A taxonomic reassessment of *Phyllachora proteae*, a leaf pathogen of Proteaceae

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Abstract: *Phyllachora proteae* is a well known leaf pathogen of *Protea* spp. In the present study this fungus was recollected from several genera and species of Proteaceae in the Western Cape province of South Africa, and its taxonomy was reassessed. Single ascospore cultures produced a *Fusicoccum* anamorph in culture, described here as *F. proteae*. A microconidial synanamorph with narrowly ellipsoidal, brown, thick-walled conidia was commonly associated with *F. proteae* in culture. Based on its bitunicate asci, as well as pseudothecial and ascospore morphology, a new combination for *P. proteae* is proposed in *Botryosphaeria*, as *B. proteae*. 

Key Words: *Botryosphaeria*, *Fusicoccum*, *Protea*, systematics

INTRODUCTION

The Proteaceae, one of the oldest plant families, is estimated to be more than 140 Myr old. The family comprises at least 1400 species, of which 330 occur in the South African Fynbos biome, and is among the most predominant groups of flowering plants in the southern hemisphere (Rebelo 1995). The unique beauty and hardiness of *Protea* flowers make them highly desirable to local and international cut-flower markets. In 1996, 4.8 million kg of fresh proteas were produced in South Africa, of which 3.3 million kg were exported, earning an estimated R64.5 million (Wessels et al 1997). However, strict phytosanitary regulations of importing countries frequently prevent blemished blooms from reaching potential export markets. Additionally, the marketing of low quality flowers results in consumer dissatisfaction, a loss in credibility of South African products and ultimately the forfeiting of markets to other exporting countries (Wessels et al 1997).

Lesions induced by plant pathogenic organisms are a major cause of foliage and bloom spoilage. A large number of fungal pathogens is known to occur on Proteaceae in South Africa (Knox-Davies et al 1987). The taxonomy of some of these has, however, changed considerably since they were first reported. The correct identification of pathogenic fungi is necessary to ensure appropriate quarantine decisions and suitable control strategies. *Phyllachora proteae* Wakef., commonly associated with leaf spots and stem cankers of *Protea* L., and *Leucospermum* R. Br. species, is an example of a pathogen that requires taxonomic reassessment. This fungus was described by Wakefield (1922) as having unilocular ascomata that develop under a very small epidermal clypeus, cylindrical ascii, pseudoparaphyses and hyaline, aseptate, ellipsoidal ascospores, 19–22 × 8–9 μm. In a reexamination of the type material, Doidge (1942) found the ascomatal wall to be continuous with, and similar in structure to the clypeus. She noted, however, that the ascomatal stromata differed from those of other South African *Phyllachora* spp. In his study of leaf pathogens of *Protea*, *Leucadendron* and *Leucospermum* spp., Van Wyk (1973) commented that the ascocarps of *P. proteae* appeared to be unilocular with pseudoparaphyses, and that the fungus should probably be transferred to *Guignardia* Viala & Ravaz or *Botryosphaeria* Ces. & De Not. The aims of this study were therefore to recollect *P. proteae*, study the type specimen, identify the anamorph, and to record new hosts and collection sites.

MATERIALS AND METHODS

Collection and isolation.—Several farms reporting proteas with severe leaf spots and stem cankers were visited. Affected plants were identified, symptoms recorded and diseased leaves and branches cut from bushes and brought back to the laboratory for study. Leaf and stem samples were incubated in Petri dishes containing moist filter paper. Single ascospore cultures were obtained from pseudothecia by squashing the contents in a drop of sterile water and spreading this onto the agar surface of dishes containing potato dextrose agar (PDA, Biolab). Alternatively, pseudoth-
were soaked in water for 2 h, attached to lids of Petri dishes, and ascospores ejected onto the agar surface of PDA plates. Single germinating ascospores were transferred to fresh PDA plates, and incubated at room temperature in the dark for 5 d. Subcultures were made from five single ascospore or conidial colonies per diseased plant.

**Morphological characterization and culture.**—To induce sporulation, two different techniques were used. In the first, cultures were transferred to divided plates containing carnation leaf agar (Fisher et al. 1982) in one half of the dish and PDA in the other. In the second technique, isolates were grown on a sterilized piece of *Leucospermum* stem in full strength V8 broth (Englander and Turbitt 1979), and placed on tap water agar (Biolab). All plates were incubated in the laboratory at room temperature (20-25°C) under cool white and near-ultraviolet light with a 12 h photoperiod. Cultures were stored on PDA slants, with or without mineral oil, at room temperature. All fungal material was mounted in lactophenol, and at least 30 structures were measured. The range of dimensions is given with the extremes in parentheses. Reference specimens have been deposited at the National Collection of Fungi in Pretoria (PREM), and cultures are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U).

Ten isolates derived from different hosts and localities were selected for cultural growth studies on PDA. Mycelial discs 5 mm diam were cut from the periphery of actively 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 7 d. Two perpendicular readings were taken for each colony, using a digital caliper. The mean growth rates for three replicates of ten isolates were plotted for each temperature tested.

**TAXONOMY**

In a reexamination of the type specimen of *Phyllachora proteae* (PREM 32915), it was found that this taxon had bitunicate ascii that were borne in thick-walled, brown pseudothecia. Contrary to the protologue for the species, no clypeus was observed. These observations suggest that this species would be better accommodated in *Botryosphaeria* than *Phyllachora*, and a new combination is therefore proposed. Cultures derived from single ascospores of *B. proteae* produced a *Fusicoccum* Corda anamorph with a microconidial state when cultured on PDA. As no anamorph has thus far been reported for *B. proteae*, the *Fusicoccum* state is described as new.

*Botryosphaeria proteae* (Wakefield) Denman et Crous, comb. nov.  
=F*Phyllachora proteae* Wakefield, Kew Bull. 1922: 164. 1922.

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**Anamorph.** *Fusicoccum proteae* Denman et Crous, sp. nov.  
Figs. 7, 8, 11

Conidiomata pycnidialia, euastromatica ad 450 μm diam atrorbrunnea, uni-ad multilocularia. Conidiophora hyalinae, laeves, ramosae subcylindricae, 1-3-septatae, 20-40 X 3-4.5 μm, paraphysibus hyalinis, septatis in multiformia. Conidiogenae cellularae holoblasticae, hyalinae, laeves, clypeocele, enteroblastice et percurrente proiferantes vel phialidos typicus perichalaler spissescentibus. Conidia hyalina, parietibus tenuibus, aseptata, laeves, clavata ad fusiformia, apice subobtusus, base truncata, (20-)22-25(-30) X (4.5-)5-6 μm.

Mycelium immersed, consisting of branched, septate, smooth, medium brown hyphae, 2.5-5 μm diam. Mycelial growth rates on PDA were maximal at 25°C, and growth virtually ceased at temperatures below 10°C and above 35°C (Fig. 1). Pseudothecia epiphyllous, separate, unilocular, initially solitary and discrete, becoming aggregated, immersed, substomatal, with a central, flattened ostiole, obovoid, slightly depressed, 200-300 μm wide, 200-240 μm high; wall consisting of 8-11 layers of brown pseudoparenchymatous texture angularis, up to 65 μm thick in upper, widest part (Figs. 2, 9). Asci fissitunicate, clavate to cylindrical, stipitate, bitunicate, 90-150 X 12-15 μm; nasse apicale visible as a notch-like indentation at the apex (Figs. 5, 10). Ascosporae uni-biseriatae, hyaline, gutulatae, smooth, ellipsoidal, clavate to fusiform, frequently widest in the upper third of the ascospore, tapering to obtuse ends, (15-)17-20(-21) X (5-)6-8(-9) μm (Figs. 4, 10). Pseudoparaphyses hyalinae, septate, branched, frequently attached to the top and base of the pseudothecial cavity, 2-3.5 μm diam (Figs. 3, 10). Conidiomata pycnidalia, eustromatic, to 450 μm diam, immersed, subepidermal, separate, dark brown, uni-

No multilocular, walls consisting of dark brown *textura angularis*, ostiolate. *Fusicoccum* anamorph: Conidiophores hyaline, smooth, branched, subcylindrical, 1–3-septate, formed from the inner layer of the locule, 20–40 × 3–4.5 μm (Fig. 11); intermingled with hyaline, septate paraphyses. Conidiogenous cells phialidic, discrete or integrated, hyaline, smooth, cylindrical, producing the first conidium holoblastically, and subsequent conidia enteroblastically, proliferating percurrently with 1–2 indistinct proliferations, or determinate, with periclinal thickening (sensu Sutton 1980), 20–30 × 2.5–3.5 μm (Fig. 11). Conidia hyaline, thin-walled, aseptate, smooth, clavate, widest in the middle or upper third of the conidium, apex subobtuse, base truncate, (20–)22–25(–30) × (4.5–)5–6 μm (Figs. 8, 11). The microconidial state occurred in the same or in separate conidiomata to the *Fusicoccum* anamorph. Microconidiophores hyaline, smooth, branched, cylindrical, 1–3-septate, formed from the inner layers of the locule, 15–25 × 2–3 μm (Fig. 13). Microconi-}

diogenous cells phialidic, discrete or integrated, hyaline, smooth, cylindrical, determinate with prominent periclinal thickening, 6–10 × 2–3 μm (Fig. 13). Microconidia medium brown, thick-walled, finely verruculose, guttulate, aseptate, subcylindrical to narrowly ellipsoid with rounded ends, (7–)8–11(–14) × 2.5–3.5 μm (Figs. 6, 13). The spermatial state occurred in conidiomata with the *Fusicoccum* anamorph, or in separate spermatogonia. Spermatiophores hyaline, smooth, branched, cylindrical, 1–3-septate, formed from the inner layer of the locule, 15–20 × 3–4 μm (Fig. 12). Spermatiogenous cells discrete or integrated, hyaline, smooth, cylindrical, proliferating via determinate phialides with periclinal thickening, 10–12 × 2–3 μm. Spermatia hyaline, smooth, aseptate, rod-shaped with rounded ends, 5–7 × 1.5–2 μm (Figs. 7, 12).

*Cultures*. Cultures were characterized morpholog-
ically after growing for 1 mo in the dark at 25 C. The colony margins were crenate to irregular and moderate to sparse, gray aerial mycelium, occasionally sectored, with black conidiomata that occurred over the entire colony surface, but aggregated in dense masses along the outer colony margins. In several plates ascomata were also observed to develop on PDA. Colony color (underneath) ranged from buff (~l"f) to olivaceous gray (~3'''''11) or iron gray (23'''k), and smoke gray (19'''i) on the surface.

Temperature requirements for growth. Min. 5 C, opt. 25 C and max 35 C. Mean daily growth rate at 25 C in the dark was 7 mm/d.

HOLOTYPES. SOUTH AFRICA. WESTERN CAPE: Klipmuts, on leaves of Protea repens (as P. mellifera). P. Van Der Bijl, No. 357 (PREM 32915, teleomorph);Grabouw, Molteno Estate, Protea grandiceps, 5 Jun. 1997, S. Denman (PREM 55769, anamorph, culture ex-type STE-U 1694).


Hosts. Protea cynaroides L.; P. eximia (Salisb. ex Knight) Fourc.; P. grandiceps Tratt.; P. magnifica Link.; P. repens (L.) L. and hybrids with cultivar names, P. aristata (E. Phillips) × P. repens cultivar "Venus", P. magnifica × P. compacta (R. Br.) cultivar "Lady Di" and a Leucospermum sp.

Known distribution. South Africa (Western Cape province) and USA (Hawaii).

DISCUSSION

In the present study we reexamined P. proteae and found that it was a species of Botryosphaeria, for which the name B. proteae is proposed. This is consistent with previous suggestions (Doidge 1942, Van Wyk 1973) that Phyllachora was not an appropriate genus for this fungus. Furthermore, we have shown that the anamorph of B. proteae is a species of Fusiculosum, now known as F. proteae. In culture as well as on host material, a microconidial state with thick-walled brown conidia is also frequently observed, accompanied by a spermatial state with spermatia that are sterile in culture.

A number of Botryosphaeria spp. have been associated with Proteaceae. These include B. dothidea (Moug.) Ces & De Not. (= B. ribis Grossenb. & Duggar; Arx and Müller 1954) on Protea, Leuocospermum and Leucadendron, B. banksiae Hansf. on Banksia (Hansford 1954), and B. gaubae Petr. on Grevillea (Petra 1968). Botryosphaeria proteae differs from B. banksiae in that it does not have paraphyses in the ostiolar region. Furthermore, ascospores of B. banksiae (13–15 µm), and B. gaubae (10–15 µm), are much wider than those of B. proteae (5–9 µm), and none are widest in the upper third of the ascospore as in the case of B. proteae (Hansford 1954, Petra 1968). Ascospores of B. dothidea are similar in size (18–23 × 7–9 µm, Arx and Müller 1954), but differ in shape, and in the anamorph produced in culture.

Botryosphaeria proteae is unusual in that the obvoid pseudothecia have a wider wall layer in the apical part, which was incorrectly referred to as a clypeus by Wakefield (1922). Furthermore, the presence of abundant pseudoparaphyses, the frequent occurrence of cylindrical asci with uniseriate ascospores, its distinct cultural characteristics, as well as the microconidial form suggest that this species may not be a typical species of Botryosphaeria.

Botryosphaeria is commonly ascribed to collections of bitunicate ascocymetes that have multi- or uniloculate, black ascomata occurring separately, or grouped to aggregated on a common basal stroma (Sivasesan 1984). Pseudothecia are ostiolate and may be embedded in the host tissue or erumpent. The centrum contains numerous filamentous pseudoparaphyses (Hanlin 1990), and although Sivasesan (1984) reported that interthecial tissues usually disintegrate, it is frequently not the case as observed in B. proteae, as well as in other species of Botryosphaeria (Pennycook and Samuels 1985). Ascospores are hyaline, one celled, often inequilateral, and may become brown and 1-2 septate with age. Some discrepancy still exists, however, regarding the presence/absence of mucilaginous caps on ascospores of Botryosphaeria and related genera. Barr (1987), in her key to the genera of the Botryosphaeriaceae, mentioned that ascospores usually lack a gel coating or appendages, thereby implying that some species may well have these features. Hanlin (1990) also stated that ascospores may have a thin gelatinous coat. However, the gelatinous sheath should be distinguished from the mucilaginous caps found in Guignardia Viala & Ravaz.
The genus *Botryosphaeria* seems to be beset with unresolved taxonomic issues. A number of authorities have thus stated that the whole complex is in urgent need of revision (Sutton 1980, Pennycook and Samuels 1985). Sivanesan (1984) treated 12 species of *Botryosphaeria*, and subsequent to his treatment several additional species have been described (Pennycook and Samuels 1985, Sivanesan and Sutton 1985, Bisset 1986, Wang 1987, Gardner and Hodges 1988, Ramesh 1991, and others). Sivanesan (1984) treated 12 species of *Botryosphaeria*, and subsequent to his treatment several additional species have been described (Pennycook and Samuels 1985, Gardner and Hodges 1988, Ramesh 1991, and others). A number of these have possibly been incorrectly assigned to *Botryosphaeria*, and there may be many more that have been incorrectly allocated to morphologically similar genera. The bulk of recent literature suggests that *Guignardia*, which has been confused with *Botryosphaeria* in the past, is clearly segregated and always associated with *Phyllosticta* Pers. (Sivanesan 1984, Hanlin 1990). The genus *Botryosphaeria*, on the other hand, has been associated with several anamorph form genera. Sivanesan (1984) listed several anamorph states of *Botryosphaeria*. These included *Botryodiplodia* (Sacc.) Sacc., *Dothiorella* Sacc., *Diplodia* Fr., *Maarophoma* (Sacc.) Ber. & Vag., and *Sphaeropsis* Sacc. Sutton (1980) placed *Maarophoma* in synonymy with *Sphaeropsis*, and stated that there are several genera available for other species originally described in *Maarophoma*. The similarities between *Dothiorella* and *Fusicoccum* were extensively discussed by Sutton (1977, 1980), and will be dealt with elsewhere (Crous and Palm unpubl). Pennycook and Samuels (1985) and Phillips and Lucas (1997) broadened the concept of *Fusicoccum* to include taxa with conidiomata ranging from unilocular pycnidia to complex multilocular eustromatic structures. Simple or branched conidiophores also produced conidia via phialides, while conidia were thin-walled, hyaline, aseptate, clavate, and had a distinct truncate base (Pennycook and Samuels 1985). The genus *Maarophoma* Petri was distinguished from *Fusicoccum* by having conidigenous cells with percurrent proliferations (annelides sensu Sutton 1980). However, Pennycook and Samuels (1985) found the same mode of conidiogenesis in specimens of *Fusicoccum*, and subsequently reduced *Maarophoma* to synonymy with it.

*Fusicoccum proteae*, the anamorph of *B. proteae*, is similar to other species in the genus that have branched conidiophores, and hyaline, thin-walled, clavate conidia. The mode of conidiogenesis by producing conidia via determinate or percurrently proliferating phialides is also more common in *Fusicoccum* than is reported in literature (Pennycook and Samuels 1985). *Botryosphaeria proteae* is an unusual species of the genus, however, in having a microconidial state with brown, thick-walled conidia.

Notwithstanding this morphological variation, it is interesting to speculate whether *B. proteae* will cluster with those taxa with typical *Fusicoccum* or typical *Sphaeropsis* Sacc. or *Diplodia* Fr. anamorphs. Molecular studies aimed at elucidating its phylogenetic position in *Botryosphaeria* and the Dothideales are currently in progress.

The occurrence of *B. proteae* on species of *Protea* and *Leucospermum* in South Africa and Hawaii, leads us to believe that this taxon may have a much wider distribution than previously thought. Presently very little is known about the distribution, host range, and pathogenicity of *B. proteae*. Further collections and inoculation trials are presently underway to characterize its importance as a pathogen of Proteaceae.

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