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Sydowia polyspora Dominates Fungal Communities Carried by Two *Tomicus* Species in Pine Plantations Threatened by *Fusarium circinatum*

E. Jordán Muñoz-Adalia [1,2,*], Antonio V. Sanz-Ros [1,3], Juan A. Flores-Pacheco [1,2,4], Jarkko Hantula [5], Julio J. Diez [1,2], Eeva J. Vainio [5] and Mercedes Fernández [1,6]

1 Sustainable Forest Management Research Institute, University of Valladolid–INIA, Avenida de Madrid 44, 34071 Palencia, Spain; asanzros@gmail.com (A.V.S.-R.); japacheco@pvs.uva.es (J.A.F.-P.); jdcasero@pvs.uva.es (J.J.D.); mffernan@agro.uva.es (M.F.)
2 Department of Vegetal Production and Forest Resources, University of Valladolid, Avenida de Madrid 44, 34071 Palencia, Spain
3 Calabazanos Forest Health Centre (Junta de Castilla y León), Polígono industrial de Villamuriel, S/N, Villamuriel de Cerrato, 34190 Palencia, Spain
4 Facultad de Recursos Naturales y Medio Ambiente, Bluefields Indian & Caribbean University-BICU, Avenida Universitaria, Apartado Postal N° 88 Bluefields, Nicaragua
5 Natural Resources Institute Finland (Luke), Viikinkaari 4, FI-00790 Helsinki, Finland; jarkko.hantula@luke.fi (J.H.); eeva.vainio@luke.fi (E.J.V.)
6 Department of Agroforestry Sciences, University of Valladolid, Avenida de Madrid 44, 34071 Palencia, Spain
* Correspondence: emigdiojordan.munoz@uva.es or ejordanmunoz@hotmail.com; Tel.: +34-979-108432

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Abstract: Bark beetles (Coleoptera, Scolytinae) carry a diverse filamentous fungal community sometimes acting as vectors or carriers of phytopathogens. In this study, mycobiota carried by two *Tomicus* species (*Tomicus piniperda* and *Tomicus destruens*) were investigated through (i) morphological and molecular identification of taxa; (ii) taxonomic richness, diversity, evenness, dominance and phoresy indices; (iii) ecological network analysis and (iv) statistical co-occurrence analysis. The studied mycobiota were formed by eleven taxa and showed a moderate fungal diversity with low evenness. The fungus *Sydowia polyspora* was significantly abundant and dominated the community. All the fungal taxa were randomly associated. Both insect species (*T. piniperda* and *T. destruens*) were collected from plantations of *Pinus radiata* infected by *Fusarium circinatum*. The ecological factors that could drive community ecology and phoretic links between fungi and bark beetles are discussed.

Keywords: COI gene; fungal diversity; mycobiota; phoresy; *Pinus radiata*; *Tomicus destruens*

1. Introduction

The ecological interactions between fungi and bark beetles (Coleoptera, Scolytinae) are complex and can range in a continuum from symbiosis to parasitism [1]. The relationship between bark beetles and potentially pathogenic fungi has been broadly studied in forest pathology [2–4], e.g., the Dutch elm disease (DED; causal agents *Ophiostoma ulmi* (Buisman) Melin & Nannf. and *Ophiostoma novo-ulmi* Brasier) and *Scolytus* spp. (Col., Scolytinae) is one of the most well-known pathosystems [5]. Despite this interesting association between fungi and insects, the community ecology approach has been scarcely applied to filamentous fungi [6].

The pine pitch canker disease (PPCD, causal agent *Fusarium circinatum* Nirenberg & O'Donnell; teleomorph *Gibberella circinata* Nirenberg & O'Donnell) is considered the most important disease of pine (*Pinus* spp.) seedlings in several countries, causing high rates of pre-, post-emergence and late

damping-off in nurseries. It also causes severe symptoms in adult trees, such as growth reduction, wilting, and the resulting bleeding cankers are dangerous because trees easily break during wind storms [7]. This pathogen is widespread around the world, establishing different phoretic relationships with several groups of invertebrates (mainly insects but also others as mollusks and crustaceans [8]). Regarding insects, thirteen species of Coleoptera and one of Diptera (*Medetera* spp.; Dolichopodidae) have been identified as carriers of PPCD fungus in the USA, as summarized by Brockerhoff et al. [9]. In Spain, all identified carriers of *F. circinatum* belong to Curculionidae, including seven species of Scolytinae, one Molytinae and one Entiminae [10,11].

The species that can effectively inoculate a pathogen in a healthy host (vectors) embody a relevant phoretic relationship in forest pathology. In the case of PPCD in the USA, some species of Scolytinae (i.e., *Ips paraconfusus* Lanier, *Ips mexicanus* Hopkins, *Ips plastographus* LeConte, *Pityophthorus setosus* Blackman, *Pityophthorus carmeli* Swaine and *Conophtorus radiatae* Hopkins), as well as *Ernobius punctulatus* (Col., Anobiidae) [9] have been proposed as vectors. In Spain, Pine shoot beetle (*Tomicus piniperda* L.; Col. Scolytinae) has recently been identified as a plausible vector of *F. circinatum* [12], with a special interest in the North where PPCD infects pine forests and plantations. This phloephagous insect can select different tree species as hosts (mainly *Pinus* sp. but also other genera of conifers such as *Picea* sp. or *Larix* sp.), and it is considered to be a secondary pest in terms of trunk infestation in their native Eurasian range [13,14]. Nevertheless, it is considered an aggressive pest on pine forests and plantations in the USA where it was introduced in 1992 [15].

In Atlantic habitats, *T. piniperda* sometimes co-occurs with Mediterranean shoot beetle (*Tomicus destruens* Wollaston) [16,17]. Mediterranean shoot beetle causes intense damages in thermophilic woodlands throughout its range, circumscribed to the Mediterranean Basin [18–20]. Despite this, the ecology of this insect in the Atlantic area and its role as a carrier or vector of forest diseases remain understudied. In consequence, more knowledge is needed of the ecology and phoretic communities of these two bark beetles in the areas where they coexist with *F. circinatum*.

We hypothesized that the fungal community carried by the studied bark beetles would be diverse and would include positive and negative interactions among fungal taxa. Additionally, we hypothesized that a few species of fungi would dominate fungal community. Therefore, the aims of this study were (i) to characterise the mycobiota carried by the two *Tomicus* spp. during their hibernation period in pine shoots; (ii) to evaluate the ecological relationships (including dominance) among fungi carried by the bark beetles and (iii) to investigate the possible presence of *Tomicus destruens* in pine stands inhabited by *T. piniperda* and damaged by PPCD.

2. Materials and Methods

2.1. Samples Collection

During November and December 2015, pine shoots bored by *Tomicus* spp. were directly collected from the ground using circular transects (three hours of sampling per plot during each fieldwork day) in two plots in the Cantabria region (North of Spain): (A) Monterey pine (*Pinus radiata* D. Don) plantations infected by *F. circinatum* (Santibañez, Cabezón de la Sal. UTM 30N, 398813; 4792690. 320 m a.s.l.) and (B) an asymptomatic mixed plantation of European black pine (*Pinus nigra* subsp. *salzmannii* (Dunal) Franco) and Scots pine (*Pinus sylvestris* L.) (San Miguel de Aguayo. UTM 30N, 419587; 4771507. 850 m a.s.l.). Insects inside shoots were removed using sterilised tweezers and stored at 4 °C. Each bark beetle was transferred to a single sterilised tube and immersed in 100 µL Tween 80 (PanReac Química, Barcelona, Spain) at 1% v/v. Then, they were sonicated (J. P. Selecta Ultrasons 2.6l; J. P. Selecta, Barcelona, Spain) for 5 s at 40 kHz to obtain spore suspensions [21]. The sampling plots showed favorable characteristics for sympatry of *T. piniperda* and *T. destruens* [17]; therefore, the body of each insect was reserved for molecular identification.

2.2. Molecular Identification of Tomicus Species

T. piniperda and *T. destruens* share a very similar morphology [22]. Therefore, molecular identification by PCR was carried out with extracted DNA from all the collected insects according to Vainio et al. [23]. The selected method for species identification was the one described by Kohlmayr et al. [24] based on amplification of cytochrome oxidase I gene (COI) using specific primer pairs. Briefly, the primer pair C1-J-2441 (5′-CCTACAGGAATTAAAATTTTTAGATGATTAGC-3′) [25] and C1-N-2937 (5′-ATATTGGAATCACTCAATTGAG-3′) was used to identify *T. piniperda* samples. Moreover, the primer pair C1-J-2441 and C1-N-2934 (5′-TTCTTGGAATCATTCAATAGAAGTC-3′) was used to ensure the identity of *T. destruens*. PCR was performed with Kapa Taq PCR Kit (Kapa Biosystems, Wilmington, MA, USA) following a protocol of 10 min at 94 °C, 36 cycles of 30 s at 94 °C, 30 s at 56 °C, 1 min at 72 °C, and a final extension of 2 min at 72 °C. The amplicons were separated in 1.60% *w*/*v* agarose gel and stained with 3x GelRed™ (Biotium, Hayward, CA, USA) after running for 18 min in an orbital shaker at 85 rpm. The amplicon size was estimated by comparison with a 100 bp ladder (Nippon Genetics Europe, Dueren, Germany).

2.3. Fungal Isolation and Identification

Fifty microliter aliquots of spore suspensions were initially cultured in a generalist culture medium (i.e., PDA; 3.90% *w*/*v* potato-dextrose-agar) amended with streptomycin sulfate salt ($C_{21}H_{39}N_7O_{12}$·1.5 H_2SO_4; 0.60 mg/L) as a broad-spectrum antibacterial agent. In the cases where bacterial colonies entirely covered the plate, a second 50 µL aliquot of spore suspension was cultured in a nutrient-deficient medium (water-agar; 1.50 mg/L agar) to reduce the number of sterile or uncolonised samples (i.e., insects that did not provide any fungal colony after culturing the spore suspension). Each colony was counted and subcultured onto MEA medium (1.45% *w*/*v* agar and 1.93% *w*/*v* malt extract) in order to get pure cultures. Fungal colonies were preliminary microscopically characterised (LEITZ DIALUX 22/22 EB; Leitz, Wetzlar, Germany) and visual inspection of colonial characteristics defined operational taxonomical units (OTUs) [26]. A representative amount of monosporic cultures per OTU from different spore suspensions (i.e., 1–10 pure cultures per OTU depending on the relative abundance; 33 isolates in total) was transferred to cellophane-membrane-covered MOS-agar medium (2.84% *w*/*v* orange serum agar, 0.85% *w*/*v* agar, 0.76% *w*/*v* malt extract and 0.76% *w*/*v* dextrose) for DNA extraction and subsequent molecular identification.

DNA extraction was carried out following the protocol described by Vainio et al. [23]. OTU homogeneity was verified by using fingerprints amplified with the M13 minisatellite primer (5′-GAGGGTGGCGGTTCT-3′) [27]. The PCR reaction was performed in a total volume of 50 µL, and it consisted of the following steps: 10 min at 93 °C followed by 45 cycles of 20 s at 93 °C, 1 min at 48 °C, 20 s at 72 °C and a final incubation step of 6 min at 72 °C. Furthermore, molecular identification of each OTU was based on rDNA sequencing (i.e., ITS region), which was amplified by using ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as a primer pair [28]. The PCR reaction was performed in a total volume of 50 µL, and it consisted of the following steps: 10 min denaturation at 96 °C followed by a stepped second phase of 13 cycles of 35 s at 95 °C, 55 s at 56 °C, 45 s at 72 °C; then, 13 cycles of 35 s at 95 °C, 55 s at 56 °C, 2 min at 72 °C and a last step of 8 additional cycles of 35 s at 95 °C, 55 s at 56 °C, 3 min at 72 °C, and finally, an elongation step for 10 min at 72 °C [28]. The amplification products were separated by electrophoresis in a gel containing 0.90% *w*/*v* each of SynerGel™ (Diversified Biotech, Dedham, MA, USA) and 1.60% *w*/*v* agarose. DNA fragments were visualized using ethidium bromide staining under UV light.

Amplicons were sent to Macrogen Europe Inc. (Amsterdam, The Netherlands) for DNA sequencing. Sequences were trimmed using Sequence Scanner v1.0 software (Thermo Fisher Scientific, Waltham, MA, USA) and then compared with those deposited in the GenBank (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) database using BLAST [29]. Fungal taxa were assigned

at species or genus level, always showing a homology equal or higher than 99%. ITS sequences representing each detected molecular variant were submitted to GenBank (Table 1).

The classification of *Fusarium* sp. cultures was done based on morphological traits as well as genetic characterization, by sequencing the ITS regions and studying their phylogenetic relationships with culture collection strains of different *Fusarium* species. The morphological characterization was carried out based on reproductive structures such as microconidia (presence/absence, number of septa, length shape and aggregation in chains, false heads, etc.), macroconidia (size, length, thickness, apical and basal cells shape, number of septa), phialides (mono- and polyphialide presence), and chlamydospores (presence/absence, ornametation, and aggregation) [30]. The phylogenetic representation (i.e., dendrogram) was computed among the *Fusarium* isolates from this study and other sequences in GenBank, always including culture collection specimen sequences for each species. The MEGA v6.06 [31] software was used to compute a tree using Neighbor-joining as the statistical method, the bootstrap method (1000 replications) as a test of phylogeny and Kimura2 [32] as the substitution method. Nevertheless, future studies based on more accurate molecular markers are required to determine the detailed taxonomical status of the *Fusarium* isolates.

2.4. Data Analysis

Diversity of fungi was analysed using Shannon (H) and Simpson (D) diversity indices, taxonomic richness (S_{obs}), Sorensen similarity index between plot A and B (I) and Shannon and Simpson taxa evenness indices (J and E, respectively) [33]. Fungal dominance was measured using Camargo's index where dominance was defined if $p_i > 1/S_{obs}$ (p_i being the number of isolates of taxon i/total number of isolates) [34]. In addition, the EstimateS v9.1.0 [35] software was used to compute sample-based rarefaction curves [36,37], and 95% confidence intervals [38]. The same software was used to calculate estimated taxonomic richness through six richness non-parametric estimators: Mao-Tau, Incidence-based Coverage Estimator of species richness (ICE), First and Second order Jackknife richness estimator (Jack 1 and 2), Chao 1 and Chao 2 [37].

The phoresy index (PI) based on the pollination probability index previously described by Ne'eman et al. [39] was also calculated for each taxa and carrier *Tomicus* sp. according to Equation (1). This index ranges from 0 where the insects did not yield any fungal isolates (null phoretic association), to 1 where only one taxon was isolated from spore suspensions (intense phoretic association). R software [40] was used to develop Kruskal-Wallis rank sum tests implemented in the "Agricolae" package [41] to analyse the PI variation among taxa. Subsequently, Dunn's test was applied for post-hoc analysis, using the "DescTools" package [42].

$$PI_{ij} = \left(\frac{n_{ij}}{n_j}\right) \times \left(\frac{N_i}{N}\right) \quad (1)$$

where PI_{ij}: phoresy index for fungal taxon i carried by insect j; n_{ij}: number of isolates of taxon i yielded by insect j; n_j: total amount of isolates yielded by insect j; N_i: number of insects that carried taxon i; N: total amount of collected insects.

The relationships between host trees, bark beetles and fungi were analysed using the package "bipartite" [43] of R software. This package was used to calculate the connectance (Cn; total number of links in the network/S_{obs}^2) [44] and the mean number of links per taxa (L) as well as to represent the sampled mycobiota in a tripartite graph. This statistical software was also used to perform a co-occurrence analysis using the package "cooccur" [45]. The function "co-occur" provides the observed and expected frequencies of co-occurrence by pairs of taxa (F_{obs} and F_{exp} respectively) and two associated probabilities (P_{gt} and P_{lt}) that could be interpreted as p-values [46]. These probabilities identify each pairwise association between fungi as follows: (a) positive association between taxa if $F_{obs} > F_{exp}$ and $P_{gt} < 0.05$; (b) negative association if $F_{obs} < F_{exp}$ and $P_{lt} < 0.05$ or (c) random association in the remaining cases.

Table 1. Data of fungal isolates from *Tomicus* species in the studied plots.

OTU	GenBank Accession Number of Best Matches	Id (%)/Qc (%)	Description of GenBank Best Match	Suggested Name for OTU	Accession Number
1	HG008754.1	100/100	*Sydowia polyspora* (Bref. & Tavel) E. Müll.	*S. polyspora*	KY081694
2	KM199339.1	99/97	*Pestalotiopsis hawaiiensis* Maharachch., K.D. Hyde & Crous	*Pestalotiopsis* sp.	KY081696
3	JX421725.1	100/100	*Phoma herbarum* Westend	*P. herbarum*	KY081697
4	KU182497.1	100/100	*Cladosporium cladosporioides* (Fresen.) G.A. de Vries	*Cladosporium* sp.	KY081699
5	JN617665.1	100/99	*Penicillium westlingii* K.M. Zalessky	*Penicillium* sp.	KY081700
6	JX421733.1	99/100	*Phaeomoniella effusa* Damm & Crous (synonym: *Aequabiliella effusa* (Damm & Crous) Crous)	*P. effusa*	KY081695
7	KU184424.1	99/100	*Ophiostoma canum* (Münch) Syd. & P. Syd.	*O. canum*	KY081698
8	AJ876490.1	99/100	*Mucor hiemali* Wehmer	*M. hiemalis*	KY081701
9	FJ403224.1	99/100	*Fusarium sporotrichioides* Sherb.	*F. sporotrichioides* *	KY081692
10	AF310978.1	100/100	*Fusarium lateritium* Nees	*F. lateritium* *	KY081690
11	KU516466.1	100/100	*Fusarium avenaceum* (Fr.) Sacc.	*Fusarium* sp. *	KY081689
12	KU516468.1	100/100	*F. avenaceum*	*Fusarium* sp. *	KY081691
13	KU516467.1	100/100	*F. avenaceum*	*Fusarium* sp. *	KY081693

OTU: Operational taxonomical unit; Id: Maximum identity of sequence; Qc: Query coverage of sequence. *: *Fusarium* species were clustered according to GenBank best matches and Neighbor-joining dendrogram results (see Figure A1). Taxonomy according to Robert et al. [47].

3. Results

3.1. Shoot Collection and Insect Identification

A total of 499 pine shoots bored by the two *Tomicus* species were collected during the study period. The main collected species were Monterey pine (58.11%; plot A) followed by European black pine and Scots pine (33.27% and 8.62% respectively; plot B). Insect occupation rate was 9.82%, with a total of 49 insects (one insect inside each shoot), while frequencies of insects in each pine species were 5.17% in Monterey pines, 18.07% in European black pines and 9.30% in Scots pines.

Both insect species, *T. destruens* and *T. piniperda*, were present in the study area, but the only two individuals captured in the pure Monterey pine stand (plot A) were *T. destruens* (13.33% plot A; 4.08% total amount of insects) while 100% of insects in the mixed stand (plot B) were *T. piniperda*. Two individuals (one from plot A and the other one from plot B) could not be identified at the species level due to the low quality of the extracted DNA. They did not provide any fungal colony information.

3.2. Fungal Community Characterisation

A total of 113 pure fungal cultures were isolated, belonging to 13 morphological OTUs (2.69 $\pm$ 0.10 isolates per insect and 1.38 $\pm$ 0.15 OTUs per insect; average values and standard error) (Tables 1 and 2). Fungal cultures were assigned to morphological OTUs based on combined results of morphological investigation and M13 fingerprinting. Species identification was conducted using ITS sequences, which revealed a total of 11 taxa (1.33 $\pm$ 0.05 taxa per insect).

Table 2. Relative abundance and phoresy index (PI (%); mean value and standard error (SE)) of fungal taxa carried by the two studied *Tomicus* species in the sampling plots.

Fungal Taxa	Relative Abundance		Total	PI (%) $\pm$ SE
	Plot A	Plot B		
Cladosporium sp.	1 (3.23%)	0 (0.00%)	1 (0.88%)	$8.33 \times 10^{-3} \pm 8.33 \times 10^{-3}$ a
Fusarium lateritium	1 (3.23%)	2 (2.44%)	3 (2.65%)	0.15 $\pm$ 0.08 a
Fusarium sporotrichioides	0 (0.00%)	3 (3.66%)	3 (2.65%)	0.04 $\pm$ 0.04 a
Fusarium sp.	2 (6.45%)	8 (9.76%)	10 (8.85%)	0.74 $\pm$ 0.35 a
Mucor hiemalis	1 (3.23%)	2 (2.44%)	3 (2.65%)	0.11 $\pm$ 0.09 a
Ophiostoma canum	0 (0.00%)	2 (2.44%)	2 (1.77%)	0.04 $\pm$ 0.04 a
Penicillium sp.	3 (9.68%)	0 (0.00%)	3 (2.65%)	0.04 $\pm$ 0.04 a
***Pestalotiopsis* sp.**	**6 (19.35%)**	1 (1.22%)	7 (6.19%)	0.29 $\pm$ 0.16 a
Phaeomoniella effusa	2 (6.45%)	3 (3.66%)	5 (4.42%)	0.15 $\pm$ 0.10 a
Phoma herbarum	0 (0.00%)	4 (4.88%)	4 (3.54%)	0.15 $\pm$ 0.08 a
Sydowia polyspora	**15 (48.39%)**	**57 (69.51%)**	**72 (63.72%)**	37.84 $\pm$ 4.30 b
Subtotal	31	82	113	
Total number of bark beetles	49			
Total uncolonised samples	7 (14.28%)			

Plot A: Plantation of *Pinus radiata* infected by *Fusarium circinatum*; Plot B: Asymptomatic plantation of *Pinus nigra* subsp. *salzmanni* and *Pinus sylvestris*. Dominant species according to Camargo's Dominance Index in bold. Small letters (a,b) denote significant differences (Dunn's test, p-value < 0.05).

In spite of the morphological differences clustered the *Fusarium* sp. isolates in five OTUs (Table 1). ITS sequences were not useful to assign species to OTUs 11, 12 and 13. Dendrogram results (Figure A1) and GenBank best matches supported the clustering of *Fusarium* spp. in three clades (i.e., *Fusarium sporotrichioides* Sherb., *Fusarium lateritium* Nees and *Fusarium* sp.) (Table 1).

Sydowia polyspora (Bref. & Tavel) E. Müll. dominated the whole community (plots A and B), but in plot A, *Pestalotiopisis* sp. was also dominant according to the Camargo's index (Table 2). PI was very low in general, showing low phoretic associations between fungi and *Tomicus* spp. This index varied significantly among fungal taxa (X^2 = 209.74; *d.f.* = 10; p-value < 0.01), but the PI of *S. polyspora* was significantly higher than that of other taxa, according to Dunn's test (p-value < 0.01 in all cases)

(Table 2). *F. circinatum* was not isolated from spore suspensions despite the fact that plot A was severely damaged by PPCD.

The sample-based rarefaction curves did not show an asymptotic development (Figure 1). A tripartite graph summarized the observed mycobiota (Figure 2) that showed four shared taxa between bark beetle species (all of them carried by *T. destruens*: *Fusarium* sp., *F. lateritium*, *Pestalotiopsis* sp. and *S. polyspora*). No phoretic fungal taxon was present exclusively in one pine species. All ecological indices calculated for fungal community are shown in Table 3.

The analysis of co-occurrence showed 100% random relationships between fungal taxa (P_{gt} and $P_{lt} \geq 0.05$ in all cases). However, the relationship between *S. polyspora* and *Fusarium* sp. showed an F_{obs} lower than the F_{exp}, and the associated probability was slightly higher than the significance level ($P_{lt} = 5.72 \times 10^{-2}$; effect size: -0.45). Results did not vary when an alternative analysis was performed excluding pairs of taxa in which F_{exp} resulted less than one (threshold use; i.e., 89.09% pairs of fungal taxa excluded from the analysis).

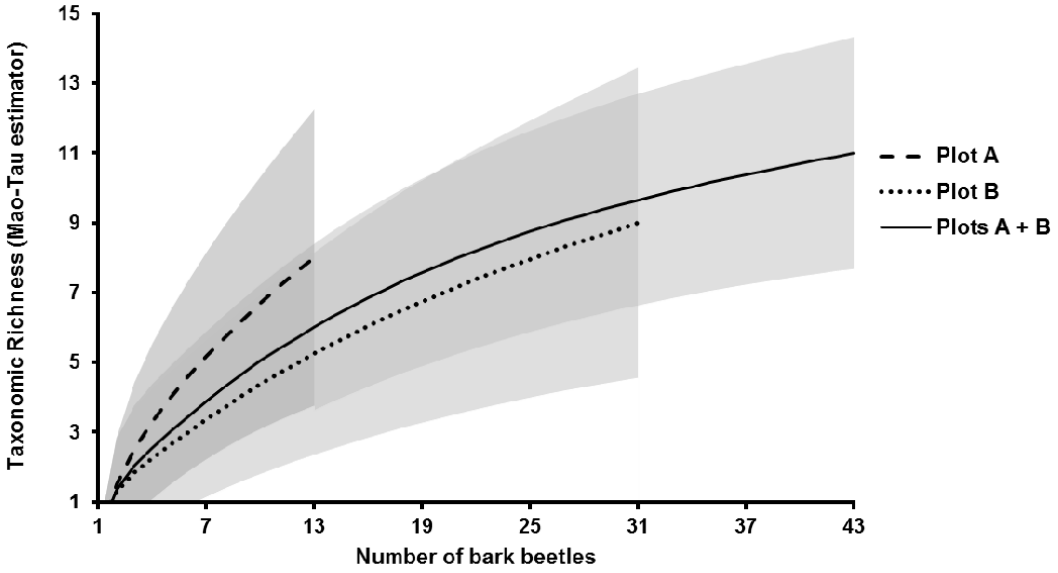

Figure 1. Taxonomic accumulation curves of fungal taxa carried by the two studied *Tomicus* species (shaded areas: 95% confidence intervals). Taxonomic richness computed as expected number of taxa (Mao-Tau estimator).

Table 3. Ecological indices calculated for fungal community.

Ecological Index	Value
Taxonomic richness (S_{obs}) (plot A/plot B/total)	8/9/11
ICE	13.78
Chao1	11.00
Chao2	12.95
Jack1	14.90
Jack2	16.86
Shannon (H)	1.43
Simpson (D)	0.42
Shannon evenness (J)	0.59
Simpson evenness (E)	0.21
Sorensen index (I)	0.70

ICE: Incidence-based Coverage Estimator of species richness.

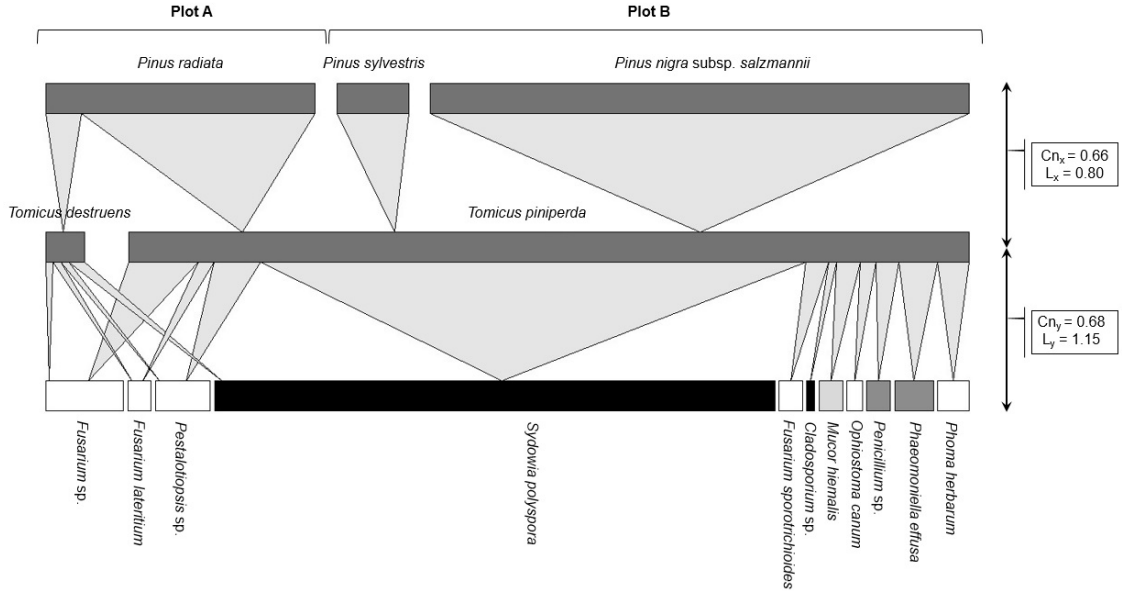

Figure 2. Tripartite graph of sampled mycobiota. Upper level: hosts (*Pinus* spp.); middle level: carrier insects (*Tomicus destruens* and *Tomicus piniperda*); lower level: phoretic fungal taxa. Rectangle width is proportional to the sum of interactions involving each taxon. Fungal classes are represented by colors in lower level boxes: white (Sordariomycetes), black (Dothideomycetes), light grey (Lecanoromycetes) and dark grey (Eurotiomycetes). Cn_x and Cn_y show the connectance for each network level. L_x and L_y show the mean number of links per taxa among levels.

4. Discussion

Phoretic fungal communities are driven by complex relationships, and the presence or absence of single involved taxa could have an ecological meaning. The observed richness (S_{obs}) was identical to the value of Chao1; meanwhile, Chao2 and ICE values increased by less than 26%. Jackknife richness estimators 1 and 2 showed values of 35.45% and 53.27% higher, respectively, than S_{obs}, suggesting that a larger sample size would have provided further information. Similarly, accumulation curves computed for the studied mycobiota suggested that more fungal species would have been obtained if more captures had been achieved. The majority of fungal species tended to appear in very low numbers (singletons and doubletons) while only few taxa were really frequent, as phoresy index values suggested (Table 2). This phenomenon seems to be common everywhere in nature, and it has been broadly reported either in fungi-insect associations or fungal endophytes [48–50]. Although pine host species were different among plots, the Sorensen index indicated a high-intermediate similarity in the mycobiota among plots (I = 0.70). In addition, the use of an identification method based on culturing in a specific medium implies a detection bias that must be taken into consideration [51–53], even though a generalist medium like PDA is selected. Considering these possible sources of richness underestimation, it is not possible to claim that the whole community was identified. Nevertheless, regarding previously discussed ecological indicators, it is possible to conclude that mycobiota were sufficiently characterised. Results from evaluating fungal community composition by their taxonomical placement agreed with other studies where Dothideomycetes and Sordariomycetes were the main taxonomic groups of fungal communities inhabiting pine species [52,54].

The observed fungal diversity carried by scolytids in this study was similar to that reported by Romón et al. [49] for bark beetles *Orthotomicus erosus* Wollaston and *Hylastes attenuatus* Erichson, associated with *F. circinatum* infected plots in the Basque Country (Spain). Meanwhile, fungal evenness was rather low, due to the high presence of a few taxa (dominance) previously commented. Interestingly, this finding accords with studies of fungal endophytes in pine twigs [54]. Moreover, lower values of fungal diversity were reported for ophiostomatoid fungi associated with *T. piniperda*,

as described by Romón et al. [55,56] in Spain. On the contrary, higher diversity values were observed in northern countries for *Hylurgus ligniperda* Fabricius [57] and even for *T. piniperda* [58,59]. An increase in fungal endophytic diversity in northern latitudes has been suggested [60], but how latitudinal gradients can affect phoretic communities composition remains understudied.

The mycobiota of two studied bark beetles included some *Fusarium* species reported in earlier studies for *Tomicus* genus [61,62]. The presence of this taxonomical group could be considered noteworthy due to the broad range of hosts and this genus pathogenic potential. Specifically, *F. sporotrichioides* is considered as an opportunistic pathogen while *F. lateritium* is not only considered as nonpathogenic but also as a possible antagonist against *F. circinatum* when *F. lateritium* performs an earlier colonization [49]. In this way, plot A showed evident symptoms of PPCD, and the presence of the pathogen in this area has been proved in previous studies [63,64]. In spite of this, *F. circinatum* was not present in collected insects either from this infected plot or from plot B. The most plausible explanation for this absence could be the previously reported low phoresy rate of *T. piniperda* [11,49], even in infected pine stands (4% according to Bezos et al. [12]). There is not much information on phoretic capacity of *T. destruens*, although it might be expected to be similar to its sister species. The absence of *F. circinatum* in plot B could suggest that this area was not yet infected by the pathogen. However, the high dispersion capacity of *F. circinatum* and the presence of plausible vector species indicate that the area cannot be ruled out as potentially threatened by PPCD. According to Bateman et al. [65], the association between *Fusarium* spp. and bark beetles could be explained by the abundant spore production of this genus (phoretic opportunism) and also by the pathogenic behavior of some *Fusarium* species, which could embody an advantage for bark beetles during tree colonization.

The most abundant species *S. polyspora* (anamorph: *Hormonema dematioides* Lagerb. & Melin) requires special attention. This fungus was a dominant species in the studied community and showed the highest and most significant value of PI. *S. polyspora* has been reported as very frequent species carried by insects [57,58] as well as an endophyte and a saprophyte [48,66,67]. Therefore, this fungus has been considered a primary coloniser of woodlands litter [68]. In contrast, Talgø et al. [69] identified the possible pathogenic traits of *S. polyspora* that could be involved in current season needle necrosis (CSNN), a disease that affects fir trees (*Abies* spp.) in Europe and the USA. With regard to the role that *S. polyspora* could play in association with bark beetles, Jankowiak and Kurek [70] proposed that it may embody an advantage during early host colonization. The aforementioned pathogenic behavior related to CSNN could support this hypothesis but, in contrast, Boberg et al. [71] reported that *S. polyspora* mainly consumes soluble compounds in pine needles, suggesting the absence of cellulolytic activity. Hence, the participation of this fungus in host colonization by bark beetles, and specifically with *Tomicus* species, remains unclear. Nevertheless, the observed abundance of *S. polyspora* can be explained by a two-way transmission that may simultaneously take place. On the one hand, the fungus could be abundant in shoots during bark beetle feeding period (endophytic way); on the other hand, insects could be loaded off spores directly from the litter (saprophytic way) where shoots had collected.

In terms of the other fungi observed, some of the genera isolated from spore suspensions have been cited as endophytes of *Pinus* spp., and consequently, could be transient members of the mycobiota. Regarding *Phaeomoniella effusa* Damm & Crous, this species has been previously isolated from old stumps of *Pinus mugo* Turra [66]. In the same way, *Pestalotiopsis* sp., which dominated in plot A as reported by Romón et al. [49], has been also considered as an endophyte of *Pinus* spp. [72,73]. *Phoma* spp. are frequent in the tissues of pines such as sapwood, needles and twigs [73,74] and are also carried by bark beetles [53,70,75]. Sanz-Ros et al. [54] found high rates of appearance of *Phoma herbarum* Westend in *P. sylvestris* supporting the endophytic role of this fungus.

Associations between ophiostomatoid fungi and bark beetles have been widely reported [70,76–79]. In this study the only member of this order was the blue stain fungus *Ophiostoma canum* (Münch) Syd. & P. Syd. This species seems to be more strongly associated with *Tomicus minor* Hartig than *T. piniperda* [62,80], which could explain the low frequency observed. However, this fungus has been also isolated from *T. piniperda* [61,81], being quite frequent among blue stain fungi carried

by this insect species [59]. *Penicillium* spp. have been frequently isolated from several species of bark beetles [53,61,82]. Anemochory is considered the main dispersion method for this genus, being the association between *Penicillium* sp. and bark beetles a consequence of contact during flight. The appearance of *Mucor hiemalis* Wehmer was probably caused by an eventual contact with insects because its spores are frequently distributed in soil and litter [58,62]. *Cladosposrium* sp. has been also cited as a random phoretic fungus even though the entomopathogenic potencial of this genus has also been reported [83,84], giving two plausible explanations of its presence in spore suspension.

Antagonism has been broadly documented among fungi [85,86], driving multiple interactions between species in nature. Curiously, the results provided by co-occurrence analysis only showed random associations among fungal taxa. However, it is noticeable that the relationship between *S. polyspora* and *Fusarium* sp. was close to being negative, according to analysis parameters. In consequence, a larger sample size supported with a confrontation assay could provide more data about the potential antagonistic effects among these fungal taxa [87].

In this study, the ecological network analysis was used as an approach to the complexity of the ecosystem starting from the observed mycobiota. The connectance was higher than previously described in other plant-beetle [88,89] and fungi-beetle [90] systems. Our results are circumscribed to the sampled mycobiota; therefore, new network studies in locations along the distribution ranges of these two *Tomicus* spp. could clarify the complexity of the complete ecological network. In addition, *T. piniperda* has been proposed as a predominant species in the study area according to some distribution models [16,18]. Nevertheless, its coexistence with *T. destruens* was observed in plot A, and it has been previously reported in *Pinus pinaster* Aiton stands in other locations of the Iberian Peninsula [16,17]. According to Lieutier et al. [91], the phenology of *T. destruens* can become similar to *T. piniperda* in cold areas. Our sampling areas were located in the North of Spain (Cantabrian Mountains) where annual rainfall is abundant and the climate is not as warm as in the Mediterranean Basin. These climatic aspects could explain why both species feed inside pine shoots during autumn and winter. The number of collected bark beetles was rather low, probably because the population is in an endemic phase in these sampling areas. Therefore, new studies should be performed in order to clarify the ecological interactions between both species in the Atlantic area.

5. Conclusions

1. *Sydowia polyspora* was the most frequent fungus carried by *T. piniperda* and *T. destruens*. This species dominated the sampled mycobiota.
2. The sampled mycobiota showed moderate taxonomic richness and diversity. However, the evenness was rather low.
3. *T. destruens* co-occurs with *T. piniperda* in Monterey pine plantations infected by PPCD in Cantabrian Mountains.

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

Appendix A

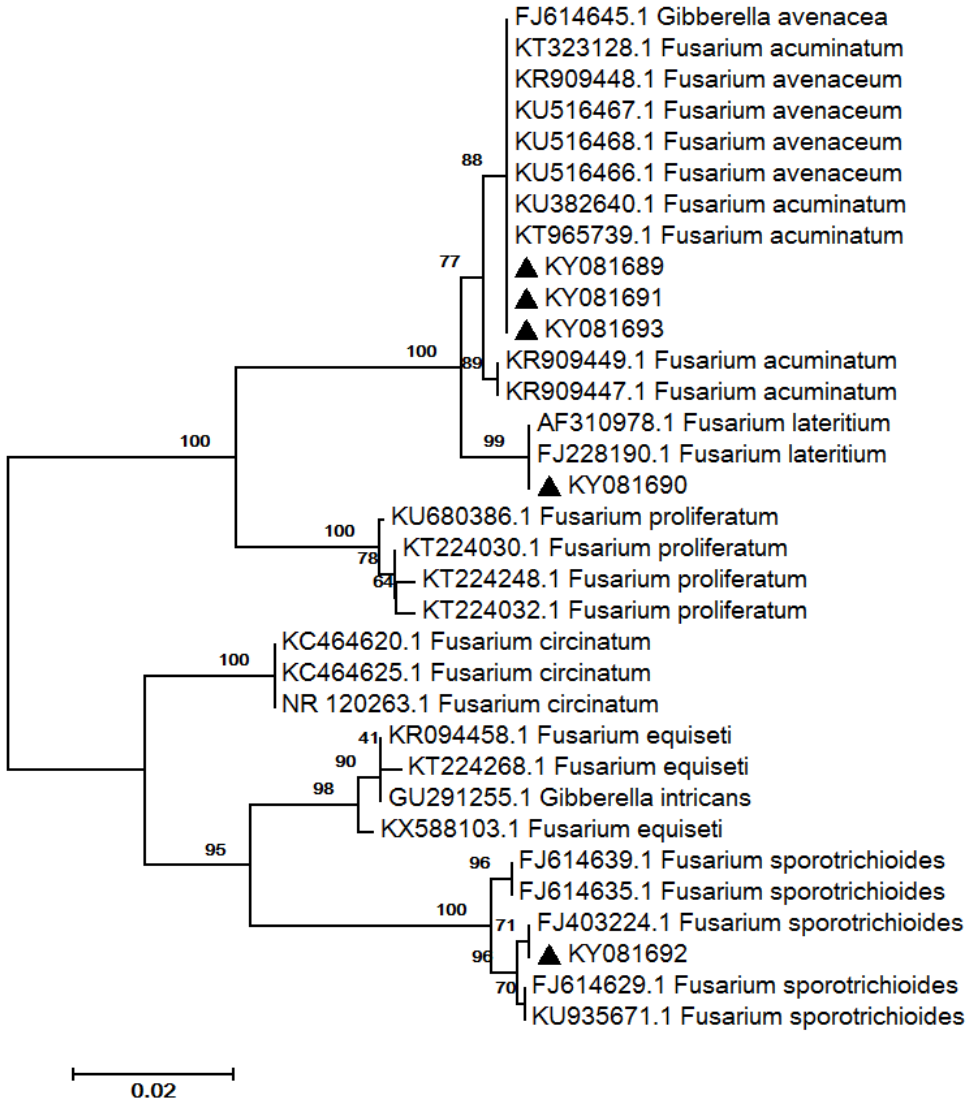

Figure A1. Neighbor-joining dendrogram of ITS showing *Fusarium* spp. clusters. The percentages of replicate trees in which the associated taxa clustered together in the Bootstrap method (1000 replications) are shown next to the branches. This dendrogram is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the dendrogram. Evolutionary distances were computed using the Kimura2 parameter method and are in the units of the number of base substitutions per site. The analysis involved 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 435 positions in the final dataset. Black triangles represent sequences of OTUs corresponding to *Fusarium* samples isolated in this study. GenBank accession numbers of each reference sample are provided.

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