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Host specificity of co-infecting Botryosphaeriaceae on ornamental and forest trees in the Western Balkans

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Summary

The Botryosphaeriaceae is a diverse family of endophytes and fungal pathogens of mainly woody plants. We considered the host range and distribution of these fungi by sampling diseased ornamental and forest trees and shrubs in Serbia, Montenegro, Bosnia and Herzegovina, spanning a Mediterranean and a Continental climatic region. In total, ten Botryosphaeriaceae species were identified in the Western Balkans and with the exception of *Sphaeropsis visci* and *Phaeobotryon cupressi*, which occurred on one host, all the species had a broader host range. *Phaeobotryon cupressi* was found only in the Mediterranean region and *S. visci*, *Dothiorella* sp., *Dothiorella sarmentorum* and *Diplodia seriata* were present only in the Continental region. Pathogenicity tests were conducted on a variety of hosts from which the Botryosphaeriaceae species were isolated. These included leaves and/or stems of seedlings of 21 hosts, and cut leaves and/or branches of six hosts. Moreover, stems of seedlings of *Chamaecyparis lawsoniana*, *Cedrus deodara*, *Picea omorika*, *Pinus patula* and *Eucalyptus grandis* were inoculated as hosts from which some or all of the Botryosphaeriaceae species used for inoculation were not isolated. Inoculations showed that the majority of these fungi could also co-infect hosts other than those from which they were isolated. The results suggest that most of the species have broad host ranges and can potentially cause disease on a broad range of tree species under certain conditions.

1 | INTRODUCTION

Species of the Botryosphaeriaceae (Ascomycota: Botryosphaeriales) have a broad host range and occur in temperate, mediterranean and tropical climates worldwide (Mehl, Slippers, Roux, & Wingfield, 2013; Phillips et al., 2013). They can exist as endophytes in asymptomatic plant tissues, but some are also pathogens of agricultural and forestry crops. The shift from an endophytic to a pathogenic lifestyle often occurs after hosts have been subjected to stress caused by environmental factors such as drought, flooding or high temperatures (Mehl et al., 2013; Slippers et al., 2007). The recent increased pathogenic activity and geographic range expansion of the Botryosphaeriaceae in Europe has been linked to warmer climate and extreme weather (Adamson, Klavina, Drenkhan, Gaitnieks, & Hanso, 2015; Fabre, Piou, Desprez-Loustau, & Marcais, 2011; Piškur et al., 2011; Zlatković, Keča, Wingfield, Jami, & Slippers, 2016a).

A number of Botryosphaeriaceae infect multiple hosts, including some that are distantly related. Other Botryosphaeriaceae species appear to have a narrow host range. Examples include *Botryosphaeria scharifii*, *Diplodia quercivora*, *Dothiorella brevicollis*, *Phaeobotryon mamane* and *Sphaeropsis visci*, which have been found associated with *Mangifera indica*, *Quercus canariensis*, *Acacia karroo*, *Sophora chrysophylla* and *Viscum album*, respectively (Jami, Slippers, Wingfield, Loots, & Gryzenhout, 2015; Phillips et al., 2013). However, patterns of host preference in the Botryosphaeriaceae are considered questionable because sampling in most studies has focused on a particular tree species or geographic area (Jami, Slippers, Wingfield, & Gryzenhout, 2014). Moreover, multiple Botryosphaeriaceae often occur on the same host, and within individual host trees, tissues or sites

(Jami et al., 2014, 2015; Linaldeddu, Scanu, Maddau, & Franceschini, 2014; Sakalidis, Slippers, Wingfield, Hardy, & Burgess, 2013; Slippers, Crous, Jami, Groenewald, & Wingfield, 2017; Slippers et al., 2007; Taylor, Barber, Hardy, & Burgess, 2009).

Botryosphaeriaceae have mostly been reported from angiosperms, which appear to be the major group of plants on which these fungi have diversified (De Wet, Slippers, Preisig, Wingfield, & Wingfield, 2008; Phillips et al., 2013; Slippers et al., 2013). However, some Botryosphaeriaceae predominantly infect conifers; that is *Diplodia sapinea* (syn. *Diplodia pinea*, *Sphaeropsis sapinea*), which shows a host preference mainly on *Pinus*, but is also found on *Abies*, *Picea* and *Pseudotsuga* (Pinales: Pinaceae); *Diplodia scrobiculata* associated with *Pinus banksiana*, *P. resinosa* and *P. greggii* (Pinales: Pinaceae); *Diplodia cupressi* isolated from *Cupressus* and *Juniperus* spp. (Pinales: Cupressaceae); *Diplodia tsugae* isolated only from *Tsuga heterophylla* (Pinales: Pinaceae); and *Phaeobotryon cupressi* isolated from *Cupressus sempervirens* and *Juniperus scopulorum* (Pinales: Cupressaceae) (De Wet et al., 2008; Phillips et al., 2013; Slippers et al., 2013; Zlatković, Keča, Wingfield, Jami, & Slippers, 2017).

There are apparently few barriers to the Botryosphaeriaceae moving from native to non-native species and *vice versa* where hosts occur in the same geographic regions. For example, *Diplodia seriata* has been isolated from native *Vitis vinifera* and introduced *Chamaecyparis lawsoniana* and *Thuja plicata* in Portugal (Alves, Barradas, Phillips, & Correia, 2013); *D. sapinea* has been found on various native *Pinus* spp. and introduced *P. radiata* in Italy (Luchi, Longa, Danti, Capretti, & Maresi, 2014); *Neofusicoccum mediterraneum* has been found associated with native *Sequoiadendron giganteum* and introduced *Pistacia vera* in the USA (Inderbitzin, Bostock, Trouillas, & Michailides, 2010); *Neofusicoccum parvum* has been found colonizing native *Syzygium cordatum* and introduced *Eucalyptus grandis* in South Africa (Pavlic, Slippers, Coutinho, & Wingfield, 2007; Pillay, Slippers, Wingfield, & Gryzenhout, 2013). Moreover, when they are isolated from native hosts of a region, Botryosphaeriaceae have also been able to infect and cause disease symptoms on introduced hosts existing in the same region in greenhouse trials (Pavlic et al., 2007).

Uncertainty regarding the taxonomy and overlapping morphological characters of the Botryosphaeriaceae have made these fungi difficult to identify (Phillips et al., 2013; Slippers et al., 2013). For this reason, the ecological role of Botryosphaeriaceae species has been poorly addressed in many ecosystems, including urban environments in which ecological interactions and evolution of species are still poorly understood (Begoude, Slippers, Wingfield, & Roux, 2010; Shochat, Warren, Faeth, McIntyre, & Hope, 2006; Slippers et al., 2007; Slippers et al., 2013; Zlatković et al., 2016a). In contrast to a large number of studies investigating the Botryosphaeriaceae diversity and impact in the Mediterranean region (MR) and in the tropics (e.g., Alves, Linaldeddu, Deidda, Scanu, & Phillips, 2014; Burgess, Barber, & Hardy, 2005; Jami et al., 2014; Rodas, Slippers, Gryzenhout, & Wingfield, 2009), less attention has been given to the Botryosphaeriaceae occurring in the Continental climate-type region (CR). Apart from some recent studies from the Balkans (e.g., Piškur et al., 2011; Zlatković, Keča, Wingfield, Jami, & Slippers, 2016b; Zlatković et al., 2016a, 2017), most research on the Botryosphaeriaceae conducted in the CR has been focused on the pine pathogen, *D. sapinea* (e.g., Jankovský & Palovćiková, 2003; Karadžić & Milijašević, 2008; Fabre et al., 2011; Adamson et al., 2015).

In the Western Balkans, *N. parvum*, *Botryosphaeria dothidea* and *D. seriata* have been found to cause disease on *V. vinifera*, *Malus domestica* and *Olea europaea* (Kaliterna, Milicevic, Bencic, & Duralija, 2013; Kaliterna, Milicevic, Ivic, Bencic, & Mesic, 2012; Latinović, Mazzaglia, Latinović, Ivanović, & Gleason, 2013; Vasić, Duduk, Vico, & Ivanović, 2013). Furthermore, a large number of Botryosphaeriaceae species have recently been found on various diseased trees in this region, including *B. dothidea*, *N. parvum*, *D. sapinea*, *Diplodia mutila*, *D. seriata*, *Dothiorella sarmentorum*, *Dothiorella* sp., *Dothiorella omnivora*, *P. cupressi* and *S. visci* (Zlatković et al., 2016a,b, 2017). These species have been found associated with both angiosperms and gymnosperms belonging to various families, that is Rosaceae, Fagaceae, Cupressaceae, Pinaceae, Oleaceae, Sapindaceae, Santalaceae, Pittosporaceae and Myrtaceae.

The aim of this study was to investigate the host range of the Botryosphaeriaceae in the Western Balkans. This was achieved by identifying species of the Botryosphaeriaceae from a broad collection of Botryosphaeriaceae-like isolates using the DNA sequence data for four gene regions. A selection of these isolates and isolates of the Botryosphaeriaceae identified in the previous study (Zlatković et al., 2016a) was then used to conduct reciprocal inoculations on the hosts from which the Botryosphaeriaceae had been isolated, as well as on *C. lawsoniana*, *Cedrus deodara*, *Picea omorika*, *E. grandis* and *Pinus patula* from which some or all of the Botryosphaeriaceae species were not isolated. The overlapping patterns of all Botryosphaeriaceae species identified in the Western Balkans (Zlatković et al., 2016a,b, 2017) were assessed at an individual plant, host species and geographic level.

2 | MATERIALS AND METHODS

2.1 | Sample collection and Botryosphaeriaceae isolation

Samples were collected from 84 trees (representing 28 species), ten shrubs (representing four species) and four seedlings (representing three species) between 2009 and 2014 in 12 cities, three villages, three forest stands and two ornamental nurseries in Serbia, Montenegro and Bosnia and Herzegovina (Tables S1, S2). Samples were collected from trees exhibiting various disease symptoms including cankers, dieback and tissues displaying abundant resin flow (Table S3). The Botryosphaeriaceae were isolated from healthy and diseased tissues, as well as from fungal fruiting bodies on 2% malt extract agar (MEA) plates acidified with lactic acid (AMEA) as described in Zlatković et al. (2016a). Fungal colonies were purified using hyphal tip transfers, and Botryosphaeriaceae-like isolates (mycelium mostly fast-growing, white to greyish olive in colour, often with a rosette appearance) were transferred to new Petri dishes. Representative isolates from each host have been maintained in the culture collection (CMW) of the

Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Sampling was conducted in regions of Serbia, Montenegro and Bosnia and Herzegovina having both continental and mediterranean climates. According to the Köppen–Geiger system (Köppen, 1936), continental climate with hot summers (Dwa) occurs in northern, eastern and central parts of Serbia, and continental climate with dry summers (Dwb) occurs in western and southern parts of Serbia, Bosnia, Herzegovina and northern parts of Montenegro. Mediterranean climate (Csa) is found in the coastal area of Montenegro.

2.2 | DNA sequence analyses

DNA was extracted from the mycelium of one-week-old cultures using PrepMan Ultra reagent (Applied Biosystems, Foster City, California) following the manufacturer's protocol. The ITS region of the rDNA operon was amplified using primer pairs ITS-1 and ITS-4 or ITS1F and ITS-4 (Gardes & Bruns, 1993; White et al., 1990); part of the TEF-1-α gene was amplified using primer pairs EF1-728F and EF1-986R (Carbone & Kohn, 1999) or EF1-F and EF2-R (Jacobs et al., 2004); the BT2 gene was amplified using primers Bt2a and Bt2b (Glass & Donaldson, 1995), and RPB2 gene was amplified using primers RPB2bot6F and RPB2bot7R (Sakaladis, 2004). The conditions and procedures for PCR amplification, PCR sequencing and sequence alignments were as those described in Zlatković et al. (2016a).

The phylogenetic analyses were performed using maximum parsimony (MP) and maximum-likelihood (ML) analyses. The MP analyses were performed in PAUP version 4.0b10 (Swofford, 2003). The ML analyses were run using an online version of PhyML 3.0 (Guindon et al., 2010), and confidence levels were determined with 1,000 bootstrap replications (Felsenstein, 1985). For ML analyses, the best nucleotide substitution model was found using jModelTest v.0.1 (Posada, 2008). Phylogenetic trees were created with MEGA v.6. The DNA sequences were deposited in GenBank (Table S2). A diagram that outlines materials and methods used in this study is presented in Figure S1.

2.3 | Botryosphaeriaceae species diversity

Alpha diversity was estimated in terms of species richness S (number of species in the region/isolated from the host), the abundance (number of isolates) and evenness J (dominance of species in the region/isolated from the host). Shannon–Wiener's index (H) was calculated with $H = \sum PilnPi$ (Shannon & Weaver, 1949) and normalized to correct for differences in isolate numbers with $H' = H/lnN$, where Pi is the proportion of individuals in the ith species and *N* is the number of isolates associated with each host. Values for H' range from 0 (single species present) to 1 (each isolate associated with the particular host represents a different species).

Beta diversity was measured using the Jaccard's similarity index with $JI = a/(a + b + c)$, where a represents the number of species occurring in both regions/gymnosperms–angiosperms, b represents the number of species restricted to region 1/gymnosperms, and c represents the number of species restricted to region 2/angiosperms. The JI values range from 0 (no species shared) to 1 (all species shared) (Kumar & Hyde, 2004). Diversity indexes were calculated in R (R Core Team, 2015), using statistical package Vegan v. 2.2.1 (Oksanen et al., 2015). Botryosphaeriaceae species diversity in the Western Balkans was assessed using the whole collection of Botryosphaeriaceae isolates from the Western Balkans region, including isolates identified in this and in previous studies (Figure S1; Zlatković et al., 2016a,b, 2017).

2.4 | Inoculations of stems and leaves of seedlings in the field

The inoculation tests were conducted during 2014, 2015 and 2016 growing seasons, from April to October and from April to June, in an open-air nursery of the Faculty of Forestry in Belgrade, Serbia. Some of the seedlings (two- or three-year-old potted plants) were obtained from the nursery of the Faculty of Forestry, and others were purchased from a commercial ornamental nursery located in Novi Sad, Serbia. Seedlings were arranged in a completely randomized design and when rainfall was insufficient they were irrigated on a daily basis (Table S4).

Inoculations were done on stems of seedlings of 21 hosts from which Botryosphaeriaceae were isolated in this and in the previous study (Zlatković et al., 2016a; Tables S5, S6). When possible, inoculations were done using two isolates of both continental and mediterranean origins of each species isolated from the given host (Tables S5, S7; Figure S1). *C. deodara* and *P. omorika* were also inoculated with *N. parvum* isolate CMW 39327, which was not isolated from *C. deodara* and *P. omorika* neither in this nor in the previous study but was shown to be very aggressive when inoculated on its natural hosts (Tables S5, S8; Figure S5; Zlatković et al., 2016a). Because *C. lawsoniana* is one of the most frequently propagated ornamental trees in Serbian nurseries and a large number of seedlings of this host were available, to test for host specificity, it was inoculated with nine Botryosphaeriaceae. Among them, six Botryosphaeriaceae species were isolated from *C. lawsoniana* in this and in the previous study, and three other Botryospaheriaceae used for inoculation were not isolated from *C. lawsoniana* (Table S5, Figure S1, Figure 5; Zlatković et al., 2016a). Inoculation experiments were repeated once.

Ten seedlings per species per isolate were inoculated 3-9 cm above the soil level of the stems by placing the mycelium in a wound made with a 3- or 6-mm-diameter cork borer, as described in Zlatković et al. (2017). The same number of control seedlings of each species was inoculated with plugs of sterile water agar (WA). Seedlings were inspected for disease symptoms and mortality each week.

Leaves of *P. laurocerasus* seedlings were lightly wounded with a sterile needle and inoculated using isolates of *N. parvum* and *D. mutila* (Table S5) as described in Zlatković et al. (2016b). Ten leaves per *P. laurocerasus* seedling and ten seedlings per isolate were inoculated, giving a total of 40 seedlings and 400 leaves.

For seedlings of *C. lawsoniana*, *Populus nigra* var. *italica*, *Prunus laurocerasus*, *P. abies*, *Q. robur*, *C. arizonica* and *Liriodendron tulipifera*, a score of 0–2, 1–3 or 1–2 was assigned to each Botryosphaeriaceae isolate used in the inoculation trial (Table 1; Figure S1). In the case

TABLE 1 Disease assessment for a variety of tree species inoculated with Botryosphaeriaceae isolates. Inoculations were conducted on leaves of *Prunus laurocerasus* seedlings in the field, on cut branches of *Aesculus hippocastanum* in laboratory conditions and on stems of seedlings of all other tree species in the field

Inoculated species	Disease assessment
Chamaecyparis lawsoniana	0 (not pathogenic) = plants looking healthy, only resinous changes in the woody tissue were observed (*B. dothidea* CMW 39301, *N. parvum*, *S. visci*, *Do. sarmentorum*, *D. mutila*, *D. seriata*, *Dothiorella* sp.)
	1 (moderately aggressive) = >50% of plants with browning of the leaves from the inoculation point upwards and downwards, elliptical cankers (measuring 1.4–2.1 cm) formed on the stems, pycnidia formed in the cankered tissue (*B. dothidea* CMW 39315)
	2 (most aggressive) = all plants with browning of the leaves, elongated, girdling, sunken cankers with vertical cracks within the canker and along the canker margins formed on the stems (measuring > 3 cm), *P. cupressi* pycnidia formed in cankers (*P. cupressi*)
Populus nigra var. italica	0 (not pathogenic) = plants looking healthy, no cankers were observed on the inoculated stems (*B. dothidea*)
	1 (less aggressive) = <50% of plants girdled and dead, necrotic lesions (measuring <3 cm) formed on stems of all remaining plants (*N. parvum* CMW 39317)
	2 (more aggressive) = >50% of plants girdled and dead; sunken, girdling cankers with cracks within the canker (measuring > 3 cm) and *N. parvum* pycnidia formed on stems of all remaining plants (*N. parvum* CMW 39327)
Prunus laurocerasus	0 (not pathogenic) = lesions did not appear on inoculated leaves (*D. mutila* CMW 39385)
	1 (least aggressive) = small (3–5 mm) lesions appeared around inoculation point but dropped out of the leaves (*D. mutila* CMW 39348)
	2 (moderately aggressive) = reddish-brown necrotic lesions (measuring 1–2.5 cm) with concentric rings surrounded by reddish borer or light green halo appeared on leaves, *N. parvum* pycnidia formed in lesions, all lesions dropped out (*N. parvum* CMW 39327)
	3 (most aggressive) = reddish-brown necrotic lesions (measuring 2.5–4 cm) with concentric rings surrounded by reddish border or light green halo appeared on leaves, <10% of lesions dropped out, >50% of leaves were completely necrotic and detached and, *N. parvum* pycnidia formed in dead leaf tissue, 20% of plants experienced disease progress towards shoots which showed dieback and *N. parvum* pycnidia formed in dead shoot tissue (*N. parvum* CMW 39317)
Picea abies	1 (least aggressive) = elongated, girdling, necrotic, resin-soaked lesions (measuring 2.5–6.5 cm) formed on stems, some plants with shoot dieback (*N. parvum* CMW 39317)
	2 (moderate aggressive) = <50% of plants girdled and dead, remaining plants with elongated, resinous, girdling necrotic lesions (measuring 2.5–7.5 cm), resin flow observed from the infected needles and stems (*B. dothidea*)
	3 (most aggressive) = all plants girdled and dead, resin flow observed from the infected needles and stems (*D. sapinea*[a], *N. parvum* CMW 39327)
Quercus robur	1 (less aggressive) = plants with elongated, girdling cankers (measuring 1.9–7.5 cm), pycnidia formed in cankered tissue (*D. seriata* CMW 39382)
	2 (more aggressive) = >50% plants girdled and dead, *D. seriata* pycnidia formed in dead plant parts, elongated, girdling cankers (measuring > 2 cm) formed on all remaining plants, *D. seriata* pycnidia produced in the cankered tissue (*D. seriata* CMW 39376)
Cupressus arizonica	1 (less aggressive) = plants with elliptical necrotic lesions (measuring < 2 cm) (*B. dothidea* CMW 39301, *D. mutila*)
	2 (more aggressive) = plants with elongated, girdling, sunken cankers with vertical cracks within the canker and along the canker margins (measuring > 4 cm), *B. dothidea* pycnidia formed in cankered tissue (*B. dothidea* CMW 39315)
Liriodendron tulipifera	1 (less aggressive) = plants with lesions measuring 5.4–8.3 cm (*B. dothidea* CMW 39315)
	2 (more aggressive) = plants with lesions measuring 6–30 cm, >50% of plants with stem dieback, *B. dothidea* pycnidia formed in dead stem tissue (*B. dothidea* CMW 39301)
Aesculus hippocastanum	1 (least aggressive) = branches with girdling lesions measuring 1.5–4 mm, *Do. sarmentorum* pycnidia formed in lesions (*Do. sarmentorum* CMW 39365)
	2 (moderately aggressive) = >50% of branches dried, girdled, with dark staining of the vascular tissue, *D. mutila* pycnidia formed in the dead branch tissue (*D. mutila* CMW 39385)
	3 (most aggressive) = all branches dried, girdled, with dark staining of the vascular tissue, pycnidia formed in the dead branch tissue (*B. dothidea*, *D. mutila* CMW 39348, *Do. sarmentorum* CMW 39364, *N. parvum*)

[a]Data taken from Zlatković et al., 2017.

of *Q. robur* and *P. nigra* var. *italica*, the results were observed after 6 weeks when more than half of the plants inoculated with one isolate were girdled and dead. In the case of *P. abies*, the results were observed after 8 weeks when one of the isolates killed all the seedlings inoculated with that isolate. As *C. lawsoniana*, *C. arizonica* and *L. tulipifera* did not experience mortality during inoculation trials, the test lasted for the whole vegetation season (6 months) for these tree species. Inoculation tests conducted on *P. lauracerasus* leaves lasted for 3 weeks and until all lesions formed by *N. parvum* isolate 39327 dropped out of the leaves. Disease assessment for seedlings of *S. giganteum*, *T. occidentalis*, *Abies concolor*, *C. sempervirens* and *P. omorika* was based on the calculation of the Area Under Disease Progress Curve (AUDPC), and the test lasted until all of the inoculated plants were killed. The AUDPC was measured using the trapezoidal integration method (Madden, Hughes, & van den Bosch, 2007):

$$\text{AUDPC} = \sum_{i=1}^{n-1} [(y_i + y_{i+1})/2] \times (t_{i+1} - t_i)$$

where "*n*" is the total number of observations, "y_i" the severity of disease at the ith observation and "t_i" time (week) at the ith observation. Seedlings of *Pittosporum tobira*, *C. deodara*, *C. atlantica*, *Ligustrum vulgare*, *Juniperus horizontalis*, *C. arizonica*, *P. laurocerasus*, *Magnolia grandifolia*, *Pseudotsuga menziesii*, *L. tulipifera*, *Taxus baccata*, *P. cerasus*, *Q. rubra*, *Platanus acerifolia* and *Forsythia europaea* were sectioned longitudinally, and the extent of resin accumulation/vascular discoloration was measured above and below the point of inoculation 6 weeks (for *P. tobira*, *C. deodara*, *T. baccata*, *P. cerasus*, *Q. rubra*, *P. acerifolia* and *F. europaea*) and 6 months (for all other species) after the beginning of the experiment. Botryosphaeriaceae were re-isolated from the margin between necrotic and apparently healthy tissue of inoculated plants. Fungal pycnidia were collected from the surface of the dead tissues and examined, as described in Zlatković et al. (2016b, 2017). Botryosphaeriaceae identity was verified based on the morphology of cultures and conidia to fulfil Koch's postulates (Zlatković et al., 2016a).

2.5 | Inoculations of cut branches and leaves of cut branches

When seedlings were not available for the experimental trials, inoculations were done on cut branches. Cut branches, 30-40 cm in length and 1.3-1.5 cm thick, were collected from healthy mature *Aesculus hippocastanum* trees growing in the experimental forest of the Faculty of Forestry in Belgrade, Serbia in May 2015. Cut branches of the same length and width were also collected from healthy *P. tremula*, *Fraxinus excelsior* and *Q. cerris* trees growing in urban forests "Ada" and "Košutnjak," in Belgrade during May 2016. *V. album* shrubs showing no symptoms of disease were collected in October 2014 from *Abies alba* trees in "Košutnjak," Belgrade. Isolates used for inoculations of cut branches were selected according to the same principle as for inoculations of seedlings (Tables S5, S7; Figure S1).

Ten branches per isolate were inoculated as described in Zlatković et al. (2017), and the same number of control branches was inoculated with sterile WA plugs. For branches of *Aesculus hippocastanum*, a score of 1–3 was assigned to each of the Botryosphaeriaceae isolates used in the inoculation trial (Table 1; Figure S1). Ten *V. album* branches were wounded using a 3-mm cork borer and inoculated with a plug of 10-day-old *S. visci* mycelium, covered with moist cotton wool and wrapped with Parafilm (Pechiney, Chicago, USA). Ten leaves of ten *V. album* branches were lightly wounded with a sterile needle and inoculated as described previously. The same number of branches and leaves was inoculated with sterile WA plugs as controls.

After 2 weeks (*V. album*) and 3 weeks (all other species), the extent of vascular discoloration on sectioned branches or lesions on leaves was measured, and re-isolations were made as described in Zlatković et al. (2017). Experiments were repeated once.

2.6 | Inoculations of stems of seedlings in the greenhouse

To test for host specificity, inoculations were also conducted on twenty potted 5-year-old *P. patula* and 3-year-old *E. grandis* seedlings as hosts from which Botryosphaeriaceae strains from this study were not isolated. Seedlings were inoculated with the two most commonly found Botryosphaeriaceae species in this study, namely *B. dothidea* and *N. parvum*. Isolates used for inoculation were selected as described above (Tables S5-S7; Figure S1). Seedlings were inoculated in the lower parts of the stems as described in Zlatković et al. (2017). Twenty control seedlings were inoculated with sterile WA plugs. Seedlings were watered daily, maintained at 25°C under natural day/night cycles, and the lesion lengths were measured after 6 weeks. *B. dothidea* and *N. parvum* were re-isolated as described in Zlatković et al. (2017).

2.7 | Statistical analyses

Statistical analyses were conducted using Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA) and IBM SPSS Statistics 20.0 (New York, USA). The normality of the data sets was checked using Kolmogorov–Smirnov test and homogeneity of variances using Leven's test. The results of the two subsequent pathogenicity tests were analysed using Student's *t* test at $\alpha = 0.05$ (data not shown). As no significant differences were found between the two repeats of the trials, a single data set was used in further analyses. The log10 transformation was used to improve normality of the data set to analyse the effect of *B. dothidea* (CMW 39315) on vascular discoloration of *L. tulipifera* seedlings. The analyses were further assessed using either one-way analysis of variance (ANOVA) followed by post hoc Fisher's Least Significant Difference (LSD) test, z test (Pocock, 2006), Student's *t* test or Kruskal–Wallis H test at $\alpha = 0.05$.

3 | RESULTS

3.1 | Botryosphaeriaceae isolation

A total of 188 Botryosphaeriaceae isolates were obtained in this study, and 120 isolates obtained in the previous studies (Zlatković et al., 2016a,b, 2017) were included in the subsequent analyses. (Tables S1, S2; Figure S1).

Among all samples collected in the Western Balkans, including those collected in this and in previous studies (Zlatković et al., 2016a,b, 2017), Botryosphaeriaceae were most frequently isolated from necrotic lesions and cankers (45.4% of the total samples), followed by resinous lesions and tissues (22%). Botryosphaeriaceae were only occasionally isolated from healthy tissues (1.3%), discoloured wood (4.9%) and margins between apparently healthy and dead stem wood of trees with top dieback (5.2%) (Table 2).

3.2 | DNA sequence analyses

Preliminary identification of the isolates obtained in this study was made using MP analyses of the ITS sequences (tree not shown). Isolates of *N. parvum* were further subjected to analyses of the combined ITS, TEF-1-α, BT2 and RPB2 gene regions (trees not shown). Based on these analyses, isolates considered in this study were identified as *D. seriata*, *D. mutila*, *B. dothidea*, *Do. sarmentorum*, *Do. omnivora* and *N. parvum*. Representative isolates from each host of each identified fungal species including four that were previously identified, namely *Dothiorella* sp., *S. visci*, *P. cupressi* and *D. sapinea* (Zlatković et al., 2016a, 2017) were further subjected to MP and ML analyses of the ITS sequence data (Figure 1; Figure S1).

The ITS data set contained 145 sequences and was rooted to *Pseudofusicoccum stromaticum* (CBS 117448, CBS 117449). The sequence data set contained 528 characters among which 150 were parsimony informative, 378 were parsimony uninformative, with CI = 0.8, RI = 0.9 and TL = 247. The model GTR + G was chosen for the ML analyses (G = 0.2050). The topologies of the trees emerging from MP and ML analyses were similar, and therefore, only ML tree is presented (Figure 1).

3.3 | Botryosphaeriaceae species diversity

Among the whole collection of isolates of Botryosphaeriaceae species from the Western Balkans, including isolates from this study and

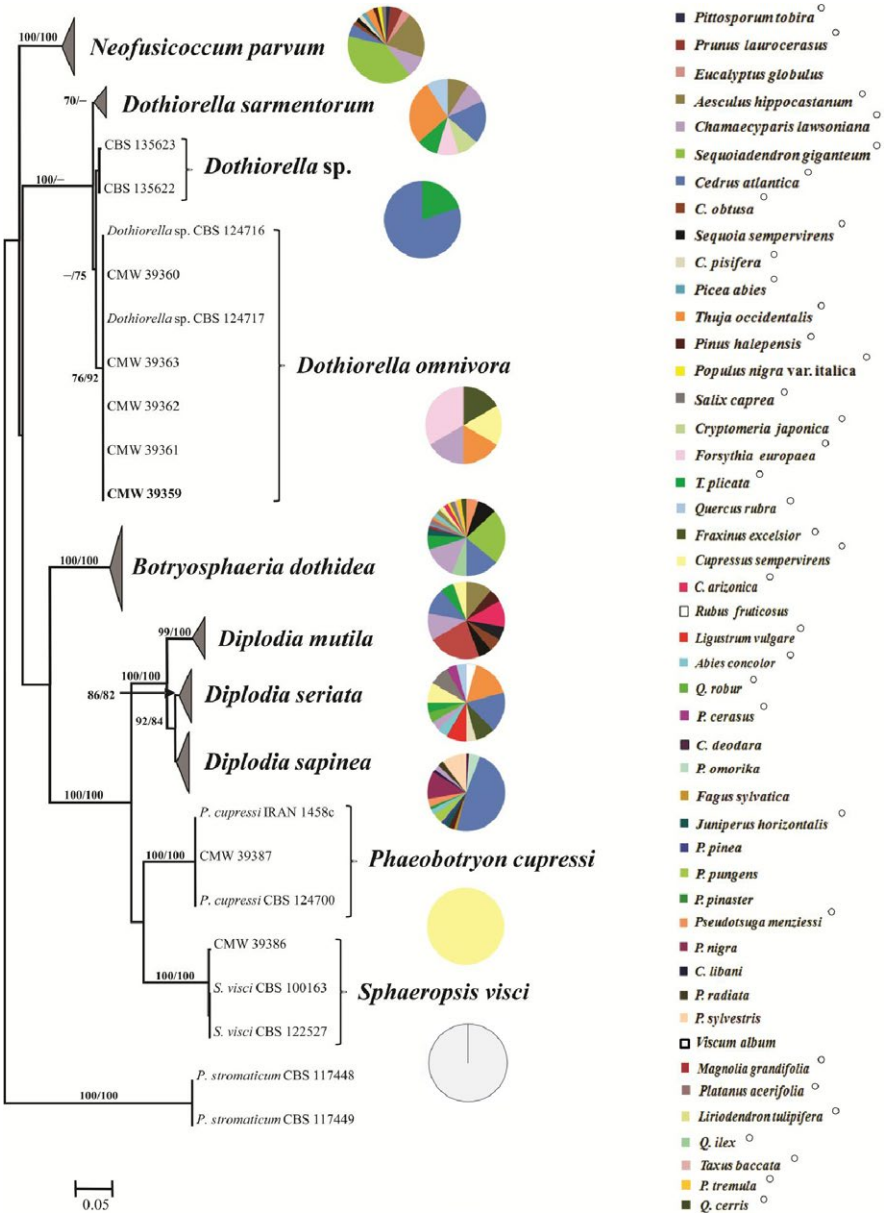

FIGURE 1 Maximum-likelihood (ML) tree of the ITS sequence data. The bootstrap support values (MP/ML ≥70%) are indicated at the nodes, and the scale bar represents the number of changes. The tree was rooted to *P. stromaticum* CBS 117448 and CBS 117449. Isolates sequenced in this study are presented in bold. Branches corresponding to Botryosphaeriaceae species that were isolated from more than five hosts are collapsed. Collapsed branches are displayed as grey triangles, and the size of a triangle is proportional to the number of hosts. Pie charts indicate the hosts from which species were isolated. Hosts from this study are indicated with the circles

from previous studies (Zlatković et al., 2016a,b, 2017), the most commonly isolated species was *B. dothidea* (32.1%), followed by *D. sapinea* (28.2%) and *N. parvum* (18.2%). *Do. omnivora* (1.9%), *Dothiorella* sp. (1.6%), *S. visci* (0.3%) and *P. cupressi* (0.3%) were isolated only occasionally. The majority of the Botryosphaeriaceae were most commonly isolated from necrotic lesions and cankers. Exceptions were *D. sapinea* that was most frequently isolated from resinous lesions and tissues (44.8%), *S. visci* isolated from pycnidia and *P. cupressi* isolated only from resinous branch lesion (Table 2).

There were significant differences ($F = 7.85$, $df = 9$, $p = 0$) in host association patterns among Botryosphaeriaceae species from the Western Balkans from this and from previous studies (Zlatković et al., 2016a,b, 2017). *P. cupressi*, *S. visci* and *Dothiorella* sp. showed the strongest host association patterns being isolated from a single host or two hosts, respectively. Each of the remaining seven Botryosphaeriaceae species showed unique host association patterns (Figure 1 When considering only isolates from this study, new host associations were found for *Do. sarmentorum*, *Do. omnivora*, *B. dothidea*, *D. mutila*, *D. seriata* and *N. parvum* (Table S9). Shannon–Weiner's diversity index (H') showed greatest Botryosphaeriaceae diversity associated with *T. occidentalis* (H' = 0.61), followed by *T. plicata* (H' = 0.53) and 27 hosts harboured a single species. High values of evenness (J = 0.58-0.91) indicated even communities of species for most hosts (Table 3). There were significant differences ($F = 1.61$, $df = 46$, $p = .009$) in the composition and diversity of Botryosphaeriaceae among different hosts (Table 3, Figure S2).

There were no significant differences (H = 0.37, $df = 1$, $p = .54$) in the diversity of Botryosphaeriaceae species associated with angiosperms (H' = 0.42) compared to gymnosperms (H' = 0.29). High values of evenness (0.76 and 0.8) showed that the communities are relatively similar. Jaccard's index showed relatively high overlap of species isolated from gymnosperms and angiosperms (J' = 0.7, Table S10). The species composition differed in such a way that *S. visci* was found only on angiosperms, *Dothiorella* sp. and *P. cupressi* were isolated only from gymnosperms, *B. dothidea* and *D. seriata* were isolated from gymnosperms and angiosperms with almost equal frequency and all the rest of the Botryosphaeriaceae species were most commonly isolated from gymnosperms (Table S10).

There were no significant differences (H = 2.28, $df = 1$, $p = .13$) of Botryosphaeriaceae species diversity in the Continental region (CR) (H' = 0.30) compared to the Mediterranean region (MR) (H' = 0.37). High values of evenness indicated almost even distribution of species (0.77 and 0.8), and Jaccard's index showed moderate overlap of species (J' = 0.5). *D. seriata*, *Do. sarmentorum*, *Dothiorella* sp. and *S. visci* were found only in the CR; *P. cupressi* was found only in the MR and five species were found in both climatic regions (Table S11, Figure S3).

Individual trees, tree parts and lesions were often colonized by several Botryosphaeriaceae species. Up to five species were found cohabiting the same diseased host tree, and up to three species were isolated from the same diseased tree parts (Table S12). *B. dothidea* and *N. parvum* were most commonly found cohabiting the same host trees (16%); *B. dothidea* was found most frequently (30.6%) in the same host tree and in same tree part (24.5%) in combination with other species

TABLE 2 Botryosphaeriaceae species isolated from different tree parts in this and in previous studies in the Western Balkans (Zlatković et al., 2016a,b, 2017). Values represent the number and percentage of samples from which Botryosphaeriaceae were isolated

Botryosphaeriaceae species	Resinous						Necrotic/Cankered										
	Needles/ Leaves	Shoot	Branch	Stem	Bark	Cone	Needles/ Leaves	Shoot	Branch	Stem	Top dieback	Wood discoloration	Fruit bodies	Dead	Healthy	Total	%
Diplodia sapinea	3	1	13	17	0	5	0	4	6	12	4	1	13	6	2	87	28.2
D. mutila	0	0	1	1	0	1	0	4	1	5	0	1	3	1	0	18	5.8
D. seriata	0	1	2	0	0	0	3	1	4	4	0	2	4	3	0	24	7.8
Dothiorella sp.	0	0	0	0	0	0	1	0	1	1	0	0	1	1	0	5	1.6
Do. omnivora	0	0	0	0	0	0	2	0	2	1	0	1	1	0	0	6	1.9
Do. sarmentorum	0	0	1	0	0	0	2	3	0	2	0	0	2	1	0	11	3.6
Botryosphaeria dothidea	2	0	4	3	2	0	20	9	11	13	10	5	11	8	1	99	32.1
Neofusicoccum parvum	0	1	6	2	0	0	11	8	2	11	2	6	2	4	1	56	18.2
Sphaeropsis visci	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0.3
Phaeobotryon cupressi	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0.3
Total	5	3	29	23	2	6	38	28	26	48	16	15	38	26	4	308	100
%	1.6	1	9.4	7.5	0.6	1.9	12.3	9.1	8.4	15.6	5.2	4.9	12.3	8.4	1.3	100	

(Table S13), and multiple Botryosphaeriaceae species were most commonly isolated from *C. atlantica* (22.2%, Table S14).

3.4 | Inoculations of stems and leaves of seedlings in the field

Botryosphaeria dothidea and *P. cupressi* formed cankers when inoculated onto *C. lawsoniana* seedlings, and *P. cupressi* was more aggressive. In contrast, *C. lawsoniana* seedlings inoculated with *B. dothidea* isolate from *C. lawsoniana* from the CR, and five other Botryosphaeriaceae showed only resinous lesions that were not significantly different to that observed in the controls (Tables S5, S8, S15; Figure S4). *D. seriata* produced girdling cankers with pycnidia of the fungus obvious on the canker surfaces when inoculated onto *Q. robur*, and the isolate from *C. pisifera* was more aggressive compared to that from *Q. robur*. *N. parvum* and *B. dothidea* produced resin-soaked girdling necrotic lesions when inoculated onto *P. abies*. Other disease symptoms were similar to those described by Zlatković et al. (2017). *N. parvum* isolated from *C. lawsoniana* was the most aggressive and it eventually killed the inoculated *P. abies* plants. *B. dothidea* and *D. mutila* produced girdling

TABLE 3 Diversity and overlap of Botryosphaeriaceae species isolated from different hosts in this and in previous studies in the Western Balkans (Zlatković et al., 2016a,b, 2017)

Host	Species richness (S)	Abundance	Shannon index (H')[a]	Evenness (J)[a]	LSD[b]
Gymnosperms					
Chamaecyparis lawsoniana	7	26	0.44	0.73	A
Cedrus atlantica	7	71	0.30	0.66	A
Cupressus sempervirens	5	6			B
Thuja plicata	5	10	0.53	0.76	B
Thuja occidentalis	5	11	0.61	0.91	B
Abies concolor	3	5			D
Sequoiadendron giganteum	3	47	0.22	0.76	D
Pinus halepensis	3	4			D
Sequoia sempervirens	3	10	0.28	0.58	D
Chamaecyparis pisifera	2	2			D
Chamaecyparis obtusa	2	2			D
Cupressus arizonica	2	4			D
Pseudotsuga menziesii	2	8			D
Juniperus horizontalis	2	5			D
Picea abies	2	2			D
Pinus nigra	1	10	0	0	E
Pinus sylvestris	1	9			E
Cedrus deodara	1	1			E
Picea pungens	1	4			E
Picea omorika	1	4			E
Cedrus libani	1	1			E
Pinus radiata	1	2			E
Pinus pinea	1	1			E
Pinus pinaster	1	1			E
Cryptomeria japonica	1	1			E
Taxus baccata	1	1			E
Angiosperms					
Aesculus hippocastanum	4	16	0.34	0.69	C
Salix caprea	2	4			D
Prunus laurocerasus	2	7			D
Fraxinus excelsior	2	3			D
Pittosporum tobira	2	2			D
Quercus rubra	3	3			D

(continues)

TABLE 3 (continued)

Host	Species richness (S)	Abundance	Shannon index (H')[a]	Evenness (J)[a]	LSD[b]
Quercus ilex	1	6			E
Ligustrum vulgare	1	2			E
Populus tremula	1	2			E
Eucalyptus globulus	1	2			E
Platanus acerifolia	1	2			E
Viscum album	1	1			E
Forsythia europaea	1	1			E
Magnolia grandiflora	1	1			E
Liriodendron tulipifera	1	1			E
Fagus sylvatica	1	1			E
Quercus robur	1	1			E
Rubus fruticosus	1	1			E
Populus nigra var. italica	2	2			E
Prunus cerasus	1	1			E
Quercus cerris	1	2			E

[a]Diversity indexes were calculated only for hosts from which at least ten isolates were obtained.
[b]Hosts with the same letter did not differ significantly in terms of Botryosphaeriaceae species composition at the α = 0.05 significance level using LSD test.

cankers and necrotic lesions when inoculated onto *C. arizonica*, and *B. dothidea* isolated from *C. lawsoniana* grown in the MR was the most aggressive to this host. *N. parvum* was able to produce lesions and eventually kill inoculated *P. nigra* var. *italica* seedlings. The *N. parvum* isolate from *S. giganteum* was more aggressive to this host compared to the isolate from *E. globulus*. In contrast, *B. dothidea* did not produce lesions when inoculated into stems of *P. nigra* var. *italica* seedlings (Tables 1 and 4; Tables S3, S8, S15; Figures S4-S8).

Botryosphaeria dothidea, *Do. omnivora* and *N. parvum* killed *S. giganteum* plants 13 weeks after inoculation. Seedlings showed initial symptoms of shoot dieback 1 week after inoculation. Other symptoms included wilting and girdling stem lesions. Control plants remained asymptomatic. The *B. dothidea* isolate from *C. lawsoniana* grown in the CR and *Do. omnivora* had the highest AUDPC values and was shown to be the most aggressive. *N. parvum* isolated from *E. globulus* was the least aggressive. *D. seriata* and *B. dothidea* killed *A. concolor* seedlings 12–20 weeks post-inoculation. Plants displayed symptoms and signs of yellowing and browning of the needles, stem dieback and needle cast followed by the appearance of *D. seriata* and *B. dothidea* pycnidia in the dead bark. *B. dothidea* was the most aggressive, and *D. seriata* isolate from *Q. robur* was the least aggressive (Table 4; Tables S3, S15; Figures 2 and 3; Figures S9, S10).

Seedlings of *T. occidentalis* displayed initial symptoms of yellowing, browning, reddening of the leaves and shoot flagging 7 weeks after inoculation with four Botryosphaeriaceae species. Other symptoms included dry, black and necrotic leaves, girdling lesions and fungal pycnidia in the dead tissues. Control plants remained asymptomatic. *D. seriata*, *N. parvum* isolated from *E. globulus* and *B. dothidea* isolated from *C. lawsoniana* grown in the CR were the most aggressive species. *D. mutila*, *D. seriata* and *B. dothidea* were able to kill *C. sempervirens*. Seedlings displayed the first symptoms of shoot flagging 2 weeks after inoculation and later exhibited dieback of stems, yellowing of the needles and needle drying. Disease symptoms were not observed on seedlings inoculated with *P. cupressi*, *Do. omnivora* or on the controls. *N. parvum* was able to kill *P. omorika*. Disease symptoms were similar to those described in Zlatković et al. (2017) and controls remained healthy (Tables 1 and 4; Table S3, Figures 4-6; Figures S11-S13).

Diplodia seriata was shown to produce vascular discoloration when inoculated onto *L. vulgare* and *B. dothidea* produced vascular discoloration on *M. grandifolia*. *B. dothidea* produced necrotic lesions when inoculated onto *L. tulipifera*, and an isolate from *C. lawsoniana* grown in the CR was more aggressive compared to an isolate from the same host grown in the MR. In contrast, *B. dothidea* did not cause lesions on seedlings of *P. menziesii*, and *N. parvum* did not cause lesions on seedlings of *C. deodara*. *N. parvum* isolated from *S. giganteum* produced sunken, girdling cankers on stems of *C. atlantica*. Other Botryosphaeriaceae and the controls produced only small resinous lesions. *B. dothidea* produced necrotic lesions on stems of *J. horizontalis*. *Do. sarmentorum* did not produce lesions on seedlings of *F. europaea*; *D. seriata*, *Do. sarmentorum* and *N. parvum* did not produce lesions on *Q. rubra*; *B. dothidea* did not produce lesions on *P. acerifolia* and *T. baccata*, and *D. seriata* did not produce lesions on *P. cerasus*. *N. parvum* produced dark brown elongated lesions on stems of *P. tobira*. Isolates of *D. mutila* and control plants displayed no lesions. *N. parvum* and *D. mutila* isolated from *C. sempervirens* produced reddish-brown elliptical cankers with cracks inside the canker and along the canker margin on stems of *P. laurocerasus* seedlings. In contrast, *P. laurocerasus* seedlings inoculated with *D. mutila* isolated from *T. plicata* showed only dark brown lesions visible after the outer bark was removed, and these lesions were not significantly larger from those produced on the controls.

Leaves of *P. laurocerasus* showed necrotic lesions 5 days after inoculation. *N. parvum* isolated from *E. globulus* was the most aggressive fungus. No lesions appeared on leaves of the control plants and plants inoculated with *D. mutila*

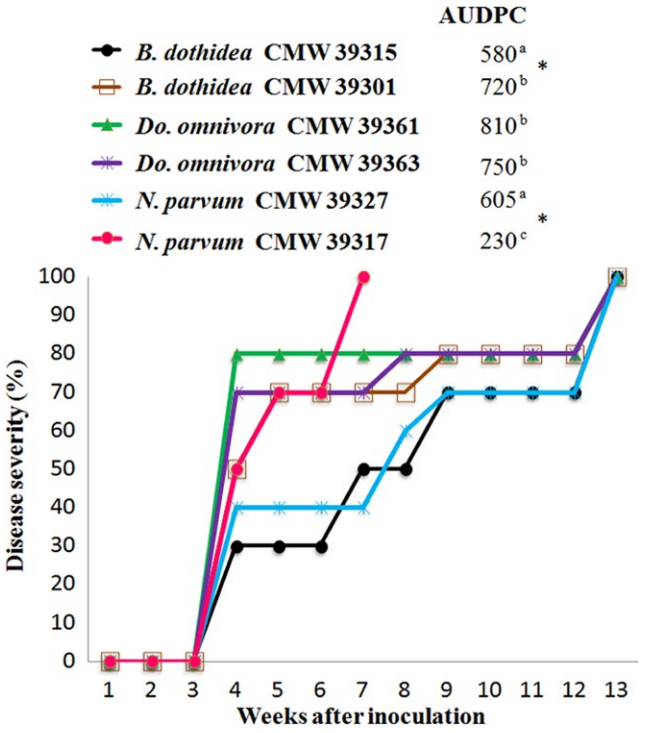

FIGURE 2 Disease progress curves and Area Under the Disease Progress Curve (AUDPC) of seedlings of *Sequoiadendron giganteum* inoculated with *Botryosphaeria dothidea*, *Dothiorella omnivora* and *Neofusicoccum parvum*. The ANOVA test indicated that there were significant differences in AUDPC values of seedlings of *S. giganteum* at $\alpha = 0.05$ level ($F = 70.57$, $df = 2$, $p = .03$). Means with the same letter did not differ significantly, as determined by the LSD test at $\alpha = 0.05$ level. "*" indicates that AUDPC values of isolates of the same species were significantly different (z test, *B. dothidea*: $z = 3.89$, $p = .0001$; *N. parvum*: $z = 12.98$, $p < .00001$)

isolated from *T. plicata* (Zlatković et al., 2016b; Table 1; Tables S3, S8; Figure S22). Botryosphaeriaceae were re-isolated and identified from symptomatic tissues of all inoculated stems and leaves of seedlings. Exceptions were resinous lesions formed on *C. lawsoniana* and *C. atlantica*, brown lesions formed on *P. laurocerasus* after inoculation with *D. mutila* isolate from *T. plicata*, seedlings which were inoculated with Botryosphaeriaceae isolates but did not show lesions, and controls (Table 4, Tables S3, S8, S15; Figures S14-S18).

3.5 | Inoculations of cut branches and leaves of cut branches

Botryosphaeria dothidea, *D. mutila* isolated from *C. sempervirens*, *Do. sarmentorum* isolated from *C. lawsoniana* and *N. parvum* were the most aggressive species when inoculated onto *A. hippocastanum* branches. *Do. sarmentorum* isolate from *T. occidentalis* was the least aggressive. *B. dothidea* produced brown, girdling lesions when inoculated onto *P. tremula* branches and brown, elongated lesions when inoculated onto *Q. cerris* branches. Control branches produced no lesions. *D. seriata* isolated from *C. pisifera* produced brown, elliptical lesions with pycnidia formed in lesions when inoculated onto *F. excelsior* branches. Control branches, branches inoculated with *D. seriata* from *Q. robur* and with *Do. omnivora* showed no lesions. *V. album* shrubs showed necrotic lesions on branches and leaves 5 days after inoculation. Two weeks post-inoculation, branches and leaves appeared chlorotic; branches were girdled and *S. visci* pycnidia were found embedded in the dead tissues. Control branches and leaves showed only minimal discoloration around the wounds (Table 1; Tables S3, S8, S15; Figures S19-S21). Re-isolations from fungal pycnidia and discoloured tissues were successful, and Koch's postulates were fulfilled in both pathogenicity tests conducted on cut branches and leaves of cut branches.

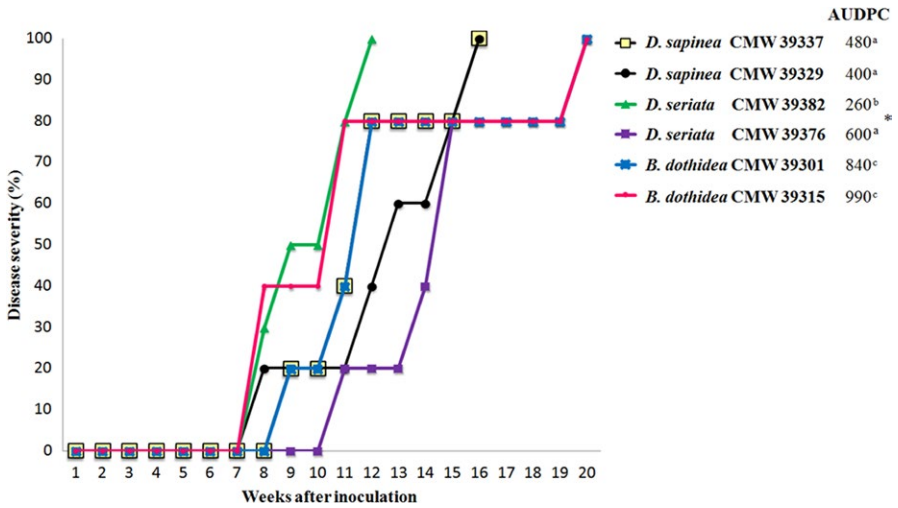

FIGURE 3 Disease progress curves and Area Under the Disease Progress Curve (AUDPC) of seedlings of *Abies concolor* inoculated with *Diplodia sapinea*, *D. seriata* and *Botryosphaeria dothidea*. The ANOVA test indicated that there were significant differences in AUDPC values of seedlings of *A. concolor* at $\alpha = 0.05$ level ($F = 19.9$, $df = 2$, $p = .02$). Means with the same letter did not differ significantly, as determined by the LSD test at $\alpha = 0.05$ level. "*" indicates that AUDPC values of isolates of the same species were significantly different (z test, *D. seriata*: $z = 11.58$, $p < .0001$). Data for *Diplodia sapinea* were retrieved from Zlatković et al., 2017 and serve for comparison with other Botryosphaeriaceae inoculated onto *A. concolor*

3.6 | Inoculations of stems of seedlings in the greenhouse

B. dothidea was less aggressive compared to *N. parvum* when inoculated onto *P. patula*. *N. parvum* isolated from *E. globulus* was the most aggressive, and the *B. dothidea* from CR was the least aggressive when inoculated onto *E. grandis*. In contrast, seedlings of *E. grandis* inoculated with *B. dothidea* isolate from MR showed lesions that did not differ obviously from those produced on the controls. Lesions formed on control seedlings were significantly smaller compared to those produced by Botryosphaeriaceae species (Table 4; Tables S3; S8, S15, Figures S23-S24). Botryosphaeriaceae were successfully re-isolated and identified from all seedlings other than from the controls.

4 | DISCUSSION

In this study, six Botryosphaeriaceae species (*B. dothidea*, *N. parvum*, *D. mutila*, *D. seriata*, *Do. sarmentorum*, *Do. omnivora*) were identified from 30 tree species and five shrubby species growing in urban areas, forests and ornamental nurseries. An additional four Botryosphaeriaceae species (*D. sapinea*, *Dothiorella* sp., *P. cupressi* and *S. visci*) were obtained in previous studies (Zlatković et al., 2016a,b, 2017), giving a total of ten Botryosphaeriaceae isolated from 47 tree and shrubby species in urban areas, forest plantations, forests and ornamental nurseries in the Western Balkans region. *P. cupressi* and *S. visci* occurred on only a single host, but *Dothiorella* sp. was found on two hosts, and the remaining Botryosphaeriaceae were associated with more than four hosts. Botryosphaeriaceae were mostly pathogenic to the hosts from which they were isolated but were also able to infect other tree species. The results of this study, based on a high number of hosts and the ability of Botryosphaeriaceae to infect both host and non-host species, illustrate the generalist nature of most of the species.

Very few Botryosphaeriaceae species appear to be host specific. The association of *S. visci* with only hemiparasitic *V. album* shrubs could be interpreted as host specificity. Other than this study, *S. visci* has been isolated only from *V. album* (Phillips et al., 2013; Zlatković et al., 2016a). Furthermore, Varga, Taller, Baltazár, Hyvönen, and Poczai (2012) showed that *S. visci* causes disease in *V. album*. *P. cupressi* was found only on *C. sempervirens* in this study but has previously also been isolated from *J. scopulorum*, both of which are Cupressaceae (Phillips et al., 2013). In our inoculation experiments, *P. cupressi* was able to infect *C. lawsoniana*, and it gave rise to large lesions. In contrast, *P. cupressi* did not cause lesions on inoculated *C. sempervirens*. This is possibly because *P. cupressi* was isolated as an endophyte from healthy tissues of *C. sempervirens* in the current study and previously by Abdollahzadeh et al. (2009). *Dothiorella* sp. was also isolated from a limited number of hosts in this study, namely from *T. plicata* and *C. atlantica*. This fungus appears to display some patterns of host preference as it was not able to cause lesions on *C. lawsoniana* despite its having been found on *C. atlantica*, on which it occurs as an endophyte.

Botryosphaeria dothidea and *N. parvum* are known to have a broad host range (Marsberg et al., 2017; Sakalidis et al., 2013). In the present study, *B. dothidea* occurred on the greatest number (22) of hosts and was shown to cause lesions on many of them. Isolations and inoculation experiments showed that this fungus is a significant pathogen of ornamental trees and shrubs. Apart from producing cankers on the inoculated seedlings and lesions on the inoculated branches, the pathogen was able to kill *S. giganteum*, *T. occidentalis*, *A. concolor*, *P. abies* plants and to girdle branches of *A. hippocastanum*. *B. dothidea* was also able to infect *P. patula* and *E. grandis* from which it was not isolated, and lesions produced on *P. patula* were significantly smaller than those caused by *N. parvum*. Similarly, in a study by Pavlic et al. (2007), *B. dothidea* isolated from native *Syzigium cordatum* produced lesions on *E. grandis* from which it has not been isolated, and it was less aggressive compared to *N. parvum*. *Neofusicoccum parvum* is an important pathogen of *Eucalyptus* spp. (Slippers et al., 2009) and its aggressiveness on this tree in the present study is thus not surprising. *N. parvum* is also a pathogen of ornamental trees and shrubs (e.g., Begoude et al., 2010; Varela, Redondo Fernández, Mansilla Vázquez, & Aguín Casal, 2011) and in the present study was shown to infect eight ornamental tree species. *N. parvum* was isolated from 16 hosts and these included ten gymnosperms and six angiosperms. Similarly,

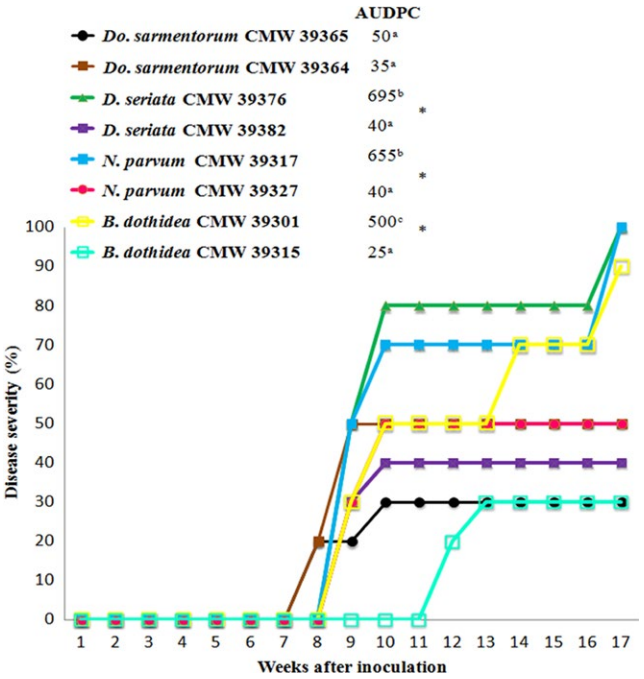

FIGURE 4 Disease progress curves and Area Under the Disease Progress Curve (AUDPC) of seedlings of *Thuja occidentalis* inoculated with *Diplodia seriata*, *Neofusicoccum parvum* and *Botryosphaeria dothidea*. The ANOVA test indicated that there were significant differences in AUDPC values of seedlings of *T. occidentalis* at $\alpha = 0.05$ level ($F = 913.15$, $df = 3$, $p = .000004$). Means with the same letter did not differ significantly, as determined by the LSD test at $\alpha = 0.05$ level. "*" indicates that AUDPC values of isolates of the same species were significantly different (z test, *D. seriata*: $z = 24.16$, $p < .00001$; *N. parvum*: $z = 23.33$, $p < .00001$; *B. dothidea*: $z = 20.73$, $p < .00001$)

Sakalidis et al. (2013) showed a lack of host specificity for this fungus and argued that the ability of *N. parvum* to exist as an endophyte in asymptomatic plants and to colonize a wide range of hosts could explain its wide distribution in many countries and continents. This is also consistent with the view that the endophytic nature of fungi such as the Botryosphaeriaceae implies that they are easily overlooked by quarantine systems (Burgess & Wingfield, 2017; Crous, Groenewald, Slippers, & Wingfield, 2016; Wingfield, Brockerhoff, Wingfield, & Slippers, 2015).

The majority of the Botryosphaeriaceae species (seven of 10) from the Western Balkans identified in this and in previous studies (Zlatković et al., 2016a,b, 2017) were isolated from multiple tree species, and numerous new host associations have been established. This is not surprising for Botryosphaeriaceae species such as the previously mentioned *B. dothidea* and *N. parvum*, which are known to occur on a large number of different tree species (Marsberg et al., 2017; Sakalidis et al., 2013). However, the previous study showed that the host range of the pine pathogen *D. sapinea* was unexpectedly broad, and the species was isolated from 16 tree and shrub species (Zlatković et al., 2017).

In this study, Botryosphaeriaceae species were shown to infect tree species having important ecological and cultural value. This adds to the threat many of these trees face already. For example, it was shown that *B. dothidea*, *D. seriata* and *D. mutila* contribute to the dieback of *C. sempervirens*, a valuable ornamental tree that is regarded as key element of the Mediterranean scenery (Xenopoulos, Andréoli, Panconesi, Pinto Ganhao, & Tusset, 1990). The "cypress canker" disease caused by *Seiridium* spp. is known as one of the most serious threats to the survival of *C. sempervirens* (Danti, Barberini, Pecchioli, Di Lonardo, & Della Rocca, 2014; Graniti, 1998) and this study adds to the understanding that there are other fungi involved in the dieback of this tree. Similarly, *N. parvum* was able to infect and kill *P. omorika*, an endemic tree that is in danger of extinction due to its limited population distribution, loss of genetic diversity and the effects of climate change (Alberto et al., 2013). Likewise, multiple Botryosphaeriaceae species were shown to infect *A. hippocastanum*, an endemic tree threatened by the leaf-mining moth *Cameraria ohridella* (Stojanović & Marković, 2004; Valade et al., 2009).

Various Botryosphaeriaceae found in this study were able to kill tree species that are economically valuable. For example, *D. seriata* was able to infect and eventually kill *Q. robur*, which is an economically valuable tree that is threatened by a change in flood regime and climate in northern Serbia (Stojanović, Levanič, Matović, & Orlović, 2015). *D. seriata* was able to produce lesions on *F. excelsior* branches. *F. excelsior* has been devastated by the fungal pathogen *Hymenoscyphus fraxineus* in many parts of Europe, including parts of

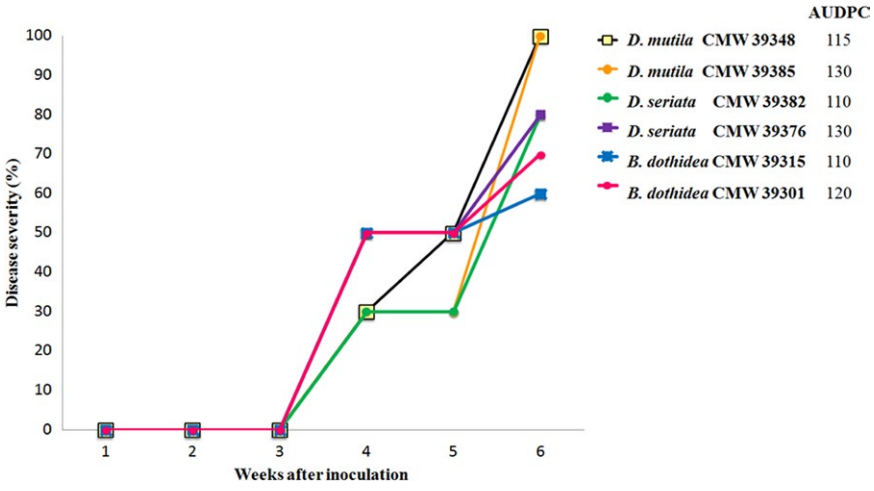

FIGURE 5 Disease progress curves and Area Under the Disease Progress Curve (AUDPC) of seedlings of *Cupressus sempervirens* inoculated with *Diplodia mutila*, *D. seriata* and *Botryosphaeria dothidea*. The ANOVA test indicated that there were no significant differences in AUDPC values of seedlings of *C. sempervirens* at $\alpha = 0.05$ level ($F = 5.97$, $df = 1$, $p = .07$)

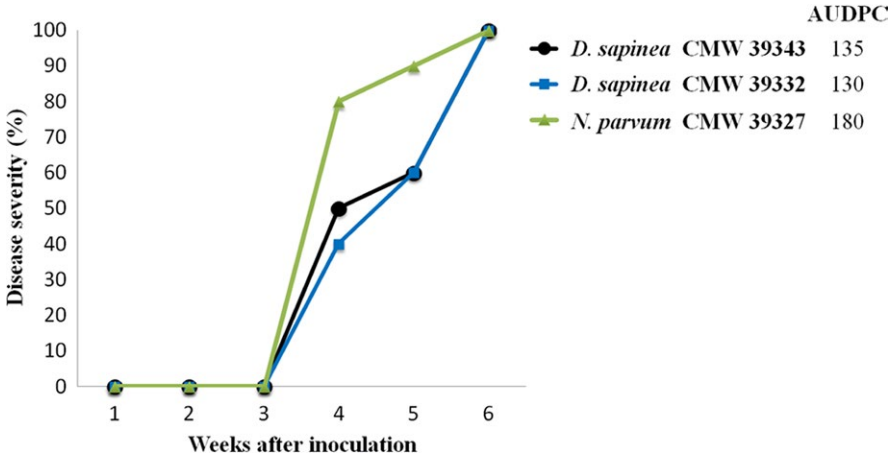

FIGURE 6 Disease progress curves and Area Under the Disease Progress Curve (AUDPC) of seedlings of *Picea omorika* inoculated with *Diplodia sapinea* and *Neofusicoccum parvum*. The *t* test indicated that there were no significant differences in AUDPC values of seedlings of *P. omorika* at $\alpha = 0.05$ level ($t = -10.97$, $df = 1$, $p = .06$). Data for *Diplodia sapinea* were retrieved from Zlatković et al. (2017) and serve for comparison with other Botryosphaeriaceae inoculated onto *P. omorika*

the Western Balkans (Keča, Kirisits, & Audrious, 2017; Milenković, Jung, Stanivuković, & Karadžić, 2017; Pautasso, Aas, Queloz, & Holdenrieder, 2013; Stanivuković, Karadžić, & Milenković, 2014), and this study shows that *D. seriata* might be also contributing to the impact of ash dieback disease.

The results from this study showed that an individual isolate of the Botryosphaeriaceae species has the potential for a wide range of aggressiveness to various often phylogenetically unrelated hosts. However, inoculations were carried out under partially controlled conditions on plants in the field and on cut branches; the level of plant stress was not measured. Botryosphaeriaceae are known as opportunistic pathogens capable of causing disease in a stressed host (Slippers et al., 2007). Thus, the aggressiveness of these fungi might have been higher than it would have been under completely controlled conditions or using living plants instead of plant parts. Moreover, a large amount of inoculum supplied with nutrients in the agar plug was placed on a wound and in contact with vascular tissues. Although this method has been considered to represent the natural mode of infection of these fungi, it would be likely that under such conditions even an opportunistic pathogen could cause the disease (Manawasinghe et al., 2016).

Multiple Botryosphaeriaceae species were commonly isolated from the same host species, trees or even the same lesions. Similarly, in a study by Pavlic et al. (2008), eight Botryosphaeriaceae were isolated from asymptomatic tissues of the native *S. cordatum* plants, and many species were obtained from the same plant. Luchi et al. (2014) reported a complex process of colonization of eight conifer species in Italy, where eleven Botryosphaeriaceae were found in symptomatic and asymptomatic tissues. Likewise, Jami et al. (2014) found no evidence that Botryosphaeriaceae species were tissue-specific. Results of the pathogenicity tests in the present study showed that Botryosphaeriaceae isolated from the same host might play different roles in the process of tree dieback. For example, seven species were isolated from *C. atlantica* and *C. lawsoniana*, but of these only a single strain of *N. parvum* and *B. dothidea* was able to cause disease symptoms on these hosts. However, given the opportunistic nature of Botryosphaeriaceae species and predicted more frequent extreme weather events in the Western Balkans (www.hidmet.gov.rs, accessed October 2017), the possibility of currently non-pathogenic Botryospaheriaceae becoming pathogenic to the particular host should not be neglected.

The results of this study confirm that the distribution of Botryosphaeriaceae is influenced by the host (Burgess et al., 2005; Fabre et al., 2011; Jami et al., 2014; Slippers et al., 2007). Host factors might have influenced the distribution of *S. visci* because shrubs of *V. album* grow only in the CR. In contrast, *Do. sarmentorum* and *Dothiorella* sp. were not isolated from the MR even though its hosts, *C. lawsoniana* and *C. atlantica*, are widely planted in both areas. Similarly, Burgess et al. (2005) showed that *N. australe* is prevalent in Western Australia but can rarely be found in Eastern Australia, even though its *Eucalyptus* host is present in both areas. *P. cupressi* was found only in the MR where its host, *C. sempervirens* is native although this tree is also occasionally planted in the CR. Differences in host genetics and the presence of both native and introduced trees could explain the different Botryosphaeriaceae community structures on this tree.

Diversity analyses from this study showed no significant differences in the Botryosphaeriaceae species diversity in the CR compared to the MR. This was surprising as previous studies showed that Botryosphaeriaceae species diversity and occurrences are also influenced by the environment in which the given host occurs (Fabre et al., 2011; Slippers et al., 2007). However, no general conclusions can be made based on the current data regarding the differences in Botryosphaeriaceae species distributions because the number of samples from the MR was unduly limited.

The dieback of trees sampled in this study could be associated with some form of stress to trees linked to either recent extreme weather patterns, a "heat island effect," other stresses affecting trees in the cities, or planting of species on suboptimal sites (Allen et al., 2010; Zlatković et al., 2016a, 2017). *P. abies* sampled in this study also occurs at the edge of its southern range, which might imply stress symptoms. Furthermore, the shallow-rooted *P. abies* is vulnerable to drought, which is already causing mortality of this tree in Central and Northern Europe. Similarly, *C. atlantica* is experiencing mass mortality in its native range in North Africa linked to climate change (Allen et al., 2010). In addition, a number of forest management practices might have contributed stress on these trees. For example, ornamental trees in Serbia are frequently propagated from locally collected seeds in urban areas of unknown provenance (www.minpolj.gov.rs) and that have not been adequately tested for planting in given environments. Also, *P. laurocerasus* is a shade demanding species, but in the cities of Serbia, these shrubs are frequently planted on open spaces.

In urban areas, exotic tree species are planted close to native species that could provide a source of inoculum for cross-infection to occur. The results of the inoculation tests confirmed that Botryosphaeriaceae isolated from introduced trees can infect native trees and *vice versa*. It also shows that Botryosphaeriaceae can move from ornamental to forest trees and *vice versa*. For example, isolates of *B. dothidea* from *C. lawsoniana* were able to infect seedlings of native *P. abies*. Similarly, close proximity of native and exotic species was hypothesized to be the reason for the occurrence of *N. eucalyptorum*, a pathogen of *Eucalyptus* spp. on native myrtaceous hosts in Uruguay (Pérez, Wingfield, Slippers, Altier, & Blanchette, 2010) and for the infection of *Eucalyptus* plantations established close to the native vegetation in South Africa (Burgess & Wingfield, 2017; Pavlic et al., 2007). *D. seriata* is a well-known pathogen of fruit trees (Slippers et al., 2007); however, in this study, isolates of *D. seriata* from *C. pisifera* and *Q. robur* were not able to produce lesions on *P. cerasus* but could produce lesions or infect and eventually kill four ornamental and two forest tree species. Population genetic analyses are needed to test the gene flow between populations of *D. seriata* on fruit, ornamental and forest trees as has recently been shown by Mehl, Slippers, Roux, and Wingfield (2017).

The Botryosphaeriaceae were most diverse on gymnosperms in this study. With the exception of *A. hippocastanum* and *S. caprea*, which harboured four and three Botryosphaeriaceae, respectively, all other angiosperms harboured one or two species. This is surprising

TABLE 4 Pathogenicity of Botryosphaeriaceae species on various hosts in this and in previous studies in the Western Balkans (Zlatković et al., 2016b, 2017)

Host	Botryosphaeriaceae species									
	Botryosphaeria dothidea	*Neofusicoccum parvum*	*Diplodia seriata*	*D. mutila*	*D. sapinea*[m]	*Dothiorella sarmentorum*	*Do. omnivora*	*Dothiorella* sp.	*Phaeobotryon cupressi*	*Sphaeropsis visci*
Chamaecyparis lawsoniana	−[e]/+[f]	−	−	−	−	−	−	−	+	−
Cedrus atlantica	−	+[g]/−[h]	−	−	−	−		−		
C. deodara		−[g]			−					
Prunus laurocerasus		+[a,g,h]/+[g,h]		−[a,k]/+[a,l]/+[g,h,k]	−					
Sequoiadendron giganteum	+	+					+			
Thuja occidentalis	+	+	+		−	+				
Pseudotsuga menziesii	−				+					
Abies concolor	+		+		+					
Juniperus horizontalis	+				+					
Cupressus arizonica	+			+						
Liriodendron tulipifera	+									
Magnolia grandifolia	+									
Ligustrum vulgare			+							
C. sempervirens	+		+	+			−		−	
Picea omorika		+[g]								
Quercus robur			+							
Pittosporum tobira		+		−						
Aesculus hippocastanum[b]	+	+		+		+				
Pinus patula[c]	+	+			+					
Eucalyptus grandis[c]	+	+			−					
P. abies	+	+			+					
P. pungens					+					
P. nigra					+					
P. sylvestris					+					
F. sylvatica[b]					+					
Viscum album[b,d]										+

(continues)

TABLE 4 (continued)

Host	Botryosphaeriaceae species								
	Botryosphaeria dothidea	*Neofusicoccum parvum*	*Diplodia seriata*	*D. mutila*	*D. sapinea*[m]	*Dothiorella sarmentorum*	*Do. omnivora*	*Dothiorella* sp.	*Phaeobotryon cupressi*	*Sphaeropsis visci*
Populus tremula[b]	+									
Fraxinus excelsior[b]			+[i]/–[j]				–			
Forsythia europaea						–				
Quercus cerris[b]	+									
Taxus baccata	–									
Prunus cerasus			–							
P. nigra var. italica	–	+								
Quercus rubra		–	–			–				
Platanus acerifolia	–									

[a]Inoculations conducted on leaves of seedlings in the field, [b]cut branch inoculations in laboratory conditions, [c]stem inoculations in the greenhouse, [d]leaf inoculations on cut branches in laboratory conditions. All other inoculations were conducted on stems of seedlings in the field. Empty spaces indicate that the species was not isolated from the host and/or inoculated into host, +The species was pathogenic, –The species was not pathogenic, [e]CMW 39301, [f]CMW 39315, [g]CMW 39327, [h]CMW 39317, [i]CMW 39376, [j]CMW 39382, [k]CMW 39348, [l]CMW 39358, [m]Zlatković et al. (2017).

and in contrast to the results of most previous studies. For example, previous authors (De Wet et al., 2008; Sakalidis et al., 2013; Slippers et al., 2013), the Botryosphaeriaceae are most common and diverse on angiosperms. In contrast, Alves et al. (2013) reported great diversity of these species associated with conifers in Portugal, but conifers were the only trees sampled in the study, and no comparison with the diversity on angiosperms was made. It is difficult to explain these patterns because sampling, host diversity, climate and other factors are not consistent across these studies. Overall, however, the results show that both gymnosperms and angiosperms can harbour substantial Botryosphaeriaceae diversity in different environments and circumstances.

This study has demonstrated that various trees and shrubs in the Western Balkans harbour a wide diversity of Botryosphaeriaceae, and with the exception of *S. visci* on *V. album*, most have broad host ranges. However, some Botryosphaeriaceae species appeared to occur predominantly on certain hosts and this suggests that those tree species posses some characteristics favourable for these fungi. The fact that some Botryosphaeriaceae were able to cross-infect taxonomically unrelated trees and eventually kill the plants emphasizes the importance of this fungal group. There is clearly a need for further research considering the pathways of introduction and spread of these fungi (Slippers et al., 2017) as well as methods (Crous et al., 2016) to understand and manage the diseases with which they are associated.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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