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

Barbara Piškur[1], Draginja Pavlic[2], Bernard Slippers[3], Nikica Ogris[1], Giorgio Maresi[4], Michael J. Wingfield[2] and Dušan Jurc[1]

(1) Department of Forest Protection, Slovenian Forestry Institute, Večna pot 2, 1000 Ljubljana, Slovenia.
(2) Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), Centre of Excellence in Tree Health Biotechnology, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, 0002, South Africa.
(3) Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), Centre of Excellence in Tree Health Biotechnology, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, 0002, South Africa.
(4) FEM-IASMA, Centre for Technology Transfer, Via E. Mach 1, 38010 San Michele all'Adige, TN, Italy.

Barbara Piškur
Email: barbara.piskur@gozdis.si

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*.

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). Ascomata 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 (~1 mm^2) and blotted dry. Asymptomatic branches were washed in running tap water for 2 h prior to isolation. Bark strips (~20 mm^2) were excised and disinfected for 1 min in 6% commercial bleach. Segments (~1 mm2) 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× Tris–acetate EDTA (TAE) 1.5% agarose gel (Agarose LE, Roche) with ethidium bromide added to a final concentration of 5 × 10−4 µg/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).

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[e]	AFLP[f]	EMBL accession No.[d]	
									ITS	EF-1α
CMW 25688	APK 5/1	*B. dothidea*	*A. platanoides*	Gorjansko, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25689[c]	APK 4/2	*B. dothidea*	*A. platanoides*	Gorjansko, SI	Necrotized bark	D. Jurc/N. Ogris	/	*	FM955373	FM955407
CMW 26223	APK 3/3	*B. dothidea*	*A. platanoides*	Gorjansko, SI	Necrotized bark	D. Jurc/N. Ogris	/	*	FM955372	FM955408
CMW 25690	APK 1/4	*B. dothidea*	*A. platanoides*	Gorjansko, SI	Necrotized bark	D. Jurc/N. Ogris	II	*	FM955374	
CMW 25691	APK 2/4	*B. dothidea*	*A. platanoides*	Gorjansko, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25692	CST 1/3	*B. dothidea*	*C. coggygria*	Gorjansko, SI	Dead bark of cut branch	D. Jurc/N. Ogris	II	*	FM955375	FM955409
CMW 25693	CST 2/3	*B. dothidea*	*C. coggygria*	Gorjansko, SI	Dead bark of cut branch	D. Jurc/N. Ogris	II	*		
CMW 25686	OPIT 17	*B. dothidea*	*O. carpinifolia*	Cast Dante, IT	Necrotized bark	G. Maresi	/		FM955381	FM955414
CMW 26221	OPIT 18	*B. dothidea*	*O. carpinifolia*	Cast Dante, IT	Necrotized bark	G. Maresi	II		FM955382	
CMW 26222	OEIT 24	*B. dothidea*	*O. carpinifolia*	Nomi, IT	Green bark	G. Maresi	II	*	FM955376	FM955410
CMW 26220	OPIT 3	*B. dothidea*	*O. carpinifolia*	S. Michele, IT	Necrotized bark	G. Maresi	II		FM955380	
CMW 25687	OEIT 25	*B. dothidea*	*O. carpinifolia*	S. Michele, IT	Green bark	G. Maresi	II	*		
CMW 25694	OPG 5/1	*B. dothidea*	*O. carpinifolia*	Gropajska Gmajna, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25695	OPG 6/1	*B. dothidea*	*O. carpinifolia*	Gropajska Gmajna, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25696[c]	OPG 7/1	*B. dothidea*	*O. carpinifolia*	Gropajska Gmajna, SI	Necrotized bark	D. Jurc/N. Ogris	/	*	FM955379	FM955413
CMW 25697	OPG 8/1	*B. dothidea*	*O. carpinifolia*	Gropajska Gmajna, SI	Necrotized bark	D. Jurc/N. Ogris	II			
CMW 25698	OPG 3/2	*B. dothidea*	*O. carpinifolia*	Gropajska Gmajna, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25699	OPK 1/1	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	II			
CMW 26700	OPK 2/1	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25701	OPK 3/1	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 26702	OPK 6/1	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	II			
CMW 25703	OPK 10/1	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	II			
CMW 25704	OPK 1/2	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	/	*		
CMW 25705	OPK 2/2	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	/	*		
CMW 25706	OPK 4/2	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	/	*		
CMW 25707	OPK 5/2	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25708	OPK 7/2 A	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25709	OPK 9/2	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	/	*		
CMW 25710	OPERK 1	*B. dothidea*	*O. carpinifolia*	Kišč, SI	Pesthecium from dead bark	D. Jurc/N. Ogris	/	*	FM955378	FM955412
CMW 25711	OPERO 4	*B. dothidea*	*O. carpinifolia*	Opatje Selo, SI	Pesthecium from dead bark	D. Jurc/N. Ogris	II	*		
CMW 25712	OPERO 5	*B. dothidea*	*O. carpinifolia*	Opatje Selo, SI	Pesthecium from dead bark	D. Jurc/N. Ogris	II	*		
CMW 25713	OPERO 6	*B. dothidea*	*O. carpinifolia*	Opatje Selo, SI	Pesthecium from dead bark	D. Jurc/N. Ogris	II	*		
CMW 25714	OPO 2/1	*B. dothidea*	*O. carpinifolia*	Opatje Selo, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25715	OPO 4/1	*B. dothidea*	*O. carpinifolia*	Opatje Selo, SI	Necrotized bark	D. Jurc/N. Ogris	II			
CMW 25716	OPO 3/2	*B. dothidea*	*O. carpinifolia*	Opatje Selo, SI	Necrotized bark	D. Jurc/N. Ogris	II			
CMW 25717	OPO 1/1	*B. dothidea*	*O. carpinifolia*	Opatje Selo, SI	Necrotized bark	D. Jurc/N. Ogris	II			
CMW 25718	OPP 1/1	*B. dothidea*	*O. carpinifolia*	Podgorje, SI	Necrotized bark	D. Jurc/N. Ogris	/	*		
CMW 25719	OPP 6/1	*B. dothidea*	*O. carpinifolia*	Podgorje, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25720	OPP 6/2	*B. dothidea*	*O. carpinifolia*	Podgorje, SI	Necrotized bark	D. Jurc/N. Ogris	II			
CMW 25721	OPR 1/1	*B. dothidea*	*O. carpinifolia*	Ravnje, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25722	OPR 3/1	*B. dothidea*	*O. carpinifolia*	Ravnje, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25723	OPR 5/1	*B. dothidea*	*O. carpinifolia*	Ravnje, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25724	OPR 6/1	*B. dothidea*	*O. carpinifolia*	Ravnje, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25725	OPR 9/1	*B. dothidea*	*O. carpinifolia*	Ravnje, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25726	OPR 4/X	*B. dothidea*	*O. carpinifolia*	Ravnje, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25727	OER 9/A1	*B. dothidea*	*O. carpinifolia*	Ravnje, SI	Green bark	D. Jurc/N. Ogris	II	*		
CMW 25728	OPS 1/1	*B. dothidea*	*O. carpinifolia*	Stolovec, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25729	OPS 4/1	*B. dothidea*	*O. carpinifolia*	Stolovec, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25730	OPS 6/1	*B. dothidea*	*O. carpinifolia*	Stolovec, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25731	OPS 5/X	*B. dothidea*	*O. carpinifolia*	Stolovec, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25732	OPS 10/X	*B. dothidea*	*O. carpinifolia*	Stolovec, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 26224[c]	OES 4/1	*B. dothidea*	*O. carpinifolia*	Stolovec, SI	Green bark	D. Jurc/N. Ogris	II	*	FM955377	FM955411
CMW 25733	OES 8/1	*B. dothidea*	*O. carpinifolia*	Stolovec, SI	Green bark	D. Jurc/N. Ogris	II	*		
CMW 25734	OPERSG 1	*B. dothidea*	*O. carpinifolia*	Sv. Gora, SI	Pesthecium from dead bark	D. Jurc/N. Ogris	II	*		
CMW 25735	OPERSG 2	*B. dothidea*	*O. carpinifolia*	Sv. Gora, SI	Pesthecium from dead bark	D. Jurc/N. Ogris	II	*		
CMW 25736	OPSG 1/1	*B. dothidea*	*O. carpinifolia*	Sv. Gora, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25737	OPSG 4/1	*B. dothidea*	*O. carpinifolia*	Sv. Gora, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25738	OPSG 2/2	*B. dothidea*	*O. carpinifolia*	Sv. Gora, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25739	OPSG 3/2	*B. dothidea*	*O. carpinifolia*	Sv. Gora, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25740	OPSG 5/2	*B. dothidea*	*O. carpinifolia*	Sv. Gora, SI	Necrotized bark	D. Jurc/N. Ogris	II	*		
CMW 25743	OEIT 28	*Dothiorella* sp.	*O. carpinifolia*	Lochere, IT	Green bark	G. Maresi	III		FM955386	FM955418
CMW 25744	OPIT 1	*Dothiorella* sp.	*O. carpinifolia*	Monte Mario, IT	Necrotized bark	G. Maresi	I		FM955387	FM955419
CMW 25745	OPIT 11	*Dothiorella* sp.	*O. carpinifolia*	Pomaretto, IT	Necrotized bark	G. Maresi	I			
CMW 25746	OPIT 13	*Dothiorella* sp.	*O. carpinifolia*	Pomaretto, IT	Necrotized bark	G. Maresi	/		FM955388	FM955420
CMW 25747	OEIT 22	*Dothiorella* sp.	*O. carpinifolia*	Pomaretto, IT	Green bark	G. Maresi	I			
CMW 25748	OPIT 2	*Dothiorella* sp.	*O. carpinifolia*	S. Michele, IT	Necrotized bark	G. Maresi	I			
CMW 25749	OPIT 6	*Dothiorella* sp.	*O. carpinifolia*	S. Michele, IT	Necrotized bark	G. Maresi	I			
CMW 25750	OEIT 27	*Dothiorella* sp.	*O. carpinifolia*	S. Michele, IT	Green bark	G. Maresi	I		FM955385	FM955417
CMW 25751[c]	CST 3/2	*Dothiorella* sp.	*C. coggygria*	Gorjansko, SI	Dead branch	D. Jurc/N. Ogris	I		FM955384	FM955416
CMW 25752[c]	BRINPK 1/1	*Dothiorella* sp.	*J. communis*	Kišč, SI	Dead branch	D. Jurc/N. Ogris	I		FM955383	FM955415
CMW 26361[c]	OPK 1/X	*Dothiorella* sp.	*O. carpinifolia*	Kišč, SI	Necrotized bark	D. Jurc/N. Ogris	/		FM955389	FM955421
CMW 26362	OPP 4/1	*Dothiorella* sp.	*O. carpinifolia*	Podgorje, SI	Necrotized bark	D. Jurc/N. Ogris	/		FM955390	FM955422
CMW 25753	OPP 9/X	*Dothiorella* sp.	*O. carpinifolia*	Podgorje, SI	Necrotized bark	D. Jurc/N. Ogris	/		FM955391	FM955423
CMW 25754[c]	OPR 8/2	*Dothiorella* sp.	*O. carpinifolia*	Ravnje, SI	Necrotized bark	D. Jurc/N. Ogris	I		FM955392	FM955424
CMW 25755	OPS 7/x	*Dothiorella* sp.	*O. carpinifolia*	Stolovec, SI	Necrotized bark	D. Jurc/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

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 of genomic DNA from selected *B. dothidea* isolates (Table 1) were performed following the guidelines of Vos *et al*. (1995). Genomic DNA, ranging in concentration from 50 to 100 ng/µl, 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 EcoRI and MseI adapter-specific primers. The success of the preamplification step was checked by electrophoresis on a 1.2% agarose gel in 1 × 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.

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

Fungal species	Isolate	GenBank		Reference
		ITS	EF-1α	
Dothiorella sp.	JL 599	EU673314	EU673281	Phillips et al. (2008)
Dothiorella sp.	CAP 187	EU673313	EU673280	Phillips et al. (2008)
Dothiorella sp.	CAA 005	EU673312	EU673279	Phillips et al. (2008)
Dothiorella moreti	MUCC 506	EF591921	EF591972	Taylor et al. (2009)
Dothiorella santali	MUCC 509	EF591924	EF591975	Taylor et al. (2009)
Dothiorella longicollis	CBS 122068	EU144054	EU144069	Pavlic et al. (2008)
Dothiorella longicollis	CBS 122067	EU144053	EU144068	Pavlic et al. (2008)
Dothiorella iberica	CBS 115041	AY573202	AY573222	Phillips et al. (2005)
Dothiorella iberica	CBS 113188	AY573198	EU673278	Phillips et al. (2005, 2008)
Dothiorella sarmentorum	CBS 115038	AY573206	AY573223	Phillips et al. (2005)
Diplodia acerina	CBS 910.73	EU673315	EU673282	Phillips et al. (2008)
Dothiorella sarmentorum	IMI 63581b	AY573212	AY573235	Phillips et al. (2005)
Diplodia coryli	CBS 242.51	EU673317	EU673284	Phillips et al. (2008)
Diplodia juglandis	CBS188.87	EU673316	EU673283	Phillips et al. (2008)
Spencermartinsia viticola	CBS 117010	AY905558	AY905561	Luque et al. (2005)
Botryosphaeria dothidea	CMW 8000	AY236949	AY236898	Slippers et al. (2004)
Botryosphaeria dothidea	CBS 110302	AY259092	AY573218	Alves et al. (2004), Phillips et al. (2005)
Botryosphaeria corticis	CBS 119047	DQ299245	EU017539	Lazzizera et al. (2008), Phillips et al. (2006)
Neofusicoccum parvum	CBS 110301	AY259098	AY573221	Alves et al. (2004), Phillips et al. (2005)
Neofusicoccum mangiferum	CMW 7024	AY615185	DQ093221	Burgess et al. (2005), Slippers et al. (2005a)
Neofusicoccum luteum	CBS 110299	AY259091	AY573217	Alves et al. (2004), Phillips et al. (2005)
Lasiodiplodia crassispora	WAC 12533	DQ103550	DQ103557	Burgess et al. (2006)
Lasiodiplodia gonubiensis	CBS 115812	DQ458892	DQ458877	Alves et al. (2006)
Lasiodiplodia parva	CBS 356.59	EF622082	EF622062	Alves et al. (2008)
Lasiodiplodia pseudotheobromae	CBS 447.62	EF622081	EF622060	Alves et al. (2008)
Lasiodiplodia theobromae	CBS 124.13	DQ458890	DQ458875	Alves et al. (2006)
Diplodia pinea	CBS 393.84	DQ458895	DQ458880	Alves et al. (2006)
Diplodia seriata	CBS 112555	AY259094	AY573220	Alves et al. (2004), Phillips et al. (2005)
Diplodia mutila	CBS 112553	AY259093	AY573219	Alves et al. (2004), Phillips et al. (2005)
Diplodia rosulata	CBS 116470	EU430265	EU430267	Phillips et al. (2008)
Spencermartinsia viticola	CBS 117009	AY905554	AY905559	Luque et al. (2005)

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.

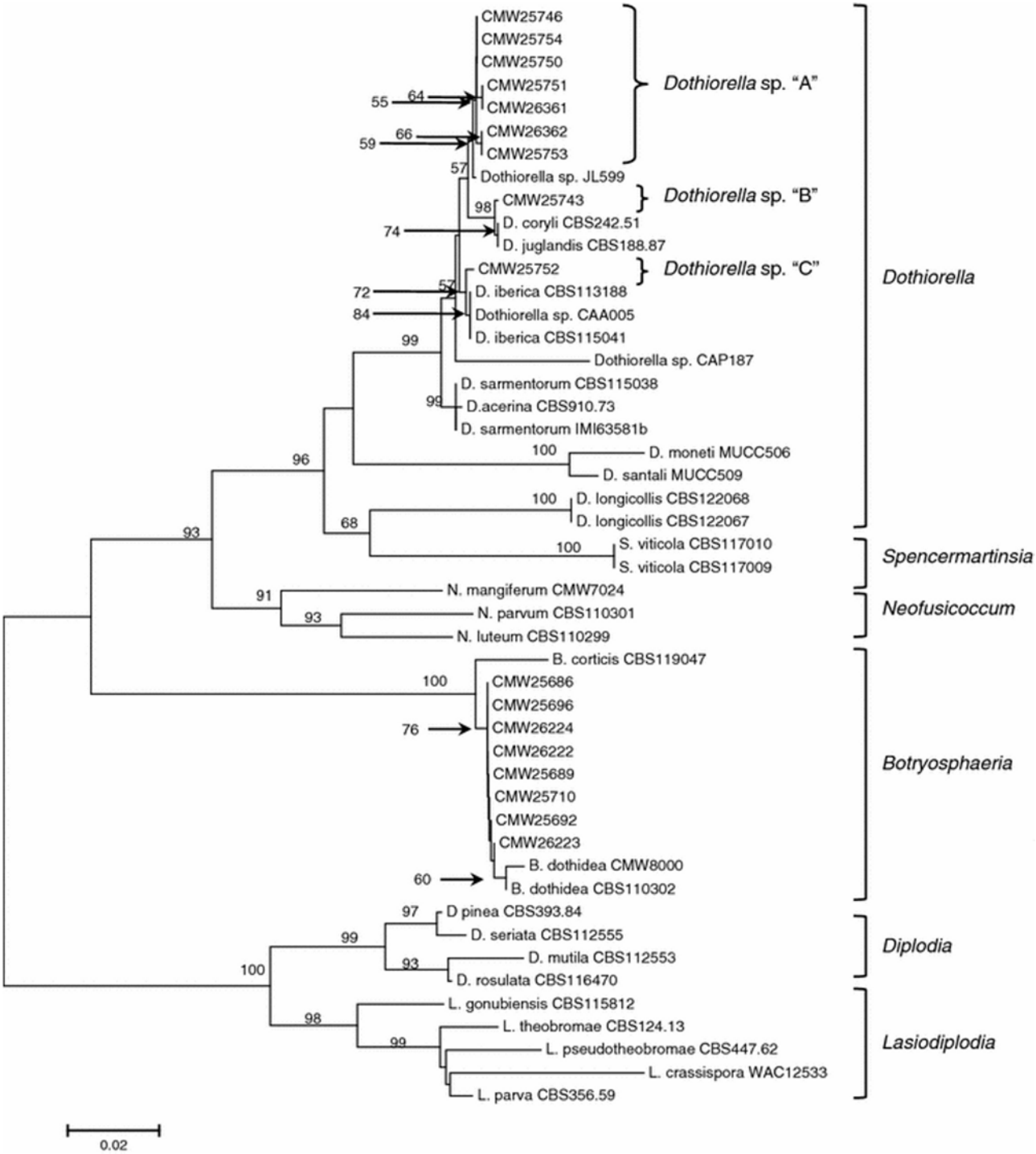

Fig. 1. Phylogenetic tree from combined EF-1α and ITS rDNA dataset. The tree is unrooted and was constructed with the neighbor-joining method. Bootstrap values (2,000 replicates) are indicated at the nodes.

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" 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. carpinifolia* 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 EF-1α 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 × 6.0 µm). *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 × 9.6 µm) 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 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".

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

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 × 9.8 µm large (average of 350 conidia) and had one to three septa (Fig. 3).

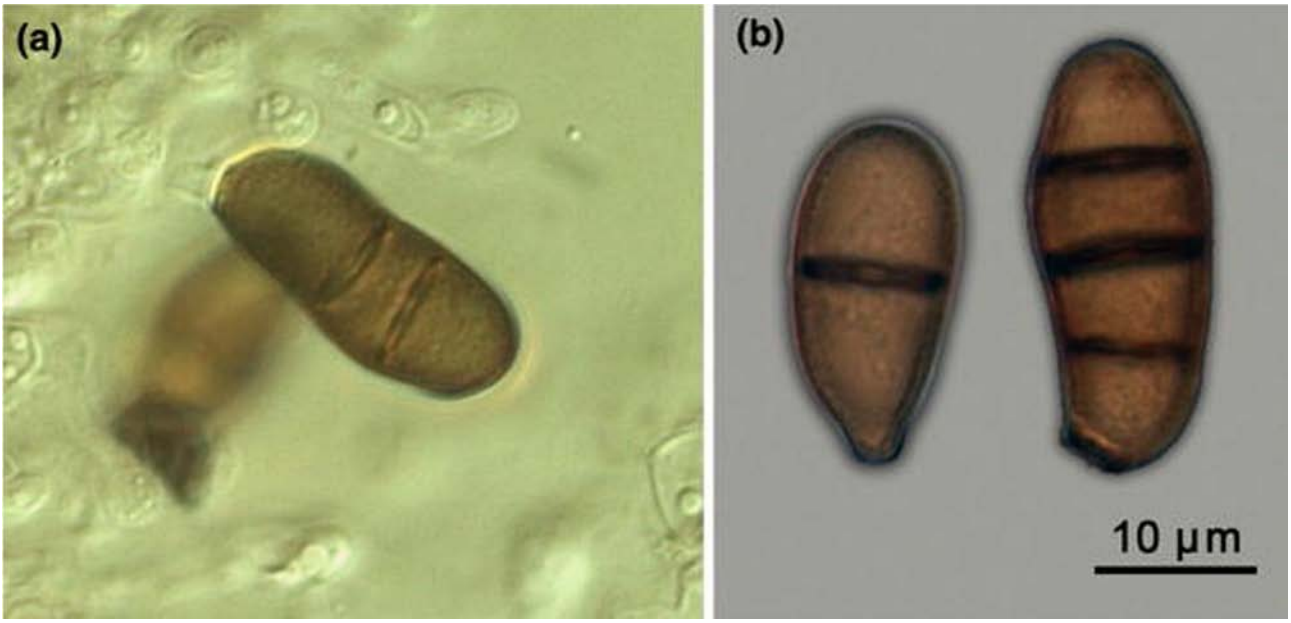

Fig. 3. *Dothiorella* sp. "A". (**a**) Developing conidia and conidiogenous cells. (**b**) Conidia with one to three septa

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.

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. coggrygria* 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% ± 0.5% for the "dry" experiment and 84.0% ± 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% ± 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% ± 0.6%) compared to the moisture levels at the start of the trial.

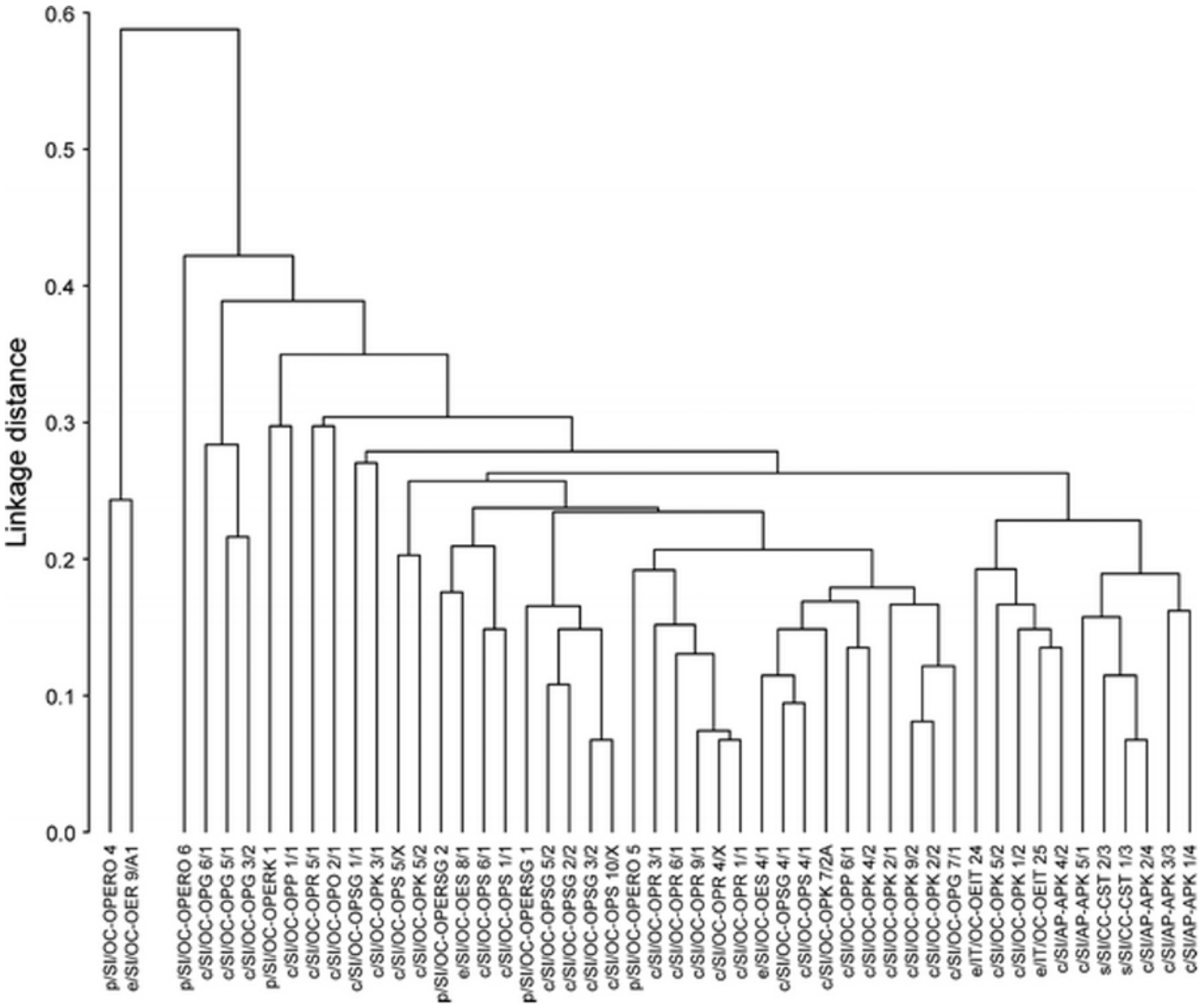

Fig. 4. Unweighted pair-group average (UPGMA) dendrogram based on 50 *Botryosphaeria dothidea* isolates and 74 AFLP polymorphic markers. Isolates are marked with their original designations (Table 1) to emphasize their origins. Abbreviations before isolate names denote the isolation material (p, perithecium 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*).

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

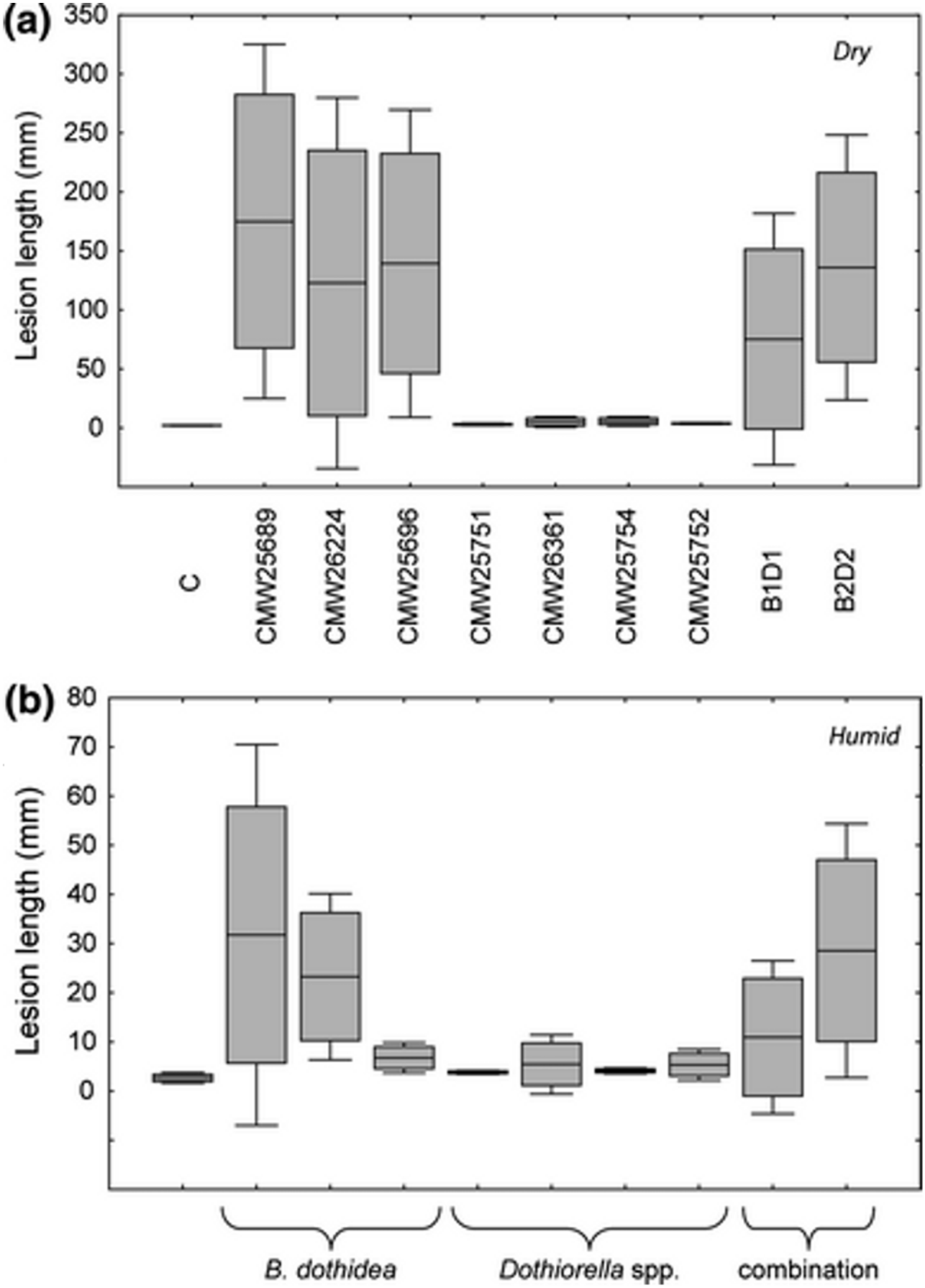

Fig. 5. Mean lesion lengths (mm) and 95% confidence intervals for each isolate of different species of Botryosphaeriaceae 4 weeks after inoculations on *Ostrya carpinifolia* cuttings at (**a**) dry experiment and (**b**) humid experiment. Control (C); *Botryosphaeria dothidea* (CMW 25689, CMW

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

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.

Botryosphaeria dothidea was the dominant species isolated from diseased *O. carpinifolia* trees in Slovenia and Italy. This species was also collected from cankers on *A. pseudoplatanus* 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). *Botryosphaeria 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) 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. *Botryosphaeria 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. *Ostrya 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*.

Ostrya 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

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