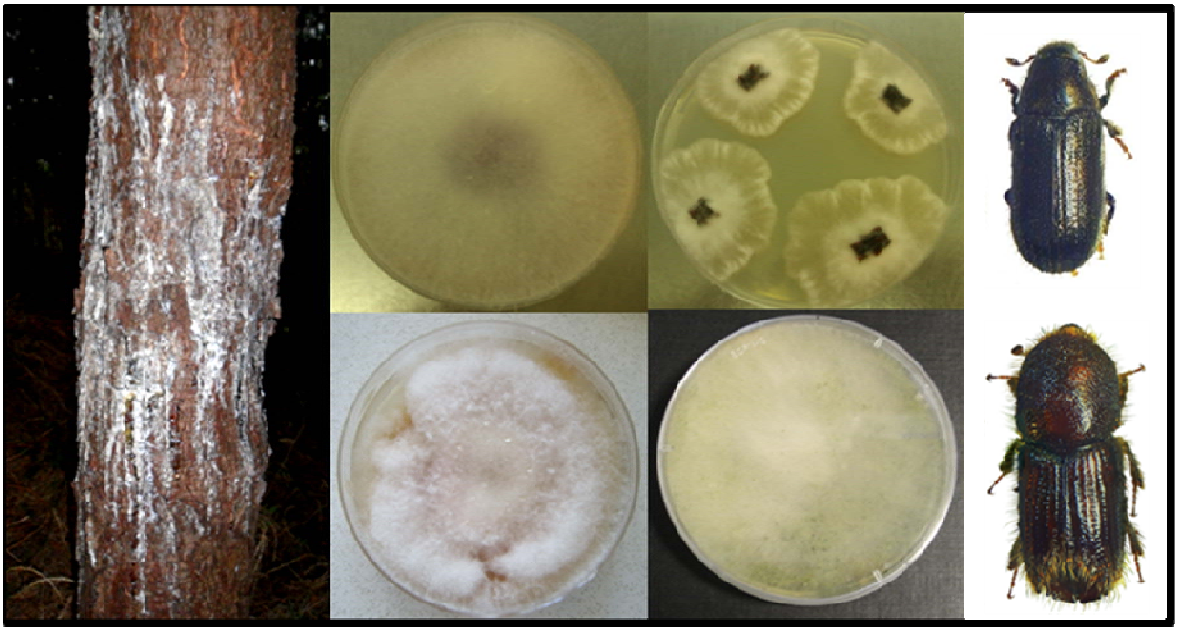
Fungi and insects diversity associated to pitch canker disease in *Pinus radiata* in northern Spain.



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ABSTRACT

Fusarium circinatum is the causal agent of pitch canker disease (PCD) on pines. This fungus is a threat to *Pinus radiata* plantations in Cantabria (Spain) due to the high susceptibility of this pine species. Several bark beetle species have been implicated as important vectors of spreading this pathogenic fungus in northern Spain and in California. These insects are also well-known for their association with other endophytic or pathogenic fungi. We hypothesize that the role of the different bark beetle species has not the same importance in the disease spreading in our forests due to the differences in their bioecology. The aims of this study are (1) to study the fungal diversity associated to both bark beetles and *P. radiata* affected trees (2) to evaluate the relationship between pruning and the diversity of fungi and bark beetles and (3) to know what is the role of Scolytinae species, regarding *F. circinatum* transportation and transmission in *P. radiata* plantations in Cantabria, Spain.

For these purposes, three kinds of traps were displayed in two *P. radiata* plots affected by *F. circinatum,* one of them was pruned and the other was unpruned. Thus, on each plot two Pityol baited funnel traps, three baited funnel traps (ethanol and α-pinene) and six pine logs were established. All of them were displayed with the aim of collecting the highest number of bark beetles species implicated on the transmission of the disease. For better understanding the role of *T. piniperda* in *F. circinatum* transmission, fresh green shoots with *Tomicus piniperda* feeding gallery were collected from the ground in 25 affected plots. Both, insects from the traps and logs were plated in culture media as well as tissues from the logs (xylem and phloem) and shoots. The presence of *F. circinatum* was confirmed by PCR with primers CIRC1A-CIRC4A. Other fungal species were identified by ITS region sequencing, but in the case of *Fusarium* spp. the region encoding the Translation Elongation Factor 1 alpha (α-TEF) was also amplified by PCR and sequenced. *Hylastes attenuatus, H. ater, H. angustatus* and *Ips sexdentatus* were the species most abundantly found in the logs whereas *H. ater* and *Xyleborinus saxeseni* were the more abundant species collected in ethanol and α-pinene traps. Some *Fusarium* spp., like *F. avenaceum*, appeared in high proportion in tissues and insects. Furthermore, several endophytic species that commonly appear in pines were isolated from both insects and tissues. Some of them, like *Trichoderma* spp., are known for their role in biological control of *Fusarium* spp. *Fusarium circinatum* was isolated from 1.05 % of the *Pityophthorus pubescens* specimens and from the 3.5 % of the shoots with *T. piniperda* feeding gallery. These results showed the important role of *T. piniperda* in the transmission of *F. circinatum* comparing with other bark beetles in our study area.

Key words: *Fusarium circinatum*, bark beetles, Monterey pine, endophytes.

RESUMEN

Fusarium circinatum es el hongo causante de la enfermedad del chancro resinoso de los pinos. La elevada susceptibilidad de *Pinus radiata* frente a este patógeno representa una amenaza para las plantaciones de la Comunidad Autónoma de Cantabria. Varias especies de barrenillos han sido descritas como importantes vectores de esta enfermedad, tanto en el norte de España como en California. Estos insectos son también importantes por su asociación con otros hongos endófitos o patógenos. La importancia de las diferentes especies de Escolítidos en la dispersión de este patógeno está directamente relacionada con su bioecología. Por lo tanto, los objetivos de este trabajo son (1) estudiar la diversidad fúngica asociada tanto con los insectos como con los pinos insignes afectados y (2) evaluar si existe relación entre poda y la diversidad de insectos y hongos (3) conocer el papel de las diferentes especies de barrenillos en lo que respecta a la transmisión de la enfermedad en plantaciones de *P. radiata* en Cantabria. Con este propósito se dispusieron tres tipos de trampas en dos parcelas de *P. radiata* afectadas por la enfermedad, en una de las ellas se habían efectuado labores de poda mientras que en la otra no. Así, en cada parcela se colocaron dos trampas multiembudo cebadas con pityol, tres trampas multiembudo cebadas con etanol y α-pineno y seis montones de trozas cebo procedentes de pinos sanos. Todas ellas con la finalidad de capturar el mayor número de especies implicadas en la transmisión. Además, se recogieron ramillos del suelo horadados por *Tomicus piniperda* en 25 parcelas afectadas con el objetivo de conocer el papel de este insecto en la transmisión de la enfermedad. Semanalmente, se recogieron tanto los insectos de las trampas como los tejidos de trozas (xilema y floema) y ramillos caídos al suelo con galería de *T. piniperda*. El material vegetal y los insectos se sembraron en medio de cultivo. Los hongos se clasificaron por morfotipos y fueron posteriormente identificados. La identificación molecular de *F. circinatum* fue llevada a cabo mediante PCR con los cebadores específicos CIRC 1A y CIRC 4A. Las demás especies de hongos se identificaron mediante amplificación por PCR con cebadores ITS 1F e ITS 4 y posterior secuenciación. Las especies pertenecientes al género *Fusarium* fueron identificadas mediante análisis morfológico así como mediante la secuenciación de la región que codifica para el Factor de Elongación de la Traducción 1 alfa (α-TEF). *Hylastes attenuatus, H. ater, H. angustatus* e *Ips sexdentatus* fueron las especies que aparecieron más frecuentemente en las trozas, mientras que *H. ater* y *Xyleborinus saxeseni* fueron las especies más abundantes en los embudos con etanol y α-pineno. Algunas especies del genero *Fusarium*, como *F. avenaceum*, y varias especies de hongos

endófitos aparecieron en elevada proporción tanto en los tejidos como en los insectos. Algunas de ellas, como *Trichoderma* spp., son importantes por su papel en el control biológico de *Fusarium* spp. *Fusarium circinatum* se aisló del 1.05 % de los espécimenes de *Pityophthorus pubescens* y del 3.5 % de los ramillos con galería de alimentación de *T. piniperda*. Estos resultados destacan el importante papel de *T. piniperda* respecto a las demás especies de barrenillos en nuestro área de estudio.

Palabras clave: *Fusarium circinatum,* barrenillos, pino de Monterrey, endófitos.

INTRODUCTION

Fusarium circinatum Nirenberg and O'Donnell (1998) (teleomorph=*Gibberella circinata*) is the causal pathogen of pitch canker disease (PCD) on pines. This fungus threatens pine plantations and natural stands throughout the world (Wingfield *et al.*, 2008), especially *Pinus radiata* D. Don plantations due to the high susceptibility of this pine species (Viljoen *et al.*, 1995), although other *Pinus* species like *P. pinaster* Ait. And *P. sylvestris* L. (Landeras *et al.*, 2005, Pérez-Sierra *et al.*, 2007) as well as *Pseudotsuga menziesii* (Gordon *et al.*, 1996) are susceptible to the pathogen. This disease leads great economical losses in wood industries due to the symptoms that spoil the trees (Pérez-Sierra *et al.*, 2007). The main symptom of PCD is the presence of pitch soaked cankers in trunks and big branches in adult trees which can girdle both trees and branches (Wikler *et al.*, 2003). Due to this girdling, seedlings can show damping-off and die. *Fusarium circinatum* is a seedborne pathogen characterised microscopically by the presence of sterile coiled hyphae, polyphialides in branched conidiophores, non-septate microconidia and multiseptate macroconidia. In culture on Potato Dextrosa Agar (PDA) *F. circinatum* has a white to greyish-violet aerial mycelium and can produce grey to dark pigmentation. It was first reported in North Carolina (Hepting and Roth, 1946) but has also been observed in Haiti (Hepting and Roth, 1953), California (McCain *et al.*, 1987), Japan (Muramoto and Dwinell, 1990), South Africa (Viljoen *et al.*, 1994), Mexico (Guerra-Santos, 1998), Chile (Wingfield *et al.*, 2002), Korea (Cho and Shin, 2004), France (EPPO, 2004), Spain (Landeras *et al.*, 2005), Italy (Carlucci *et al.*, 2007), Uruguay (Alonso and Bettucci, 2009) and Portugal (Bragança *et al.*, 2009).

Monterey pine is a widely planted conifer, with 199.000 ha in Spain mainly planted in the northern coast where it provides a lot of benefits due to its rapid growth. This pine species´ wood is well-known for its use like structural material in construction (Hermoso *et al.*, 2007).

Several insect vectors have been implicated as important means of spreading this pathogenic fungus in *P. radiata* plantations in northern Spain, e.g. *Pityophthorus pubescens* (Marsh.) and *Ips sexdentatus* (Börner) were found in Basque Country transporting *F. circinatum* (Romón *et al.*, 2007a). In California the importance of *Pityophthorus* spp. as vectors of *F. circinatum* has also been observed (Sakamoto *et al.*, 2007) because any fresh wound in the trees, like those caused by insects, provides an infection court for the pathogen (Dwinell *et al.*, 2001). Insects´ infection with *F. circinatum* spores may occur in pitch canker diseased trees or dead branches, thus, insects carrying the spores can inoculate them when

they feed or breed on healthy trees. Also, insects can act as wounding agents contributing to airborne spores' infection even if they do not carry *F. circinatum* spores. But not only the presence of wounding agents like insects is a relevant factor for the spreading and establishment of the disease, as specific environmental and moisture conditions are required for the pathogen infection (Gordon *et al.*, 2001). Another important factor concerning the relevant role of bark beetles in the disease spreading is the fact that populations levels may increase until epidemic levels (Raffa and Berryman, 1983). Although insect species have an important role on *F. circinatum* spreading, this dispersal is not exclusively dependant on them since it can be also dispersed by wind and water.Since *F. circinatum* was first reported in Spain, several studies has been carried out to identify which factors are influencing its distribution on the northern coast (Iturritxa *et al.*, 2011) although little is known about the role of other bark beetle species (Curculionidae: Scolitynae) like *Tomicus piniperda* L.. We hypothesize that the role of the different insect species has not the same importance in the disease spreading in our forests due to the differences in their bioecology, since *P. pubescens* attacks dead branches whereas *T. piniperda* feed on the shoots of healthy trees.

These vectors are also well known for their association with other endophytic (Romón *et al.*, 2008) or pathogenic fungi like *Ophiostoma* species (Giordano *et al.*, 2012, Bueno *et al.*, 2010). *Fusarium* species, which are widespread and abundant in living and dead plants, also appear commonly related to bark beetles (Teetor-Barsch and Roberts, 1983), e.g. *Fusarium solani* (Martius) (teleomorph=*Nectria haematococca*) that has a symbiotic relationship with *Hypothenemus hampei* (Ferrari) colonizing coffee beans (Morales-Ramos *et al.*, 2000) and with *Xyleborus ferrugineus* (Fabricius) in the work carried out by Baker and Norris (1968). Some *Fusarium* spp. have been reported as entomopathogenic fungi although they can also colonize the dead insects as saprophytes, for instance, in the pine beetle *Dendroctonus frontalis* (Zimmerman) infection with *F. solani* resulted in 90% dead insects in 5 days (Teetor-Barsch and Roberts, 1983), and was also isolated as a weak enthomopathogen from the bark beetle *Scolytus scolytus* F. (Barson, 1976).

Pines also appear associated with several fungi species, not only plant-pathogenic species but also endophytic ones which do not cause any damage to the tree. Some of these endophyte species, like *Trichoderma viride* Bissett could be used for biological control of *Fusarium* spp. (Martínez-Álvarez *et al.*, 2012). The genus *Fusarium* is important as a plant pathogen affecting forest species and agriculture ones because of the production of different types of mycotoxins. *Fusarium oxysporum* spp. complex show disease symptoms in a large number of vegetable crops (Román-Avilés *et al.*, 2011), although some *Fusarium* species

appear as endophytes or as saprophytes, like *F. verticilliodes* (Saccardo) (Teleomorph= *Gibberlla moniliformis*) (Summerell and Leslie, 2011).

The aims of this study are (1) to study the fungal diversity associated to both insects and *P. radiata* found in pitch canker affected stands, regarding especially the presence of *Fusarium* species (2) to evaluate the relationship between pruning and the diversity of fungi and bark beetles and (3) to know what is the role of several Scolytinae species regarding *F. circinatum* transmission in *P. radiata* plantations in Cantabria (Spain).

MATERIALS AND METHODS

Sampling

The sampling was carried out in two *P. radiata* plots affected by *F. circinatum*, located in Vejorís (Cantabria), one of the plots had been pruned before this work started and the other remained still unpruned.

In each plot three kinds of traps were displayed: 6 baited logs (3 thin logs from branches and 3 thick logs from trunks), two pityol (an aggregation pheromone of the genus *Pityophthorus* that attracts both males and females) baited funnel traps and three ethanol + α-pinene baited funnel traps. *P. pubescens* is the only species of this genus known in *P. radiata* stands in Basque Country (López Romero, 2007) and in Cantabria, as it was observed in this study.

For better understanding the role of *T. piniperda* in *F. circinatum* transmission, fresh fallen green shoots with *T. piniperda* feeding gallery (Figure 1) were collected from the ground in 25 plots. *Tomicus piniperda* is a phloephagous species that feed on shoots of healthy pine trees. A total of 285 *P. radiata* shoots with *T. piniperda* entrance hole and feeding gallery were collected from the ground in 25 plots located in *F. circinatum* affected areas. These plots were located in *F. circinatum* affected areas along a total of 8 different municipalities.

Figure 1. *Tomicus piniperda* within a shoot feeding gallery.

Tissues (xylem and phloem) from the breeding galleries, shoots and insects were collected weekly from June to October 2010 and carried to the laboratory.

Fungi identification

Vegetal samples from logs and shoots were plated in PDAS (PDA with streptomycin sulfate) culture media after surface sterilization (1´ tap water, 1´ethanol 70%, 1´ sodium hypochlorite 20%, 1´destilled sterilized water). Moreover, a total of 458 insects were plated on *Fusarium* selective media (FSM: 15gr bactone peptone, 1gr KH_2PO_4 monobasic, 0.5gr $MgSO_4.7H_2O$, 20gr agar, 0.2gr PCNB and 0.3 streptomycin sulfate) for avoiding bacterial contamination. All specimens of all the species were cultured with the exception of *P. pubescens* where 97 individuals, from the 143 collected, were cultured.

Fungi isolated from plant tissues and insects were classified into morphotypes on the basis of cultural characteristics, thus mycelia were classified into morphological units (Lacap *et al.*, 2003). Those fungi growing on PDAS from plant tissues were classified into 30 morphotypes whereas those growing on FSM from insects were classified into 17 different ones. Single hyphae cultures were made from one isolate of each morphotype for proper molecular identification, with the exception of those that were red, orange, yellow, violet or pink. From them, 40 isolates were selected for molecular identification.

DNA extraction with phenol was made from the colony (Vainio *et al.*, 1998). Once the DNA was extracted the polymerase chain reaction (PCR) was run to amplify the Internal Transcribed Spacer region (ITS) of the rDNA with primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA- 3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC- 3′) (Gardes and Bruns, 1993). For amplification the thermal cycling program was: 10 min denaturation at 95ºC followed by 13 cycles of 35 sec at 95ºC, 55 sec at 55ºC and 45 sec at 72ºC; 13 cycles of 35 sec at 95ºC, 55 sec at 55ºC and 2 min at 72ºC; 9 cycles of 35 sec at 95ºC, 55 sec at 55ºC and 3 min at 72ºC; and a final elongation 7 min at 72ºC. PCR product was sent to sequencing (Secugen, Madrid) after purification (Nuecleo Spin Gel and PCR Clean up, Macherey Nagel). The ITS region sequences were corrected with Genious Pro 5.6.5 software package for proper search with Blast in the Gen Bank data base.

Since ITS region is not specific for identifying *Fusarium* species at species level, those fungi that apparently were *F. circinatum* were identify with the specific primers CIRC 1A (5′-CTTGGCTCGAGAAGGG-3′) and CIRC 4A (5′-ACCTACCCTACACCTCTCACT-3′) as

described by Schweigkofler *et al.* (2004). Agarose gel electrophoresis at 1% was run to observe a 360 bp band.

Furthermore, microscopic morphological and morphometric identification of *Fusarium* spp. was carried out. For this purpose, 29 isolated fungi that according to their ITS region belonged to the genus *Fusarium* were plated on Spezieller Nahrstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA), both media specifically used for *Fusarium* species identification (Leslie and Summerell, 2006). After 10 to 20 days, SNA diagnostic characters including shape of the macroconidia, presence or absence of microconidia, shape and mode of formation of microconidia, shape of conidiogenous cells and presence or absence of chlamydiospores were observed, colour and size of the sporodochia were observed in CLA (Figure 2). This samples were amplified with the primers EF1 (forward primer; 5´-ATGGGTAAGGA(A/G)GACAAGAC-3´) and EF2 (reverse primer; 5´-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3´) (O'Donnell *et al.*, 1998) for sequencing the Translation Elongation Factor (α-TEF) region, which encodes an essential part of the protein translation machinery, and is highly informative at the species level in *Fusarium* (Geiser *et al.,* 2004). PCR reactions were done in reaction volumes of 50 µL: 1 µL template DNA, 1x reaction buffer, 200 mM dNTPs ,0.4 µM forward and reverse primers , 1U of Kapa Taq DNA polymerase. The thermal profile of PCR was one cycle of 10 min 94ºC followed by 36 cycles of 30 sec at 94ºC, 55 sec at 62º C and 1 min at 72ºC and a final 10 min extension at 72º (modified from Pérez-Sierra *et al.*, 2007). The α-TEF region was sequenced and the corrected sequences were search in the Gene Bank by BLAST and FUSARIUM ID sequence data base.

Once α-TEF region sequences of the identified *Fusarium* spp. had been corrected, multiple sequences alignment was done using Geneious Pro. The algorithm used was the one provided by the software. Tamura-Nei genetic distance model was used for calculating distance matrix and the tree was built following Neighbor-joining method.

Figure 2. *Fusarium* spp. samples cultured in different media (from left to right): PDAS, CLA and SNA.

Insects molecular identification

Tomicus piniperda and *T. destruens* (Woll.) are morphologically difficult to distinguish, although they have differences in their live cycles (Gallego and Galian, 2001). Thus, 17 *Tomicus* samples collected from the feeding galleries were sent to a specialized laboratory for molecular identification (Department of Animal Biology, Faculty of Veterinary, University of Murcia).

Statistical analyses

Chi square test was performed using Vegan package of R software to evaluate differences between pruned and unpruned plots regarding the insects species´ distribution.

Ips sexdentatus samples and their galleries in logs were analyzed in order to search for differences between the associated fungal communities. Dissimilarities matrix (Jaccard method) was constructed before Analyses of Similarities (ANOSIM) was performed. In these analyses 2 factors were included: source (Insect or plant tissue) and tissue (xylem or phloem). These fungal species were fitted in Principal Component Analysis (PCA) after data logarithmic transformation.

Hylastes attenuatus samples and their galleries in logs were analyzed to search for differences between the associated fungal communities Dissimilarities matrix (Jaccard method) was constructed before ANOSIM was performed. In these analyses 3 factors were included: Source (Insect or plant tissue), tissue (xylem or phloem) and prune (pruned or unpruned). These fungal species were fitted in a Canonical Correspondance Analysis (PCA) after data logarithmic transformation.

Ips sexdentatus and *H. attenuatus* were selected for these analyses among all the insect species collected in logs for being the most abundant ones and for its association with *F. circinatum*.

Regarding the fungal community associated to those shoots collected in Vejorís (pruned and unpruned plots), dissimilarities matrix (Jaccard method) was constructed before ANOSIM was performed. Factor prune (pruned or unpruned) was included in this analysis to know if the associated communities were different depending on the plot

Those species that only appeared in one sample (singletons) were removed from the analyses.

RESULTS

Collected insects

A total of 504 bark beetles of 12 different species were collected in the traps and shoots. Of them, 486 came from the traps located in Vejorís (157 were collected in the pruned plot and 329 from the unpruned one). Regarding the shoots sampling, 19 specimens of *T. piniperda* were found within the shoots feeding galleries in the 25 plots (6.6% of occupation) only one of them was found in a shoot from the unpruned plot in Vejorís.

Pityophthorus pubescens was the more abundant species followed by *I. sexdentatus* and *H. attenuatus* (Table 1). All *P. pubescens* specimens were collected in pityol baited funnel traps, whereas *I. sexdentatus* was always collected from thick logs located in the unpruned plot. Regarding *H. attenuatus* results, it appeared most commonly in logs than in other kinds of traps. From the remaining species, less than 30 individuals were collected (Table 2) in the different kind of traps, most species were phloephagous with the exception of two species found in ethanol traps (*Xyleborinus saxeseni* and *Xyleborus dispar*) which are xylomycetophagous.

Table 1. Insects species collected from logs, baited funnel traps (ethanol and pityol) and fallen shoots.

Insects Species	Total number	Logs	Ethanol	Pityol	Shoots
Pityophthorus pubescens	143	0	0	143	0
Ips sexdentatus	116	116	0	0	0
Hylastes attenuatus	86	83	2	0	1
Orthotomicus erosus	30	30	0	0	0
Crypturgus mediterraneus	26	26	0	0	0
Hylastes ater	25	20	5	0	0
Hylastes angustatus	23	23	0	0	0
Xyleborinus saxeseni	22	1	21	0	0
Hylurgops palliatus	9	9	0	0	0
Xyleborus dispar	4	0	4	0	0
Hylastes linearis	1	1	0	0	0
Tomicus piniperda	19	0	0	0	19

Table 2. Number of insect of each species collected in Vejorís from pruned (P) and unpruned plots (UP).

Species	Pruned	Unpruned	Total
Pityophthorus pubescens	77	66	143
Ips sexdentatus	0	116	116
Hylastes attenuates	39	47	86
Orthotomicus erosus	1	29	30
Crypturgus mediterraneus	0	26	26
Hylastes ater	10	15	25
Hylastes angustatus	10	13	23
Xyleborinus saxaseni	17	5	22
Hylurgops palliatus	1	8	9
Xyleborus dispar	2	2	4
Hylastes linearis	0	1	1
Tomicus piniperda	0	1	1
Total	157	329	486

Chi square test indicated that there were significant differences between pruned and unpruned plot regarding insects species abundances distribution (χ^2 = 142.02, d.f.=11, *p-value*<0.001). The insect species distribution can be observed in figure 3, where each column represents the proportion of insects collected in each plot and the wide of the column represent the proportion of insects of each species.

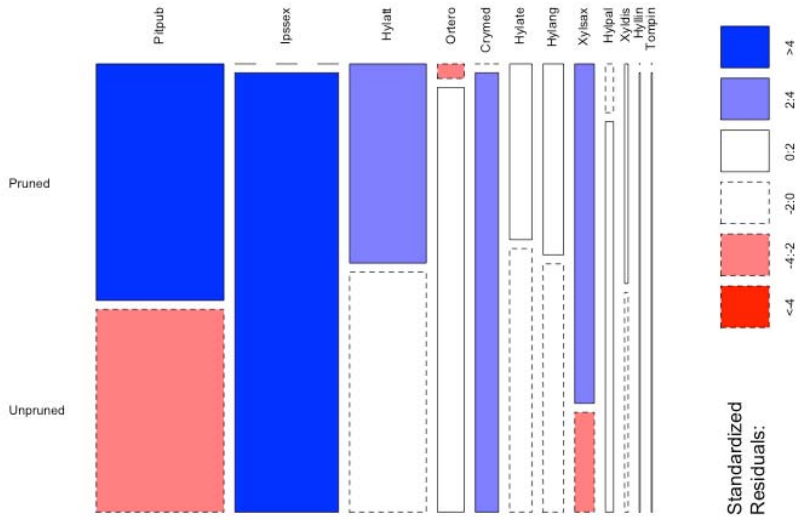

Figure 3. Mosaic plot for the insects species distribution in pruned and unpruned plots. Different colours represent how each residual value differs from the expected one. (Pitpub: *Pityophthorus pubescens*, Ipssex: *Ips sexdentatus,* Hylatt: *Hyalstes attenuatus,* Ortero*: Orthotomicus erosus,* Crymed: *Crypturgus mediterraneus,* Hylate: *H. ater*, Hylang: *H.angustatus,* Xylsax: *Xyleborinus saxaseni,* Hylpal: *Hylurgops palliatus,* Xyldis: *Xyleborus dispar,* Hyllin*: H. linearis,* Tompin: *Tomicus piniperda*).

Fungi Identification

Fusarium circinatum was isolated from 3 bark beetle species: *H. attenuatus* collected from logs (1.20 %), *P. pubescens* from pityol funnel traps (1.05%), and from *I. sexdentatus* (0.86%). Moreover, other species from the genus *Fusarium* were also isolated from the insects. From the 14 isolates identified, six *Fusarium* species were identified: four isolates were *F. oxysporum* spp. complex, one was *F. anthophilum* (A. Braun) Wollenw, six were *F. avenaceum* (Fries) Saccardo (Teleomorph= *Gibberella avenacea*), one *F. sambucinum* Fuckel (Teleomorph= *Gibberella pulicaris*), one *F. tricinctum* (Corda) (Teleomorph=*Gibberella tricincta*) and another one *F. konzum* (Teleomorph=*Gibberella konza)*. *Candida fructicans*, *Neonectria radicicola* (Gerlach & L. Nilsson) Mantiri & Samuels, *Penicullium thomi* Maire, *Trichoderma atroviride* and *Gliocadium roseum* Bainier were also identified. Other seven fungi species remained unidentified (Fungal species from 1 to 7). *Fusarium* spp. ware the most frequently isolated species, highlighting *F. avenaceum*. It is also interesting to notice that the totality of the species of the insects carried at least one *Fusarium* spp. (Table 3), and in some cases one insect species was associated to more than one *Fusarium* (Table 4).

Any fungus species was isolated from some insect samples when plated in FSM (Figure 4) presumably due to the presence of the fungicide PCNB, as in the case of *Ips sexdentatus*, where the 58.6% of the insects were not associated to any fungal species.

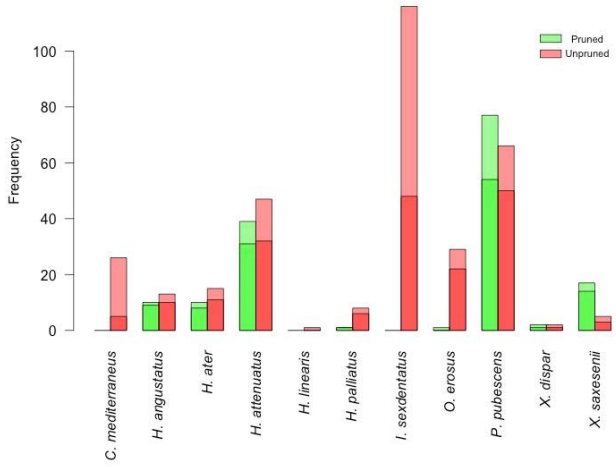

Figure 4: Graphic representation of collected insect species in pruned plot (green columns) and unpruned plot (pink columns) associated with some fungus species (dark green and dark pink bars, respectively).

Tabla 3. Percentage of fungal species isolated from different insect species.

Species	*P. pubescens*	*I. sexdentatus*	*H. attenuatus*	*O. erosus*	*C. mediterraneus*	*H. ater*	*H. angustatus*	*X. saxeseni*	*H. palliatus*	*X. dispar*
Candida fructicans	25.7	0.9	8.1	10	-	8	4.3	27.3	11.1	25
Fusarium spp.	20.6	34.48	32.5	56.6	12	64	39.1	31.8	55.5	25
Fusarium circinatum	1.05	0.9	1.6	-	-	-	-	-	-	-
Gliocadium roseum	-	-	2.3	-	-	-	-	-	-	-
Neonectria radciicola	5.1	-	2.3	-	-	-	4.3	22.3	-	-
Penicillium thomi	16.5	-	-	-	-	-	8.7	4.5	-	-
Trichoderma atroviride	1.03	0.9	3.48	3.3	8	-	4.3	-	-	-
Sp1	25.7	0.9	8.1	10	-	8	4.3	27.3	11.1	25
Sp 2	1.03	-	11.6	-	-	-	8.7	-	-	-
Sp 3	-	-	4.6	-	-	8	4.3	-	11.1	-
Sp 4	1.03	1.7	1.16	-	-	-	-	-	-	-
Sp 5	1.03	1.7	6.96	-	-	-	8.7	-	-	-
Sp 6	-	1.7	2.3	6.6	-	4	-	4.5	-	-
Sp 7	2.06	0	5.81	-	4	-	-	4.5	-	-

Regarding plant tissue (xylem and phloem) cultured from the logs breeding galleries, *F. circinatum* appeared in a 0.43% of the xylem samples and in the 0.85% of the phloem ones. *Hylastes angustatus´* galleries were the most frequently associated with *F. circinatum* (5.5% of the xylem samples and 5.2% of the phloem ones) whereas the phloem on *H. attenuatus´* galleries presented *F. circinatum* in the 1.36% of the samples. Other *Fusarium* spp. were isolated from xylem and phloem. From the seven identified isolates, we got four species: one isolate belonged to *F. oxysporum* spp. complex, one was *F. sporotrichioides* Sherbakoff from phloem, two were *F. beomiforme* (Nelson, Toussoun & Burgess) from both, phloem and xylem, and three were *F. avenaceum* isolated from xylem (Table 3). Other endophytes were also identified: two *Pestalotiopsis* different species., *Mucor* sp, *Trichoderma harzianum* Rifai and two other *Trichoderma* spp., *Diplodia pinea* (Desm.), *Peniophora pini* (Schleich.) Boidin and *Penicullium glabrum* (Wehmer) Westling.

Fungal communities associated to *Ips sexdentatus* and those associated to its galleries were significantly different (*p-value*<0.01, R= 0.63). Regarding xylem and phloem there were no significant differences in the associated fungal communities (*p-value*>0.05). This data were represented in PCA (Figure 5). The effect of the factor prune could not be analyzed because of the absence of *I. sexdentatus* samples in the pruned plot.

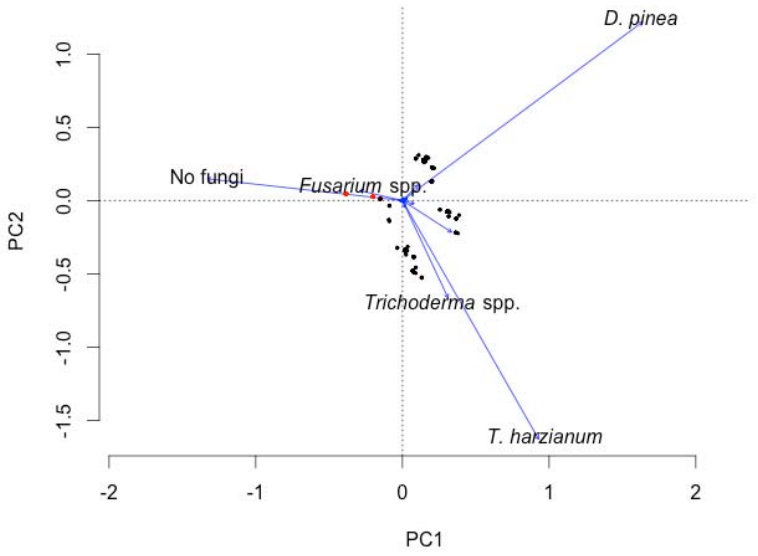

Figure 5. PCA of *I. sexdentatus* associated fungi. Black spots represent plant tissue samples and red ones represent insect samples.

Hylastes attenuatus was the insect species that more different fungal species carried. Fungal communities associated to *H. attenuatus* and those associated to its galleries were significantly different ($p<0.01$, R= 0.38). Regarding the fungi associated to both, insects and galleries, there were significant differences between pruned and unpruned plots (*p-value*<0.01, R= 0.028). These data were represented in CCA (Figure 6).

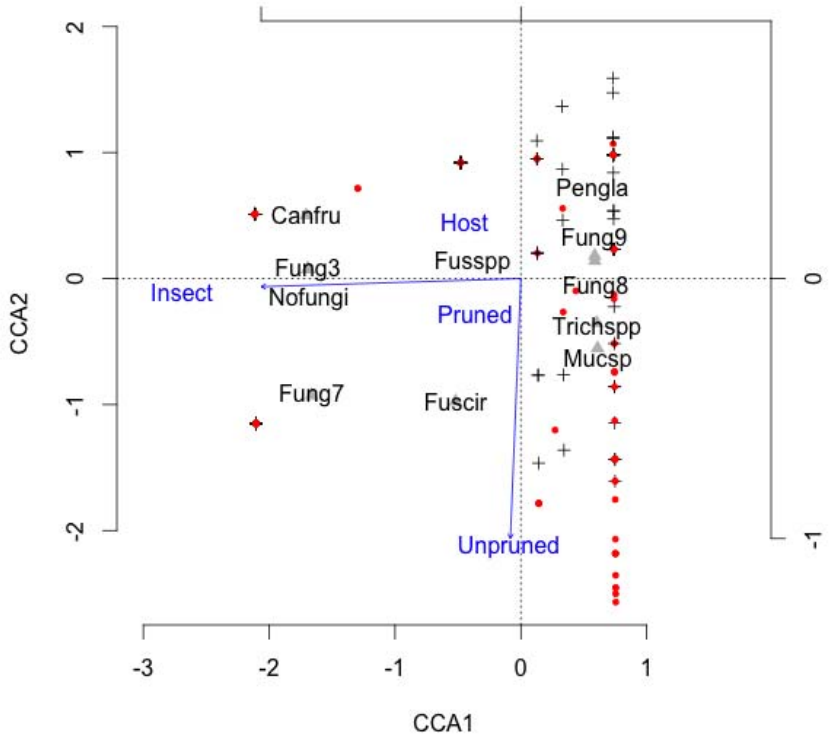

Figure 6: CCA representation of insect samples (red spots), plant tissue samples (black cross).(Canfru: *Candida fructicans*, Fung3: Species 3, Fung7: Species 7, Fusspp: *Fusarium* spp, Fuscir: *F. circinatum*, Pengla: *Penicillium glabrum*, Fung 8: Species 8, Fung 9: Species 9, Trichspp: *Trichoderma* spp. , Mucsp: *Mucor* sp.)

From the 285 fallen shoots, 8 were collected in June, 5 in July, 14 in August, 164 in September and 94 in October (Figure 7) taking into account the 25 plots. Of them 81 were collected in both prune and unpruned plots in Vejorís. Regarding the total number of collected shoots, *F. circinatum* was isolated from the 3.5% of the tissues coming from *T. piniperda* shoots feeding galleries. September was the month in which *F. circinatum* was more frequently isolated from the shoots. Four *Fusarium* species (22.1%) were isolated from these shoots when eight isolates were analyzed: three isolates were *F. avenaceum*, three were *F. tricinctum*, one isolate belonged to *F. cortaderiae* and another one was *F. sporotrichioides* (Table 4). Other fungi species were isolated and identified: *Diplodia pinea* (88.4%), three *Pestalotiopsis* spp. (24.5%), *Gliocadium roseum* (12.2%), *T. harzianum* (11.9%) and two other *Trichoderma* spp. (9.1%), *Penicullium glabrum* (11.2%). *Mucor* sp.

(3.8%), *Botritys cinerea* (6.3%), *Epicoccum nigrum* (1.7%) and *Trichoderma atroviride* (1.4%). From plant tissues (xylem, phloem and shoots) six fungal species remain unidentified (Fungal species from 8 to13).

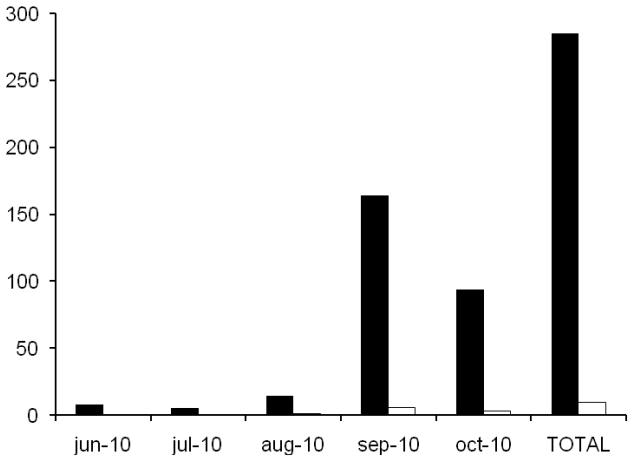

Figure 7. Number of shoots collected per month (black) and number of shoots with presence of *F. circinatum* (white).

Fungal communities associated to the 81 shoots collected in Vejorís did not show any differences between pruned and unpruned plot (*p-value*> 0.05). This data were fitted in a CCA (Figure 8).

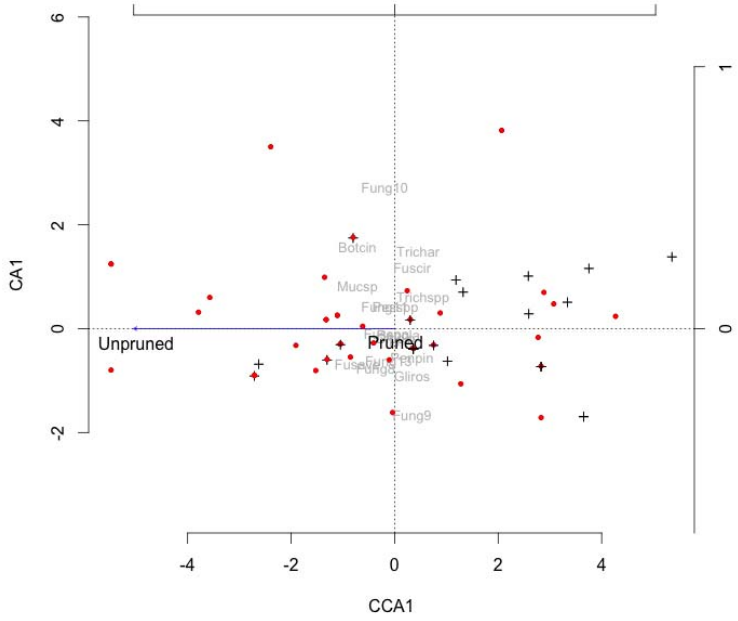

Fig 8: CCA of shoots collected from the unpruned plot (red spots) and shoots collected from the pruned one (black crosses) and fungal species. (Botcin: *Botritis cinerea*, Fung3: Species 3, Fung7: Species 7, Fusspp: *Fusarium* spp, Fuscir: *F. circinatum*, Pengla: *Penicillium glabrum*, Fung 8: Species 8, Fung 9: Species 9, , Fung 10: Species 10, Trichspp: *Trichoderma* spp. Triaatr: *T. atroviride*, Mucsp: *Mucor* sp., Penpin: *Peniophora pini*, Gliros: Gliocadium roseum).

Table 4. Identified *Fusarium* isolates, comparing results obtained from different methodologies.

N. Isolate	Origin	Collected in	ITS	Morphology	TEF	Species
1	*Hylastes ater*	Logs	*Fusarium lateritium*	*F. avenaceum*	-	*F. avenaceum*
2	*H. attenuatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
3	*H. attenuatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
4	*H. attenuatus*	Logs	*Fusarium* sp.	*F. anthophilum*	*F. avenaceum*	*F. anthophilum*
5	*H. attenuatus*	Logs	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
6	*Ips sexdentatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
7	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
8	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
9	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. tricinctum*	-	*F. tricinctum*
10	*I. sexdentatus*	Logs	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
11	*Orthotomicuserosus*	Logs	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
12	*Pityophthorus pubescens*	Funnel	*Fusarium* sp.	*F. sambucinum*	*F. sambucinum*	*F. sambucinum*
13	*Xyleborinus saxesni*	Funnel	*Fusarium* sp.	*F. konzum*	-	*F. konzum*
14	Phloem	Logs	*Fusarium* sp.	*F. sporotrichioides*	*F. sporotrichioides*	*F. sporotrichioides*
15	Phloem	Logs	*Fusarium* sp.	*F. beomiforme*	-	*F. beomiforme*
16	Phloem	Logs	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
17	Xylem	Logs	*Fusarium* sp.	*F. beomiforme*	-	*F. beomiforme*
18	Xylem	Logs	*Fusarium* sp.	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
19	Xylem	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
20	Xylem	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
21	Xylem	Logs	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
22	Shoot	Shoots	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
23	Shoot	Shoots	*Fusarium* sp.	*F. tricinctum*	*F. tricinctum*	*F. tricinctum*
24	Shoot	Shoots	*Fusarium* sp.	*F. cortaderiae*	*F. cortaderiae*	*F. cortaderiae*
25	Shoot	Shoots	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
26	Shoot	Shoots	*Fusarium* sp.	*F. avenaceum*	*F. avenaceum*	*F. avenaceum*
27	Shoot	Shoots	*Fusarium* sp.	*F. tricinctum*	*F. tricinctum*	*F. tricinctum*
28	Shoot	Shoots	*Fusarium* sp.	*F. tricinctum*	*F. tricinctum*	*F. tricinctum*
29	Shoot	Shoots	*Fusarium* sp.	*F. sporotrichioides*	*F. sporotrichioides*	*F. sporotrichioides*

Genetic distances between *Fusarium* sequences were represented in a dendrogram. It is interesting to notice that the sequences are grouped by species and in some cases they are even more related depending on the origin of the fungi isolate, e.g. *Fusarium avenaceum* coming from shoots collected in the unpruned plot in Vejorís or *F. avenaceum* from *I. sexdentatus*. *Fusarium oxysporum* identified from *I. sexdentatus* and phloem found in the same gallery of the thick logs is closely related branches in the dendrogram (Figure 9).

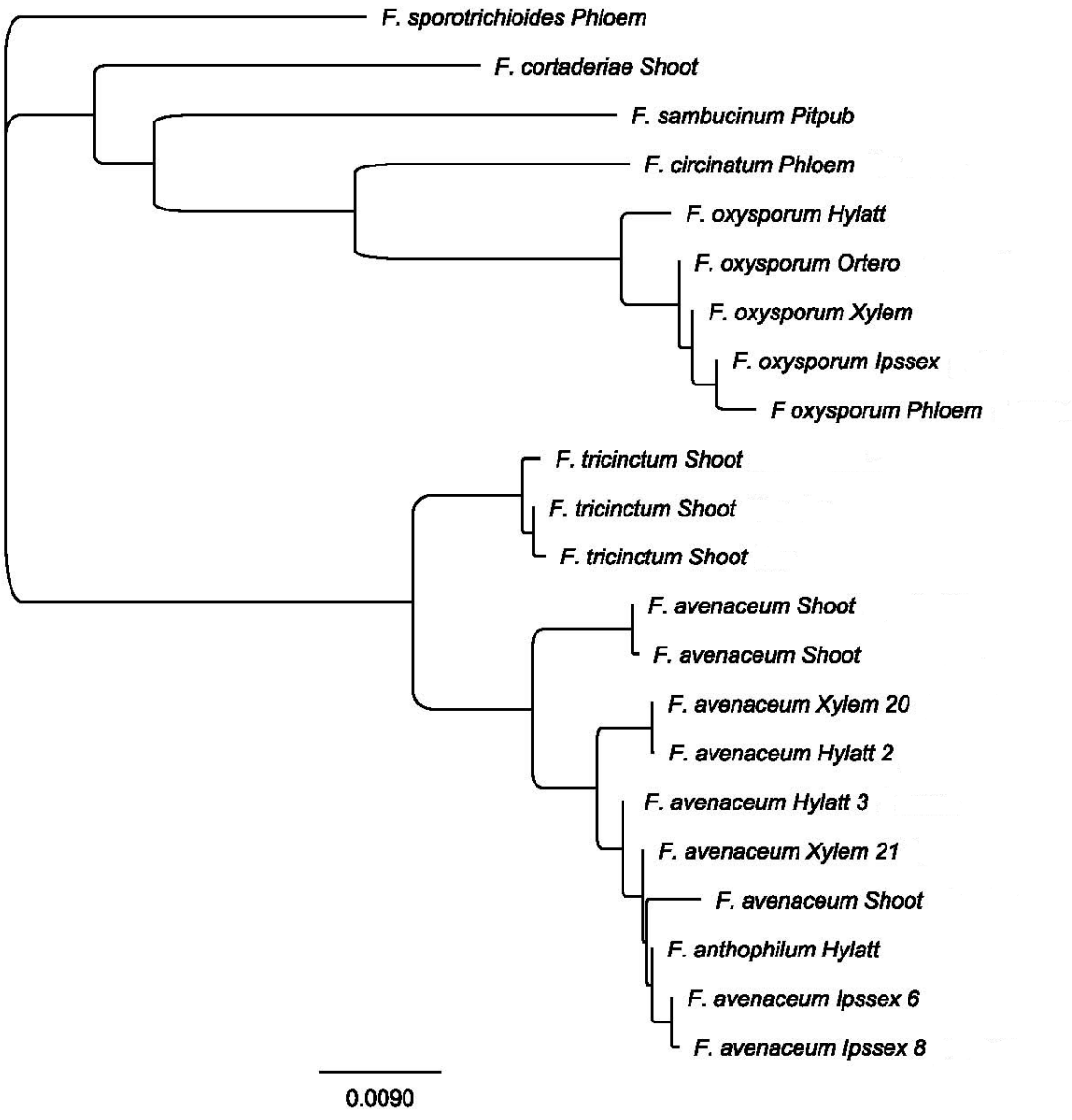

Figure 9. Dendrogram of *Fusarium* spp sequences from α-TEF region in a genetic distances tree. The origin of the sample is represented by: Xylem, Phloem, Shoot or insect species (Hylatt for *H. attenuatus*, Pitpub for *P. pubescens* and Ipssex for *I. sexdentatus*) followed by the number of isolate when necessary.

Insects molecular identification

After making the molecular analyses, we found that all the specimens belonged to the species *T. piniperda*.

DISCUSSION

The role of the different insects species in *F. circinatum* spreading depends on their bioecology. *Tomicus* piniperda is the most important candidate due to its shoot-feeding maturation behavior in the crowns of healthy pine trees (Jacobs *et al.*, 2004). *Fusarium circinatum* appeared in the 3.5% of the fallen shoots bored by *T. piniperda*. This insect species has also been related to some other pathogenic fungi like *Leptographium wingfieldii* Morelet in North America and in Europe (Jacobs *et al.*, 2004), but it has never been reported its relationship with the pitch canker fungus *F. circinatum*. The role of this bark beetle species could be also related to forest management, thus, in the study carried out by Långström and Hellqvist (1993) *T. piniperda´*s breeding galleries appeared more frequently related to those trees that had been pruned one month before beetles´ flight than in those that had been prune one year before. This could be an important issue to take into account due to the relationship between pruning and pitch canker disease (Bezos *et al.*, 2012), although in the present study no differences were found between pruned and unpruned plot regarding fungal diversity associated to shoots with *T. piniperda* feeding gallery.

The 1.05% of the captured specimens of *Pityophthorus pubescens* carried the pathogen. Bonello *et al.* (2001) reported the ability of *Pityophthorus* spp. in discriminating between healthy and pitch canker diseased branches due to the increasing ethylene emission in symptomatic branches. The importance of other species of this genus has been noticed in several countries; in California it was shown the importance of *P. carmeli* Swaine and *P. setosus* Blackman in *F. circinatum* spreading when they wound the potential host trees for testing their suitability (Sakamoto *et al.*, 2007).

Other bark beetle species captured in this study showed a relationship with *F. circinatum*: it was isolated from the 0.9% of the *I. sexdentatus* specimens, all of them collected in thick logs. *Ips sexdentatus* can inoculate the pathogen as a secondary bark beetle that can attack healthy trees when the population reach epidemic levels (Etxebeste and Pajares, 2011). In Basque Country (Spain) the 8.57% of the *I. sexdentatus* analyzed by Romón *et al.*, (2007a) carried the pathogen. In California other species of this genus, like

I. mexicanus and *I. paraconfusus,* were reported as vectors of the pitch canker fungus in Santa Cruz County (Fox *et al.*, 1991). Some authors explain this association between insects and pathogenic fungi because it allows them to beat tree resistance (Lieutier *et al.*, 2009), but, as bark beetles at epidemic levels can kill healthy trees without carrying any phytopathogenic fungi some other authors state that insect-pathogen interactions could only benefit the fungus species which get to trees they could never reach without this association (Six and Wingfield, 2011).

Other fungi were isolated in this study from the insect galleries in logs and shoots, like *Diplodia pinea, Penicillium* spp., *Pestalotiopsis* spp., *Trichoderma* spp. and *Fusarium* spp. *Diplodia pinea* was the most frequently isolated species in plant tissue samples, although it did not appear in the insects´ bodies. This fungus is important due its pathogenicity causing shoot blight and dieback on pine trees, been previously recorded associated to pine species (Botella *et al.*, 2010) and also to bark beetles in Spain (Romón *et al.*, 2007b). *Penicillium* spp. had been previously isolated from pine species as pure saprobes in pines, as they rarely occur as endophytes in healthy tissues (Zamora *et al.*, 2008). *Pestalotiopsis* is an ubiquitous genus that appear as endophyte, saprobe and pathogenic in different hosts in a wide geographical distribution (Jeewon *et al.*, 2004). It has been reported in pine species in Spain, being present in healthy tissues (Zamora *et al.*, 2008) but some species like *Pestalotiopsis funerea* can cause damping off in other conifers (Bajo *et al.*, 2008). Four morphotypes were identified as *Trichoderma* spp. in this study, but two of them could not be identified at species level using ITS region, that means that other molecular markers would be necessary for this purpose. *Trichoderma harzianum*, *T. viride* and *T. atroviride* have been reported as effective biological control of pathogenic *Fusarium* spp. (Alves-Santos and Diez, 2012). *Trichoderma atroviride* parasitizes a large variety of phytopathogenic fungi thanks to the production of hydrolytic enzymes. This has allowed its use as a biological control agent (Olmedo-Monfil *et al.*, 2002).

Eleven species were identified growing when insects were plated in FSM, five of them belonged to the genus *Fusarium* as this culture media was specific for this fungi isolation. Bark beetles are known to be associated with some other phytopathogenic fungi, (Kirisits, 2004). In this study, *Neonectria radicicola* was isolated from four bark beetle species. This fungus species has been observed as endophytic in pine roots without causing any damage in Norway (Ndobe, 2012), although the genus *Neonectria* is known for causing neonectria canker in subalpine fir in Denmark (Talgø *et al.*, 2011) and stem cankers in *P. radiata* in Chile (Morales, 2009).

Nine morphotypes from both insects and plant tissue were identified as *Fusarium* spp. The genus *Fusarium* is a polyphyletic group that can appear as endophyte or as pathogen depending on the species and on the plat host, due to the specificity of this genus regarding plant-pathogen interactions. The species of *Fusarium* have a wide geographical distribution. Several *Fusarium* spp. are associated with insects and lots of them are known for being entomopathogenic, although those *Fusarium* species associated with Coleoptera are weakly entomopathogenic and frequently mutualistic (Teetor-Barsch and Roberts, 1983).

In this study, 10 species of this genus were identified following both molecular and morphological criteria; morphological and morphometric identification of *Fusarium* species were necessary for distinguish *F. anthophilum* where the DNA sequence seemed to belong to *F. avenaceum*, but the presence of napiform microconidia allowed us to define the isolate as *F. anthophilum*. So, morphological identification is strongly recommended for identifying species from the genus *Fusarium*. *Fusarium avenaceum* was the most commonly identified species of this genus, it was isolated from *I. sexdentatus* and *H. ater*, it also appeared in the galleries made by *T. piniperda* in shoots and in those made by *H. ater* in logs. *F. avenaceum* commonly occurs associated to crops and soils. *Fusarium tricinctum* was isolated from *I. sexdentatus* and from *T. piniperda* feeding galleries, this fungus usually occurs as a saprophyte or weak parasite in Europe and North America (Leslie and Summerell, 2006). *Fusarium oxysporum* spp. complex appeared associated to *O. erosus* and *H. attenuatus* and also isolated from *Ips sexdentatus* galleries in logs. This fungus is a saprophyte and pathogen soil species complex with a wide plant host range divided in many *formae specialis* depending on the host specificity. *Fusarium sporotrichioides, F. konzum* and *F. beomiforme* has not been described as plant pathogens or are very weak (Leslie and Summerell, 2006).

CONCLUSIONS

1. A total of 12 different bark beetle species were found in this study, from them 20 different fungal species were isolated.

2. In this study it was found an important association between bark beetles and *Fusarium* species. This fungal species did not only appear in the insect bodies but also associated to plant tissue from their galleries. This association should be more deeply studied in order to better understand the role of the different insect species in this genus distribution.

3. A total of 28 fungal species were associated to the insects galleries from logs and shoots. Of them 23 were identified, with high occurrence of *D. pinea* and *Fusarium* spp.

4. No association between pruning and fungal diversity was found in the fallen shoots collected in the plots located in Vejorís.

5. In *Fusarium circinatum* spreading, *T. piniperda* had the most important role among all the collected species due to its shoot-feeding maturation behavior.

6. *Pityophthorus pubescens* and *I. sexdentatus* were also associated to *F. circinatum* spores as well as *H. attenuatus*. The importance of these three species in the pathogen distribution has to be related with their population levels and stand conditions as they are not primary species. For these reasons, knowing the necessary conditions for the different bark beetles species to spread the disease is of big relevance.

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