Fungal Genetics and Biology 175 (2024) 103937

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

New simple sequence repeat markers reveal undetected diversity in Spanish and Californian *Diplodia sapinea* populations

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ARTICLE INFO

Keywords:
Genotypic diversity
Genetic markers
Population structure
Diplodia tip blight
SSR

ABSTRACT

Diplodia sapinea is the causal agent of Diplodia shoot blight, an emerging disease affecting pine forests worldwide. The range expansion of this pathogen in northern Europe has been suggested to be partially facilitated by recent warmer conditions. Although *D. sapinea* has been studied extensively, critical aspects of its infection biology and population structure remain unexplored. In this study, we developed nine simple sequence repeat (SSR) markers mined from *D. sapinea* genomes to assess the genetic diversity at higher resolution. Isolates from northern Spain, an area formerly regarded as having low genetic diversity and samples from a Californian population that was formerly regarded as clonal, were analysed in the study. In Spain, the nine SSR markers identified 56 genotypes in 285 samples. Isolates from symptomatic shoots, cones and asymptomatic tissues collected from different stands, suggested admixture between local populations. The same genotype tended to dominate within a single cone, and the same genotypes were usually found in both symptomatic and asymptomatic shoot tissues. The nine new SSR markers developed in this study revealed a high level of genetic diversity in both the northern Spanish and northern Californian populations than previously anticipated. Analyses using these nine SSR markers should contribute to a better understanding of the epidemiology, evolution and origin of *D. sapinea*, a pathogen that is gaining prominence in many parts of the world.

1. Introduction

Diplodia sapinea is the causal agent of Diplodia shoot blight (also known as Diplodia tip blight), an emerging global disease affecting natural and exotic stands of mainly *Pinus* spp. (Swart and Wingfield, 1991; Zwolinski et al., 1995). The pathogen induces necrosis in current-year shoots that causes shoots to dry out from the tips and progresses down the shoot. Asexual conidia are produced on infected tissues (Brookhouser, 1971). The fungus survives as a saprotroph in dead needles and branches (Stanosz et al., 2007) and is also commonly found on cones (Müller et al., 2019; Munck and Stanosz, 2009).

Diplodia sapinea in not only considered as a facultative pathogen but also an endophyte (Maresi et al., 2007; Smith et al., 1996; Stanosz et al., 2005). The fungus can be isolated from asymptomatic tissues where it remains dormant until the host is subjected to stressful conditions, when it emerges as a pathogen (Slippers and Wingfield, 2007). Drought (Blodgett et al., 1997; Blodgett and Stanosz, 1998; Stanosz et al., 2001), and hail damage (Caballol et al., 2022b; Zwolinski et al., 1995, 1990) are common triggers for disease development. Disease emergence is affected by competition with other microbes as well as by differences in the susceptibility of different pine species (Oliva et al., 2020). Although *Pinus* spp. are the most common hosts of *D. sapinea*, several other tree genera, including *Abies*, *Cedrus* and mangrove trees (such as *Avicennia marina* and *Bruguiera gymnorrhiza*) have also been recorded as hosts of the pathogen (Osorio et al., 2017; Zlatković et al., 2017).

Interest in *D. sapinea* has recently increased due to unprecedented outbreaks of Diplodia tip blight in areas such as Sweden, Finland and Estonia, which were previously thought to be free of the pathogen (Brodde et al., 2019; Hanso and Drenkhan, 2009; Müller et al., 2019). Historically, *D. sapinea* has been found in areas of the world that have

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https://doi.org/10.1016/j.fgb.2024.103937
Received 26 June 2024; Received in revised form 14 September 2024; Accepted 9 October 2024
Available online 11 October 2024

temperate climates (Caballol et al., 2024; De Wet et al., 2000; Swart and Wingfield, 1991). Therefore, an increase in drought spells and hailstorms, and higher average temperatures over the past decades due to climatic change are thought to be possible causes that have led to the appearance of *D. sapinea* and related disease outbreaks in Northern Europe (Brodde et al., 2019; Fabre et al., 2011). Nevertheless, further research, for instance by looking at endophytic fungal diversity in herbarium specimens, is needed to clearly link Diplodia shoot blight emergence to climatic change given that *D. sapinea* has been known to be present as a pathogen for many years in other areas of the world with moderate climates and cold winters (Palmer, 1987; Swart et al., 1993).

An improved understanding of the biology and epidemiology of *D. sapinea* is required to better grasp the observed changes in its patterns of occurrence. To achieve this, the tools that are currently used to assess genetic diversity in populations of the pathogen at a global scale require reconsideration. Although the fungus is known to be genetically variable in areas such as Australia and South Africa (Bihon et al., 2012b; Burgess et al., 2004, 2001a, 2001b), previous studies have shown that it has a low genetic variation or even clonality in other areas such as Europe (Adamson et al., 2021; Brodde et al., 2019) and California (USA) (Barnes et al., 2014). New markers that better capture the diversity in the Northern Hemisphere populations could improve understanding of genetic diversity in this region. Future research on the pathogen's evolutionary potential and the threat it poses to European forests will be built upon the improved understanding of *D. sapinea*'s genetic diversity and how it correlates with virulence (McDonald and Linde, 2002).

The aim of this study was to develop a set of simple sequence repeat (SSR) markers using available genomic data that better capture the true diversity in the global *D. sapinea* population. The markers were used to assess the genetic diversity in a population from Spain and to reevaluate the clonality of a Californian population.

2. Materials and Methods

2.1. Sampling and fungal isolation

The Spanish population of *D. sapinea* consisted of 285 isolates, which were collected from five different *Pinus nigra* ssp. *salzmannii* stands in the Southern Pyrenees in northeast Spain (Table S1). Three of these stands showed typical symptoms of Diplodia tip blight associated with drought. The other two stands were in areas affected by hailstorms and showed severe shoot dieback. The average distance between stands was 2.5 km (ranging from 0.6 to 3.5 km). In each stand, one shoot was collected from five different trees. Cones were collected from three stands. In each stand, 20 recently fallen cones showing signs of infection were collected. Cones and shoots were bagged and kept at 4 °C until sample preparation.

Isolations from cones were carried out by placing each cone in a plastic cup with 250 mL of distilled water to release conidia from pycnidia. Conidia were then re-suspended in sterile water with 0.01 % Tween-20; approximately 50 conidia of *D. sapinea* were plated onto potato dextrose agar (PDA, Merck, Darmstadt, Germany) amended with 100 mg L^{-1} of chloramphenicol (Sigma, Saint Louis, MO, USA) at 28 °C. Three monosporic isolates were collected from each cone and cultured on PDA for further analysis.

Isolations were made from the symptomatic and the asymptomatic part of the shoot, which were distinguished by the necrotic limit. Needles were removed, then shoots were surface sterilized with 70 % ethanol for 1 min, after which the bark was removed. Three woody tissue samples (approximately 2 × 3 mm) 2 cm apart were cut from the symptomatic and from the asymptomatic part of each shoot (i.e., above and below the necrotic limit, respectively). Samples were contiguous to each other and the exact position with respect to the necrotic front was noted to understand whether the same genotype was present in both the necrotic and the immediate asymptomatic tissues or even further apart. Each sample was plated onto PDA and incubated at 28 °C for 48 h. Colonies showing the characteristic mycelium of *D. sapinea* were plated onto water agar media at 28 °C for 24 h, from which pure isolates were obtained by hyphal tip isolation. One single pure isolate from each tissue sample was grown on PDA and retained for further analysis.

The Californian population of *D. sapinea* was isolated from samples collected from mature *Pinus ponderosa* trees showing Diplodia shoot blight symptoms growing at five sites in northern California (Table S1). Five branches showing typical shoot-tip dieback symptoms were collected from each tree, placed in paper bags and transported to a laboratory for further processing. Five isolations were made from each branch sample at regular intervals from discoloured xylem tissue. Tissue samples were plated onto 2 % malt extract agar (MEA, Biolab, Midrand, Johannesburg, South Africa) and incubated at 20 °C. Fungi growing from tissue samples that showed typical mycelial characteristics of *D. sapinea* were plated onto water agar with autoclaved *Pinus radiata* needles placed on the surface. These plates were incubated at 20 °C under near UV light to induce sporulation (Smith et al., 1996). Conidia were collected from emerging pycnidia and plated onto PDA to obtain monosporic cultures. All the Californian cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Isolates selected for DNA extraction and downstream analyses were grown on 2 % MEA. A total of 399 isolates were analysed using the SSR markers designed by Bihon et al. (2011a). A randomly selected subset of 35 isolates was used to compare the resolution of the existing SSR markers designed by Bihon et al. (2011a) with the new SSR markers developed in this study.

2.2. Development of SSR markers

Two *D. sapinea* genomes (NCBI accession numbers ASM72994v1 and ASM2308738v1) of isolates obtained from samples collected in South Africa and China, respectively, were used for *in silico* SSR marker development. The genomes were screened with Krait (Du et al., 2018) using default parameters to identify SSR regions and to design flanking primers. The screening identified 4736 (ASM72994v1) and 6008 (ASM2308738v1) SSR regions with corresponding primers. To determine whether the SSR regions of the two genomes were polymorphic, the SSR regions were compared using R language script. Only those loci that had the same forward primer, reverse primer and motif, but that differed in terms of their motif repeat number, were retained. After this filtering process, the remaining 677 loci that were retained were further filtered to retain only those SSR that were approximately 150 bp in length to facilitate amplification. Markers with longer repeat motifs were favoured because of the potential for a higher allele number. Furthermore, trinucleotide repeats were chosen over dinucleotide repeats to facilitate screening. Generally, only one marker from each contig was selected to reduce the risk of linkage between markers. The primers of the resulting 74 markers were inspected with Beacon Designer (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1) to avoid secondary structures such as self/cross dimers and hairpin loops. In total, a subset of 46 SSR markers were obtained.

2.3. DNA isolation and D. sapinea identification

Fungal DNA was extracted from 200 mg of pure culture following the protocol described by Oliva et al. (2017) and using the commercial kit NucleoSpin Plant II (Macherey-Nagel, Düren, Germany) as described in the manufacturer's recommendations. Species-specific primers (Smith and Stanosz, 2006) were used to confirm isolates as *D. sapinea*. PCR amplifications were performed in a total volume of 25 μL containing 1x DreamTaq Green PCR Master Mix (ThermoFisher, Austin, TX, USA), 0.4 μM of each specific primer and 10 ng of DNA. The cycling conditions were as follows: an initial denaturation step of 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min, followed by an extension step of 72 °C for 6 min. The size of the fragments was checked on a 1 % agarose gel in a sodium borate buffer (1x SB) using a 1 kb GeneRuler ladder (ThermoFisher).

Table 1
Characteristics of the nine *Diplodia sapinea* simple sequence repeat (SSR) markers designed in this study with the fluorophore attached to the forward primers ("*" indicates the location of the fluorophore). The recommended combinations if used when pooling products from singleplex PCRs are DS_SSR2, DS_SSR13 and DS_SSR16 (panel 1); DS_SSR4, DS_SSR6 and DS_SSR20 (panel 2); and DS_SSR21, DS_SSR11 and DS_SSR10 (panel 3).

Locus	Primer sequences (5′–3′)	Fluorophore	Repeat motif	Size range (bp)	No. of alleles	Allele diversity (*H*)
DS_SSR2	*TGTGGCATCCGAACGGTATG TTGATACCACTGCACACCTCC	FAM	TCT	118–175	14	0.70
DS_SSR4	*TCCTAATGTCGCGGAAGACG TGAGTGGTTACCGGAATGCC	FAM	CA	161–181	8	0.48
DS_SSR6	*GCTCTTCGAGTCCAAGCAAC GGAGCATGAGCAGCTTGC	HEX	CAA	193–253	15	0.67
DS_SSR10	*CGAACCGAACCATGCATCC CACTGTGGCTTCTGCTCATG	ATTO550	AGA	177–192	5	0.37
DS_SSR11	*TAGACACGGCACTCTGCTTC TGGATGATGATGGTGCCTCG	HEX	TGC	145–178	5	0.66
DS_SSR13	*CTGCTGTTGCTGTTGCTGG CATCGACGACCTCTTCCTCG	HEX	GTT	178–217	6	0.56
DS_SSR16	*CACACGGCAATCAACTTCGG CCTCCTCTTCCGTTAGACCG	ATTO550	GAA	199–211	5	0.85
DS_SSR20	*AATGGTGACCACGCGCTC GGAGATTGTTGGCGTGCTTG	ATTO550	CAA	196–205	2	1.00
DS_SSR21	*TCGATGGACGACGGACGG AAGGTGCGGATGGCCTGTAG	FAM	CTT	122–161	4	1.35

2.4. Selection of SSR markers

From the 46 markers obtained following genome mining, a subset of 35 SSR markers were tested on seven monosporic isolates to assess amplification success and to verify that the polymorphism observed was due to length differences in the microsatellite repeat and not due to indels in the flanking region (Fig. S1 and Table S2). PCR amplification reactions and cycling conditions were conducted as previously described except that 1x DreamTaq PCR Master Mix was used, the annealing temperature was set at the optimal temperature of 58 °C, and the product size in all cases was approximately 200 bp. Simple sequence repeat markers showing clear visual differences on a 2.5 % agarose gel with 1x SB that was run for 2–3 h at 80 V were considered good candidates. To check whether length differences corresponded to variations in sequence repeat numbers, amplification products were purified using a MicroElute Cycle kit (Omega Bio-Tek, Norcross, GA, USA) and sequenced at Macrogen (Madrid, Spain).

From the subset of 35 markers, nine SSR markers were selected and used to analyse the genetic diversity of the Spanish and Californian *D. sapinea* isolates. Markers were fluorescently labelled with FAM, HEX and ATTO550 (Integrated DNA Technologies, B.V., Leuven, Belgium). The PCR products were pooled after amplification using a maximum of three SSRs at a time, as recommended by the supplier of the fragment analysis service, and then separated using a DS-30 matrix (Table 1). Isolates were genotyped at Macrogen using an ABI 3730XL DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). The size of the PCR products was assessed against a 400HD internal standard (ThermoFisher) and electropherograms were evaluated using the Microsatellite Analysis App (Applied Biosystems).

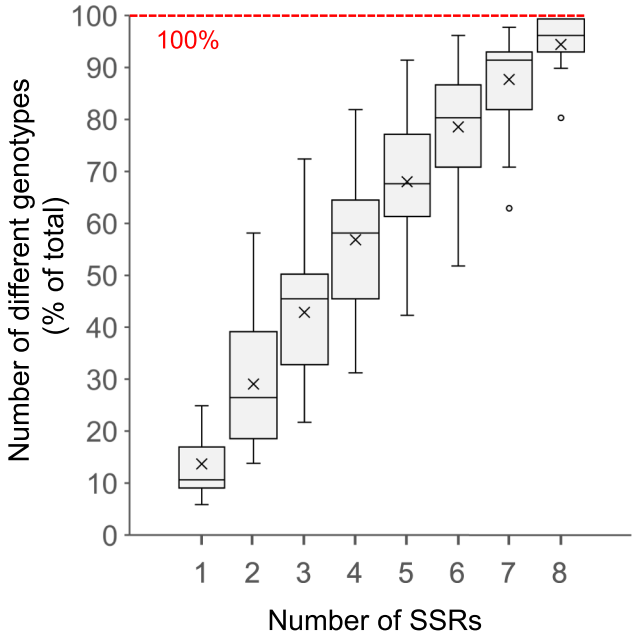

Fig. 1. Genotype accumulation curve for 320 Spanish and Californian isolates of *Diplodia sapinea* genotyped over nine simple sequence repeat (SSR) markers. The vertical axis shows the percentage of unique multilocus genotypes (MLGs) found in the Spanish and Californian populations. The horizontal axis shows the minimum number of SSR markers needed to differentiate each observed MLG. The genotype accumulation curve was obtained by performing 100 randomizations of the analysed data.

2.5. Data analysis

The multilocus genotype (MLG) of each isolate was identified based on the allele at each of the nine polymorphic loci. Allele diversity at each locus was calculated for a clone-corrected matrix. The clone-corrected matrix was established by removing the same genotype from the same cone or shoot to avoid considering vegetative growth rather than clonal reproduction. A genotype accumulation curve was performed to determine the minimum number of SSR markers that would be needed to distinguish between individuals in a population without experiencing modifications if additional markers were included. The accumulation curve was obtained using the genotype_curve function in the R package poppr V.2.9.5. (Kamvar et al., 2014).

For each sampling level (i.e., population, stand, cone/shoot or symptomatic/asymptomatic tissue), the average number of MLGs, the expected multilocus genotype (eMLG) based on rarefaction, the Shannon index of diversity (*H*) and the evenness were assessed using the locus_table function in the poppr package. The standardised index of association (rbarD) was calculated to estimate the population linkage disequilibrium. All analyses were conducted with and without clone-correction.

To visualise relationships among MLGs, we constructed a minimum spanning network (MSN) based on Bruvo's distance using the bruvo.msn function. Different MSN trees included different types of isolates. For instance, when visualizing differences between the Spanish and Californian populations, all isolates were included. When visualizing

Table 2
Genetic diversity of *Diplodia sapinea* populations in a clone-corrected matrix. The number of multilocus genotypes (MLG) found in the population, the expected number of MLG at the lowest common sample size (eMLG), the Shannon index (*H*) of diversity, the evenness index adapted from the Simpson Index, and the standardized index of association (rbarD) are shown for each population. "Symp." and "Asymp." refer to symptomatic and asymptomatic shoots, respectively.

Parameters	Country		Spanish stands					Sample type		
	USA	Spain	1	2	3	4	5	Cones	Symp. shoots	Asymp. shoots
Sample size (*n*)	35	129	7	42	34	40	6	44	37	19
MLG (G)	7	56	6	28	18	20	5	36	22	17
eMLG	7.00	21.00	6.00	8.48	7.49	7.36	5.00	12.2	8.42	9.47
Diversity (*H*)	1.39	3.37	1.75	3.07	2.57	2.61	1.56	3.50	2.90	2.80
Evenness	0.61	0.52	0.94	0.67	0.71	0.66	0.93	0.88	0.82	0.95
rbarD	0.85	0.22	0.36	0.26	0.19	0.17	0.66	0.15	0.25	0.27

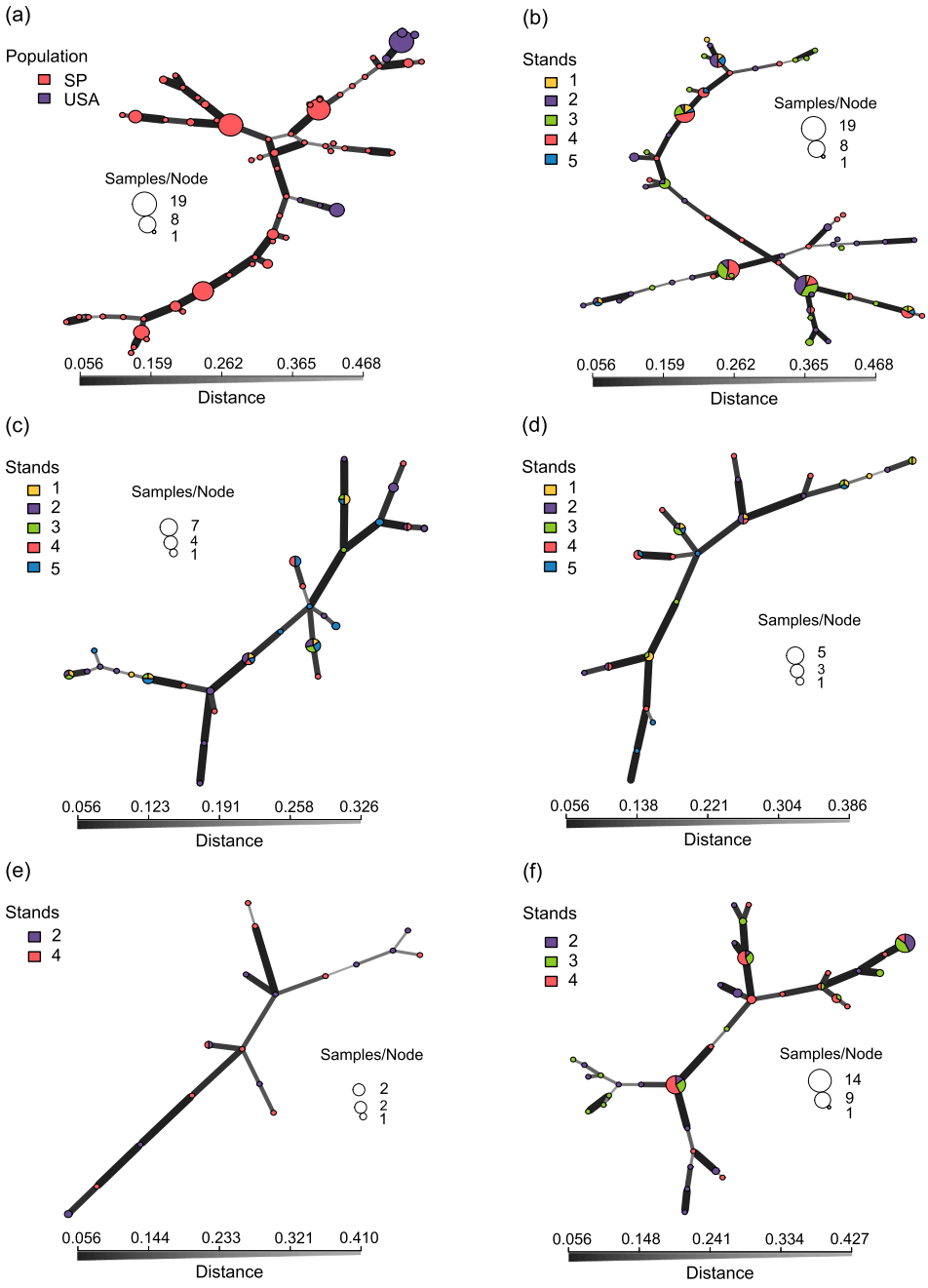

Fig. 2. Minimum spanning network (MSN) of *Diplodia sapinea* isolates from different populations and tissues. (a) Representation of 129 isolates from Spain (SP) and 35 isolates from a highly clonal population in California (USA). MSNs showing the distribution of 285 isolates collected from five different *Pinus nigra* ssp. *salzmannii* stands in Spain considering: (b) both shoot and cone isolates together; (c) only shoot isolates; (d) only symptomatic shoot isolates; (e) only asymptomatic shoot isolates; or (f) only cone isolates.

Table 3
Analysis of molecular variance (AMOVA) of *Diplodia sapinea* populations hierarchically partitioned. "df" refers to the degrees of freedom of the analysis. Negative values from variance and the percentage of variation were excluded.

Variance components	With clone-correction				Without clone-correction			
	df	Variance	Percentage of variation	*p*-value	df	Variance	Percentage of variation	*p*-value
Spanish and USA populations								
Both cones and shoots								
Between countries	1	2.92	41.54	0.001	1	2.99	42.33	0.001
Among stands	4	0.04	0.95	0.17	4	0.15	3.44	0.001
Between sample types (cones and shoots)	7	0.02	0.55	0.34	7	0.20	4.69	0.001
Spanish population								
Cones								
Among stands	2	0.02	0.38	0.35	2	0.07	1.75	0.02
Among cones	26	0.32	7.25	0.053	59	3.61	85.13	0.02
Shoots								
Among stands					4	0.17	4.29	0.01
Among shoots	24	0.76	17.85	0.008	24	2.36	58.28	0.001
Between symptomatic and asymptomatic shoots					9	0.25	6.23	0.01
Asymptomatic shoots (endophytic isolates)								
Among stands					1	0.10	2.31	0.18
Among shoots	9	0.76	15.56	0.14	9	2.29	49.92	0.001

differences between shoots or cones in different stands in Spain, only shoot and cone isolates were shown. In addition, differentiation of the Spanish and Californian populations was also visualized by multidimensional scaling analysis (MDS) using the cmdscale function in the stats package. Minimum spanning network and MDS analyses were conducted with clone-correction. In addition, the percentage of variation between and within populations and each of the hierarchical levels therein were determined using Analysis of Molecular Variance (AMOVA) with 999 permutations using the poppr.amova function. All AMOVA analyses were conducted with and without clone-correction. AMOVA analyses rendering negative variance values were discarded.

3. Results

3.1. SSR amplification

We genotyped 320 isolates using nine newly developed SSRs (DS_SSR2, DS_SSR4, DS_SSR6, DS_SSR10, DS_SSR11, DS_SSR13, DS_SSR16, DS_SSR20 and DS_SSR21). The number of alleles per locus ranged from 2 to 15, with SSR2 and SSR6 generating the highest level of polymorphism (Table 1). A total of 63 genotypes were detected. When more than seven SSRs were used, the genotype accumulation curve showed a tendency to saturate; however, it also indicated that all nine SSRs were needed to achieve a good genotypic resolution, i.e., 5 % of the genotypes could be missed if only eight SSR markers were used rather than nine (Fig. 1).

3.2. Population diversity per country

The Spanish population was more diverse than the northern Californian (USA) population (Shannon index values of 3.37 and 1.39, respectively, using a clone-corrected matrix) (Table 2). A higher rbarD value was obtained for Californian samples than for Spanish samples (0.85 and 0.22, respectively), indicating a higher level of clonal reproduction among isolates in northern California. The Spanish population comprised 56 MLGs (based on the analysis of 129 isolates) whereas the Californian population comprised seven MLGs (based on the analysis of 35 isolates) (Table 2). Although some alleles of several loci were found in both populations, the MSN analysis showed that were no MLGs that were present in both populations (Fig. 2a and Fig. S2). The Californian population comprised two distinct groups, both of which seemed to be genetically more similar to Spanish isolates than to each other. One of the Californian groups was composed of four MLGs with three private alleles and the other of three MLGs with one private allele. AMOVA revealed that country explained a large amount of the genetic diversity observed amongst isolates, indicating the possibility that geographically separated populations could be differentiated (Table 3).

The number of genotypes in the Californian population was assessed using the existing SSR markers designed by Bihon et al. (2011a) and the nine new SSR markers. When using the markers developed by Bihon et al. (2011a), the Californian population of 399 isolates showed a large dominance of one haplotype, which was present in 80 % of the isolates. Another three haplotypes were also present but at a much lower frequency (10 %, 2 % and 0.5 %) (Fig. S3a). In a random subset of the Californian population, which comprised 35 isolates, only two genotypes were detected. The most frequent genotype was detected in 77 % of the population whereas the other genotype was detected in only 23 % of the population (Fig. S3b and Table S3). However, when the subset population was analysed using the nine new SSR markers, seven different genotypes were detected (Fig. S3c and Table S4). The most dominant of these seven genotypes was detected in half of the analysed isolates.

3.3. Population diversity per stand

The number of MLGs detected per stand in the five Spanish stands ranged from 5 to 28 (Table 2). A single genotype was shared among all five stands and five genotypes were shared among three or more stands (Fig. 2b), presumably indicating asexual spread. All five of the MLGs present in stand 5 were also found in the other stands. Isolates from the different stands were distributed throughout the MSN, and no relationship between stands or location was detected (Fig. 2b). Overall, AMOVA analyses showed that there was little genetic differentiation between forest stands (Table 3).

3.4. Population diversity per tissue type

Across the five sampled stands in northeast Spain, based on the analysis of the 129 isolates, 36, 22 and 17 MLGs were detected in cones, symptomatic and asymptomatic shoots, respectively (Table 2). In several stands, the same genotype was found in both cones and shoots. AMOVA showed that cones or shoots within stands explained a larger share of the variation than stands (Table 3).

On average, in 80 % of the studied shoots, isolates from the asymptomatic and symptomatic parts of the shoot had the same genotype. In some cases, a single isolate spanned the entire length of the shoot (i.e., both asymptomatic and necrotic tissue) (Fig. 3a). Hail-induced necrotic tissues tended to be dominated by the same genotype

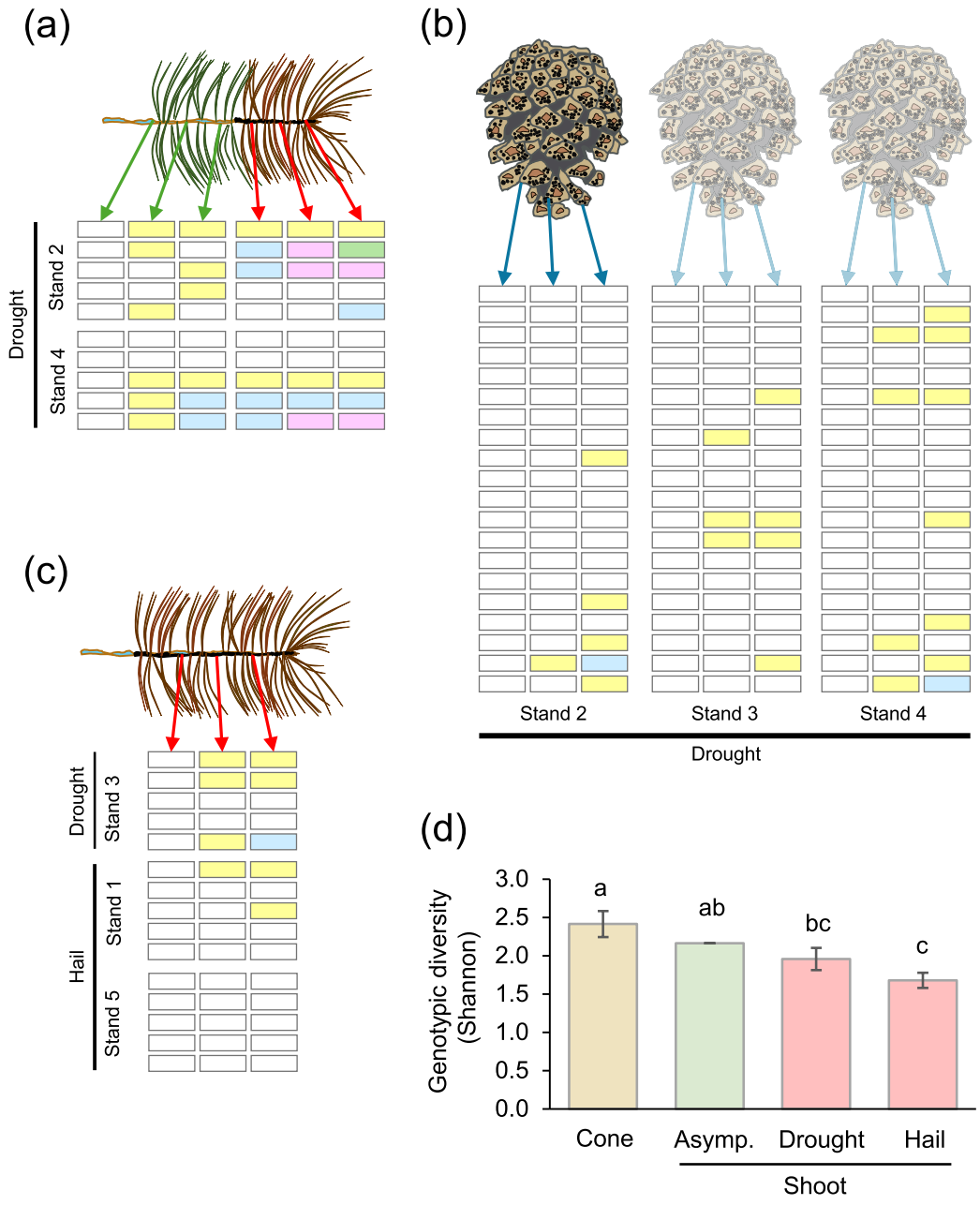

Fig. 3. Representation of the different *Diplodia sapinea* genotypes isolated from shoots and cones in Spain. Each of the different colours (white, yellow, blue, pink or green) shown in each row represents a different multilocus genotype found in a single shoot or cone. (a) Genotypes of isolates obtained from different pieces of a drought-induced necrotic shoot sample taken from asymptomatic (shown with green arrows) and symptomatic (shown with red arrows) parts of the shoot. Rectangles represent contiguous samples located at 2 cm from each other spanning over the necrotic front (b) Genotypes of isolates from cones collected from stands showing Diplodia tip blight associated with drought spells. Twenty cones were sampled per stand (shown in rows) and three different isolates (shown in columns) were obtained from each cone. (c) Genotypes of isolates obtained from different pieces of a necrotic shoot from stands showing Diplodia tip blight associated with drought spells and stands affected by hailstorms. (d) Genotypic diversity using the Shannon index for the different populations obtained from cones and shoots using a clone-corrected matrix. "Asymp." refers to asymptomatic shoots. Data shown are mean values ± the standard deviation. Different lowercase letters above the bars indicate a significant difference in genotypic diversity ($p < 0.05$).

(Fig. 3c).

Cones tended to harbour a higher diversity of genotypes than symptomatic tissues (i.e., shoots with drought- or hail-induced necrosis); however, no significant differences in *D. sapinea* genotype diversity were found between cones and asymptomatic shoots (Fig. 3d). MSN analysis showed an admixture of genotypes across shoot isolates, symptomatic shoot isolates and asymptomatic shoot isolates within and between stands (Fig. 2c–e). Cones tended to be dominated by a single genotype (a single MLG was found in 80 % of cones) (Fig. 3b). The same genotype was also commonly found across cones from the same stand (28 % of cone isolates from the same stand shared the same genotype) (Fig. S4). MSN analysis showed a mixed distribution of genotypes across cones within and between stands (Fig. 2f).

4. Discussion

In this study, existing genomes of *D. sapinea* were used to develop nine SSR markers to expand the toolbox for population biology studies. The new set of markers were used to analyse Spanish and northern Californian isolates. We were able to show high levels of genetic

variation in these two distant populations. Our findings differed from those of previous studies that had assessed isolates from these regions using existing SSR markers (Bihon et al., 2011a; Burgess et al., 2001b). Although high levels of genotypic diversity in South African populations have previously been reported (Bihon et al., 2012b, 2012a, 2011a; Burgess et al., 2004, 2001a), much lower levels of genetic diversity have been reported in studies considering European (Adamson et al., 2021; Brodde et al., 2019; Doğmuş-Lehtijärvi et al., 2014; Manzanos et al., 2019; Zlatković et al., 2019), USA (Aragonés et al., 2021; Barnes et al., 2014) or western Asia populations (Adamson et al., 2021). However, our findings suggest that some of these populations are more variable than previously believed. For example, when we re-evaluated the Californian population, which was considered to be predominantly clonal based on analyses with existing SSR markers (Barnes et al., 2014), we detected seven different genotypes, whereas only four genotypes had previously been found.

Previous studies on *D. sapinea* have reported low levels of genetic differentiation for populations of the pathogen within European countries, but large differences between countries (Adamson et al., 2021; Brodde et al., 2019; Burgess et al., 2004). For example, Adamson et al. (2021) observed that country-level sub-populations were mainly dominated by one haplotype (more than 45 % of the isolates), indicating that the European population of *D. sapinea* was highly clonal. However, when screening 425 samples from 15 different European countries, they found 27 private alleles, most of which originated from one specific population in Germany (Adamson et al., 2021). By contrast, in this study, which considered only a relatively small area in northern Spain, we identified 56 different genotypes, none of which appeared to be a clearly dominant genotype. Furthermore, our analyses revealed that the northern Californian population was genetically diverse with seven private alleles in 35 isolates. Thus, our analyses of both the Spanish and Californian populations revealed that diversity levels were similar to those reported for populations in the Southern Hemisphere (Bihon et al., 2011a; Burgess et al., 2001b). Based on these observations, it would be interesting to re-assess other previously studied European populations to determine whether this same pattern occurs across Europe. Previous studies showing that Spanish populations are less diverse than North American populations (Aragonés et al., 2021) may now need to be re-evaluated.

In addition, our findings have increased available knowledge regarding the infection biology of *D. sapinea*. For example, our analyses suggest that shoot mortality induced by drought did not involve or benefit from the expansion of particular *D. sapinea* genotypes. The same haplotype was often found in both the symptomatic and the asymptomatic parts of the shoot. This is similar to the findings of studies involving other Botryosphaeriaceae species (Jami et al., 2022) that are also well-recognized endophytes (Slippers and Wingfield, 2007). Furthermore, the diversity of haplotypes in samples excised from asymptomatic tissues was similar to that of symptomatic tissues. However, when shoots had been damaged by a hailstorm, the same genotype usually dominated the entire symptomatic area of the shoot. Hailstorm-triggered outbreaks of *D. sapinea* blight are usually more severe than those induced by drought (Blodgett et al., 1997).

Pine cones can be heavily colonized by *D. sapinea* and they are considered to be important sources of inoculum (Feci et al., 2003; Palmer, 1987; Smith et al., 1996). In this study, cones harboured a higher diversity of *D. sapinea* isolates than other tissues, perhaps indicating that they are more susceptible to infection than shoots. Even though different cones showed infection by different genotypes, in most cases, the same pathogen individual colonized the entire cone. This could imply that cone colonization occurs either due to vegetative spread from endophytic colonies or due to infections during early stages of cone development (Bihon et al., 2011b). Nevertheless, more than one genotype was occasionally found in the same cone, this does not rule out the possibility that infections could also take place later during cone maturation.

Given that the same *D. sapinea* genotype was found infecting different cones within the same pine stand, this could support the existence of horizontal spread, i.e., asexual conidia originating from cones or shoots infecting neighbouring susceptible tissues. Although there is evidence of such horizontal spread between canopy trees and regenerating seedlings (Caballol et al., 2022a), there is no evidence that the same genotypes are present in canopy trees and in recruits. Whether present or not, local spread is probably not predominant because neither clustering by stand nor evidence of isolation by distance were detected in the present study. The results of this study suggest that long-distance dispersal of *D. sapinea* is occurring in the sampled areas, both in Spain and California. Admixture, as a result of mixing two or more populations, seemed to dominate, at least at the scale of this study. Similar results were obtained with existing SSR markers in northwest Spain (Manzanos et al., 2019) and Sweden (Brodde et al., 2019) where no structure within the different geographical locations was found. However, in these cases, the lack of structure within locations could be due to the low resolution of the markers used in these studies.

The new and, in some cases, aggressive emergence of Diplodia tip blight on *Pinus* spp. in Europe could represent one of the best examples of climate-change driven range expansion of a forest pathogen (Bosso et al., 2017; Brodde et al., 2019; Caballol et al., 2024; Ghosh et al., 2022). Our study provides a new set of SSR markers that will be useful when exploring key biological questions concerning *D. sapinea*, such as those involving biogeography, infection biology and reproduction mode, amongst others.

CRediT authorship contribution statement

Laura Vilanova: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Maria Caballol:** Writing – review & editing, Software, Formal analysis. **Ke Zhang:** Writing – review & editing, Methodology. **Åke Olson:** Writing – review & editing, Methodology, Conceptualization. **Irene Barnes:** Writing – review & editing, Resources, Methodology, Conceptualization. **Michael J. Wingfield:** Writing – review & editing, Resources, Methodology. **Jonàs Oliva:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was partly funded by CLARA grant (PID2021-127328OB-I00) from the Ministry of Science and Innovation of Spain and TOFU grant (2023 CLIMA 00109) from the Ministry of Research and Universities of Catalonia. M.C. was supported by an AGAUR FI fellowship 2021FI_B00223 from the Secretariat for Universities and Research of the Ministry of Business and Knowledge of the Government of Catalonia and the European Social Fund. We thank Francesc Serradó for support in the field work, Alexandra Ceausu for her assistance with DNA extractions and Donald Owen, previously of the California Department of Forestry and Fire Protection, for support collecting samples in California.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2024.103937.

Data availability

Data will be made available on request.

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