

***Botryosphaeria dothidea*: A latent pathogen of global importance to
woody plant health**

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Summary

Botryosphaeria dothidea is the type species of *Botryosphaeria* (Botryosphaeriaceae, Botryosphaeriales). Fungi residing in this order are amongst the most widespread and important canker and dieback pathogens of trees worldwide, with *B. dothidea* one of the most common species on a large number of hosts. Its taxonomic circumscription has undergone substantial change in the past decade, making it difficult to interpret the large volume of literature linked to the name *B. dothidea*. This pathogen profile synthesises the current understanding of *B. dothidea* pertaining to its distribution, host associations and its role as a pathogen in managed and natural woody environments. The prolonged latent infection or endophytic phase is of particular importance as it implies that the fungus can easily pass undetected by quarantine systems in traded living plants, fruits and other plant parts. Infections typically become obvious only under conditions of host stress, when disease symptoms develop. This study also considers the knowledge emerging from the recently sequenced *B. dothidea* genome elucidating previously unknown aspects of the species, including mating and host-infection strategies. Despite more than 150 years of research on *B. dothidea*, there is clearly much to be learned regarding this global tree pathogen. This is increasingly important given the stresses imposed on various woody hosts due to climate change.

Taxonomy: *Botryosphaeria dothidea* (Moug. ex Fr) Ces. & De Not, 1863. Kingdom Fungi, Phylum Ascomycota, Class Dothideomycetes, Order Botryosphaeriales, Family Botryosphaeriaceae, Genus *Botryosphaeria*, Species *dothidea*

Host range: Confirmed on more than 24 host genera, including woody plants such as *Acacia* (= *Vachellia*), *Eucalyptus*, *Vitis* and *Pistachio*.

Disease symptoms: Associated with twig, branch and stem cankers, tip and branch dieback, fruit rot, blue stain and plant death

Useful websites:

- The *Botryosphaeria* site for detailed morphological descriptions (http://www.crem.fct.unl.pt/botryosphaeria_site/);
- Systematic Mycology and Microbiology Laboratory Fungal Database for all literature and associated hosts (<https://nt.ars-grin.gov/fungaldatabases/>);
- TreeBASE link for the combined ITS and TEF-1 α tree [in progress] (<http://purl.org/phylo/treebase/phyloids/study/TB2:S18906>);

(For review purposes TreeBase link: <http://purl.org/phylo/treebase/phyloids/study/TB2:S18906?x-access-code=e18e07712d9441650a1234fd14c5798c&format=html>)

- DOE Joint Genome Institute, JGI Mycocosm for the *Botryosphaeria dothidea* genome (http://genome.jgi.doe.gov/Botdo1_1/Botdo1_1.home.html)

1. Introduction

The ascomycete fungus *Botryosphaeria dothidea* is the type species of *Botryosphaeria* (Botryosphaeriaceae, Botryosphaeriales) residing in the Dothideomycetes. Since the original description of *B. dothidea* in 1853 (Cesati and De Notaris, 1853), numerous morphologically similar specimens have been reported as representing this pathogen. In retrospect, however, many of these specimens have been shown to belong other species. The advent of DNA-based identification has

largely resolved this important problem due to the epitypification of this species (Slippers *et al.*, 2004a). As a consequence of previous taxonomic confusion, reports of this fungus on a wide range of hosts and countries prior to the application of DNA sequencing techniques are either incorrect, or must at least be viewed with circumspection.

Botryosphaeria dothidea and other species in the Botryosphaeriaceae were considered as wound-infecting pathogens for many years. During the course of the last few decades, however, these fungi have been recognised primarily as endophytes that infect healthy tissue of woody plants and remain dormant until the onset of stress conditions (Fisher *et al.*, 1993, Johnson *et al.*, 1992, Maresi *et al.*, 2007, Perez *et al.*, 2010, Petrini & Fisher, 1988, Sakalidis *et al.*, 2011a, Smith *et al.*, 1996a, Smith *et al.*, 1996b, Stanosz *et al.*, 2005). In the case of *B. dothidea*, the distinction between endophyte and latent pathogen is of little value and simply reflects a snapshot in time of the complex lifestyle of this plant-infecting fungus. This is true for many pathogens that have a latent phase during a part of their lifecycle (Hyde & Soyong, 2008, Rai & Agarkar, 2014, Schulz & Boyle, 2005).

Climate change is expected to increase the stress on many plant communities, including trees in natural woody ecosystems, managed forests and agriculture (Kirilenko & Sedjo, 2007, Lavallo *et al.*, 2009, Sturrock *et al.*, 2011). Consequently, the potential impact of the Botryosphaeriaceae in general, but specifically *B. dothidea*, which is a widespread pathogen already present as an endophyte in numerous plant communities in various parts of the world might be exacerbated (Desprez-Loustau *et al.*, 2006). Already, the impact of the pathogen in relation to climatic conditions and stress has been documented in plant communities in parts of

Southern Europe (Piškur *et al.*, 2011), Australia (Dakin *et al.*, 2010), South Africa (Van Der Linde *et al.*, 2012, Van Der Linde *et al.*, 2011) and others (Brown and Hendrix, 1981, Ma *et al.*, 2001, Sturrock *et al.*, 2011).

Reports prior to the application of DNA sequence data and phylogenetic inference treated *B. dothidea* as a conglomerate of taxa. Therefore, the scientific literature regarding this fungus must be interpreted with care. In this review, we address this issue by considering the extensive knowledge regarding *B. dothidea*, and the family in which it resides, as it has been updated during the course of the past decade through accurate DNA-based identification. We also utilise currently available public data to characterise its confirmed host associations and geographic distribution. Available knowledge regarding the epidemiology of this latent pathogen, its role in the environment and how this might be influenced by climate change are considered. Furthermore, key insights emerging from the recently sequenced genome of *B. dothidea*, are highlighted. This is done with the specific intention of laying a foundation for future studies aimed at understanding the ability of the fungus to avoid host defence systems and to establish continuing endophytic infections.

2. Taxonomy and identification

Prior to its epitypification by Slippers *et al.* (2004a), there was confusion pertaining to the taxonomy and identification of *B. dothidea*. Cesati and De Notaris (1863) described the genus *Botryosphaeria* Ces. & De Not., with the addition of *B. berengeriana* De Not., by De Notaris (1863). No type species was selected for the genus at the time, but Barr (1972) designated *B. dothidea* (Moug.: Fr.) Ces. & De Not. (= *Sphaeria dothidea* Moug. ex Fr.) as the lectotype species for the genus. Subsequently, many additional *Botryosphaeria* species have been described, often

based on the fact that they were isolated from different hosts and despite morphological similarities between specimens. Von Arx and Müller (1954) reduced many of these species to synonymy with *B. quercuum* and *B. dothidea* based on the morphology of their sexual morphs. Von Arx and Müller (1975) and von Arx (1987) later restricted *B. dothidea* to isolates that were pathogenic on roses, and considered *B. berengeriana* (including *B. ribis*) to be polyphagous and widespread. Pennycook and Samuels (1985), however, recognised that *B. dothidea* was a complex of species that could be distinguished based on morphological differences of the asexual *Fusicoccum* states, and separated *F. aesculi* Corda, *F. parvum* Pennycook & Samuels and *F. luteum* Pennycook & Samuels from *B. dothidea* (Pennycook & Samuels, 1985).

Jacobs and Rehner (1998) produced the first DNA-based phylogeny for *Botryosphaeria* and related asexual genera, based on sequences for the ITS rDNA locus. The *Botryosphaeria* spp. included in their study formed clades corresponding with species having dark, *Diplodia*-like conidia and those with hyaline, *Fusicoccum*-like conidia (Jacobs & Rehner, 1998). Subsequent DNA-based studies supported these clades and suggested that *Botryosphaeria* spp. with hyaline, fusoid conidia should reside in *Fusicoccum* and those with dark to opaque and ellipsoid mature conidia should be placed in *Diplodia*, *Lasiodiplodia*, or in the sections *Hyalia* and *Brunnea* (Denman *et al.*, 2000, Zhou & Stanosz, 2001). These developments provided the basis for further resolution of the species complex grouped under the name *B. dothidea*. *Neofusicoccum luteum* was the first species to be separated from *B. dothidea* based on colony morphology and DNA sequence data (Phillips *et al.*, 2002). The taxonomic confusion surrounding *B. dothidea* was finally resolved by the epitypification of the species by Slippers *et al.* (2004a). Based on a collection from

the same region (Crocifisso, Switzerland) and host (*Prunus* sp.) where the original type specimen had been collected, the epitype specimen was linked to a culture and DNA barcode. This made it possible to connect subsequent studies to a reliable name (Slippers *et al.*, 2004a).

Subsequent to the designation of the epitype, a number of species and even families have been separated from *B. dothidea* (Phillips *et al.*, 2013, Crous *et al.*, 2006, Damm *et al.*, 2007, Phillips *et al.*, 2008, Slippers *et al.*, 2013). Despite the narrower species delimitation after epitypification, reports of *B. dothidea* during the last decade have confirmed that it is one of the most widespread and important endophytes or latent pathogens occurring on a large number of plants important in agriculture, forestry and in natural forest ecosystems (Jami *et al.*, 2013a, Piškur *et al.*, 2011, Slippers & Wingfield, 2007, Pavlic *et al.*, 2007, Phillips *et al.*, 2005, Qiu *et al.*, 2008, Tang *et al.*, 2012, Xu *et al.*, 2015).

Morphological characters have been important in the identification of fungi in the past. The ascospores of *B. dothidea* are unicellular, hyaline, 17-22 µm long and are fusoid to ovoid with tapered ends. Conidia are unicellular, narrowly or irregularly fusiform with rounded ends. Conidia are also 17-22 µm long, hyaline, and rarely form a septum before germination (Slippers *et al.*, 2004a, Phillips *et al.*, 2013). In culture, colonies are olivaceous becoming dark grey and black in reverse as the colony ages. The mycelial mat is moderately dense with smooth margins (Slippers *et al.*, 2004a, Phillips *et al.*, 2013). Pycnidia produced on water agar and sterilized host twigs or pine needles are solitary, globose and covered by mycelium. Pycnidia contain a single ostiole with white to creamy contents. Conidia are similar to those produced in nature, except that they are 20-30 µm in length and are more narrowly

fusiform (Slippers *et al.*, 2004a). These morphological characteristics are no longer routinely used in the identification of *B. dothidea*, given the variation amongst isolates and the fact that they overlap with other species in the Botryosphaeriaceae.

DNA-based identification of *B. dothidea* typically relies on the sequences linked to the ex-type isolate (CMW8000 / CBS115476) for β -Tubulin (BT, AY236927), rDNA Internal Transcribed Spacer (ITS, AY236949), and Translation Elongation Factor-1 α (TEF-1 α , AY236898) (Slippers *et al.*, 2004a). As of November 2015, more than 2,000 sequences have been generated for *B. dothidea* and deposited into GenBank (NCBI; www.ncbi.nlm.nih.gov), most of them for the three above-mentioned loci. The majority of the sequences in GenBank are of the ITS rDNA locus, which has been shown to clearly distinguish this species from its closest known relatives (Phillips *et al.*, 2013). Given that cryptic species have regularly been discovered in this family during the course of the last decade, it is recommended, however, that sequence data of the ITS be combined with that of TEF-1 α and at least one other phylogenetic marker where possible.

3. Distribution and host associations

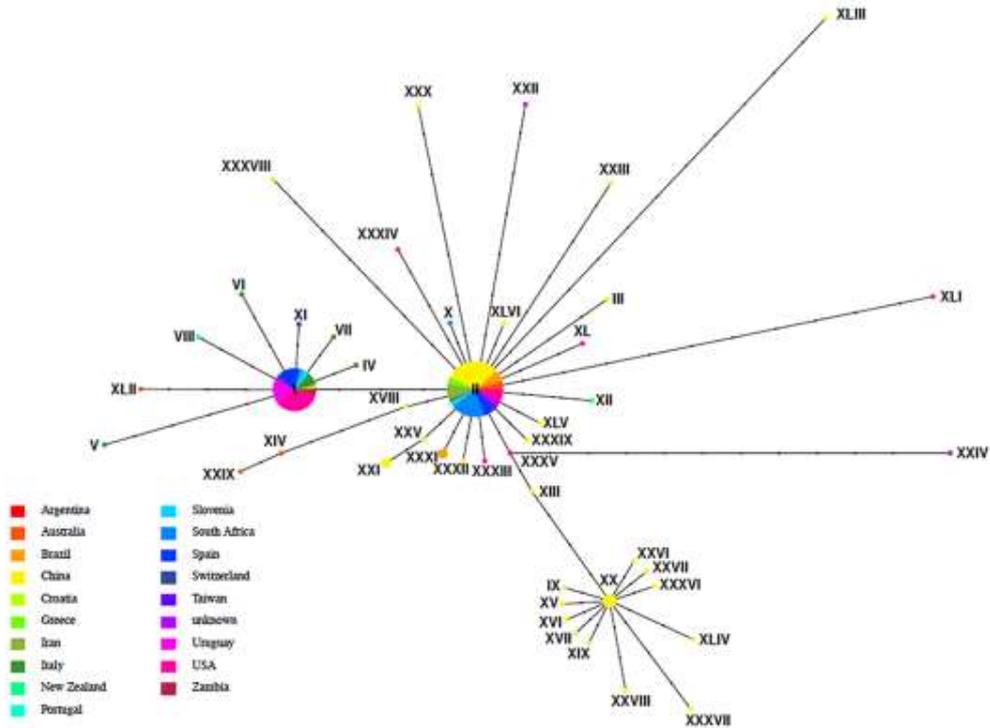
Botryosphaeria dothidea has been reported from hundreds of plant species with a broad global distribution. For example there are 856 fungal-host reports and 239 literature records of *B. dothidea* and its synonyms listed in the Systematic Mycology and Microbiology Laboratory Fungus-Host Distributions Database (Farr & Rossman, 2015). Many of these reports are of uncertain validity due to the previously confusing taxonomy for the species. In order to gain a more realistic perspective of the host range of *B. dothidea* as it is currently defined, GenBank was mined for sequences of *Botryosphaeria* for the ITS, TEF-1 α and BT regions. Sequences for these loci were

downloaded for the genus *Botryosphaeria* (ITS: 1434; TEF-1 α : 398; BT: 272) in November 2015. A multigene phylogeny was inferred for isolates (424) that were represented by the ITS and TEF-1 α DNA regions because the combination would give more robust haplotypes than ITS alone. There was also no overlap between the BT and TEF-1 α datasets (see Supplemental Information 1).

The sequences for this analysis represented specimens that originated from 24 host genera (representing 17 families) and 18 different countries spread over 6 continents (Supplementary Table 1). The 240 isolates representing *B. dothidea* from GenBank clustered in 46 haplotypes, whereby two haplotypes contained the vast majority of sequences (Figure 1). However, with the exception of a group of haplotypes representing Rosaceae (i.e. *Malus* and *Pyrus* spp.) in China (Figure 1), the minimum spanning tree did not separate specimens according to host genera or country of origin.

The low host specificity and broad geographical distribution of haplotypes for *B. dothidea* confirmed previous studies. This is surprising, as most studies that scrutinized pathogen species with low host specificity often recover many host specific cryptic species (Filipe *et al.*, 2012, Stergiopoulos & Gordon, 2014). Our data indicate that *B. dothidea* does not show any host preference. This was confirmed through single haplotypes that exist on many different hosts, although their wide distribution was somewhat surprising. Conidia and ascospores of *B. dothidea*, as for other Botryosphaeriaceae, are considered to be dispersed by wind and rain over relatively short distances (Ahimera *et al.*, 2004, Amponsah *et al.*, 2009, Swart & Wingfield, 1991, Sutton, 1981, Úrbez-Torres *et al.*, 2010, Pusey, 1989, van Niekerk *et al.*, 2010). Such limited dispersal must be assumed to result in a population structure

A



B

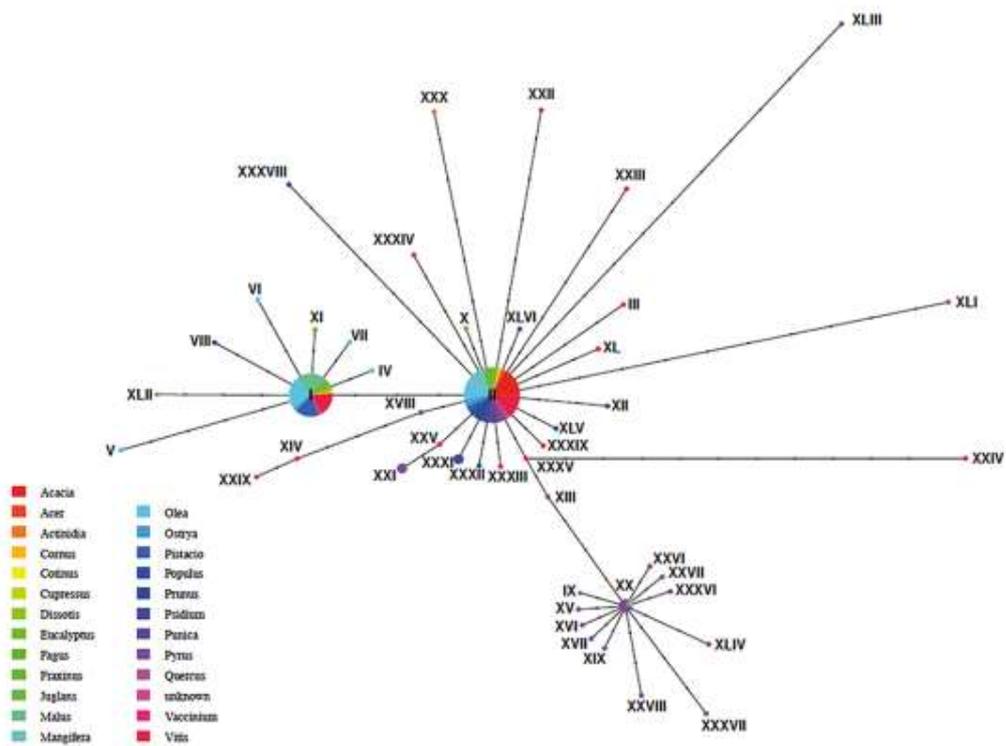


Figure 1. (A) Haplotype network representing 240 *Botryosphaeria dothidea* isolates from 18 different countries divided into 46 haplotypes. (B) Haplotype network representing the 24 host genera from which *B. dothidea* was isolated. Isolates could not be separated by country of origin or host genera.

that is shaped by geographical distance. But the low host specificity could in part contribute to low genetic structure between geographic locations, by allowing the fungus to colonise many hosts in a given area. Many of the hosts in our analysis are agricultural, forestry or horticulture-related plants. The current widespread distribution of a few haplotypes of *B. dothidea* globally has most likely resulted from anthropogenic long-distance dispersal via global trade of plants and plant products, illustrating the phytosanitary implications of undiscovered plant pathogens.

4. Epidemiology and host infection

Species in the Botryosphaeriaceae are considered to be stress-associated pathogens (Ma *et al.*, 2001b, Smith *et al.*, 1994, Mehl *et al.*, 2013, Czernemmel *et al.*, 2015, Zhang *et al.*, 2013, Desprez-Loustau *et al.*, 2006, Piškur *et al.*, 2011, Slippers & Wingfield, 2007, Stanosz *et al.*, 2001, van Niekerk *et al.*, 2011). Disease expression is more often associated with abiotic stresses such as drought, physical damage, waterlogging, frost and unsuitable growing environments (Bostock *et al.*, 2014, Desprez-Loustau *et al.*, 2006, Slippers & Wingfield, 2007, Zhang *et al.*, 2013, Stanosz *et al.*, 2001, Sakalidis *et al.*, 2011b, Ragazzi *et al.*, 1999, Ma *et al.*, 2001b, van Niekerk *et al.*, 2011). When these stressful conditions occur there are often a variety of Botryosphaeriaceae isolated from different plant tissues, potentially all contributing to the observed symptoms, albeit to varying degrees (e.g, Jami *et al.* 2015).

Disease symptoms (Figure 2) include twig, branch and stem cankers, tip and branch dieback, fruit rots, blue stain or in extreme cases, the death of the host plant (Michailides, 1991, Slippers & Wingfield, 2007, Smith *et al.*, 1994, Swart & Wingfield, 1991). While the association of *B. dothidea* with such symptoms before 2004 must remain uncertain, recent studies have linked *B. dothidea* to similar

symptoms on a variety of hosts. These include apple ring rot (Kim *et al.*, 2004, Tang *et al.*, 2012, Xu *et al.*, 2015), fruit rot of olives (Phillips *et al.*, 2005), grapevine trunk disease (Qiu *et al.*, 2008, van Niekerk *et al.*, 2006, Li *et al.*, 2010), leaf spots and lesions on horticultural plants (Cunnington *et al.*, 2007) as well as dieback and stem cankers on acacia (Gezahgne *et al.*, 2004), eucalypt (Burgess *et al.*, 2005, Gezahgne *et al.*, 2004, Mohali *et al.*, 2007), European hop hornbeam (Jurc *et al.*, 2006), pine (Gezahgne *et al.*, 2004), mango (Slippers *et al.*, 2005), poplar (Grasso & Granata, 2010, Slippers *et al.*, 2004b) and stone fruit (Wang *et al.*, 2011, Inderbitzin *et al.*, 2010, Slippers *et al.*, 2004b). Many more host associations and specific symptoms have been reported, but not confirmed using sequence data (Farr & Rossman, 2015).

Prior to its epitypification in 2004, it was assumed that *B. dothidea* could infect its hosts through either wounds (Michailides, 1991) or endophytically through natural openings (Michailides, 1991, Kim *et al.*, 1999, Smith, 2001). Direct penetration was also possible on apple fruits through the formation of appressoria (Kim *et al.*, 1999, Jurick II *et al.*, 2013). Much is still unknown regarding the infection biology of *B. dothidea*, especially how it penetrates without causing a host response. Preliminary data, for example, indicates that the fungus is capable of infecting *Eucalyptus grandis* leaves by direct penetration via the formation of appressorium-like structures at the germ tube tips, without causing a host response and persisting endophytically (Marsberg *et al.*, unpublished data). Future infection studies on hosts from which *B. dothidea* is isolated as an endophyte are required to characterise the various stages of this process. Questions that remain to be answered include the location of the endophytic hyphae within infected plant tissues, as well as the level of activity within this tissue.

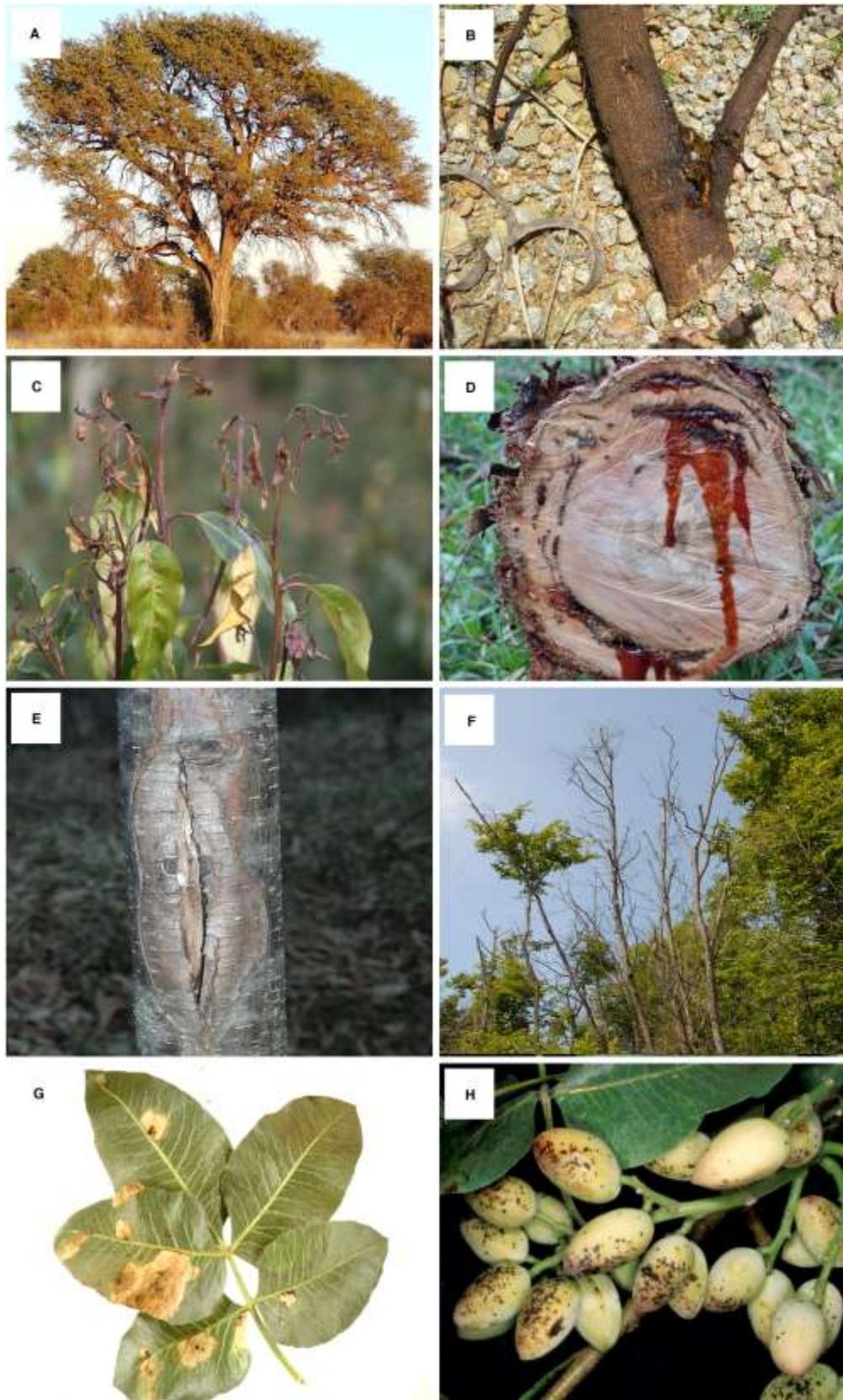


Figure 2. Different disease symptoms caused by *Botryosphaeria dothidea*, representing four different hosts spanning four different continents. (A) Healthy *Acacia erioloba* (= *Vachellia*), a native host in

Africa. (B) Kino secretions from the trunk of *Acacia*. (C) Dieback symptoms on *Eucalyptus*, a non-native host in South America. (D) Kino secretions from the trunk of *Eucalyptus*. (E) Canker on the trunk of *Ostrya carpinifolia*, a native host in Europe. (F) Dieback symptoms on *Ostrya carpinifolia*. (G) Leaf lesion on the leaves of *Pistachio*, a non-native host in North America. (H) Early disease symptoms on the fruit of *Pistachio*.

5. Latent pathogens and quarantine

Botryosphaeria dothidea is known to be a serious plant pathogen and is listed in many countries as a quarantine organism. The fact that it frequently exists as an endophyte in asymptomatic tissue for prolonged periods of time poses a significant quarantine challenge (Slippers & Wingfield, 2007). Neither *B. dothidea* nor its synonyms, currently appear on the European and Mediterranean Plant Protection Organization (EPPO) A1 and A2 lists of pests recommended for regulation as quarantine pests (<http://www.eppo.int/QUARANTINE/quarantine.htm>), but it does occur in the EPPO global database (<https://gd.eppo.int/taxon/BOTSDO>). Infected plant tissues and seed can easily be moved between countries and regions without any visible indications of infection. Inspection-based quarantine procedures that target only symptomatic material are therefore not able to detect it.

The low host specificity of *B. dothidea* increases the likelihood that it will spread to new hosts once it is introduced into a new area. It is thus not surprising that various studies have provided evidence that *B. dothidea* infects both native and non-native trees in areas where it is found (Mohali *et al.*, 2007, Pavlic *et al.*, 2007, Slippers *et al.*, 2009, Jami *et al.*, 2013a, Jami *et al.*, 2013b). In some cases, native and non-native hosts grow in close proximity, e.g. *Pistachio* and native hosts in California (Inderbitzin *et al.*, 2010, Ma *et al.*, 2001a). In other cases, *B. dothidea* is common both on native and non-native hosts that are not in close proximity, such as the

isolated native stands of the widely distributed *Acacia* (= *Vachellia*) trees in the dry northern regions of South Africa (Jami *et al.*, 2015). Therefore, *B. dothidea* could be equally threatening to both commercial enterprises and native ecosystems. The same situation is most likely also true for other Botryosphaeriaceae, such as the important

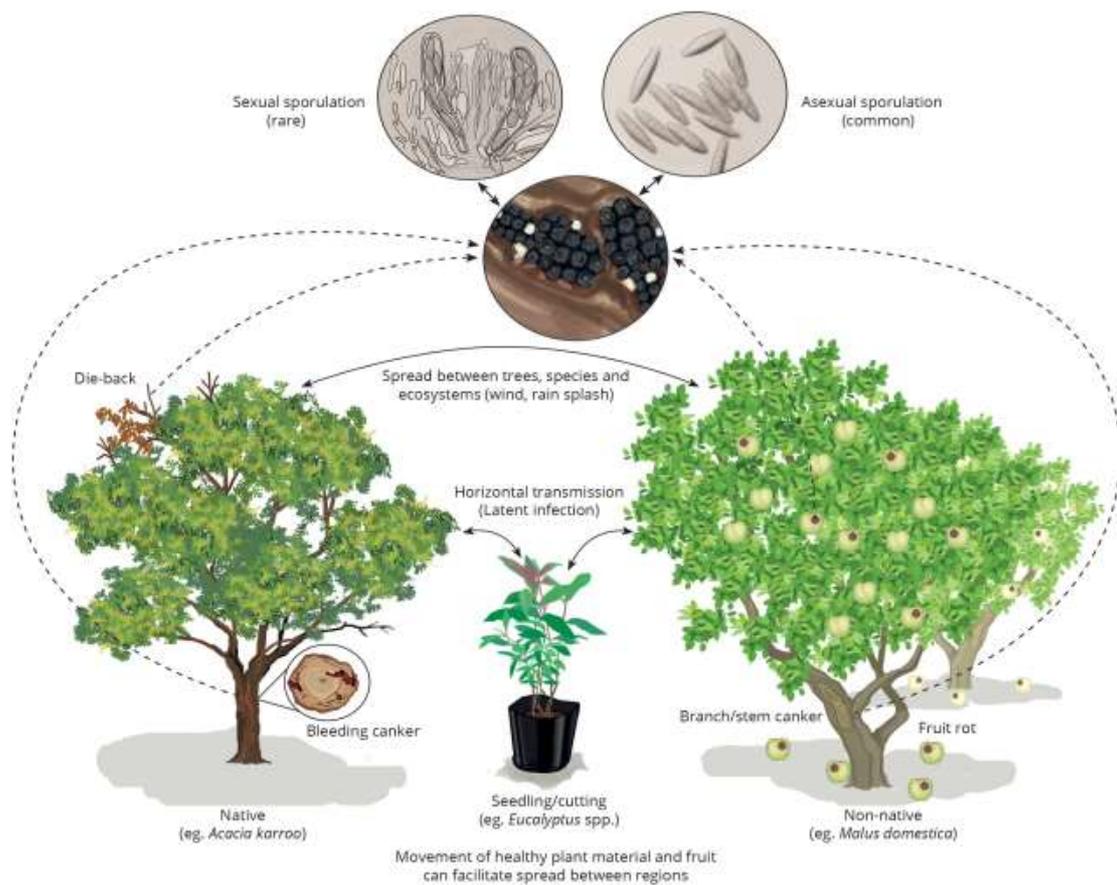


Figure 3. An illustrative example of typical disease symptoms, host associations, sporulation and potential spread of *Botryosphaeria dothidea*. The illustration depicts infection of the fungus on three hosts, as observed in South Africa, including native (*Acacia karroo*) and non-native (*Eucalyptus* and *Malus domestica*) hosts. Typical disease symptoms include dieback, cankers and fruit rot. Fruiting structures containing sexual and/or asexual spores are often found associated with the disease symptoms and these are dispersed through wind or rain splash. Movement of latent or endophytically infected plant material to new regions poses a threat to quarantine systems. Given the broad host range and geographical distribution of *B. dothidea*, all three hosts could be replaced by various others, as could the geographical setting.

conifer pathogen *Diplodia sapinea* (Slippers & Wingfield, 2007, Burgess *et al.*, 2001, Smith, 2001, Smith *et al.*, 1996a). Figure 3 broadly encompasses the dispersal of *B. dothidea* between trees, species and regions with the potential threat to quarantine systems.

Our haplotype analysis (Figure 1) suggests that the same *B. dothidea* genotypes occur in different regions of the world, implying that quarantine measures, as they are currently applied, are often futile. However, there is little understanding of the distribution and diversity of important traits, such as aggressiveness, on specific hosts. Efforts to reduce the spread of this potentially important pathogen as well as other latent pathogens consequently represent a sensible goal.

6. Population genetics and mating

Surprisingly, for a pathogen of such wide distribution and importance, few population genetic studies have been conducted on *B. dothidea*. Where these have been undertaken, they have mostly focussed on disease outbreaks. In one Californian study a low level of diversity was observed in isolates from *Pistachio* (Ma *et al.*, 2001a). A subset of isolates collected from the same location in different years was identical, suggesting that the *B. dothidea* population in California is highly clonal (Ma *et al.*, 2001a). The *B. dothidea* isolates from the non- *Pistachio* hosts had a greater diversity than those collected from *Pistachio* trees in the same area. This suggests that the pathogen had most likely become established in the area long before *Pistachio* was commercialised in California, and that it is possibly native to the area (Ma *et al.*, 2001a). An alternative explanation could be that the fungus was introduced into the area multiple times and/or from various sources. This latter scenario is possible in the light of the apparently extensive human-mediated movement of the fungus on crops

and other plants globally, but does not explain the lack of diversity on *Pistachio* (see section 3). Furthermore, *B. dothidea* has been found to occur on almond, olive and blackberry in California (Inderbitzin *et al.*, 2010). Because the fungus is known to occur on almond, the almond orchards were thought serve as sources of inoculum to the neighbouring vegetation, but the opposite situation could also have applied.

Piškur *et al.* (2011) studied the population diversity of an outbreak of *B. dothidea* on European hop hornbeam (*Ostrya carpinifolia*) in Slovenia and northern Italy, using amplified fragment length polymorphism (AFLP) markers. The population linked to the outbreak was highly diverse and did not appear to be the result of a recent introduction or due to a particularly pathogenic clone. Rather, the population resembled what would be expected from an established, possibly native, latent pathogen that had emerged to cause widespread dieback when extreme weather conditions (drought and heat) imposed undue stress on the plant communities in these regions. The diversity structure did not correlate to specific tissues or geographic location across Slovenia to Italy (Piškur *et al.*, 2011). The possibility of *B. dothidea* being native to Europe is supported by its first isolation there in 1863 by Cesati and De Notaris, although should be seen in the light of the majority of fungal taxonomy work at the time being done in Europe.

Both sexual and asexual stages have been reported for *B. dothidea* (Slippers *et al.*, 2004a), but it is not known whether the species is homo- or heterothallic. The sexual stage is rarely encountered in nature, sometimes co-occurring with the asexual stage, and it has never been recovered from culture. The more common asexual stage of *B. dothidea* is thus believed to play the most prominent role in dispersal and structuring diversity in populations. Understanding these dynamics is important in order to

inform future studies on population genetics, spread and adaptive ability of the pathogen.

Genomics research is beginning to shed light on questions relating to the reproductive strategies in the Botryosphaerales. The first *MAT* gene sequence for a species in the Botryosphaerales was described for *Diplodia sapinea*, a well-known pathogen of *Pinus* spp. (Bihon *et al.*, 2014, Bihon *et al.*, 2012, Swart & Wingfield, 1991).

Because a sexual stage has never been observed in *D. sapinea* (Bihon *et al.*, 2012, Bihon *et al.*, 2014), it was surprising to find the complete set of *MAT* genes necessary for heterothallic reproduction in complementary isolates. This finding, as well as the diversity of alleles at these loci, suggests a rarely observed, heterothallic sexual cycle (Bihon *et al.*, 2014).

In the present study, the *MAT* genes of *B. dothidea* were compared with those of *D. sapinea*. As in *D. sapinea*, the *MAT1-1* genes in *B. dothidea* consisted of the *MAT1-1-1* and *MAT1-1-4* genes while the *MAT1-2* genes consisted of the *MAT 1-2-1* and *MAT1-2-5* genes. However, unlike *D. sapinea*, both *MAT1-1* and *MAT1-2* genes were present in a single individual. In fact, all four characterized *MAT* genes grouped together in the genome at a single locus (Figure 4), indicating that *B. dothidea* has a homothallic mating system. The genes adjacent to the *MAT* genes on this locus are inverted when comparing *B. dothidea* to *D. sapinea* (Figure 4).

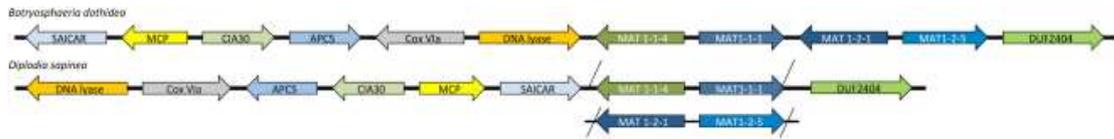


Figure 4. Comparison of the genomic architecture of the *MAT* locus and surrounding genes between *Botryosphaeria dothidea* and *Diplodia sapinea*. All four characterized *MAT* genes group together at a single locus, suggesting a homothallic mating-type system in *B. dothidea*. Arrows represent gene order and orientation, but genes and intergenic regions are not to scale. Abbreviations: CIA30, complex I intermediate-associated protein 30; Cox VIa, cytochrome *C* oxidase subunit VIa; DUF2404, putative integral membrane protein containing DUF2404 domain; MCP, mitochondrial carrier protein; SAICAR, SAICAR (phosphoribosylaminoimidazolesuccinocarboxamide) synthase.

7. Genomics of host infection and future research

The *B. dothidea* genome has been sequenced as part of the Assembling the Fungal Tree of Life Project and curated as part of the 1,000 fungal genomes initiative of the Fungal Genomics Program (FGP) of the Department of Energy (DoE) Joint Genomics Institute (JGI; <http://1000.fungalgenomes.org>). It establishes an opportunity to investigate one of the most intriguing questions relating to the infection biology of *B. dothidea*, namely understanding the mechanisms of host infection without resulting in symptom development for a prolonged period.

In order to provide a foundation for future studies, we analysed potential pathogenic factors of the *B. dothidea* genome in a simple comparison with that of the most closely related genome that has been annotated and analysed, namely *Zymoseptoria tritici* (synonym: *Mycosphaerella graminicola*; *Mycosphaerellaceae*) (Goodwin *et al.*, 2011). These pathogens both have a latent infection phase, although for *Z. tritici* it is typically much shorter and transitory in nature compared to *B. dothidea*. The genomes have similar sizes of ~38Mb (*Z. tritici*) and ~34Mb (*B. dothidea*). However,

the genome structure of the two fungi appears to be different in key aspects related to host-pathogen interactions. For example, the carbohydrate active enzyme (CAZyme) repertoire of *B. dothidea* (n=623) is expanded when compared to *Z. tritici* (n=440) (Supplementary Table 2). For pathogens, the CAZymes are key in plant interactions, as they are responsible for the breakdown of plant cell wall components, namely cellulose, hemicellulose, xylan, xyloglucan, mannan and pectin (Gan *et al.*, 2013, Knogge, 1996).

The mechanism of penetration and avoidance of host reactions by endophytic fungi, such as *B. dothidea*, is poorly understood. It has been suggested that the reduced number of genes coding for cell wall degrading enzymes in *Z. tritici* could be an evolutionary adaptation linked to “stealth” penetration during the latent, biotrophic phase of infection (Goodwin *et al.*, 2011, Kema *et al.*, 2008). The CAZyme repertoire of *B. dothidea* suggests that it uses a different mechanism for avoidance of host defences. The expanded CAZyme repertoire of *B. dothidea* can possibly be explained by the tissue it infects. In this regard it is quite different from *Z. tritici* that infects the leaves of its cereal hosts (Poaceae) whereas *B. dothidea* is mainly known to be a canker pathogen and a coloniser of woody tissue, although it has been observed in leaves.

Analysis of the genome showed that *B. dothidea* has three distinctive glycoside hydrolase families (GH27, GH33 and GH75), of which GH33 is not present in the *Z. tritici* genome. The GH33 hydrolase family consists of sialidases that hydrolyse the glycosidic linkages of terminal sialic residues in oligosaccharides. Sialidases can act as pathogenicity factors, which can assist in host adaptation by avoiding host recognition or by inhibiting host defence responses (Alviano *et al.*, 2004). This is an

example of a potential mechanism that could be investigated using these genome data to understand how *B. dothidea* infects without resulting in symptoms and exists as an endophyte.

It is clear from this simple comparison of *B. dothidea* and *Z. tritici* that it is unreasonable to assume that latent pathogens with an endophytic life stage share a similar repertoire of genes. Comparing processes across different host-pathogen systems will be needed to clarify whether there are common patterns to this process. As with other pathogen-host interactions (Teixeira *et al.*, 2014), a genomics and dual RNA sequencing approach of both *B. dothidea* and its host(s) are required to provide insight into the mechanisms launched by *B. dothidea* that are countered by the host and *vice versa*. In the case of *B. dothidea*, the genome sequence of its host *E. grandis* has recently become available (Myburg *et al.* 2014). This offers an opportunity to examine induced defence responses to *B. dothidea* (Naidoo *et al.* 2014) to further qualify its endophytic and latent lifestyle.

8. Conclusions

Considerable taxonomic confusion has characterised studies of *B. dothidea* in the past. Although the epitypification of *B. dothidea* aided in clarifying confusion and misperceptions, many still prevail despite the number of confirmed host and geographical reports of the pathogen. The importance of *B. dothidea* as a pathogen has been underestimated and this is largely due to its endophytic nature. Under adverse environmental conditions, the fungus is capable of making the switch from “friend” to “foe” with potentially serious consequences for the hosts that it infects. As a group, the Botryosphaeriaceae provide an apt illustration that the distinction between endophytes and pathogens is artificial. This is mainly because these

definitions are based on different stages in a lifecycle rather than on distinct lifecycles.

The latent stages of fungal plant infection must be considered in terms of quarantine and this is clearly not the case at present. This is particularly important because new species, genotypes or mating types can be introduced as endophytes. As quarantine systems generally rely on visual inspection, these potential pathogens are nearly impossible to detect. Human actions have clearly and unwittingly introduced new genotypes of tree pathogens into new areas and hosts on a massive scale (Wingfield *et al.*, 2015), a problem vividly illustrated by the distribution of shared haplotypes of *B. dothidea* across the globe. Because many of the hosts of *B. dothidea* have not co-evolved with the fungus, it can be speculated that at least some of these plants have resistance mechanisms or recognition systems that are not adapted to the fungus. For the same reasons, native populations of *B. dothidea* also pose a risk to introduced (non-native) trees propagated for commercial purposes.

Climate change could affect stress associated canker-forming and dieback pathogens of plants. The Botryosphaeriaceae has been highlighted as one of the possible threats in this regard (Desprez-Loustau *et al.*, 2006). As climate change affects environmental conditions, it equally affects both pathogens and their hosts (Ahanger *et al.*, 2013). This could have an effect on the expansion of the geographical distribution and host range of some pathogens because conditions may become favourable in areas where the pathogen was not previously detected (Sturrock *et al.*, 2011, Chakraborty, 2013). Because *B. dothidea* has a wide host range, any potential expansion in its distribution due to environmental changes can result in previously uninfected hosts being affected. Adverse conditions may also cause the host plant to

become stressed, which in turn would afford latent pathogens, such as *B. dothidea*, the opportunity to cause disease (Sturrock *et al.*, 2011).

The available genome sequence for *B. dothidea* provides an important resource to gain an understanding of the endophytic and latent pathogenic nature of *B. dothidea*. There is a particularly important opportunity for transcriptome analysis during infection to identify secreted molecules that induce plant defence response. Such studies have the potential to inform general mechanisms of endophytic infections, which despite their common occurrence in all plants, are very poorly understood.

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Supplementary material

Methods S1 Haplotype methodology.

Sequences were aligned using MAFFT v7.215 (Kato *et al.*, 2002, Kato & Standley, 2013) using the L-INS-I option. A maximum likelihood phylogeny was inferred using RAxML v8.1.22 (Stamatakis, 2014) invoking the GTRGAMMA option and a rapid bootstrap with 100 replications. Based on this phylogeny, sequences for ITS and TEF-1 α that grouped with the ex-type isolate of *B. dothidea*, and did not belong to other species for which a type specimen is available, were extracted. The retained sequences were realigned under the same options using MAFFT, sequences with many leading or trailing gaps were removed and the resulting alignments were cropped to remove leading and trailing gaps. The two alignments were concatenated; indels and sites with infinite site violations were removed using Map in the SNAP workbench (Price & Carbone, 2005, Aylor *et al.*, 2006). Minimum spanning trees of haplotypes were calculated in the pegas package (Paradis, 2010) in R v3.1.3 (R Core Team, 2015).

Supplementary Table 1: Country and host range of the 240 *Botryosphaeria*

dothidea isolates used **in the haplotype analysis**.

Supplementary Table 2: CAZyme comparisons of *Botryosphaeria dothidea* and

Zymoseptoria tritici.