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Severity of Diplodia shoot blight (caused by *Diplodia sapinea*) was greatest on *Pinus sylvestris* and *Pinus nigra* in a plantation containing five pine species

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Summary. The severity of Diplodia shoot blight in *Pinus nigra* and *P. sylvestris* seed orchards, and in *P. taeda*, *P. pinaster* and *P. radiata* industrial plantations was examined in two 20 × 20 m plots of each tree species. All 159 trees assessed in the plots showed symptoms of *Diplodia sapinea* infections. Average disease severity in the tree crowns varied from 42 to 68% (mean = 53.8%) over all tree stands. Fungus isolates obtained from affected trees were identified as *D. sapinea*, based on morphological characteristics of cultures and conidia grown on agar plates. The identifications were confirmed by sequence analysis of the ITS rDNA of a subsample of isolates. Multilocus genotyping with RAMS primers showed that 13 *D. sapinea* genets were present in the plots. *Diplodia sapinea* had serious impacts on *P. sylvestris* and *P. nigra* in the seed orchards Marmara Region of Turkey, and is likely to have been the main causal agent of shoot blight and reductions in seed production at this location.

Keywords. Disease severity, RAMS, pathogenicity, pine plantations.

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INTRODUCTION

Diplodia sapinea (Desmaz.) J. Kickx [syn. *Sphaeropsis sapinea* (*Diplodia pinea*) (Fr.: Fr.) Dyko & Sutton] causes a range of disease symptoms on conifer trees, including stunting, browning of needles, shoot blight, twig and branch dieback, crown wilt and bark cankers, root disease, and damping-off and root rot of seedlings. In addition, the fungus causes blue stain of sapwood in fallen or freshly cut timber, in which it is considered a saprotroph (Brookhouser and Peterson, 1971; Peterson, 1977). The fungus has wide distribution in temperate and tropical regions. *Pinus nigra* Arnold, *Pinus radiata* Don. and *Pinus sylvestris* L. are particularly susceptible to infec-

tion (Wingfield and Know-Davies, 1980; Palmer and Nicholls, 1985; Swart *et al*., 1987; Chou and Mackenzie, 1988; Rees and Webber, 1988; Nicholls and Ostry, 1990; Stanosz and Cummings, 1996; Stanosz *et al*., 2001). Predisposing factors such as drought stress or physical wounding through forestry practices may have important roles in increasing tree susceptibility to *D. sapinea* infections (Swart and Wingfield, 1991; Blodgett and Stanosz, 1997; Adams *et al*., 2002; Blodgett and Bonello, 2003).

Although *D. sapinea* was first reported in Turkey in 1993 on *Pinus pinea* L. and *Pinus pinaster* Ait. (Ünligil and Ertaş, 1993), little is known about the incidence and severity of Diplodia diseases, or genetic variation in the pathogen populations, in Turkish forests. Some of the most severe damage from Diplodia shoot blight has occurred in the southern part of Turkey. The pathogen was first noted on dead twigs or canker samples of *Pinus brutia* var. *eldarica* (Medw.) Silba and *Pinus brutia* Ten. in Kahramanmaraş, Turkey, in 2000 (Sümer, 2000). *Diplodia sapinea* was later shown to be the main causal agent of shoot blight of *P. brutia* in the Isparta region, in the western Taurus Mountains (Doğmuş Lehtijärvi *et al*. 2007). In 2013, similar symptoms were reported in *Pseudotsuga menziesii* (Mirb.) Franco plantations in İzmit province (Aday Kaya *et al*., 2014), and *D. sapinea* was suggested as the likely causal agent.

Genetic variation in *D. sapinea* populations has previously been determined based on morphological characteristics and virulence of the isolates (De Wet *et al*., 2002). In recent years, however, molecular genetic analyses of several populations worldwide have been performed. The results of these studies indicate high variation in the genetic diversity among *D. sapinea* populations (Burgess *et al*., 2004; Bihon *et al.,* 2012 a, b). The pathogen is predominantly asexual and wound-associated die-back appears to be caused by clones of the pathogen occurring in narrow time frames (Bihon *et al*., 2012b). However, endophytic infections show high levels of genetic diversity (Bihon *et al*., 2011, Bihon *et al.,* 2012 a, b), probably resulting from a cryptic, heterothallic sexual cycle (Bihon *et al.,* 2014).

To date, genetic differentiation and pathogenicity in Turkish *D. sapinea* isolates has not been examined. Random amplified microsatellites (RAMS) and internal simple sequence repeat (ISSR) are reliable tools for analysis of genetic variation in several important forest pathogens (Hantula *et al*., 1996). Using RAMS, Doğmuş Lehtijärvi *et al*. (2014) demonstrated that 60 isolates of *D. sapinea* in *P. nigra* and *P. sylvestris* seed orchards were genetically identical.

The objectives of the study reported here were: i) to estimate the disease severity caused by *D. sapinea* in *P. nigra* and *P. sylvestris* seed orchards, and in *P. taeda*, *P. pinaster* and *P. radiata* industrial plantations in Turkey, ii) to characterize isolates of the fungus using a combination of morphological and molecular techniques, iii) to investigate pathogenicity, host specialization and virulence of isolates, and iv) to determine genetic variation within the *Diplodia* isolates.

MATERIALS AND METHODS

Disease severity

Surveys were conducted in October 2014 in two seed orchards planted with *Pinus nigra* and *P. sylvestris*, and in industrial plantations of *P. taeda*, *P. pinaster* and *P. radiata* in the Kerpe Research Forest, İzmit province, Marmara Region of Turkey. These pine plots (sections) were located 130 to 870 m apart from each other (Table 1). Soil types of the stands were brown forest soil, light hydromorphic grey brown podzolic brown soil, and andesite brownstone soil on the main rock. For each tree species, two 20 × 20 m plots were surveyed. Diameter at breast height (DBH), tree height and disease severity caused by *D. sapinea* were recorded for all trees in the plots, and mature, open cones remaining on shoots were collected from each tree. Severity of Diplodia shoot blight was scored using a 1-5 scale (Table 2), and calculated using the Townsend-Heuberger formula (Townsend-Heuberger, 1943):

$$\text{Disease Severity (\%)} = [\Sigma(n.V)/Z.N] \times 100$$

Where
n = number of samples that are in different disease scales,
V = scale value,
Z = greatest scale value,
N = total number of samples

Estimation of inoculum densities in cones

A total of 477 cones were collected from sampled trees in the investigated plots. Three cones were taken from each tree to estimate *D. sapinea* inoculum densities. Cone samples from each tree were pooled in a 100 mL plastic beaker containing 80 mL of deionized water with two drops of Tween®80 (Sigma-Aldrich, Inc.), which was then shaken for 3 h at 24°C at 110 rpm. After shaking, the cones were removed from the beakers and the

Table 1. Locations of pine plantings assessed for Diplodia shoot dieback.

Tree species	Coordinates	Section ID	Type of stand	Size of section (ha)	Origin of tree species	Soil
Pinus nigra	41° 9' 5.83" N 30 °12'19.39" E 41° 8' 59.03" N 30°12'20.47" E	10c	Plantation	8.4	Turkey- Dursunbey	Brown forest soil
P. sylvestris	41° 9' 26.06" N 30°13'3.77" E 41° 9' 23.21" N 30°13'3.02" E	18e1, 19b	Seed orchard	34	Turkey- Sarıkamış	Brown forest soil
P. taeda	41° 9' 5.75" N 30°12'27.06" E 41° 8' 58.88" N 30°12'27.91" E	15r	Plantation	28.1	USA	Light hydromorphic gray brown podzolic brown soil
P. pinaster	41° 9' 11.73" N 30°12' 21.62" E 41° 9' 9.05" N 30°12' 25.03" E	9g	Plantation	19.1	France	Light hydromorphic gray brown podzolic brown soil
P. radiata	41° 9' 27.19" N 30°12' 29.55" E 41° 9' 23.50" N 30°12' 28.66" E	9a	Plantation	9.0	Australia	Andesite brownstone land on the main rock

Table 2. Disease severity scores used for assessments in pine stands.

Score	Damage class	Symptoms used for scoring
1	No damage	-
2	Light damage	≤five main branches with advanced defoliation
3	Moderate damage	Several (≥five) main branches or a whole crowns, groups of branches seriously damaged.
4	Severe damage	Most of the main branches seriously damaged.
5	Dead	-

volume of water in each beaker adjusted to 100 mL with deionized water. The numbers of conidia in the suspensions were enumerated with five replicate counts using a compound microscope and a haemocytometer.

Identification and assessment of genetic variation of the pathogen

Cones taken from pines were examined for the presence of pycnidia typical of *D. pinea* under a dissecting microscope (Table 3). Pycnidia were transferred to potato dextrose agar (PDA Merck 1.10130) and incubated at 24°C for 5 d. In total, 106 isolates were obtained. Morphological features of the conidia collected from pynidiaon cones were examined under a binocular microscope, and cultural characteristics of the isolates were examined using pure cultures grown on fresh PDA.

Isolates were sub-cultured to cellophane membranes covering PDA and incubated at 24°C for 7 d. The mycelium was then scraped off the membrane surfaces and ground in liquid nitrogen using a mortar and pestle. DNA was extracted using DNeasy Plant Mini Kits (Qiagen) following the manufacturer's instructions. PCR amplification of the internal transcribed spacer (ITS) region of the rDNA gene was performed using the primer set ITS1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3') (White *et al.* 1990) in 50 µL reactions. Each reaction containing 50 ng genomic DNA, 250 nM of each primer, 200 µM of each dNTP, 25 mM $MgCl_2$, 1U *Taq* polymerase, 1 × Q solution, and 1 × PCR buffer (Promega Corporation). PCR was conducted in a Biorad MJ Mini Personal Thermal Cycler. PCR conditions were: denaturing at 95°C for 10 min, followed by 30 cycles of amplification (20 s denaturation at 94°C, 25 s annealing at 55°C, and 2 min extension at 72°C). PCR products were separated by electrophoresis in 1% (w/v) agarose.

Genetic variation among the isolates was determined using multilocus genotyping with the M13 marker (Zamponi *et al.* 2007) and RAMS-primers VDH $(TCG)_5$, DDB$(CCA)_5$ and DDB$(CGA)_5$, where B = G/T/C, D = G/A/T, H = A/T/C and V = A/C/G (Hantula *et al.* 1996).

Table 3. Diplodia shoot dieback severity assessments.

Stand No.	Tree species	No. of trees	Damage scale and disease severity (%)[1)]									
			No damage (1)		Light damage (2)		Moderate damage (3)		Severe damage (4)		Dead (5)	
			Tree No.	%	Tree No.	%	Tree No.	%	Tree No.	%	Tree No.	%
1	*Pinus sylvestris* 1	11	0	0	5	18.7	6	32.7	0	0	0	0
2	*Pinus sylvestris* 2	10	0	0	3	12	6	26.0	1	8	0	0
		21		0		15.1		34.3		4		0
3	*Pinus nigra* 1	10	0	0	0	0	3	18.0	6	48.0	1	10.0
4	*Pinus nigra* 2	9	0	0	0	0	3	20.0	6	53.3	0	0.0
		19		0		0		19.0		50.6		5.0
5	*Pinus pinaster* 1	26	0	0	0	0	12	27.7	14	43.0	0	0
6	*Pinus pinaster* 2	17	0	0	8	18.8	9	31.7	0	0.0	0	0
		43		0		9.4		29.7		21.5		0
7	*Pinus radiata* 1	21	0	0	6	11.4	8	22.8	6	22.8	1	4.8
8	*Pinus radiata* 2	12	0	0	0	0	4	20.0	8	53.3	0	0
		33		0		5.7		21.4		38.0		2.4
9	*Pinus taeda* 1	22	0	0	7	12.7	10	27.2	5	18.0	0	0
10	*Pinus taeda* 2	21	0	0	0	0	21	80.0	0	0	0	0
		43		0		6.3		53.6		9.0		0

Primers were synthesized by IonTek. A total of 50 isolates was analysed, with ten isolates from each filed plot examined. DNA was extracted from each isolate as described above and subjected to PCR. DNA was denatured at 95°C for 10 min, after which 37 cycles of amplification were carried out (30 s denaturation at 95°C, 45 s annealing at the primer dependent temperature and 2 min extension at 72°C), followed by 7 min at 72°C (Maresi *et al*. 2007).

Amplification products were separated by electrophoresis (6 V cm^{-1} for 180 min) in 1.0% agarose gels (Biobasic Inc.) and 1.0% SynerGel gels (Diversified Biotech) in TAE buffer, and lengths of the products was estimated using DNA molecular size markers with 100 bp repeats (Thermo Fisher Scientific).

The presence or absence of amplification products was scored, with only clear and reproducible markers considered for the analysis. To estimate genetic variation, analysis of molecular variance (AMOVA) was calculated based on RAMS markers. Variation was assessed among and within isolate groupings based on the host species.

Genetic variation between *D. sapinea* isolates was computed using Jaccard's coefficient of similarity in Arlequin V 6.1 of NTSYSpc 2.1 software (Exeter Software Co.) and GenAlEx 6.5 (Peakall and Smouse, 2006).

Pathogenicity, host specialization and virulence of isolates

Seedling inoculations

Pathogenicity of the *D. sapinea* isolates was assessed using potted seedlings of *P. sylvestris*, *P. nigra*, *P. radiata*, *P. taeda* and *P. pinaster*, which were obtained from local forest nurseries. Three-year-old seedlings growing in a mixture of 60% clay, 20% sand and 20% humus were placed in a growth chamber and incubated at 20°C for 28 d prior to inoculation and during incubation. Seedlings were irrigated with tap water at 48 h intervals. Twenty-five isolates of *D. pinea* grown on PDA were used for the inoculations. Seedlings were inoculated 2 cm above the root collar. The inoculation point was cleaned with 70% (v:v) ethanol. A circular 3 mm diam. wound was made in the stem of each seedling using a sterilized cork borer to remove the bark. An equal-sized agar plug colonized by *D. sapinea* was inserted into each wound and wrapped with Parafilm M®. Experimental controls were mock-inoculated with non-colonized agar plugs. Five seedlings were inoculated for each isolate-host combination, and a randomized complete block experimental design used in the trial. Inoculated seedlings were incubated in the growth chamber for 4 weeks, after which lesion lengths were measured. Random re-isolations were made onto fresh PDA to confirm *Diplodia* sp. as the causal agent of the lesions.

Cross inoculations on twigs

Host specialization and virulence of isolates were also tested on 30 cm long twigs of the five host species. All twigs were obtained from İzmit-Kerpe Research forests. The same 25 isolates as in the seedling inoculation assay were grown for 7 d on PDA and then used for twig inoculations. Twigs were under bark-inoculated 8 cm above the base of each twig using a method similar to that described above for seedling inoculations. Five twigs were used for each fungal isolate-host species combination.

In addition, five control twigs were inoculated with sterile PDA discs. Inoculated twigs were kept at 20°C in a growth chamber for 1 month before recording lesion lengths using the methods described above.

Analysis of variance (ANOVA) was performed on lesion length data using the SPSS MGLM procedure (SPSS Inc.), and differences among mean values were assessed using Duncan's multiple range test ($P < 0.05$).

RESULTS

Disease severity

All 159 trees assessed in the plots had symptoms of *Diplodia* infections. Needles on recently killed shoots were reddish brown. Needles that were dead for longer periods were greyish brown or dark grey.

The average disease severity varied from 42 to 68% (mean = 53.8%) in the stands (Table 2). The predominant disease severity class was moderate, whereas high severity was less common (Figure 1). Comparisons of the damage classes between host species indicated that light damage (score = 2) occurred more frequently in *P. sylvestris* stands, whereas moderate damage (score = 3) was greater in *P. taeda* stands. Frequencies of severely damaged and dead individuals were greatest in the *P. nigra* stands (Table 3).

Estimation of inoculum densities in cone samples

Cones from all sampled stands yielded conidia characteristic of *D. sapinea*. Pycnidia were abundant on the cones and shoots of the specimens examined. The number of conidia extracted from cones varied between tree species. Numbers of conidia were greater from cones of *P. nigra* and *P. sylvestris*, particularly when compared with those obtained from cones of *P. radiata* or *P. taeda*. There was no significant difference in the numbers of conidia (overall mean = 1.3×10^6) obtained from *P. sylvestris* and *P. pinaster* cones ($P \leq 0.05$) (Table 4).

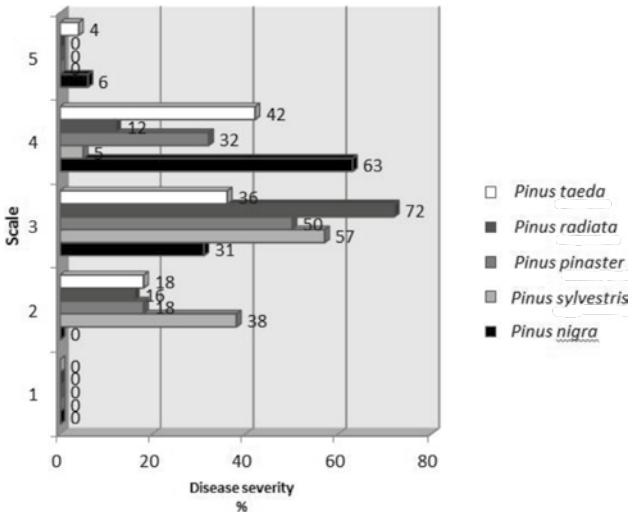

Figure 1. Percentage disease severity in each disease scale group for different *Pinus* spp.

Identification and genetic diversity in the pathogen populations

A total of 106 isolates was obtained from shoot and cone samples, and identified as *D. sapinea*. Conidia were brown to dark brown, thick-walled, with a mean width of 18.0 µm (SD ± 2.6) (range 9 to 22 µm) and mean length of 35.0 µm (SD ± 5.1) (range 20–41 µm) ($n = 100$).

Morphological identifications of the isolates were confirmed by sequence analysis of the ITS rDNA of representative isolates. The sequences showed homologies > 99% with GenBank accessions of *D. sapinea*.

Amplification of genomic DNA of 50 isolates of *D. sapinea* using the M13 and three RAMS markers produced 78 fragments of which 13 were polymorphic. All fragments were clear and reproducible, with sizes ranging from 350 to 870 base pairs (Table 5). The variation was analysed according to the presence or absence of the markers.

Table 4. Occurrence of *Diplodia sapinea* in cones of pine species and recovery of conidia in suspensions.

Host species	No. of cones examined	*Diplodia sapinea* positive (%)	Conidia per cone ($\times 10^6$)
Pinus sylvestris	54	81	1.3
P. nigra	51	88	1.1
P. pinaster	129	76	1.3
P. radiate	129	79	0.5
P. taeda	99	54	0.6
	Total = 462	Mean = 75.6	Mean = 0.96

Table 5. Presence/absence (1/0) vector of *Diplodia sapinea* isolates tested with RAMS and M13 primers.

Sample ID	Population name	CCA-870	CCA-800	CGA-750	M13-650	M13-600	CCA-570	CGA-550	CGA-510	CCA-480	TCG-440	TCG-420	CCA-400	CCA-370
D1	Pn	1	1	0	1	1	0	0	0	1	0	1	1	1
D2	Pr	0	1	0	1	0	0	0	1	1	1	1	0	1
D3	Pt	0	1	0	0	0	1	0	0	0	0	0	0	0
D4	Pp	1	1	1	1	0	1	0	0	0	1	1	0	1
D5	Ps	0	1	0	0	0	0	0	0	1	0	1	1	1
D6	Ps	0	1	0	0	0	0	0	0	1	0	1	1	1
D7	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D8	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D9	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D10	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D11	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D12	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D13	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D14	Ps	0	1	0	0	1	0	0	0	1	0	1	1	1
D15	Pn	0	1	0	1	1	0	1	0	1	0	1	1	1
D16	Pn	0	1	0	0	0	0	1	0	1	1	0	1	1
D17	Pn	0	1	0	0	0	0	1	0	1	1	0	1	1
D18	Pn	0	1	0	0	0	0	1	0	1	1	0	1	1
D19	Pn	0	1	0	0	0	0	1	0	1	1	0	1	1
D20	Pn	0	1	0	1	0	0	1	0	1	1	0	1	1
D21	Pn	0	1	0	1	0	0	1	0	1	1	0	1	1
D22	Pn	0	1	0	0	0	0	1	0	1	1	0	1	1
D23	Pn	0	1	0	1	0	0	1	0	1	1	0	1	1
D24	Pp	0	1	0	1	0	0	1	0	0	0	0	0	0
D25	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D26	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D27	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D28	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D29	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D30	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D31	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D32	Pp	0	1	0	1	0	0	1	1	0	1	1	0	1
D33	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D34	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D35	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D36	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D37	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D38	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D39	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D40	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D41	Pt	0	1	0	1	1	1	1	1	1	1	1	0	1
D42	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D43	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D44	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D45	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D46	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D47	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D48	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D49	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1
D50	Pr	0	0	0	1	0	0	0	1	1	1	1	0	1

ANOVA showed that variation among and within *D. sapinea* isolates obtained from different pine species amounted to, respectively, 44 and 56% of the total variation. Population divergence measured with F_{ST} also indicated differentiation in *D. sapinea* among *P. nigra*, *P. taeda* and *P. pinaster* (F_{ST} = 2.432) (Table 6).

Thirteen *D. sapinea* genotypes were present in all host species (Table 7). Genotypes of *D. sapinea* infecting *Pinus sylvestris* and *P. taeda* were unique to those hosts, whereas *P. nigra*, *P. pinaster* and *P. radiata* shared nine genets.

Genotype size was also estimated based on the RAMS analysis, and ranged from a single tree to approx. 115 m^2 (15 trees). Two genotypes were shared within two stand replicates. The largest genotype was located in a *P. radiata* stand.

Table 6. Genetic variation within *Diplodia sapinea* populations.

Variation source	df	SS	MS	Est. Var.	%
Between populations	4	58.640	14.660	1.359	56
Among populations	45	48.300	1.073	1.073	44
Total	49	106.940		2.432	100

Table 7. Genet size of *Diplodia sapinea* populations.

Tree species	Stand number	Genet code	Number of isolates/per genet	Estimated genet size m^2
Pinus nigra	1	1.genet	2	20
		2.genet	3	5
	2	3.genet	3	38
		4. genet	2	16
P. sylvestris	1	5.genet	2	6
		6.genet	3	18
	2	6.genet	5	100
P. pinaster	1	7.genet	1	1
		8.genet	1	1
		9.genet	3	55
	2	9.genet	5	68
P. taeda	1	10.genet	1	1
		11.genet	4	18
	2	11.genet	5	106
P. radiata	1	12.genet	1	1
		13.genet	4	114
	2	13.genet	5	93
Total		13	50	661

Pathogenicity, host specialization and virulence of isolates

Seedling inoculations

Diplodia sapinea inoculations caused dark brown to black discolouration around the inoculation points in seedlings of all pine species tested. Infection frequency in the inoculated seedlings was 100%. The pathogen was successfully re-isolated from symptomatic stem tissues, thus fulfilling Koch's postulates. Susceptibility of the tree species to *D. sapinea*, and the virulence of the *D. sapinea* isolates among those host species, differed significantly. Compared to the other *Pinus* species tested, *P. radiata* was highly susceptible to *D. sapinea*. There were also significant differences in the extents of the lesions produced by *D. sapinea* on the host species tested ($P < 0.05$) (Table 8). The longest lesions were on *P. radiata* and *P. sylvestris*. Lesion lengths caused by isolates were greater on *P. radiata*, *P. sylvestris* than *P. nigra* seedlings than on ($P < 0.05$).

Cross inoculations on twigs

Most of the isolates used in the inoculations caused lesions on the twigs of the five host species. Differences between the isolates obtained from the sampled pine species, in terms of ability to induce lesions on all tested twigs were significant ($P < 0.05$). In general, isolates from *P. taeda* caused longer lesions on *P. radiata* twigs (Table 8) than on the other host species. *Pinus radiata*, *P. sylvestris* and *P. nigra* twigs were susceptible to all isolates. MLGM analyses indicated that there was no host specificity from the cross inoculations (Table 9).

DISCUSSION

The studies reported in this paper have confirmed the presence of *D. sapinea* on *Pinus nigra*, *P. sylvestris*,

Table 8. Mean lesion lengths caused by *Diplodia sapinea* on seedlings of different *Pinus* spp. hosts.

Host	Mean lesion length (mm) (± S.D.)
Pinus radiata	18.1±0.580 A
P. sylvestris	16.5± 0.564 B
P. nigra	12.5± 0.564 B
P. taeda	5.4± 0.591 C
P. pinaster	5.4± 0.593 C

Means within a column followed by different letter are significantly different ($P < 0.05$) based on Duncan's Multiple Range test.

Table 9. Results of *Diplodia sapinea* cross inoculation of twigs.

Pinus. radiata isolates		*P. taeda* isolates		*P. pinaster* isolates		*P. sylvestris* isolates		*P. nigra* isolates	
P. sylvestris	23.8a	*P. radiata*	30.0a	*P. nigra*	13.0a	*P. nigra*	17.6a	*P. sylvestris*	15.5a
P. radiata	21.8a	*P. sylvestris*	19.3b	*P. radiata*	12.7a	*P. radiata*	14.8a	*P. nigra*	10.8b
P. nigra	10.8b	*P. nigra*	10.3bc	*P. sylvestris*	11.5ab	*P. sylvestris*	12.9ab	*P. radiata*	9.4bc
P. taeda	5.9c	*P. taeda*	5.8c	*P. pinaster*	5.3b	*P. pinaster*	6.0b	*P. pinaster*	6.3c
P. pinaster	5.3c	*P. pinaster*	4.4c	*P. taeda*	5.1b	*P. taeda*	4.5c	*P. teada*	5.7c

Means within a column followed by different letter are significantly different ($P < 0.05$) based on Duncan's Multiple Range test.

P. taeda, *P. pinaster* and *P. radiata* in the Kerpe Research Forest, in İzmit province of Turkey. On the basis of morphological characteristics of the pathogen in culture, ITS sequencing of isolates obtained from sampled trees with the symptoms and the pathogenicity tests performed on excised twigs of same host species, it was clear that *D. sapinea* was responsible for shoot blight and reductions in seed production in *Pinus* spp. trees at this location.

Typical Diplodia shoot blight symptoms (Maresi *et al*., 2007) were observed, scattered throughout the crowns of the trees. The most serious damage occurred on the middle and upper crowns. It is not clear why there was such high incidence of Diplodia shoot dieback in the Kerpe Research Forest, but there were no records to suggest that this was connected with predisposing factors such as wounding or drought. However, the trees were planted in unsuitable climatic conditions.

The design of the Kerpe research forest was not optimal for critical comparison of the tree species for disease incidence and severity, as the trees were not planted in blocks homogenous for site factors. *Pinus sylvestris* and *P. nigra* were growing on brown forest soil, *P. pinaster* and *P. taeda* on light hydromorphic grey brown podzolic soil and *P. radiata* on andesite brownstone soil on the main rock (Table 1). For tree species pairs growing on the same soil type, the disease was more severe on *P. nigra* and *P. pinaster* than on *P. sylvestris* and *P. taeda*. (Table 3).

Colony morphology of the isolates obtained here was identical to that of the *D. sapinea* described in De Wet *et al*. (2002). The presence of abundant pycnidia on branches, stems and cones of infected trees illustrated the potential of this fungus to cause epidemics when conditions are favourable for infection and disease development (Nicholls and Ostry, 1990).

In previous research, cones collected from tree canopies yielded conidia more frequently than cones collected from the ground (Doğmuş Lehtijärvi *et al*., 2014). In the present study, therefore, we sampled only from the tree canopies. It is very likely that cones in the canopies are more important for spread of the fungus than cones on the ground, and tree cones should be used for estimation of inoculum potential at particular sites (Munck and Stanosz, 2009).

Diplodia sapinea has been detected in the seeds of many *Pinus* species, including *P. radiata*, *P. nigra*, *P. sylvestris*, *P. resinosa*, as well as *P. patula*, *P. wallichiana*, *P. elliottii*, *P. taeda*, *P. oocarpa*, *P. caribaea*, *P. michoacana* and other tropical pines (Bihon *et al*., 2011). However, despite the probable critical importance of seed infection in the epidemiology of disease caused by this fungus, and implications for dissemination via plant material (e.g. from seed orchards or through international trade), seed and plant disemination is likely to be very important (Bihon *et al*., 2012a).

Diplodia sapinea causes degeneration of edible seeds on *P. pinea,* with direct economic consequences (Vagniluca *et al*., 1995; Santini *et al*., 2008). Moreover, the frequent occurrence of *Diplodia* symptoms on pine trees planted in urban landscapes and recreational areas has raised awareness of this pathogen among public administrations.

Multilocus genotyping with RAMS primers indicated that the trees in the sampled stands were colonized by thirteen *D. sapinea* genets. Genets found in *P. taeda* and *P. sylvestris* occurred only on these hosts, while the remaining genets were shared among other host species. Previous work in 2012 with *D. sapinea* in the same seed orchard showed that one genet occurred within *P. nigra* and *P. sylvestris* stands (Doğmuş Lehtijarvi *et al*., 2014). This indicates that the disease in the stand had possibly started with wound infections by one or a few clones of the pathogen (cf. Bihon *et al*., 2012b). In the present study, there were six distinguishable genets on the same hosts. The difference in number of genets could be due to spread of *Leptoglossus occidentalis* Heidemann to the study area. This alien invasive insect is a new vector of the fungus in Europe (Luchi *et al*., 2012), and could possibly have introduced more genets to the site. *Leptoglossus occidentalis* was first detected in Turkey in 2009 in Istanbul (Arslangündoğdu and Hızal, 2010), approx. 100 km from the Kerpe Research Forest. Owing to the rapid

increase in the known distribution of this insect around the Marmara Region over 2 to 3 years (Öztemiz and Doğanlar, 2015), it is possible that arrival and establishment of *L. occidentalis* in the area of the present study has affected the *D. sapinea* population, as the 13 genotypes were all closely related.

Bihon *et al.* (2012a), in examination of genetic differences between single *D. sapinea* populations from South Africa, Ethiopia, Argentina and Australia using microsatellite markers, found genetic variation between continents and within Australia.

Similarities between isolates of *D. pinea* were high when analysed with RAPD markers and isoenzyme analysis (Stanosz *et al.*, 1999). Burgess *et al.* (2004) studied six different populations of *D. pinea* isolated from *P. radiata* in New Zealand using SSR markers, and found small variations and diversity amongst populations. In the present study, the greatest numbers of polymorphic loci were in the pathogen population from *P. nigra*, with the lowest numbers of loci in the *D. sapinea* isolates from *P. radiata*.

The genetic similarities between *D. sapinea* genotypes and populations across continents suggests that the fungus has had a long asexual history of dispersal. Clones generally accumulate unique alleles in geographically isolated populations (Taylor *et al.*, 2000). In addition, plant genetic material such as seeds or seedlings for breeding programmes, has been exchanged widely between Turkey and other countries. Because *D. sapinea* is a latent pathogen, genotypes of the fungus have probably been spread with these materials (Burgess *et al.*, 2001; Flowers *et al.*, 2001).

The present study used three RAMS and M13 markers to detect genetic variation between *D. sapinea* isolates obtained from five different host pine species, although the isolate sampling was made at one site. Further analyses using SSR markers or other microsatellite markers are required to confirm these results and to determine if there is host specialization in *D. sapinea*. In the results presented here, cross inoculations suggested that there was no host specialization of this pathogen for the isolates tested.

Pathogenicity of *D. sapinea* did not relate to isolate origin or tested host in this study. This is similar to the results of Chou (1976), who found no differences in pathogenicity amongst New Zealand isolates of *D. pinea*. Doğmuş Lehtijärvi *et al.* (2009) previously reported the ability of a single isolate of *D. sapinea* to infect a range of coniferous species in Turkey, including *Cedrus libani* and *P. nigra*. In the present study, inoculations with 25 *D. sapinea* isolates resulted lesions on the tested coniferous tree species, with the greatest virulence on *P. nigra* and *P. sylvestris* and least on *P. taeda* and *P. pinaster*.

Outbreaks of disease caused by *D. sapinea* have occurred periodically wherever pines are grown. No direct and effective control measures are available for this pathogen in plantations and seed orchards. However, irrigation of seed orchards may prevent drought stress that can precede outbreaks of Diplodia shoot blight. Wounding of trees should also be minimized during forestry management to reduce infections of *D. sapinea*. Most importantly, foresters need to consider the risk of planting *P. sylvestris* and *P. nigra* in regions with unsuitable climate and location, such as low altitude, which may subject trees to stresses that predispose them to *Diplodia* infections.

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