Distribution and genetic diversity of *Dothistroma septosporum* in *Pinus brutia* forests of south-western Turkey.

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Abstract

Dothistroma needle blight (DNB) is a serious disease of the Pinaceae, mainly *Pinus* species, caused by the fungi *Dothistroma septosporum* and *Dothistroma pini*. Both species are regarded as invasive forest pathogens worldwide with rising incidence in central and northern Europe over the last three decades. Increasing numbers of reports of DNB in Mediterranean countries and those bordering the Black Sea in recent years, suggest that the prevalence and severity of the disease is increasing in this region. In this work, 29 sites were investigated between 2013 and 2015 in south-western Turkey. Morphological examination of needles confirmed DNB infection (i.e. *Dothistroma* conidiospores observed) at 18 sites, and a total of 108 *Dothistroma* sp. isolates were obtained from 11 of the sites. Host age seemed to be an important factor in both occurrence and severity of DNB in *Pinus brutia* forests. Continuous

rainy days, especially in December, may increase severity of disease, however extreme rain events may reduce available conidiospores on plant tissues or in the air.

Species-specific mating type primers showed that all isolates were *Dothistroma septosporum; D. pini* was not detected. The mating type ratio was close to 1:1 indicating sexual recombination was occurring. Eleven microsatellite markers revealed 59 unique multilocus haplotypes (MLHs) among the 73 isolates originating from different conidiomata. The majority of MLHs were represented by a single isolate (n=52) and only one MLH was shared between two localities. Analyses showed high genetic diversity, isolation-by-distance, and clear population clusters. These findings suggest that *D. septosporum* is well-established in south-western Turkey and is probably not a recent introduction.

Keywords

Red band needle blight, *Dothistroma septosporum*, Population genetics, microsatellites, SSR, Mediterranean pine forests, invasive forest pathogens

1. Introduction

Dothistroma needle blight (DNB) is one of the most damaging foliage diseases of pine in plantations and natural forests worldwide; the disease has increased in prevalence and in severity over the last 20 – 30 years (Drenkhan *et al*., 2016).

Although DNB came to prominence after severe outbreaks in the Southern Hemisphere on exotic pine plantations during the 1950s and 1960s, it was almost unrecognised in the Northern Hemisphere, including Europe, until the 1990s (Drenkhan *et al*., 2016). Rising incidence and severity of DNB in the Northern Hemisphere have been linked to changing

climatic conditions, increases in trade and movement of plant material between countries and within regions and planting of susceptible, non-native pines in climates suitable for the pathogen (Drenkhan *et al.,* 2016).

The disease was thought to be caused by a single pathogen species until Barnes *et al*. (2004) demonstrated the presence of two distinct species: *Dothistroma septosporum* (Dorog.) Morelet. and *Dothistroma pini* Hulbary. *Dothistroma septosporum* and *D. pini* produce identical symptoms on host trees and differentiation of the two species based solely on morphology is virtually impossible (Barnes *et al*., 2004). However, an increasing range of molecular methods can be used to identify and differentiate the DNB pathogens, either from cultures or directly from needles (Barnes *et al.,* 2004, Ioos *et al*., 2010, Schneider *et al.,* 2019, Groenewald *et al.,* 2007), and microsatellite markers are available to assess genetic diversity within and between populations (Barnes *et al*., 2008, Siziba *et al*., 2016).

A recent review reported the presence of DNB in 76 countries, 35 of which are in Europe, and on 109 Pinaceae host taxa, 95 of in the genus Pinus (Drenkhan *et al*., 2016). Yet the number of known host taxa and geographical distribution continue to increase (Mullett *et al.,* 2018, Ondruskova *et al.,* 2018). *Dothistroma septosporum* has a worldwide distribution and a broad host range (Drenkhan *et al*., 2016). Conversely, *D. pini* is assumed to have a more limited distribution and host range, although recent studies utilizing molecular detection tools have suggested that it is more common than previously thought (Mullett *et al*., 2018). Investigations of the environmental requirements of *D. septosporum* and *D. pini* suggested the two species may have different climatic requirements, with *D. pini* present in slightly warmer climates. Nevertheless, both *Dothistroma* species sometimes co-occur in the same geographic locality even within the same host needle (Drenkhan *et al*., 2016, Schneider *et al*., 2019).

Dothistroma needle blight is known from a wide range of climates, from boreal to tropical (Drenkhan *et al*., 2016; Adamson *et al*., 2018), and large areas of the globe are projected to be suitable for the disease (Watt *et al*., 2009). Nonetheless, reports of DNB from countries or regions with a Mediterranean climate are scarce, or limited to a few locations (Lazarevic *et al*., 2014; Drenkhan *et al*., 2016; Marchi and Ghelardini, 2017) and with low disease severity, probably due to low moisture and humidity limiting disease development, yet damage can occur in particular microclimates, especially plantations, suggested by recent reports from Mediterranean regions of Spain (Mullett *et al*., 2018). Increasing reports of DNB in recent years from Mediterranean and neighbouring regions, including Turkey and countries bordering the Black Sea, suggest that the disease situation may be changing in the region (Mullett *et al*., 2018).

Western and southern Turkey have a Mediterranean climate with hot dry summers and moderate, wet winters (Csa, Köppen-Geiger Climate Classification). Among the Mediterranean basin countries, Turkey has the second largest forest cover of 216,000 km^2, corresponding to 27.6% of total land area. Most of this forested area is dominated by naturally regenerating pine forests, with Turkish pine (*Pinus brutia* Ten.) as the main species of the Turkish Mediterranean belt. *Pinus brutia* is a low-elevation Mediterranean forest species that is well adapted to drought and alkaline soils and, along with Aleppo pine (*Pinus halepensis* Mill.), forms a distinct group within the Eurasian hard pines. While the largest area covered by *P. brutia* occurs in Turkey (5.4 million ha), natural populations of *P. brutia* also occur in other countries with Mediterranean climates, including Greece, Cyprus, Syria and Lebanon, and are also present in Iraq and Iran (Boydak, 2004). Although *P. brutia* is considered highly susceptible to DNB, confirmed reports of the disease in the native range of *P. brutia* are few

(Drenkhan *et al*., 2016). *Dothistroma septosporum* was reported on *P. brutia* in a plantation in Greece (Tsopelas *et al*., 2013) and from natural forest stands in south-western Turkey (Doğmuş-Lehtijärvi *et al*., 2013; Tunalı *et al*., 2018).

Under current climatic conditions several temperate and Mediterranean regions, including Turkey, are predicted to be suitable for infection by species of *Dothistroma* and development of DNB (Watt *et al*., 2009). In line with these predictions, an outbreak of DNB resulting in severe needle discolouration and defoliation of *Pinus brutia* stands was reported from south-western Turkey in 2013 (Doğmuş-Lehtijärvi *et al*., 2013). However, it is unclear how widespread DNB is in *P. brutia* forests of south-western Turkey. Nor is it clear which species of *Dothistroma* is responsible for the damage. Preliminary results indicated the causal agent to be *D. septosporum*, however *D. pini* occurs in many countries bordering the Black Sea and it is unknown whether this species is present in Turkey. Due to a lack of forest health monitoring and surveillance programmes in Turkey, many pests, particularly pathogens of forest trees, are poorly documented and have probably gone unnoticed for a considerable time. For example, uncorroborated observations by forest protection department personnel suggest areas around the 2013 Turkish DNB outbreak displayed similar symptoms as far back as 2004 (Arif Yılmaz, 2013 Personal Communication).

Both *Dothistroma* species have long been assumed to be non-native in Europe, with unknown origins. However, recent studies have revealed new insights into the possible origins of *D. septosporum*, although not yet for *D. pini*. Studies using dendrochronology, herbarium specimens and population genetics have suggested that *D. septosporum* is endemic on pines in parts of Europe and North America (Dale *et al*., 2011, Fabre *et al*., 2012, Drenkhan *et al*., 2013, Barnes *et al*., 2014). Recent population genetic studies focusing on Northern Europe

(i.e. the Baltic states, Sweden, Finland, European Russia, Norway), Britain and Ukraine found the greatest genetic diversity of *D. septosporum* within the native range of *P. sylvestris* and suggested this host and its native range could be within the centre of origin of the pathogen (Mullett *et al*., 2017, Adamson *et al*., 2018).

When confirmed in *P. brutia* forests in 2013, *D. septosporum* was also assumed to be non-native to Turkey in parallel with what was then accepted in Europe. Determining the extent of DNB in Turkish pine forests and exploring the population genetics of the causal agent will shed light on the nature of the disease in Turkey. The aim of this work, therefore, was to determine: 1) the prevalence and the impact of climate on severity of DNB in epidemic outbreak areas and surrounding forests in south-western Turkey; 2) the *Dothistroma* species occurring; 3) the diversity and population genetic structure of the causal agent(s); and 4) the likelihood of sexual recombination in the population.

2. Materials and Methods

2.1 Surveys and sample collection

The first epidemic outbreaks of DNB in Turkey were detected in two forest districts, Ağlasun and Pamucak, in the Isparta regional forestry management unit in south-western Turkey (Doğmuş-Lehtijärvi *et al*., 2013). In order to determine the extent of DNB in and around these areas, a survey focusing on forest districts in adjacent regions (Isparta, Antalya, Denizli and Muğla; Figure 1) was undertaken between 2013 and 2015.

At the individual survey sites, *Pinus brutia* stands were observed at a crown level for the presence of symptoms resembling DNB, such as discoloration and defoliation starting from

the lower branches. If crown level symptoms were seen, a closer examination of needles for typical DNB symptoms followed. These symptoms included needle tip dieback, reddish-brown banding on needles and small black fruit bodies on necrotic lesions on the needles. When needle symptoms were observed, needle samples from 1 to 13 trees were collected for further laboratory examination. Trees were sampled randomly from the stands. Samples were collected in May 2013, June and December 2014 and May 2015 and stored at -20°C prior to further processing.

In the laboratory, symptomatic needles were examined by microscopy to detect conidiomata typical of *Dothistroma* species. Subsequently, the presence of characteristic fusiform to short clavate, hyaline, smooth and thin-walled 1-5 septate conidiospores was used to confirm infection by *Dothistroma*. The survey site was considered positive for *Dothistroma* sp. when conidiospores of *Dothistroma* sp. were detected from at least one needle.

Severity of DNB was assessed following Bulman *et al*. (2004) with modifications; 1 to 13 randomly selected individual trees (trees from which needle samples were collected) within the survey site were assessed by scoring the percentage of the total crown volume with symptoms of DNB in 10% steps. The mean score across trees was considered as the severity of DNB in that survey site.

2.2. Impact of climate and site related factors on Dothistroma needle blight

Linear regression analysis was used to assess the impact of climate on the severity of the disease in surveyed sites. Fourteen climate variables (Table S1.1), each for 12 months (in total 168 variables) covering the 20-year period (1982 to 2012) were acquired from the link

180 "http://globalweather.tamu.edu/" as Climate Forecast System Reanalysis (CFSR) data in
181 SWAT file format. Data were acquired from 8 virtual stations within the survey area. In
182 addition to the climate variables, the average age of trees within a site (stand age) and the
183 altitude of sampling sites was also incorporated into the statistical analysis.
184
185 To compare climatic features as well as stand age and altitude between the sites with and
186 without DNB (present/absent) a Mann–Whitney U test was applied, since some of the
187 variable distributions differed significantly from a normal distribution.
188
189 Linear regression models were created with the arcsine square root transformed severity data
190 as the response variable, and climate variables and stand age as explanatory variables. All
191 statistical analyses were carried out using SPSS© 20 (IBM, version 20).
192
193
194 **2.3 Isolation of *Dothistroma* sp. and DNA extraction**
195
196 Single spore isolates (SSI) of the fungus were obtained from conidiospores released from a
197 single mature conidiomata on a needle. To obtain isolates, needles bearing mature
198 conidiomata of *Dothistroma* sp. were first gently cleaned by wiping with a 70% ethanol-
199 soaked tissue. A single mature conidiomata was removed gently with a sterile scalpel or
200 dissecting needle, placed into a 1.5 ml microcentrifuge tube containing 1 ml sterile distilled
201 water and vortexed to release the spores. The resulting spore suspension was spread onto 1%
202 water agar in Petri dishes and incubated at room temperature for 3 – 5 days. To ensure single
203 spore isolations, germinated spores were located under a microscope and excised separately
204 using a glass Pasteur pipette before transferring to a Petri dish containing 2% malt extract agar

(MEA). Isolate preparation and DNA extraction was performed following Mullett *et al*. (2017).

2.4 Species identification and mating type determination

The species-specific mating type primers developed by Groenewald *et al*. (2007) were used to determine the species and mating type of each isolate. Isolates were first tested using the *D. septosporum* specific mating type primers. If bands were absent or weak, PCRs were repeated using both *D. septosporum* and *D. pini* primer sets. Each reaction contained 1x (i.e. 12.5 µl) GoTaq Green Master Mix (Promega, Madison, WI, USA), 0.1 µM each primer, 10.5 µl PCR H_2O (Promega), and 1 µl template DNA in a total volume of 25 µl. The thermal cycler conditions followed those of Groenewald *et al*. (2007), and reactions were carried out on a GeneAmp 9700 thermocycler (Applied Biosystems). PCR products were visualised and scored on a 1.5% agarose gel stained with GelRed nucleic acid stain (Biotium, Hayward, CA, USA) after running for 40 minutes at 120V. For both species, amplification of the MAT1-1 idiomorph produces an amplicon of size c. 820 bp while the MAT1-2 idiomorph produces an amplicon of c. 480 bp.

2.5 Haplotype determination

Eleven microsatellite markers (Barnes *et al*., 2008) were used to determine the multilocus haplotype of each isolate. Multiplex PCR of the markers (Doth_DS1, Doth_DS2, Doth_E, Doth_F, Doth_G, Doth_I, Doth_J, Doth_K, Doth_L, Doth_M, Doth_O) and fragment analysis were conducted as described by Mullett *et al*. (2015). The two panels of PCR

products were analysed using an Applied Biosystems 3130XL genetic analyser along with a LIZ 600 size standard (Applied Biosystems), and alleles scored using GENEMAPPER v5.0 (Applied Biosystems, Carlsbad, USA).

Gene diversity was plotted against the number of loci using MULTILOCUS 1.3b (Agapow and Burt, 2001) in order to assess whether scoring more loci would enable greater discrimination of gene diversity. Individuals with identical multilocus haplotypes (MLHs, *i.e.* alleles identical at all 11 loci) were considered clones. Two data sets were created: one containing all individuals (non-clone-corrected data set), the second containing only one individual of each multilocus haplotype per population (clone-corrected data set).

2.6 Genetic diversity and population clustering

The non-clone-corrected dataset was used to evaluate genotypic diversity, richness and evenness calculated in the R packages poppr (Kamvar *et al*., 2014) and vegan (Oksanen *et al*., 2013). The following measures i) Genotypic richness, the expected number of multilocus genotypes (eMLG); ii) Shannon-Wiener index, H; iii) Stoddart and Taylor's index, G; iv) Simpson's index λ; v) Genotypic evenness, $E_{5;}$ and vi) the clonal fraction (CF) were calculated as described in Mullett *et al.* (2017).

The clone-corrected dataset was used to calculate Nei's gene diversity, H_{exp} (Nei, 1978), in poppr and allelic richness (A_R) and private allele richness (PA_R) in ADZE (Szpiech *et al*., 2008). A_R (*i.e.* the number of distinct alleles in a group) and PA_R (*i.e.* the number of alleles unique to a particular group) were computed using a rarefaction procedure to adjust A_R and PA_R to a specific sample size, allowing comparisons between populations with different

sample sizes. Calculations were standardised to a uniform size corresponding to the size of the smallest group.

To assess the population structure of the *Dothistroma* isolates two methods were applied to the clone-corrected dataset: 1) Discriminant Analysis of Principal Components (DAPC) and 2) a Bayesian, model-based, clustering algorithm implemented in STRUCTURE.

DAPC was used to identify clusters (K) of genetically related individuals (Jombart *et al*., 2010). The optimal number of clusters was assessed using a sequential K-means procedure followed by an assessment of the Bayesian information criterion (BIC) conducted in the R package ADEGENET (Jombart, 2008, Jombart *et al*., 2010). Cross-validation was used to determine the optimal number of principal components retained in the analysis.

STRUCTURE 2.3.4 (Falush *et al*., 2003) was used to assign individuals to a specified number of clusters (K). To estimate the optimal number of clusters, 30 independent runs of K=1-10 were carried out in STRUCTURE using no priors (i.e. no information on geographical location or host was provided). Each run had a burn in of 100,000 iterations followed by 500,000 data-collecting iterations, using a model of correlated allele frequencies and with admixture among populations allowed. CLUMPAK (Kopelman *et al*., 2015) was used to determine the optimal value of K using the ln(Pr(X|K)) method suggested by Pritchard *et al*. (2000). CLUMPAK was used to align all optimum K STRUCTURE runs to the permutation with the highest H-value. The DISTRUCT programme (Rosenberg, 2004) was used to visualise the CLUMPP output.

279 Hierarchical Analysis of Molecular Variance (AMOVA) was carried out on the clone-
280 corrected data set to test hypotheses of population differentiation using GENALEX 6.5
281 (Peakall and Smouse, 2012). Isolates were grouped by original locality, by DAPC and
282 STRUCTURE clusters, and by altitude of the stand (250-350 metres above sea level vs 600-
283 800 metres above sea level).
284
285 To test for isolation by distance and examine the relationship between genetic and geographic
286 distance, a Mantel test was conducted followed by a spatial principal component analysis
287 (sPCA). The Mantel test was carried out in GENALEX 6.5 using linear genetic distance and
288 10,000 randomizations of the data (Peakall and Smouse, 2012) while the sPCA was conducted
289 in the R package ADEGENET (Jombart, 2010). sPCA is a modification of PCA which relies
290 on no specific population models or assignment of individuals to discrete subpopulations, but
291 takes into account both genetic variance between individuals and their spatial autocorrelation.
292 A minimum distance neighbouring graph was chosen, as suggested by Jombart *et al.* (2008).
293 Significance of the spatial principal components was tested by the global and local Monte
294 Carlo tests of Jombart *et al.* (2008) using 10,000 permutations.
295
296 **2.7 Mating type and sexual recombination**
297
298 In order to investigate the possibility of sexual recombination, two tests were conducted on
299 both the clone-corrected and non-clone-corrected datasets. Isolates were grouped by locality,
300 DAPC cluster, and STRUCTURE cluster. Firstly, an exact binomial test, using two tailed p-
301 values, was used to determine whether groups differed significantly from the null hypothesis
302 of a 1:1 ratio of mating type idiomorphs (http://www.biostathandbook.com/exactgof.html).
303 An equal proportion of mating type idiomorphs indicates that sexual reproduction could be

frequent enough to maintain equilibrium. Secondly, the index of association (I_A) together with its associated measure ($\overline{r_d}$) were calculated using poppr (Kamvar *et al*., 2014). The I_A is a measure of multilocus linkage disequilibrium and $\overline{r_d}$ is a modification of I_A that removes dependency on the number of loci used, thus facilitating comparisons between studies (Agapow and Burt, 2001). Clonal populations are expected to have significant disequilibrium due to linkage among loci while sexual populations are expected to have linkage equilibrium due to no linkage among loci. The I_A and $\overline{r_d}$ from the observed data were compared to values obtained after 1,000 randomizations to simulate random mating.

3. Results

3.1 Surveys

Surveys were conducted across 13 forest districts in four administrative forest regions and field examinations were conducted at 29 individual sites (Table 1, Figure 1). Most of the sites consisted of young, naturally regenerated *P. brutia* stands, approximately 15 to 25 years-old, although six mature (i.e. over c. 30 year old) stands and four sites where trees of different ages were present around the stands were also surveyed (Table 1). Four sites were plantations of *P. brutia* (Ağlasun S1, Sütçüler S15, Pamucak S19, Acıpayam S23), with an additional site being a *Pinus pinea* plantation surrounded by naturally regenerated *P. brutia* stands (Ağlasun, S3) (Table 1).

Foliage symptoms resembling those of DNB on a crown or needle level were observed and needle samples collected at 25 of the 29 surveyed sites in ten of the 13 forest districts (Table 1). At each site, samples were collected from 1 to 13 trees (Table 1) with the distance between sampled trees ranging from 2 to 20 m within the same stand. In sites consisting of multiple

328 stands (e.g. S29, Cerle Valley) the distance between sampled trees ranged from 2 to ca. 400
329 m.
330
331 Morphological examination of needles in the laboratory confirmed DNB infection (i.e.
332 *Dothistroma* conidiospores observed) at 18 sites. *Dothistroma* sp. was isolated from 11 out of
333 the 18 sites within 6 forest districts: Ağlasun, Pamucak, Söğütdağı, Çandır, Gündoğmuş and
334 Sağırın (Table 1). At four of the 29 surveyed sites inspection of foliage under the microscope
335 indicated shoot blight caused by *Diplodia pinea* was the cause of damage, not DNB. DNB
336 was mainly detected in young stands, whereas no DNB infection was observed on older trees
337 (over c. 30 years old).
338
339 **3.2. Impact of climate and site related factors on Dothistroma needle blight**
340 The Mann-Whitney U test revealed 32 out of 168 climate variables differed significantly
341 between sites with and without DNB (Table S1.2). In addition to climate variables, altitude
342 and stand age also differed significantly between sites with and without DNB; trees in sites
343 with DNB infections were significantly younger than those in sites without DNB infections
344 (U= 19.00, p <0.001; Table S1.2). The probability of a wet day following a dry day in
345 December (PR_W1_12) was a significant, yet weak, variable in predicting the severity of
346 DNB in infected sites among the 168 climate variables (N = 29, R^2=0.425, F=19.973, p=
347 0.000; Table S1.3). When stand age and altitude were incorporated into the analysis (i.e. 170
348 variables in total), a significant model was obtained which largely explained the total
349 variation in severity of DNB with two predictors: stand age and the most extreme 30-minute
350 rainfall intensity recorded in June (Age + RAINHHMX6; N =29, R^2=0.798, F=47.41, p=
351 0.000); Table S1.4).
352

353

354 **33 Identification of *Dothistroma* spp.**

355

356 A total of 181 single spore isolates were obtained from the *P. brutia* needle samples taken
357 from 11 sampling sites within 6 forest districts (Table 1). Species-specific mating type
358 primers showed that all isolates were *D. septosporum. Dothistroma pini* was not detected at
359 any of the sites examined.

360

361 **3.4 Haplotype determination**

362

363 For population analysis, isolates from the 11 sites were grouped into 7 localities (Table 1).
364 Isolates were grouped into the same locality if needle samples were collected from the same
365 forest district. This process resulted in each forest district being considered a locality, except
366 for two localities in Sağırın forest district where the sites were c. 8 km apart.

367

368 The clonal isolates obtained from the same conidioma were removed from the primary dataset
369 and in total 73 single spore *D. septosporum* isolates were subjected to multilocus haplotyping
370 and subsequent population analysis.

371

372 Ten of the 11 microsatellite markers were polymorphic, yielding a total of 98 different alleles
373 ranging from 1 at Doth_G to 20 at Doth_M. A plot of gene diversity against number of loci
374 showed that five markers accounted for 99.07% of the variation, while ten markers accounted
375 for 99.92% (data not shown). Therefore the 11 markers used were deemed sufficient for
376 population genetic analyses.

377

Based on the 11 microsatellite loci a total of 59 unique multilocus haplotypes (MLHs) were detected in the 73 isolates. The majority of MLHs were represented by a single isolate (n=52), six MLHs were represented by two isolates, and one MLH was represented by nine isolates. Only one MLH was shared between two localities, Ağlasun (L1) and Pamucak (L4).

At each locality, the number of trees from which needle samples were collected ranged between 1 and 13. Even though the isolates were derived from a single tree in Söğütdağı (L2), only two isolates were identical, whereas in Pamucak (L4) the number of identical isolates from 11 trees was 9 out of 17 isolates (Table S2a).

3.5 Genetic diversity and population statistics

The Ağlasun locality (L1) had the highest diversity (H, G and λ) and Gündoğmuş (L5) the lowest (Table S2a). Pamucak (L4) had the lowest evenness (E_5) and the highest clonal fraction (Table S2a), primarily influenced by nine isolates of the same MLH (out of a total of 17 isolates). Gene diversity (H_{exp}), allelic richness (A_R) and private allele richness (PA_R) were highest in Dipyurt (L6) when standardized to the smallest sample size of two, yet the total number of private alleles was highest in Cerle valley (L7).

DAPC and assessment of the BIC indicated that four clusters best described the dataset (Figure 2). The number of isolates, number of MLHs and their diversity statistics are given in Table S2. The genotypic and gene diversity were broadly similar for the clusters (Table S2b). DAPC cluster 1 had the highest diversity statistics (H, G and λ) while DAPC cluster 2 had the lowest, as well as having the lowest A_R and PA_R. The highest A_R and PA_R were found in DAPC cluster 4, which also had the highest total number of private alleles (Table S2b).

The STRUCTURE analysis also revealed four clusters when the ln(Pr(X|K)) method was used to determine the best K. (Figure 3, Figure S1). The Δ K method of determining the best K suggested five clusters (Figure S2); however, inspection of the barplot revealed artificial splitting of individual membership probabilities to accommodate this cluster (Figure S3) and five clusters were therefore deemed highly unlikely; the four clusters determined using the ln(Pr(X|K)) method were therefore retained. STRUCTURE cluster 1 had the highest diversity statistics (H, G and λ) as well as A_R and PA_R, having 36 private alleles (Table S2c).

Grouping the isolates by locality, altitudinal level, DAPC cluster and STRUCTURE clusters was highly significant (AMOVA; Table S3).

The Mantel test showed that isolation-by-distance was significant in the dataset, with a correlation between linear genetic and geographic distance ($R^2 = 0.0476$, $p < 0.001$). Further investigation of spatial patterns via the sPCA revealed both global structure (global test, max(t) = 0.0410; $p \leq 0.001$) and local structure (local test, max(t) = 0.0280; p = 0.0032) (Figure S4). Global structure relates to clines or patches of relatedness among individuals while local structure refers to strong differences between neighbouring individuals.

3.6 Sexual recombination

Both mating types of *D. septosporum* were found in south-western Turkey, in a near perfect 1:1 ratio in both the non-clone-corrected and clone-corrected datasets (Table 2). The I_A and $\overline{r_d}$ tests, however, did not support random mating in the entire dataset. Each of the seven localities had both mating types and none differed significantly from a 1:1 ratio using both the

non-clone-corrected and clone-corrected datasets. The I_A and $\bar{r}_d$ tests on the clone-corrected dataset supported random mating in four of the localities: Ağlasun (L1), Sögütdağı (L2), Dipyurt (L6), Cerle valley (L7). These tests were not possible on the Gündoğmuş (L5) samples as only 2 MLHs occurred.

The four DAPC and the four STRUCTURE clusters each contained both mating types and none of the clusters significantly deviated from a 1:1 ratio of mating types (Table 2b, c). Random mating was suggested by the I_A and $\bar{r}_d$ tests in all of the clone-corrected clusters except for STRUCTURE cluster 3. In contrast, random mating was only supported in the non-clone-corrected datasets of DAPC cluster 4 and STRUCTURE cluster 2 by the I_A and $\bar{r}_d$ tests.

4. Discussion

Dothistroma needle blight is more widespread in south-western Turkey than previously believed with numerous areas of native forest affected. The causal agent of DNB in the *P. brutia* forests of south-western Turkey is *D. septosporum*. *Dothistroma pini* was not found in the region, although it is present in neighbouring Georgia and Ukraine (Matsiakh *et al*., 2018) and may be present in other regions of Turkey. This paper presents the results of the first analysis of *D. septosporum* populations in Turkey, in a region with a Mediterranean climate from which limited reports of DNB were available, and with a historical role as a bridge between Asia and Europe. The findings of high genetic diversity, sexual recombination, isolation-by-distance, and clear population clusters suggest *D. septosporum* is well-established and widespread in south-western Turkey and is not a recent introduction.

Dothistroma needle blight was confirmed in 17 naturally regenerated young (up to approximately 25-year-old) *P. brutia* stands and in a sixteen-year-old experimental plantation, which together comprised 62% of surveyed sites. Symptoms were most apparent in the lower crown. No symptoms were observed in mature stands where trees were over c. 30 years old. The age of trees in sites with DNB infections (average age of trees approximately 17 years old) was significantly younger than the sites without DNB infections (average age of trees approximately 35 years old). This finding suggests that the susceptibility of *P. brutia* to Dothistroma infections may decrease with age. For example, it is known that *P. radiata* is less susceptible to infections after 15 years of age (Bulman et al., 2004).

The severity of DNB symptoms ranged from 40 to 80%. The most severe infections were observed in stands located in valleys, where the formation of mist during spring and winter is common (as occurs in the Pamucak survey sites; S17-S21), and in riparian forests where relative humidity remains high due to the flowing river (as at the Cerle Valley site, S29). A similar severity pattern was also reported from Greece where heavier attacks were observed in a *P. brutia* plantation in the humid lower parts of a valley (Tsopelas *et al*., 2013). In Poland, Boron *et al*. (2016) also observed high rates of infection of *P. nigra* and the less susceptible *P. sylvestris*, above river rapids and concluded that the high humidity caused by the rapids increased microclimatic suitability for DNB. Additionally, survey sites with a dense understory of shrubs (such as Ağlasun S2) or growing under a closed canopy layer (such as Cerle Valley site, S29) also tended to have high disease incidence. Leaf wetness plays a crucial role in infection and symptom development of DNB (Gadgil, 1977). Therefore, longer periods of intense mist may increase the efficiency of the infection and disease development process.

The regression analysis used to assess the impact of climate using 168 climate variables revealed that as the probability of a wet day following a dry day in December increased

disease severity increased. This effect suggests a requirement for continuous wetness in the winter months (December), it is thus possible that this period is related with spore dispersal or infection development of the fungus in this region. However, spore dispersal of the fungus is known to occur primarily from April to October in many regions in Europe and North America. Thus, continuous wetness in December probably represents a critical period for disease development instead of pathogen dispersal in this region. On the other hand, when site related factors (stand age and altitude) were included in the analysis, the most extreme 30-minute rainfall intensity recorded in June together with stand age were better able to explain the variation in severity of DNB in surveyed *P. brutia* forests. Extreme 30-minute rainfall amount (mm) in June was negatively correlated with disease severity. Intensive rainfall is likely to wash conidiospores out of the air and off needle surfaces. However, the climatic data used in the study were unable to adequately represent the small scale microclimatic conditions in these sites and thus a relationship for the microclimatic conditions and disease severity remains anecdotal. This is not surprising given the limited number of sites and their proximity regarding climatic variables; climate data for 29 sites was accrued from 8 virtual stations. For example, the climatic variables were the same for sites S1 to S12, which included sites with no disease and severe disease. Furthermore, the climate data used were from the 20-year period 1982 to 2012. Although suitable for the examination of long term trends, the data are less suited to the investigation of recent changes in local climate that may be related to an increase in disease. It is known that the severity of DNB is highly sensitive to yearly differences in weather, as well as being site dependent (Fraser et al., 2016).

Nevertheless, the observations and statistical analyses still suggest that DNB in south-western Turkey is closely associated with specific microclimatic conditions favouring high humidity and leaf wetness. This situation is also likely to be the case for other Mediterranean pine forests and for pine forests elsewhere where the specific moisture regime needed for *D.*

septosporum to become an appreciable problem only occurs in particular years or at specific locations. Such a scenario helps explain the scarcity of DNB reports from the Mediterranean region, which were hitherto generally limited to a few locations, with low levels of disease severity (Lazarevic *et al*., 2014; Drenkhan *et al*., 2016; Marchi and Ghelardini, 2017). More severe damage is known to occur in particular microclimates, however, especially plantations as suggested by recent reports in Mediterranean Spain (Mullett *et al*., 2018).

The current survey represents the widest DNB survey in Turkey to date. Nonetheless, it was limited in the area and number of locations surveyed. As *P. brutia* forests alone cover approximately 6 million hectares in Turkey it is impractical to survey the full extent of DNB in pine forests using only ground level observations. In addition, a lack of striking DNB symptoms in a stand or tree does not necessarily confirm that *Dothistroma* is not present. Indeed, needles of healthy appearance at some locations (Ağlasun and Pamucak) were shown to be infected with *D. septosporum* using direct in-planta PCR detection (Tunalı *et al*., 2018). With the aid of molecular methods, such as conventional PCR or quantitative PCR (qPCR), which allow sensitive and specific detection and identification of DNB agents (Ioos *et al*., 2010; Schneider *et al*., 2019), it is possible to detect pathogens in stands or trees with low levels of infection or early in the disease cycle. Therefore, for future studies, we recommend aerial surveillance of large areas followed by ground level truthing of specific locations using molecular diagnostic methods.

Examination of 181 *Dothistroma* isolates obtained from *P. brutia* needles from 11 sites revealed that the causal agent of DNB in the Turkish locations examined was *D. septosporum*. While *D. septosporum* is the most important and widespread DNB-causing pathogen worldwide, the number of reports on the occurrence of *D. pini* has increased, particularly in

527 the last few years in Europe (i.e., Mullett *et al*., 2018; Ondrušková *et al*., 2018). Given this
528 increase in *D. pini* findings, we speculated whether *D. pini* might also be present in Turkey,
529 particularly as this species tends to favour warmer areas (Fabre *et al*., 2012). However, *D. pini*
530 was not detected in the survey area. Since the sampling did not cover the entire geographic
531 range of Turkey and focused on native *P. brutia*, it is possible that *D. pini* is present
532 elsewhere in the country. However, based on the surveyed area of *P. brutia* forests in 13
533 forest districts it can be concluded that *D. pini* is not involved in the current DNB epidemic in
534 south-western Turkey.
535
536 Multilocus haplotyping of the *D. septosporum* isolates demonstrated a high level of diversity
537 in the Turkish population. Fifty-nine unique multilocus haplotypes (MLHs) were detected in
538 the 73 isolates from separate conidiomata included in microsatellite analysis (out of 181
539 isolates obtained in this work), with the majority of MLHs represented by a single isolate.
540 This situation is not common for populations of this fungal pathogen; for example, only 81
541 unique MLHs were found in 282 isolates collected from Brittany, France (Mullett *et al*., 2015)
542 and 382 MLHs from 1194 isolates in Britain (Mullett *et al*., 2017). However, similar levels of
543 haplotypic diversity have been found elsewhere e.g. in British Columbia, Canada (Dale *et al*.,
544 2011) and northern Europe (Adamson *et al*., 2018) and it was concluded that the pathogen
545 could potentially be native in these areas. With such high levels of haplotypic diversity, it is
546 unsurprising that sexual reproduction was found to occur in the *D. septosporum* populations
547 of Turkey. Mating types were present in equal proportions in the overall dataset and at each of
548 the individual localities, using both the clone-corrected and non-clone-corrected datasets.
549 Random mating, a clear indication of sexual recombination, was found to occur at four of the
550 seven localities (Ağlasun, Sögütdağı, Dipyurt, Cerle valley) using the clone-corrected dataset.
551

552 Analysis of the population structure using clustering software (STRUCTURE and DAPC)
553 showed that distinct groupings of the isolates occurred, with many isolates belonging clearly
554 to one cluster or another. However, while certain clusters predominated at particular
555 localities, there were no striking geographical patterns, with most localities being composed
556 of isolates from two to all four of the clusters. However, AMOVA also showed that
557 populations at individual localities were significantly different from each other and the Mantel
558 test was highly significant for isolation-by-distance, thus geographically close isolates were
559 also close genetically, with more geographically distant isolates more distinct. Isolation-by-
560 distance is often associated with well-established, wide-ranging populations and is not
561 typically seen in recently introduced species, where a single population of low genetic
562 diversity dominates a large geographical area. Taken together, these findings of high genetic
563 diversity, sexual recombination, isolation-by-distance, and clear population clusters suggest
564 *D. septosporum* is well-established in south-western Turkey and is probably not a recent
565 introduction.
566
567 One of the localities (Pamucak, L4) included an experimental plantation (S19), the only
568 plantation included in the *Dothistroma* population analyses. The plantation was established
569 using seedlings from 5 different *P. brutia* provenances and was planted close to other sampled
570 sites (S20 and S21) within the Pamucak locality. Pamucak had the highest number of *D.*
571 *septosporum* clones and thus the lowest evenness (E_5). Four of the six isolates from the
572 plantation site (S19) were the same MLH, and this haplotype also comprised five of the eight
573 isolates from the adjacent site (S20) only about three hundred metres away. While this result
574 could be purely by chance it may also be due to distribution of identical *D. septosporum*
575 haplotypes through plantation material, as has been reported for other countries (e.g. Mullett
576 *et al.*, 2017). However, as these data were from a single plantation site the result is not

conclusive and more plantation sites, along with nurseries, should be sampled to provide more evidence of *D. septosporum* transport via plantation material in Turkey. Plantation forestry in Turkey uses plants raised from seeds obtained from natural forest stands or seed orchards derived from natural stands within the country, usually from the same region. Thus, the most important international introductory pathway of forest pathogens, trade in plant materials, is unlikely to be the source of the current *D. septosporum* outbreak in *P. brutia* forests of south-western Turkey. In addition, international trade of live plants to Turkey is concentrated on ornamental species and rarely includes pines or other Pinaceae. Crucially, the south-western Turkish pine stands where *D. septosporum* was detected represent mostly naturally regenerated forests. Thus, it is unlikely that the pathogen was introduced and spread via the international plant trade. Nevertheless, as *D. septosporum* was also detected in an experimental plantation site in this region, the movement of infected material within the country could still be a possibility.

Invasions by alien forest pathogens have strong anthropogenic dimensions (Santini *et al*. 2013) and *D. septosporum* is probably an important example in this regard (Barnes *et al*., 2014). Although trade in live plants, especially pines, is not a major current concern in Turkey, the historical role of the country as a bridge between Asia and Europe should not be ignored. Generally, population analyses of this pathogen have lacked representatives from Asian countries. However, Adamson *et al*. (2018), in a rare study to include Asian isolates, showed that populations from Asia (Far East Russia and Bhutan) had lower genetic diversity compared with those of Northern Europe and that gene flow was more likely to be from Europe to Asia rather than vice versa. Barnes *et al*. (2014) also found similar results for Asia using limited numbers of isolates from Bhutan, the same as those used by Adamson *et al.* (2018). Therefore, when analysing European-Asian gene flow and movement of *D.*

septosporum, the inclusion of populations from Turkey and other areas of Asia would be extremely valuable.

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TABLE AND FIGURE CAPTIONS

Table 1. Dothistroma needle blight survey sites in south-western *Pinus brutia* forests in Turkey.

Table 2. Mating type ratio and index of association tests for the *Dothistroma septosporum* isolates grouped by a) locality, b) DAPC4 cluster and c) STRUCTURE K4 cluster; Bold p-values (i.e. those that are non-significant) indicate random mating is supported by the test.

Figure 1. Map of Turkey showing Dothistroma needle blight survey sites, forest districts, and forest regions.

Figure 2. (a) Scatterplot of the discriminant analysis of principal components (DAPC) on Turkish *Dothistroma septosporum* multilocus haplotypes. Numbers and colours represent the 4 groups delineated after assessment of the Bayesian information criterion (BIC) (see main text for details). Individual multilocus haplotypes are represented by dots and clusters as inertia ellipses. The inset at the bottom right represents the cumulated variance (%) axes. **(b)** Map showing *Dothistroma septosporum* population localities. Pie charts represent assignment likelihood to each DAPC cluster at each locality. The clone-corrected (by locality) sample sizes are: Ağlasun 17; Çandır 10; Söğütdağı 6; Pamucak 9; Cerle Valley 13; Dipyurt 3; Gündoğmuş 2.

Figure 3. (a) STRUCTURE clustering (K=4) of *Dothistroma septosporum* multilocus haplotypes from Turkey. Each multilocus haplotype is represented by a horizontal line partitioned into coloured sections that represent the isolate estimated membership fractions in each cluster. Black lines separate the sampling localities. **(b)** Map showing *Dothistroma*

septosporum population localities. Pie charts represent assignment likelihood to each STRUCTURE cluster at each locality. The clone-corrected (by locality) sample sizes are: Ağlasun 17; Çandır 10; Söğütdağı 6; Pamucak 9; Cerle Valley 13; Dipyurt 3; Gündoğmuş 2.

SUPPORTING INFORMATION LEGENDS

Table S1.1 – S1.4. Data and results in analysis of impact of climate and site related factors on Dothistroma needle blight

Table S2. Number of *Dothistroma septosporum* isolates and summary statistics for groupings a) by locality; b) by DAPC4 cluster; c) by STRUCTURE K4 cluster.

Table S3. Hierarchical analysis of molecular variance (AMOVA) for groupings of *Dothistroma septosporum* isolates.

Figure S1. Choice of the best K using the ln(Pr(X|K)) method. The highest probability is for K=4.