

Diversity and pathogenicity of *Botryosphaeriaceae* on declining *Ostrya carpinifolia* in Slovenia and Italy following extreme weather conditions

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Received: 26 March 2010/Revised: 5 July 2010/Accepted: 11 August 2010
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Abstract Unusual and extensive dieback of European hop hornbeam (*Ostrya carpinifolia*) has been observed in western Slovenia and northern Italy in recent years, when extreme drought and higher temperatures were recorded. A preliminary study identified *Botryosphaeria dothidea* as a potential causative agent of the dieback. Further characterization of intra- and interspecies diversity of Botryosphaeriaceae collected from *O. carpinifolia* and other tree species in the affected area was achieved based on anamorph morphology, sequence data from the ITS rDNA and EF1- α , PCR-RFLP analysis and AFLP profiles. The majority of the isolates analyzed were identified as *B. dothidea*, and in vitro pathogenicity tests re-confirmed the fungus to be an important cause of the disease. Insight into the *B. dothidea* population, diversity based on

AFLP markers indicates that the dieback observed in *O. carpinifolia* is probably associated with a heterogenous population of *B. dothidea*, which emerged from a latent state in response to changed climate conditions. Isolates with *Dothiorella*-like conidia were also identified during the survey, but these were collected more rarely and appear to represent undescribed species. Isolates from *Dothiorella* genus expressed low pathogenicity in pathogenicity tests and proved no impact on the pathogenic ability of *B. dothidea*.

Keywords *Botryosphaeria dothidea* · *Dothiorella* · European hop hornbeam · Latent pathogen · Dieback · Climate change

Communicated by R. Matyssek.

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Introduction

The European hop hornbeam, *Ostrya carpinifolia* Scop. (Betulaceae) is a southern European species, found on dry and degraded sites, in warm, sunny spots, and it is considered resistant to drought. These characteristics make *O. carpinifolia* an important pioneer species. This tree species is the autochthonous in the western karst parts of Slovenia named Kras (Kotar and Brus 1999) and in both the Alps and the Apennines in Italy, where this study was conducted. During the course of the last decades, *O. carpinifolia* has invaded vast areas of abandoned agricultural land, and it is regarded as an ecologically and economically valuable foundation for future forests.

O. carpinifolia is known to be resistant to various diseases (Kotar and Brus 1999), but an unusual dieback has been observed in Slovenia since 1997. Especially extensive damage and high mortality occurred in 2003, when unusual weather conditions and extreme drought were experienced

(Jurc et al. 2006). The disease occurred patchily throughout Slovenian Submediterranean forests (approximately 76,000 ha), where hop hornbeam is present over approximately 60% of the area. The dieback was noted in about 90% of the hop hornbeam range, and severe dieback was reported in 26% of the area (Zafran et al. 2006). The intensive dieback of hop hornbeam is considered a major threat to the reforestation of the Slovenian Submediterranean karst. In Italy, damage due to cankers on branches and stems was reported in 2001 in Province of Trento during Forest Tree Damage Monitoring (FTDM) surveys (Salvadori et al. 2002). In subsequent years, cankers were observed in declining *Ostrya* wood in Friuli, and they were also recorded in Lombardy and the Apennines, but not related to dieback symptoms (Maresi, personal observation).

Necrotized bark on branches and trunks is the first visual symptom of the disease. The necrosis can spread rapidly throughout the whole trunk and branches of the tree, which subsequently die. Alternatively, the disease ceases to spread and perennial cankers form in the following years (Jurc et al. 2006). Ascospores of *Botryosphaeria dothidea* (Moug.) Ces. & De Not. were observed in the dead bark of *O. carpinifolia* in the winter, and conidiomata of the anamorph *Fusicoccum aesculi* Sacc. were seen during the growing season (Jurc et al. 2006).

Members of the Botryosphaeriaceae produce asci and ascospores in pseudothecia (teleomorph). Their conidia produced in conidiomata are either pigmented and *Diplodia*-like or hyaline and *Fusicoccum*-like (anamorph). This fungal family has had a confusing taxonomic history, where names were initially linked to hosts and based on morphological characteristics. The emergence of DNA-based phylogenetic inference has allowed for a more natural classification of the Botryosphaeriaceae (Crous et al. 2006; Slippers et al. 2004). The teleomorphs of species in this group are rarely observed in nature or under laboratory conditions, and they consequently have little taxonomic value due to overlapping morphological characteristics (Slippers et al. 2004). Anamorph morphology, usually adopted to identify species of Botryosphaeriaceae, is also unable to resolve closely related species (Denman et al. 2000; Slippers et al. 2004). Comparisons of DNA sequences for various gene regions have led to re-evaluation of the Botryosphaeriaceae, and numerous new genera and species have been proposed (Crous et al. 2006; Denman et al. 2000; Pavlic et al. 2009; Phillips et al. 2005, 2008; Slippers et al. 2004, 2005a, 2007).

Species of the Botryosphaeriaceae have a wide global distribution on virtually all woody hosts examined to date (Burgess et al. 2005, 2006; Ma et al. 2001a; Mohali et al. 2006, 2007; Pavlic et al. 2007; Slippers et al. 2005a, 2007; Slippers et al. 2005b; Slippers and Wingfield 2007; Taylor et al. 2005). Different species of Botryosphaeriaceae can be

isolated from diseased and healthy tissues of the same host (Mohali et al. 2006; Pavlic et al. 2007; Slippers and Wingfield 2007). Endophytic isolates obtained from healthy material have been shown to cause disease symptoms in greenhouse trials (Pavlic et al. 2007). Many of these species are known as opportunistic pathogens with a latent endophytic stage, causing disease symptoms on stressed plants (Slippers and Wingfield 2007; Swart and Wingfield 1991). Pathogenic activity of the Botryosphaeriaceae, as latent opportunistic pathogens, is expected to increase due to climate changes (Desprez-Loustau et al. 2006; Slippers and Wingfield 2007). Extremely dry weather conditions are believed to provoke these quiescent facultative parasites, living in dead parts or as latent endophytes in living tissues to cause disease (Desprez-Loustau et al. 2006).

The study was conducted following preliminary surveys showing that *B. dothidea* was connected to dieback of *O. carpinifolia* in Slovenia (Jurc et al. 2006). Additional investigation has revealed further diversity and potentially other Botryosphaeriaceae that might be involved in the disease. The primary aim was to consider whether the disease observed on *O. carpinifolia* in Slovenia and Italy is due to a recently introduced pathogenic species and alternatively whether this is associated with a native population of opportunistic pathogens induced to cause disease due to altered environmental factors. To achieve this goal, intra- and interspecies diversity of Botryosphaeriaceae associated with dieback of *O. carpinifolia* and other tree species in Slovenia and Italy was characterized. This was accomplished via analyses of sequence data for the ITS rDNA and translation elongation factor 1- α (EF1- α), PCR-RFLP and AFLP profiles, morphological characteristics and pathogenicity tests.

Materials and methods

Isolates

The majority of isolates included in this study were obtained during a 2005 and 2006 disease survey undertaken in the western part of Slovenia (Kras) and including *O. carpinifolia*, *Acer platanoides* L., *Juniperus communis* L. and *Cotinus coggygria* Scop. trees. The isolates from Italy were obtained in the provinces of Trento and Bologna in 2006. Isolates were taken from necrotic bark, dead branches and from asymptomatic tissues. Healthy tissues were washed in tap water and treated with more concentrated disinfecting solutions comparing with diseased and more degraded tissues. One-millimeter-wide strips were excised from the margins of the necrotic tissue and surface disinfected by submerging for 3 min in commercial bleach (NaOCl, 6% free Cl), diluted to contain 3% active chlorine.

Strips were cut into segments ($\sim 1 \text{ mm}^2$) and blotted dry. Asymptomatic branches were washed in running tap water for 2 h prior to isolation. Bark strips ($\sim 20 \text{ mm}^2$) were excised and disinfected for 1 min in 6% commercial bleach. Segments ($\sim 1 \text{ mm}^2$) of the disinfected strips were placed on 2% malt extract agar (MEA; 2% malt extract, 1.5% agar; Biolife) and incubated at 24°C. Some isolates were obtained directly from the perithecia embedded in the diseased bark. All isolates used in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

DNA extraction

DNA was extracted from pure cultures of all isolates using PrepMan[®] Ultra Sample Preparation Reagent (Applied Biosystems). The nucleic acid concentrations in DNA extracts were determined with an ND-1000 Spectrophotometer (NanoDrop Technologies). The original DNA extracts were diluted with distilled water to concentrations of 30–60 ng/ μl and used in further reactions unless otherwise specified.

PCR amplification

The amplification of the ITS rDNA region, comprising the 3' end of the 18S rRNA gene, the first internal transcribed spacer ITS1, the 5.8S rRNA gene, ITS2 and the 5' end of the 26S rRNA gene, was performed with the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). A part of elongation factor 1- α (EF1- α) was amplified using the primer pair EF-Af (5'-CATCGAGAAGTTCGAGAA-3') and EF-Br (5'-CRATGGTGATACCRCGCTC-3') (Sakalidis 2004). The PCR mixtures and cycling parameters were as described by Slippers et al. (2004). The PCR products were separated in a 1 \times Tris-acetate EDTA (TAE) 1.5% agarose gel (Agarose LE, Roche) with ethidium bromide added to a final concentration of $5 \times 10^{-4} \mu\text{g/ml}$. The bands were visualized under UV light, and the sizes of PCR products were estimated using molecular weight marker (O'Gene Ruler[™] 100 bp DNA Ladder Plus, Fermentas).

PCR–RFLP analysis

The ITS rDNA PCR–RFLP analyses were performed for all isolates collected in this study. For PCR–RFLP analysis, the ITS rDNA PCR products were digested with the restriction endonuclease *CfoI* (Roche Diagnostics). The reaction mixture contained 10 or 12 μl of PCR product, 0.3 μl of 10 U/ μl *CfoI*, 2 μL of matching enzyme buffer

and an adequate volume of distilled water to reach the final volume of 15 μl . Restriction digests were performed at 37°C for 12 h. The fragments were separated on a 3% agarose gel as previously described for PCR products. The resulting restriction profiles were compared with those of Slippers et al. (2007).

Morphological characterization

Selected isolates, representing different localities, hosts, morphological characteristics and RFLP profiles, were transferred onto 2% water agar (WA; Biolab) with sterilized pine needles and incubated at 25°C under near-UV light, to induce sporulation. Released conidia were collected and mounted in 85% lactic acid on microscope slides. Slides were examined, and spores photographed and measured under a light microscope with an Axiocam digital camera and accompanying software (Carl Zeiss Ltd).

DNA sequencing and analyses

The ITS rDNA and EF1- α regions of selected isolates, representing different localities, hosts and morphological characteristics, were sequenced (Table 1). The PCR products were cleaned using Centri-Sep Spin Columns (Applied Biosystems) with Sephadex[®] G-50 (Sigma–Aldrich) or using the High Pure PCR Product Purification Kit (Roche Diagnostics). Sequencing was performed in both the forward and reverse directions using the same primers as for the PCR. The ABI PRISM[™] Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer) was used for sequencing reactions according to the manufacturer's instructions. Sequencing reactions were run on an ABI PRISM 3100[™] automated DNA sequencer (Perkin–Elmer).

All sequences were checked manually, and indistinct nucleotides were clarified by comparing sequences from both strands. Sequence data were analyzed using Chromas Lite Version 2.01 (Technelysium Pty Ltd) and aligned with ClustalW, available within the program pack MEGA Version 4.0.2 (Tamura et al. 2007).

Phylogenetic analysis was conducted in MEGA 4.0.2 (Tamura et al. 2007). Phylogenetic relationships were estimated using neighbor-joining (NJ) analysis (Saitou and Nei 1987) performed using the maximum composite likelihood method and pairwise deletion options. Bootstrapping (2,000 replicates) was performed to assess the confidence level at each branch. Sequences used for phylogenetic analyses are listed in Tables 1 and 2.

AFLP analyses

AFLP analyses of genomic DNA from selected *B. dothidea* isolates (Table 1) were performed following the guidelines

Table 1 Fungal isolates subjected to anamorph morphology characterization, DNA sequence analyses, phylogenetic studies, PCR-RFLP, pathogenicity trials and AFLP analyses

| Culture No. ^{a,b} | Other No. | Identity | Host | Location | Isolated from | Collector | PCR-RFLP profile ^c | AFLP ^f | EMBL accession No. ^d | |
|------------------------------|-----------|--------------------|-------------------------|----------------------|-------------------------|-------------------|-------------------------------|-------------------|---------------------------------|---------------|
| | | | | | | | | | ITS | EF-1 α |
| CMW 25688 | APK 5/1 | <i>B. dothidea</i> | <i>A. platanoides</i> | Gorjansko, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25689^e | APK 4/2 | <i>B. dothidea</i> | <i>A. platanoides</i> | Gorjansko, SI | Necrotized bark | D. Jurec/N. Ogris | / | * | FM955373 | FM955407 |
| CMW 26223 | APK 3/3 | <i>B. dothidea</i> | <i>A. platanoides</i> | Gorjansko, SI | Necrotized bark | D. Jurec/N. Ogris | / | * | FM955372 | FM955408 |
| CMW 25690 | APK 1/4 | <i>B. dothidea</i> | <i>A. platanoides</i> | Gorjansko, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | FM955374 | |
| CMW 25691 | APK 2/4 | <i>B. dothidea</i> | <i>A. platanoides</i> | Gorjansko, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25692 | CST 1/3 | <i>B. dothidea</i> | <i>C. coggygria</i> | Gorjansko, SI | Dead bark of cut branch | D. Jurec/N. Ogris | II | * | FM955375 | FM955409 |
| CMW 25693 | CST 2/3 | <i>B. dothidea</i> | <i>C. coggygria</i> | Gorjansko, SI | Dead bark of cut branch | D. Jurec/N. Ogris | II | * | | |
| CMW 25686 | OPIT 17 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Cast Dante, IT | Necrotized bark | G. Maresi | / | | FM955381 | FM955414 |
| CMW 26221 | OPIT 18 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Cast Dante, IT | Necrotized bark | G. Maresi | II | | FM955382 | |
| CMW 26222 | OEIT 24 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Nomi, IT | Green bark | G. Maresi | II | * | FM955376 | FM955410 |
| CMW 26220 | OPIT 3 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | S. Michele, IT | Necrotized bark | G. Maresi | II | * | FM955380 | |
| CMW 25687 | OEIT 25 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | S. Michele, IT | Green bark | G. Maresi | II | * | | |
| CMW 25694 | OPG 5/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Gropajska Gmajna, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25695 | OPG 6/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Gropajska Gmajna, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25696^e | OPG 7/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Gropajska Gmajna, SI | Necrotized bark | D. Jurec/N. Ogris | / | * | FM955379 | FM955413 |
| CMW 25697 | OPG 8/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Gropajska Gmajna, SI | Necrotized bark | D. Jurec/N. Ogris | II | | | |
| CMW 25698 | OPG 3/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Gropajska Gmajna, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25699 | OPK 1/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | II | | | |
| CMW 26700 | OPK 2/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25701 | OPK 3/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 26702 | OPK 6/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | II | | | |
| CMW 25703 | OPK 10/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | II | | | |
| CMW 25704 | OPK 1/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | / | * | | |
| CMW 25705 | OPK 2/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | / | * | | |
| CMW 25706 | OPK 4/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | / | * | | |
| CMW 25707 | OPK 5/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25708 | OPK 7/2 A | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25709 | OPK 9/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jurec/N. Ogris | / | * | | |

Table 1 continued

| Culture No. ^{ab} | Other No. | Identity | Host | Location | Isolated from | Collector | PCR-RFLP profile ^e | AFLP ^f | EMBL accession No. ^d | |
|------------------------------|-----------|--------------------|-------------------------|-----------------|----------------------------|-------------------|-------------------------------|-------------------|---------------------------------|---------------|
| | | | | | | | | | ITS | EF-1 α |
| CMW 25710 | OPERK 1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Križ, SI | Perithecium from dead bark | D. Jurec/N. Ogris | / | * | FM955378 | FM955412 |
| CMW 25711 | OPERO 4 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Opatje Selo, SI | Perithecium from dead bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25712 | OPERO 5 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Opatje Selo, SI | Perithecium from dead bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25713 | OPERO 6 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Opatje Selo, SI | Perithecium from dead bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25714 | OPO 2/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Opatje Selo, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25715 | OPO 4/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Opatje Selo, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25716 | OPO 3/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Opatje Selo, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25717 | OPO 1/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Opatje Selo, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25718 | OPP 1/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Podgorje, SI | Necrotized bark | D. Jurec/N. Ogris | / | * | | |
| CMW 25719 | OPP 6/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Podgorje, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25720 | OPP 6/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Podgorje, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25721 | OPR 1/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Ravnje, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25722 | OPR 3/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Ravnje, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25723 | OPR 5/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Ravnje, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25724 | OPR 6/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Ravnje, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25725 | OPR 9/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Ravnje, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25726 | OPR 4/X | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Ravnje, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25727 | OER 9/A1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Ravnje, SI | Green bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25728 | OPS 1/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Stolovec, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25729 | OPS 4/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Stolovec, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25730 | OPS 6/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Stolovec, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25731 | OPS 5/X | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Stolovec, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25732 | OPS 10/X | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Stolovec, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 26224^c | OES 4/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Stolovec, SI | Green bark | D. Jurec/N. Ogris | II | * | FM955377 | FM955411 |
| CMW 25733 | OES 8/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Stolovec, SI | Green bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25734 | OPERSG 1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Sv. Gora, SI | Perithecium from dead bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25735 | OPERSG 2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Sv. Gora, SI | Perithecium from dead bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25736 | OPSG 1/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Sv. Gora, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25737 | OPSG 4/1 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Sv. Gora, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |
| CMW 25738 | OPSG 2/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Sv. Gora, SI | Necrotized bark | D. Jurec/N. Ogris | II | * | | |

Table 1 continued

| Culture No. ^{a,b} | Other No. | Identity | Host | Location | Isolated from | Collector | PCR-RFLP profile ^c | AFLP ^f | EMBL accession No. ^d | |
|------------------------------|------------|------------------------|-------------------------|-----------------|-----------------|------------------|-------------------------------|-------------------|---------------------------------|---------------|
| | | | | | | | | | ITS | EF-1 α |
| CMW 25739 | OPSG 3/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Sv. Gora, SI | Necrotized bark | D. Jure/N. Ogris | II | * | | |
| CMW 25740 | OPSG 5/2 | <i>B. dothidea</i> | <i>O. carpiniifolia</i> | Sv. Gora, SI | Necrotized bark | D. Jure/N. Ogris | II | * | | |
| CMW 25743 | OEIT 28 | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | Lochere, IT | Green bark | G. Maresi | III | | FM955386 | FM955418 |
| CMW 25744 | OPIT 1 | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | Monte Mario, IT | Necrotized bark | G. Maresi | I | | FM955387 | FM955419 |
| CMW 25745 | OPIT 11 | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | Pomarolo, IT | Necrotized bark | G. Maresi | I | | | |
| CMW 25746 | OPIT 13 | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | Pomarolo, IT | Necrotized bark | G. Maresi | / | | FM955388 | FM55420 |
| CMW 25747 | OEIT 22 | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | Pomarolo, IT | Green bark | G. Maresi | I | | | |
| CMW 25748 | OPIT 2 | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | S. Michele, IT | Necrotized bark | G. Maresi | I | | | |
| CMW 25749 | OPIT 6 | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | S. Michele, IT | Necrotized bark | G. Maresi | I | | | |
| CMW 25750 | OEIT 27 | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | S. Michele, IT | Green bark | G. Maresi | I | | FM955385 | FM55417 |
| CMW 25751^e | CST 3/2 | <i>Dothiorella</i> sp. | <i>O. coggygia</i> | Gorjansko, SI | Dead branch | D. Jure/N. Ogris | I | | FM955384 | FM955416 |
| CMW 25752^e | BRINPK 1/1 | <i>Dothiorella</i> sp. | <i>J. communis</i> | Križ, SI | Dead branch | D. Jure/N. Ogris | I | | FM955383 | FM955415 |
| CMW 26361^e | OPK 1/X | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | Križ, SI | Necrotized bark | D. Jure/N. Ogris | / | | FM955389 | FM955421 |
| CMW 26362 | OPP 4/1 | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | Podgorje, SI | Necrotized bark | D. Jure/N. Ogris | / | | FM955390 | FM955422 |
| CMW 25753 | OPP 9/X | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | Podgorje, SI | Necrotized bark | D. Jure/N. Ogris | / | | FM955391 | FM955423 |
| CMW 25754^e | OPR 8/2 | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | Ravnje, SI | Necrotized bark | D. Jure/N. Ogris | I | | FM955392 | FM955424 |
| CMW 25755 | OPS 7/x | <i>Dothiorella</i> sp. | <i>O. carpiniifolia</i> | Stolovec, SI | Necrotized bark | D. Jure/N. Ogris | I | | | |

^a Culture collection: CMW = Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa

^b Anamorph morphology of isolates given in bold was examined microscopically

^c Isolates selected for pathogenicity trials

^d Nucleotide sequences, sequenced in this study and deposited in the EMBL Nucleotide Sequence database

^e Different RFLP profiles of ITS rDNA PCR products with the restriction enzyme *CfoI* are marked with I, II and III; slash sign (/) indicates isolates where RFLP reactions were not successful

^f *B. dothidea* isolates included in the AFLP analyses

Table 2 Nucleotide sequences obtained from the GenBank nucleotide database and used in phylogenetic analyses

| Fungal species | Isolate | GenBank | | Reference |
|---------------------------------------|------------|----------|---------------|--|
| | | ITS | EF-1 α | |
| <i>Dothiorella</i> sp. | JL 599 | EU673314 | EU673281 | Phillips et al. (2008) |
| <i>Dothiorella</i> sp. | CAP 187 | EU673313 | EU673280 | Phillips et al. (2008) |
| <i>Dothiorella</i> sp. | CAA 005 | EU673312 | EU673279 | Phillips et al. (2008) |
| <i>Dothiorella moneti</i> | MUCC 506 | EF591921 | EF591972 | Taylor et al. (2009) |
| <i>Dothiorella santali</i> | MUCC 509 | EF591924 | EF591975 | Taylor et al. (2009) |
| <i>Dothiorella longicollis</i> | CBS 122068 | EU144054 | EU144069 | Pavlic et al. (2008) |
| <i>Dothiorella longicollis</i> | CBS 122067 | EU144053 | EU144068 | Pavlic et al. (2008) |
| <i>Dothiorella iberica</i> | CBS 115041 | AY573202 | AY573222 | Phillips et al. (2005) |
| <i>Dothiorella iberica</i> | CBS 113188 | AY573198 | EU673278 | Phillips et al. (2005, 2008) |
| <i>Dothiorella sarmentorum</i> | CBS 115038 | AY573206 | AY573223 | Phillips et al. (2005) |
| <i>Diplodia acerina</i> | CBS 910.73 | EU673315 | EU673282 | Phillips et al. (2008) |
| <i>Dothiorella sarmentorum</i> | IMI 63581b | AY573212 | AY573235 | Phillips et al. (2005) |
| <i>Diplodia coryli</i> | CBS 242.51 | EU673317 | EU673284 | Phillips et al. (2008) |
| <i>Diplodia juglandis</i> | CBS188.87 | EU673316 | EU673283 | Phillips et al. (2008) |
| <i>Spencermartinsia viticola</i> | CBS 117010 | AY905558 | AY905561 | Luque et al. (2005) |
| <i>Botryosphaeria dothidea</i> | CMW 8000 | AY236949 | AY236898 | Slippers et al. (2004) |
| <i>Botryosphaeria dothidea</i> | CBS 110302 | AY259092 | AY573218 | Alves et al. (2004), Phillips et al. (2005) |
| <i>Botryosphaeria corticis</i> | CBS 119047 | DQ299245 | EU017539 | Lazzizzera et al. (2008), Phillips et al. (2006) |
| <i>Neofusicoccum parvum</i> | CBS 110301 | AY259098 | AY573221 | Alves et al. (2004), Phillips et al. (2005) |
| <i>Neofusicoccum mangiferum</i> | CMW 7024 | AY615185 | DQ093221 | Burgess et al. (2005), Slippers et al. (2005a) |
| <i>Neofusicoccum luteum</i> | CBS 110299 | AY259091 | AY573217 | Alves et al. (2004), Phillips et al. (2005) |
| <i>Lasiodiplodia crassispora</i> | WAC 12533 | DQ103550 | DQ103557 | Burgess et al. (2006) |
| <i>Lasiodiplodia gonubiensis</i> | CBS 115812 | DQ458892 | DQ458877 | Alves et al. (2006) |
| <i>Lasiodiplodia parva</i> | CBS 356.59 | EF22082 | EF622062 | Alves et al. (2008) |
| <i>Lasiodiplodia pseudotheobromae</i> | CBS 447.62 | EF622081 | EF622060 | Alves et al. (2008) |
| <i>Lasiodiplodia theobromae</i> | CBS 124.13 | DQ458890 | DQ458875 | Alves et al. (2006) |
| <i>Diplodia pinea</i> | CBS 393.84 | DQ458895 | DQ458880 | Alves et al. (2006) |
| <i>Diplodia seriata</i> | CBS 112555 | AY259094 | AY573220 | Alves et al. (2004), Phillips et al. (2005) |
| <i>Diplodia mutila</i> | CBS 112553 | AY259093 | AY573219 | Alves et al. (2004), Phillips et al. (2005) |
| <i>Diplodia rosulata</i> | CBS 116470 | EU430265 | EU430267 | Phillips et al. (2008) |
| <i>Spencermartinsia viticola</i> | CBS 117009 | AY905554 | AY905559 | Luque et al. (2005) |

of Vos et al. (1995). Genomic DNA, ranging in concentration from 50 to 100 ng/ μ l, was cut with the restriction enzymes *Eco*RI and *Mse*I. Corresponding *Eco*- and *Mse*-specific oligonucleotide adapters were used in the subsequent ligation step. Preselective amplification was performed with zero-base-addition *Eco*RI and *Mse*I adapter-specific primers. The success of the preamplification step was checked by electrophoresis on a 1.2% agarose gel in 1 \times TBE buffer (30 min at 80 V). Three different primer pair combinations of labeled *Eco*RI (E) and unlabeled *Mse*I (M) were used for final selective amplifications (M-02/E-AA, M-02/E-AAC and M-06/E-ACC). Primer E was 5' labeled with infrared dye (IRDye 700 or IRDye 800, LI-COR). PCR conditions and reaction mixtures were as

described by De Vos et al. (2007). An equal volume of loading solution (LI-COR) was added to each selective amplification reaction, followed by denaturation at 95°C for 3 min. The samples were placed on ice before gel loading. Electrophoresis and detection of AFLP fragments were performed on a LI-COR IR² (model 4200S) automated DNA analyzer, as described by Myburg et al. (2001). The electrophoresis run parameters were set to 1,500 V, 35 mA, 35 W, 45°C, motor speed 3 and signal filter 3. The electrophoresis prerun time was set to 30 min, and the run time was set to 4 h. The Quantar Pro 1.1 Program (KeyGene) was used for gel analysis. Visible and polymorphic AFLP bands were scored as present '1' or absent '0' at each position. The statistical program

STATISTICA Version 9 (Statsoft, USA) was used to make a dendrogram, using the cluster procedure with unweighted pair-group averages and percent disagreement.

Pathogenicity trials

Three *B. dothidea* isolates, three isolates representing *Dothiorella* sp. “A” group and one isolate from the *Dothiorella* sp. “C” group (Fig. 1) were selected to represent different localities, hosts, morphological and molecular characteristics and used in a pathogenicity trial

under laboratory conditions at 24°C (Table 1). Two combinations, CMW 25696 × CMW 25751 and CMW 25689 × CMW 25754, representing *B. dothidea* and *Dothiorella* sp. “A”, respectively, were used to test the synergistic effect of these species on disease development. *O. carpinifolia* cuttings, 35 cm long with a mid-diameter of around 30 mm were inoculated.

Additionally, the pathogenicity trials were designed to test the relative turgidity of bark as a possible factor that might influence pathogenicity. Therefore, two different trials were performed, named the “humid” and “dry”

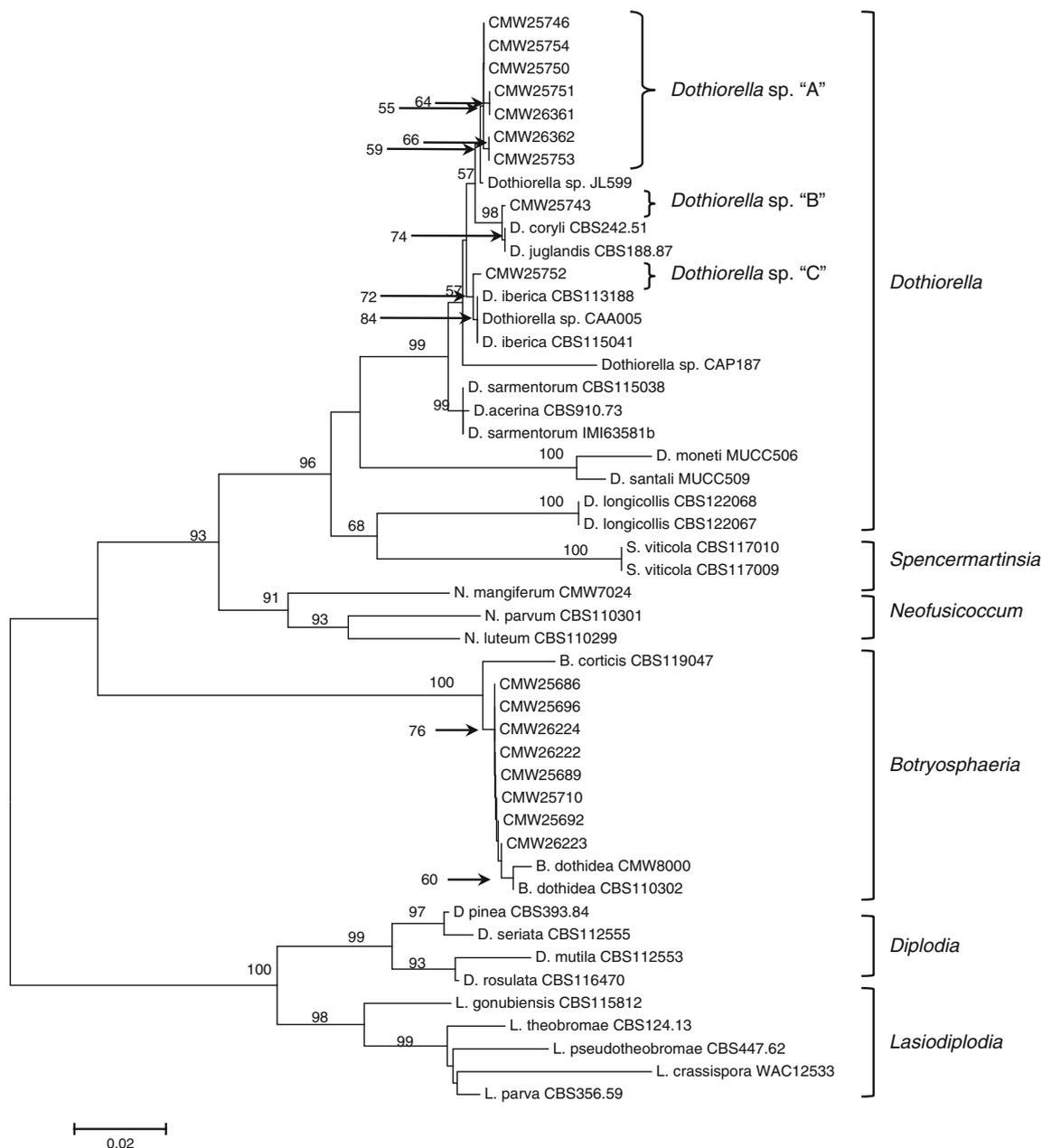


Fig. 1 Phylogenetic tree from combined EF-1 α and ITS rDNA dataset. The tree is unrooted and was constructed with the neighbor-joining method. Bootstrap values (2,000 replicates) are indicated at the nodes

experiments. In each experiment, ten cuttings were inoculated with each of the selected isolate (one inoculation per cutting), with a combination of the two isolates or with sterile MEA plugs as controls. All cuttings included in pathogenicity tests were harvested at one location in the western part of Slovenia, near Kozina, at 535 m above sea level, and each cutting was cut from a different tree.

Inoculations were performed in the late summer (August 2007) with one-week-old fungal cultures grown on MEA at 24°C. The cuttings were wounded with an 8-mm diameter cork borer to remove the bark. Mycelium plugs (8 mm in diameter) were placed into the wounds with the mycelium surface facing the cambium. For combined inoculations, halves of mycelial plugs of the selected isolates were placed in the same wound. Inoculation points were covered with laboratory film to prevent desiccation and contamination (Parafilm M, Pechiney Plastic Packaging). The “humid” experiment was performed using fresh *O. carpiniifolia* cuttings with the upper ends sealed with paraffin and the bottom ends freshly cut and placed in damp vermiculite, supplied with water weekly. In the “dry” experiment, the relative turgidity of the bark was lowered by drying. Inoculated cuttings, with both ends sealed with paraffin, were placed in a plastic bag in a woodpile arrangement with sheets of coarse filter paper between the layers and stored at room temperature. The filter papers were changed every week to ensure constant removal of humidity. The relative turgidity of the bark was determined for each cutting used in both experiments, at the beginning and at the end of the experiment following the method described by Weatherley (1950) and Bier (1959, 1961). Relative turgidity estimates involved the measurement of fresh weight, saturated weight and dry weight of bark samples. The relative turgidity is expressed as the ratio between the amount of water in a fresh bark sample and the amount of water required to saturate the same bark sample.

The pathogenicity was determined by measuring the lengths of lesions after one month. Two randomly selected cuttings representing inoculated isolates or uninoculated controls were selected for fungal re-isolations from the lesion edges. Statistical analyses (Duncan’s multiple comparison procedure) were performed with Statgraphics Plus (version 5.1, Statistical Graphics Corp.). The differences between means were determined at a 95% confidence level.

Results

PCR–RFLP analysis

Three different banding patterns were obtained after restriction with *Cfo*I. The restriction profile of the majority

of isolates resembled that of *B. dothidea*, as reported by Slippers et al. (2007). The remaining two profiles could not be matched with any of the patterns presented in the previous study (Table 1). The restriction reactions were not successful for a group of isolates, and some of these isolates were selected for further sequence analyses in order to determine their identity.

Morphological and molecular characterization

Regions approximately 550 (ITS rDNA) and 300 (EF1- α) base pairs (bp) in length were amplified and sequenced for selected isolates (Table 1). The sequence data for the ITS rDNA regions and EF1- α were combined and used for phylogenetic analyses. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. There were a total of 915 positions in the final dataset.

The grouping of isolates included in this comparison was consistent with the analysis performed by Phillips et al. (2008). Isolates obtained during the 2005 and 2006 surveys in Slovenia and Italy were grouped into *Botryosphaeria* and *Dothiorella* clades, based on the combined sequence data for the ITS rDNA and EF1- α gene regions (Fig. 1).

All but one of the selected isolates (CMW 25743) formed pycnidia on sterilized pine needles on WA after 2–3 weeks. No teleomorph structures were observed. Based on conidial morphology, including shape, color, septation and size, isolates were separated into two distinct groups. These included those (1) with *Diplodia*-like conidia that were brown and had one to three septa and conidia becoming pigmented and septate while still attached to conidiogenous cells and (2) those with *Fusicoccum*-like conidia that were hyaline and aseptate. These groupings were consistent with those emerging from the PCR–RFLP profiles and comparisons of the ITS rDNA and EF1- α sequences. Isolates with *Fusicoccum*-like conidia were identified as *B. dothidea*. These conidia were narrowly fusiform, and some were irregularly shaped with granular contents (average of 150 conidia: 24.1 \times 6.0 μ m). *Diplodia*-like conidia were pigmented, thick-walled and had rounded ends, one of which was usually truncated. Isolates with *Diplodia*-like conidia fell into the *Dothiorella* group of the Botryosphaeriaceae, where further divisions were made using DNA sequence comparisons. Isolate CMW 25752 (average of 50 conidia: 25.2 \times 9.6 μ m) was grouped close to *Dothiorella iberica* A.J.L. Phillips, J. Luque & A. Alves, but it resided a separate clade in the phylogenetic tree based on two DNA regions (Fig. 1). Definitive identification as *D. iberica* could not be obtained due to discrepancies in the conidial morphology among isolates. Conidia of isolate CMW 25752 were found to have one to three septa (Fig. 2), while *D. iberica* is



Fig. 2 *Dothiorella* sp. “C”. Conidia with one to three septa

reported to have smaller and wider conidia with one septum (Phillips et al. 2005). Consequently, this isolate was not assigned a name and it is referred to as *Dothiorella* sp. “C”.

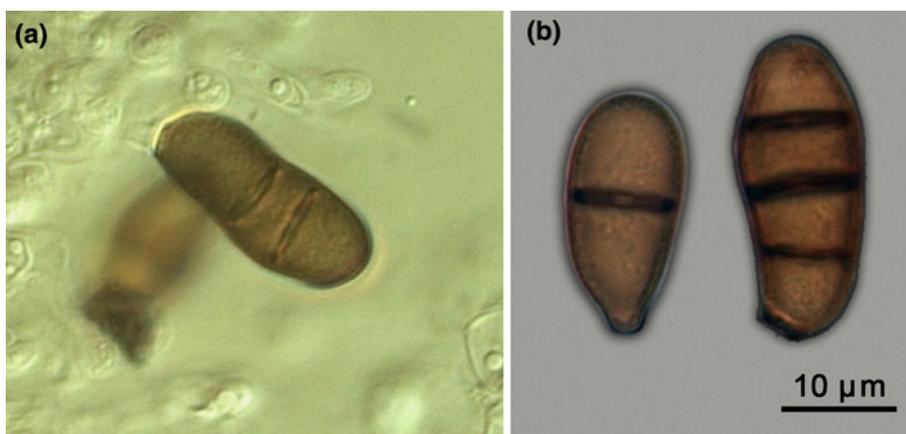
Seven of the isolates obtained during this study form a subclade within the genus *Dothiorella* (Fig. 1) and most probably represent a new species. These isolates were designated as *Dothiorella* sp. “A”. Conidia from these isolates were $20.9 \times 9.8 \mu\text{m}$ large (average of 350 conidia) and had one to three septa (Fig. 3).

One isolate, CMW 25743, showed a different PCR–RFLP profile to the other isolates included in this research and grouped with *Diplodia coryli* Fuckel and *Diplodia juglandis* Fr. in the phylogenetic tree (Fig. 1); it was designated as *Dothiorella* sp. “B”. The isolate failed to sporulate, as was also reported for the *D. coryli* and *D. juglandis* (Phillips et al. 2008).

AFLP analyses

Three primer combinations, selected to screen the genetic diversity of 50 *B. dothidea* isolates, yielded a total of 74 visible and polymorphic AFLP bands.

Fig. 3 *Dothiorella* sp. “A”. (a) Developing conidia and conidiogenous cells. (b) Conidia with one to three septa



Clustering analysis revealed two groups of isolates (Fig. 4). The larger group included the majority of *B. dothidea* isolates (48), isolated throughout the Kras region in Slovenia and Italy from asymptomatic and diseased *O. carpinifolia* and other hosts. Within this group, further groupings are evident. Isolates from *C. coggygria* and *A. platanoides*, except for APK 4/2 (CMW 25689), were grouped together. Isolates from *O. carpinifolia* were dispersed into different subgroups with no clear lineage linked to the locality or origin of isolation (plant tissue, symptomatic/asymptomatic). The other group included only two isolates, one isolated from a perithecium found on dead bark of *O. carpinifolia* in Opatje Selo, Slovenia and the other obtained from green bark of asymptomatic *O. carpinifolia* in Ravnje, Slovenia.

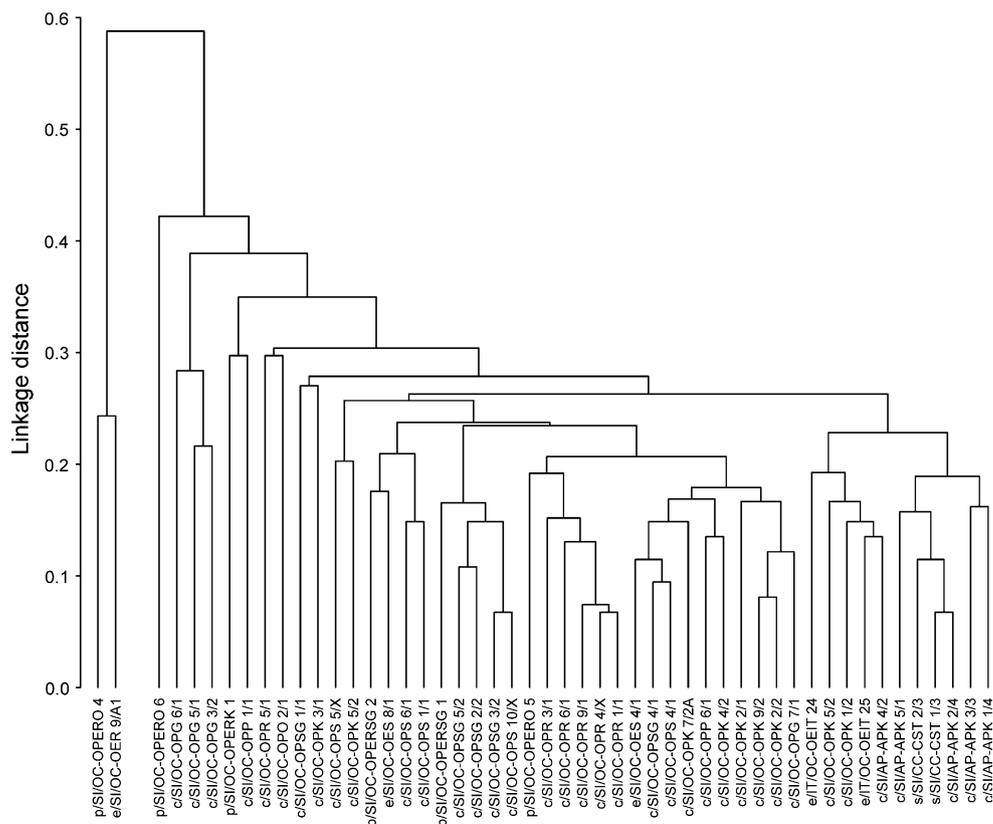
Pathogenicity

All isolates tested for pathogenicity on the *O. carpinifolia* cuttings produced bark lesions within four weeks, and the inoculated fungi were successfully re-isolated from selected inoculated cuttings. No lesions developed on control cuttings inoculated with sterile MEA plugs, and no Botryosphaeriaceae were obtained from these control inoculations.

At the beginning of experiments, the mean relative turgidity of the bark samples was $84.0\% \pm 0.5\%$ for the “dry” experiment and $84.0\% \pm 0.6\%$ for the “humid” experiment (at the 95% confidence level). The relative turgidity of the bark was significantly lower at the end of the dry experiment ($76.1\% \pm 0.8\%$), indicating a successful lowering of the bark moisture and thereby causing water stress to the inoculated cuttings. In the “humid” experiment, higher final moisture levels were established ($85.0\% \pm 0.6\%$) compared to the moisture levels at the start of the trial.

In both pathogenicity experiments, there was a great variation in the size of the lesions produced by individual

Fig. 4 Unweighted pair-group average (UPGMA) dendrogram based on 50 *Botryosphaeria dothidea* isolates and 74 AFLP polymorphic markers. Isolates are marked with their original designations (Table 1) to emphasize their origins. Abbreviations before isolate names denote the isolation material (p, perithecium from dead bark; c, canker-necrotized bark; e, green bark), country (SI, Slovenia; IT, Italy) and host (OC, *Ostrya carpinifolia*; AP, *Acer platanoides*; CC, *Cotinus coggygria*)



isolates (Fig. 5a, b). The mean lesion lengths for the *B. dothidea* isolates differed significantly from the control, except for isolate CMW 25696 in the “humid” experiment (Fig. 5b). The comparison between the results of the “humid” and “dry” experiments showed that lower relative bark turgidity generally results in longer lesions.

In both pathogenicity experiments, the isolates of *B. dothidea* produced extensive lesions and displayed higher levels of pathogenicity on *O. carpinifolia* cuttings than those of *Dothiorella* spp. (Fig. 5a, b). The *Dothiorella* spp. included in the pathogenicity trials produced lesions that were not statistically different from those of the uninoculated control, even though they were able to infect and cause lesions.

A *t*-test indicated that the mean lesion lengths produced by *B. dothidea* isolate were not significantly different to those of the corresponding inoculated combination with *Dothiorella*-like isolates in either the “dry” (Fig. 5a) or the “humid” experiment (Fig. 5b). Thus, there was no evidence of synergistic or antagonistic impacts of *Dothiorella* isolates on the pathogenic activity of *B. dothidea*.

Discussion

Among the 75 isolates collected from trees grown in autochthonous forests in Slovenia and Italy, different

Botryosphaeriaceae species were identified based on ITS rDNA and EF1- α sequence data, PCR-RFLP and anamorph morphology. The majority of the isolates were identified as *B. dothidea*, while others grouped in the genus *Dothiorella*. The isolates showed different degrees of pathogenicity on *O. carpinifolia* in laboratory trials, and *B. dothidea* was the most pathogenic of the fungi tested. Insight into the diversity of *B. dothidea* based on AFLP markers revealed a heterogeneous collection of isolates without patterns linked to geographic origin.

B. dothidea was the dominant species isolated from diseased *O. carpinifolia* trees in Slovenia and Italy. This species was also collected from cankers on *A. pseudo-platanus* trees and from pruned branches of *Cotinus coggygria* that had been left for few months in the vicinity of the shrub. Observations in the field also revealed branch dieback on *Cornus mas* L. and *Carpinus betulus* L., and fungal isolates were determined to be anamorphs of *B. dothidea* (unpublished observations). *B. dothidea* was also isolated from healthy branches, with no visual signs of disease, on *O. carpinifolia* both in Italy and Slovenia. Isolates of *B. dothidea* obtained from the same geographic location were dispersed throughout the AFLP marker-based tree. This distribution indicates that a heterogeneous *B. dothidea* population is present in the affected area. Additionally, isolates obtained from the same tree (e.g. CMW 25705 = OPK 2/2 and CMW 26700 = OPK 2/1)

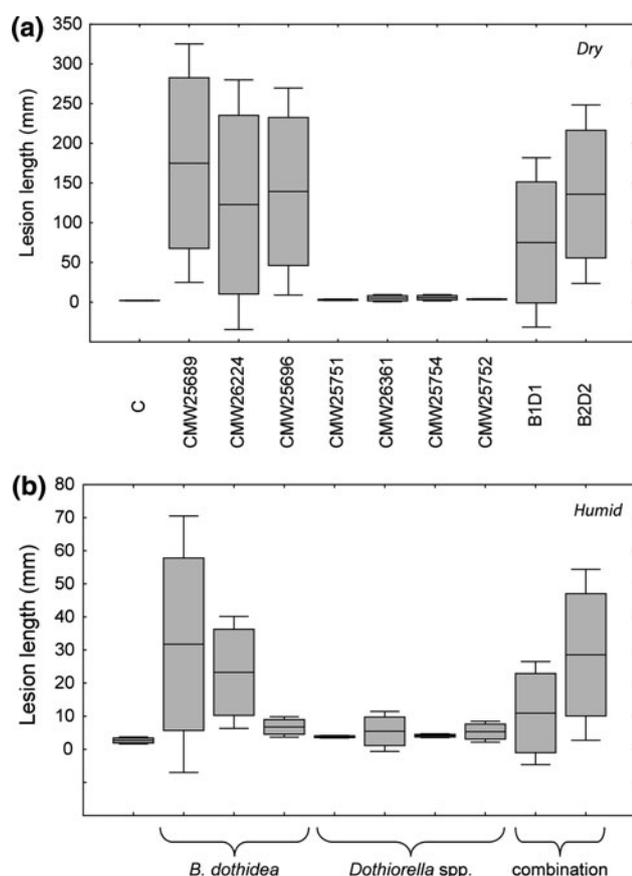


Fig. 5 Mean lesion lengths (mm) and 95% confidence intervals for each isolate of different species of Botryosphaeriaceae 4 weeks after inoculations on *Ostrya carpinifolia* cuttings at (a) dry experiment and (b) humid experiment. Control (C); *Botryosphaeria dothidea* (CMW 25689, CMW 25696, CMW 26224); *Dothiorella* sp. “A” (CMW 25751, CMW 25754, CMW 26361); *Dothiorella* sp. “C” (CMW 25752); B1D1 = combination of CMW 25696 × CMW 25751, B2D2 = combination of CMW 25689 × CMW 25754

did not group close together (Fig. 4), which suggests that a community of various different fungal individuals most likely exists within the same tree and wound. The large number of isolates obtained during the course of this study and reports of *B. dothidea* on other plants (e.g. *A. pseudoplatanus*, *Fraxinus excelsior* L., *Ostrya* sp., *Platanus* spp., *Populus nigra* L., *P. tremula* L., *Prunus* sp., *Vitis vinifera* L., *Quercus rubra* L., *Q. robur* L., *Q. suber* L., *Q. ilex* L.) in Europe (Alves et al. 2007; Grasso and Granata 2010; Slippers et al. 2004; Turco et al. 2006; van Niekerk et al. 2004) suggest a wide distribution of the species in this part of the world. Furthermore, the AFLP data from the *B. dothidea* population collected in this study in Slovenia and Italy showed no geographic or tissue-specific lineages, but groupings according to host were revealed. These results, together with the knowledge that *B. dothidea* is common on various native European trees, suggest that *O. carpinifolia* dieback in western Slovenia

and northern Italy is associated with a native population of *B. dothidea*.

An isolate obtained from diseased *A. pseudoplatanus* was able to successfully infect *O. carpinifolia* cuttings. This is consistent with previous observations for other members of the Botryosphaeriaceae (Ma et al. 2001a; Pavlic et al. 2007), where cross-infection and movement of *B. dothidea* among different hosts are possible and most likely also occur in affected areas. Different forecasts for the movement of Botryosphaeriaceae between native and introduced hosts and the expansion of hosts susceptible to Botryosphaeriaceae following climate changes have been made (Desprez-Loustau et al. 2006; Pavlic et al. 2007; Slippers and Wingfield 2007). Even though we observed a relatively small range of susceptible hosts in the field, we speculate that further extreme weather conditions will reduce resistance to *B. dothidea* infections and numerous woody host species could be affected.

All three *B. dothidea* isolates tested in this study were able to infect *O. carpinifolia* cuttings in in vitro pathogenicity trials and were successfully re-isolated from lesions. There were significant differences in the pathogenicity of the *B. dothidea* isolates on cuttings with retained moisture content. Interestingly, very low levels of pathogenicity were found for isolate CMW 25696, which originated from necrotic bark. In contrast, isolate CMW 26224, originating from healthy *O. carpinifolia* as an endophyte, was pathogenic. *B. dothidea* isolates analyzed on cuttings subjected to drying did not group into different virulence groups, even though they originated from different hosts and localities and from diseased or asymptomatic tissues. Pathogenicity of isolates collected as endophytes is not unusual and is consistent with the results of previous studies using other species of the Botryosphaeriaceae, recognized as endophytes, saprophytes and latent pathogens (Pavlic et al. 2007; Slippers and Wingfield 2007).

Various studies suggest that global climate change will cause a shift in potential areas and activity of pathogens and also in host susceptibility (Coakley et al. 1999; Desprez-Loustau et al. 2006). Periods of water stress were reported to increase susceptibility of trees to various latent pathogens, for example *Diplodia pinea* (Desm.) J. Kickx f. (Stanosz et al. 2001) and *Biscogniauxia mediterranea* (De Not.) Kuntze (Jurc and Ogris 2006; Vannini et al. 1996). Botryosphaeriaceae are known to be latent pathogens, and their pathogenic impact is also predicted to increase under stress-related conditions, such as those of drought (Crist and Schoeneweiss 1975; Ma et al. 2001b; Slippers and Wingfield 2007). Ma et al. (2001b) have investigated the relationship between water availability and *B. dothidea* growth. They observed that this pathogen grows more rapidly in pistachio leaves under water stress. Our comparison of pathogenicity under humid and dry

conditions clearly shows that lowering of the bark water content promotes pathogenicity of *B. dothidea*. As has been suggested elsewhere, stress most likely affects plant resistance to disease development and accelerates the growth of some pathogens (Desprez-Loustau et al. 2006). Bier (1959, 1961, 1964) showed a close correlation between the development of bark cankers caused by facultative parasites and moisture content of the bark. A correlation between bark moisture and pathogenic activity of tested isolates was also found in this study. The dry conditions in the laboratory experiment imitating drought resulted in extensive lesions and uniformity in expressed pathogenicity in different isolates of *B. dothidea*.

Slovenia and Italy have been confronted with changes in climatic conditions, similar to those also found in other parts of the world. *O. carpinifolia* dieback caused by species of the Botryosphaeriaceae was unknown until 1997 (Jurc et al. 2006), when the first reports of damages to hop hornbeam appeared. In 2007, European aspen (*Populus tremula* L.) was reported to be affected by *B. dothidea* in Italy (Grasso and Granata 2010). These reports together with the genetic heterogeneity of the *B. dothidea* population established during this study and the results of pathogenicity tests indicate that an existing pool of native endophytic fungi can become serious pathogens when higher temperatures and drought conditions occur.

A genetically diverse collection of *Dothiorella* spp. was obtained from *O. carpinifolia*, *C. coggygria* and *J. communis* in the affected area. Among the isolates of *Dothiorella* spp., discrepancies in conidial size and number of septa in conidia were observed. Color and septation were detected in conidia still attached to conidiogenous cells, which is typical for *Dothiorella* species (Phillips et al. 2005). Isolates were obtained from green bark of *O. carpinifolia*, dead branches of *C. coggygria*, and the majority of isolates were from necrotized bark of *O. carpinifolia*. Preliminary inoculation experiments with *Dothiorella* sp. “A” in forests under conditions of extreme drought showed development of extensive bark necroses (unpublished results). Inoculated isolates of *Dothiorella* spp. on *O. carpinifolia* cuttings in the pathogenicity tests reported in this study produced lesions not statistically different from those in control inoculations. However, these experiments did show its ability to infect bark and to cause lesions. It is questionable whether these fungi could be defined as potential pathogens. *Dothiorella* spp. and *B. dothidea* were isolated consistently from the necrotic bark that developed on the same tree. *B. dothidea* isolates displayed relatively high levels of pathogenicity on *O. carpinifolia* cuttings. We assume that this fungus has the main role in the disease development. Co-isolations of *Dothiorella* spp. and *B. dothidea* from the diseased tissues raise questions about the possible role of *Dothiorella* in disease development. However, pathogenicity tests with *B. dothidea*

in combination with isolates of *Dothiorella* sp. “A” showed no evidence that the latter fungus might inhibit or stimulate the pathogenic activity of *B. dothidea*.

O. carpinifolia dieback has been previously associated with *B. dothidea*, and this research confirms those findings. The disease occurrence is believed to be connected to extreme weather conditions that have provoked endophytic *B. dothidea* population to act pathogenic on stressed trees. The unforeseen disease occurrence and the insight into the diversity of the *B. dothidea* population causing the *O. carpinifolia* dieback follow predictions regarding the influence of global warming on disease occurrence in forest ecosystems.

Acknowledgments The study was financed by the Slovenian Research Agency and the Ministry of Agriculture, Forestry and Food of Republic of Slovenia through the research program P4-0107, projects L4-9585 and V4-0493, and Young Researcher Scheme for B. Piškur. Molecular studies were done in laboratories of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa and funded by the DST/NRF Centre of Excellence in Tree Health Biotechnology.

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