Comparison of Diplodia Tip Blight Pathogens in Spanish and North American Pine Ecosystems

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Abstract: Diplodia tip blight is the most ubiquitous and abundant disease in Spanish Pinus radiata plantations. The economic losses in forest stands can be very severe because of its abundance in cones and seeds together with the low genetic diversity of the host. Pinus resinosa is not genetically diverse in North America either, and Diplodia shoot blight is a common disease. Disease control may require management designs to be adapted for each region. The genetic diversity of the pathogen could be an indicator of its virulence and spreading capacity. Our objective was to understand the diversity of Diplodia spp. in Spanish plantations and to compare it with the structure of American populations to collaborate in future management guidelines. Genotypic diversity was investigated using microsatellite markers. Eight loci (SS9–SS16) were polymorphic for the 322 isolates genotyped. The results indicate that Diplodia sapinea is the most frequent Diplodia species present in plantations of the north of Spain and has high genetic diversity. The higher genetic diversity recorded in Spain in comparison to previous studies could be influenced by the intensity of the sampling and the evidence about the remarkable influence of the sample type.

Keywords: Sphaeropsis sapinea; Diplodia tip blight; dieback; SSRs; Diplodia scrobiculata

1. Introduction

Pinus radiata D. Don. is the most widespread exotic forest species susceptible to the fungal pathogen Diplodia sapinea (Fr.) Fuckel (syn. Diplodia pinea (Desm.) Kickx., Sphaeropsis sapinea (Fr.:Fr./Dyko and Sutton)) in Spain. The first record of the introduction of P. radiata D. Don in Spain is its presence in a garden in Lekeitio (Bizkaia) in the mid-19th century [1]. It was considered an appropriate candidate for forestry in Spain based on previous acclimatization studies [2]. The first plantations of this tree species in Spain were established at the end of the 19th century in the Basque Country. A policy of reforestation of public lands [3] led to an increase in the area covered by plantations, which reached...
over 160,000 ha by the 1970s [3–5]. Due to the environmental requirements of P. radiata, cold sensitivity and high humidity, its distribution within the northern Iberian Peninsula is limited mainly to the Cantabrian coast, which has an Atlantic climate, where this species is an important feature of the landscape [4,6]. It is difficult to determine the origin of P. radiata germplasm at any particular location in Spain [3]. P. radiata seeds were obtained from collections performed during the first thinnings of the Basque Country pine forests and from providers located in New Zealand, the USA, Chile, France and Denmark. However, a study of the population of P. radiata growing in Spain showed low genetic diversity [7]. The local landrace and the three Californian natural provenances (Año Nuevo, Monterey and Cambria) have been compared using genetic diversity analysis by molecular markers (RAPDs), growth and characteristics morphological and survival. The local population was found to be most similar to the Año Nuevo provenance. The Año Nuevo and the local landrace showed the lowest mortality (2.1 and 8.1%, respectively). Mortality was greater for Monterey provenance (29.3%) and particularly high for the Cambria provenance (52.6%). The differential genotype adaptation to local conditions of northern Spain and survival there may explain in part the detected low genetic diversity.

Diplodia tip blight caused by D. sapinea is the most ubiquitous and abundant disease in Spanish P. radiata plantations, which are monocultures and susceptible to various diseases. In a field survey performed in 2009 in the Basque Country [8], the incidence of Diplodia tip blight in surveyed plots was 100%, compared to 17% for pitch canker disease caused by Fusarium subglutinans f. sp. pini. The severe economic losses in forest stands of P. radiata can be attributed to both the low genetic diversity of the host [7,9] and abundance of the pathogen in cones and seeds [8,10–12] which are the major sources of inoculum in the area [13].

Similar to P. radiata in Spain, P. resinosa Ait. is one of the least genetically diverse conifer species in North America [14]. The native range of P. resinosa is a narrow latitudinal band running east–west across southeastern Canada and the northeastern USA. This range extends north to Maine (USA), southern Quebec (Canada), New Brunswick (Canada), and Nova Scotia (Canada), west to central Ontario (Canada), and south to Minnesota (USA), Wisconsin (USA), Michigan (USA), northern Pennsylvania (USA), northern New Jersey (USA), Connecticut (USA), and western Massachusetts (USA) [15]. In addition, separated patches of endemic P. resinosa also occur in Newfoundland (Canada), northern Illinois (USA), and eastern West Virginia (USA) [15]. Red pine is naturally found in pure stands or more commonly in mixtures with Pinus strobus (eastern white pine) or Pinus banksiana (jack pine) on well-drained sandy soils [15].

Red pine was widely planted in the 1930s to 1960s to stabilize abandoned agricultural lands [15]. Today, red pine is one of the most commonly planted trees in the northern USA and Canada [15]. Diplodia shoot blight, collar rot and canker are common diseases in these red pine plantations [16–22]. The persistence of D. sapinea in seed orchards and forest nurseries may have contributed to the widespread dissemination of this pathogen [13,23–25]. Diplodia sapinea was considered a variable organism, both in morphological and virulence of different strains but a recent study confirms that the European D. sapinea population is homogeneous and little differentiated except for subpopulations from Italy and Georgia [26]. Historically, three morphotypes were differentiated [27–29]. In 2003, de Wet et al. [30], however, proposed the separation of morphotypes into species. Based on comparison of multiple genes and microsatellite markers, they concluded that strains of morphotypes A and C corresponded to D. sapinea. For the morphotype B strains, they proposed a new taxon called Diplodia scrobiculata J. de Wet, Slippers & M. J. Wingf. Both species have been detected in Spain on P. radiata [31] and North America on P. resinosa [32,33].

Control of D. sapinea is complicated because it is capable of surviving on needles, branches, shoots, wood and pine cones for long periods [20,23,34–37]. It is commonly isolated from seedlings, needles, cones, branches, seed scales, seeds and pits of cones and mature wood [25,38–43]. Furthermore, D. sapinea can persist asymptomatically as a latent pathogen [10,11]. Only after trees experience a stress event such as drought, physical
damage or hail may the characteristic Diplodia tip blight symptoms develop [44–47]. In addition, its genetic diversity could be an indicator of its potential virulence and capacity to spread.

The frequent importation of *P. radiata* seeds in Spain from different suppliers of uncertain origin and the negative impact of these diseases on the productivity of our forest stands led us to carry out this study. Our objective was to understand the diversity of *Diplodia* spp. in Spanish *P. radiata* plantations and to compare the structure of American *D. sapinea* populations with populations obtained from *P. radiata* in northern Spain. Most of the North American *D. sapinea* isolates used in this study came from *P. resinosa* plantations, which are similar to *P. radiata* plantations in the low genetic variation of the hosts and potential spread of the pathogen via nursery stock. Improved knowledge about the genetic diversity and mode of reproduction in the Basque Country *D. sapinea* populations might help to design specific management options at a local scale. In addition, the potential influence of the sampling strategy (density and sample type) will be discussed.

### 2. Materials and Methods

#### 2.1. Fungal Collection and Isolation

Symptomatic (dieback) *P. radiata* trees were sampled in the major pine-growing regions of the Basque Country during spring and summer of 2016–2020. Plantations located in Laukiniz (P1), Sollano (P2), Hernani (P3), Luyando (P4) and Oiartzun (P5) were intensively sampled. Otherwise, only one strain of *Diplodia* spp. per plot was isolated. Fragments of cone scales bearing pycnidia were soaked in 30% commercial bleach (1.6% sodium hypochlorite) for 1 min and rinsed with sterile water. A single pycnidium from the cone surface was transferred to water agar medium (Panreac, Barcelona, Spain), and a single conidium was selected to initiate a monosporic culture. Single conidial isolates were grown on potato dextrose agar (Panreac, Barcelona, Spain) in petri plates in darkness at 20 ± 3 °C for 4 to 6 days. Fungal species were initially identified by colony and conidium morphology [30,48]. *Diplodia* species were confirmed by molecular methods. All isolates were maintained in the Culture Collection of the Forestry Department, Neiker, BRTA Granja Modelo Arkaute, Vitoria-Gasteiz, Spain.

Roots were carefully washed under tap water to remove any adhered soil particles. For surface disinfestation, roots were dipped into 70% EtOH for 1 min, submerged in a 30% commercial bleach with Tween 20 (1 drop/100 mL) solution for 15 min and rinsed twice in sterile distilled water. The thinnest roots (less than 1 mm diameter) were immersed in the same commercial bleach solution but for 10 min instead of 15 min. These thin surface disinfested roots were aseptically transferred to sterilized filter paper and when dried, transversally cut into 5 mm long segments and placed on PDA petri dishes. The thickest roots were first longitudinally divided into two pieces and then cut into 5 mm long pieces. In addition, branches, needles, pieces of wood from cankers and cores were sampled. Cores were collected from tree trunks with a Pressler’s 5-mm-diameter increment borer at 130 cm height [49]. Needles, fragments of branches and wood were separately collected in paper bags, and cores were introduced into sterilized tubes. All the tools in contact with the samples were disinfected before and after sampling with 70% EtOH. All the samples were labelled and stored at 4 °C. In the laboratory, the samples were immersed for 2 min in a sodium hypochlorite solution (1% active chlorine) and rinsed with sterile water. Thin disks cut from whole cross sections of the cores and branches were placed on potato dextrose agar (Panreac, Barcelona, Spain) and cultivated under the same conditions as the conidial cultures.

Three to six petri dishes per sample were used. Dishes were incubated in darkness at 25 °C and evaluated every 3 days. Putative colonies of *D. sapinea* were transferred to potato dextrose agar (PDA) dishes, which were incubated for 7 days at 25 °C, and mycelial growth characteristics were observed. Isolates were then grown on 2% water agar with sterilized pine needles at 25 °C under near-ultraviolet light (near-UV light) to induce sporulation.
North American isolates were obtained from an extensive collection at the University of Wisconsin-Madison and *P. resinosa* cones collected in New England, USA. Isolates U1–U50 were obtained as part of a study evaluating the factors and effects associated with widespread red pine mortality. *Pinus resinosa* branch and cone samples were obtained from asymptomatic trees and trees expressing crown dieback symptoms associated with *Matsucoccus matumurae* Kuwana (pine bast scale) infestation within both plantations and natural stands, the latter of which were located in Hancock County, Maine.

*Pinus resinosa* cones from New England were sampled using methods described previously [50]. Cones were bagged, placed on ice in a cooler for transportation, and stored in a freezer until processed in the laboratory. Conidia were extracted from each cone, and *Diplodia* species were identified by morphological and molecular methods described below. Isolates were sent to Neiker’s lab to implement the molecular work.

2.2. Species Identification

Morphological and molecular methods were used to identify isolates. Conidial shape, color, presence of septa, width and length were observed, as well as mycelial growth. Mycelium grown on medium in petri dishes was scraped off and collected in a 2 mL tube with five sterile tungsten carbide beads (300 µM diameter). The fungal material was disrupted using a Qiagen-Retsch MM300 Tissuelyser (Qiagen, Hilden, Germany) at a speed of 30 m/s for 3 min at room temperature. In all cases, fungal DNA was extracted from 200 µg pure monosporic cultures using a DNA Plant Mini Kit (Analytik Jena AG, Life Science). Extractions were performed following the manufacturer’s instructions. DC-PCR with species-specific primers was used to differentiate *D. sapinea* DpF (5′-CTTATATATCACAATATGCTTGA-3′) and *D. scrobiculata* DsF (5′-CTTATATATCACAATATGCTTGA-3′); a Botryosphaeria-specific primer was used as the reverse primer BotR (5′-GCTTACACTTTCATTTATAGACC-3′) [18] and was used for the identification of species. PCR amplification was performed in a total volume of 25 µL containing 1 x reaction buffer, 2 mM MgCl₂, 0.25 mM dNTPs, 0.8 µM of each specific primers, 40 ng of DNA and 1.25 U Platinum Taq polymerase (Roche Diagnostic GmbH, Mannheim, Germany). The cycling profile was as follows: denaturation at 94 °C for 60 s, followed by 35 cycles at 94 °C for 30 s, 67 °C for 30 s, and 72 °C for 30 s, and a final extension at 70 °C for 5 min. Fragment sizes were verified on 0.7% agarose gels in Tris-boric acid-EDTA buffer (TBE) with DNA loading buffer, 5× DNA (Bioline Merdidian Bioscience, London, UK).

2.3. PCR Amplification of SSR Loci and Data Analysis

Ten microsatellite loci, SS1-5-9-10-11, previously described by Burgess et al. [51], and SS12-14-15-16, described by Bihon et al. [52], were amplified for 322 *D. sapinea* isolates (Table 1). Positive controls with known DNA and negative controls without DNA were included. All SSR-PCR products were multiplexed and run in a single lane. SSR-PCR was conducted with a PCR mixture containing 1× QIAGEN® Multiplex PCR kit, 0.15 µM (each) primer, 15 ng of DNA template and water to a final volume of 13 µL. The reactions were carried out in a thermocycler (Eppendorf, Hamburg, Germany) programmed for an initial denaturation of 1 min at 95 °C, followed by 2 min at 94 °C, 15 cycles of 30 s at 58 °C, 45 s at 60 °C and 1 min at 72 °C, and 20 cycles of 55 °C. Dilution of 1:50 for the thermocycler products was conducted before multiplex analysis to avoid detection error. The forward primers were labelled with a phosphoramidite fluorescent dye indicated as FAM, NED, PET and VIC.

One µL of these multiplexed PCR products was separated on an ABI Prism 3130 Genetic Analyser (Applied Biosystems, Foster, CA, USA). The amplicon peaks were determined based on the four fluorescent dyes used and the sizes of the DNA fragments. The mobility of SSR products was compared to those of internal size standards (LIZ-500), and allele sizes were estimated by GeneMapper 4.0 computer software (Applied Biosystems, Foster, CA, USA). A reference sample was run on every gel to ensure reproducibility.
For each population defined by country of origin (the Basque Country in Spain and USA), nursery location within the Basque Country and sample type, the total number of alleles at each SSR locus was estimated. A multilocus genotype (MLG) was constructed for each isolate by combining data for single SSR alleles, and the expected multilocus genotype (eMLG) based on rarefaction was calculated using the R package poppr V.2.3.0 [53,54]. Given the clonality observed analyses were conducted for the clone-corrected dataset, with only one isolate of each MLG considered. Stoddart and Taylor’s diversity index (G) [55] and evenness index E5 [56] were calculated using the same R package.

The standardized index of association (rbarD) as an estimate of linkage disequilibrium was calculated to investigate the mode of reproduction [54,57]. The expectation of rbarD for a randomly mating population is zero, and significant deviation from this value would suggest clonal reproduction. Significance was tested based on 1000 permutations and conducted in the R package poppr using the clone-corrected data [54].

The standardized measure of genetic differentiation G’st described by Hedrick [58] was calculated to estimate subdivision among populations. This index ranges from 0 to 1, independent of the extent of population genetic variations and locus mutation rates [58]. Pairwise G’st values within the clone-corrected data were calculated using the R packages strata G V.1.0.5 [59] and mmod V.1.3.3 [60]. Statistical significance was calculated based on 1000 permutations.

Discriminant analysis of principal components (DAPC) was performed to infer clusters of populations without considering previous geographic/nursery location/isolation tissue-based assignment criteria [61]. DAPC was conducted with the R package adegenet V.2.0.1 [62] using the Bayesian information criterion (BIC) to infer the optimal number of groups. Important advantages of DAPC are that it maximizes variation between the groups, minimizes the within-group genetic variability and does not require assumptions regarding evolutionary models [61].

To assess the relationships among MLGs, minimum spanning networks (MSNs) were constructed from the clone-corrected dataset. Bruvo’s genetic distance matrix and MSNs were generated using the R package poppr V.2.3.0 [53,54]. The genetic distance described by Bruvo et al. [63] takes the SSR repeat number into account, with a distance of 0.1 equivalent to one mutational step (one repeat).

### 2.4. DNA Sequencing and Phylogenetic Analysis

Based on different MLGs, 47 *D. sapinea* isolates were selected. The internal transcribed spacer (ITS) region was amplified using the primers ITS1 and ITS4 [64], and translation elongation Factor 1-α (TEF1-α) was amplified using the primers EF1-728F and EF1-986R [65]. PCRs for each region contained 20 ng DNA, 3 µL 10× PCR Complete KCl reaction buffer (IBIAN Technologies) containing 15 mM MgCl2, 200 nM of each primer, 200 µM of each dNTP and 1 U IBIAN-Taq DNA polymerase (IBIAN Technologies, Zaragoza, Spain). The PCR profile for the ITS region was as follows: 94 °C for 10 min, 35 cycles at 94 °C for 30 s, 58 °C for 45 s, 72 °C for 60 s, and 72 °C for 10 min. For the TEF1-α region, the same PCR conditions were used, but the annealing temperature was set at 52 °C. PCR products were sequenced by Macrogen (Seoul, South Korea).

Sequence data were edited using FinchTV software version 1.4.0 (https://finchtv.software.informer.com/1.4/, accessed on 8 January 2021) and aligned, and a phylogenetic tree was constructed from the aligned sequences with MEGA X software version 10.0.4 (https://www.megasoftware.net/, accessed on 3 October 2021).

### 3. Results

#### 3.1. Species Identification

The presence of *Diplodia scrobiculata* was detected only in a single tree, from a wood core sample, of all the 253 analyzed trees in the Basque Country. *Diplodia scrobiculata* detection is considered something exceptional in this region where both *Diplodia* species
co-occurred in the same tree. Only *D. sapinea* was isolated from red pine cones collected in New England. Table 1 shows the strains identified as *D. sapinea*.

| Origin                     | Pinus Species    | Sample Type | Sample ID          | Origin                     | Pinus Species    | Sample Type | Sample ID          |
|-----------------------------|------------------|-------------|--------------------|-----------------------------|------------------|-------------|--------------------|
| Baja Deba, Spain            | Pinus pinaster   | Canker      | BC12 P1 (Laukiniz) | Pinus Radiata              | Canker          | BC120       |
| Plentzia-Mungvia, Spain     | Pinus radiata    | Root        | BC15 P1 (Laukiniz) | Pinus radiata              | Canker          | BC150       |
| Alto Deba, Spain            | Pinus radiata    | Cone        | BC14 P1 (Laukiniz) | Pinus radiata              | Canker          | BC140       |
| Estribaciones del Gorbea,   | Pinus radiata    | Cone        | BC13 P1 (Laukiniz) | Pinus radiata              | Canker          | BC130       |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC17 P1 (Laukiniz) | Pinus radiata              | Canker          | BC170       |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC18 P2 (Sollano)  | Pinus radiata              | Cone            | BC180       |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC19 P2 (Sollano)  | Pinus radiata              | Cone            | BC190       |
| Alto Deba, Spain            | Pinus radiata    | Cone        | BC39 P2 (Sollano)  | Pinus radiata              | Cone            | BC390       |
| Alto Deba, Spain            | Pinus radiata    | Cone        | BC40 P2 (Sollano)  | Pinus radiata              | Cone            | BC400       |
| Goerri, Spain               | Pinus nigra      | Cone        | BC41 P2 (Sollano)  | Pinus radiata              | Cone            | BC410       |
| Montaña Alavesa, Spain      | Pinus nigra      | Cone        | BC42 P2 (Sollano)  | Pinus radiata              | Cone            | BC420       |
| Llanada Alavesa, Spain      | Pinus radiata    | Cone        | BC44 P2 (Sollano)  | Pinus radiata              | Cone            | BC440       |
| Llanada Alavesa, Spain      | Pinus radiata    | Cone        | BC45 P2 (Sollano)  | Pinus radiata              | Cone            | BC450       |
| Gernika Bermeo, Spain       | Pinus radiata    | Cone        | BC46 P2 (Sollano)  | Pinus radiata              | Cone            | BC460       |
| Gernika Bermeo, Spain       | Pinus radiata    | Cone        | BC49 P2 (Sollano)  | Pinus radiata              | Cone            | BC490       |
| Gernika Bermeo, Spain       | Pinus radiata    | Cone        | BC50 P2 (Sollano)  | Pinus radiata              | Cone            | BC500       |
| Gernika Bermeo, Spain       | Pinus radiata    | Cone        | BC51 P2 (Sollano)  | Pinus radiata              | Cone            | BC510       |
| Gernika Bermeo, Spain       | Pinus radiata    | Cone        | BC52 P3 (Hernani)  | Pinus radiata              | Cone            | BC520       |
| Duranguesudo, Spain         | Pinus radiata    | Cone        | BC53 P3 (Hernani)  | Pinus radiata              | Cone            | BC530       |
| Markina-Ordarroa, Spain     | Pinus radiata    | Cone        | BC54 P3 (Hernani)  | Pinus radiata              | Cone            | BC540       |
| Donostia San Sebastian,     | Pinus radiata    | Cone        | BC55 P3 (Hernani)  | Pinus radiata              | Cone            | BC550       |
| Valles Alaveses, Spain      | Pinus attenuata  | Cone        | BC56 P3 (Hernani)  | Pinus radiata              | Cone            | BC560       |
| Gernika Bermeo, Spain       | Pinus radiata    | Cone        | BC57 P3 (Hernani)  | Pinus radiata              | Cone            | BC570       |
| Gernika Bermeo, Spain       | Pinus radiata    | Cone        | BC76 P3 (Hernani)  | Pinus radiata              | Cone            | BC760       |
| Tołosa, Spain               | Pinus radiata    | Cone        | BC77 P3 (Hernani)  | Pinus radiata              | Cone            | BC770       |
| Tołosa, Spain               | Pinus radiata    | Cone        | BC78 P3 (Hernani)  | Pinus radiata              | Cone            | BC780       |
| Tołosa, Spain               | Pinus radiata    | Cone        | BC79 P3 (Hernani)  | Pinus radiata              | Cone            | BC790       |
| Tołosa, Spain               | Pinus radiata    | Cone        | BC80 P3 (Hernani)  | Pinus radiata              | Cone            | BC800       |
| Tołosa, Spain               | Pinus radiata    | Cone        | BC81 P3 (Hernani)  | Pinus radiata              | Cone            | BC810       |
| Tołosa, Spain               | Pinus radiata    | Cone        | BC82 P3 (Hernani)  | Pinus radiata              | Cone            | BC820       |
| Tołosa, Spain               | Pinus radiata    | Cone        | BC83 P3 (Hernani)  | Pinus radiata              | Cone            | BC830       |
| Tołosa, Spain               | Pinus radiata    | Cone        | BC84 P3 (Hernani)  | Pinus radiata              | Cone            | BC840       |
| Tołosa, Spain               | Pinus radiata    | Cone        | BC85 P3 (Hernani)  | Pinus radiata              | Cone            | BC850       |
| Donostia San Sebastian,     | Pinus radiata    | Cone        | BC87 P4 (Luyando)  | Pinus radiata              | Root            | BC870       |
| Valles Alaveses, Spain      | Pinus radiata    | Cone        | BC90 P4 (Luyando)  | Pinus radiata              | Root            | BC900       |
| Donostia San Sebastian,     | Pinus radiata    | Cone        | BC91 P4 (Luyando)  | Pinus radiata              | Root            | BC910       |
| Donostia San Sebastian,     | Pinus radiata    | Cone        | BC92 P4 (Luyando)  | Pinus radiata              | Root            | BC920       |
| Donostia San Sebastian,     | Pinus radiata    | Cone        | BC93 P4 (Luyando)  | Pinus radiata              | Core            | BC930       |
| Donostia San Sebastian,     | Pinus radiata    | Cone        | BC94 P4 (Luyando)  | Pinus radiata              | Root            | BC940       |
| Donostia San Sebastian,     | Pinus radiata    | Cone        | BC95 P4 (Luyando)  | Pinus radiata              | Root            | BC950       |
| Urola Costa, Spain          | Pinus radiata    | Cone        | BC96 P4 (Luyando)  | Pinus radiata              | Core            | BC960       |
| Urola Costa, Spain          | Pinus radiata    | Cone        | BC97 P5 (Ozarzun)  | Pinus radiata              | Core            | BC970       |
| Urola Costa, Spain          | Pinus radiata    | Cone        | BC98 P5 (Ozarzun)  | Pinus radiata              | Core            | BC980       |
| Bajo Deba, Spain            | Pinus radiata    | Cone        | BC99 P5 (Ozarzun)  | Pinus radiata              | Core            | BC990       |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC100 P5 (Ozarzun) | Pinus radiata              | Core            | BC1000      |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC101 P5 (Ozarzun) | Pinus radiata              | Core            | BC1010      |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC102 P5 (Ozarzun) | Pinus radiata              | Core            | BC1020      |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC103 P5 (Ozarzun) | Pinus radiata              | Core            | BC1030      |
| Encartaciones,Spain         | Pinus radiata    | Cone        | BC104 P5 (Ozarzun) | Pinus radiata              | Core            | BC1040      |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC105 P5 (Ozarzun) | Pinus radiata              | Core            | BC1050      |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC106 P5 (Ozarzun) | Pinus radiata              | Core            | BC1060      |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC107 P5 (Ozarzun) | Pinus radiata              | Core            | BC1070      |
| Encartaciones, Spain        | Pinus radiata    | Cone        | BC108 P5 (Ozarzun) | Pinus radiata              | Core            | BC1080      |
| Encartaciones, Span         | Pinus radiata    | Cone        | BC109 P5 (Ozarzun) | Pinus radiata              | Root            | BC1090      |
| Estribaciones del Gorbea,   | Pinus radiata    | Cone        | BC112 P5 (Ozarzun) | Pinus radiata              | Core            | BC1120      |
| Arratia Nervion, Spain      | Pinus radiata    | Cone        | BC113 P5 (Ozarzun) | Pinus radiata              | Root            | BC1130      |
| Arratia Nervion, Spain      | Pinus radiata    | Cone        | BC114 P5 (Ozarzun) | Pinus radiata              | Root            | BC1140      |
| Cantábrica Alavesa, Spain   | Pinus radiata    | Cone        | BC115 P5 (Ozarzun) | Pinus radiata              | Root            | BC1150      |
| Cantábrica Alavesa, Spain   | Pinus radiata    | Cone        | BC116 P5 (Ozarzun) | Pinus radiata              | Root            | BC1160      |
| Valles Alaveses, Spain      | Pinus radiata    | Cone        | BC120 P5 (Ozarzun) | Pinus radiata              | Root            | BC1200      |
| Valles Alaveses, Spain      | Pinus radiata    | Cone        | BC121 P5 (Ozarzun) | Pinus radiata              | Root            | BC1210      |
Table 1. Cont.

| Origin                      | Host Species | Sample Type | Sample ID   | Origin                     | Host Species | Sample Type | Sample ID   |
|-----------------------------|--------------|-------------|-------------|----------------------------|--------------|-------------|-------------|
| Llanada Alavesa, Spain      | Pinus radiata| Cone        | BC129       | Itasca St. Park, Minnesota USA | Pinus resinosa| Unknown     | W192        |
| Spain                       | Pinus nigra  | Cone        | BC131       | Dallas County, Texas USA     | Pinus eldarica| Cone        | W194        |
| Llanada Alavesa, Spain      | Pinus nigra  | Cone        | BC132       | Georgia USA                 | Pinus taeda  | Needle      | W206        |
| Llanada Alavesa, Spain      | Pinus radiata| Cone        | BC133       | Wisconsin USA               | Pinus banksiana| Needle     | W210        |
| Llanada Alavesa, Spain      | Pinus radiata| Cone        | BC134       | Wisconsin USA               | Pinus banksiana| Needle     | W211        |
| Markina-Ondarroa, Spain     | Pinus radiata| Cone        | BC135       | Waukesha County, Wisconsin USA| Pinus resinosa| Needle      | W212        |
| Llanada Alavesa, Spain      | Pinus radiata| Cone        | BC137       | Portage County, Wisconsin USA| Pinus banksiana| Needle     | W213        |
| Llanada Alavesa, Spain      | Pinus radiata| Cone        | BC138       | Portage County, Wisconsin USA| Pinus resinosa| Needle      | W214        |
| Montaña Alavesa, Spain      | Pinus radiata| Cone        | BC141       | Wood County, Wisconsin USA   | Pinus resinosa| Needle      | W215        |
| Alto Deba, Spain            | Pinus radiata| Cone        | BC142       | Adams County, Wisconsin USA  | Pinus resinosa| Needle      | W216        |
| Bajo Deba, Spain            | Pinus radiata| Cone        | BC143       | Marathon County, Wisconsin USA| Pinus resinosa| Needle      | W217        |
| Bajo Deba, Spain            | Pinus radiata| Cone        | BC144       | Wallowa County, Oregon USA   | Pinus ponderosa| Cone        | W218        |
| Bajo Deba, Spain            | Pinus radiata| Cone        | BC145       | Bennington County, Vermont USA| Pinus ponderosa| Needle     | W219        |
| Markina Ondarroa, Spain     | Pinus radiata| Cone        | BC146       | Stem tip                    | Pinus resinosa| Stem        | W220        |
| Estribaciones del Gorbea, Spain | Pinus radiata| Cone        | BC147       | Sawyer County, Wisconsin USA| Pinus banksiana| Stem        | W221        |
| Aratta Nervión, Spain       | Pinus radiata| Cone        | BC148       | Wood County, Wisconsin USA   | Pinus banksiana| Stem        | W222        |
| Aratta Nervión, Spain       | Pinus radiata| Cone        | BC149       | Vilas County, Wisconsin USA  | Pinus resinosa| Unknown     | W223        |
| Encartaciones, Spain        | Pinus radiata| Cone        | BC150       | South Dakota                | Pinus ponderosa| Unknown     | W224        |
| Encartaciones, Spain        | Pinus radiata| Cone        | BC151       | Dallas County, Texas USA     | Pinus nigra  | Cone        | W225        |
| Encartaciones, Spain        | Pinus radiata| Cone        | BC152       | Bayfield County, Wisconsin USA| Pinus resinosa| Needle      | W226        |
| Duranguesado, Spain         | Pinus radiata| Cone        | BC154       | Sumter County, Alabama USA   | Pinus taeda  | Cone        | W227        |
| Gernika Bermeo, Spain       | Pinus radiata| Cone        | BC155       | Adams County, Wisconsin USA  | Pinus sylvestris| Bark        | W228        |
| Gernika Bermeo, Spain       | Pinus radiata| Cone        | BC156       | Vilas County, Wisconsin USA  | Pinus ponderosa| Unknown     | W229        |
| Markina-Ondarroa, Spain     | Pinus radiata| Cone        | BC157       | Vilas County, Wisconsin USA  | Pinus resinosa| Unknown     | W231        |
| Markina-Ondarroa, Spain     | Pinus radiata| Cone        | BC158       | Pine County, Minnesota USA   | Pinus resinosa| Unknown     | W232        |
| Duranguesado, Spain         | Pinus radiata| Cone        | BC159       | Morgantown, WV              | Pinus nigra  | Needle      | W233        |
| Gernika Bermeo, Spain       | Pinus radiata| Cone        | BC160       | Jackson County, Wisconsin USA| Pinus resinosa| Needle      | W234        |
| Spain                       | Pinus radiata| Cone        | BC161       | Dane County, Wisconsin USA   | Pinus nigra  | Needle      | W235        |
| Plentzia-Munguia, Spain     | Pinus radiata| Cone        | BC162       | Northern Highland American Legion State Forest, Wisconsin USA | Pinus banksiana| Twig        | W236        |
| Goierrí, Spain              | Pinus radiata| Cone        | BC163       | Marquette County, Wisconsin USA| Pinus resinosa| Needle      | W238        |
| Tolosa, Spain               | Pinus radiata| Cone        | BC164       | Trempealeau County, Wisconsin USA| Pinus resinosa| Needle      | W239        |
| Tolosa, Spain               | Pinus radiata| Cone        | BC165       | Fairfield County, Connecticut USA| Pinus sylvestris| Needle     | W240        |
| Urola Costa, Spain          | Pinus radiata| Cone        | BC166       | Adair County, Iowa USA       | Pinus nigra  | Needle      | W241        |
| Markina-Ondarroa, Spain     | Pinus radiata| Cone        | BC169       | Codington County, South Dakota USA| Pinus sylvestris| Needle     | W242        |
| Cantàbrica Alavesa, Spain   | Pinus radiata| Cone        | BC196       | Polk County, Iowa USA        | Pinus nigra  | Needle      | W243        |
| Aratta Nervión, Spain       | Pinus radiata| Cone        | BC197       | Lacrosse County, Wisconsin USA| Pinus resinosa| Needle      | W244        |
| Aratta Nervión, Spain       | Pinus radiata| Cone        | BC198       | Wood County, Wisconsin USA   | Pinus resinosa| Stem        | W245        |
| Aratta Nervión, Spain       | Pinus radiata| Cone        | BC199       | Centre County, Pennsylvania USA| Pinus nigra  | Needle      | W246        |
| Aratta Nervión, Spain       | Pinus radiata| Cone        | BC200       | Upshur County, West Virginia USA| Pinus sylvestris| Needle     | W247        |
| Duranguesado, Spain         | Pinus radiata| Cone        | BC201       | Lafayette County, Wisconsin USA| Pinus resinosa| Needle      | W248        |
| Gran Bilbao, Spain          | Pinus radiata| Cone        | BC202       | Monroe County, Wisconsin USA  | Pinus banksiana| Needle      | W250        |
| Origin          | Host Species | Sample Type | Sample ID | Origin                        | Host Species | Sample Type | Sample ID |
|-----------------|--------------|-------------|-----------|-------------------------------|--------------|-------------|-----------|
| Plentzia-Munguia, Spain | Pinus radiata | Core        | BC203     | Cheboygan County, Michigan USA | Pinus banksiana | Needle      | W251      |
| Plentzia-Munguia, Spain | Pinus radiata | Stem        | BC208     | Manistee National Forest, Michigan USA | Pinus resinosa | Unknown     | W252      |
| Plentzia-Munguia, Spain | Pinus radiata | Stem        | BC209     | Jefferson County, West Virginia USA | Pinus nigra | Needle      | W253      |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC2       | Stanislaus National Forest, California USA | Pinus ponderosa | Unknown     | W254      |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC3       | Morgan County, West Virginia USA | Pinus syleestris | Needle     | W255      |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC4       | Marion County, Indiana USA | Pinus nigra | Cone        | W256      |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC5       | Foxborough, Massachusetts, USA | Pinus resinosa | Cone       | U1        |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC6       | Washington, Vermont, USA | Pinus resinosa | Cone       | U2        |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC7       | Hancock County, Maine, USA | Pinus resinosa (natural) | Cone       | U3        |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC8       | Hancock County, Maine, USA | Pinus resinosa (natural) | Cone       | U4        |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC9       | Andover, Massachusetts, USA | Pinus resinosa | Cone       | U5        |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC10      | Hancock County, Maine, USA | Pinus resinosa (natural) | Cone       | U6        |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC11      | Washington, Vermont, USA | Pinus resinosa | Cone       | U7        |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC12      | Andover, Massachusetts, USA | Pinus resinosa | Cone       | U8        |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC13      | Foxborough, Massachusetts, USA | Pinus resinosa | Cone       | U9        |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC14      | Shrewsbury, Vermont, USA | Pinus resinosa | Cone       | U10       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC15      | Hancock County, Maine, USA | Pinus resinosa (natural) | Cone       | U11       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC16      | Shrewsbury, Vermont, USA | Pinus resinosa (natural) | Cone       | U12       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC17      | Hancock County, Maine, USA | Pinus resinosa (natural) | Cone       | U13       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC18      | Shrewsbury, Vermont, USA | Pinus resinosa (natural) | Cone       | U14       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC19      | Hudson, Massachusetts, USA | Pinus resinosa | Cone       | U15       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC20      | Hudson, Massachusetts, USA | Pinus resinosa | Cone       | U16       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC21      | Shrewsbury, Vermont, USA | Pinus resinosa | Cone       | U17       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC22      | Hancock County, Maine, USA | Pinus resinosa | Cone       | U18       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC23      | Hancock County, Maine, USA | Pinus resinosa | Cone       | U19       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC24      | Shrewsbury, Vermont, USA | Pinus resinosa | Cone       | U20       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC25      | Hudson, Massachusetts, USA | Pinus resinosa | Cone       | U21       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC26      | Washington, Vermont, USA | Pinus resinosa | Cone       | U22       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC27      | Foxborough, Massachusetts, USA | Pinus resinosa | Cone       | U23       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC28      | Hudson, Massachusetts, USA | Pinus resinosa | Cone       | U24       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC29      | Hudson, Massachusetts, USA | Pinus resinosa | Cone       | U25       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC30      | Washington, Vermont, USA | Pinus resinosa | Cone       | U26       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC31      | Andover, Massachusetts, USA | Pinus resinosa | Cone       | U27       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC32      | Washington, Vermont, USA | Pinus resinosa | Cone       | U28       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC33      | Foxborough, Massachusetts, USA | Pinus resinosa | Cone       | U29       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC34      | Washington, Vermont, USA | Pinus resinosa | Cone       | U30       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC35      | Hancock County, Maine, USA | Pinus resinosa | Cone       | U31       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC36      | Hudson, Massachusetts, USA | Pinus resinosa | Cone       | U32       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC37      | Hancock County, Maine, USA | Pinus resinosa (natural) | Cone       | U33       |
| P1 (Laukiniz), Spain | Pinus radiata | Core        | BC38      | Andover, Massachusetts, USA | Pinus resinosa | Cone       | U34       |
Table 1. Cont.

| Origin       | Host Species | Sample Type | Sample ID | Origin       | Host Species         | Sample Type | Sample ID |
|--------------|--------------|-------------|-----------|--------------|----------------------|-------------|-----------|
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM172    | Hancock County, Maine, USA | Pinus resinosa | Cone        | U35       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM174    | Andover, Massachusetts, USA | Pinus resinosa | Cone        | U36       |
| P1 (Laukiniz), Spain | Pinus radiata | Root        | BCM182    | Hancock County, Maine, USA | Pinus resinosa | Cone        | U37       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM184    | Hancock County, Maine, USA | Pinus resinosa | Cone        | U38       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM185    | Foxborough, Massachusetts, USA | Pinus resinosa | Cone        | U39       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM186    | Andover, Massachusetts, USA | Pinus resinosa | Cone        | U40       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM188    | Washington, Vermont, USA | Pinus resinosa | Cone        | U41       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM189    | Washington, Vermont, USA | Pinus resinosa | Cone        | U42       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM190    | Hancock County, Maine, USA | Pinus resinosa | Cone        | U43       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM191    | Hancock County, Maine, USA | Pinus resinosa | Cone        | U44       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM192    | Washington, Vermont, USA | Pinus resinosa | Cone        | U45       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM193    | Hudson, Massachusetts, USA | Pinus resinosa | Cone        | U46       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM194    | Washington, Vermont, USA | Pinus resinosa | Cone        | U47       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM195    | Washington, Vermont, USA | Pinus resinosa | Cone        | U48       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM196    | Shrewsbury, Vermont, USA | Pinus resinosa | Cone        | U49       |
| P1 (Laukiniz), Spain | Pinus radiata | Canker      | BCM197    | Shrewsbury, Vermont, USA | Pinus resinosa | Cone        | U50       |

3.2. PCR Amplification of SSR Loci and Data Analysis

All primer pairs evaluated successfully amplified SSR loci for *D. sapinea* from the Basque Country and the USA. Eight loci (SS9, SS10, SS11, SS12, SS13, SS14, SS15 and SS16) were polymorphic for the 322 isolates genotyped. The number of observed alleles per locus ranged from two to nine (Table 1), resulting in a total of 48 MLGs (Table 2). The Basque Country population exhibited 19 MLGs, the USA population exhibited 34 MLGs, and both populations shared five MLGs (Figure 1). A clone correction of the dataset was performed to remove the bias of resampled MLG in the analysis, resulting in a total of 53 representative isolates.

Table 2. Genetic diversity and linkage disequilibrium among loci based on the standardized index of association (rbarD) of *Diplodia sapinea* populations defined by country of origin, by nursery location in the Basque Country and by sample type.

| Parameters | Country | Nursery Location in the Basque Country | Sample Type |
|------------|---------|---------------------------------------|-------------|
|            | Spain (Basque Country) | USA | Laukiniz | Sollano | Hernani | Luyando | Oiarztun | Canker | Root | Cone | Core |
| Sample size (N) | 216 | 106 | 58 | 14 | 16 | 9 | 12 | 28 | 13 | 145 | 30 |
| MLG/Diversity (G) | 19 | 34 | 6 | 4 | 8 | 4 | 4 | 5 | 5 | 19 | 4 |
| eMLG | 19 | 19 | 6 | 4 | 8 | 4 | 4 | 5 | 5 | 10 | 4 |
| Evenness (E) | 2.94 | 3.53 | 1.79 | 1.39 | 2.08 | 1.39 | 1.39 | 1.61 | 1.61 | 2.94 | 1.39 |
| Diversity (H) | −0.074 | 0.087 | −0.080 | −0.316 | −0.066 | −0.115 | −0.333 | −0.056 | −0.194 | −0.074 | −0.115 |
| p-value | 0.999 | 0.001 |

*a* The set of nonredundant indices of genotypic diversity recommended by Arnaud-Haond et al. [66] was calculated for each population clone-corrected dataset. b MLG, number of multilocus genotypes observed; G, Stoddart and Taylor’s diversity [55] genotypic diversity; eMLG, expected multilocus genotypes based on rarefaction; E, evenness index adapted from Simpson diversity; H, Shannon-Weiner diversity index [67]; rbarD, standardized index of association; p-value for rbarD. c Sample size before clone correction.
Figure 1. Minimum spanning network from the clone-corrected data showing the relationships among the individual multilocus genotypes (MLGs) found among the populations defined by country of origin (the Basque Country in Spain and USA). Each node represents a different MLG. The distances and thicknesses of the lines between nodes are proportional to Bruvo’s distance [63]. Node colors and sizes correspond to the population studied and the number of individuals, respectively.

The USA population showed higher genetic diversity ($G = 34$) than the Basque Country population ($G = 19$). Similarly, the Shannon-Weiner diversity index ($H$) for the USA population was higher (3.53) than that observed for the Basque Country population (2.94) (Table 2). When considering the population defined by the nursery location within the Basque Country, Laukiniz and Hernani showed the highest genetic diversity based on values of $G$ (six and eight, respectively), evenness (0.427 and 0.407, respectively) and $H$ (1.79 and 2.08, respectively) (Table 2). Among the populations defined by sample type within the Basque Country, the cone population showed the highest genetic diversity based on the same indices (Table 2). The Basque Country population showed no significant deviation in the $r_{bar}D$ value from the null hypothesis of recombination, supporting sexual reproduction ($r_{bar}D = -0.0739; p = 0.999$).

Pairwise $G^{\prime}st$ values calculated on the clone-corrected data showed very low genetic differentiation among Basque Country and USA populations ($G^{\prime}st = 0.161; p > 0.01$). In general, low genetic differentiation was also observed among populations defined by nursery location within the Basque Country or by sample type. $G^{\prime}st$ values were above 0.04 when the Laukiniz population was compared with Sollano and Oiartzun (0.1303 and 0.0809, respectively). The results of the population subdivision analysis based on $G^{\prime}st$ were consistent with those obtained by AMOVA. Analysis of molecular variance on the clone-corrected data revealed only 9.5% variation between nursery populations ($p = 0.024$).
None of the calculated values were statistically significant, showing that further sampling is likely needed.

In the Basque Country, 11 out of the 19 MLGs identified were present in the populations defined by nursery location, and three MLGs were shared among all populations (Figure 2A). The Sollano population showed one exclusive MLG, while the Laukiniz population showed two exclusive MLGs and one shared with the Hernani population. Luyando and Oiartzun populations also showed one MLG shared with the Hernani population. This last population also showed two exclusive MLGs.

![Minimum spanning network from the clone-corrected data showing the relationships among the individual multilocus genotypes (MLGs) found among populations defined by A. geographical location of the nurseries within the Basque Country and B. type of material. Each node represents a different MLG. The distances and thicknesses of the lines between nodes are proportional to Bruvo’s distance [63]. Node colors and sizes correspond to the population studied and the number of individuals, respectively.](image)

**Figure 2.** Minimum spanning network from the clone-corrected data showing the relationships among the individual multilocus genotypes (MLGs) found among populations defined by A. geographical location of the nurseries within the Basque Country and B. type of material. Each node represents a different MLG. The distances and thicknesses of the lines between nodes are proportional to Bruvo’s distance [63]. Node colors and sizes correspond to the population studied and the number of individuals, respectively.

For populations in the Basque Country defined by sample type (canker, root, cone and core), among the 19 MLGs, only three were shared by all populations (Figure 2B). The canker population showed one exclusive MLG and one shared with the cone population. The root population showed an MLG shared with the cone population and an MLG shared with the core population. Finally, the cone population showed 12 exclusive MLGs. Of all the samples analyzed, a higher isolation of *D. sapinea* strains and density of pycnidia were always observed in the samples obtained from cone scales.

3.3. DNA Sequencing and Phylogenetic Analysis

Spanish *D. sapinea* isolates belong to one ITS (531 aligned nucleotides) and one TEF1-α (408 aligned nucleotides) haplotype that was equivalent to *D. sapinea* CAA892; Portugal [68].

4. Discussion

*Diplodia sapinea* is, by far, the most frequent *Diplodia* species present in *P. radiata* stands in northern Spain. The presence of *D. scrobiculata*, confirmed by morphological and molecular methods, has been reported in only a single tree of all the analyzed samples. This is consistent with what was described by Burgess et al. [69], in which *D. scrobiculata* (formerly known as the B morphotype of *D. pinea*) was considered to have a much more limited distribution in the USA and Mexico, where it was found to coexist with *D. sapinea*. *D. scrobiculata* has only been reported sporadically in Europe in Mediterranean areas [27,70]. It was only detected on *P. radiata* in Corsica, France [12] and Bizkaia, Spain [31]. Although the distribution of *D. scrobiculata* in Spain detected in this study was extremely limited, the hypothesis that the replacement of this species by the more aggressive *D. sapinea* seems unlikely. Inoculation trials have shown that some of the *D. scrobiculata* isolates were as virulent as those of *D. sapinea* on *P. radiata* and *Pinus elliottii* Engelm [31,52]. Other studies
have shown that D. sapinea isolates were more aggressive than D. scrobiculata isolates to young P. resinosa, P. banksiana, Pinus sylvestris L., Pinus mugo Turra, Picea pungens Engelm, Pseudotsuga menziesii (Mirb.) Franco, and Abies balsamea (L.) Mill. trees in a greenhouse setting [33,71].

Diplodia sapinea is also the most frequently encountered Diplodia species in samples collected from P. resinosa in the northeastern USA. This result is consistent with predominance of D. sapinea in North America. For example, in previous studies in Wisconsin, most cones and asymptomatic shoots from which Diplodia species were identified, were positive for D. sapinea, with only <13% of P. resinosa cones collected from the canopy and asymptomatic shoots were positive for D. scrobiculata [50,72,73]. Red pine cones collected in New England for the current study yielded only D. sapinea isolates. Both studied Diplodia species may have a preference in host range, as D. scrobiculata is more frequently isolated from P. banksiana than P. resinosa, whereas D. sapinea is more frequently isolated from P. resinosa than from P. banksiana cones from mature trees [72].

Expansion of the know range of D. sapinea to the northern regions in Europe is well documented. It has recently been detected in relatively cold areas such as Estonia [74], Sweden [75], Finland [76] and northwestern Russia [77]. In the current study, D. sapinea was isolated from wood, roots and mainly from cones of P. radiata, in which the fruiting bodies of this species are found more easily and abundantly than in the rest of the tree parts. The fungus is commonly found as a saprophyte in cone bracts [78–80]. In Finland, the frequency of cones with pycnidia of D. sapinea varied from 1% to 12% in the infested P. sylvestris stands [76]. No symptoms of Diplodia tip blight or resinous cankers were detected in trees in the Finnish stands where the cones were collected. Since fruiting bodies on cones are easily seen and cones can be collected from the soil without having to climb the tree, it is an effective source of fungal material. In some studies, the frequency of cone colonization by D. sapinea was considered a measure of the level of pathogen presence in the stands [12,50]. However, this type of extrapolation is susceptible to errors derived from a sampling bias [81].

The genetic diversity, population structure and mode of reproduction of D. sapinea were assessed using previously developed microsatellite markers. Analyzed populations were defined based on the country of origin (the Basque Country in Spain and USA), sampling intensity stands within the Basque Country and sample type. The population from the USA showed a higher number of genotypes compared to those in Spain, as expected from a well-established pathogen in a country, taking into account that USA isolates came from a wider area and different pine species. However, the genetic diversity observed in the Basque Country was high relative to the low variability found in previous studies in Spain [82]. An important difference between the previous and the present study is the sampling that was intensively performed within Basque Country plantations. Nevertheless, this result contrasts with other studies of the fungus showing that genetic diversity among D. sapinea populations is low at the global scale [27,83,84]. This contradiction may be due to methodological differences. The low genetic diversity has been explained based on the success of some genotypes as endophytes. The close association of D. pinea as an endophyte with pines suggests the ability to overcome pressure regardless of the external environmental conditions [84]. This fact coupled with no evidence of sexual reproduction would explain the clonality found within global populations of D. pinea, although recent studies showed a cryptic sexual stage in this species [82].

Regarding the pathogen mode of reproduction in Spain, linkage disequilibrium analysis showed evidence of recombination in the D. sapinea population of the Basque Country. This result contrasts with the idea of predominantly asexual reproduction of the pathogen in Europe based on the clonal structure found across the continent [82,83].

When considering the population based on the geographical location of the nurseries within the Basque Country, Laukiniz and Hernani showed the highest genetic diversity based on the estimated parameters. Among intensively sampled locations, the highest diversity was expected to be detected in Laukiniz since this location was a nursery that
received \textit{P. radiata} material, mainly seeds, from different countries and distributed the material through different surrounding locations. This fact is supported by the population subdivision results showing an overall lack of structure based on geographic location within the Basque Country. However, measures of genetic differentiation showed higher values in pairwise comparisons between Laukiniz and Sollano and Laukiniz and Oiartzun. Subdivision among Laukiniz and other populations within the Basque Country might be explained by the fact that the main sources of \textit{P. radiata} seeds in this location were imported from countries such as the USA, Chile and New Zealand, and seeds are a common disease propagation system for this species.

Among the populations defined by sample type within the Basque Country, the cone population showed the highest genetic diversity based on the estimated parameters. These results are in accordance with the success and stability of \textit{D. sapinea} as an endophyte in healthy trees [51]. From the results obtained in this study, it can be inferred that the plant material should be considered for the sampling strategy. Sampling carried out based on exclusively cones could give problems of biasing the results since the population structure may differ depending on the type of sample. Despite the fact that in certain situations, global scaling could potentially provide better results than local scaling [85], this study once again emphasizes the importance of enhancing hierarchical studies, including intensive sampling at the local scale and matching scales of disease monitoring with scales of management systems, avoiding scale discordance. Forest managers are affected by forest policies formulated at the subnational, national, regional and global levels. National climate change policies are influenced by global factors and regional policies but adapted to local circumstances [FAO, http://www.fao.org/3/i3383e/i3383e.pdf, accessed on 10 October 2021]. Forest managers should be aware of forest ecosystem issues at global and local scales that will affect them directly or indirectly, such as the disease impact, epidemiology and genetic diversity of \textit{D. sapinea}, which will lead to disease outbreaks when trees are physiologically stressed [52]. Disease management should focus on reducing inoculum pressure (inoculum present in pruning and felling remains, pine cones, etc. colonized by \textit{D. sapinea}) and new sources of entry of genetic diversity of the pathogen.

Pathogens impose strong selective pressures on their hosts. The way in which fungal populations respond to adverse conditions, such as those generated by control strategies, determines the risk of management failure due to pathogen adaptation conditioned by the type of genetic variability available.

Changes in weather and climate may have implications for development of Diplodia tip blight. In northern Spain, spring temperatures have shown an increase over recent decades [86]. According to the Intergovernmental Panel on Climate Change (IPCC), climate change could increase average temperatures by 2–4 °C in Europe over the next 50 years and cause considerable changes in regional and seasonal patterns of precipitation. This will alter the environmental conditions to which forest trees in Europe are adapted and expose them to new and old diseases. The increased temperature could also increase trees’ susceptibility to disease due to increased exposure to drought. Generation of disease databases at local and global scales in regional climate change scenarios is one of the fundamental starting points in assessing impacts, vulnerability and future needs with respect to adaptation to Diplodia tip blight forest outbreaks [16,44,82,87].

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