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# Genetic uniformity characterizes the invasive spread of *Neofusicoccum parvum* and *Diplodia sapinea* in the Western Balkans

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## Abstract

In the past decade, trees and shrubs in the Western Balkans region have been damaged by canker and die-back disease caused by Botryosphaeriaceae species. These pathogens include *Neofusicoccum parvum* and *Diplodia sapinea*. In this study, we determine genetic diversity and structure between populations of *N. parvum* and *D. sapinea* from Serbia and Montenegro (Western Balkans) using DNA sequence data of the internal transcribed spacer rDNA, translation elongation factor 1-alpha, β-tubulin-2 and microsatellite markers. The relationship of both pathogens was compared for populations from the Continental (CR) and Mediterranean (MR) regions and for isolates of *D. sapinea* from *Cedrus* spp. and *Pinus* spp. *Neofusicoccum parvum* and *D. sapinea* were shown to have a low gene and genotypic diversity across the regions and hosts. All genotypes of *D. sapinea* found on *Pinus* spp. were also present on *Cedrus* spp. The CR and MR populations of both species were found to be only slightly separated from one another by a geographical barrier. Low genetic diversity and dominance of *N. parvum* and *D. sapinea* on non-native trees suggests that these species have most likely been introduced into Western Balkans, possibly through the movement of infected plants.

## 1 | INTRODUCTION

Biological invasion by "alien" plant pathogens represents an important driver of tree disease epidemics worldwide (Brasier, 2008; Desprez-Loustau et al., 2007; Santini et al., 2013; Wingfield, Brockerhoff, Wingfield, & Slippers, 2015). Classic examples include the chestnut blight epidemic on *Castanea* spp. in the USA and Europe caused by *Cryphonectria parasitica* (Murr.) Bar (Anagnostakis, 2001); Dutch elm disease epidemics on American and European *Ulmus* spp. caused by *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* (Brasier) (Brasier & Buck, 2001); canker stain disease of plane in Europe on *Platanus* spp. caused by *Ceratocystis platani* (Walter) Engelbrecht & Harrington (Tsopelas, Santini, Wingfield, & Wilhelm de Beer, 2017), spread of the pitch canker disease caused by *Fusarium circinatum* Nirenberg & O'Donnell from its origin in Central America (Wingfield et al., 2008), *Phytophthora* spp. outbreaks in the USA and Europe (Gruenwald, Garbelotto, Goss, Heungens, & Prospero, 2012; Hardham & Blackman, 2018) and the recent emergence of the ash die-back pathogen *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya in Europe (Keča, Kirisits, & Audrious, 2017; Milenković, Jung, Stanivuković, & Karadžić, 2017; Pautasso, Aas, Queloz, & Holdenrieder, 2013).

  

Increased international trade and travel is considered to be the main driver of invasive alien fungi (Desprez-Loustau, 2008; Ghelardini, Pepori, Luchi, Capretti, & Santini, 2016; Vannini, Franceschini, & Vettraino, 2012; Wingfield et al., 2015). Trade in plants and plant products are considered amongst the most common means for the introduction of alien fungal pathogens (Ghelardini et al., 2016; Liebhold, Brockerhoff, Garrett, Parke, & Britton, 2012; Migliorini, Ghelardini, Tondini, Luchi, & Santini, 2015; Vannini et al., 2012). Amongst these, rough wood packaging and large mature specimen trees ("instant trees") traded with bark, root balls and soil attached are considered to be the most important risk pathways for invasive pathogens (Brasier, 2008; Ghelardini et al., 2016; Santini et al., 2013).

Pathogens that move with their hosts as "hitchhikers" as saprophytes, endophytes or as resting spores in the soil are especially difficult to detect and to prevent their introduction (Burgess, Crous, Slippers, Hantula, & Wingfield, 2016; Crous, Groenewald, Slippers, & Wingfield, 2016; Ghelardini et al., 2016; Migliorini et al., 2015; Santini et al., 2013). A typical example is fungi in the Botryosphaeriaceae, which are known as endophytes that remain latent in asymptomatic plant tissue for long periods of time. Some of these endophytes may become important pathogens where they are introduced (Burgess & Wingfield, 2017; Slippers & Wingfield, 2007; Wingfield et al., 2015).

Risks of host range expansion of introduced pathogen are considered greatest in urban areas with gardens and parks comprising dense assemblages of exotic and native plants. Moreover, urban trees are grown in "locally heated islands," experience stress from, that is, pollution, soil compaction and such conditions may predispose trees to pathogen attack, increasing the likelihood that an alien pathogen will successfully establish (Santini et al., 2013; Walther et al., 2009). After establishment, invasive pathogens can cause severe ecological, social and economic impacts and destabilize entire ecosystems by affecting, that is, hydrology, recreation, carbon and nitrogen cycles (Brasier, 2008; Mitchell et al., 2014; Stenlid, Oliva, Boberg, & Hopkins, 2011). Invasive pathogens are also affected by other drivers of global change, such as climate change, which may increase their invasion potential (Ghelardini et al., 2016; Ramsfield, Bentz, Faccoli, Jactel, & Brockerhoff, 2016; Walther et al., 2009).

Despite various phytosanitary measures and regulations (e.g., plant passport, ISPM-15, 36, "EU black list," EPPO A1 and A2 lists, sentinel plantings), the problem of invasive pathogens is especially evident in Europe. Santini et al. (2013) reported 60 alien invasive forest pathogens in Europe compared to only 17 found in the USA by Aukema et al. (2010). A long history of colonialism and extensive planting of non-native trees are important factors considered to be responsible for the high number of alien pathogens being established on this continent (Brasier, 2008; Santini et al., 2013).

*Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips and *Diplodia sapinea* (Fr.) Fuckel (syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton) are plant pathogens that commonly occur in temperate, Mediterranean and tropical climates worldwide (Phillips et al., 2013; Slippers, Crous, Jami, Groenewald, & Wingfield, 2017; Slippers & Wingfield, 2007). *Neofusicoccum parvum* has been found associated with a wide range of tree species, including grapevine, fruit and forest trees, whereas *D. sapinea* is known as a pathogen of conifers, mostly pines, but it also infects spruces and firs (Slippers et al., 2017; Swart & Wingfield, 1991; Zlatković, Keča, Wingfield, Jami, & Slippers, 2017). These fungi are latent pathogens, causing disease when trees are subjected to stresses (e.g., climate extremes) (Mehl, Slippers, Roux, & Wingfield, 2013; Slippers & Wingfield, 2007). *Diplodia sapinea* is thought to be native in North America and Eurasia, and it has been introduced into countries of the Southern Hemisphere where pines are exotic (Bihon, Slippers, Burgess, Wingfield, & Wingfield, 2012; Burgess, T., Wingfield,B. D., & Wingfield, M. J., 2001, 2004; Smith, Wingfield, Wet, & Coutinho, 2000). *Neofusicoccum parvum* has been speculated to be native to Southern Africa, where populations exhibit high genetic diversity (Sakalidis, Slippers, Wingfield, Hardy, & Burgess, 2013).

*Neofusicoccum parvum* and *D. sapinea* have recently been shown responsible for disease on various trees and shrubs in the Western Balkans region (Zlatković, Keča, Wingfield, Jami, & Slippers, 2016a; Zlatković et al., 2017; Zlatković, Wingfield, Jami, & Slippers, 2018), but nothing is known about the population genetics or biology of these species in this region. In this study, we determined genetic diversity and structure between different populations of *N. parvum* and *D. sapinea*. The relationships between isolates from the Continental (CR) and Mediterranean (MR) regions and between isolates of *D. sapinea* from *Cedrus* spp. and *Pinus* spp. were also considered. This was achieved using the DNA sequence data for the internal transcribed spacer (ITS) rDNA, translation elongation factor 1-alpha (TEF 1-α), β-tubulin-2 (BT2), RNA polymerase II gene (RPB2) and microsatellite markers.

## 2 | MATERIALS AND METHODS

### 2.1 | Fungal isolates

The isolates used in this study were collected during a survey of trees showing disease symptoms such as die-back, cankers and resin exudation as previously described (Zlatković et al., 2016a) in Serbia and Montenegro between 2009 and 2014. Symptomatic samples were mostly collected in the cities, but also in plantations, forest stands, nurseries of ornamental plants and two isolates of *D. sapinea* were collected from *Pinus radiata* D. Don trees grown on Mt. Athos in Greece (Supporting Information Table S1; Zlatković et al., 2016a; Zlatković et al., 2017; Zlatković et al., 2018).

From each tree, isolations were made from symptomatic and asymptomatic tissues as described previously by Zlatković et al. (2016a). Fifty-six isolates of *N. parvum* and 87 isolates of *D. sapinea* were identified in previous studies based on the DNA sequence data of the ITS region, TEF-1-α, β-tubulin, large subunit rRNA and RPB2 (Zlatković et al., 2016a, 2017, 2018). Haploid cultures established from hyphal-tip transfers used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

## 2.2 | DNA extraction, microsatellite-PCR amplification and genotyping

Microsatellite analyses were conducted on a subset of *N. parvum* and *D. sapinea* isolates identified in the previous studies, including 46 isolates of *N. parvum* and 85 isolates of *D. sapinea* (Zlatković et al., 2016a, 2017, 2018). Fungal cultures were grown on malt extract agar in Petri dishes for seven days, and DNA was extracted from the mycelium as previously described (Zlatković et al., 2016a). Six of the seven fluorescently labelled primer pairs previously designed for species of *Neofusicoccum* spp. (Slippers et al., 2004) successfully amplified isolates of *N. parvum*. Primer pairs BOT21 and BOT22 could not amplify these isolates and were excluded from subsequent analyses. Primer pairs BOT15 and BOT16 produced numerous stutter peaks that would hamper the reliable interpretation of the genotypes and were also excluded from further analyses. Thirteen microsatellite loci of *D. sapinea* were amplified using primer pairs specifically designed for this fungus by Burgess, T., Wingfield, B. D., and Wingfield, M. J. (2001) and Bihon, Burgess, Slippers, Wingfield, and Wingfield (2011). However, primer pairs TB19 and TB 20–2, TB37 and TB38 and WB1-a and WB1-b were discarded from the analyses due to the excessive stuttering (Supporting Information Table S2).

The 25 μl PCR mixtures contained 2.5 μl of 10 mM PCR buffer (PCR buffer with $MgCl_2$), 1 μl of 100 mM of each dNTPs, 0.25 μl of 10 mM of each primer, 2 ng of genomic DNA, 0.2 μl (1U) of Fast Start Taq polymerase (Roche Molecular Biochemicals, Indianapolis) and 18.8 μl of sterile distilled Sabax water. In most cases, the PCR mixture contained an additional 1–3 μl of 25 mM $MgCl_2$ (data not shown). PCRs were performed using the following protocol: 95°C for 3–6 min initial denaturation followed by ten cycles of 95°C for

**TABLE 1** Allele size (bp) and frequency at five loci for *Neofusicoccum parvum* and at ten loci for *Diplodia sapinea*

| Locus | Allele (bp) | Pop 1[a] (Continental) | Pop 2[a,b] (Mediterranean) | Pop 3[c] (*Cedrus* spp.) | Pop 4[c] (*Pinus* spp.) | All[d] |
|---|---|---|---|---|---|---|
| *Neofusicoccum parvum* | | | | | | |
| BotF11 | 429 | 1.000 | + | | | 1.000 |
| BotF17 | 232 | 1.000 | + | | | 0.935 |
| | 248 | - | + | | | 0.043 |
| | 260 | - | + | | | 0.022 |
| BotF18 | 244 | - | + | | | 0.022 |
| | 245 | 1.000 | + | | | 0.913 |
| | 247 | - | + | | | 0.022 |
| | 250 | - | + | | | 0.043 |
| BotF23 | 423 | 1.000 | + | | | 0.065 |
| | 424 | - | + | | | 0.065 |
| BotF35 | 223 | 1.000 | + | | | 1.000 |
| *Diplodia sapinea* | | | | | | |
| SS1 | 411 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| SS2 | 195 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| SS7 | 384 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| SS8 | 280 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| SS10 | 313 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| SS11 | 173 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| SS13 | 149 | 0.078 | - | 0.091 | 0.040 | 0.059 |
| | 151 | 0.094 | - | 0.023 | 0.160 | 0.071 |
| | 157 | 0.828 | 1.000 | 0.886 | 0.800 | 0.129 |
| SS14 | 149 | 0.125 | - | 0.091 | 0.040 | 0.094 |
| | 161 | 0.797 | 0.762 | 0.795 | 0.160 | 0.788 |
| | 164 | 0.047 | 0.238 | 0.120 | 0.091 | 0.094 |
| | 171 | 0.031 | - | 0.023 | - | 0.024 |
| SS15 | 68 | 0.938 | 0.714 | 0.795 | 0.040 | 0.882 |
| | 71 | 1.000 | 1.000 | 0.205 | 0.040 | 1.000 |
| SS16 | 108 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

[a]Populations 1 and 2 = geographically defined populations of *N. parvum* and *D. sapinea* originating from Continental and Mediterranean climate-type region [b]Allele frequencies for population 2 of *N. parvum* were not calculated because of the small sample size [c]Populations 3 and 4 = isolates of *D. sapinea* originating from *Cedrus* spp. and *Pinus* spp. [d]All isolates of *N. parvum*/*D. sapinea*, + Allele was present in a population.

30 s, 52–65°C for 45 s, 72°C for 1 min; then by 20–30 cycles of 95°C for 30 s, 52–65°C for 45 s, 72°C for 1 min +0.05 s/cycle increase and 60°C for 30 min final extension (to reduce stutters in the microsatellite peaks). Where the above-mentioned PCR protocol failed to amplify in some isolates, PCRs were performed using touchdown thermal cycling programs (Don, Cox, Wainwright, Baker, & Mattick, 1991) with a 6°C to 10°C span of annealing temperatures, ranging from 68°C to 62°C or 65°C to 55°C. Amplifications were carried out alongside a control containing sterile water in place of the DNA. Details of the cycling parameters and PCR conditions for each primer set are shown in Supporting Information Table S2.

PCR products were separated by electrophoresis on 2% agarose gels, stained with GelRed (Biotium, Hayward, California, USA) and visualized under ultraviolet light. Sizes of PCR products were estimated by comparison with a DNA molecular weight marker (Gene Ruler $^{TM}$ 100 bp DNA Ladder, Fermentas). Fluorescently labelled PCR products were multiplexed for each species separately, and 1 µl of these multiplexed PCRs was separated on ABI Prism 3,500 Genetic analyzer. Allele sizes of labelled microsatellite-PCR products were compared against a LIZ-500 internal size standard and analysed with the GeneScan 2.1 and GeneMapper 3.7 softwares (Applied Biosystems).

## 2.3 | Microsatellite analyses

The multilocus genotypes (MLGs) for each isolate were generated by coding the alleles at different loci using alphabetical letters for each individual allele (e.g., AAAAAABBAA). Prior to analyses, data for isolates of each species were separated into two collections representing the climatic region from which they originated (CR and MR). Additionally, data for isolates of *D. sapinea* were separated into two host populations that originated either from *Cedrus* spp. or from *Pinus* spp. Allele frequencies were determined for the entire populations, as well as for predefined geographic and host sub-populations (Table 1). For each population, the observed number of alleles, number of unique alleles, the observed number of multilocus genotypes (MLGs, g) and percentage of polymorphic loci (P) were evaluated. *Diplodia sapinea* data sets were clone-corrected by removing the duplicate MLGs, and microsatellite analyses were conducted on both original and clone-corrected data sets. Population genetic parameters for the *N. parvum* population were calculated only on the original data sets because the number of individuals after clone correction was less than ten.

Gene diversity of each population was estimated by calculating the Nei's unbiased gene diversity (corrected for sample size) (Hexp; Nei, 1978). Genotypic diversity was evaluated using the Shannon's diversity index of MLG diversity (H), which was calculated with $H = \sum PiInPi$ and corrected for differences in isolate numbers with $H' = H/ln(g)$, where Pi is the frequency of the $i^{th}$ MLG in a given host population and g is the number of MLGs observed in each population (Grünwald, Goodwin, Milgroom, & Fry, 2003; Shannon, 2001). Stoddart and Taylor's index of MLG diversity (G) was estimated using the equation $G = 1/\sum Pi^2$ and corrected for unequal sample sizes with G' = G/g where Pi is the observed frequency of the $i^{th}$ genotype in the population and g is the observed number of MLGs in the population (Grünwald et al., 2003; Stoddart & Taylor, 1988). Values for H' and G' range from 0 (single genotype present) to 1 (each isolate represents a different genotype). Genotypic evenness ($E_5$) was calculated with $E_5 = (G-1)/(e^{H'}-1)$ (Grünwald et al., 2003). Values of E can range from 0 to 1, with lower values indicating that a certain genotype dominates in the collection of isolates from a particular host population. Clone corrections, estimations of gene diversity, genotypic diversity and genotypic evenness were performed in R v. 3.2.3 (R Core Team, 2015) using population genetics package "poppr" v. 2.1.0 (Kamvar, Tabima, & Grünwald, 2014). Allelic richness (Ar) was computed in R using package "hierfstat" v. 004–22 (Goudet & Jombart, 2015). The package implements a rarefaction approach allowing for comparison of the values for a standardized sample size corresponding to the smallest sample size across populations. Other analyses were not conducted because of the clonal structure and small sample sizes of each population. Values of the genetic diversity for the *N. parvum* and *D. sapinea* populations were compared using z-test (Pocock, 2006) at the 5% significance level ($z > 1.96$, $p < 0.05$).

## 2.4 | DNA sequence haplotype networks and phylogeographic relationships with haplotypes outside Western Balkans

DNA sequence haplotype analyses were conducted using all isolates of *N. parvum* and *D. sapinea* identified in the previous studies ie 56 isolates of *N. parvum* and 87 isolates of *D. sapinea* (Zlatković et al., 2016a, 2017, 2018). Data sets containing sequences of ITS, TEF-1-α, β-tubulin and RPB2 for isolates of *N. parvum* and ITS, TEF-1-α and β-tubulin for isolates of *D. sapinea* were aligned separately as described in Zlatković et al. (2016a). Because incongruence between the studied genes was not detected following the partition homogeneity test (Farris, Kallersjo, Kluge, & Bult, 1995) using PAUP v. 4 (Swofford, 2003), combined data sets for each species were included in the subsequent analyses. Haplotypes were determined from the aligned data in DnaSP 5.10.1 (Librado & Rozas, 2009). Unique haplotypes were then pooled with unique haplotypes of *N. parvum* and *D. sapinea* obtained from GenBank covering a large portion of the known geographic range of each species. Relationships between the haplotypes for each gene region separately and for the combined datasets were inferred via a median-joining network calculated using NETWORK 4.6.1.2 (http://www.fluxus-engineering.com, Supporting Information Table S3).

# 3 | RESULTS

## 3.1 | Genetic and genotypic diversity of *Neofusicoccum parvum*

All five microsatellite loci were monomorphic for the *N. parvum* CR population, and three loci were polymorphic in the MR population

**TABLE 2** Measures of genetic diversity based on the analysis of five microsatellite loci for *Neofusicoccum parvum* and ten loci for *Diplodia sapinea*

| | *Neofusicoccum parvum* | | | *Diplodia sapinea* | | | | |
|---|---|---|---|---|---|---|---|---|
| | Pop 1 (Continental)[a] | Pop 2 (Mediterranean)[b] | Total isolates | Pop 1 (Continental)[a] | Pop 2 (Mediterranean)[b] | Pop 3 (*Cedrus*)[c] | Pop 4 (*Pinus*)[d] | Total isolates |
| No. of isolates | 40 | 6 | 46 | 64 | 21 | 44 | 25 | 85 |
| No. of alleles | 5 | 11 | 11 | 16 | 12 | 16 | 15 | 16 |
| No. of private alleles | 0 | 6 | N/A | 4[e] | 0 | 1[f] | 0 | N/A |
| g | 1 | 5 | 6 | 7 | 3 | 7 | 6 | 7 |
| P (%) | 0 | 60 | 60 | 30 | 20 | 30 | 30 | 30 |
| Ar | 2[g] | | 2.7[g] | 2.33[h] | 2.2[h] | 2.38[i] | 2.33[i] | 2.33[h]/2.37[i] |
| Ar(c) | | | | 2.6[h] | 2.2[h] | 2.6[i] | 2.5[i] | 2.6[h,i] |
| Hexp | 0 | | 0.084 | 0.078 | 0.081 | 0.09 | 0.072 | 0.081 |
| Hexp(c) | | | | 0.152 | 0.133 | 0.152 | 0.153 | 0.152 |
| H | 0 | | 0.591 | 1.43 | 1.05 | 1.5 | 1.24 | 1.47 |
| H(c) | | | | 1.95 | 1.1 | 1.95 | 1.79 | 1.95 |
| H' | 0 | | 0.33 | 0.73 | 0.96 | 0.77 | 0.69 | 0.76 |
| H'(c) | | | | 1 | 1.78 | 1 | 1 | 1 |
| G | 1 | | 1.32 | 2.82 | 2.74 | 3.38 | 2.47 | 3 |
| G(c) | | | | 7 | 3 | 7 | 6 | 7 |
| G' | 0 | | 0.22 | 0.4 | 0.91 | 0.48 | 0.41 | 0.43 |
| G'(c) | | | | 1 | 1 | 1 | 1 | 1 |
| $E_5$ | | | 0.392 | 0.57 | 0.93 | 0.68 | 0.6 | 0.6 |
| $E_5$(c) | | | | 1 | 1 | 1 | 1 | 1 |

*Note*. g: Observed number of multilocus genotypes (MLGs); P: Number of polymorphic loci; Ar: Allelic richness using non-clone-corrected data, Ar (c). Allelic richness using clone-corrected data; Hexp: Nei's unbiased gene diversity using non-clone-corrected data (Nei, 1978), Hexp (c). Nei's unbiased gene diversity using clone-corrected data; H: Shannon–Wiener index of MLG diversity using non-clone-corrected data (Shannon, 2001), H(c). Shannon–Wiener index of MLG diversity using clone-corrected data; H': Corrected Shannon–Wiener index of MLG diversity using non-clone-corrected data (Grünwald et al., 2003), H'(c). Corrected Shannon–Wiener index of MLG diversity using clone-corrected data; G: Stoddart and Taylor's index of MLG diversity using non-clone-corrected data (Stoddart & Taylor, 1988), G(c). Stoddart and Taylor's index of MLG diversity using clone-corrected data; G'(c): Corrected Stoddart and Taylor's index of MLG diversity using non-clone-corrected data (Grünwald et al., 2003), G(c). Corrected Stoddart and Taylor's index of MLG diversity using non-clone-corrected data; $E_5$: Genotypic evenness using non-clone-corrected data (Grünwald et al., 2003), $E_5$(c) Genotypic evenness using clone-corrected data; Comparison of the measures of genetic diversity of population1 of *N. parvum* and *N. parvum* entire isolate collection, and *D. sapinea* populations 1 and 2, 3 and 4, 2 and *D. sapinea* entire isolate collection was not significant at $p < 0.05$ (z-test). Population genetic parameters for the *N. parvum* population were calculated only on the original data sets because the number of individuals after clone correction was less than ten. Some of the population genetic parameters for the *N. parvum* population were not calculated due to a small sample size.
[a]Isolates collected in the Continental climate-type region. [b]Isolates collected in the Mediterranean climate-type region. [c]Isolates obtained from *Cedrus* spp. [d]Isolates obtained from *Pinus* spp., [e]Compared to isolate collection from the Mediterranean region. [f]Compared to isolate collection from *Pinus* spp. [g]Standardized for 40 isolates. [h]Standardized for 21 isolates. [i]Standardized for 25 isolates.

(Tables 1 and 2). For the entire data set of 46 *N. parvum* isolates, a total of 11 alleles were observed across five loci examined. There were five alleles detected in the CR population, and 11 alleles were found in the MR population. Five alleles were shared among the two populations, and there were six private alleles in the MR population. Low gene diversity was observed across in the total *N. parvum* population (Hexp = 0.084). The CR population was completely clonal.

Among the 46 isolates of *N. parvum* examined, a total of six microsatellite MLGs were found (Figure 1; Table 2). Of these, one MLG was detected in the CR population and five MLGs were detected in the MR population. No MLGs were shared between the two populations. Corrected Shannon–Wiener genotypic diversity (H') and Stoddart and Taylor genotypic diversity (G') values were low for the entire data set of *N. parvum* isolates (0.33, 0.22).

The MLG S1 was shared among the highest number of hosts (ten), MLG S4 was shared among the two hosts and all the remaining MLGs were associated with a single host species. *Cedrus atlantica* (Endl.) Manetti ex Carrière, *Prunus laurocerasus* L. and *Eucalyptus globulus* Labill. each harboured two MLGs and ten other hosts harboured a single MLG. With the exception of *Pittosporum tobira* (Thunb.) W.T.Aiton, all the hosts had one MLG shared with at least one other host. Ten hosts shared a MLG with two other hosts and two hosts shared a MLG with one other host (Figure S1;

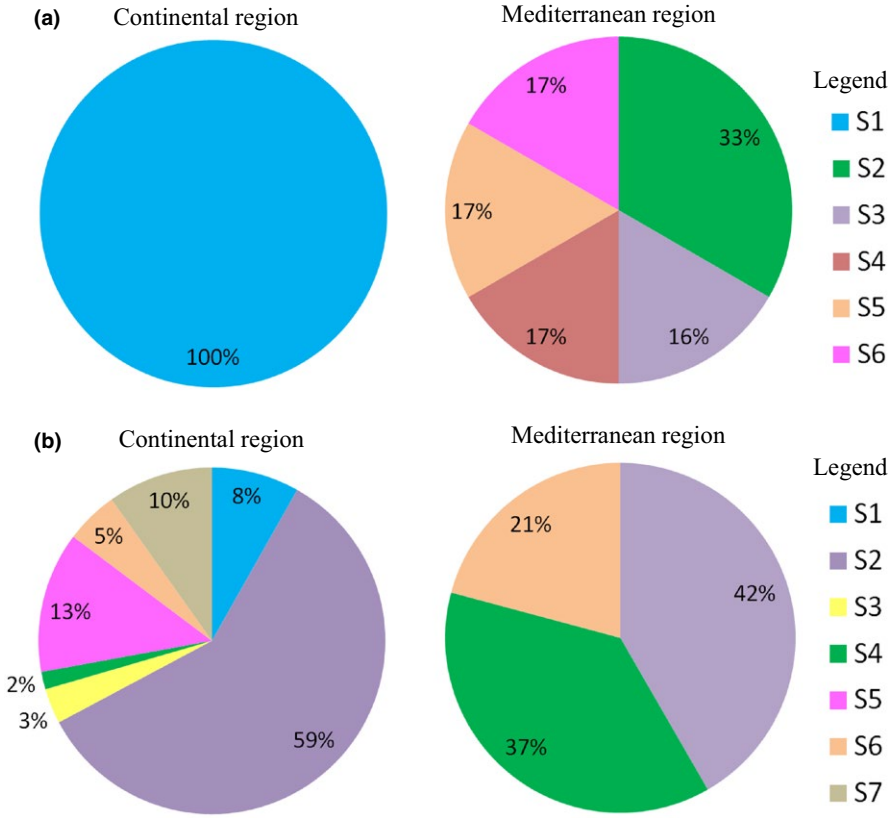


**FIGURE 1** Pie charts representing genotypic diversity of *Neofusicoccum parvum* and *D. sapinea* populations from the Continental climate-type region and Mediterranean climate-type region along the Adriatic cost. (a) *N. parvum*; (b) *Diplodia sapinea*. Different multilocus genotypes are indicated as S1–S6 for *N. parvum* and S1–S7 for *D. sapinea*

Supporting Information Table S4). Six MLGs were found on hosts in urban areas, and one was also detected on seedlings in ornamental plant nurseries (Supporting Information Table S4; Figure S2).

### 3.2 | Genetic and genotypic diversity of *Diplodia sapinea*

Two of the loci were polymorphic in the MR population of *D. sapinea* and three of the loci were polymorphic in the remaining analysed populations (Table 2). For the *D. sapinea* data set of 85 isolates, a total of 23 alleles were observed across ten loci examined (Table 1). Sixteen alleles were detected in the CR population, in the population from *Cedrus* spp. and for the entire data set. Fifteen alleles were identified in the population from *Pinus* spp. and 12 alleles were detected in the MR population. Twelve alleles were shared among the two geographically defined populations and 15 alleles were shared among the populations from *Cedrus* spp. and *Pinus* spp. There was one private allele in the CR population and four in the *D. sapinea* population from *Cedrus* spp. The corresponding Ar was low for the total *D. sapinea* population (2.2–2.38, Table 2). Low gene diversity was observed across all isolates of *D. sapinea*, as well as across geographically and host defined *D. sapinea* populations (Hexp = 0.072–0.09).

Seven MLGs were observed among 85 isolates of *D. sapinea* from the CR, and three MLGs were identified among isolates from the MR. Seven MLGs were found among isolates from *Cedrus* spp., and six MLGs were detected among isolates from *Pinus* spp. (Figures 1 and 2; Table 2). Three MLGs were shared between populations from the CR and MR, and six MLGs were shared between populations from *Cedrus* spp. and *Pinus* spp. Corrected Shannon–Wiener genotypic diversity (H') and Stoddart and Taylor genotypic diversity (G') values did not differ significantly among *D. sapinea* populations. As reflected by the $E_5$ values, the frequencies of the MLGs in all isolate collections were not evenly distributed. For example, in a population from the CR, MLG S2 comprised 60% of the population ($E_5$ = 0.57). The only exception was the population from MR that was characterized by high $E_5$ value ($E_5$ = 0.93), suggestive of a more even within-population distribution of MLGs.

The MLG S2 was shared among the highest number of hosts (11); MLG S7 was shared among four hosts, MLGs S4, S5 and S6 were shared among three hosts, whereas MLGs S1 and S3 were shared among the two hosts. *Cedrus atlantica* harboured the greatest number of MLGs (7), *Picea pungens* Engelm., *Pinus nigra* J. F. Arnold and *Pinus sylvestris* L. each harboured three MLGs, *Picea omorika* (Pančić) Purk. and *Pinus halepensis* Miller harboured two MLGs and five other hosts harboured a single MLG. Each host had at least one MLG shared with at least one other host. *Cedrus atlantica* shared all seven MLGs with other hosts, three hosts shared three MLGs with other hosts, two hosts shared two MLGs with other hosts and the rest of the hosts had a single MLG shared with other host species. *Cedrus atlantica* shared MLGs with all other hosts (15), 10 hosts shared MLGs with five or six other hosts and five hosts shared MLGs with two or one other host (Figure S1; Supporting Information Table S4). Seven MLGs were found on hosts in urban areas. Among them, one MLG was also detected in both pine plantations and forest stands and two other MLGs were

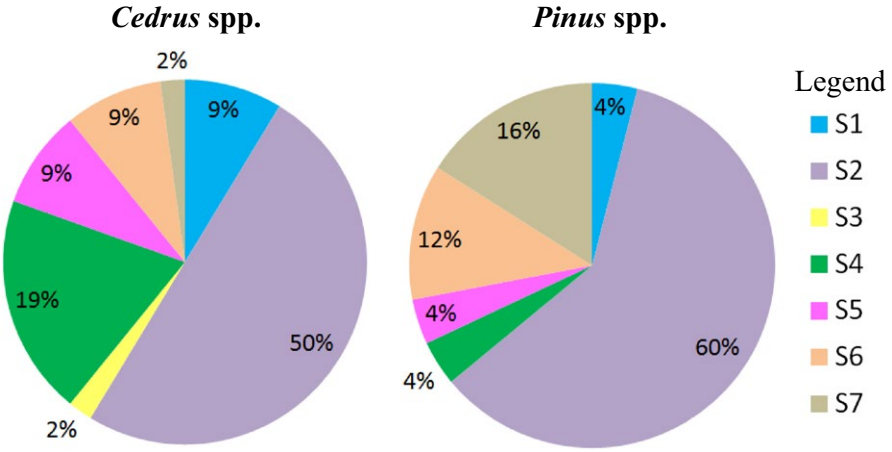


**FIGURE 2** Pie charts representing genotypic diversity of the *Diplodia sapinea* populations from *Cedrus* spp. and *Pinus* spp. Different multilocus genotypes are indicated as S1–S7

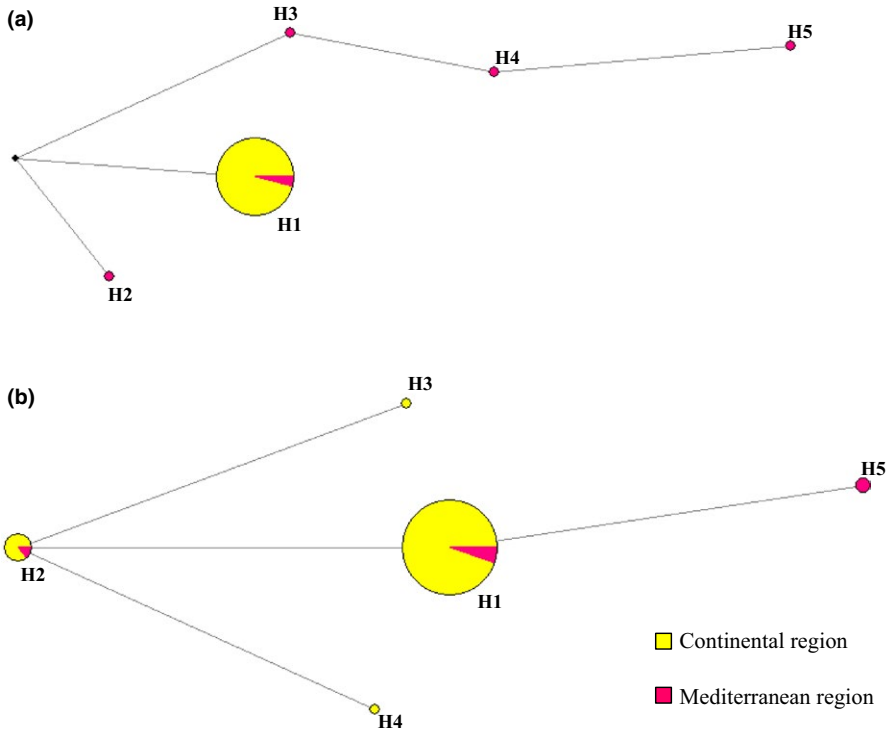


**FIGURE 3** Median-joining network for the multilocus haplotypes of (a) internal transcribed spacer (ITS), translation elongation factor 1-alpha (TEF-1-α), β-tubulin (BT2) and RNA polymerase II gene for *Neofusicoccum parvum*; (b) ITS, TEF-1-α and BT2 for *Diplodia sapinea*. Each circle represents a haplotype and circle size is shown proportional to haplotype frequency. Colours indicate the geographic origin of haplotypes. Median vectors (small black dots) represent missing or not sampled haplotypes. Branch lengths are approximately equal to inferred mutational steps. Haplotype codes according to those are represented in Table S1

shared with either pine plantations or forest stands (Supporting Information Table S4; Figure S3). Up to three *D. sapinea*, genotypes were found coexisting in the same host tree and in the same lesion or tree part. All but one of the trees and tree parts had genotype S2 coexisting with other genotypes (Supporting Information Table S5).

### 3.3 | DNA sequence haplotype networks

A total of five multilocus haplotypes were detected in the *N. parvum* population (Figure 3; Supporting Information Table S1). Haplotype 1 (H1) was the most common, being present in 52 of 56 isolates. H1 was the only haplotype detected in the CR and all five haplotypes were found in the MR. Three of five MR haplotypes were separated from one another by a single point mutation giving a haplotype network a chain-like pattern. H1 was found on 14 of 15 hosts (Supporting Information Table S1).

Five multilocus haplotypes were found in the *D. sapinea* population (Figure 3; Supporting Information Table S1). Among them, three haplotypes were found in the MR and four haplotypes were detected in the CR. Two haplotypes were shared among the regions. H1 was the most common and it was found in 76 of 87 isolates. This haplotype was shared among the greatest number of hosts (14 of 16) (Supporting Information Table S1).

### 3.4 | Phylogeographic relationships with haplotypes outside Western Balkans

*Neofusicoccum parvum* haplotype networks showed a star-like pattern with 1–3 dominating haplotypes and multiple less frequent haplotypes with mostly short branches and only one or few longer branches (Figure 4). In the ITS network isolates from Serbia and Montenegro belonged to H9, which was the most common haplotype

found in 18 countries and on four continents (Figure 4a). In the TEF-1-α network, isolates from Serbia and Montenegro represented haplotype H19 and its closest relative was haplotype H13, which was found in isolates from Iran, Kenya and Uganda (Figure 4b). In the network based on β-tubulin haplotypes, isolates from Serbia belonged to descendant haplotype H8, which was related to dominant

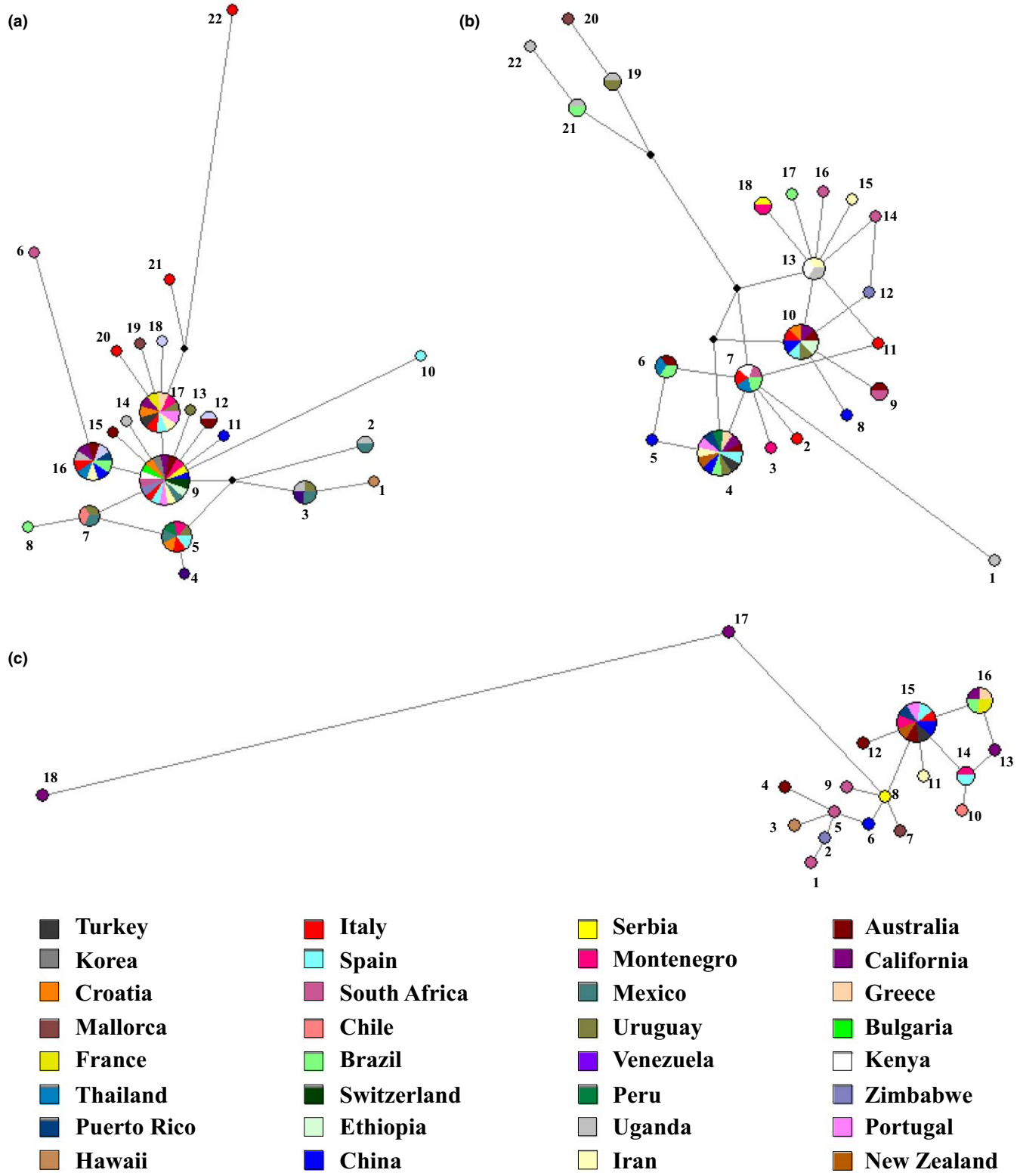


**FIGURE 4** Median-joining networks for the multilocus haplotypes of (a) internal transcribed spacer; (b) translation elongation factor 1-alpha; (c) β-tubulin for *Neofusicoccum parvum*. Each circle represents a haplotype and circle size is shown proportional to haplotype frequency. Colours indicate the geographic origin of haplotypes. Median vectors (small black dots) represent missing or not sampled haplotypes. Branch lengths are approximately equal to inferred mutational steps

haplotype H15. Isolates from Montenegro belonged to dominant haplotype H15, which was found in nine countries and on four continents and to haplotype H14 that was also found in Spain. Haplotype H14 was in close proximity to haplotype H10 which was found in Chile, and its close relative was H13 that was found in California (Figure 4c).

There was no apparent geographical structure in the relationships among the *D. sapinea* haplotypes (Figure 5). With the exception of TEF-1-α network, the *D. sapinea* haplotype networks showed star-like shape with multiple descendant haplotypes arising from the single dominating haplotype. In the ITS network, isolates from Serbia and Montenegro belonged to H5 which was a descendant haplotype and its closest relative was the most frequent haplotype H13, which was found in 21 countries and on five continents. Haplotype H5 was in close proximity to haplotypes H4 and H3 which were found in isolates from Central Europe. In the TEF-1-α network, isolates from Serbia and Montenegro belonged to haplotype H5 which was also found in isolates from Iran. The closest relatives of this haplotype were, that is, haplotype H4 which was found in isolates from the Netherlands, Belgium, Italy and South Africa and haplotype H6 that was found in isolates from the USA. In the β-tubulin network, isolates from Serbia and Montenegro belonged to H3, which was the most frequent and dominant haplotype being found in nine countries and on three continents.

## 4 | DISCUSSION

This study represents the first attempt to determine genetic diversity and structure of *N. parvum* and *D. sapinea* in the Western Balkans. It is also the first study to consider genetic diversity and structure of *N. parvum* in Europe. Microsatellite markers revealed low gene and genotypic diversity for these fungi in the region. The low diversity across the region and on a diversity of trees suggests that *N. parvum* and *D. sapinea* have probably been introduced. Shared genotypes between native and introduced tree species suggested that

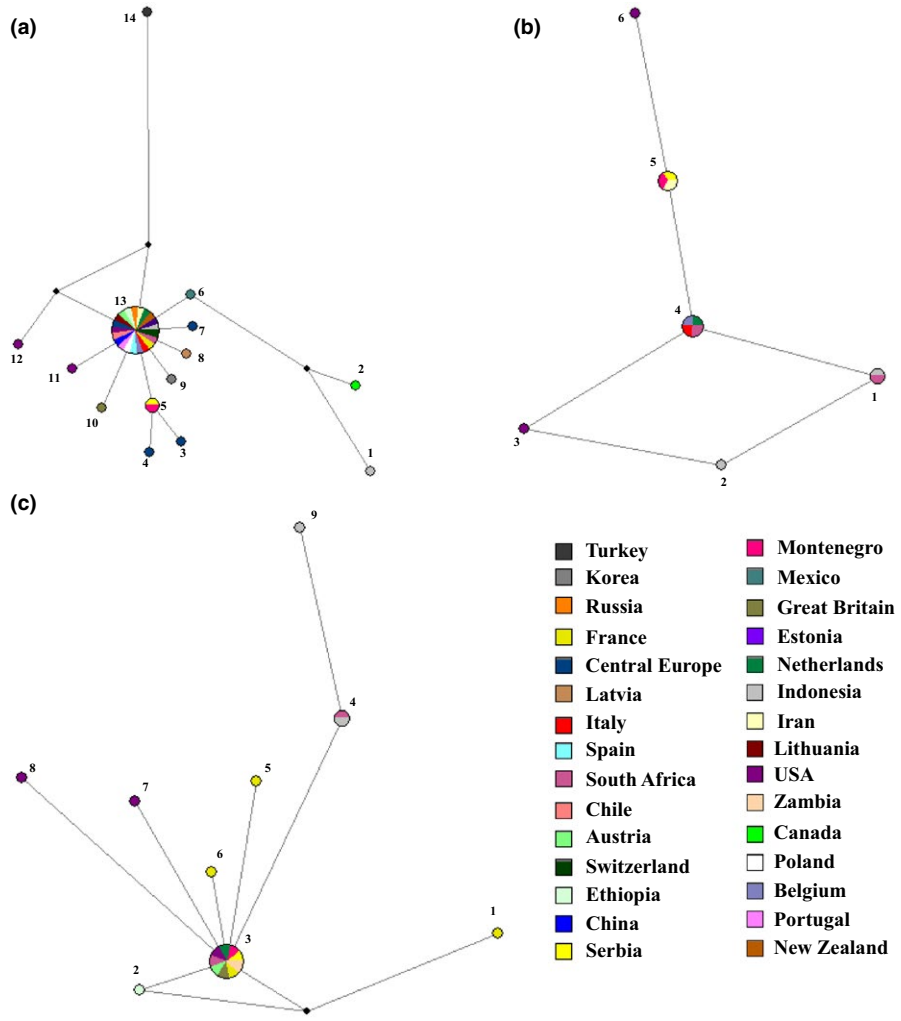


**FIGURE 5** Median-joining network for the multilocus haplotypes of (a) internal transcribed spacer; (b) translation elongation factor 1-alpha; (c) β-tubulin for *Diplodia sapinea*. Each circle represents a haplotype and circle size is shown proportional to haplotype frequency. Colours indicate the geographic origin of haplotypes. Median vectors (small black dots) represent missing or not sampled haplotypes. Branch lengths are approximately equal to inferred mutational steps

*N. parvum* and *D. sapinea* can move between them, and this is especially true for *D. sapinea* populations on *Cedrus* spp. and *Pinus* spp. The results also showed that there are shared genotypes between trees in urban areas, pine plantations, forest stands and nurseries. Moreover, multiple genotypes of *D. sapinea* were shown to exist on a single infected tree and interestingly within a single lesion.

The allelic richness, number of private alleles, number of MLGs and per cent polymorphic loci reflected a low level of gene and genotypic diversity for *D. sapinea* in the Western Balkans. Nei's (1978) unbiased gene diversity (Hexp) for the geographically and host defined populations for this fungus was low, ranging from 0.072 to 0.09. Similarly, there was low genotypic diversity in all of the populations of *D. sapinea*, ranging from 0.4 to 0.91. Likewise, Burgess, Wingfield, and Wingfield (2004) reported only 1.45 genotypic diversity of *D. sapinea* populations collected in forests and plantations in France and Switzerland. These authors also found little diversity in *D. sapinea* populations from North America and Southern Hemisphere. Moreover, Luchi, Longa, Danti, Capretti, and Maresi (2014) using DAMD-PCR markers found an almost clonal population of *D. sapinea* in Italy. In contrast, in the study of Bihon et al. (2012) in populations of *D. sapinea* from South Africa, Ethiopia and Argentina, the genetic diversity was moderate to high, indicating extensive introductions most likely linked to the trade of living plants or plant tissues.

Low levels of gene and genotypic diversity were found in all populations of *N. parvum* from this study. Gene and genotypic diversity ranged from 0 to 0.084 and 0 to 0.33, respectively. Likewise, Baskarathevan, Jaspers, Jones, Cruickshank, and Ridgway (2012) using UP-PCR markers found low genetic diversity of *N. parvum* from grapevine in Australia, South Africa and California. In contrast, high levels of genetic diversity were detected in populations of *N. parvum* from various hosts in New Zealand, China, Colombia, Hawaii, Australia and South Africa. In these studies, high genetic diversity of *N. parvum* was explained by introductions of multiple genotypes over time and movement of this pathogen between native and non-native hosts (Mehl, Slippers, Roux, & Wingfield, 2017a; Pavlic-Zupanc, Wingfield, Boissin, & Slippers, 2015; Sakalidis et al., 2013).

The low gene and genotypic diversity of *N. parvum* and *D. sapinea* is not surprising as these fungi are believed to mostly reproduce asexually (Bihon et al., 2012; Mehl et al., 2013; Slippers et al., 2017; Slippers & Wingfield, 2007). Although larger founding populations and multiple introductions from distinct genetic sources can increase genetic diversity of asexual populations, they are typically expected to have low genotypic diversity (Dlugosch & Parker, 2008; Gladieux et al., 2015). In this study, evidence for clonal reproduction was observed in the form of identical MLGs among several isolates, especially those collected from the same tree. Likewise, Bihon et al. (2011) using microsatellite markers and vegetative compatibility groups markers found high genotypic diversity of *D. sapinea* within individual asymptomatic trees in South Africa. Similarly, Pavlic-Zupanc et al. (2015) reported the existence of numerous *N. parvum* isolates with identical multilocus haplotypes in different populations of *N. parvum* in the same country.

Low genetic diversity across the region and on a diversity of trees suggests that *D. sapinea* has probably been introduced to Western Balkans. Low diversity of this pathogen could be explained by founder effects where a reduced number of individuals carrying a fraction of the diversity of the original population establish a new population in a new area (Barrès et al., 2008; Dlugosch & Parker, 2008). Similarly, Burgess at al. (2004) suggested that a single source of the genotype MS1 could explain its predominance and low genetic variation of *D. sapinea* in France and Switzerland. The possibility of introduction of *D. sapinea* to the region is further supported by the star-like topology of the DNA sequence haplotype networks depicted by the *D. sapinea* haplotypes indicating a pattern of large-scale dispersal of this fungal species (Posada & Crandall, 2001). Moreover, isolates of *D. sapinea* from Serbia and Montenegro belonged to dominant haplotypes or their closest relatives shared with many countries and continents.

The lack of structure and low genetic diversity of *N. parvum* indicated that this pathogen has probably been introduced to the region. This is expected considering the wide host range, broad geographical distribution of *N. parvum* haplotypes and the lack of structure amongst a global collection of *N. parvum* isolates (Sakalidis et al., 2013; Slippers et al., 2017; Zlatkovic et al., 2017). The broad geographical distribution of *N. parvum* haplotypes was confirmed in this study, and DNA sequence haplotype networks showed a star-like topology with isolates of *N. parvum* from Serbia and Montenegro belonging to dominant haplotypes shared with many countries and continents. Low levels of genetic diversity and the lack of structure have also been found in other Botryosphaeriaceae species that are reported to have been moving globally. For example, Ma, Boehm, Luo, and Michailides (2001) reported a highly clonal population of *B. dothidea* from pistachio in California. Moreover, Marsberg et al. (2017) reported a lack of phylogeographic structure for the global collection of *B. dothidea* isolates and dominance of identical multilocus haplotypes on distant continents. Mehl, Wingfield, Roux, and Slippers (2017b) showed the lack of structure in *L. theobromae* isolates obtained from a large number of hosts and in many countries of the world.

The shared multilocus haplotypes in *N. parvum* and *D. sapinea* from Serbia and Montenegro with many countries and continents suggest assisted dispersal and introduction of these fungi into the region. Spores of these fungi are thought to be predominantly dispersed by wind and rain and are not expected to be naturally spread over large distances, including continents (Swart & Wingfield, 1991; Mehl et al., 2013, 2017b). Therefore, their introduction into the region could be facilitated by human-associated global trade in plants and plant products (Burgess et al., 2016; Santini et al., 2013; Wingfield et al., 2015). In this regard, the majority of the plant hosts from which isolates of *N. parvum* and *D. sapinea* in this study were obtained are traded globally as ornamentals (e.g., *Chamaecyparis* spp., *Thuja occidentalis*, *C. atlantica*, *Pinus* spp.). Moreover, these fungi are well known as endophytes in plants and endophytic infections of Botryosphaeriaceae are symptomless and would be easily overlooked by phytosanitary systems

(Burgess et al., 2016; Crous et al., 2016). Likewise, Burgess et al. (2004) suggested that *D. sapinea* movement across continents is probably assisted by human activities. Sakalidis et al. (2013) concluded that the worldwide dispersal of *N. parvum* is probably due to repeated introductions of plant material into new growing areas. Pavlic-Zupanc et al. (2015) showed that *N. parvum* is a more effective invader in human-disturbed environments, such as plantations, orchards and urban environments.

Microsatellite data and DNA sequence haplotype networks displayed only slight geographic separation between populations of *N. parvum* and *D. sapinea* from the CR and MR. This result was expected given the wide geographic distribution of these pathogens and increase in global trade (Sakalidis et al., 2013; Santini et al., 2013; Slippers et al., 2017). However, it is in contrast to studies of organisms other than fungi for which almost 3,000 m high Dinaric Alps have shown to act as an effective natural geographic barrier that causes reduction in gene flow and environmental isolation in natural populations (e.g., Temunović et al., 2012; Lacković, Bertheau, Stauffer, Pernek, & Avtzis, 2015). Given the limited number of isolates obtained from the MR, the lack of diversity for *N. parvum* and *D. sapinea* and small number of molecular markers employed for *N. parvum* population in this study, it is possible that genetic diversity was underestimated and additional sampling would be required to draw a valid conclusion.

The similarity in gene frequency and shared genotypes between the *D. sapinea* isolates from *Cedrus* spp. and *Pinus* spp. indicates that the pathogen spreads between these two hosts and between native and non-native trees. This is not unexpected and a similar pattern has been observed in other Botryosphaeriaceae. For example, Mehl, et al., 2017a found significant gene flow between populations of *N. parvum* on native marula and non-native mango trees in South Africa. The authors suggested that the ability to infect multiple hosts and to migrate amongst them facilitates the establishment and spread of *N. parvum* and other Botryosphaeriaceae in new areas. Moreover, *C. atlantica* trees are often found in close proximity to pine trees in urban areas and could have served as inoculum reservoirs for the infections of *Pinus* spp. and *vice versa* (Zlatković et al., 2017). In addition, because in urban environments *Cedrus* and *Pinus* trees are surrounded by various other known hosts for *D. sapinea*, such as firs, spruces and junipers (Zlatković et al., 2017), these conifers could have provided a source of this fungus, but the opposite situation could also have applied.

The results of this study support the suggestion (Burgess & Wingfield, 2017; Slippers & Wingfield, 2007; Wingfield et al., 2015) that plant trade is an important source for the spread of Botryosphaeriaceae species. The international plant health regulations, including Serbian plant health policies, rely mostly on visual inspection of plants (Slippers & Wingfield, 2007; Law on Plant Health of the Republic of Serbia, 2009; Santini et al., 2013; Crous et al., 2016). This would allow latent pathogens, such as species in the Botryosphaeriaceae, to be imported with asymptomatic plants. The lack of host specificity for *N. parvum* and *D. sapinea* was confirmed, with the same genotypes found on different host species. Multilocus sequencing analyses and microsatellite markers suggest that these fungi were most likely introduced into the Balkan region, and together with data from previous studies (Zlatković, Keča, Wingfield, Jami, & Slippers, 2016b; Zlatković et al., 2017, 2018), this suggests that aggressive genotypes of *N. parvum* and *D. sapinea* have been spreading in the Western Balkans. The present study also emphasizes the need for precautionary measures where plants and plant parts are traded globally.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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