Taxonomy, phylogeny and identification of Botryosphaeriaceae associated with pome and stone fruit trees in South Africa and other regions of the world

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Abstract

Species of *Botryosphaeria* are well-recognized pathogens of pome and stone fruit trees. The taxonomy of these fungi, however, has been confused in the past. Recent taxonomic changes to the Botryosphaeriaceae further influence the literature pertaining to these fungi. This study reviews the taxonomic status of Botryosphaeriaceae associated with fruit tree diseases, identifies them in South Africa and elsewhere, and develops a reliable identification technique for them. Comparisons were made using DNA sequence data from the nuclear ITS rRNA operon and anamorph morphology. These analyses distinguished six clades amongst isolates associated with fruit tree diseases, corresponding to *Neofusicoccum ribis* (= *B. ribis*), *N. parvum* (= *B. parva*), *N. australe* (= *B. australis*), *B. dothidea*, *Diplodia mutila* (= *B. stevensii*) and '*Botryosphaeria*' *obtusa* (the genus *Botryosphaeria* is no longer available for the fungus known as *B. obtusa*, but a new name has not been proposed yet). Isolates from fruit trees in South

Africa were grouped in the *N. australe* and '*Botryosphaeria' obtusa* clades. This is the first report of *N. australe* from fruit trees. PCR-RFLP analysis using the restriction endonucleases *Cfo*I and *Hae*III distinguished the major clades. However, two groups of closely related species, *N. ribis* and *N. parvum*, and *N. australe* and *N. luteum* (= *B. lutea*), had identical RFLP profiles. Using RFLP, it was shown that '*Botryosphaeria*' *obtusa* is the dominant species on fruit trees in the Western Cape Province of South Africa. These results and methods will be useful in future epidemiological studies and disease management of Botryosphaeriaceae from fruit trees.

Introduction

Species of *Botryosphaeria* Ces. and De Not. are important pathogens of pome and stone fruit trees, causing fruit rots (e.g. black and white rot of apple), frogeye leaf spot, stem and branch cankers, gummosis, die-back and in some cases tree death (Weaver, 1974; Brown & Britton, 1986; Britton *et al.*, 1990; Pusey, 1993; Parker & Sutton, 1993a). Infection takes place either through wounds, or directly through the stomata and other openings (Brown & Hendrix, 1981; Smith & Hendrix, 1984; Britton & Hendrix, 1989; Kim *et al.*, 2001). These fungi can then persist in healthy tissue. Successful infection and susceptibility of infected trees is closely linked to environmental conditions, where high temperatures, water logging and other forms of stress favour infection (Holmes & Rich, 1969; Wene & Schoeneweiss, 1980; McGlohon, 1982; Arauz & Sutton, 1989, 1990a; Ahimera *et al.*, 2003).

Effective management of diseases on fruit trees caused by species of *Botryosphaeria* is achieved through integrated control strategies, which take into account cultivar susceptibility, environmental conditions, tree management and chemical applications (Holmes & Rich, 1969; Drake, 1971; Starkey & Hendrix, 1980; Arauz & Sutton, 1990b; Parker & Sutton, 1993b; Brown-Rytlewski & McManus, 2000; Beckman *et al.*, 2003). Effective control requires knowledge regarding the taxonomy and epidemiology of the pathogen involved. Furthermore, due to enhanced quarantine requirements, correct identification of the fungal pathogens that affect these crops has become increasingly important in the export of fruit products (Palm, 1999).

The *Botryosphaeria* spp. most commonly associated with diseases of pome and stone fruit are *Botryosphaeria dothidea* (Moug.: Fr.) Ces. and De Not., *B. obtusa* (Schwein.) Shoemaker and *B. stevensii* Shoemaker (Shoemaker, 1964; Laundon, 1973; Sutton, 1980; Brown & Britton, 1986; Proffer & Jones, 1989; Britton *et al.*, 1990; Pusey, 1993; Brown-Rytlewski & McManus, 2000). Although the teleomorph names are preferably used, the

anamorph fruiting structures of these fungi are frequently encountered and play an important role in their identification. These anamorphs are *Fusicoccum aesculi* Corda, a *Diplodia* sp. (also reported as a *Sphaeropsis* sp.) and *D. mutila* (Fr.) Mont., respectively. Despite considerable research on the *Botryosphaeria* spp. on fruit trees, the taxonomy of these fungi is incomplete and often confused (Brown & Britton, 1986; Ogata *et al.*, 2000).

Some *Botryosphaeria* spp. reported on pome and stone fruit trees are less well known as pathogens of these trees. *Botryosphaeria parva* Pennycook and Samuels (anamorph = *F. parvum* Pennycook and Samuels) and *B. lutea* A.J.L. Phillips (first reported as its anamorph = *F. luteum* Pennycook and Samuels) were initially described from kiwifruit, poplar and apple in New Zealand and later from vines in Portugal (Pennycook & Samuels, 1985; Phillips *et al.*, 2002). There have been no other reports of their occurrence or influence on pome and stone fruit trees. *Botryosphaeria rhodina* (Berk. and M.A. Curtis) Arx (anamorph = *Lasiodiplodia theobromae* (Pat.) Griffiths and Maubl.) has been associated with peach tree gummosis, but is less frequently isolated from these symptoms than *B. dothidea and B. obtusa* (Brown & Britton, 1986; Britton *et al.*, 1990; Pusey, 1993). Recently, an apparently emerging disease, caused by *Sphaeropsis pyriputrescens* Xiao and Rogers, was described from rotting pears in Washington state, USA (Xiao & Rogers, 2004).

Problems in distinguishing *Botryosphaeria* spp. from each other arise from the overlapping morphological characteristics for many species (Von Arx & Müller, 1954; Shoemaker, 1964). Recent studies, however, using both morphological characteristics and molecular data, have clearly defined species within the genus (Jacobs & Rehner, 1998; Denman *et al.*, 2000; Smith & Stanosz, 2001; Zhou & Stanosz, 2001; Phillips *et al.*, 2002; Slippers *et al.*, 2004a). Isolates previously classified as *B. dothidea* (*sensu* von Arx & Müller, 1954) have been shown to represent three taxa (Jacobs & Rehner, 1998; Smith & Stanosz, 2001; Slippers *et al.*, 2004a). These include *B. dothidea*, *B. parva and B. ribis*. Similarly, isolates previously identified as *B. obtusa*, *B. stevensii and B. quercuum* do not always conform to groups identified based on sequence data (Zhou & Stanosz, 2001; Alves *et al.*, 2004).

A recent phylogenetic study separated multiple lineages within the Botryosphaeriaceae, and described several new genera to represent these lineages (Crous *et al.*, 2006). The taxonomic changes suggested included a number of name changes for fungi mentioned above, associated with pome and stone fruit and tree diseases. The genus *Botryosphaeria* is restricted to *B. dothidea* and closely related species. Crous *et al.* (2006) described the genus *Neofusicoccum* Crous, Slippers and A.J.L. Phillips to accommodate

Botryosphaeriaceae with *Fusicoccum*-like anamorphs, such as *N. ribis* (Slippers, Crous and M.J. Wingf.) Crous, Slippers and A.J.L. Phillips (= *B. ribis* Grossenb. and Dugg. teleomorph and *F. ribis* Slippers, Crous and M.J. Wingf.) and others. A single generic name was provided for the latter genus, accommodating the holomorph concept. Botryosphaeriaceae with *Diplodia*-like anamorphs, will still be accommodated in the genus *Diplodia*, but the teleomorph name, *Botryosphaeria*, is no longer available for them. For example, *B. obtusa* and *B. stevensii* will formally only be known by their *Diplodia* anamorphs. For ease of reference, the traditional names are still used where no new names are available, but then in inverted commas (e.g. '*Botryosphaeria*' *obtusa*).

Botryosphaeria dothidea and '*Botryosphaeria*' *obtusa* have both been recorded on pome and stone fruit and trees in South Africa (Combrink *et al.*, 1984; Crous *et al.*, 2000), but confusion regarding the overall taxonomy of *Botryosphaeria* has reduced the value of these reports. The aim of this study was to determine the identity of species of *Botryosphaeria* from pome and stone fruit trees in South Africa, using DNA-based techniques and morphological characteristics. Species of *Botryosphaeria* occurring on fruit trees in other parts of the world were also compared with the South African isolates and an efficient molecular identification protocol was devised for all of these fungi.

Materials and Methods

Isolates and morphology

A total of 50 isolates, mainly from diseased pome and stone fruit trees in South Africa, were used in this study (Table 1). Of these, 22 isolates were from apple (*Malus domestica*), nine from pear (*Pyrus communis*), five from peach (*Prunus persica*), 10 from plum (*Prunus domestica*) and four from other hosts in the Western Cape Province. Isolates were obtained from diseased material between 1995 and 1999 by the second author and are representative of *Botryosphaeria*-like fungi from diseases associated with pome and stone fruit in the Western Cape Province, which is the primary deciduous fruit production area of South Africa.

The conidial morphology of the isolates from South Africa was studied using a light microscope, and an Axiocam digital camera and accompanying software (Zeiss). To induce sporulation, isolates were grown at 25°C on water agar (WA) (2% Biolab agar) to which sterilized pine needles had been added as a substrate. Spores were mounted in clear lactophenol and spore length, width, wall texture, shape and colour of the spores recorded. Cultures were maintained on malt and yeast extract agar (MYA) (2% malt extract, 0·2% yeast extract and 2% agar; Biolab) at 25°C and stored on this medium at

4°C. All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), **University** of **Pretoria**, South Africa. Representative strains have also been deposited in the National Collection of Fungi (PREM) and the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

In order to test for a possible link between '*Botryosphaeria*' *obtusa and* the anamorph name, *D. malorum* Fuckel, type specimens (two samples both marked as type #1706) of the latter taxon were obtained from the Conservatoire et Jardin Botaniques de la Ville de Genève, Genève, Switzerland (G). Conidia from these specimens were studied using the same equipment and techniques, described above.

DNA isolation and amplification

A modified version of the method of Raeder & Broda (1985) (as described in Slippers *et al.* (2004a)) was used to isolate DNA from the fungi. The primers ITS1 and ITS4 (White *et al.*, 1990) were used to amplify the ITS rDNA region, which included the 3' end of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5·8S rRNA gene, the second ITS (ITS2) and the 5' end of the 26S (large subunit) rRNA gene. The PCR reaction mixtures and reaction conditions were the same as those described by Slippers *et al.* (2004a). PCR products were run on 1% agarose gels, stained with ethidium bromide and visualized under UV illumination. Size estimates were made against a 100 bp or λ standard size markers.

DNA sequencing and analysis

Eleven of the 50 isolates from the Western Cape Province of South Africa, representing the different hosts and conidial types encountered, were selected for sequencing (Table 2). PCR products were cleaned using High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). Both strands of the amplicons were sequenced using the primers ITS1 and ITS4. Reactions were performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied BioSystems) as recommended by the manufacturer and run on an ABI PRISM 377 Autosequencer (Perkin-Elmer Applied BioSystems).

Sequence data were analysed using Sequence Navigator version 1·0·1™ (Perkin Elmer Applied Biosystems) and manually aligned by inserting gaps. Phylogenetic analyses were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4·0b10 (Swofford, 2003). Gaps were treated as a fifth character and all characters were unordered and of equal weight, but uninformative characters were excluded. Heuristic searches were done using stepwise (simple) addition and tree bisection and reconstruction (TBR) as 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 using 1000 bootstrap replicates (Felsenstein, 1985).

In order to determine the relationships between the fungi from fruit trees in South Africa and the rest of the world, 32 sequences mainly of Botryosphaeriaceae occurring on fruit and fruit trees in previous studies (Jacobs & Rehner, 1998; Ogata *et al.*, 2000; Zhou & Stanosz, 2001) were obtained from GenBank (Table 2). Sequences of Botryosphaeriaceae commonly encountered on other hosts in South Africa were also included (Table 2). Trees were rooted to *Mycosphaerella africana* Crous and M.J. Wingf. and *Guignardia bidwellii* (Ellis) Viala and Ravaz., which are closely related to the Botryosphaeriaceae ingroup.

PCR-RFLP analyses

ITS sequence data of the different species were used to determine polymorphisms in restriction sites of known restriction endonucleases (RE) (using Webcutter 2·0: http://rna.lundberg.gu.se/cutter2/). These analyses showed that *Cfo*I would distinguish the species of the Botryosphaeriaceae from fruit and other hosts in South Africa. *Cfo*I was thus used to screen all 50 of the isolates collected from the pome and stone fruit trees in the Western Cape region of South Africa (Table 1). Hypothetical restriction maps were also determined from GenBank sequence data for species where DNA was not available. Restriction maps using the RE *Hae*III were developed for species of the Botryosphaeriaceae on fruit outside South Africa that were not distinguishable with *Cfo*I.

Results

Isolates and morphology

Most of the 50 isolates from the Western Cape Province sporulated profusely on WA supplemented with sterilized pine needles. Isolates later identified as *N. australe* (Slippers, Crous and M.J. Wingf.) Crous, Slippers and A.J.L. Phillips (= *B. australis* Slippers, Crous and M.J. Wingf.) produced very few fruiting structures in culture. Based on spore morphology, isolates could be separated into two distinct groups. One group had *Diplodia*-like conidia that were initially hyaline and aseptate, becoming light to dark brown and occasionally 1-septate, with age. These conidia were ovoid and 20–26 × 10–12 µm in size and are characteriztic of the anamorph of *B. obtusa* (Shoemaker, 1964; Table 3, Figs 1–5). The second group had *Fusicoccum*-like conidia that were hyaline, aseptate and fusiform and 17–25 × 5–7 µm in size. These species represent *Neofusicoccum.* Some isolates also produced a yellow pigment in culture and conidia

which is characteriztic of *N. australe* and *N. luteum* (Pennycook and Samuels) Crous, Slippers and A.J.L. Phillips (= *B. lutea* Pennycook and Samuels) (Pennycook & Samuels, 1985; Phillips *et al.*, 2002; Slippers *et al.*, 2004c).

Two samples of *D. malorum* marked as '*typus*' from the Fuckel collection (G) were studied. Both samples contained similar material of dried apple fruit containing numerous fruiting structures. Two types of conidia were, however, observed in the two samples (A and B) (Figs 6–7). Conidia from both samples were discoloured and single septate. The walls of conidia from both samples were 0·8–1·2 µm thick, but those from the first sample (A) were smooth, while those from the second sample (B) were rough. Sample A had smaller conidia than those reported for the *Diplodia* sp. associated with '*Botryosphaeria*' *obtusa*, while those of sample B were larger (Table 3, Figs 6–7). The conidia of the first sample (A) also contained depressions that appeared like vacuoles in the middle of each cell. This feature is probably due to the age and dehydrated state of these conidia. From the above data it appeared that two *Diplodia* species possibly co-occur on these samples.

PCR and phylogenetic analyses

PCR products of ~580 bp were obtained for all isolates used in this study, of which ~520 bp were used for phylogenetic analyses. Of the total dataset (after alignment) of 549 characters, 178 were parsimony-informative. After heuristic searches in PAUP, 343 most parsimonious trees of 328 steps were retained [consistency index (CI) = 0·81; retention index (RI) = 0·97; g1 = −0·37] (Fig. 8). The overall topology of these trees was identical as the rearrangements were only within the major clades and not between these clades.

The isolates used in the phylogenetic analyses resided in eight clades (I−VIII) (Fig. 8). All the South African isolates from fruit trees used in this study grouped into either clade IV (CMW586 from apple, CMW980 from pear, CMW1133 from plum, CMW1187 from almond) with an ex-type isolate of *N. australe*; or clade VIII (CMW568 from apple, CMW918, CMW986 and CMW1050 from pear, CMW1069 from peach, CMW1159 from plum) which contains isolates of a *Diplodia* sp. (= '*Botryosphaeria*' *obtusa*).

PCR RFLP analyses

Using the RE *Cfo*I, unique banding patterns were obtained for isolates representing most of the major clades in the phylogenetic analyses. Isolates residing in Clade VII [*D. mutila* (= *B. stevensii*)] and clade VIII ['*Botryosphaeria*' *obtusa*] (Figs 9a, 10) could not be identified using this RE. However, isolates in these groups could be distinguished using *Hae*III (Fig. 9b). Only one profile was produced for isolates from clades *N. ribis* (Clade I) and *N. parvum* (Pennycook and Samuels) Crous, Slippers and A.J.L. Phillips (Clade II),

and N. luteum (Clade III) *and N. australe* (Clade IV), respectively. These species are best distinguished using sequence data and morphology.

All isolates of the *Botryosphaeriacea* from fruit and other hosts in South Africa were screened using this RFLP method. The identity of the isolates that belong to Clade IV and VIII based on sequence data were thus confirmed. The remainder of the 50 isolates screened, all represented the (‘*Botryosphaeria*’ *obtusa*) (VIII).

Discussion

In this study, the Botryosphaeriaceae from pome and stone fruit trees across the world could be separated into six distinct groups. These included clades for *N. ribis*, *N. parva*, *N. australe*, *B. dothidea*, *D. mutila and* ‘*Botryosphaeria*’ *obtusa.* Isolates from pome and stone fruit trees in South Africa were identified as representing either *N. australe* or ‘*Botryosphaeria*’ *obtusa*. These identifications were supported by ITS-rDNA sequence data, PCR-RFLP analysis and morphological characteristics.

This study represents the first record of *N. australe* (= *B. australis* teleomorph *and F. australis* anamorph) from apple, pear, plum and almond. This species has been recently reported from grapevines in South Africa (Van Niekerk *et al.*, 2004). The fruit trees and vineyards are often planted in close proximity, so this is not totally unexpected. *Neofusicoccum australe* also occurs on native hosts in both South Africa and Australia. It was first described from native *Acacia* spp. in eastern Australia, where it was the only species of the Botryosphaeriaceae present (Slippers *et al.*, 2004c). It is also the dominant Botryosphaeriaceae infecting native *Eucalyptus* in Western Australia, where it also occurs on grapevines (Burgess *et al.*, 2005; Taylor *et al.*, 2005). In South Africa, *N. australe* occurs on native *Widdringtonia* and *Syzygium* (Slippers *et al.*, 2005; D. Pavlic, unpublished data). *Neofusicoccum australe* was not frequent in this study, with only four representative isolates from a collection of 50 isolates assembled over a 5-year period. This species, therefore, appears to be a foreign pathogen on pome and stone fruit, and currently of minimal importance on this host in South Africa and elsewhere.

No isolates of *N. luteum* (= *B. lutea* teleomorph and *F. luteum* anamorph), which is the sister species of *N. australe*, were found on pome and stone fruit trees in South Africa. This absence is curious, because *N. luteum* has recently been reported from this area on grapevines (Van Niekerk *et al.*, 2004). The fungus was first reported (as *Fusicoccum luteum*) from kiwifruit, apple and pear in New Zealand and thus appears only to affect fruit trees in that region (Pennycook & Samuels, 1985).

'*Botryosphaeria*' *obtusa* (Clade VII) was the dominant *Botryosphaeria* species isolated from diseased pome and stone fruit trees in this study. Isolates of this species represented over 90% of those collected over a 5-year period in South Africa. The '*Botryosphaeria*' *obtusa* was also the dominant species of this genus found on grapevines in this area (Van Niekerk *et al.*, 2004). While no pathogenicity studies were conducted during this study for this species, Koch's postulates have been demonstrated by others on fruit trees (Britton *et al.*, 1990, Brown-Rytlewski & McManus, 2000). Efforts to control Botryosphaeriaceae-associated diseases in the area should thus concentrate on this species. The phylogenetic clade representing this species included isolates from many parts of the world, and these findings support those of other studies (Shoemaker, 1964; Laundon, 1973; Sutton, 1980; Brown & Britton, 1986; Proffer & Jones, 1989; Britton *et al.*, 1990; Pusey, 1993; Brown-Rytlewski & McManus, 2000; Ogata *et al.*, 2000) showing that it is one of the most important species of the Botryosphaeriaceae that affect pome and stone fruit trees in all these regions.

'*Botryosphaeria*' *obtusa* has a wide host range. Punithalingam & Waller (1973) listed 34 hosts for this pathogen. In the present study, this *Diplodia* sp. was identified from species of *Malus, Populus, Prunus*, *Pyrus, Ribes and* an unidentified hardwood. It appears that this species has been moved around the world on agricultural hosts.

An appropriate, existing taxon to accommodate the anamorph of '*Botryosphaeria*' *obtusa* could not be determined in this study. Some misconceptions regarding this fungus, however, do deserve discussion. The anamorph is generally referred to as a species of *Sphaeropsis* or *Diplodia*. The distinction between these two genera is supposedly found in the proliferation of the conidiogenous cells and the time of septation. Denman *et al.* (2000), however, argued that representatives of both *Sphaeropsis and Diplodia* have concurrently proliferating conidia and that septation occurs widely and at varying stages among many anamorphs of *Botryosphaeria*, making this character inordinately variable to distinguish groups. Molecular data also support this view. For these reasons the authors consider the anamorph of '*Botryosphaeria*' *obtusa* as a species of *Diplodia* and not *Sphaeropsis*.

The illegitimate name *Sphaeropsis malorum* Peck is sometimes used to described the anamorph of '*Botryosphaeria*' *obtusa* (Shear *et al.*, 1925; Stevens, 1925; Laundon, 1973; Brown-Rytlewski & McManus, 2000). Shoemaker (1964) and Punithalingam & Waller (1973) noted that Peck did not describe the name *S. malorum,* and that the older name, *S. malorum* (Berk.) Berk., is a synonym of *D. mutila.* This name should thus not be used for the anamorph of '*Botryosphaeria*' *obtusa. Diplodia mutila* is regarded as the anamorph of '*B. stevensii*' (Shoemaker, 1964; Alves *et al.*, 2004).

Diplodia malorum Fuckel is a more appropriate name for the anamorph of '*Botryosphaeria*' *obtusa* than *S. malorum*. This possibility, however, is rejected based on studies of the type material of *D. malorum* in the present study. *Diplodia malorum* was considered to be the anamorph of *Physalospora cydoniae* Arn., which is now accepted as a synonym of *B. obtusa* (as *P. obtusa*) (Laundon, 1973). Descriptions of *D. malorum* (Saccardo, 1884; Grove, 1937) are indistinguishable from those of the anamorph of *P. cydoniae* (= *Sphaeropsis malorum* Peck) (Stevens, 1925) and '*Botryosphaeria*' *obtusa* (Shoemaker, 1964; Punithalingam & Waller, 1973). The type material of *D. malorum*, however, appears to contain spores of two species of *Diplodia*. The morphology of both these types of conidia differed from the anamorph of '*Botryosphaeria*' *obtusa* in size and wall texture. For the present, it would be most appropriate to use the genus *Diplodia* for the anamorph of '*Botryosphaeria*' *obtusa* and not to allocate a species name to it until further type studies can determine whether another of the >1200 *Diplodia* spp. listed in *Index Fungorum* (http://www.indexfungorum.org/) fits the current concept of the anamorph of '*Botryosphaeria*' *obtusa*.

Two sequences from *Malus* represent *D. mutila* (= *B. stevensii*) (Clade VII), together with an isolate from *Fraxinus.* This taxon is well known from these hosts (Shoemaker, 1964; Laundon, 1973; Sutton, 1980). This species can be confused with the *Diplodia* anamorph of '*Botryosphaeria*' *obtusa*. Both species have been regarded as synonyms of '*B. quercuum*' (von Arx & Müller, 1954) [which is no longer considered a true species of *Botryosphaeria*: see Crous *et al.* (2006)], before being described as separate taxa (Shoemaker, 1964). On fruit trees, '*Botryosphaeria*' *obtusa and D. mutila* have also been known as *Physalospora obtusa* (Schwein.) Cooke and *P. mutila* Stevens (Laundon, 1973), respectively. The ascospores of these fungi are similar and can easily be mistaken for one another. '*Botryosphaeria' quercuum* is, however, not known from *Malus and Fraxinus*. *Diplodia mutila and* the *Diplodia* sp. (= *B. obtusa*) are easily distinguished based on conidia. Those of *D. mutila* have thick (1–2 µm), glassy walls, and become septate before discoloration. In contrast, the conidia of '*B. obtusa*' have thinner, rough walls, and discolour more commonly than *D. mutila, and* this also occurs before septation.

Three species, *B. dothidea*, *N. ribis* (as *B. ribis*) and *B. mali* Putt. [considered a synonym of *B. dothidea* by Von Arx & Müller (1954)] have previously been reported from pome and stone fruit trees in South Africa (Putterill, 1919; Combrink *et al.*, 1984; Crous *et al.*, 2000). Although none of these taxa was identified in the current study, these previous reports cannot be discounted because isolates representing those species were collected in areas of South African not included in this study. It is also possible that the fungi identified in these previous reports represented either *N. australe*, *N. ribis* or *N. parvum*,

because the conidial features reported in the studies above overlap with the morphology of all these last named species (Putterill, 1919; Combrink *et al.*, 1984; Pennycook & Samuels, 1985; Phillips *et al.*, 2002; Slippers *et al.*, 2004a, c).

Neofusicoccum parvum (Clade II) and *B. dothidea* (Clade V) are common pathogens of pome and stone fruit trees world-wide. These species have previously both been treated as *B. dothidea* (Denman *et al.*, 2000; Smith & Stanosz, 2001; Zhou & Stanosz, 2001; Slippers *et al.*, 2004a). Sequences from isolates from fruit trees in Japan reside in both groups. Only sequences of *B. dothidea* (Clade V), however, were identified from fruit trees in the USA. Based on ITS data from GenBank, one sequence from apple from the USA grouped with *N. ribis* (Clade I). The difference between *N. ribis* (Clade I) and *N. parvum* (Clade II) based on ITS data, however, is inordinately small (Slippers *et al.*, 2004a) and additional data will be required to confirm the identity of isolates residing in ITS Clades I and II. No isolates of *N. parvum* or *B. dothidea*, however, were isolated from South African pome and stone fruit trees during this study.

The name *B. berengeriana* has recently been used for isolates from fruit trees in Asia (Sassa *et al.*, 1998; Ogata *et al.*, 2000; Al-Haq *et al.*, 2002). This name, however, has been reduced to synonymy with *B. dothidea* (Clade V) (Von Arx & Müller, 1954; Slippers *et al.*, 2004a). Isolates from Asia group into both Clade II and Clade V and either represent *N. parvum* or *B. dothidea*.

Sexual structures of Botryosphaeriaceae are rare in the field and often insufficient to provide reliable identifications. Furthermore, isolates made from diseased tissue do not readily produce sexual fruiting bodies in culture. While conidia are better for identification and are more common in the field and in culture, they also overlap between species, making identification difficult for the non-specialist. There is consequently a great need for an efficient means to identify large numbers of isolates of these fungi, reasonably rapidly and reliably. In this study, it was possible to distinguish the species associated with pome and stone fruits with PCR-RFLP fingerprints generated using two restriction enzymes. Slippers *et al.* (2004b) recently used this same technique to distinguish Botryosphaeriaceae from *Eucalyptus*. Alves *et al.* (2005) used a larger region, including part of the LSU and the ITS regions to develop a PCR-RFLP identification technique to distinguish various Botryosphaeriaceae and permitting resolution between more species (e.g. *N. ribis* and *N. parvum*) than was achieved here. Such PCR-RFLP techniques thus provide quick and reliable identification of Botryosphaeriaceae, especially when dealing with a species from a specific host group (e.g. on fruit trees).

The PCR-RFLP profiling reported here was not without short-comings. Only one profile was produced for *Neofusicoccum ribis and N. parvum*, *and N. australe and N. luteum*,

respectively. Isolates from these clades must be further distinguished using sequence data and conidial morphology (Slippers *et al.*, 2004a, c). There was sequence variation among isolates from Clade II (*N. parvum*), Clade V (*B. dothidea*) and Clade VIII [*Diplodia* sp. (= *B. obtusa*)]. This variation did not influence the RFLP patterns, except in the case of Clade V for which three RFLP patterns were determined. Despite the variation in RFLP patterns of *B. dothidea*, this species could still be distinguished from the other species.

Results of this study have provided various options to resolve previously encountered problems in the identification of species of the Botryosphaeriaceae from pome and stone fruits. It also addresses recent taxonomic changes that have been suggested for these fungi. This will be important where reliable identifications are needed for quarantine purposes and where conflicts arise relating to exports and biosecurity. It should now be possible to easily distinguish species relatively rapidly using RFLP banding patterns. Where more time is available, the species have been sufficiently well characterized to be able to identify them based on conidial characteristics and DNA sequence data. In combination, these approaches will hopefully also facilitate a better understanding of diseases associated with pome and stone fruits.

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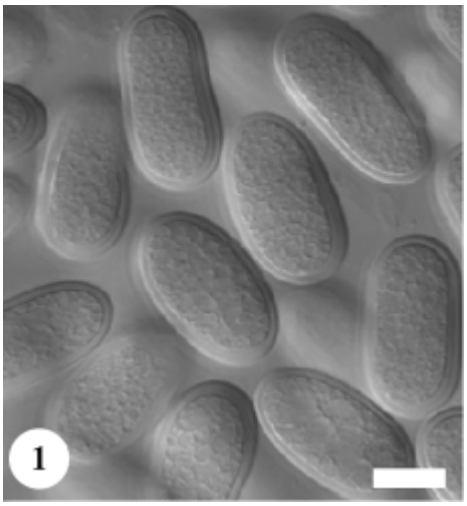
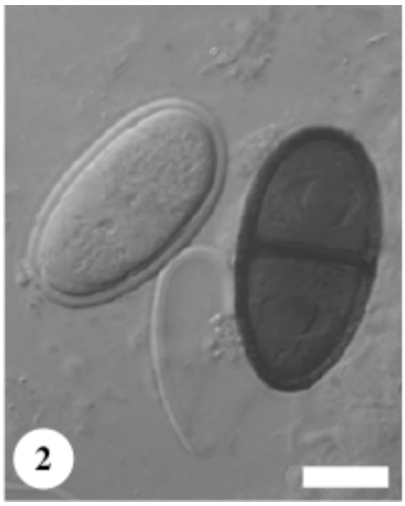
Figures and Tables

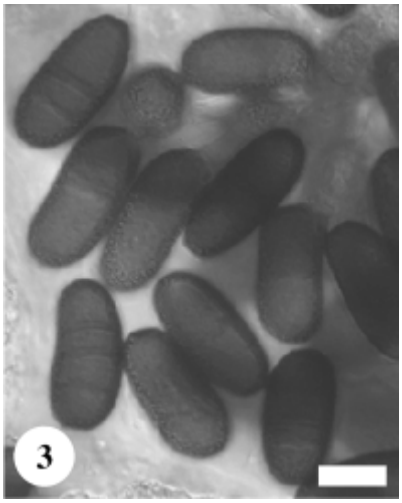
Figures 1 DIC compound-microscope micrographs of some anamorphs of *Botryosphaeria* spp. Hyaline, aseptate and pigmented, septate conidia of *D. mutila*.



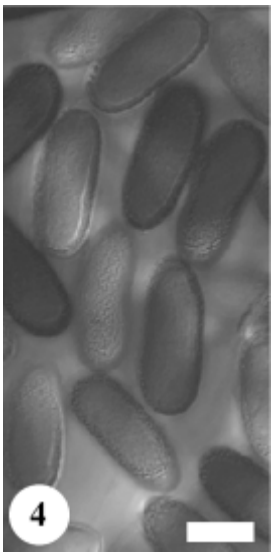
Figures 2 DIC compound-microscope micrographs of some anamorphs of *Botryosphaeria* spp. Hyaline, aseptate and pigmented, septate conidia of *D. mutila*.



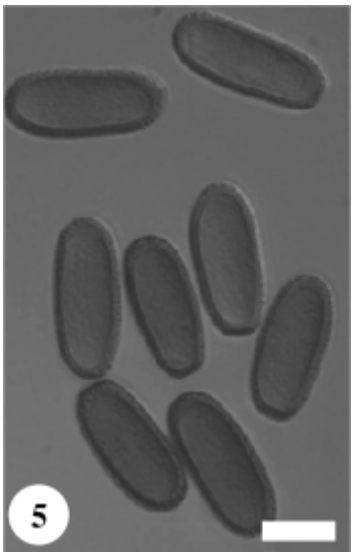
Figures 3 DIC compound-microscope micrographs of some anamorphs of *Botryosphaeria* spp. Hyaline and pigmented, aseptate conidia, as well as pigmented, 1–2-septate conidia with rough walls of the *Diplodia* anamorph of '*Botryosphaeria' obtusa*.



Figures 4 DIC compound-microscope micrographs of some anamorphs of *Botryosphaeria* spp. Hyaline and pigmented, aseptate conidia, as well as pigmented, 1–2-septate conidia with rough walls of the *Diplodia* anamorph of '*Botryosphaeria' obtusa*.



Figures 5 DIC compound-microscope micrographs of some anamorphs of *Botryosphaeria* spp. Hyaline and pigmented, aseptate conidia, as well as pigmented, 1–2-septate conidia with rough walls of the *Diplodia* anamorph of '*Botryosphaeria' obtusa*.



Figures 6 DIC compound-microscope micrographs of some anamorphs of *Botryosphaeria* spp. Dark, septate conidia from herbarium material of *Diplodia malorum*. There were two types of conidia: sample A, shown in Fig. 6 and sample B, shown in Fig. 7 (see text). Bars = 10 µm.

Figures 7 DIC compound-microscope micrographs of some anamorphs of *Botryosphaeria* spp. Dark, septate conidia from herbarium material of *Diplodia malorum*. There were two types of conidia: sample A, shown in Fig. 6 and sample B, shown in Fig. 7 (see text). Bars = 10 µm.

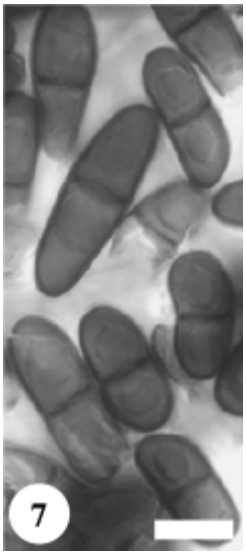

7

Figure 8 One of 343 most parsimonious trees of Botryosphaeriaceae sequences, mainly from isolates from fruit trees in South Africa, New Zealand, Japan, Spain and the USA, generated with heuristic searches of 549 characters (including gaps) of the ITS1, 5·8S and ITS2 region of the nuclear rRNA operon. Bootstrap values are based on 1000 bootstrap replicates and values greater than 65% are indicated above, below or next to the branches. The trees are rooted to sequences from *Guignardia bidwellii and Mycosphaerella africana*. Main clades are identified as I–VIII and isolate numbers are representative of those in Table 2.

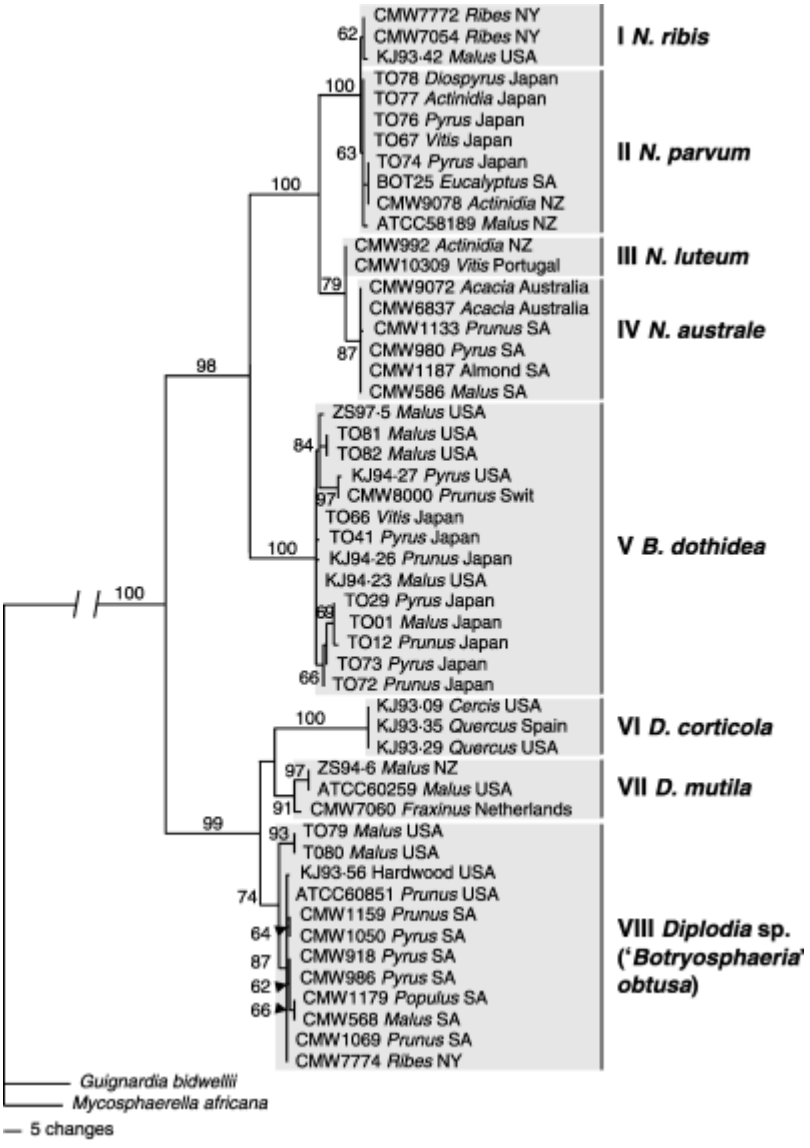


Figure 9 PCR-RFLP fingerprint maps of the ITS1, 5·8S and ITS2 region of isolates representative of the clades (I–VIII) in the phylogenetic analysis, for RE *Cfo*I (a) and *Hae*III (b). Arrows above the line indicate RE restriction sites, numbers below the line are DNA fragment lengths, and numbers in parentheses at the end of the line represent the total fragment length.

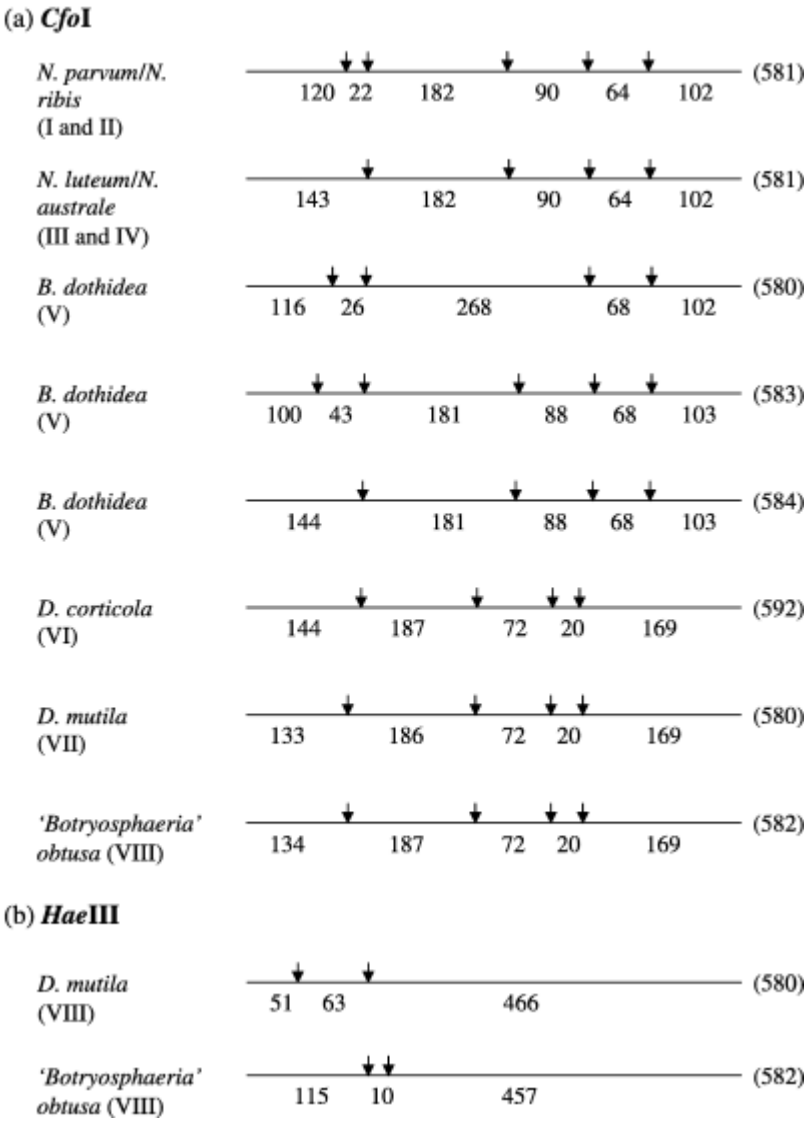


Figure 10 Example of PCR-RFLP fingerprints produced from the ITS1, 5·8S and ITS2 region of Botryosphaeria spp. that occur in South Africa, using the RE CfoI.
Lt = *Lasiodiplodia theobromae*; Np/r = *N. ribis*/*N. parvum* (I or II), Ne = *N. eucalyptorum*; Na/l = *N. luteum*/*N. australe* (III or IV); Bo = '*Botryosphaeria' obtusa* (VIII). 100 bp markers (M) are run on either side.

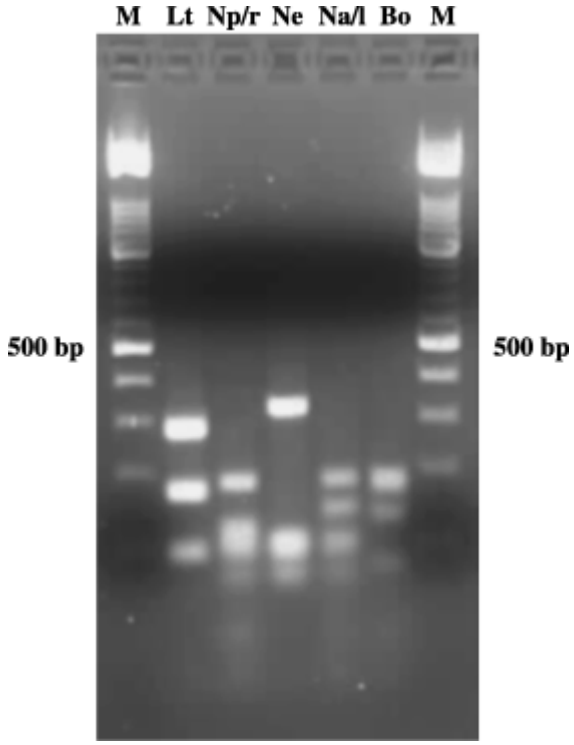


Table 1 Isolates of Botryosphaeriaceae from pome and stone fruit trees, and other hosts, from the Cape Province, South Africa that were used in this study

CMW no. [a]	BO no. [b]	Host	Location	Date isolated
242	5	*Malus domestica* (apple)	Bethlehem	9/97
258	27	‘‘	Koue Bokkeveld	‘‘
308	29	‘‘	Vyeboom	1/97
324	41	‘‘	Vyeboom	‘‘
370	31	‘‘	Ceres	96
388	35	‘‘	Grabouw	‘‘
427	43	‘‘	‘‘	‘‘
432	45	‘‘	‘‘	‘‘
442	152	‘‘	‘‘	‘‘
443	53	‘‘	Stellenbosch	96
447	65	‘‘	‘‘	11/95
474	69	‘‘	Elgin	96
509	123	‘‘	‘‘	‘‘
568	80	‘‘	Ceres	1/98
586	83	‘‘	Elgin	2/98
588	101	‘‘	Vyeboom	9/98
612	137	‘‘	Koue Bokkeveld	5/99
660	143	‘‘	Misgund	85
681	147	‘‘	Ceres	9/97
689	155	‘‘	Grabouw	11/97
893	160	‘‘	Joubertinia	97
913	227	‘‘	Krakeelrivier	5/99
915	24	*Pyrus communis* (pear)	Villiersdorp	9/96
916	47	‘‘	Grabouw	4/97
918	55	‘‘	Villiersdorp	9/96

CMW no. [a]	BO no. [b]	Host	Location	Date isolated
933	71	‘‘	Stellenbosch	96
980	76	‘‘	Hermanus	1/98
986	85	‘‘	‘‘	‘‘
1030	88	‘‘	Tulbach	6/98
1049	90	‘‘	Hamlet	9/98
1050	149	‘‘	Koue Bokkeveld	11/97
1066	22	*Prunus persica* (peach)	Robertson	9/96
1069	145	‘‘	‘‘	‘‘
1078	111	‘‘	Joubertinia	10/98
1084	134	‘‘	Bien Donné	5/99
1085	141	‘‘	Ceres	1/97
1086	19	*Prunus domestica* (plum)	Klapmuts	9/97
1087	25	‘‘	Stellenbosch	96
1088	33	‘‘	Villiersdorp	10/98
1126	142	‘‘	‘‘	‘‘
1133	37	‘‘	Klapmuts	96
1143	39	‘‘	Franschhoek	95
1148	57	‘‘	Grabouw	2/97
1149	63	‘‘	Elgin	96
1154	105	‘‘	Dennesig	9/98
1159	108	‘‘	Swellendam	10/98
1162	59	*Prunus* sp. (stone fruit)	Stellenbosch	96
1175	73	‘‘	N/a	97
1179	75	*Populus* sp.	Ceres	1/98
1187	86	*Prunus dulcis* (almond)	Hermanus	6/98

[a]Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa. Isolates shown in boldface type are *N.*

CMW no. [a]	BO no. [b]	Host	Location	Date isolated
australe. All other isolates represent '*Botryosphaeria*' *obtusa.*				
[b]Botryosphaeriaceae culture collection of WA Smit, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa.				

Table 2 Isolates considered in the phylogenetic study

Culture no. [a]	Other no. [a]	Identity [b]	Host	Location	Collector	GenBank [c]
CMW 7772	CBS 115475	*Neofusicoccum ribis*	*Ribes* sp.	New York, USA	B Slippers/G Hudler	AY236935
CMW 7054	CBS 121·26	*N. ribis*	*Ribes rubrum*	New York, USA	NE Stevens	AF241177
	KJ 93·42	*N. ribis/N. parvum*	*Malus* sp. (apple)	Washington DC, USA	KA Jacobs	AF027741
CMW 994	ATCC 58189	*N. parvum*	*M. sylvestris* (apple)	New Zealand	GJ Samuels	AF243395
CMW 9078	ICMP 7925	*N. parvum*	*Actinidia deliciosa* (kiwifruit)	New Zealand	SR Pennycook	AY236940
BOT 25		*N. parvum*	*Eucalyptus grandis*	Swaziland	H Smith	AF283679
	TO 67	*N. parvum*	*Vitis* sp. (grapevine)	Okayama Pref., Japan	H Nasu	AB034815
	TO 74	*N. parvum*	*Pyrus communis* (pear)	Tokushima Pref., Japan	H Yamatao	AB034818
	TO 76	*N. parvum*	*P. communis* (pear)	Tokushima Pref., Japan	T Ogata	AB034819
	TO 77	*N. parvum*	*A. deliciosa* (kiwifruit)	Tokushima Pref., Japan	H Yamato	AB034820
	TO 78	*N. parvum*	*Diospyrus kaki* (persimmon)	Tokushima Pref., Japan	H Yamato	AB034821

Culture no. [a]	Other no. [a]	Identity [b]	Host	Location	Collector	GenBank [c]
CMW 992/3	KJ93·52	*N. luteum*	*A. deliciosa* (kiwifruit)	New Zealand	GJ Samuels	AF027745
CMW 10309	CAP002	*N. luteum*	*Vitis vinifera* (grape)	Portugal	AJL Phillips	AY339258
CMW 9072		*N. australe*	*Acacia* sp.	Melbourne, Australia	J Roux/D Guest	AY339260
CMW 6837		*N. australe*	*Acacia* sp.	Batemans Bay, Australia	MJ Wingfield	AY339262
CMW 586	BO83	*N. australe*	*M. domestica* (apple)	Elgin, SA	WA Smit	DQ836719
CMW 980	BO76	*N. australe*	*P. communis* (pear)	Hermanus, SA	WA Smit	DQ836717
CMW 1133	BO37	*N. australe*	*Prunus salicina* (plum)	Klapmuts, SA	WA Smit	DQ836716
CMW 1187	BO86	*N. australe*	*Prunus dulcis* (almond)	Hermanus, SA	WA Smit	DQ836718
	TO 1	*B. dothidea*	*Malus* sp. (apple)	Fukusima Pref., Japan	S Hayashi	AB034808
	TO 12	*B. dothidea*	*Prunus persica* (peach)	Fukusima Pref., Japan	S Kanematsu	AB034809
	TO 29	*B. dothidea*	*P. communis* (pear)	Nagasaki Pref., Japan	T Ogata	AB034810
	TO 41	*B. dothidea*	*P. communis* (pear)	Nagano Pref., Japan	T Ogata	AB034813
	TO 66	*B. dothidea*	*Vitis* sp. (grapevine)	Okayama Pref., Japan	H Nasu	AB034814

Culture no. [a]	Other no. [a]	Identity [b]	Host	Location	Collector	GenBank [c]
	TO 72	*B. dothidea*	*P. persica* (peach)	Fukushima Pref., Japan	T Ogata	AB034816
	TO 73	*B. dothidea*	*P. communis* (pear)	Tokushima Pref., Japan	H Yamato	AB034817
	TO 81	*B. dothidea*	*Malus* sp. (apple)	USA	TB Sutton	AB034823
	TO 82	*B. dothidea*	*Malus* sp. (apple)	USA	TB Sutton	AB034811
	KJ94·23	*B. dothidea*	*M. sylvestris* (apple)	Georgia, USA	PL Pusey	AF027747
	KJ94·26	*B. dothidea*	*Prunus persica* (peach)	Japan	PL Pusey	AF027749
	KJ94·27	*B. dothidea*	*P. persica* (peach)	Georgia, USA	PL Pusey	AF027761
	ZS 97–5	*B. dothidea*	*Malus* sp. (apple)	Wisconsin, USA	P McManus	AF241173
CMW 8000	CBS 115476	*B. dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B Slippers	AY236949
	KJ 93·09	*D. corticola*[3]	*Cercis canadensis*	Washington DC, USA	KA Jacobs	AF027752
	KJ 93·35	*D. corticola*[3]	*Quercus suber*	Spain	KA Jacobs	AF027754
	KJ 93·29	*D. corticola*[3]	*Quercus* sp.	California, USA	E Hecht-Poinar	AF027753
	ZS 94–6	*D. mutila*	*M. pumila* (apple)	New Zealand	N Tisserat	AF243407
CMW 7060	CBS 431·82	*D. mutila*	*Fraxinus excelsior*	Netherlands	HA van der Aa	AY236955
	ATCC 60259	*D. mutila*	*M. pumila* (apple)	Unknown	HJ Boesewinkel	AF243406
	TO 79	'*Botryosphaeria*'	*Malus* sp. (apple)	USA	TB Sutton	AB034822

Culture no. [a]	Other no. [a]	Identity [b]	Host	Location	Collector	GenBank [c]
		obtusa				
	TO 80	'*B.*' *obtusa*	*Malus* sp. (apple)	USA	TB Sutton	AB034812
CMW 568	BO 80	'*B.*' *obtusa*	*Malus* sp. (apple)	Ceres, SA	WA Smit	DQ836726
CMW 918	BO 55	'*B.*' *obtusa*	*P. communis* (pear)	Villiersdorp, SA	WA Smit	DQ836721
CMW 986	BO 85	'*B.*' *obtusa*	*P. communis* (pear)	Hermanus, SA	WA Smit	DQ836722
CMW 1050	BO 149	'*B.*' *obtusa*	*P. communis* (pear)	Koue Bokkeveld, SA	WA Smit	DQ836723
CMW 1069	BO 145	'*B.*' *obtusa*	*P. persica* (peach)	Robertson, SA	WA Smit	DQ836724
CMW 1159	BO 108	'*B.*' *obtusa*	*P. salicina* (plum)	Swellendam, SA	WA Smit	DQ836720
CMW 1179	BO 75	'*B.*' *obtusa*	*Populus* sp. (chinese poplar)	Ceres, SA	WA Smit	DQ836725
CMW 7774		'*B.*' *obtusa*	*Ribes* sp.	New York, USA	B Slippers/ G Hudler	AY236953
	ATCC 60851	'*B.*' *obtusa*	*P. persica* (peach)	Georgia, USA	KO Britton	AF243408
	KJ93·56	'*B.*' *obtusa*	Hardwood shrub	New York, USA	GJ Samuels	AF027759
		Guignardia bidwellii	*Vitis* sp.	New York, USA	AB Baudoin	AF216533
CMW 4945		*Mycosphaerella africana*	*E. viminalis*	Stellenbosch, SA	PW Crous	AF 283690

[a]Designation of isolates and culture collections: BO = *Botryosphaeria* collection of WA Smit, ARC Infruitec-Nietvoorbij, South Africa; BOT and CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CAP = Culture collection of AJL Phillips, Lisbon, Portugal; CBS = Centraalbureau voor

Culture no. [a]	Other no. [a]	Identity [b]	Host	Location	Collector	GenBank [c]
Schimmelcultures, Utrecht, Netherlands; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; KJ = Jacobs & Rehner (1998); ATCC = American Type Culture Collection, Fairfax, VA, USA; TO = Ogata *et al*. (2000); ZS = Zhou & Stanosz (2001). Isolates from South Africa sequenced in this study are in bold.						
[b]Identities as used in this study, following conventions of Crous *et al*. (2006).						
[c]Includes isolates originally identified as *B. obtusa*, *B. stevensii* and *B. quercuum* by Jacobs & Rehner (1998), but redescribed in Alves *et al*. (2004).						

Table 3 Conidial measurements of '*Botryosphaeria*' *obtusa* and *D. mutila* that occur together on fruit trees and are sometimes confused (See also Figs 1–7)

Identity	Culture No.	Conidial measurements [a] (µm)	L/W	Wall	Host	Location
D. mutila	Shoemaker, 1964	(20–)25–27 × 10–12(−16)	2·3	1·5–2	*Fraxinus, Vitis*, etc.	Europe, Canada
	Alves *et al.*, 2004	(23·5–)25·4(−27·4) × (12·4–)13·4(−14·3)	1·9		*Fraxinus, Vitis, Malus, Populus*	Europe
Diplodia sp. ('*Botryosphaeria*' *obtusa*)	This study [b] (see Table 1, 2)	20–26 × 10–12	2·2	0·5–1	*Malus, Pyrus, Prunus*	Cape Province, South Africa
	Shoemaker, 1964	22–26 × 10–12		0·5	*Vitis, Malus, Pyrus, Ribes*, etc.	Europe, Canada, USA
D. malorum [c]	G. 1706 (A)	(16·5–)21(−26) × (7·5–)8·5(−12)	2·5	1	*Malus*	Germany
	G. 1706 (B)	(21–)27·9(−35·5) × (9–)11·7(−14·5)	2·4	1	*Malus*	Germany

[a]Extreme measurements in brackets are actual ranges. Averages are given between extreme values, and are representative of 15–60 conidia.

[b]Conidia produced *in vitro* as described in materials and methods. Other values are from field-collected samples.

[c]Measurements from type specimens *D. malorum* (two samples under this name marked as *typus* in herbarium G), which has been linked to '*B. obtusa*' (see text), are also included.