | G.J. Samuels | Jacobs and Rehner 1998 |
| STE-U 4373 | AF452556 | *B. obtusa* | *P. magnifica* | South Africa | Endophyte | S. Denman | Present study |
| STE-U 2271 | AF452562 | *B. proteae* | *Protea* cv. Lady Di | Hawaii, USA | Leaf spot | P.W. Crous | Present study |
| STE-U 4399 | AF452557 | *B. proteae* | *P. cynaroides* | Madeira | Leaf spot | S. Denman | Present study |
| STE-U 4358 | AF196299 | *B. proteae* | *P. cynaroides* | South Africa | Endophyte | L. Swart | Denman et al 2000 |
| STE-U 4354 | AF196300 | *B. proteae* | *P. cynaroides* | South Africa | Endophyte | L. Swart | Denman et al 2000 |
| STE-U 2269 | AF452563 | *B. proteae* | *P. laurifolia* | Hawaii, USA | Stem canker | P.W. Crous | Present study |
| STE-U 4400 | AF452559 | *B. proteae* | *P. repens* | Portugal | Leaf necrosis | S. Denman | Present study |
| STE-U 4359 | AF196302 | *B. proteae* | *P. repens* | South Africa | Leaf necrosis | S. Denman | Denman et al 2000 |
| STE-U 4355 | AF196301 | *B. proteae* | *P. repens* | South Africa | Leaf necrosis | S. Denman | Denman et al 2000 |
| STE-U 4378 | AF452560 | *B. proteae* | *Protea* sp. | Australia (USDA interception) | Leaf spot | M.E. Palm | Present study |
| STE-U 4385 | AF452561 | *B. proteae* | *Protea* sp. | Australia (USDA interception) | Leaf spot | M.E. Palm | Present study |
| STE-U 4386 | AF452558 | *B. proteae* | *Protea* sp. | Australia (USDA interception) | Leaf spot | M.E. Palm | Present study |

TABLE II. Continued

| Accession No. | GenBank No. | Species | Host[a] | Locality | Symptom | Collector | Reference |
|---|---|---|---|---|---|---|---|
| STE-U 1775 | AF452539 | *B. protearum* | *Ldn.* cv. Silvan Red | South Africa | Die-back | S. Denman | Present study |
| STE-U 1776 | AF452538 | *B. protearum* | *Ldn.* cv. Silvan Red | South Africa | Stem canker | S. Denman | Present study |
| STE-U 2930 | AF452528 | *B. protearum* | *Leucadendron* sp. | Australia | Leaf necrosis | P.W. Crous | Present study |
| STE-U 4398 | AF452531 | *B. protearum* | *Leucadendron* sp. | Portugal | Die-back | S. Denman | Present study |
| STE-U 4397 | AF452530 | *B. protearum* | *Ldn. tinctum* | Madeira | Die-back | S. Denman | Present study |
| STE-U 2147 | AF452534 | *B. protearum* | *P. cynaroides* | Hawaii, USA | Leaf necrosis | P.W. Crous | Present study |
| STE-U 4384 | AF452535 | *B. protearum* | *P. cynaroides* | South Africa | Die-back | S. Denman | Present study |
| STE-U 4360 | AF195774 | *B. protearum* | *P. eximia* | South Africa | Stem canker | S. Denman | Denman et al 2000 |
| STE-U 4357 | AF196296 | *B. protearum* | *P. magnifica* | South Africa | Stem canker | S. Denman | Denman et al 2000 |
| STE-U 1802 | AF196298 | *B. protearum* | *P. magnifica* | South Africa | Leaf necrosis | S. Denman | Denman et al 2000 |
| STE-U 4363 | AF452540 | *B. protearum* | *P. magnifica* | South Africa | Stem canker | S. Denman | Present study |
| STE-U 4387 | AF452546 | *B. protearum* | *P. magnifica* | South Africa | Endophyte[b] | S. Denman | Present study |
| STE-U 4388 | AF452532 | *B. protearum* | *P. magnifica* | South Africa | Endophyte | S. Denman | Present study |
| STE-U 4389 | AF452529 | *B. protearum* | *P. magnifica* | South Africa | Endophyte | S. Denman | Present study |
| STE-U 4390 | AF452533 | *B. protearum* | *P. magnifica* | South Africa | Endophyte | S. Denman | Present study |
| STE-U 4392 | AF452545 | *B. protearum* | *P. magnifica* | South Africa | Endophyte | S. Denman | Present study |
| STE-U 4391 | AF452541 | *B. protearum* | *P. magnifica* | South Africa | Endophyte | S. Denman | Present study |
| STE-U 2988 | AF452537 | *B. protearum* | *P. magnifica* | Australia | Leaf necrosis | P.W. Crous | Present study |
| STE-U4365 | AF452547 | *B. protearum* | *P. magnifica* | South Africa | Stem canker | S. Denman | Present study |
| STE-U 4356 | AF196294 | *B. protearum* | *P. magnifica* | South Africa | Leaf necrosis | S. Denman | Denman et al 2000 |
| STE-U 4361 | AF196295 | *B. protearum* | *P. magnifica* | South Africa | Stem canker | S. Denman | Denman et al 2000 |
| STE-U 4362 | AF196297 | *B. protearum* | *P. magnifica* | South Africa | Leaf necrosis | S. Denman | Denman et al 2000 |
| STE-U 4366 | AF452543 | *B. protearum* | *P. neriifolia* | South Africa | Stem canker | S. Denman | Present study |
| STE-U 4367 | AF452544 | *B. protearum* | *P. neriifolia* | South Africa | Stem canker | S. Denman | Present study |
| STE-U 4369 | AF452536 | *B. protearum* | *P. repens* | South Africa | Stem canker | S. Denman | Present study |
| STE-U 4368 | AF452542 | *B. protearum* | *P. repens* | South Africa | Stem canker | S. Denman | Present study |
| STE-U 3036 | AF452519 | *B. ribis* | *Buckinghamia* sp. | Australia | Stem canker | P.W. Crous | Present study |
| STE-U 2055 | AF452523 | *B. ribis* | *Ldn.* cv. Safari Sunset | Hawaii, USA | Stem canker | P.W. Crous | Present study |

TABLE II. Continued

| Accession No. | GenBank No. | Species | Host[a] | Locality | Symptom | Collector | Reference |
|---|---|---|---|---|---|---|---|
| STE-U 2051 | AF452526 | *B. ribis* | *Leucospermum* sp. | Hawaii, USA | Stem canker | P.W. Crous | Present study |
| KJ 94.09 | AF027741 | *B. ribis* | *M. quinquenervia* | Florida, USA | — | M. Rayachhetry | Jacobs and Rehner 1998 |
| STE-U 4382 | AF452520 | *B. ribis* | *P. cynaroides* | Zimbabwe | Stem canker | C. Saywood | Present study |
| STE-U 2057 | AF452524 | *B. ribis* | *P. cynaroides* | Hawaii, USA | Stem canker | P.W. Crous | Present study |
| STE-U 4379 | AF452525 | *B. ribis* | *P. cynaroides* | Zimbabwe | Stem canker | C. Saywood | Present study |
| STE-U 4381 | AF452522 | *B. ribis* | *P. cynaroides* | Zimbabwe | Stem canker | C. Saywood | Present study |
| STE-U 4371 | AF452518 | *B. ribis* | *P. cynaroides* | Hawaii, USA | Stem canker | P.W. Crous | Present study |
| STE-U 4376 | AF452527 | *B. ribis* | *P. cynaroides* | Hawaii, USA | Stem canker | P.W. Crous | Present study |
| STE-U 2100 | AF452521 | *B. ribis* | *Telopea* sp. | Hawaii, USA | Stem canker | P.W. Crous | Present study |
| — | AF216533 | *Guignardia bidwellii* | *Vitis* sp. | New York, USA | — | A. B. Baudoin | Crous et al 2001 |
| STE-U 794 | AF283690 | *Mycosphaerella africana* | *E. viminalis* | South Africa | Leaf spot | P.W. Crous | Crous et al 2001 |

[a] *A.* = *Actinidia*; *E.* = *Eucalyptus*; *Ldn.* = *Leucadendron*, cvs Safari Sunset = *Ldn. salignum* × *Ldn. laureolum*, Silvan Red = *Ldn. laureolum* × *Ldn. salignum*; *M.* = *Melaleuca*; *P.* = *Protea*, cv. Lady Di = *P. magnifica* × *P. compacta*.

[b] Endophytes were isolated from asymptomatic leaves.

216533), which have been shown to be useful outgroup taxa (Denman 2002).

## RESULTS

*Sequence comparisons.*—PCR products of ca 580 base pairs (bp) were obtained for the 46 isolates (TABLE II). Of these, 51 variable characters were parsimony uninformative and 208 were parsimony informative and used to obtain 159 most parsimonious trees of 470 steps, using heuristic searches (CI = 0.821, RI = 0.952, RC = 0.782) (FIG. 1). Sequence data of isolates were deposited in GenBank (TABLE II) and the alignment in TreeBase (S779; M1234).

Five distinct clades emerged and, by comparing them with the sequences obtained from GenBank, four of the clades could be identified (TABLE II). The GenBank sequence of *Botryosphaeria ribis* Grossenb. & Duggar (AF27741, Jacobs and Rehner 1998) grouped with a clade of 10 isolates from Proteaceae (99% bootstrap support). Sequence data for eight isolates corresponded to *Fusicoccum luteum* Pennycook & Samuels sequence in GenBank (AF27745, Jacobs and Rehner 1998) (94% bootstrap support). A single isolate grouped with *Botryosphaeria obtusa* (Schwein.) Shoemaker (100% bootstrap support), and seven isolates grouped with *B. proteae* (Wakef.) S. Denman & Crous (AF196299, AF196300, AF196301, AF196302, Denman et al 2000), with 100% bootstrap support.

Twenty of the isolates formed a separate, strongly supported clade (100% bootstrap support). This clade was clearly distinct from those of any other species of *Botryosphaeria* and is considered a new taxon. Previously, representative sequences from this clade had been reported to form part of *Botryosphaeria dothidea* (Moug : Fr.) Ces. & De Not. complex (Denman et al 2000). However, results of this study show that this group is closer to *B. ribis* than to *B. dothidea* but is clearly separate from both.

No isolates of *B. ribis* were found in the material from South Africa or Madeira Islands. All but one of the *F. luteum* isolates were from Australian Proteaceae (*Banksia* L.f. and *Buckinghamia* F.Muell.), growing in Australia (TABLE II). The single *F. luteum* isolate (STE-U4393), which was not obtained from Australia,

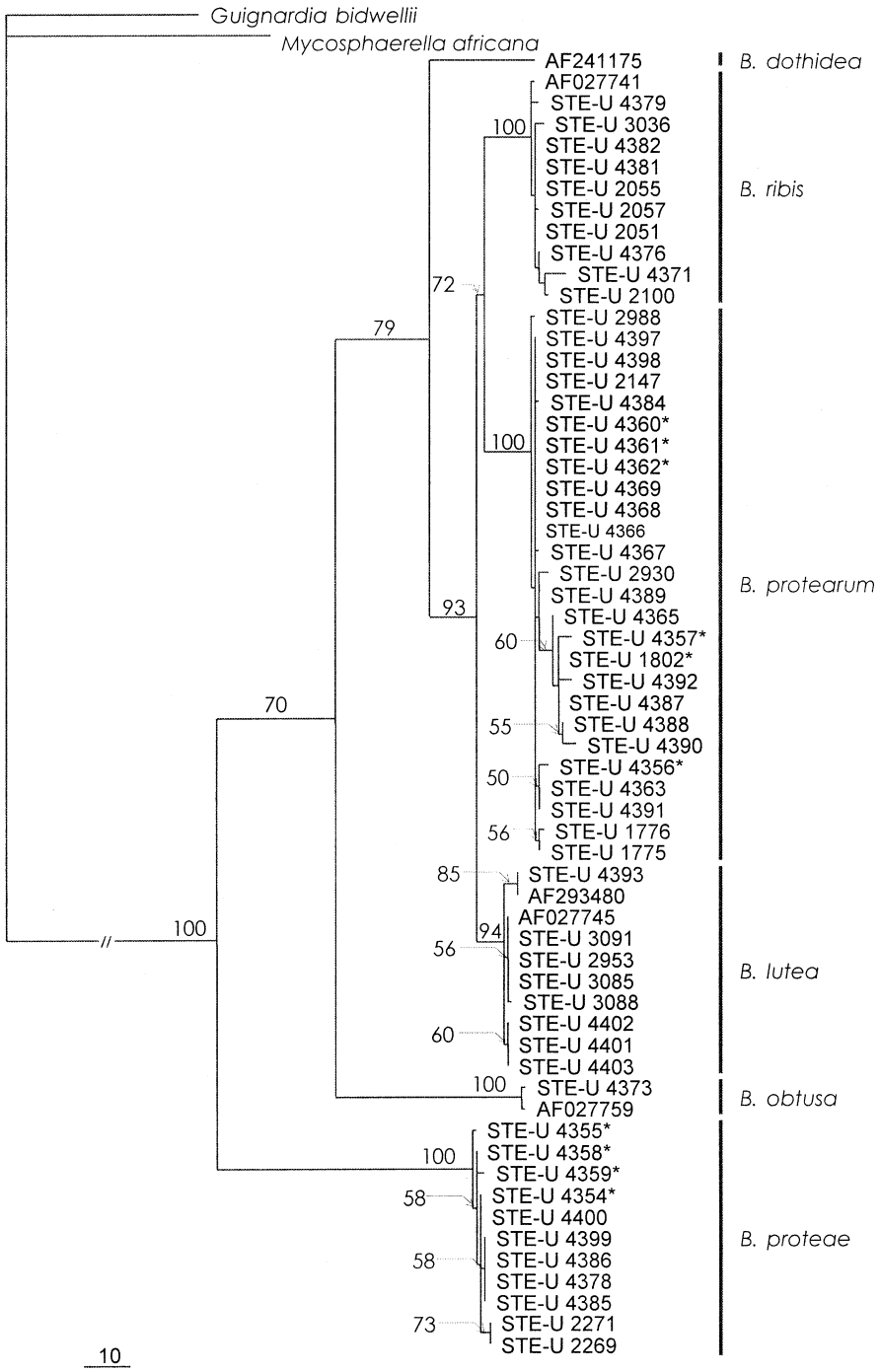



FIG. 1. The phylogram of one of the 159 most-parsimonious trees derived from the alignment of ITS1 5.8S rDNA and ITS2 sequence data of *Botryosphaeria* isolates from Proteaceae. The topology of the different trees differed only in the arrangements of isolates within the identified clades, not between them. The tree is rooted to *Mycosphaerella africana* Crous & M.J. Wingf. and *Guignardia bidwellii* (Ellis) Viala & Ravaz. Branch support is given above the branches based on 1000 bootstrap replicates. The bar represents 10 changes.

occurred as an endophyte on *Protea cynaroides* L. in South Africa (TABLE II).

All isolates of the unidentified *Botryosphaeria* sp., which we believe represents a new taxon, were from South African Proteaceae. However, they were obtained from many parts of the world, including Australia, Madeira Islands, Portugal, and South Africa (TABLE I). Similarly, *B. proteae* was restricted to South African Proteaceae, but was present in many countries, including Australia, Hawaii (USA) and

Portugal. The single isolate of *B. obtusa* was obtained from a wild *Protea* sp. in a nature reserve in South Africa.

## TAXONOMY

**Botryosphaeria protearum** Denman & Crous, sp. nov. FIGS. 2–14

*Anamorph*. **Fusicoccum protearum** Denman & Crous, sp. nov.

Ascomata in contextu hospitis inclusa, usque ad 600 μm diametro, erumpescentia, solitaria, botryosa, stromatiformia, atrobrunnea vel nigra, cum ostiolis centralibus nigris. Asci clavati ad subcylindricati, inter paraphyses filiformes interspersi, 110–200 × 15–21 μm, octosporati, bitunicati. Ascosporae irregulariter biseriatae, hyalinae, unicellulares, granulares, cum aetate pallide brunnescens, (25–)26–33(–37) × (9–)10–12(–13) μm, juventute inaequilaterae, fusiformes, medio latissimae.

Pycnidia in contextu hospitis inclusa, solitaria vel botryosa, stromatiformia, globosa, usque ad 500 μm diametro; paries pycnidii e stratis 4–8 formata, e textura angulari brunnea composita, ad intima hyalinescens. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 7–12 × 3–5 μm, percurrenter cum 1–2 proliferationibus prolificentes, vel in plano eodem periclinaliter minuter incrassatae. Conidia hyalina, granularia, ovoidea vel clavata vel fusoidea, (20–)25–30(–40) × 7–8(–10) μm.

Ascomata pseudothecial, embedded in host tissue, up to 600 μm diam, becoming erumpent, solitary or botryose, stromatic, dark brown to black, with central, black ostioles; pseudothecial wall 6–15 cell layers thick, composed of brown *textura angularis*. Asci clavate to subcylindrical, 110–200 × 15–21 μm, 8-spored, bitunicate with a well-developed apical chamber that becomes inconspicuous at maturity. Pseudoparaphyses filiform, branched, septate, 3–5 μm wide. Ascospores irregularly biseriate, hyaline, nonseptate, granular, becoming light brown with age, (25–)26–33(–37) × (9–)10–12(–13) μm, fusiform, widest in the middle with obtuse ends, sometimes inequilateral.

Pycnidia embedded in host tissue, solitary or botryose, stromatic, globose, up to 500 μm diam, pycnidial wall 4–8 cell layers thick, composed of brown *textura angularis*, becoming hyaline towards the inner region. Conidiophores 0–1-septate, hyaline, subcylindrical, rarely branched, 7–20 (–30) × 3–5 μm. Conidiogenous cells holoblastic, hyaline, subcylindrical, 7–12 × 3–5 μm, rarely proliferating percurrently with 1–2 proliferations, proliferating predominantly at the same level with minute (inconspicuous) periclinal thickening, which becomes more prominent in older conidiogenous cells. Conidia hyaline, granular, ovoid to clavate when young, becoming irregularly fusoid when mature, widest in the middle with an obtuse apex and bluntly rounded or slightly flattened base (inconspicuous in older, permanent mounts), (20–)25–30(–40) × 7–8(–10) μm *in vivo*. Spermatial state produced in conidiomata with the *Fusicoccum* anamorph, or in separate spermatogonia. Spermatiophores hyaline, smooth, branched, cylindrical, 0–2-septate, straight, unbranched or branched above, 12–17 × 2–3 μm. Spermatiogenous cells discrete or integrated, hyaline, smooth, cylindrical, proliferating via determinate phialides with periclinal thickening, 5–12 × 1.5–2.5 μm. Spermatia hyaline, smooth, aseptate, rod-shaped with rounded ends, 3–6 × 1–1.5 μm. Cultures producing colonies that are initially translucent to white, gradually darkening from the center, olive green to gray after 4–7 d, becoming charcoal black after 14–21 d. Initially forming aerial mycelium which eventually resulting in flat colonies with rims of loose aerial mycelium at the edge of the dish. Colony color, based on the color charts of Rayner (1970) was greenish black (33‴″k) underneath and olivaceous gray (23‴″i) to iron gray (25‴″k) on the surface. Aerial mycelium at the edge of the dish smoke gray (21‴f) to pale olivaceous gray (21‴″f). Black conidiomatal initials sometimes were formed, beginning in the center of colonies and spreading over the entire colony surface. Sporulation not observed *in vitro*. Cardinal temperatures for growth were min below 5 C, opt 25 C, max above 35 C, no growth at 40 C. The mean daily growth rate at 25 C was 25.5 mm/d (FIG. 15)

*Specimens examined*. SOUTH AFRICA. WESTERN CAPE: Porterville, Baanbreek Farm, on stems of *Protea magnifica* Link, 27 Jul 1997, *S. Denman,* (HOLOTYPE of *B. protearum*: PREM 57329; ex-type culture STE-U 4361); Stellenbosch, Devon Valley, Protea Heights Farm, on stems of *Leucadendron salignum* P.G. Bergius × *L. laureolum* (Lam.) Fourc. cv. Silvan Red, 31 Oct 1997, *S. Denman* and *J. Taylor,* (HOLOTYPE of *F. protearum*: PREM 57330, ex-type culture STE-U 1775); Stellenbosch, Elsenburg, on leaves of *Protea eximia* (Salisb. ex Knight) Fourc., 22 Jul 1997, *S. Denman,* STE-U 4360 (teleomorph); Porterville, Osdam Farm, on leaves of *P. magnifica*, 16 Jun 1997, *S. Denman,* PREM 57331 (teleomorph), Porterville, Baanbreek Farm, on leaves of *P. magnifica*, 29 Jul 1997, *S. Denman,* STE-U 1802, STE-U 4362 (anamorph); MADEIRA. Florialis Estate, on leaves of *Protea compacta* R. Br. × *P. susannae* E. Phillips cv. Pink Ice, Apr 2000, *S. Denman* and *J. Taylor,* STE-U 4397 (anamorph).

*Hosts*. *Protea compacta* × *P. susannae* cv. Pink Ice, *P. cynaroides*, *P. eximia*, *P. magnifica*, *P. neriifolia* R. Br., *P. repens* (L.) L., *Leucadendron salignum* × *L. laureolum* cv. Silvan Red, *L. tinctum* I. Williams and other *Leucadendron* spp.

*Known distribution*. Australia (Queensland); Madeira Islands, Portugal; Portugal; South Africa (Western Cape Province) (TABLE I).

## DISCUSSION

This study represents the first comprehensive characterization of the species of *Botryosphaeria* associat-

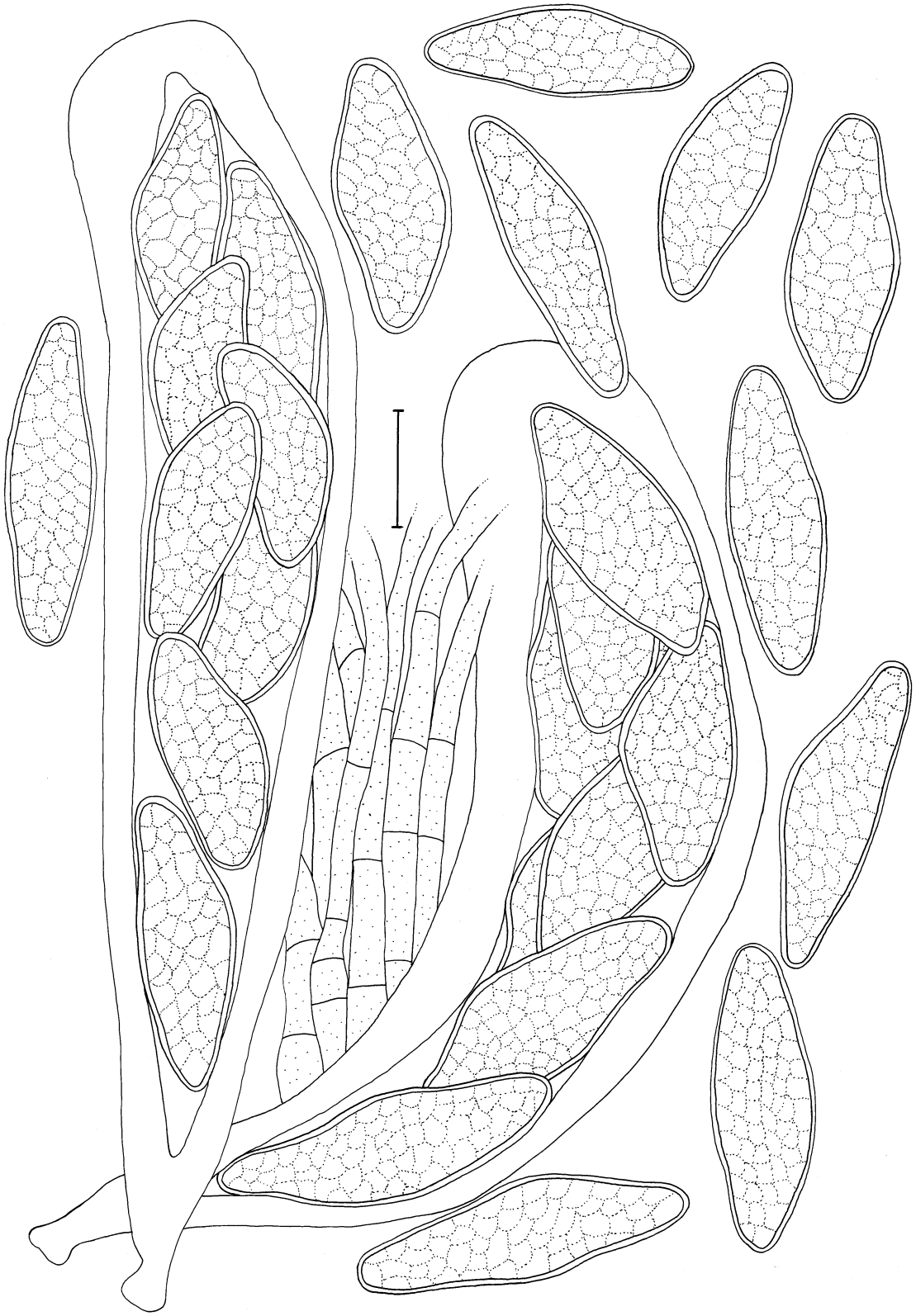
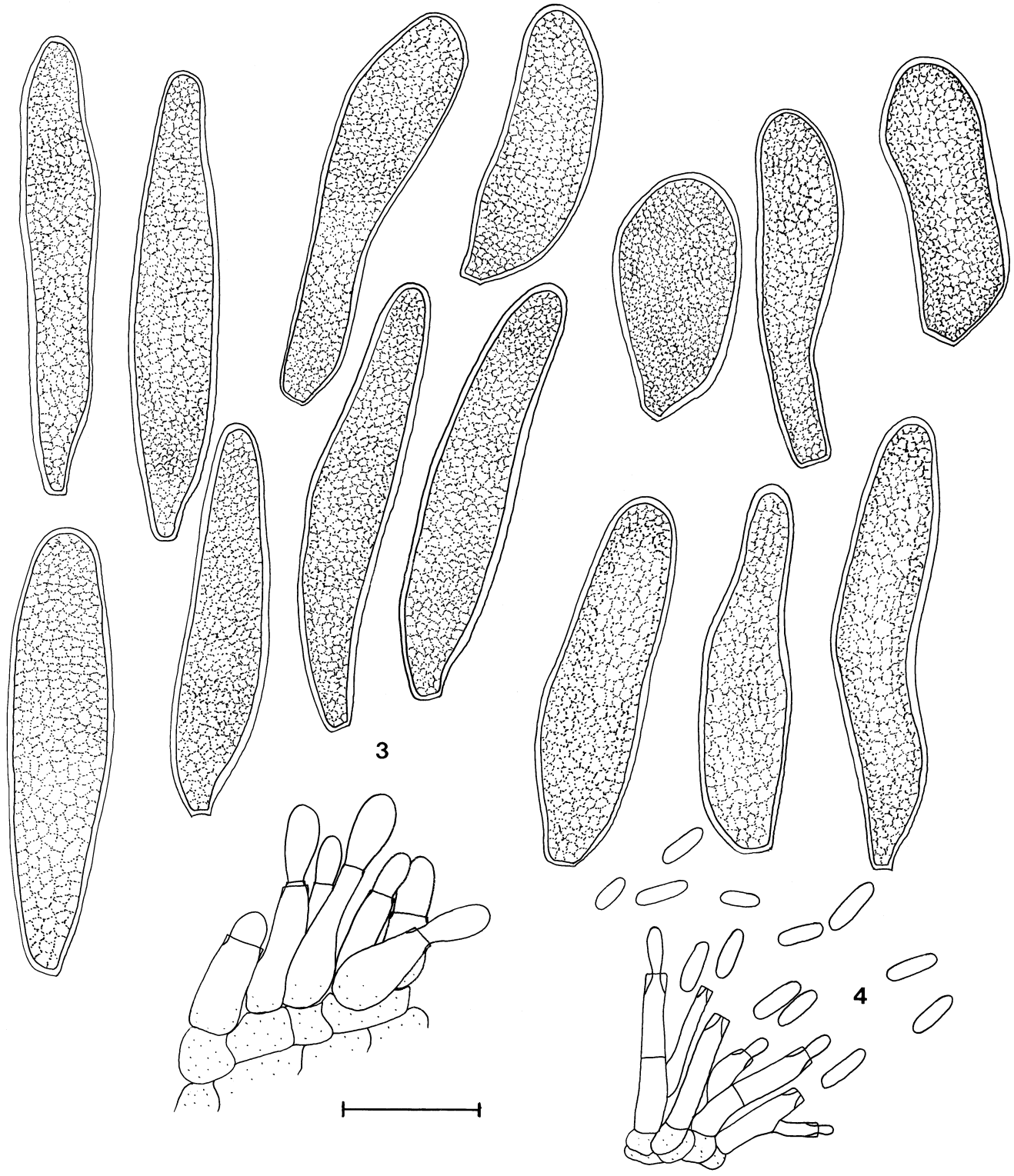
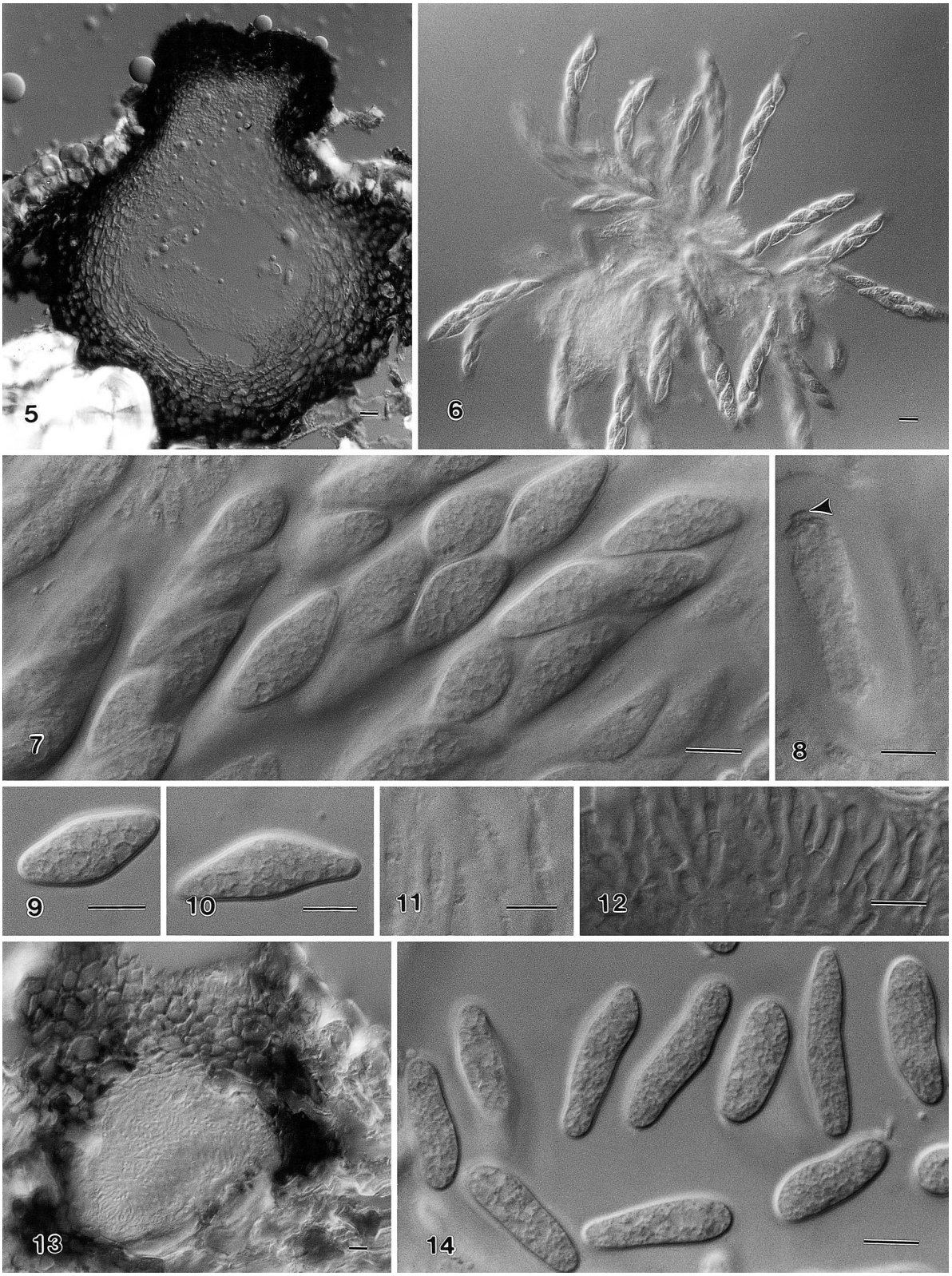


FIG. 2. Asci, ascospores and pseudoparaphyses of *Botryosphaeria protearum*. Bar = 10 μm.




FIGS. 3–4. *Fusicoccum protearum*. 3. Conidia and conidiophores. 4. Spermatia and spermatiophores. Bar = 10 μm.

ed with Proteaceae and provides a foundation for future pathological and biogeographical studies of these fungi. *Fusicoccum luteum* is newly reported on Australian Proteaceae (*Buckinghamia*) in Australia and on *P. cynaroides* in South Africa. Zhou and Stanosz (2001) also reported an isolate from *Banksia* in Australia, misidentified as *B. ribis*, to be representative of *F. luteum*. Results of this study confirm the




FIGS. 5–14. *Botryosphaeria protearum* and its anamorph *Fusicoccum protearum*. 5. Vertical section through a pseudothecium. 6, 7. Asci and ascospores. 8. Ascus with apical chamber (arrowed). 9, 10. Ascospores. 11. Pseudoparaphyses. 12. Conidiophores. 13. Vertical section through a pycnidium. 14. Conidia. Bars = 10 μm.

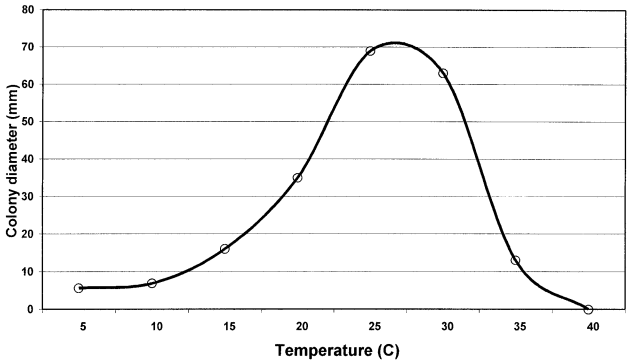



FIG. 15. Temperature-growth relationship of *B. protearum* cultured on PDA for 4 d.

presence of *F. luteum* on *Banksia* and extend the host range to *Buckinghamia*. Shearer et al (1995) described a devastating disease of *Banksia coccinea* R. Brown caused by *B. ribis* on the southwestern coast of Australia. In light of the findings by Zhou and Stanosz (2001) and results of this work, it seems likely that the pathogen described by Shearer et al (1995) is *F. luteum*. It also appears that *F. luteum* is widely distributed on Australian Proteaceae.

The single collection of *F. luteum* on Proteaceae in South Africa probably is not representative of the relative occurrence of this fungus on Proteaceae in the country. This view is based on the fact that the fungus appears to be common on Proteaceae in Australia and on kiwifruit, apple and pear in New Zealand (Pennycook and Samuels 1985). *Fusicoccum luteum* has been found commonly on grapevines in Portugal by Phillips et al (2002), who recently described the teleomorph of this fungus as *Botryosphaeria lutea* A.J.L. Phillips. In the Western Cape Province of South Africa, this fungus also has been commonly associated with grapevines as well as stone and pome fruit trees, which are cultivated alongside Proteaceae orchards (P.W. Crous unpubl). *Fusicoccum luteum* was isolated as an endophyte of *P. cynaroides* and thus cannot be viewed as a pathogen of South African Proteaceae. This isolate formed its teleomorph in culture, a feature not previously recorded for this fungus.

The newly described *Botryosphaeria protearum* was found on South African Proteaceae in their native habitat, as well as other areas where these plants are cultivated. It also is reported for the first time from Australia, Madeira Islands, Portugal and South Africa. The exclusive association with South African Proteaceae suggests that *B. protearum* is indigenous to South Africa and most likely was introduced into other countries on South African protea germplasm. In this paper the anamorph of *B. protearum* (*Fusicoccum protearum*) has been named because the taxonomy of *Botryosphaeria* largely is dependent on its anamorphs, and it is usually the anamorph that is encountered (Hanlin 1990).

The family Proteaceae comprises two subfamilies, namely the Proteoideae and the Grevilleoideae. Members of the former occur mainly in southern Africa while members of the latter group occur primarily in Australia (Rebelo 1995). Current results, as well as those from previous studies (Crous et al 2000c, Taylor et al 2001a, b), confirm that *B. proteae* is associated with only South African Proteaceae. Both *B. protearum* and *B. proteae*, therefore, seem to be specific at subfamily level to South African Proteoideae. The results of this study support those of Crous et al (2000c) and Taylor et al (2001a, b), who suggested that many of the fungal pathogens of Proteaceae are host specific.

This is the first report of *B. ribis* from South African and Australian Proteaceae cultivated in Hawaii and from *P. cynaroides* in Zimbabwe. This pathogen has been reported from *Grevillea robusta* Cunn. in Gautamala (Schieber and Zentmeyer 1978) and in South Africa from *Leucadendron* R.Br. (Olivier 1951). However, in view of the confusion about the identity of *B. ribis* (Witcher and Clayton 1963, Maas and Uecker 1984, Rumbos 1987, Rayachhetry et al 1996, Zhou and Stanosz 2001, Zhou et al 2001), earlier reports must be interpreted with some circumspection. In this study *Botryosphaeria ribis* was not found on Proteaceae in South Africa, despite previous reports to the contrary (Olivier 1951, Crous et al 2000b). The South African samples included in this work were collected from Proteaceae in the cool, winter rainfall region in Western Cape Province, which could explain the absence of *B. ribis* from these samples. Previous records of *B. ribis* from various hosts in South Africa show that the hosts were growing in warm, humid climates (Schieber and Zentmeyer 1978, Herbert and Grech 1985, Crous et al 2000b). This suggests that warm, humid climatic conditions might be a prerequisite for infection by this pathogen. Further evidence supporting this hypothesis is presented in this study, where *B. ribis* occurred only on Proteaceae in Australia (Queensland), Hawaii and Zimbabwe, which are areas with high temperature and humidity. In South Africa, the cultivation of Proteaceae is expanding into the warm, humid, summer rainfall regions, and this might lead to the appearance of *B. ribis* on Proteaceae in South Africa.

The isolated incidence of *B. obtusa* on *P. magnifica* is difficult to explain. This fungus, however, has been commonly isolated from apples (Stevens and Jenkins 1924), a host that is cultivated close to the Groot Winterhoek Nature Reserve where this sample was col-

lected. Wider sampling might reveal broader distribution of this fungus on Proteaceae.

This study has clarified the current global distribution of *Botryosphaeria* spp. associated with Proteaceae. A key to identify the taxa associated with Proteaceae is thus provided to alleviate taxonomic confusion.

KEY TO *BOTRYOSPHAERIA* SPP. ASSOCIATED WITH PROTEACEAE

1. Conidia pigmented at maturity . . . . . 2
1. Conidia hyaline at maturity . . . . . 3
   2. Conidia sienna brown at maturity, ovoid to subcylindric with truncate base and obtuse apex, 20–26 × 9–12 μm, 0(–1)-septate, walls warty or finely roughened, 0.5–1 μm thick, synanamorph absent; ascospores hyaline, broadly fusiform, widest in the middle, granular, smooth, 25–33 × 7–12 μm; colony with moderate to rapid growth rate (>40 mm/wk on PDA at 25 C) colony margins smooth and uniform, gray-brown . . . . . ***B. obtusa***
   2. Conidia medium brown, subcylindrical, 7–14 × 2.5–3.5 μm, aseptate, walls finely verruculose; synanamorph: conidia hyaline, fusiform, 20–30 × 4.5–6 μm; ascospores hyaline, ellipsoidal, clavate–fusiform, frequently widest in the upper one third of the ascospore, tapering to obtuse ends, guttulate, smooth, 15–21 × 5–9 μm; colony slow growing (<40 mm/wk on PDA at 25 C), colony margins crenate to irregular, occasionally sectored, buff to iron gray . . . . . ***B. proteae***
3. Colony slow growing (<40 mm/wk on PDA at 25 C); conidia fusiform, 20–30 × 4.5–6 μm, aseptate, walls smooth; synanamorph: conidia medium brown, subcylindrical, 7–14 × 2.5–3.5 μm, aseptate, walls finely verruculose; ascospores hyaline, ellipsoidal, clavate–fusiform, frequently widest in the upper one third of the ascospore, tapering to obtuse ends, guttulate, smooth, 15–21 × 5–9 μm; colony slow growing (<40 mm/wk), colony margins crenate to irregular, mycelium moderate, occasionally sectored, buff to iron gray . . . . . ***B. proteae***
3. Colony growth fast (>90 mm/wk on PDA at 25 C) . . . . . 4
   4. Colonies producing a yellow pigment in young cultures; conidia fusiform–ellipsoidal, base truncate or bluntly rounded, 14–32 × 4.5–9 μm, aseptate, walls smooth; synanamorph absent; ascospores hyaline, guttulate, smooth, oval to broadly fusiform, widest in the upper one third of the ascospore, tapering to obtuse base and apex, 18–28.5 × 7.5–12 μm; colony rapid growing (>90 mm/wk on PDA at 25 C) colony margins smooth and uniform, mycelium moderate, gray to dark gray ***B. lutea***
   4. Colonies not producing yellow pigment in culture . . . . 5
5. Conidia on average <25 μm in length, ovoid, apex rounded, base tapered, 17–24 × 7–11 μm; ascospores hyaline, ovoid, widest in the upper one third of the ascospore 17–28 × 7–12 μm; mycelium thick, woolly, gray (14–21 d on PDA at 25 C) . . . . . ***B. ribis***
5. Conidia on average >25 μm in length, irregularly fusoid, apex obtuse, base bluntly rounded, 20–40 × 9–13 μm; ascospores hyaline, becoming light brown with age, fusiform, widest in the middle with obtuse ends, sometimes inequilateral, 25–37 × 9–13 μm; mycelium flattened in the center, with a rim of loose aerial mycelium at the edge of the dish (14–21 d on PDA at 25 C) . . . . . ***B. protearum***

ACKNOWLEDGMENTS

The authors acknowledge the South African *Protea* Producers and Exporters Association (SAPPEX) and the South African National Research Foundation (NRF) for financial support.

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