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Distribution and Characterization of *Armillaria* Complex in Atlantic Forest Ecosystems of Spain

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Abstract: *Armillaria* root disease is a significant forest health concern in the Atlantic forest ecosystems in Spain. The damage occurs in conifers and hardwoods, causing especially high mortality in young trees in both native forests and plantations. In the present study, the distribution of *Armillaria* root disease in the forests and plantations of the Basque Country is reported. *Armillaria* spp. were more frequently isolated from stands with slopes of 20–30% and west orientation, acid soils with high permeability, deciduous hosts, and a rainfall average above 1800 mm. In a large-scale survey, 35% of the stands presented *Armillaria* structures and showed disease symptoms. Of the isolated *Armillaria* samples, 60% were identified using molecular methods as *A. ostoyae*, 24% as *A. mellea*, 14% as *A. gallica*, 1% as *A. tabescens*, and 1% as *A. cepistipes*. In a small scale sampling, population diversity was defined by somatic compatibility tests and Universally Primed-PCR technique. Finally, the pathogenicity of *A. mellea,* the species with the broadest host range, was determined on different tree species present in the Atlantic area of Spain in order to determine their resistance levels to *Armillaria* disease. A significant difference in disease severity was observed among tree species ($p < 0.001$), with *Pinus radiata* being the most susceptible tree species and *Cryptomeria japonica* the most resistant to *A. mellea.*

Keywords: *Armillaria* spp.; distribution; diversity; environmental factors; host susceptibility; UP-PCR

1. Introduction

About 40 species of *Armillaria* are known worldwide [1]. Seven are present in Europe: *A. mellea* (Vahl) P.Kumm., *A. gallica* Marxm. & Romagn., *A. ostoyae* (Romagn.) Herink, *A. tabescens* (Scop.) Emel, *A. cepistipes* Velen., *A. borealis* Marxm. & Korhonen, and *A. ectypa* (Fr.) Lamoure [2,3]. Many of these are pathogens that cause root and butt rot disease on a broad range of trees, shrubs and some herbaceous plants [4]. This disease is characterized by some general symptoms such as chlorotic leaves, progressive thinning of the crown, slower leader growth, and excess cone production. Evidence of infection may also include subcortical white mycelial fans, clusters of golden-brownish mushrooms near the tree base, rhizomorphs, rotten stringy-yellow wood with black lines (pseudosclerotia), rapid tree death without the loss of foliage, and/or basal resin or gum exudates [5,6].

In general, conifers seem to be more susceptible to infection than hardwoods [7], although susceptibility depends on the specific *Armillaria* species involved [2,8]. The extent of damage caused by *Armillaria* is variable and is determined by factors such as the fungal species, the vigor of the host, interaction with other diseases, soil properties, climate, plantation management, and previous land uses, among others [9–14]. Therefore, forest susceptibility cannot be generalized [15]. The composition of the stand can also influence the range of infection; lower density of susceptible tree

species and higher species diversity in different forest strata reduce the possibility of disease transmission [16,17]. Management procedures such as selective logging, early thinning, and the continued planting of susceptible or moderately susceptible species that are not very well adapted to the location increase the inoculum sources and the potential for spread of infection [14,18–20]. Thus, in comparison with natural forests the damage in exotic tree plantations is usually greater [8].

In field conditions, *Armillaria* can colonize different hosts by direct contact between an infected source and roots by way of hyphae, or by advancing through the ground from an infection point by way of rhizomorphs [21]. This is a short distance spreading mechanism but it is considered the most important even if, for some *Armillaria* species, formation of rhizomorphs in the field is not common [3,15]. The capacity to create new infection points by releasing basidiospores varies from one species to another [22] but, in general, this seems to be the least frequent source of infection [23].

The genotypic diversity within a population is an important parameter for the determination of the epidemiology of a disease. Many different small somatic compatibility (SC) groups in a stand imply that spread is predominantly by basidiospores, and a single extensive SC group implies spread by vegetative mycelium. By means of SC tests, *Armillaria* population structure in the field can be determined [24]. Even though SC tests are usually reliable, sometimes they do not differentiate among closely related individuals of *Armillaria* [2]. Molecular techniques such as Universally primed-PCR (UP-PCR) [25] could provide more information in these cases. UP-PCR has been used for the characterization of fungal populations at interspecies and/or intraspecies level [26–28]. In this technique, the entire genome of an organism is targeted with a single primer or a combination of primers that will anneal to multiple regions resulting in a multiband profile which differs among different genotypes. Universal primers consist of a stable minisatellite-like region, with high GC content, designed using as a template genome regions of different organisms, which allows primer annealing at high temperatures resulting in reproducible PCR product patterns, and a variable, randomly designed region. Highly variable intergenic regions are usually targeted which enables differentiation between closely related isolates [29].

Armillaria spp., including the primary parasites *A. ostoyae* and *A. mellea*, have been previously reported in Northern Spain [30–32] including the Basque Country [33]. The main tree species in Basque Country plantations is *Pinus radiata* D. Don. (covering an area of 132,084 ha), followed by *Eucalyptus* spp. (15,197 ha), *P. nigra* Arn. (13,701 ha), *Larix* sp. (8011 ha), *P. pinaster* Ait. (7238 ha), *Pseudotsuga menziessi* Franco (6537 ha), *Chamaecyparis lawsoniana* Parl. (3414 ha), *Quercus rubra* L. (3328 ha) and *Picea abies (*L.*)* H. Karst. (525 ha). The main native forest species are *Fagus sylvatica* L. (53,835 ha), *Q. faginea* Lam. (26,652 ha), *Q. ilex* L. (26,152 ha), *Q. robur* L. and *Q. petraea (*Matt.) Liebl. association (16469 ha), and *Q. pyrenaica* Willd. (13,039 ha) [34]. All of these tree species have been reported to be susceptible to different *Armillaria* spp. in the literature, however, *Armillaria* spp. distribution, species diversity, and dispersal mechanisms are not known in the Basque Country, where the wood industry is valued at 1 billion euros per year [35]. Thus, the objectives of this study were to determine: (i) the *Armillaria* species distribution and population diversity in this region; (ii) factors affecting *Armillaria* spp. Distribution; and (iii) host susceptibility. This will contribute to a better understanding of the impact of *Armillaria* spp. and establishment of management practices.

2. Materials and Methods

2.1. Collection of Fungi

Fungal samples and data related to forest characteristics were collected from native forests and plantations of the Basque Country, northern Spain, focusing on trees in pockets of mortality and decayed trees that displayed symptoms resembling those of root rot diseases. For the first sample set (Set 1), the stands were surveyed by systematic random sampling in which samples were collected systematically from randomly chosen focal points [36]. A total of 709 foci of tree mortality were examined for the presence of *Armillaria* spp., and fungal samples and ecosystem characteristics related to infection were collected. The second sampling (Set 2) was carried out to determine the genetic diversity and population structure patterns of the fungus within a plot, and consisted of a

detailed survey of three specific areas of approximately 500 × 500 m with high presence of *Armillaria* spp., located in Otxandiano (Vizcaya), Amunategi (Vizcaya) and Altube (Álava) (Figure 1). In both sample sets, all the surveyed points, including sites of fungus collection, were georeferenced using an Oregon 300 Garmin GPS.

Samples of *Armillaria* spp. basidiocarps, rhizomorphs and mycelia were collected and placed in separate polyethene bags, transported to the laboratory, and stored at 4 °C. Fungi were cultured on benomyl-dichloran-streptomycin agar (BDS) [37] and grown at 20 °C in the dark. Pure cultures were obtained, and routinely grown on malt extract agar (MEA) (PanReac Química, Barcelona, Spain). For preservation of the pure cultures, mycelial fragments were placed in 50% glycerol and, after incubating at 4 °C for 24 h, maintained at −20 °C [38].

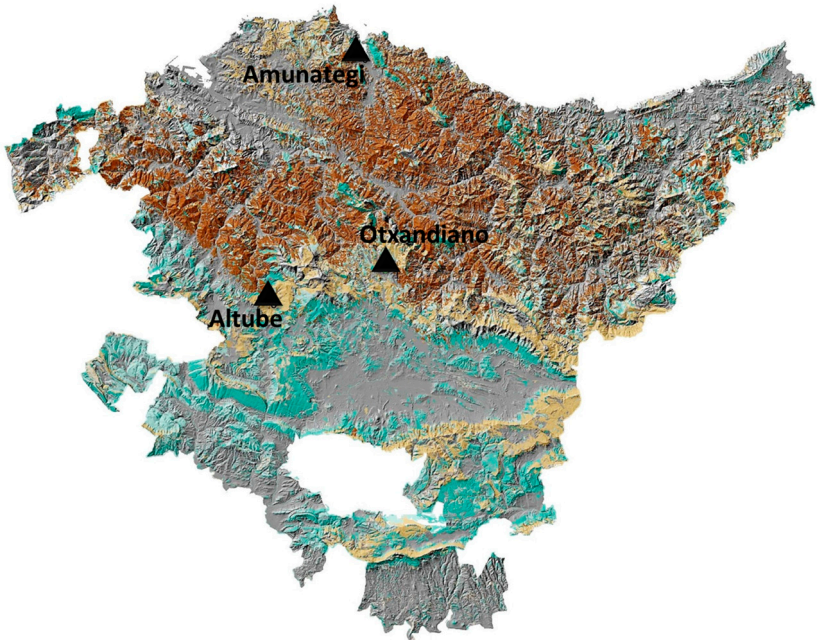


Figure 1. Location of Amunategi, Altube, and Otxandiano stands.

2.2. Description of Armillaria-Infected Ecosystems

To determine environmental factors of *Armillaria* spp. habitats, a dataset of the environmental variables of the studied ecosystems was constructed based on information supplied by the Environment Department of the Basque Government (http://www.ingurumena.ejgv.euskadi.eus/r49-579/es//publicaciones_c.htm). The variables compiled were stand slope, stand orientation, soil pH, soil permeability, average rainfall, average temperature, tree types, and host optimal conditions. Variables were categorized as shown below. *Armillaria* spp. presence was coded as a binomial variable being 0 for absence and 1 for presence.

As a preliminary exploratory analysis, multiple correspondence analysis (MCA) was applied to represent the relationships among the categorical variables, including *Armillaria* spp. presence. MCA projects the variables in a reduced space, facilitating visual interpretation of large datasets. This analysis converts a matrix of data into a graphical display known as factor planes. The rows and columns of the matrix are plotted as points in the factor planes and allow a geometrical representation of the information [39].

This procedure was complemented with contingency tables testing separately each categorical variable including its categories against *Armillaria* presence or absence. Pearson's chi square test was used to determine the independence between row and column variables, i.e., to determine if *Armillaria* spp. were more frequently detected than expected by chance in certain categories. For calculating the strength of association between categorical variables Cramer's V was used; Cramer's V ranges between 0 (no relationship) and 1 (perfect relationship). Adjusted standardized residuals were checked in order to determine the significant differences between categories; adjusted residuals

are standardized values allowing comparisons among different cells, and follow a standard normal frequency (with mean zero and standard deviation one) so it can be assumed that if their value lies outside of ±1.96 then it is significant at $p < 0.05$, if it lies outside ±2.58 then it is significant at $p < 0.01$ and if it lies outside ±3.29 then it is significant at $p < 0.001$ [40]. All of the analyses were carried out in SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

2.3. Fungus Identification and Diversity Analysis

2.3.1. Fungal Species Identification by RFLP-PCR

Restriction Fragment Length Polymorphism (RFLP)-PCR was used to confirm the species identity of the *Armillaria* isolates in sample sets 1 and 2 at the species level. DNA from two-week-old pure cultures was extracted with DNeasy Plant Mini Kit (QIAGEN Gmb, Hilden, Germany) in accordance with the manufacturer's instructions. A region of the intergenic spacer (IGS) of the rDNA was amplified using the primer pair LR12R (5′-CTGAACGCCTCTAAGTCAGAA-3′) and O-1 (5′-AGTCCTATGGCCGTGGAT-3′) [41]. The PCR mixture contained 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.5 µM each primer, 2 U Taq polymerase (Netzyme, Molecular Netline Bioproducts, Valencia, Spain) and 1 µL template DNA in a final volume of 50 µL. The cycling conditions consisted of 90 s at 95 °C, 35 cycles of 30 s at 95 °C, 40 s of annealing at 60 °C, and 2 min at 72 °C, and a final 10 min at 72 °C. The obtained DNA fragment was directly digested with the restriction enzyme Alu I (Invitrogen Life Technologies, Carlsbad, CA, USA), Nde I (Takara Bio Inc., Kusatsu, Japan) or Bsm I (Hoffmann-La Roche Ltd., Basel, Switzerland) [34]. The restriction fragments were separated in 3 % agarose gels (agarose D-1 Low EEO, Conda, Madrid, Spain). Species were identified based on the restriction patterns determined by Harrington and Wingfield [41] and Sierra et al. [42].

2.3.2. Fungal Population Analysis

In order to determine mechanisms of dispersal of *Armillaria* spp. at the stand level, genetic diversity and population structure patterns of the *Armillaria* isolates from Set 2 were analyzed. *Armillaria* species were determined by RFLP-PCR (as described above) and intraspecies differentiation was determined by SC tests and UP-PCR. For determination of SC groups (SCGs), diploid isolates from the same sampling area were paired in all possible combinations. Approximately 4 mm^2 of mycelia were placed 0.5 cm apart on MEA plates, and incubated at 20 °C for six weeks. When mycelia of co-cultured isolates fused and grew with a uniform morphology, they were considered to belong to the same species and genet, and the pairings were considered somatic compatible. When a line of demarcation was apparent, isolates were considered somatic incompatible [43].

UP-PCR reactions were carried out following the procedure described by Tyson et al. [44], in 25 µL volumes containing 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.8 µM primer (Table 1), 50 ng genomic DNA (extracted as for RFLP analysis), and 1.25 U Taq DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). The cycling conditions were 5 min at 94 °C, 5 cycles of 50 s at 94 °C, 2 min at primer specific annealing temperature (Table 1), and then 1 min at 72 °C, followed by 30 cycles of 50 s at 94 °C, 90 s at primer specific annealing temperature, and 1 min at 72 °C, and a final extension at 72 °C for 7 min [44]. UP-PCR primers were tested on a representative group of *Armillaria* isolates consisting of different species and genets of *Armillaria* (as determined by RFLP-PCR and mating tests), and the primers with the best capacity to distinguish between different SCGs and species were chosen for the analysis of all the isolates. Following gel electrophoresis of the UP-PCR amplicons, the band pattern for each isolate was assessed for the presence (1) or absence (0) of each band and represented in a binomial matrix. Similarities between strains were calculated using a simple matching coefficient [45] and represented on dendrograms constructed by average linkage between groups in SPSS software (SPSS Inc., Chicago, IL, USA). To determine the consistency between the similarity matrix and dendrograms, the cophenetic correlation coefficient was calculated [46].

Table 1. Oligonucleotide sequence of the UP-PCR primers and their respective annealing temperatures [44].

Primer	Primer Sequence	Annealing Temp. (°C)	References
0.3-1	5′-CGAGAACGACGGTTCT-3′	50	[47]
3.2	5′-TAAGGGCGGTGCCAGT-3′	52	[47]
L45	5′-GTAAAACGACGGCCAGT-3′	51	[29]
AS15inv	5′-CATTGCTGGCGAATCGG-3′	52	[48]
AA2M2	5′-CTGCGACCCAGAGCGG-3′	50	[49]
AS4	5′-TGTGGGCGCTCGACAC-3′	55	[49]
Fok1	5′-GGATGACCCACCTCCTAC-3′	52	[49]
L15/AS19	5′-GAGGGTGGCGGCTAG-3′	52	[50]
L21	5′-GGATCCGAGGGTGGCGGTTCT-3′	58	[51]
M13	5′-GAGGGTGGCGGTTCT-3′	52	[52]
AS15	5′-GGCTAAGCGGTCGTTAC-3′	52	[47]

2.4. Host Resistance

To assess the susceptibility to *A. mellea* of different tree species present in the Basque Country, 2-year-old trees of different species, including *P. radiata*, *P. nigra* subsp. salzmannii var. corsicana, *P. sylvestris* L., *Fagus sylvatica*, *Prunus avium*, *Q. petraea*, *Q. ilex*, *Cryptomeria japonica* (Thunb. ex L.f.) D.Don, *Q. robur*, *Sequoiadendron giganteum* (Lindl.) J.Buchholz, and *Eucalyptus nitens* H.Deane & Maiden (Explotaciones Forestales Jiménez Araba s. l. Nursery, Vitoria, Spain), were infected with the *A. mellea* strain AMVac isolated from an *Acer pseudoplatanus* L. (the fungal strain is maintained in a collection at Neiker Tecnalia, Arkaute, Spain). For the preparation of *A. mellea* inoculum, pieces of fungal mycelia were placed on BDS agar with autoclaved *Quercus* spp. acorns and incubated for approximately one month at room temperature in the dark [53]. Fifty trees of each species were grown in 53 × 53 × 180 mm pots (300 cc volume) using a mix of peat moss (2/3 peat, 1/3 perlite and fertilizer NPK; N = 200–450 mg/L, P_2O_5 = 200–500 mg/L, K_2O = 300–550 mg/L) and, after an adaptation period of two weeks, half acorns infected with *A. mellea* mycelium was placed in contact with tree roots. The seedlings were maintained for 4 months in a biosafety level 2 greenhouse at a mean temperature of 18 ± 5 °C, with a relative humidity of 55–60% and without supplemental light. After this period, roots were cleaned with tap water and lengths of stems, main roots and secondary roots were measured. *A. mellea* mycelial colonization was determined after removing the bark from the stem and roots. Plants were scored as healthy (without symptoms of infection) or with lesions (when *A. mellea* mycelium was present under the bark); the length of the lesions was determined by removing the bark of stem and roots and measuring the extent of *Armillaria* damage with an electronic caliper.

The differences in *Armillaria* disease severity among different tree species was determined by Pearson's chi-square. The strength of association between categorical variables (tree species and healthy or lesion containing trees) was measured with Cramer's V; adjusted standardized residuals were checked in order to determine the significant differences between categories. Differences in the size of the fungal lesions among tree species was analyzed by Brown-Forsythe and Welch statistics, and Games-Howell test was chosen for the post hoc analysis. The data was not normally distributed and therefore a base10- log transformation was applied.

3. Results

3.1. Distribution of Armillaria spp.

The presence of *Armillaria* spp. was detected in 248 (34.97%) of the 709 foci surveyed (Figure 2). Basidiocarps were mainly found in the root collars of dead and living trees, and stumps; some were found growing from superficial roots. Rhizomorphs were found in the aforementioned tree

structures and also in the soil. The tree species on which *Armillaria* spp. was present were *P. radiata*, *P. nigra*, *P. pinaster*, *Q. robur*, *Q. pirenaica*, *Fraxinus excelsior* L., *Alnus glutinosa* (L.) Gaertn., *F. sylvatica*, *E. globulus* Labill., *P. abies*, *Larix kaempferi* (Lamb.) Carr., *P. sylvestris*, *Populus alba* L., *Pseudotsuga menziesii* (Mirb.) Franco, *Q. faginea*, *Q. ilex*, *Robinia pseudoacacia* L., and *C. lawsoniana*.

Of the total of isolates obtained from the surveyed plots (Set 1), 60% were identified by RFLP-PCR patterns as *A. ostoyae*, 24% as *A. mellea*, 14% as *A. gallica*, 1% as *A. tabescens* and 1% as *A. cepistipes*. *A. ostoyae* was detected mainly in *Pinus* spp. (*P. radiata*, *P. nigra* and *P. pinaster*). The host range for *A. mellea* was more varied. *A. mellea* pattern 1 (PCR fragment sizes: 320 and 155 bp) was found on *P. radiata*, *Quercus* spp., *F. excelsior*, and *C. lawsoniana*, and corresponded to 53% of the *A. mellea* isolates, while the remaining 47% were identified as pattern 2 (fragment sizes: 320, 180, and 155 bp) and appeared on *Q. pyrenaica* and *P. radiata*. *A. gallica* was found on *A. glutinosa*, *P. radiata,* and *Q. robur*, *A. cepistipes* was detected on *P. radiata*, and *A. tabescens* on *Q. robur*.

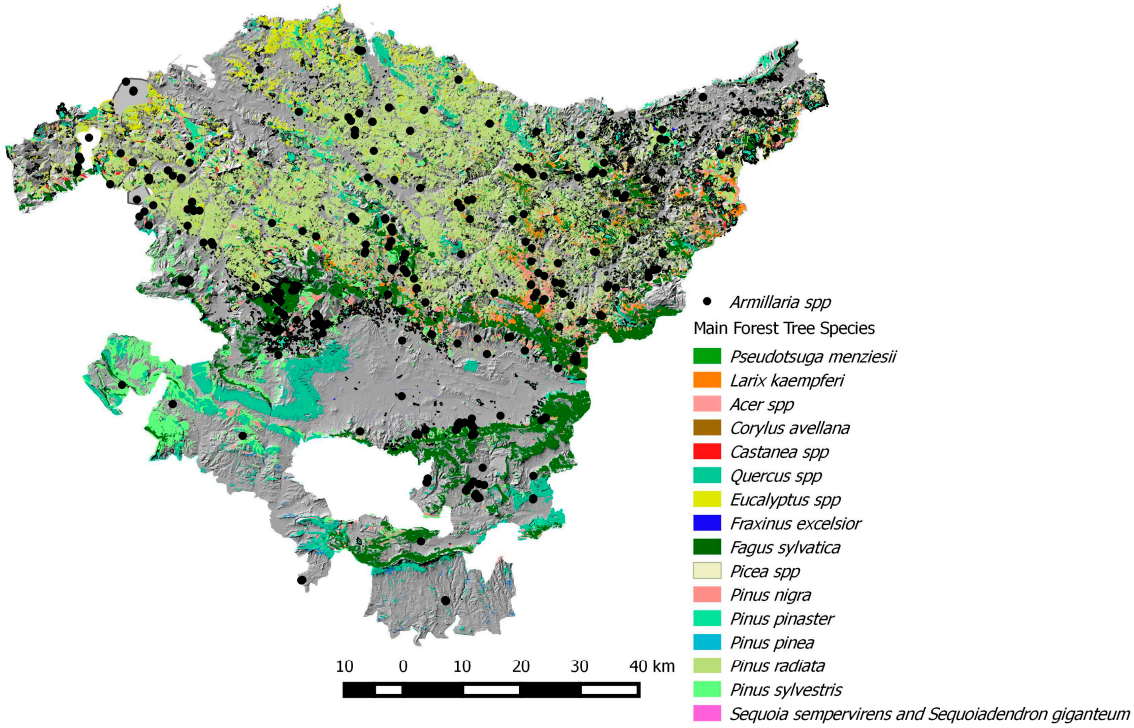


Figure 2. Distribution of *Armillaria* spp. and main forest tree species of the Basque Country.

3.2. Determination of Ecosystem Characteristics in Which Armillaria spp. Were Detected

Environmental characteristics were associated with each of the surveyed points and their relationships with the presence of *Armillaria* spp. was assessed (Table 2). The spread of the category variables for all characteristics is represented in a MCA that reflects the relationships among the variables in each dimension. MCA revealed that the first horizontal dimension explained 23.7% of the total inertia (variance), as the first factor plane represents the largest inertia, while the second vertical dimension explained 22.7%. A measure of the importance of each variable (squared component loading) is computed for each dimension. This measure is also the variance of the quantified variable in that dimension. The variables with higher variance in the first dimension were average rainfall (Rain), optimal conditions for host growth (Hoc), slope, *Armillaria* spp., soil permeability, and stand orientation (Figure 3). The variables with higher variance in the second dimension were orientation, average temperature (Temperature), average rainfall (Rain), slope, and soil acidity (Soil) (Figure 3). *Armillaria* spp. detection was related to categories such as west, northwest, and northeast stand orientation, slopes between 20% and 50%, basic soils, high average rainfalls (>1800 mm), and high soil permeability (Figure 4). *Armillaria* spp. absence was related to

categories such as south and southeast stand orientation, slopes less than 20% and soils with medium permeability (Figure 4).

Table 2. Characteristics of the plots included in this study.

Characteristics	Category code	*Description	Detection of *Armillaria* disease		Number of foci
			Negative (Arm-)	Positive (Arm+)	
Slope (%)	<10	<10	170	29	199
	10–20	10<20	133	57	190
	20–30	20<30	89	111	200
	30–40	20<40	48	34	82
	40–50	40<50	18	13	31
	50–60	50<60	3	4	7
Tree Type	Deciduous	Deciduous	28	36	64
	Conifers	Conifers	433	212	645
Orientation	N	North	45	24	69
	NE	Northeast	46	26	72
	E	East	53	32	85
	SE	Southeast	39	26	65
	S	South	75	28	103
	SW	Southwest	133	26	159
	W	West	45	73	118
	NW	Northwest	25	13	38
Soil (Acidity)	acid	acid	260	184	444
	-acid	-acid	118	31	149
	-basic	-basic	46	17	63
	basic	basic	37	16	53
Permeability	Imper	Impermeable	31	2	33
	low	Low	4	5	9
	medium	Medium	364	175	539
	high	High	62	66	128
Rain (average, mm)	1	<1000	34	7	41
	2	1000–1400	86	44	130
	3	1400–1800	295	141	436
	4	>1800	46	56	102
Temperature (average, ºC)	1	<10.5	20	5	25
	2	10.5–11.5	130	74	204
	3	11.5–12.5	162	94	256
	4	>12.5	149	75	224
HOC (Host Optimal Conditions)	+Op	Favorable	241	125	366
	Op	Acceptable	171	103	274
	-Op	Unfavorable	23	13	36
	--Op	Very Unfavorable	26	7	33

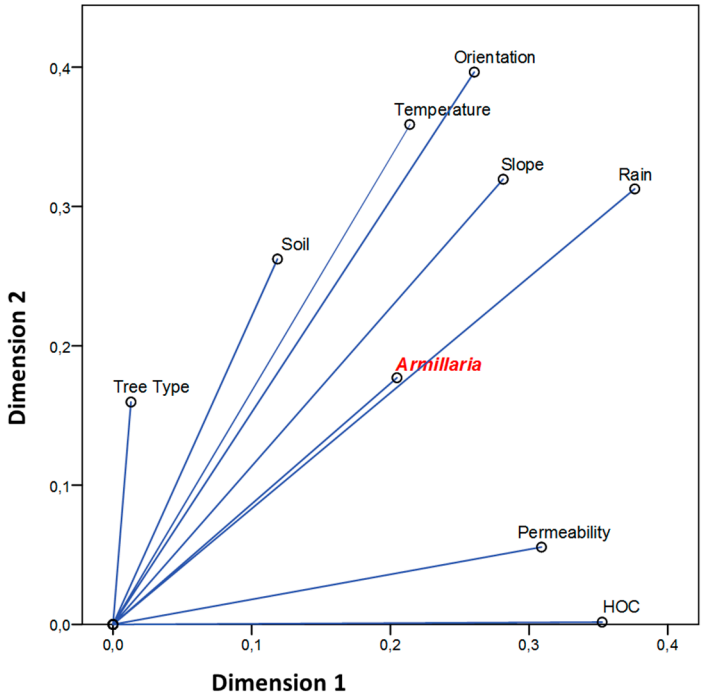


Figure 3. Measure of the variance of each variable for each dimension. The variables with higher variance in the first dimension were mainly average rainfall (Rain), optimal conditions for host growth (Hoc), slope, *Armillaria* spp., soil permeability and stand orientation (Figure 3). The variables with higher variance in the second dimension were orientation, average temperature (Temperature), average rainfall (Rain), slope, and soil acidity (Soil).

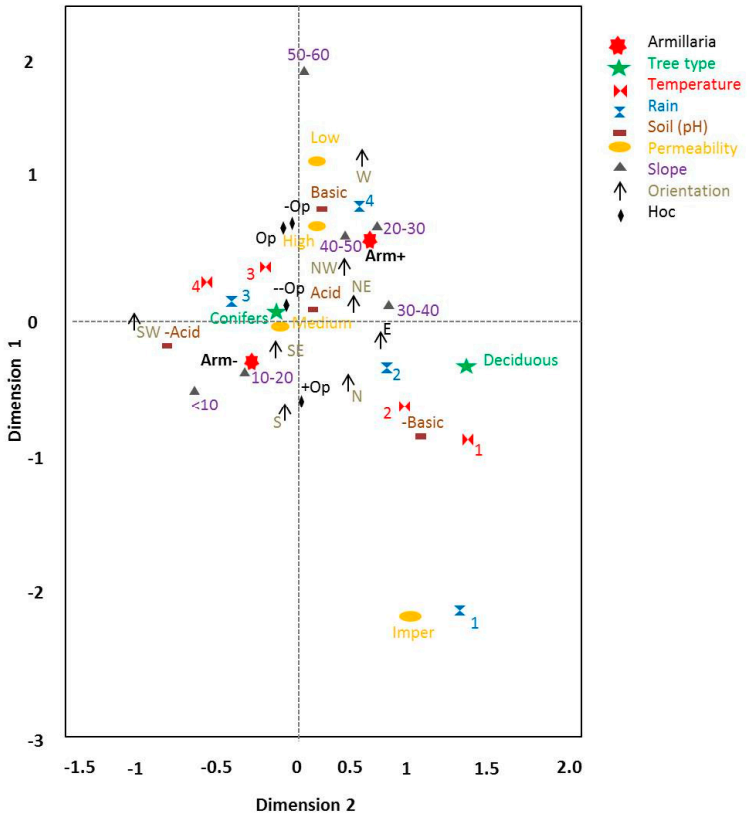


Figure 4. Location in an Euclidean space of the presence or absence of *Armillaria* spp. and environmental categories. The first two dimensions of the Euclidean space of the MCA are plotted to

examine the associations among categories. The values on the axes indicate the coordinates within the Euclidean space in which categories are located. Variable description can be found in Table 2.

The significance of the associations among fungal presence and the environmental variables was determined using Pearson's chi square test and the strength of the association was determined by Cramer's V. A significant association was observed between *Armillaria* spp. and slope, tree type, soil acidity, soil permeability, and rainfall average (Table 3). When the adjusted standardized residuals were examined, *Armillaria* spp. were significantly present in stands with slopes of 20–30% ($z = 7.2$; $p < 0.001$); stands with western orientation ($z = 6.7$; $p < 0.001$); deciduous hosts ($z = 3.7$; $p < 0.001$); acid soils ($z = 4.7$; $p < 0.001$); high permeability soils ($z = 4.3$; $p < 0.001$), and rainfall average (mm) >1800 ($z = 4.6$; $p < 0.001$) (Figure 5). *Armillaria* spp. were significantly absent in stands with slopes <10% ($z = -7.1$; $p < 0.001$); stands with southwestern orientation ($z = -5.6$; $p < 0.001$); coniferous hosts ($z = -3.7$; $p < 0.001$); moderately acid soils ($z = -4.1$; $p < 0.001$); medium permeability soils ($z = -2.5$; $p < 0.01$), impermeable soils ($z = -3.6$; $p < 0.01$), and rainfall average (mm) <1000 ($z = -2.5$; $p < 0.05$) (Figure 5).

Table 3. Environmental variables for which a significant association with *Armillaria* spp. was observed. Pearson's chi square value (χ^2), degrees of freedom (df), *p*-value, Cramer´s V value, and effect size are shown for each environmental variable.

Environmental Variables	df	χ^2	*p*-Value	Cramer's V	Effect Size
Slope (%)	5	79.2	<0.001	0.334	medium-large
Orientation	7	65.5	<0.001	0.304	medium-large
Tree type	1	14.0	<0.001	0.140	small-medium
Soil acidity	3	23.6	<0.001	0.183	small-medium
Soil permeability	3	30.8	<0.001	0.208	small-medium
Rainfall average (mm)	3	25.0	<0.001	0.188	small-medium

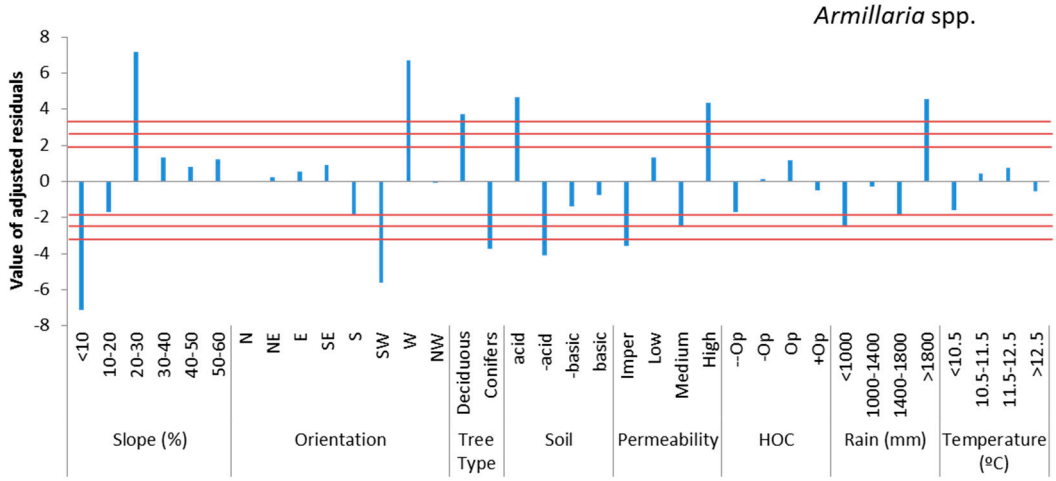


Figure 5. Values of the adjusted residuals (blue bars) for each category within a categorical variable for the presence of *Armillaria* spp. Red lines represent the value the adjust residuals must have to consider a category significant; if y = ±1.96 then it is significant at $p < 0.05$, if y = ±2.58 then it is significant at $p < 0.01$, and if y = ±3.29 then it is significant at $p < 0.001$ (Field, 2009).

3.3. Fungal Population Analysis by RFLP, SI and UP-PCR

Analysis of fungal samples (Set 2) for determining the mechanism of *Armillaria* spp. dispersal in three specific plots in Otxandiano, Amunategi, and Altube (Figure 1) revealed three different *Armillaria* species, *A. mellea*, *A. ostoyae* and *A. gallica*. The 21 samples collected in the stand located in Otxandiano belonged to the same SCG and were identified as *A. ostoyae* (data not shown). They were found in *Q. robur* stumps and trees, *C. lawsoniana*, *Crataegus monogyna* Jacq., and grassland. In the stand located in Amunategi, four of the 19 isolates were classified as *A. mellea* RFLP pattern 2 that belonged to two SCGs, both present in *R. pseudoacacia*, and 15 isolates as *A. gallica* in a more complex

population structure located in *R. pseudoacacia*, *Salix alba* L. and stumps of deciduous trees (Figure 6). In the stand located in Altube, nine of the 17 samples were identified as *A. ostoyae*, separated in 3 SCGs, in *F. sylvatica* and *Q. robur*, seven as *A. mellea* pattern 2, separated in 3 SCGs, in *F. sylvatica* and *C. monogyna*, and one as *A. mellea* pattern 1 in *F. sylvatica* (Figure 6). The larger size of *A. ostoyae* SCGs indicates dispersal predominantly by vegetative mycelium. In contrast, the smaller SCGs obtained for *A. mellea* and *A. gallica* indicate dispersal by basidiospores and vegetative mycelium. The location in the stands from which the samples were collected, the groups obtained by SC tests and their extension are depicted in Figure 7.

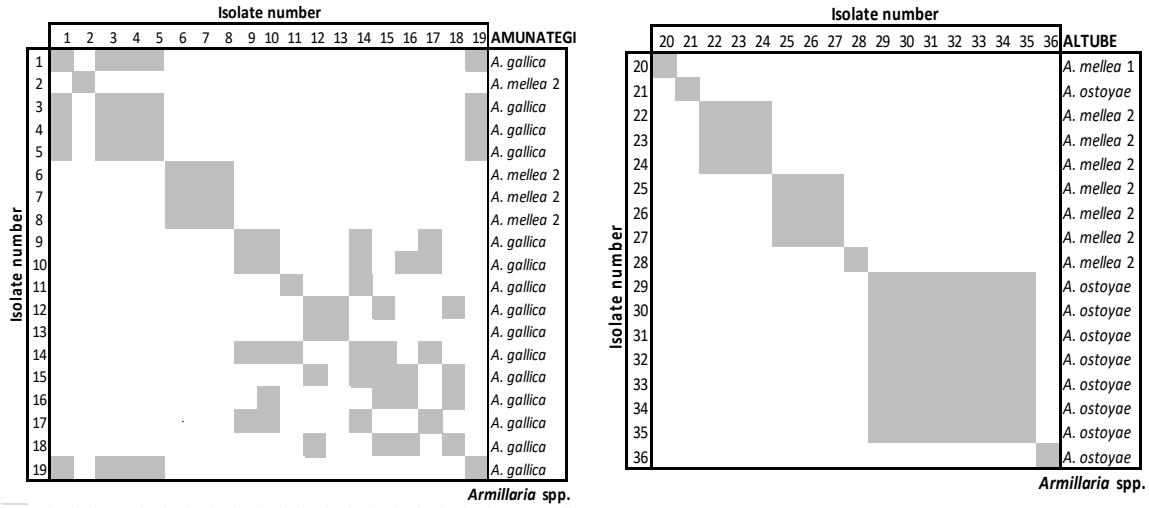
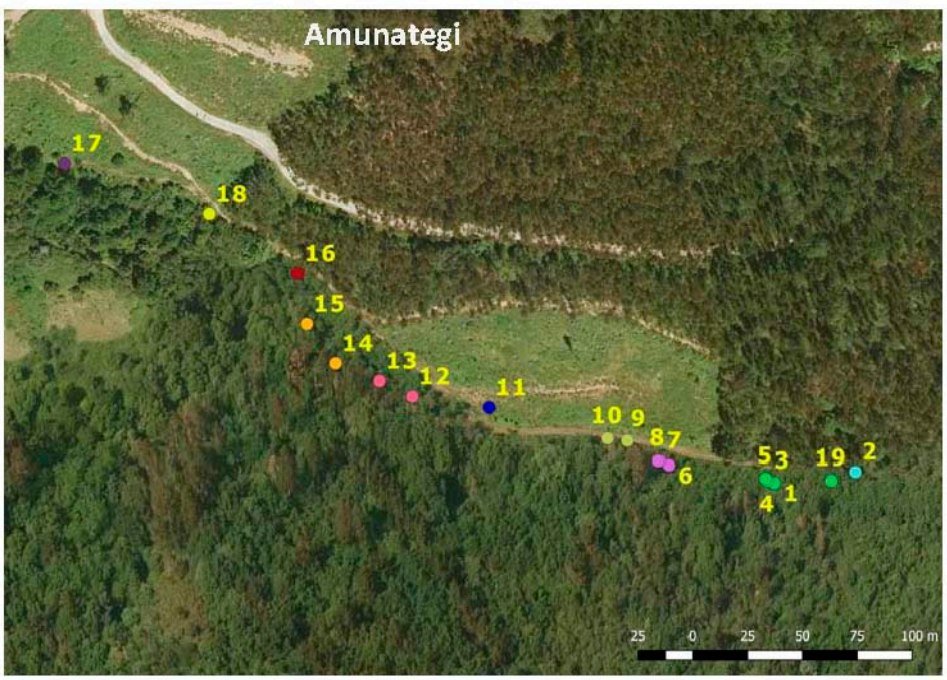


Figure 6. Fungal population analysis by mycelial pairings. Grey squares indicate strains belonging to the same SCG; white squares correspond to non-compatible strains. Paired sets shown on the left correspond to samples collected in Amunategi, paired sets shown on the right correspond to samples collected in Altube.



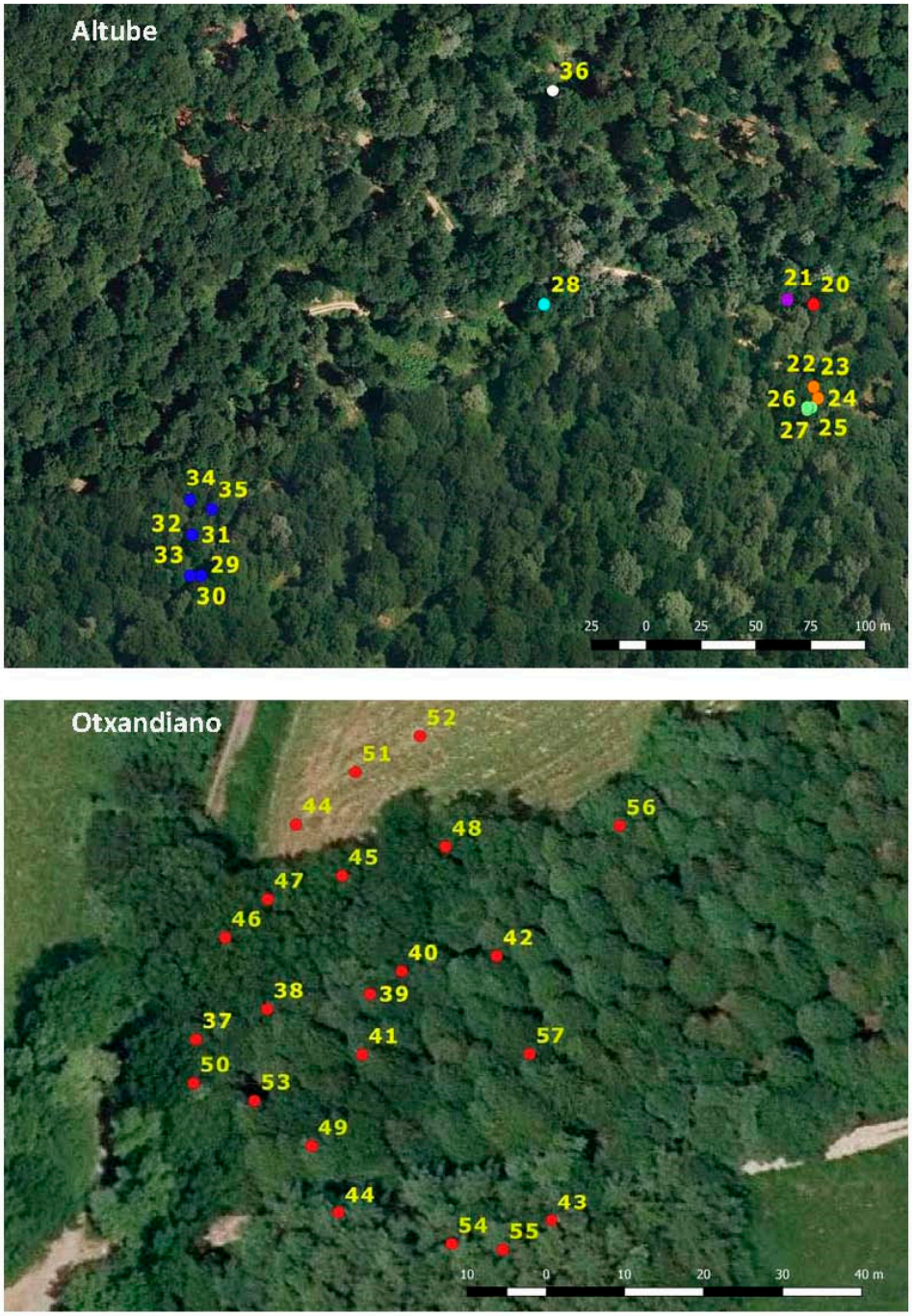


Figure 7. Spatial distribution of the infected trees and *Armillaria* spp. genotypes. Numbers correspond to *Armillaria* spp. isolates. Same genotype is marked with the same color. From top to bottom, stand located in Amunategi, stand located in Altube, and stand located in Otxandiano.

UP-PCR primer AS4 showed good ability to distinguish the fungal strains at the interspecies and intraspecies levels. Although primer L15/AS19 showed good discrimination in the initial screen, it did not yield specific banding profiles when all the samples where tested (data not shown). The best differentiation patterns were obtained for *A. ostoyae* strains and in general the clusters were comparable to those generated from mycelia matings (Figure 8). The cophenetic correlation coefficient between the similarity matrix and the dendrogram was 0.886, meaning that the clustering had a good fit.

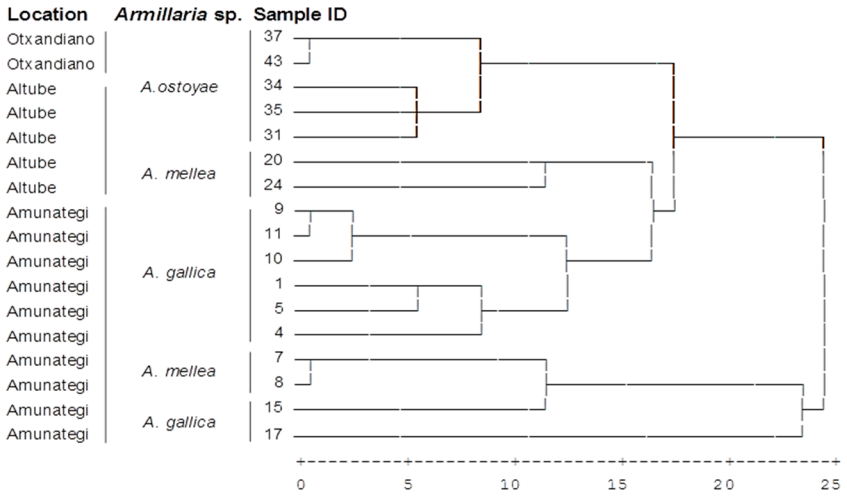


Figure 8. Genetic relationships among *Armillaria* strains. The dendrogram was generated by the average-linkage method of clustering using the distances calculated by simple matching coefficient among the binomial matrix obtained from UP-PCR banding patterms using AS4 universal primers.

3.4. Host Resistance

A significant difference in disease severity was observed among tree species (χ^2 (10) = 83.154, p < 0.001), and a Cramer's V of 0.454 indicated a medium-large effect size between health state and tree species. *P. radiata*, *P. sylvestris* and *P. nigra* seedlings were the most susceptible to *A. mellea* with 59.6%, 57.9% and 48% of plants containing lesions, respectively (Figure 9). *C. japonica*, *Q. robur* and *S. giganteum* were the least susceptible species with 0%, 5% and 5.6% of plants infected, respectively. When the adjusted standardized residuals were examined, *P. radiata*, *P. sylvestris* and *P. nigra* had significant positive values in the lesion category, meaning that more plants than expected by chance had a lesion, and significant negative values in the healthy category, meaning that fewer plants than expected were healthy (z = ±5.4, p < 0.001, z = ±3.0, p < 0.01 and z = ±3.4; p < 0.001, respectively). *C. japonica*, *Q. robur* and *S. giganteum* had significant positive values in the healthy category, and significant negative values in the lesion category (z = ±4.2, p < 0.001; z = ±3.4, p < 0.001; z = ±3.1, p < 0.01, respectively). *F. sylvatica*, *Prunus avium*, *Q. petraea*, *Q. ilex*, and *E. nitens* plants did not show any significant difference between the expected and the observed value (Figure 9).

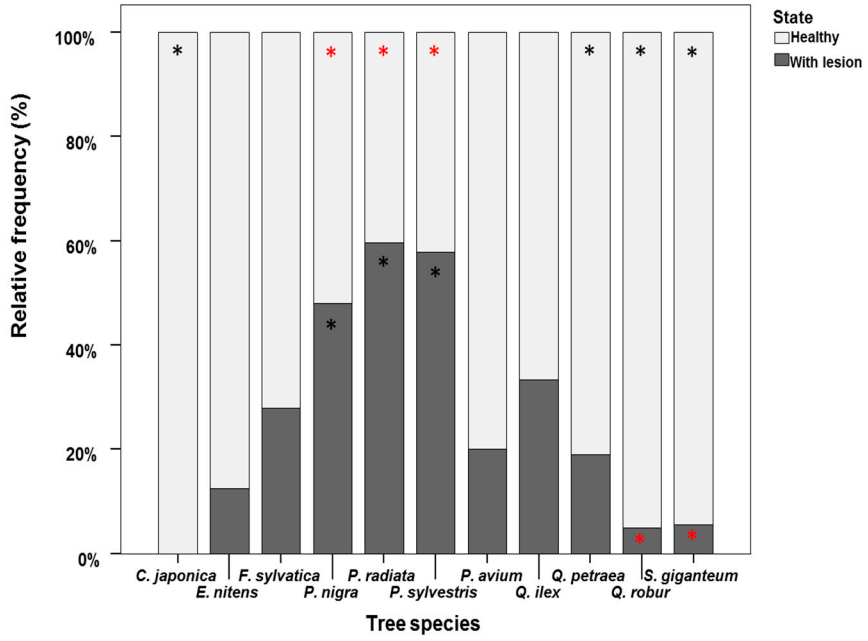


Figure 9. Susceptibility of several tree species found in the Basque Country to *A. mellea* infection. The relative frequency of healthy young trees and those with fungal lesions was determined four months after infection with *A. mellea* and growth under greenhouse conditions. Counts are represented as percentage of the total number of plants for each tree species. Black asterisks indicate positive significant *z* scores ($p < 0.05$). Red asterisks indicate negative significant *z* scores ($p < 0.05$).

Tree species with more than three trees containing lesion, and therefore suitable for the Brown-Forsythe and Welch tests, were *P. radiata*, *P. nigra*, *P. sylvestris*, *Q. ilex* and *F. sylvatica*. Significant differences in lesion size were found among the tree species (Brown-Forsythe F (4, 39.364) = 9.235, $p < 0.001$ and Welch F (4, 35.030) = 7.255, $p < 0.001$). The mean lesion length was highest for *P. nigra* (16.18 ± 5.03 cm), followed by *P. radiata* (12.70 ± 6.95 cm), *P. sylvestris* (12.66 ± 5.67 cm), *Q. ilex* (9.23 ± 9.25 cm), and *F. sylvatica* (6.75 ± 4.32 cm) (Figure 10).

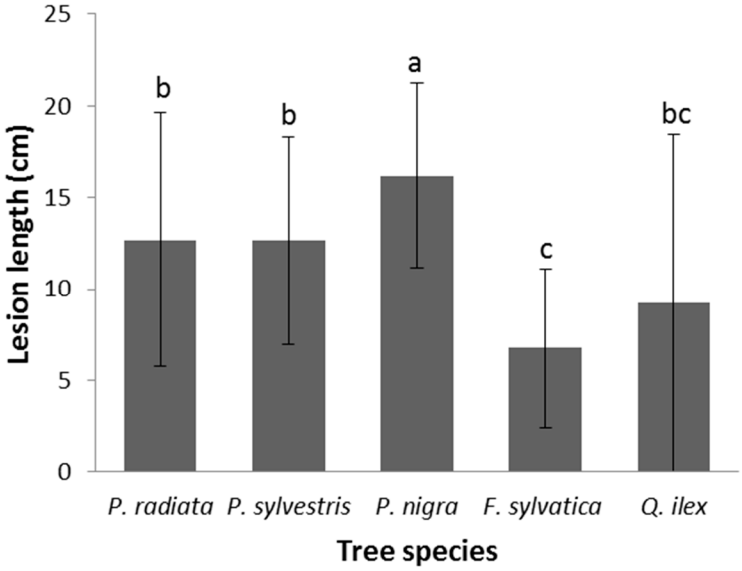


Figure 10. Length of lesions (cm) caused by *A. mellea* in different tree species. Error bars show the standard deviation of the means. Statistically significant differences of $p < 0.05$ between tree species are presented with different lowercase letters.

4. Discussion

In the present study, we report the broad distribution of *Armillaria* spp. in native forests and plantations of the Basque Country and the ecosystem characteristics that may foster the occurrence of this fungal complex. The diversity of the *Armillaria* population in three of the infected plots was assessed in order to determine their interspecies and intraspecies diversity and potential dispersal mechanisms. Finally, host susceptibility to *A. mellea* was determined in a set of native and exotic forest species selected on the basis of their presence in the Atlantic area of Spain.

In the large and small scale surveys, *A. ostoyae* was the predominant species, mainly detected in conifers, but also in native forests of *F. sylvatica* and *Q. robur*. The wide host range displayed by *A. mellea* in this study has been thoroughly referenced [2,8], and *A. gallica* was also found in both conifers and deciduous trees.

In the stands infected with *Armillaria* spp., *A. ostoyae* was distributed in larger clonal clusters than *A. mellea* and *A. gallica,* which were found in medium and small clonal clusters, respectively. Differences in genet size among *Armillaria* spp. has been previously reported [54–56]. Genet size is influenced by basidiocarp formation, basidiospore abundance and survival, and formation of diploids, which are related to moisture and temperature. The presence of larger genets, which indicates dispersion by vegetative mycelium, is more common in dry and/or cold forests than in moist-warm forests, where dispersion by basidiospores would be favored [54–56]. In the present

study the stands with the largest genets, Otxandiano and Altube, experience colder temperatures. However, *A. mellea* genets were of similar sizes in stands with cold and warmer climate, so differences in behavior among species may also be a factor.

Most of the genets determined for *Armillaria* spp. by UP-PCR using AS4 primer and SI tests were consistent. In the case of the genet from Otxandiano, no difference in UP-PCR band patterns were apparent between *A. ostoyae* samples, but when *A. ostoyae* samples located in Altube were assessed, different band patterns were detected among isolates of the same SCG. Different band patterns were also detected among *A. gallica* isolates of the same SCG. This could mean that some genets were composed of sib-related genets, meaning that basidiospores could be more important in the dispersion process than observed in the SC tests [56]. UP-PCR may provide more information about the genets obtained by SC tests in *Armillaria* spp. Dodd et al. [57] also used UP-PCR for determining polymorphism within and between different species of *Armillaria* (*A. limonea* (G. Stev.) Boesew. and *A. novae-zelandiae* (G. Stev.) Boesew.), and in their study primer AS4 also provided consistent results.

Although *Armillaria* spp. were present in forests in the study area with a wide range of environmental conditions, they were more frequently detected in stands with 20–30% slopes, with a westerly orientation, deciduous forests, acid soils with high permeability, and rainfall average values above 1800 mm. *Armillaria* spp. were less abundant in stands with slopes less than 10%, southwest orientation, rainfall average values below 1000 mm, and coniferous forests with moderately acidic and medium permeability or impermeable soils. In general, species of the *Armillaria* complex have been isolated from a wide range of soil and environmental conditions but disease foci are also influenced by host adaptability and stress [7].

A. mellea is recognized as a primary pathogen of a broad range of tree species. In the present study, *A. mellea* had the highest host diversity at the stand level. This was also observed by Pintos et al. [58] when different soils from Pontevedra, Galicia, Spain were analyzed for the presence of *Armillaria* spp., and in general for European populations [2]. To confirm the host range in trees that are predominant in the Basque Country, and to identify resistance species, their susceptibility was tested under greenhouse conditions. All the tested tree species except *C. japonica* were susceptible to *A. mellea*. Melo et al. [59] determined that resistance of *C. japonica* was due to the presence of polyphenolic compounds in the heartwood [60]. *Pinus* species were the most affected by *A. mellea* infection. Aguín et al. [61] reported that *Pinus* spp. infected with *A. mellea* were the first to show aerial symptoms of disease in comparison to those infected with other species of *Armillaria*, such as *A. ostoyae* and *A. gallica*. In this study, *P. radiata* had the most infected plants and *P. nigra* had the largest average lesion size. *P. radiata* is known to be very susceptible to other *Armillaria* spp. such as *A. novae-zelandiae* [62]. *C. japonica* has been reported tolerant to *Fusarium circinatum* Nirenberg & O'Donnell [63], since both diseases are present in this region and the area shows optimal growth conditions for this tree species it could be considered a promising alternative to *P. radiata* in monoculture plantations. However, in the present susceptibility assay only *A. mellea* pathogenicity was examined in greenhouse conditions, so this result should be considered as preliminary findings.

While *Armillaria* was found to be associated with mortality in the forests of the Basque Country, the impact of the fungus can be difficult to evaluate because it is influenced by other biotic and abiotic elements of the ecosystem. *Armillaria* spp. can cause mortality in susceptible tree species, however, sometimes their effects are less visible and infection does not always have a negative impact on tree development. The outcome of infection is also influenced by forest management strategies and this study is a first attempt to define factors that contribute to the development of *Armillaria* disease in the area, bearing in mind the complex etiology of this pathosystem.

5. Conclusions

Although *Armillaria* was frequently detected in the studied native and plantation forests, control measures have been restricted to urban trees and recreational parks, and implemented to prevent civilian and structural damage that may be caused by instability of affected trees. The control of *Armillaria* complex in forests is more difficult as it is spread over a wider area, often in areas that are difficult to access. The best way to reduce the vigor of the fungus, which is strongly dependent on

the availability of food sources, is by pulling out infected stumps and roots. However, this measure can also disrupt beneficial microbial populations [64], which may act as a natural control of pathogens [65]. Fungicidal treatments can kill the fungus in the soil; however, the economic and environmental costs are high. By contrast, early detection of disease and treatment with effective biological antagonists, and planting *Armillaria*-tolerant tree species are recommended for forests, plantations, urban areas and recreational parks [66]. In typical modern plantations, control measures are probably economically justified if mortality from *Armillaria* spp. is severe early in the previous rotation. It is, therefore, important to keep good stand records that will point out the impact of different factors when a decision may be necessary prior to planting a new species. Even if action is not taken, forest owners should be aware of the presence of the fungi, especially if there is a chance that a change of management practice could inadvertently lead to an increase in disease impact [14,18,20].

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Conflicts of Interest: The authors declare no conflict of interest.

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