

An overview of the taxonomic history of *Botryosphaeria*, and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny

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Abstract: The taxonomic history of *Botryosphaeria* is reviewed and the genus is circumscribed and distinguished from other morphologically similar genera. Several anamorph genera have been linked to *Botryosphaeria*. Based on morphological observations and phylogenetic analysis of ITS rDNA sequence data, two groups of anamorphs are recognised. Anamorphs with conidia that are pigmented when mature are placed in *Diplodia*, while those with hyaline conidia are accommodated in *Fusicoccum*. *Botryosphaeria proteae*, a species with both conidial types, should be excluded from *Botryosphaeria* based on its ascomatal wall anatomy, anamorph morphology and ITS rDNA phylogeny.

Key words: *Botryosphaeria*, *Diplodia*, *Fusicoccum*, *Botryodiplodia*, ITS rDNA

Introduction

Botryosphaeria Ces. & De Not. is a species-rich genus with a cosmopolitan distribution (Barr, 1987). Species are saprophytic, occasionally parasitic and endophytic (Smith *et al.*, 1996; Denman, unpublished), and can cause die-back and canker diseases of woody hosts (von Arx, 1987). They occur on a wide range of monocotyledonous, dicotyledonous and gymnospermous hosts, on woody branches, herbaceous leaves, stems of grasses, on twigs and in the thalli of lichens (Barr, 1987).

The taxonomy of *Botryosphaeria* is problematic at several levels of classification. The position of the genus in the higher classification of ascomycetes has not yet been resolved (von Arx & Müller, 1975; Sivanesan, 1984; Barr, 1987; Silva-Hanlin & Hanlin, 1999). Secondly, in *Botryosphaeria*, much confusion has occurred regarding the allocation of species to the genus. The reasons for this are many. Teleomorphs are uncommonly encountered in nature (Shoemaker, 1964; Laundon, 1973; Jacobs & Rehner, 1998), or are difficult to induce in culture (Laundon, 1973), and there is insufficient diversity of teleomorph fea-

tures to allow unequivocal differentiation at the species level (Shoemaker, 1964; Laundon, 1973). Furthermore, concepts defining morphological features of the teleomorph (e.g. perithecial or pseudoperithecial ascomata, uni- or bitunicate asci, hamathecium) have been historically slow to develop. Ontogenic studies on the majority of species allocated to *Botryosphaeria* have not been conducted (Sivanesan, 1984), which has also hindered the correct placement of many species. Therefore, the assignment of many species to this genus is questionable (see synonymies in von Arx & Müller, 1954). A number of species of *Botryosphaeria* were reduced to synonymy (von Arx & Müller, 1954), only later to be recognized as distinct (Shoemaker, 1964; Laundon, 1973).

The taxonomy of *Botryosphaeria* is thus largely dependent upon the taxonomy of its anamorphs, which are the more commonly encountered morphs (Hanlin, 1990). Morgan-Jones & White (1987) maintained that the identification of *Botryosphaeria* species is by no means a simple task because the characters used to circumscribe anamorph genera associated with *Botryosphaeria* are poorly described

and inconsistently applied. Morphological changes in conidia as they age also make identification difficult (Laundon, 1973).

The recent advent of molecular techniques and application of ribosomal RNA genes to fungal phylogenetic studies have contributed greatly to the phylogenetic reconstruction of fungi (Bruns *et al.*, 1991; Lee & Taylor, 1991; Berbee & Taylor, 1992; O'Donnell & Gray, 1995; O'Donnell *et al.*, 1997). Among the variable regions of rDNA, the internal transcribed spacers (ITS), which often vary between and within species (Lee & Taylor, 1991; Peterson & Kurtzman, 1991), have been successfully used to investigate phylogenies of *Pezizales* (Momol & Kimbrough, 1994), *Leptosphaeria* Ces. & De Not. (Morales *et al.*, 1993, 1995), *Alternaria* Nees (Jasalovich *et al.*, 1995) and *Mycosphaerella* Johanson (Stewart *et al.*, 1999). Jacobs & Rehner (1998) used ITS sequence data to link several anamorphs to *Botryosphaeria*, which in turn helped to clarify the taxonomy of the genus.

In this paper, we provide an overview of the taxonomy of *Botryosphaeria*, and additional molecular data that support two morphological groups within the genus. We also place special emphasis on characters of the anamorphs, which we regard as important in defining the genus.

Historical review

TAXONOMIC HISTORY OF HIGHER CLASSIFICATION OF *BOTRYOSPHAERIA*

The genus *Botryosphaeria* was introduced in 1863 by Cesati and De Notaris, who designated *Botryosphaeria dothidea* (Moug.) Ces. & De Not. as the type species (Johnson, 1992). At this time, the taxon was placed under the group name *Sphaeria* (Munk, 1953) and anamorph genera were not explicitly linked to teleomorphs. In the late nineteenth century, two systems of classification emerged. In *Sylloge Fungorum*, Saccardo grouped species based on spore-shape, septation and colour, an artificial system that was very practical to use (Munk, 1953), but did not reveal phylogenetic relationships. Another system, proposed by Lindau (1897), attempted to place fungi in natural (phylogenetic) groups. This system, considered by some (Luttrell, 1951; Wehmeyer, 1975) as the earliest significant classification, is the system upon which higher taxonomy of the Ascomycetes is currently based. *Botryosphaeria* was allocated to the *Melogrammataceae* in the *Sphaeriales* (Lindau, 1897). At that time, the *Sphaeriales* included fungi with clearly differentiated carbonaceous ascumata with or without a stroma, while the *Dothideales* were

characterized by the formation of asci in locules embedded in stromata, and not in distinct peridia. A single family, the *Dothideaceae*, which was restricted to compound (multiloculate) forms, was placed in this order. Von Höhnell (1907) established the family *Pseudosphaeriaceae* to accommodate taxa with single-locule, multiascal ascostromata, and placed *Botryosphaeria* in this family, which was later allocated to the order *Dothideales* (Von Höhnell, 1909).

In the period from 1909–1928, the classification of *Botryosphaeria* was subjected to much rearrangement. Theissen & Sydow (1915) created a subfamily *Botryosphaerieae* and placed *Botryosphaeria* into this subfamily in the *Pseudosphaeriaceae*. This family was treated as an 'anhang' and was not placed in any order. A year later, Theissen (1916) allocated the *Pseudosphaeriaceae* to the *Myriangiales*. However, by 1917 Theissen & Sydow thought that the *Pseudosphaeriaceae* should be united with the *Dothideaceae* (Luttrell, 1951). A year later, Theissen & Sydow (1918) created a subclass the *Dothideineae* into which the order *Pseudosphaeriales*, family *Botryosphaeriaceae*, and the genus *Botryosphaeria* were assigned. Petrak (1923) rejected Theissen & Sydow's classification and placed *Botryosphaeria* in the subfamily *Pseudosphaerieae*, which he put in the *Pleosporaceae* (*Sphaeriales*). One of the main reasons for this reshuffling of the classification of *Botryosphaeria* was confusion regarding ontogeny and morphology of true perithecia, ascostromata and interthelial tissues.

Miller (1928) showed that there was a fundamental difference between the tissues forming the perithecium and those forming the boundary of the locule. He also showed how these different tissue types were correlated with features of the centrum. Taxa allocated to the *Sphaeriales* had true perithecia and paraphyses (or in some cases periphyses) while those assigned to the *Dothideales* had ascostromatic ascumata and lacked paraphyses. Thus, *Botryosphaeria* species (*Pseudosphaeriaceae*) were allocated to the *Dothideales* because they lacked true perithecial walls (Miller, 1928).

Nannfeldt (1932) re-grouped the Euscomycetes into three orders. The ascostromatic forms, where asci formed in cavities in pre-formed stromata, were accommodated in the *Ascoloculares*. The true *Sphaeriales*, i.e. species in which the asci developed in a hymenium, were accommodated in the *Ascohymeniales*. Although these groups were not accepted by many at the time, they were consistent with the bitunicate and unitunicate groups later proposed by Luttrell (1955).

Concepts based on morphological features resulting from the ontogeny of the perithecial wall and the development of centrum tissues were further developed by Miller (1938) and three orders were recognized. The *Sphaeriales* had perithecia and paraphyses, the *Dothideales* encompassed ascostromatic forms without paraphyses and the *Pseudosphaeriales* included ascostromatic forms with interthecial threads that appeared in the ascotal cavity before the asci arose. Although details of the development of hamathelial tissues were beginning to take form, conflicting opinions regarding the taxonomic value of these structures predominated. Miller (1938) established a new order, the *Pseudosphaeriales*, and retained the position of *Botryosphaeria* in the family *Pseudosphaeriaceae*. Thus, *Botryosphaeria* was placed in the *Pseudosphaeriales* and not the *Dothideales*, where Miller had classified the genus in 1928.

Luttrell (1951) recognized two major morphological groups in the pyrenomycetous fungi. He also emphasized the significance of ontogenetic characters of ascomata in classification. The two major morphological groups were those with single-walled asci or unitunicate ascomycetes, and those with double-walled asci, the Loculoascomycetes, commonly referred to as bitunicate ascomycetes (Luttrell 1955). Luttrell also identified eight types of centrum development and highlighted the taxonomic value of sterile interthecial tissues. He provided an explanation why the original name of the order *Pseudosphaeriales* was no longer tenable. The type of the family *Pseudosphaeriaceae*, and the type of the genus *Pseudosphaeria*, had been transferred to the *Dothideales*. Therefore, Luttrell (1955) replaced the name *Pseudosphaeriales* with *Pleosporales*, based on the most important genus in the group with that type of centrum development, and assigned *Botryosphaeria* to the *Pleosporales*.

Luttrell's views were promoted by Barr (1972, 1976, 1979, 1983, 1987). In Barr's earlier work (1972, 1976), she had not studied specimens of *B. dothidea* in which the interthecial tissues were clearly visible and, despite the clear demonstration by Parguey-Leduc (1966) that *B. dothidea* exhibited a *Pleospora* centrum-type, she classified *Botryosphaeria* in the *Dothideales*. Later, however, Barr (1979) acknowledged that *Botryosphaeria* species had a centrum typical of the *Pleosporales*, and she concluded that the genus should reside in this order. This view was retained in later publications (Barr, 1983, 1987).

The orders proposed by Luttrell (1955, 1973) and Barr (1979, 1987) were not accepted by von Arx & Müller (1975) and von Arx (1987). They felt the ordinal boundaries did not enable the correct group-

ings of related genera. The orders comprised a mixture of unrelated genera (von Arx, 1987) and there was overlap of some features amongst the orders (von Arx & Müller, 1975). Furthermore, von Arx & Müller (1975) did not support the placement of what they considered closely related genera, such as *Guignardia* Viala & Ravaz and *Botryosphaeria*, in different orders (*Dothideales* and *Pleosporales*, respectively) (Luttrell, 1973). They delimited a single order, the *Dothideales*, which comprised two sub-orders and 24 families. They felt that this was a more appropriate means of dealing with the taxonomy of this very large, heterogeneous group, at least until a more natural method of classification could be developed. Thus, *Botryosphaeria* was maintained in the *Botryosphaeriaceae*, but was placed once again in the *Dothideales*. Hence the two major systems of classification that prevailed at the end of 1975, and which remain in common use, are those of Barr (1987), in which *Botryosphaeria* was placed in the *Pleosporales*, and von Arx & Müller (1975), who placed the genus in the *Dothideales*.

Eriksson (1981) emphasized that *Botryosphaeria* species have a centrum typical of the *Pleosporales* with pseudoparaphyses and pseudothecia. Currently, however, the widely accepted classification of *Botryosphaeria* is that it is a member of the family *Botryosphaeriaceae* accommodated in the *Dothideales* (Hawksworth *et al.*, 1995).

BOTRYOSPHAERIA SPECIES DESCRIBED TO DATE

The Index to Saccardo's *Sylloge Fungorum* lists 116 *Botryosphaeria* species recorded up to 1920, including varieties, subspecies and *formae speciales*. Twenty-three of these species were either redispersed to other genera or reduced to synonymy (Reed & Farr, 1993). Petrak's Lists, numbers 1-8, 1920-1939, record 10 species of *Botryosphaeria*, six of which are not in Saccardo's lists as presented by Reed & Farr (1993). Prior to 1954, another two new species were added (Index of Fungi vol. 1). Von Arx & Müller (1954) examined 183 taxa reducing them to 11 species. Only 31 of the 183 taxa examined by Von Arx & Müller (1954), were *Botryosphaeria* spp. Out of the 31 *Botryosphaeria* spp. examined, 24 appeared previously in Saccardo's lists as presented by Reed & Farr (1993), one in Petrak's Lists and the other six are unaccounted for. Nine of the 11 species treated by Von Arx & Müller (1954) were new, and thus up to 1954, 85 *Botryosphaeria* species could be accounted for. Between 1954 and 1997 a further 58 *Botryosphaeria* spp. were listed in the Index of Fungi. Thus, to date, the genus *Botryosphaeria* comprises 143 species. However, until the concept of the genus

is clearly defined and all the types have been re-examined it is impossible to assess the validity of all the species described to date.

Taxonomic Part

Botryosphaeria Ces. & De Not., Comm. Soc. Critog. Ital. 1: 211. 1863; emend. Sacc., *Michelia* 1: 42. 1877.

= *Melanops* Nitschke ex Fuckel, *Symb. Mycol.*: 225. 1870.

= *Thuemenia* Rehm, *Flora* 62: 123. 1879.

= *Coutinia* J.V. Almeida & Sousa da Câmara, *Rev. Agric. Lisboa* 1: 392. 1903 (non Vellozo, 1799).

= *Phaeobotryosphaeria* Speg., *An. Mus. Nac. Buenos Aires* 17: 120. 1908.

= *Cryptosporina* (Henn.) Höhn., *Sitzungsber. Akad. Wiss. Wien* 120: 437. 1911.

= *Amerodothis* Theiss. & Syd., *Ann. Mycol.* 13: 295. 1915.

= *Phaeobotryon* Theiss. & Syd., *Ann. Mycol.* 13: 664. 1915.

= *Epiphyma* Theiss., *Verh. Zool.-Bot. Ges. Wien* 66: 306. 1916.

= *Pyreniella* Theiss., *ibid.*: 371. 1916.

= *Desmotascus* F. Stevens, *Bot. Gaz.* 68: 476. 1919.

= *Rostrisphaeria* Tehon & E.Y. Daniels, *Mycologia* 19: 112. 1927.

= *Neodeightonia* C. Booth, *Mycol. Pap.* 119: 19. 1969.

= *Caumadothis* Petr., *Sydowia* 24: 276. 1971.

The features that characterize *Botryosphaeria* include the production of ascostromatic pseudothecia, described as uniloculate by Sivanesan (1984) but generally considered multiloculate (Hanlin, 1990). Pseudothecia are ostiolate, solitary or botryose, on a common basal stroma and may be embedded in the host tissue or erumpent. Cellular pseudoparaphyses are prevalent in the centrum (Dennis, 1981; Hanlin, 1990). The asci are bitunicate, stalked or sessile, clavate and contain eight hyaline ascospores (Dennis, 1981; Sivanesan, 1984; Hanlin, 1990). Ascospores have a uni- to bi-seriate arrangement, are aseptate and they vary from ovoid to fusoid to ellipsoid in shape. They are often inequilateral with the widest part in the middle. Ascospores may become brown and 1–2-septate with age, are smooth and thin-walled, but can occasionally be slightly verruculose after discharge (Sivanesan, 1984; Hanlin, 1990). They may possess evanescent hyaline appendages (Sivanesan, 1984; Pennycook & Samuels, 1985) or a thin gelatinous coat (Hanlin, 1990).

CLOSELY RELATED TELEOMORPH GENERA

Barr (1987) differentiated *Botryosphaeria* from *Auerswaldiella* Theiss. & Syd., *Discochora* Höhn., *Dothidotthia* Höhn., *Neodeightonia* C. Booth and the lichenicolous genus *Homostegia* Fuckel based on a number of morphological features. For instance, the

ascomata of *Auerswaldiella* species (with amerosporous ascospores) and *Homostegia* species (phragmosporous) are borne in a pulvinate stroma. *Dothidotthia* species are separated from *Botryosphaeria* species by their 1-septate, yellow-brown to dark-brown ascospores.

Another genus that has been confused with *Botryosphaeria* is *Discochora*. Barr (1987) placed *Guignardia* in *Discochora*. Although the name *Discochora* pre-dates *Guignardia* (Bissett, 1986), the name *Guignardia* has subsequently been conserved. Therefore, Barr's reference to *Discochora* being closely related to *Botryosphaeria* actually pertains to *Guignardia*. *Guignardia* species are separated from *Botryosphaeria* species by having smaller ascospores, distinct mucilaginous caps on the apices of the ascospores, and *Phyllosticta* Pers. anamorphs (van der Aa, 1973; Punithalingam, 1974; Hanlin, 1990). Barr et al. (1986) used *Neodeightonia* for *Dothidotthia*, but *Neodeightonia* had been reduced to synonymy with *Botryosphaeria* by von Arx & Müller (1975). Barr (1987) supported the latter synonymy.

Physalospora is a long-standing name that was misapplied to *Botryosphaeria* species, probably because the concept of unitunicate and bitunicate asci was only developed in 1951 by Luttrell (Luttrell, 1951, 1955). *Physalospora* differs from *Botryosphaeria* in that the species have unitunicate asci with non-septate ascospores and a hamathecium composed of paraphyses (Hanlin, 1990). Many *Physalospora* species were placed in *Botryosphaeria* by von Arx & Müller (1954). Since then, another 32 *Physalospora* species have been relocated to *Botryosphaeria* (Index of Fungi, vols. 1–6).

Species of *Othia* Nitschke have short-stalked, cylindrical, bitunicate asci (Dennis, 1981; Sivanesan, 1984). Ascospores are hyaline when young but become brown and 1-septate when mature, and are slightly constricted at the septum (Dennis, 1981; Sivanesan, 1984). Booth (1958) reviewed the history of the genus and designated *Othia spiraeae* (Fuckel) Fuckel as the lectotype species. Booth (1958) identified *Diplodia sarmentorum* (Fr.) Fr. as the anamorph of *O. spiraeae*. Laundon (1973) expressed doubt over the tenability of *Othia*, because the anamorphs were clearly related to those of *Botryosphaeria*. Von Arx (1974) listed *Othia* as the teleomorph of *Aplosporella* Speg. However, von Arx's concept for *Aplosporella* is indistinguishable from the anamorph of *B. obtusa* (Schw.) Shoemaker, i.e. *Sphaeropsis* Lév. sp. Hawksworth et al. (1995) described *Aplosporella* as being stromatic and having 1-celled, brown, holoblastically produced conidia. The description of *Aplosporella* given by Sutton (1980) is also very similar to

that of *Diplodia sarmentorum*. Furthermore, Booth (1958) studied the type material of *Othia quercus* Fuckel, and its measurements are identical to those of *Botryosphaeria quercuum* (Schw.) Sacc. It seems, therefore, that *Aplosporella* is indistinguishable from *Sphaeropsis* Sacc. It therefore seems likely that *Othia* should be synonymized with *Botryosphaeria*, but further morphological and molecular studies need to be conducted to confirm this.

ANAMORPH GENERA ASSOCIATED WITH *BOTRYOSPHERIA*

Botryosphaeria species are pleomorphic ascomycetes with coelomycetous anamorphs (Barr, 1987), which traditionally include the genera *Botryodiplodia* (Sacc.) Sacc., *Diplodia* Fr., *Dothiorella* Sacc., *Lasiodiplodia* Ellis & Everh., *Macrophoma* (Sacc.) Berl. & Voglino and *Sphaeropsis* (Sivanesan, 1984). More recently, *Fusicoccum* Corda species have been recorded as anamorphs of some *Botryosphaeria* species (Sutton, 1980; Pennycook & Samuels, 1985; Samuels & Singh, 1986; Morgan-Jones & White, 1987; Denman *et al.*, 1999). Other anamorph genera have also been associated occasionally with *Botryosphaeria*. These include *Chaetodiplodia* P. Karst., *Colletotrichella* Höhn., *Diplodiella* Petr., *Kabatia* Bubák, *Pellionella* (Sacc.) Petch, *Placosphaeria* (De Not.) Sacc., *Rhynchodiplodia* Briosi & Farneti, *Selenophoma* Maire, *Striodiplodia* Zambett., and *Strionemadiplodia* Zambett. (Barr, 1987). *Phyllosticta* was linked to *Botryosphaeria* by von Arx (1987) and Jacobs & Rehner (1998), but this genus should be reserved for anamorphs of *Guignardia* (van der Aa, 1973; Punithalingam, 1974; Sivanesan, 1984; Hanlin, 1990).

Anamorph genera with hyaline conidia

Fusicoccum Corda, in Sturm, Deutschlands Flora 2: 111. 1829.

= *Macrophomopsis* Petr., Ann. Mycol. 22: 108. 1924.

A revised description of *Fusicoccum* is provided by Crous & Palm (1999).

According to Sutton (1980), *Fusicoccum* includes coelomycetes with fusiform, hyaline, non-septate conidia produced holoblastically in stromatic conidiomata. He regarded *Fusicoccum* as the genus best suited to accommodate the anamorphs of the *B. ribis* Grossenbacher & Duggar/*B. dothidea* complex, an opinion later also shared by Maas & Uecker (1984). Pennycook & Samuels (1985) accepted this concept but commented that the specimen examined by Sut-

ton (1980) (Saccardo in PAD, now the neotype designated by Crous & Palm, 1999), is immature, with most conidiogenous loci appearing to produce only one holoblastic conidium. Based on observations of cultures and older material, Pennycook & Samuels (1985) expanded the generic circumscription of *Fusicoccum* to include species with pycnidial conidiomata with enteroblastic phialidic conidiogenesis (Pennycook & Samuels, 1985), with proliferation occurring at the same level, resulting in periclinal thickening, or percurrently resulting in annellations (Crous & Palm, 1999). Pennycook & Samuels (1985) examined the type specimen of *Macrophomopsis* and found this genus indistinguishable from the earlier described *Fusicoccum*. Consequently they recommended that *Macrophomopsis* be placed in synonymy under *Fusicoccum*.

Sutton (1980) noted that Petrak (1922) first made the link between *Botryosphaeria* (*B. berengeriana* De Not.) and *Fusicoccum*, but at that time Petrak referred to *Fusicoccum* as *Dothiorella*. This probably marks the beginning of an extended confusion regarding the application of the name *Dothiorella* to specimens that have hyaline spores (Petrak, 1922; von Arx & Müller, 1954; Luttrell *et al.*, 1962; Bezuidenhout & Marasas, 1978; Johnson, 1992), which should probably have been referred to as *Fusicoccum*. Recently, Crous & Palm (1999) re-examined the type of *Dothiorella*, and considered it synonymous with the earlier-described genus *Diplodia* (see below). Thus, taxa with hyaline conidia previously referred to as *Dothiorella* and associated with *Botryosphaeria* teleomorphs will need to be carefully re-examined to confirm their correct taxonomic placement.

Anamorph genera with pigmented conidia

Diplodia Fr., Ann. Sci. Nat. Bot., Sér. 2, 1: 302. 1834.

= *Sphaeropsis* Sacc., *nom. cons.*, Michelia 2: 105. 1880.

= *Dothiorella* Sacc., Michelia 2: 5. 1880.

= *Macrophoma* (Sacc.) Berl. & Voglino, Atti Soc. Venet.-Trent. Sci. Nat. 10: 4. 1886, and Sacc., Syll. Fung. Addit. 1-4: 306. 1886.

= *Phoma* Westend. subgen. *Macrophoma* Sacc., Syll. Fung. 3: 65. 1884.

= *Lasiodiplodia* Ellis & Everh., Bot. Gaz. 21: 92. 1896.

(additional synonyms listed in Sutton, 1980)

Type: *D. mutila* Fr., Ann. Sci. Nat. Bot., Sér. 2, 1: 302. 1834.

Mycelium immersed or superficial, branched, septate, heavily melanized, dark brown. *Conidiomata* pycnidial, ostiolate, formed in uni- or multi-loculate stromata, comprising single, thin-walled pycnidia to large erumpent pustules containing up to 20 pycnidial

locules, each with a prominent ostiole, immersed or erumpent, separate or aggregated. *Paraphyses* present or lacking. *Conidiophores* (where present) hyaline, simple, occasionally septate, rarely branched, cylindrical, arising from the inner layers of the pycnidial cavity. *Conidiogenous cells* holoblastic, hyaline, cylindrical, determinate or proliferating percurrently, borne on flattened, pale brown cells lining the inside of the pycnidial cavity. *Conidia* variable in colour, ornamentation and septation; initially hyaline, thick-walled, smooth or granular, aseptate with a central guttule, becoming 1-euseptate in some cases; mature conidia light to dark brown with melanin often being deposited on the inner surface of the outer wall (i.e. irregularly verruculose), in some species longitudinal striations evident. Both young and mature conidia can occur concurrently in the same pycnidium, resulting in a mixture of hyaline and dark conidia.

The above description of *Diplodia* is an amended version of several descriptions, and represents a new generic concept proposed in the present paper. According to Sutton (1980), the original generic description of *Diplodia* was compiled by Fries based on a sample collected by Montagne in 1834, and identified as *Diplodia mutila* Fr. The teleomorph of *D. mutila* was discovered by Stevens (1936) who cited it as *Physalospora mutila* (Fr.) N.E. Stevens. However, von Arx & Müller (1954) reduced *P. mutila* to synonymy under *Botryosphaeria quercuum* (Schw.) Sacc. Shoemaker (1964) renamed *P. mutila* as *Botryosphaeria stevensii* Shoem., and separated it from *B. quercuum* based on conidial characteristics. He did not, however, name the anamorphs "because this would have raised the problem of taxonomic distinctions between *Diplodia* Fr., *Sphaeropsis* Lév., and *Dothiorella* Sacc." (Shoemaker, 1964). Sivanesan (1984) accepted *D. mutila* as the anamorph of *B. stevensii* and, because *Diplodia* predates *Sphaeropsis* and *Lasiodiplodia* (Sutton, 1980), this genus should be adopted to accommodate the dark-spored anamorphs of *Botryosphaeria*.

The following anamorph genera are here considered synonyms of *Diplodia*:

Macrophoma (Sacc.) Berl & Vogl. has been commonly applied to *Botryosphaeria* anamorphs with hyaline conidia (Tehon & Daniels, 1927; Funk, 1964; Smerlis, 1970). Sutton (1980) re-examined the type specimen and found it to be a later name for *Sphaeropsis*. Phillips & Lucas (1997) examined the causal agents of excoriosis on grapevines in Portugal, namely *Macrophoma flaccida* (Viala & Ravaz) Cav. and *Macrophoma reniformis* Viala & Ravaz, and reported

that these species represented later names for *Fusicoccum aesculi* Corda, the anamorph of *B. dothidea*.

Because many *Botryosphaeria* species with *Macrophoma* anamorphs have conidia described as hyaline to pale brown (Funk, 1964; Bezuidenhout & Marasas, 1978), it is unclear whether they would be better accommodated in *Fusicoccum* or in *Diplodia*. They all need to be re-examined to determine their correct generic placement.

Dothiorella Sacc. A great deal of confusion has surrounded the type specimen and generic concept of *Dothiorella*, which was discussed in detail by Sutton (1977) and Crous & Palm (1999). *Dothiorella pyrenophora* Sacc., the type species, is typified by Berkeley's English material of *Dothiora pyrenophora* Fr. (Berk. Exs. No. 282, K 54913). Crous & Palm (1999) re-examined this specimen, and found conidiomata to be variable (unilocular to multilocular, eustromatic). Conidiophores were branched, septate, holoblastic, and gave rise to smooth to finely verruculose, brown, 1-euseptate conidia, indistinguishable from those of *Diplodia*. *Dothiorella* should therefore be considered a synonym of *Diplodia*, and all anamorphs of *Botryosphaeria* that were placed in *Dothiorella* need to be re-examined.

Lasiodiplodia Ellis & Everh. Laundon (1973) stated that anamorphs of *Botryosphaeria* have to be studied at two stages, namely at conidial dehiscence from conidiogenous cells, when conidia are regarded as 'mature', and after discharge from the pycnidium, when conidia are regarded as 'aged'. Conidia vary greatly in septation, ornamentation and colour at these two stages. Young conidia are hyaline, and become pigmented with age. Conidial septation and ornamentation also develop with age. In the case of the type species, *L. theobromae* (Pat.) Griffon & Maubl., Uduebo (1975) clearly illustrated that the conidial wall ornamentation is made up of deposits of melanin on the inside of the wall, creating the illusion of striations on surfaces of conidia. The paraphyses observed in conidiomata of *L. theobromae* are characteristic of this species, but not unique, as they also occur in other anamorphs of *Botryosphaeria* (Zambettakis, 1954). Thus on the basis of these observations, there is justification for including *Lasiodiplodia* as a synonym of *Diplodia* until it can be proven otherwise.

Sphaeropsis Sacc. The distinction between *Diplodia* and *Sphaeropsis* has never been clear (Hesler, 1913; Shoemaker, 1964). Percurrent proliferation seen in conidiogenous cells has been regarded more typical of *Sphaeropsis* as defined by Sutton (1980) than of *Diplodia*. However, isolates of *Diplodia* also produce percurrent proliferation in conidiogenous

cells. Conidial septation is another feature that has been used to separate these genera. In *Sphaeropsis*, conidia are initially aseptate but become euseptate prior to germination (Sutton, 1980), but in *Diplodia* conidia become 1-euseptate as they mature (Sutton, 1980). There are a number of reports of 'ageing' conidia of *Botryosphaeria* anamorphs developing septa (Witcher & Clayton, 1963; Shoemaker, 1964; Maas & Uecker, 1984; Pennycook & Samuels, 1985; Samuels & Singh, 1986; Rayachhetry *et al.*, 1996). Thus the concept pertaining to maturity of conidia is vague, and we question the value of using septation to distinguish the two genera.

Anamorphs excluded from Botryosphaeria

Botryodiplodia Sacc. The name *Botryodiplodia* was first used as a subdivision of *Diplodia* by Saccardo (1880). This same treatment was presented by Saccardo in 1883, where he listed "*Diplodia juglandis* Fr. (Summa Veg. Sc. p. 417), based on *Sphaeria juglandis* Fr. (Syst. Mycol. II, p. 493)" in the group *Sphaeriae Compositae*. When Saccardo (1884) published *Botryodiplodia* Sacc., he cited "Mich. II p. 7 (Saccardo, 1880)" as the place of publication, and listed *Botryodiplodia juglandicola* (Schw.) Sacc. as the first species. *Diplodia juglandis* was treated under *Diplodia*, rather than with *Botryodiplodia*. Saccardo (1884) thus changed the concept of his subgenus and erected this new genus based on a different species. Therefore, the correct generic citation is *Botryodiplodia* Sacc. (1884). *Botryodiplodia juglandicola* (Schw.) Sacc. (basonym *Sphaeria juglandicola* Schw.), the first species listed, has been considered the type of *Botryodiplodia* (see Sutton, 1977). Crous & Palm (1999) re-examined authentic Schweinitz specimens of *S. juglandicola* (U.S.A. Pennsylvania: Bethlehem, Collins Collection No. 113 (PH, BPI US800048); Salem-Bethlehem, "1328-181-Syn. Fung." (PH); *Hicoria* sp., Shear types and rarities (annotated by Shear as *Eutypella*), Schweinitz Mtd. Coll. no 1328 (BPI US800046); on *Carya* sp. ex herb. Schweinitz #1328, in Michener Collection [annotated by Shear as *Eutypella*] (BPI US800047), and observed only a valsoid fungus to be present. Cooke (1884) examined Berkeley's specimen 8846 of *S. juglandicola*, and treated this fungus as *Valsa juglandicola* Schw. In so doing, he established a new combination which Saccardo correctly cited as *Valsa juglandicola* (Schw.) Cooke (Saccardo, 1886). Because the type specimen of *Botryodiplodia* represented a valsoid ascomycete and not a coelomycete, to which it has been applied in the past, it was declared a *nomen dubium* (Crous & Palm, 1999).

Experimental part

Materials and methods

ISOLATE COLLECTION AND EXAMINATION

Fresh plant material infected with *Botryosphaeria* species was obtained from species of the *Proteaceae* sampled in the Western Cape, South Africa. Material was treated as described in Denman *et al.* (1999). Slide preparations were made by squashing fruiting structures in a drop of water or lactophenol, and examining them under a Zeiss Axioskop light microscope. When necessary, ascomata and conidiomata were rehydrated, and sections made using a Leica CM 1100 cryostat freezing microtome.

DNA SEQUENCING AND PHYLOGENETIC ANALYSIS

The methods employed in DNA isolation, PCR amplification, sequencing and phylogenetic analysis are identical to those used by Crous *et al.* (this volume). Data were compared with those generated by Jacobs & Rehner (1998), in which a *Dothidea* sp. was used as outgroup.

Results

PHYLOGENETIC ANALYSIS

DNA sequences were determined for approximately 470 bp of ITS1, 5.8S and ITS2 regions of the rRNA gene of *Botryosphaeria* isolates included in this study (Table 1). These sequences were algorithmically (Clustalw) aligned with those generated by Jacobs & Rehner (1998) (Table 1), and manually adjusted for improvement. The alignment (data not shown, available from corresponding author) contains 369 constant characters and 242 variable characters, of which 215 were parsimony-informative. A single most parsimonious tree (Fig. 1) was generated using PAUP 4* (Swofford, 1999) with the branch and bound option and 1000 bootstrap replicates using the sequences of *Dothidea insculpta* Wallr. and *D. hippophaës* (Pass.) Fuckel as outgroups. The neighbour-joining method included in PAUP* 4 (Swofford, 1999) was also used for the analysis, and produced an identical tree topology. In the phylogenetic tree (Fig. 1), isolates of *B. proteae* formed a clade paraphyletic to other *Botryosphaeria* isolates with 100% bootstrap support. The major *Botryosphaeria* clade is composed of dark- and hyaline-conidial groups, and has 100% bootstrap support. All the pigmented (dark) conidial isolates (Jacobs & Rehner, 1998) formed a clade with 99% bootstrap support. Three subclades were found in the dark-conidial clade. The hyaline-conidial group was only supported by a bootstrap value of 54%. Jacobs &

Table 1. Isolates used in phylogenetic studies

Species	Reference	GenBank	Strain number
<i>Diplodia quercina</i>	Jacobs & Rehner, 1998	AFO 27753	K.J. 93-29 ¹
<i>Sphaeropsis</i> sp. (<i>B. obtusa</i>)	Jacobs & Rehner, 1998	AFO 27759	K.J. 93-56
<i>Sphaeropsis sapinea</i>	Jacobs & Rehner, 1998	AFO 27758	K.J. 94-07
		AFO 27757	K.J. 94-05
		AFO 27756	K.J. 93-31
<i>Lasiodiplodia theobromae</i> (<i>B. rhodina</i>)	Jacobs & Rehner, 1998	AFO 27761	K.J. 93-27
		AFO 27760	K.J. 93-40
		AFO 27762	K.J. 93-41
<i>Fusicoccum aesculi</i> (<i>B. dothidea</i>)	Jacobs & Rehner, 1998	AFO 27741	K.J. 93-42
		AFO 27743	K.J. 94-09
		AFO 27742	K.J. 93-03
		AFO 27744	K.J. 94-11
<i>Fusicoccum luteum</i>	Jacobs & Rehner, 1998	AFO 27745	K.J. 93-52
<i>Fusicoccum</i> sp. (<i>B. dothidea</i> -complex)	Present study	AF 195774	BOT 6-1 ²
		AF 196294	BOT 8-4
		AF 196295	BOT 9-3
		AF 196296	BOT 10-2
		AF 196297	BOT 11-1
		AF 196298	BOT 12-3
<i>Fusicoccum aesculi</i> (<i>B. dothidea</i>)	Jacobs & Rehner, 1998	AFO 27746	K.J. 93-12
		AFO 27751	K.J. 93-23
		AFO 27750	K.J. 93-54
		AFO 27749	K.J. 94-26
		AFO 27748	K.J. 94-27
		AFO 27747	K.J. 94-23
<i>Fusicoccum proteae</i> (<i>B. proteae</i>)	Present study	AF 1962996	BOT 2-2
		AF 1963007	BOT 3-3
		AF 1963018	BOT 15-4
		AF 1963029	BOT 17-5

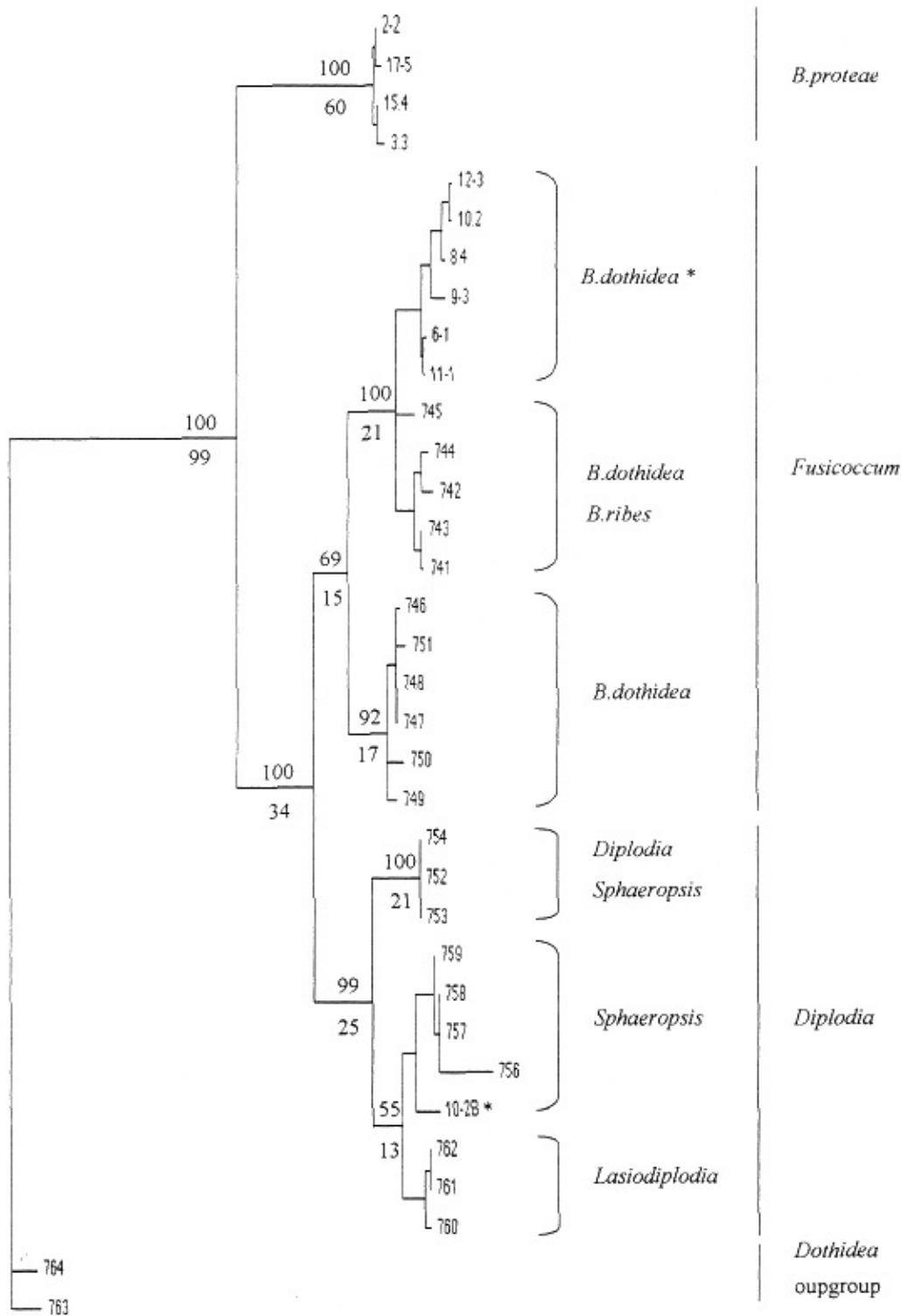
¹ K.J. = cultures of K. Jacobs (Jacobs & Rehner, 1998).

² BOT = Cultures of S. Denman, maintained at the Department of Plant Pathology, University of Stellenbosch, STE-U.

Rehner (1998) demonstrated 83% bootstrap support for the hyaline clade, so the decline in support with addition of more *B. dothidea* isolates suggests that the hyaline strains may not represent a true division in *Botryosphaeria*. Two subclades were formed in the hyaline group, the *B. dothidea* isolates (Group 3) from Jacobs & Rehner (1998) with a 96% bootstrap support, and a second subclade (99% bootstrap support) comprising three groups of strains. The *B. dothidea* strains from *Protea* formed one of the subclades with 99% bootstrap support. *Fusicoccum luteum* Pennycook & Samuels (Group 2 in the Jacobs & Rehner, 1998) and two *B. ribis* and two *B. dothidea* strains (Group 1 in the Jacobs & Rehner, 1998) formed the other subclades.

The significance of the phylogenetic differences between the hyaline-conidial clades remains uncertain, but they might represent species.

Jacobs & Rehner (1998) discussed the phylogenetic information obtained from ITS sequence data for *Botryosphaeria*. Doubts were raised where morphological characters and ITS phylogeny were contradictory. In our alignment, however, we observed sufficient informative characters that supported the phylogenetic tree topology (Fig. 1), which correlated with morphological characters used to distinguish species in *Botryosphaeria*. More sequence data and other gene trees will be required, however, to support the observations made in these studies.



* Host: *Protea*

Fig. 1. The single most parsimonious tree with a tree length of 440 steps (CI = 0.777, RI = 0.915, RC = 0.712) derived from a branch and bound search in PAUP* 4 (Swofford, 1999) with 1000 randomizations of sequence input orders and 1000 bootstrap replications using ITS1, 5.8S and ITS2 data. The tree is rooted with outgroups *Dothidea inculpta* and *D. hippophaës*. The bootstrap values and branch lengths are indicated above and below the branches.

General discussion

Anamorphs of *Botryosphaeria* can essentially be

differentiated into two groups based on conidium colour, namely a hyaline group typified by *Fusicoccum*, and a dark-conidial group represented by *Diplodia*. Our

view is that anamorphs of *Botryosphaeria* should either be placed in *Fusicoccum* or *Diplodia*. Species of *Fusicoccum* are those with hyaline conidia that can become translucent brown and septate prior to germination. *Diplodia* anamorphs of *Botryosphaeria* have 0–1-euseptate conidia that are opaque brown when mature and they can have prominent melanin deposits on the insides of the conidial walls, which give the impression of striations, or the conidial walls can be smooth.

Results from this study suggest that *Botryosphaeria* is monophyletic. The clade representing the hyaline *Fusicoccum* anamorphs received only 54% bootstrap support, and this may reduce even further as more taxa are added. The question then arises if two separate anamorph genera should be retained for *Botryosphaeria*. In culture, hyaline conidia of several *Fusicoccum* species are known to turn brown with age, making them similar to those of *Diplodia* species. Given the present impetus to merging anamorph and teleomorph genera in accordance with the true phylogeny, the future may see us moving to a system where only one anamorph name is available for asexual *Botryosphaeria* species. If the monophyly of *Botryosphaeria* also holds with other data sets, the older, valid name for anamorphs of *Botryosphaeria* would be *Fusicoccum* (1829), and not *Diplodia* (1834).

Of special interest in our study was the clustering of isolates of the recently described *Botryosphaeria proteae* (Denman *et al.*, 1999). This species is rather unusual because it has a *Fusicoccum* anamorph, as well as a dark-spored synanamorph (or microconidial state) and spermatia. Isolates commonly form both anamorphs in culture. Both synanamorphs also occur on diseased host material. If the argument presented in this paper, where there are two anamorph groups associated with *Botryosphaeria*, is correct, the placement of *B. proteae* raises serious problems. Our molecular data (Fig. 1) show, however, that isolates of *B. proteae* reside in a clade outside *Botryosphaeria*. The correct generic placement of *B. proteae* is thus uncertain, and it will have to be compared to other, similar genera to try and resolve its correct generic affinity.

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