Ophiostoma species (Ascomycetes: Ophiostomatales) associated with bark beetles (Coleoptera: Scolytinae) colonizing *Pinus radiata* in northern Spain

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Abstract: Bark beetles (Coleoptera: Scolytinae) are known to be associated with fungi, especially species of *Ophiostoma* sensu lato and *Ceratocystis*. However, very little is known about these fungi in Spain. In this study, we examined the fungi associated with 13 bark beetle species and one weevil (Coleoptera: Entiminae) infesting *Pinus radiata* in the Basque Country of northern Spain. This study included an examination of 1323 bark beetles or their galleries in *P. radiata*. Isolations yielded a total of 920 cultures, which included 16 species of *Ophiostoma* sensu lato or their asexual states. These 16 species included 69 associations between fungi and bark beetles and weevils that have not previously been recorded. The most commonly encountered fungal associates of the bark beetles were *Ophiostoma ips*, *Leptographium guttulatum*, *Ophiostoma stenoceras*, and *Ophiostoma piceae*. In most cases, the niche of colonization had a significant effect on the abundance and composition of colonizing fungi. This confirms that resource overlap between species is reduced by partial spatial segregation. Interaction between niche and time seldom had a significant effect, which suggests that spatial colonization patterns are rarely flexible throughout timber degradation. The differences in common associates among the bark beetle species could be linked to the different niches that these beetles occupy.

Key words: Pinus radiata, bark beetles, ophiostomatoid fungi diversity, new insect–fungal associations, colonization patterns.

Résumé : Les scolytes (Coleoptera : Scolytinae) sont des insectes ravageurs associés aux champignons, spécialement aux espèces *Ophiostoma* sensu lato et *Ceratocystis*. Cependant, on sait peu de choses sur ces champignons en Espagne. Dans cette étude, nous avons examiné les champignons associés à 13 espèces de scolytes et une espèce d'entiminae infestant le pin de Monterey (*Pinus radiata*) au Pays Basque au nord de l'Espagne. Cette étude comprenait l'examen de 1323 scolytes ou de leurs galeries, trouvés chez *P. radiata*. Les isolements ont résulté en un total de 920 cultures qui incluaient 16 espèces d'*Ophiostoma* sensu lato ou de leurs formes asexuées. Ceci incluait 69 associations entre des champignons et des scolytes jamais encore répertoriées. L'association fongique la plus fréquente impliquait les scolytes et *Ophiostoma ips*, *Leptographium guttulatum*, *Ophiostoma stenoceras* et *Ophiostoma piceae*. Dans la plupart des cas, la niche de colonisation avait un effet significatif sur l'abondance et la composition des champignons colonisateurs. Ceci confirme que l'empiètement entre les espèces est réduit par une ségrégation spatiale. L'interaction entre la niche et le temps avait rarement d'effet significatif, suggérant ainsi que les patrons de colonisation spatiale étaient rarement flexibles au cours de la dégradation du bois. Les différences entre les associations communes impliquant les scolytes pourraient être liées aux différentes niches que ces insectes occupent.

Mots-clés : Pinus radiata, scolytes, diversité des champignons ophiostomatoïdes, nouvelles associations insecte–champignon, patrons de colonisation.

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Introduction

Bark beetles (Coleoptera: Scolytinae) that infest coniferous trees are among the most destructive forest pests, and they represent a continuous threat to forests. Economic losses due to bark beetle infestations are difficult to quantify; approximate estimates exist for only a few species. For example, during 1989, massive outbreaks of *Tomicus piniperda* caused losses of 92 million Euros in the Basque Country of northern Spain (Amezaga 1993).

Very little is known about the bark beetles that occur on *Pinus* spp. in northern Spain. Seven major bark beetle species, *Hylurgops palliatus*, *Hylastes attenuatus*, *Ips sexdentatus*, *Dryocoetes autographus*, *Orthotomicus erosus*, *Hylastes ater*, and *T. piniperda*, have been reported on mature *Pinus radiata* stands in northern Spain. Other bark beetle species also occur in this area, but at lower population densities. These include *Hylurgus ligniperda*, *Pityogenes calcaratus*, *Pityophthorus pubescens*, *Hypothenemus eruditus*, *Xyleborus dispar*, and *Xyleborus dryographus* (Goldarazena 2004). Although they are generally considered secondary pests, *T. piniperda* and *I. sexdentatus* can cause serious infestations that result in tree mortalities under favourable conditions (Gil and Pajares 1986).

Ophiostomatoid fungi represent an artificial grouping of morphologically similar genera, including *Ophiostoma*, *Grosmannia*, *Ceratocystiopsis*, *Ceratocystis*, *Gondwanamyces*, and *Cornuvesica*. These genera represent two large phylogenetically unrelated groups, one with *Ophiostoma* close to the Dothideales and the other with *Ceratocystis* in the Microascales (Spatafora and Blackwell 1993; Zipfel et al. 2006). Anamorph genera associated with these teleomorph genera include *Pesotum*, *Leptographium*, *Sporothrix*, and *Hyalorhinocladiella* that are related to *Ophiostoma* whereas *Graphium* and *Thielaviopsis* are related to *Ceratocystis* (Upadhyay 1981; de Hoog 1993; Seifert and Okada 1993; Wingfield 1993; Wingfield et al. 1993).

Many ophiostomatoid fungi are pathogens of plants and, especially, trees (Harrington 1993). Others are saprophytic but they include some of the most important causal agents of sapstain and wood degrade (Seifert 1993). In northern Spain, sapstain fungi degrade high-quality pine logs, causing significant financial losses to the local forestry industry each year. These losses can amount to a 50% price reduction in wood assigned to the furniture industry in the Basque Country (Maderas Elorriaga Company, 2004, personal communication).

Many sapstain fungi, especially ophiostomatoid fungi, are associated with bark beetles. The association between bark beetles and fungi suggests that there is mutual benefit to both partners, although this matter is the subject of considerable debate (Paine et al. 1997). It has been demonstrated that some ophiostomatoid fungi help the bark beetle kill trees by reducing water conduction through the phloem (Lieutier 1993, 2004; Solheim et al. 2001); however, other blue-staining ophiostomatoid fungi compete with bark beetles for uncolonized host tissue and inhibit the development of mycangial fungi essential for larval feeding (Barras 1970).

Information about conifer bark beetle-associated ophiostomatoid fungi in the Iberian Peninsula is very limited (De Ana Magán 1982, 1983; Fernández et al. 2004; Villarreal et al. 2005). There have been only two studies published dealing with the taxonomy of these fungi in the area. One (De Ana Magán 1983) erroneously described a new species, *Leptographium gallaeiciae*, which was later identified as *Ophiostoma serpens* (Jacobs and Wingfield 2001). Another fungus in this group, *Ophiostoma sejunctum*, has recently been described (Villarreal et al. 2005), suggesting that these fungi deserve more study. The primary aims of this study were therefore to conduct surveys of ophiostomatoid fungi associated with several bark beetle species that infest *Pinus radiata* in northern Spain and to define the relationship between them and their vectors. A second objective was to consider spatial colonization patterns and temporal dynamics within populations and communities of these fungi.

Materials and methods

Collection of bark beetles and galleries

During 2004, beetles and galleries of *Hylurgops palliatus*, *Hylastes attenuatus*, *I. sexdentatus*, *Dryocoetes autographus*, *Orthotomicus erosus*, *T. piniperda*, *Hylastes ater*, *Pityogenes calcaratus*, *Hylurgus ligniperda*, *Pityophthorus pubescens*, *Hypothenemus eruditus*, *X. dryographus*, *X. dispar* (Coleoptera: Scolytinae), and *Brachyderes incanus* (Coleoptera: Entiminae) were collected from baiting logs in three stands of *Pinus radiata*, with a mean age of 21 years, in Biscay province (northwestern Morga and Muxika, and central Urkiola). These stands were sampled to characterize the dominant bark beetles and their fungal associates. In January 2004, 60 trap logs, 1.5 m long and 0.2 m in diameter (bark thickness about 2 cm), were set in each stand, using the technique described by Tribe (1992). Half the logs from each locality were partially buried, at an angle of 90°, and the other 30 were placed on the ground surface. Approximately every 6 weeks, three partially buried and three nonburied logs at each site were inspected for the presence of beetle entrance holes. Bark surrounding the entrance holes was cut and peeled away from the logs. All beetles from a single gallery were removed with sterilized tweezers, placed individually in sterile bottles, and morphologically identified with a LEICA MZ95 dissecting microscope to determine, with several taxonomic keys (Balachowsky 1949; Gil and Pajares 1986; Pfeffer 1995), taxonomic characteristics located in the pronotum, scutellum, elytra, metapisternum, and antennal funiculum. Complete galleries, including the cambium up to 2 cm away from the tunnel, were removed and placed in separate clean paper bags. The galleries, together with the beetles in them, were treated as single samples.

Isolation and identification of fungi

Beetles were removed from storage bottles with sterilized tweezers, and squashed onto the surface of a selective medium for *Ophiostoma* spp. (20 g malt extract, 20 g agar, and 1 L distilled water, amended with 0.05% cycloheximide and 0.04% streptomycin). Beetles from different galleries were incubated in separate Petri dishes at 25 °C in the dark for 2 weeks, during which time they were regularly examined for fungal growth and sporulation. Cultures were purified by transferring hyphal tips from the edges of individual colonies, or spore masses from the apices of emerging peri-

Table 1. Isolation number and frequencies (in parentheses) of fungi associated with bark beetles and their galleries in *Pinus radiata* in

	Hylurgops palliatus	*Hylastes attenuatus*	*Ips sexdentatus*	*Dryocoetes autographus*	*Orthotomicus erosus*	*Tomicus piniperda*	*Hylastes ater*
Ophiostoma ips	14 (3%)	8 (5%)	**73 (69.5%)**	**79 (58.5%)**	**43 (19.6%)**	3 (5.4%)	**4 (4.1%)**
Leptographium guttulatum	**85 (18.2%)**	**29 (17.6%)**	1 (1%)	10 (7.4%)	11 (5%)	**15 (27.2%)**	**9 (9.2%)**
Ophiostoma stenoceras	24 (5.1%)	**26 (16%)**	8 (7.6%)	11 (8.1%)	**46 (21%)**	—	1 (1%)
Ophiostoma piceae	**39 (8.3%)**	**21 (13%)**	—	—	4 (2%)	**12 (22%)**	2 (2%)
Ophiostoma pluriannulatum	**31 (6.6%)**	—	4 (4%)	—	11 (5%)	4 (7.2%)	**3 (3%)**
Ophiostoma piliferum-like	**41 (8.7%)**	**13 (8%)**	—	1 (0.7%)	—	—	1 (1%)
Ophiostoma quercus	21 (4.5%)	11 (6.7%)	—	—	—	—	**5 (5.15)**
Leptographium wingfieldii	**25 (5.3%)**	8 (5%)	—	—	1 (0.4%)	1 (2%)	**3 (3%)**
Ophiostoma olivaceum	10 (2.1%)	9 (5.5%)	5 (4.7%)	1 (0.7%)	1 (0.4%)	—	1 (1%)
Leptographium truncatum-like	18 (4%)	5 (3%)	1 (1%)	—	—	—	1 (1%)
Ophiostoma rectangulosporium-like	1 (0.2%)	—	1 (1%)	16 (12%)	3 (1.3%)	—	2 (2%)
Pesotum fragrans	10 (2.1%)	3 (2%)	—	—	1 (0.4%)	—	—
Diplodia pinea	4 (1%)	3 (2%)	—	—	—	1 (2%)	—
Ophiostoma minus	—	—	1 (1%)	2 (1.5%)	—	—	—
Ophiostoma floccosum	—	—	—	—	2 (1%)	—	1 (1%)
Ophiostoma canum-like	—	—	—	—	2 (1%)	—	—
Sporothrix schenckii-like	—	—	—	—	—	—	—
Total no. samples	467	164	105	135	219	55	98
Total no. isolates	323	136	94	120	125	36	33
H	2.24	2.16	0.84	1.11	1.61	1.25	2.20
S	13	11	8	7	11	6	12
E	0.87	0.90	0.40	0.57	0.67	0.69	0.88

Note: Values in bold represent dominant species for each bark beetle-associated fungal community. A species is considered dominant if $P_i > 1/S$, where The Shannon–Weaver index (*H*) indicates the biodiversity degree of a certain fungal community. Evenness (*E*) is a measure of the relative abundance of

thecia or conidiophores, to fresh 2% malt extract agar. Pure sporulating cultures were examined and identified with a LEICA DM4500B microscope. Fungi were assigned to putative genera and distinct taxa on the basis of hyphal morphology and characteristics of anamorph fruiting structures.

Bark beetle galleries were maintained in humid chambers at 25 °C and 70% relative humidity in the dark for 3–4 weeks. During this period, galleries were carefully examined under a dissecting microscope. Spore masses accumulating at the apices of perithecia or conidiophores produced in the galleries were carefully lifted out with a fine sterile needle and transferred onto 2% malt extract agar. These cultures were incubated at 25 °C in the dark for 2 weeks and purified, where necessary, as previously described. Isolates with anamorphs resembling those of *Ophiostoma* spp. were grown on 2% water agar (20 g of agar and 1000 mL of distilled water) with autoclaved pine twigs to induce the production of perithecia. Perithecia and conidiophores were mounted in lactophenol on glass slides and characterized using light microscopy.

From each sample, only one isolate per fungal species was recorded and subsequently used for frequency calculations. Frequencies of occurrence of fungi were computed using the formula of Yamaoka et al. (1997): $F = (NF/NT)100\%$, where *F* represents the frequency of occurrence (%) of each fungus from each niche, NT represents the total number of samples from which isolation attempts were made, and NF represents the number of samples from which each fungus was isolated. All cultures obtained in this study have been accessioned in the CMW culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; and in the OPH culture collection of the Basque Institute of Agricultural Research and Development.

DNA extraction, PCR amplification, and sequencing

Flasks containing 50 mL of malt extract broth (2% *m*/*v*) were inoculated by transferring hyphal tips from the edges of individual colonies of each putatively taxonomic unit. After 10 days of static incubation at room temperature (25 °C), mycelium was harvested by filtration through Whatman No. 1 filter paper, and freeze-dried. Freeze-dried fungal tissue was ground into a fine powder in liquid nitrogen with a mortar and pestle, and homogenized in extraction buffer (200 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, 25 mmol/L EDTA (pH 8.0), and 0.5% SDS). Phenol (500 μL) and chloroform (300 μL) were added to the suspension, and the mixture was mixed and then centrifuged in a Heraeus Biofuge 3325 rotor (12 000 r/min for 60 min at 4 °C). The upper aqueous layer was transferred to sterilized Eppendorf tubes. Phenol (200 μL) and an equal volume of chloroform were added, mixed, and then centrifuged for 5 min. The aqueous phase was transferred to new Eppendorf tubes, and chloroform extraction (400 μL) was repeated once or twice until the interface was clear. Nucleic acid was then overnight precipitated with 0.1 volume of 3 mol/L NaAc (pH 5.4) and 1 volume of isopropanol. The nucleic acid was pelleted using centrifugation (12 000 r/min for 30 min at 4 °C), and the salt was removed by washing with 70% ethanol once. After vacuum-drying in a DNA Mini speed-vac (Heto) for 5 min, the dried pellet was resuspended in 50 μL sterile water, and 2 μL RNase (10 mg/mL, Roche Applied Science) was added to digest any RNA. The reaction was incubated in a water bath overnight at 37 °C. This protocol is a modified version of the extraction method developed by Raeder and Broda (1985).

PCR amplification was performed with primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-

northern Spain (in order of total relative abundance).

Pityogenes calcaratus	*Hylurgus ligniperda*	*Pityophthorus pubescens*	*Hypothenemus eruditus*	*Xyleborus dryographus*	*Xyleborus dispar*	*Brachyderes incanus*	Collection No.; GenBank acc. No.
1 (20%)	—	1 (9.1%)	—	—	—	—	CMW22835; DQ539541
3 (60%)	3 (60%)	**2 (18.2%)**	—	—	—	4 (9.5%)	Z2-1230; EF104911
—	—	—	—	—	—	—	CMW22804; DQ539509
—	—	—	—	**3 (43%)**	—	**12 (28.5%)**	CMW22844; DQ539550
—	—	—	**2 (40%)**	—	2 (40%)	**10 (24%)**	CMW22803; DQ539508
—	—	**2 (18.2%)**	—	—	—	—	CMW22824; DQ539531
—	—	—	1 (20%)	—	—	1 (2.4%)	CMW22815; DQ539522
—	—	—	—	—	—	—	CMW22854; DQ539502
—	—	—	—	2 (28.5%)	—	—	CMW22809; DQ539516
—	—	—	—	—	—	—	CMW22857; DQ539512
—	—	—	—	—	—	—	CMW22829; DQ539535
—	—	—	1 (20%)	—	—	—	CMW22849; DQ539557
—	—	—	—	—	2 (40%)	—	OPH-DIP15; DQ674377
—	—	—	—	—	—	—	CMW22800; DQ539505
—	—	—	—	—	—	—	CMW22807; DQ539514
—	—	—	—	—	—	—	CMW22833; DQ539539
—	—	—	—	1 (14.3%)	—	—	CMW22859; DQ539556
5	5	11	5	7	5	42	
4	3	5	4	6	4	27	
0.56	0.00	1.05	1.04	1.01	0.69	1.13	
2	1	3	3	3	2	4	
0.80	0.00	0.95	0.94	0.91	0.99	0.81	

P_i is proportion of total sample represented by species i, and S (species richness) is number of competing species in the community (Camargo 1993). fungal species within each community (see Materials and methods for mathematical descriptions of H and E.

3′) (White et al. 1990) to amplify the ITS1–5.8S–ITS2 region of the rDNA. Template DNA was amplified in a 50 μL PCR volume, consisting of 0.5–2.5 μL DNA solution, 0.5 μL *Taq* DNA polymerase (2.5 U) (Invitrogen Corporation), 5 μL of 10× PCR buffer, 3 μL $MgCl_2$ (25 mmol/L), and 1.5 μL each primer (10 mmol/L). PCR mixtures were overlaid with mineral oil, and reactions were performed, on a RoboCycler Gradient 96 robotic-armed thermocycler (Stratagene), with an initial denaturation step of 4 min at 95 °C. This step was followed by 40 cycles of denaturation at 95 °C (1 min), annealing at 55 °C (1 min), and elongation at 72 °C (2 min). A final extension was conducted for 10 min at 72 °C. A negative control, using sterile water, was included with each PCR. PCR products were visualized under UV illumination on a 1% agarose gel stained with ethidium bromide (10 mg/mL), run about 15 min in a Run-One Horizontal Electrophoresis System (EmbiTech) and then digitized in a ChemiDoc XRS Gel Documentation System (BioRad) with Quantity One 1-D Analysis Software 4.4.1 (BioRad). Amplification products were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences).

Sequencing reactions were performed on an ABI PRISM 377 Autosequencer in accordance with the instructions of the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems). PCR products were sequenced with the same primers used for PCR and two additional internal primers: CS2 (5′-CAATGTGCGTTCAAAGATTCG-3′) (Wingfield et al. 1996) and ITS3 (5′-GCATAGATGAAGAAGCAGC-3′) (White et al. 1990). All sequences were aligned using MEGA 3.1 and MAFFT v. 5.667 (Katoh et al. 2002), and compared using the services provided by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the internally maintained sequence database at the Department of Microbiology and Plant Pathology of the FABI, University of Pretoria.

Statistical analyses of fungus–insect associations

The effects of time within the logs and niches on the number and species composition of colonizing fungi within plantations were analyzed to study colonization density variations throughout the timber degradation processes. Overall sources of variation in the abundance of fungi collected from trap logs were analyzed using analysis of variance (ANOVA). Because the interaction terms between log and time were significant in some cases, a split-plot analysis (SPSS® 11.5, SPSS Inc. Chicago, Illinois), including logs within time period as the whole plot, niche as the split plot, and site as a random effect, was applied.

The Shannon–Weaver diversity index was also used to compare the diversity of fungal taxa on different insects and niches. This index, $H = -\sum(P_i \times \ln P_i)$, combines measurements of richness with those of evenness, so that rare species carry less weight. P_i is the proportion of the total sample represented by species i (Hill et al. 2003). Evenness (E), a measure of the relative abundance of species, is expressed as $E = H/H_{max}$, where $H_{max} = -\ln(S)$. Dominance or subordinance in fungal communities was judged using Camargo's index ($1/S$) (Camargo 1993), where S represents species richness (the number of competing species in the community), and dominant species have the relative abundance $P_i > 1/S$.

The relationship between fungal communities and environmental factors was analysed using canonical correspondence analysis (Ter Braak and Prentice 1988; CANOCO 4.0 software: Ter Braak and Smilauer 1998), the suitability of which was determined with a DCA (detrended canonical

Table 2. Isolation number and frequencies (in parentheses) of fungi associated with bark beetles and their galleries in buried and protruding *Pinus radiata* baiting logs in northern Spain.

	Buried wood	Protruding wood	Collection No.; GenBank acc. No.
Ophiostoma ips	—	**219 (33%)**	CMW22835; DQ539541
Leptographium guttulatum	**167 (25.4%)**	5 (0.7%)	Z2-1230; EF104911
Ophiostoma stenoceras	30 (4.5%)	**86 (13%)**	CMW22804; DQ539509
Ophiostoma piceae	**75 (11.4%)**	18 (2.7%)	CMW22844; DQ539550
Ophiostoma pluriannulatum	—	**67 (10%)**	CMW22803; DQ539508
Ophiostoma piliferum-like	**49 (7.4%)**	9 (1.3%)	CMW22824; DQ539531
Ophiostoma quercus	39 (6%)	—	CMW22815; DQ539522
Leptographium wingfieldii	30 (4.5%)	8 (1.2%)	CMW22854; DQ539502
Ophiostoma olivaceum	—	29 (4.3%)	CMW22809; DQ539516
Leptographium truncatum-like	25 (4%)	—	CMW22857; DQ539512
Ophiostoma rectangulosporium-like	—	23 (3.4%)	CMW22829; DQ539535
Pesotum fragrans	15 (2.3%)	—	CMW22849; DQ539557
Diplodia pinea	—	10 (1.5%)	OPH-DIP15; DQ674377
Ophiostoma minus	—	3 (0.4%)	CMW22800; DQ539505
Ophiostoma floccosum	3 (0.4%)	—	CMW22807; DQ539514
Ophiostoma canum-like	2 (0.3%)	—	CMW22833; DQ539539
Sporothrix schenckii-like	—	1 (0.1%)	CMW22859; DQ539556
Total no. samples	657	666	
Total no. isolates	442	478	
H	1.89	1.69	
S	11	12	
E	0.78	0.68	

Note: Values in bold represent dominant species for each niche-associated fungal community. A species is considered dominant if $P_i > 1/S$, where P_i is the proportion of total sample represented by species *i*, and *S* (species richness) is the number of competing species in the community (Camargo 1993). The Shannon–Weaver index (*H*) indicates the biodiversity degree of a certain fungal community. Evenness (*E*) is a measure of the relative abundance of fungal species within each community (see Materials and methods for mathematical descriptions of *H* and *E*.

analysis). Statistical significance ($P \leq 0.05$) was calculated using the Monte Carlo permutation test.

Results

After collecting and identifying 1323 bark beetles and their galleries, isolations yielded a total of 920 cultures, which included 16 species of *Ophiostoma* sensu lato or their asexual states. These included, in order of total relative abundance, *Ophiostoma ips*, *Leptographium guttulatum*, *Ophiostoma stenoceras*, *Ophiostoma piceae*, *Ophiostoma pluriannulatum*, *Ophiostoma piliferum*-like, *Ophiostoma quercus*, *Leptographium wingfieldii*, *Ophiostoma olivaceum*, *Leptographium truncatum*-like, *Ophiostoma rectangulosporium*-like, *Pesotum fragrans*, *Ophiostoma minus*, *Ophiostoma floccosum*, *Ophiostoma canum*-like, and *Sporothrix schenckii*-like (Table 1). One nonophiostomatoid fungus, *Diplodia pinea* (*Sphaeropsis sapinea*), was occasionally isolated from bark beetle galleries in this survey. This taxon is a well-known pathogen of *Pinus* spp., causing shoot blight and sapstain (Swart and Wingfield 1991). GenBank accession Nos. of the ITS1-5.8S-ITS2 sequences of each fungal species are presented in the Table 1.

Ophiostoma ips was the most frequently encountered fungus on *I. sexdentatus* and *Dryocoetes autographus*, whereas *L. guttulatum* was commonly found on *T. piniperda*, *Hylurgops palliatus*, and *Hylastes attenuatus*. Frequency of occurrence of *Ophiostoma ips* from *I. sexdentatus* was 69.5% and from *Dryocoetes autographus* was 58.5%. *Leptographium guttulatum* occurred on 27.2%, 18.2%, and 17.6% of *T. piniperda*, *Hylurgops palliatus*, and *Hylastes attenuatus* individuals, respectively. *Ophiostoma stenoceras* was the most frequently encountered fungus on *Orthotomicus erosus,* although this bark beetle species was also commonly associated with *Ophiostoma ips*. *Hylastes attenuatus* and *Hylurgops palliatus* had high percentages of association with *Ophiostoma stenoceras*, *Ophiostoma piceae*, and *Ophiostoma piliferum*-like. *Ophiostoma pluriannulatum* was most commonly associated with *Hypothenemus eruditus*, *T. piniperda*, and *Hylurgops palliatus*. Frequencies of occurrence of each fungal species are presented in Table 1.

The fungal community associated with *Hylurgops palliatus* had the highest biodiversity and species-richness values; it was dominated by *L. guttulatum*, *Ophiostoma piliferum*-like, *Ophiostoma piceae*, *Ophiostoma pluriannulatum*, and *L. wingfieldii* (Table 1). The fungal community associated with buried *Pinus radiata* baiting logs had slightly more diversity than the aerial community, and they were, respectively, dominated by *L. guttulatum* – *Ophiostoma piceae* – *Ophiostoma piliferum*-like and *Ophiostoma ips* – *Ophiostoma stenoceras* – *Ophiostoma pluriannulatum* (Table 2).

The results of interactions among the ophiostomatoid fungi, their bark beetle vectors, and environmental factors (site, time, niche) are presented in Table 3. All ophiostomatoid species considered had a significant general spatial segregation pattern, but their colonization density did not

Table 3. Analysis of variance for ophiostomatoid fungi collected from *Pinus radiata* stands with (A) all variables (site, time, log, and interactions) as a source of variation and with (B) a separate analysis by time of colonization.

	Ophiostoma ips		*Leptographium guttulatum*		*Ophiostoma pluriannulatum*		*Ophiostoma stenoceras*		*Ophiostoma piceae*		*Ophiostoma piliferum*-like		*Ophiostoma olivaceum*		*Ophiostoma rectangulosporium*-like	
Source of variation	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
(A) All variables.																
S	2.229	0.127	3.078	0.063	1.839	0.178	5.258	**0.012**	2.267	0.123	5.167	**0.013**	0.046	0.955	2.554	0.096
T	0.323	0.860	0.512	0.727	0.397	0.809	0.156	0.958	1.983	0.128	0.858	0.503	0.641	0.638	1.083	0.386
L	40.115	**0.001**	22.212	**0.001**	10.527	**0.003**	11.930	**0.002**	7.382	**0.011**	9.372	**0.005**	10.588	**0.003**	4.421	**0.045**
S × *T*	0.205	0.985	0.306	0.952	0.541	0.808	0.143	0.995	0.636	0.737	0.251	0.973	0.747	0.652	0.413	0.896
S × *L*	4.335	**0.025**	3.926	**0.033**	2.954	0.071	22.787	**0.001**	0.923	0.411	6.157	**0.007**	0.133	0.876	3.500	**0.046**
T × *L*	0.362	0.833	0.444	0.775	0.492	0.741	0.060	0.993	1.664	0.198	0.601	0.667	1.200	0.342	1.300	0.304
(B) Separate analysis by time of colonization.																
March																
S	0.401	0.701	0.845	0.511	0.520	0.640	0.877	0.501	1.000	0.465	1.000	0.465	0.500	0.650	0.655	0.581
L	6.646	0.061	3.818	0.122	3.769	0.124	1.421	0.299	1.000	0.374	1.000	0.374	4.000	0.116	2.579	0.184
April																
S	0.806	0.524	0.296	0.763	1.000	0.465	0.725	0.553	—	—	2.778	0.208	0.059	0.944	1.000	0.465
L	3.580	0.131	15.031	**0.018**	1.000	0.374	1.142	0.345	—	—	0.529	0.507	49.000	**0.002**	1.000	0.374
May																
S	0.256	0.790	0.810	0.523	0.700	0.563	0.527	0.637	0.547	0.627	1.027	0.457	3.000	0.192	1.000	0.465
L	12.843	**0.023**	3.314	0.143	2.286	0.205	3.692	0.127	6.942	0.058	1.952	0.235	0.500	0.519	1.000	0.374
June																
S	0.276	0.776	0.943	0.481	0.808	0.524	1.073	0.445	1.730	0.316	0.949	0.479	1.000	0.465	—	—
L	11.769	**0.027**	2.432	0.194	1.714	0.261	1.724	0.259	1.364	0.308	2.761	0.172	1.000	0.374	—	—
August																
S	0.619	0.596	0.909	0.491	0.658	0.580	0.273	0.778	0.779	0.534	0.433	0.683	1.000	0.465	1.000	0.465
L	5.829	0.073	2.127	0.218	2.560	0.185	9.000	**0.040**	4.600	0.099	4.923	0.091	1.000	0.374	1.000	0.374

Note: *S*, site; *T*, time; *L*, log; *F*, *F*-value; *P*, *P*-value; bold values represent significant *P*-values. —, insufficent data.

Table 4. Summary statistics of the canonical correspondence analysis ordinations of the ophiostomatoid fungi community and dummy variables for the following sources of variation. (A) Interaction between bark beetle species and niche. (B) Split-plot analysis of the effect of interaction between date and niche on fungal-community colonization density, by bark beetle species.

			Cumulative percentage variance of species		Sum of all eigen values	
Axes	Eigen value	Species–environment correlation	Data	Environment relation	Unconstrained	Canonical
(A) Bark beetle species × niche						
1	0.801	0.940	11.1	39.7		
2	0.700	0.885	20.8	74.4		
3	0.338	0.719	25.5	91.2		
4	0.124	0.468	27.2	97.3		
Total inertia	7.210				7.210	2.017
Explained variation					27.97%	
(B) Date × niche effect by bark beetle species						
Hylurgops palliatus						
1	0.398	0.790	19.2	51.5		
2	0.315	0.754	34.3	92.2		
3	0.039	0.491	36.2	97.2		
4	0.020	0.268	37.2	99.8		
Total inertia	2.078				2.078	0.774
Explained variation					37.24%	
Hylastes attenuatus						
1	0.879	0.960	26.2	62.5		
2	0.278	0.830	34.4	82.4		
3	0.152	0.573	39.0	93.2		
4	0.089	0.466	41.6	99.5		
Total inertia	3.359				3.359	1.405
Explained variation					41.82%	
Ips sexdentatus						
1	0.131	0.925	32.2	53.9		
2	0.076	0.799	50.7	84.9		
3	0.029	0.497	57.8	96.8		
4	0.008	0.642	59.7	100.0		
Total inertia	0.408				0.408	0.244
Explained variation					59.80%	
Dryocoetes autographus						
1	0.521	0.726	32.2	76.4		
2	0.109	0.607	38.9	92.5		
3	0.046	0.401	41.8	99.2		
4	0.005	0.340	42.1	100.0		
Total inertia	1.618				1.618	0.681
Explained variation					42.08%	
Orthotomicus erosus						
1	0.949	0.974	19.4	54.5		
2	0.383	0.648	27.2	76.5		
3	0.254	0.631	32.5	91.1		
4	0.129	0.537	35.1	98.5		
Total inertia	4.889				4.889	1.742
Explained variation					35.63%	

respond significantly to temporal succession steps of timber degradation. Although only two species, *Ophiostoma stenoceras* and *Ophiostoma piliferum*-like, showed significant differences related to the geographic distribution of sampling stands, *Ophiostoma ips*, *L. guttulatum*, and *Ophiostoma rectangulosporium*-like responded significantly to the interaction between stand and niche. Split-plot analysis by time of colonization showed that niche partitioning was significant in May and June for *Ophiostoma ips*, in April for *L. guttulatum* and *Ophiostoma olivaceum*, and in August for *Ophiostoma stenoceras*, whereas, for the other species separation was only significant when all the data were taken into account.

The results of canonical correspondence analysis for the

Fig. 1. Canonical correspondence analysis ordination of the ophiostomatiod fungal community in *Pinus radiata* stands and dummy variables for different bark beetle species and niches (A) and different colonization times and niches for *Hylurgops palliatus* (B), *Hylastes attenuatus* (C), *Ips sexdentatus* (D), *Dryocoetes autographus* (E), and *Orthotomicus erosus* (F). The fungal species presented in bold accepted the restriction rule of visibility of "fit" = 2 and "weight" = 1 to avoid, respectively, very widespread species and rare species.

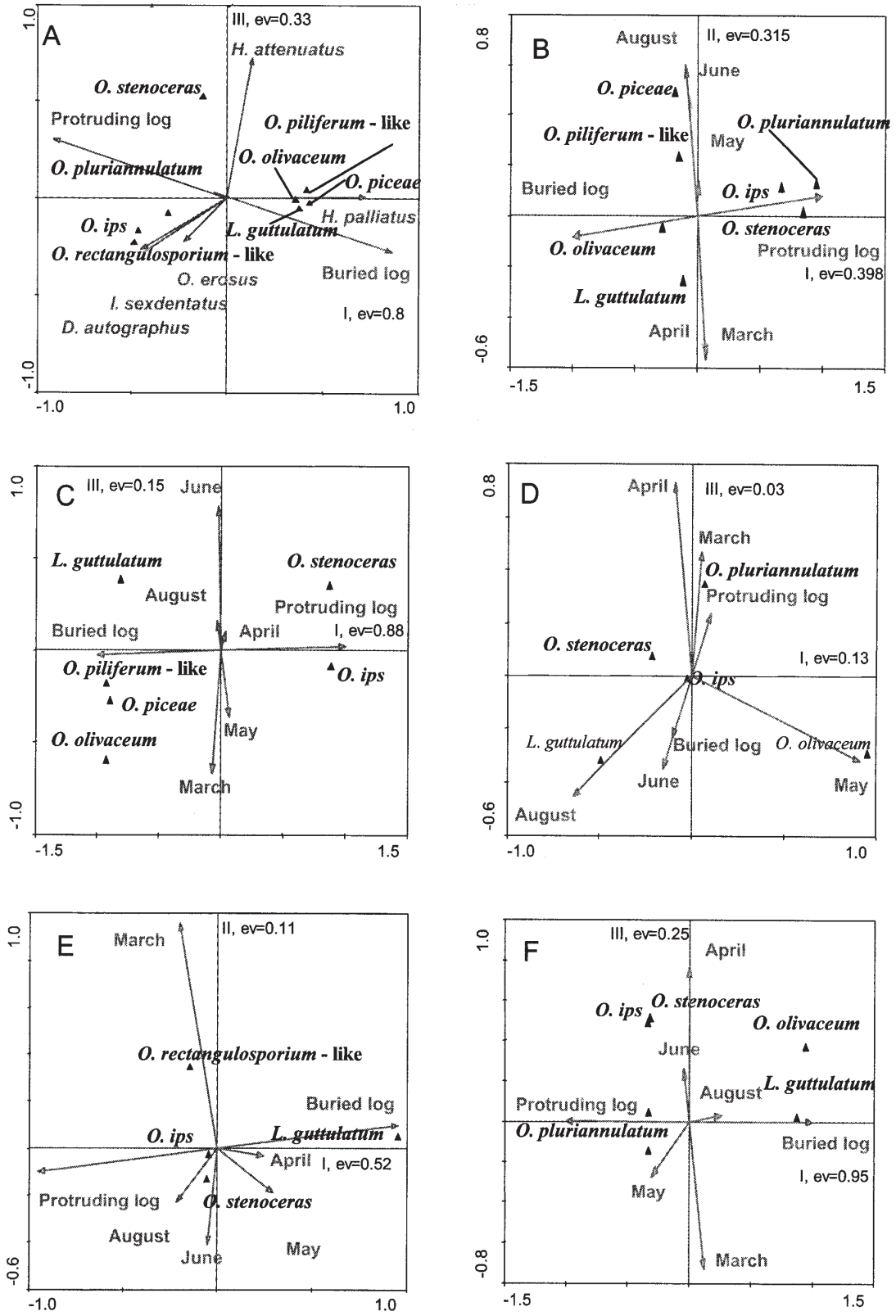

Table 5. New associations of fungi with bark beetles (Coleoptera: Scolytinae), weevils (Coleoptera: Entiminae), and *Pinus radiata* (Coniferales: Pinaceae).

Beetle species	Fungal species
Coleoptera: Scolytinae	
Hylurgops palliatus	*Ophiostoma piliferum*-like, *Ophiostoma pluriannulatum, Ophiostoma ips, Ophiostoma olivaceum, Ophiostoma quercus, Ophiostoma rectangulosporium*-like, *Pesotum fragrans, Diplodia pinea*
Hylastes attenuatus	*Leptographium guttulatum, L. wingfieldii, L. truncatum*-like, *Ophiostoma ips, Ophiostoma olivaceum, Ophiostoma piliferum*-like, *Ophiostoma stenoceras, Ophiostoma piceae, Ophiostoma quercus, Pesotum fragrans, Diplodia pinea*
Ips sexdentatus	*Leptographium guttulatum, L. truncatum*-like, *Ophiostoma olivaceum, Ophiostoma pluriannulatum, Ophiostoma stenoceras, Ophiostoma rectangulosporium*-like
Dryocoetes autographus	*Leptographium guttulatum, Ophiostoma ips, Ophiostoma olivaceum, Ophiostoma minus, Ophiostoma piliferum*-like, *Ophiostoma stenoceras, Ophiostoma rectangulosporium*-like
Orthotomicus erosus	*Leptographium guttulatum, L. wingfieldii, Ophiostoma olivaceum, Ophiostoma stenoceras, Ophiostoma floccosum, Ophiostoma canum*-like, *Ophiostoma rectangulosporium*-like, *Pesotum fragrans*
Tomicus piniperda	*Ophiostoma pluriannulatum, Diplodia pinea*
Hylastes ater	*Leptographium wingfieldii, Ophiostoma olivaceum, Ophiostoma pluriannulatum, Ophiostoma piliferum*-like, *Ophiostoma stenoceras, Ophiostoma quercus, Ophiostoma floccosum, Ophiostoma rectangulosporium*-like
Pityogenes calcaratus	*Leptographium guttulatum, Ophiostoma ips*
Hylurgus ligniperda	*Leptographium guttulatum*
Pityophthorus pubescens	*Leptographium guttulatum, Ophiostoma ips, Ophiostoma piliferum*-like
Hypothenemus eruditus	*Ophiostoma pluriannulatum, Ophiostoma quercus, P. fragrans*
Xyleborus dryographus	*Ophiostoma olivaceum, Ophiostoma piceae, Sporothrix schenckii*-like
Xyleborus dispar	*Ophiostoma pluriannulatum, Diplodia pinea*
Coleoptera: Entiminae	
Brachyderes incanus	*Leptographium guttulatum, Ophiostoma pluriannulatum, Ophiostoma piceae, Ophiostoma quercus*
Coniferales: Pinaceae	
New records on *Pinus radiata**	*Leptographium guttulatum, L wingfieldii, Ophiostoma olivaceum, Ophiostoma minus, Ophiostoma canum*-like, *Ophiostoma rectangulosporium*-like, *Sporothrix schenckii*-like
Not recorded in *P. radiata* stands in Spain†	*Ophiostoma coronatum, Ophiostoma galeiforme, Ophiostoma huntii, Ophiostoma nigrocarpum, Ophiostoma perfectum, Ophiostoma setosum, Ophiostoma radiaticola, Leptographium procerum, Leptographium bistatum*

*This study.
†Kim et al. 2005; Thwaites et al. 2005.

ophiostomatoid fungal community are provided in Table 4. General analysis of the interaction between vector and niche explained about 28% of the community's variability, whereas mentioned independent sources of variation explained only about 22% and 10%, respectively. Split-plot analysis of the interaction between date and niche explained approximately 37%, 42%, 60%, 42%, and 35% of fungal communities associated with *Hylurgops palliatus*, *Hylastes attenuatus*, *I. sexdentatus*, *Dryocoetes autographus*, and *Orthotomicus erosus* (Figs. 1B–1F). However, most separated fungal communities responded significantly only to the ecological niche effect, explaining about 19%, 26%, 30%, and 19% for fungi associated with *Hylurgops palliatus*, *Hylastes attenuatus*, *Dryocoetes autographus*, and *Orthotomicus erosus*, respectively. In contrast, the fungal community associated with *I. sexdentatus* responded significantly only to colonization time, explaining approximately 56% of the variability of colonization density (Table 4; Fig. 1D).

Discussion

In this study, 16 species of ophiostomatoid fungi, including *Ophiostoma*, *Leptographium*, *Pesotum*, and *Sporothrix* spp., were isolated from bark beetles and weevils. Niche had a significant effect on abundance and composition of most colonizing fungi. This confirms that resource overlap between species is reduced by partial spatial segregation. Interaction between niche and time seldom had a significant effect, suggesting that spatial colonization patterns are rarely flexible throughout timber degradation. This is the first comprehensive survey of the fungi associated with these insects in Spain, and the results represent numerous new records of fungi for the region. The most commonly encountered fungal associates of the bark beetles considered were *Ophiostoma ips*, *L. guttulatum*, *Ophiostoma stenoceras*, and *Ophiostoma piceae*.

Ophiostoma ips was most commonly associated with *I. sexdentatus*, which colonizes in the above-ground parts of trees. This association was previously recorded by Lieutier et al. (1991). The fungus is also a well-known associate of other bark beetles that infest the above-ground parts of trees, such as *Orthotomicus erosus* (Zhou et al. 2001; de Beer et al. 2004) and *Ips grandicollis* (Stone and Simpson 1987). It appears to be specifically associated with bark beetles that infest the above-ground parts of trees, and it is also known to have a relatively high level of pathogenicity (Lieutier et al. 1989; Fernández et al. 2004).

In contrast to *Ophiostoma ips*, *L. guttulatum* is a common

associate of root-infesting bark beetles, including species of *Hylastes*. Thus, the association of this and other *Leptographium* spp. in this study with *Hylastes attenuatus* and *Hylurgops palliatus* was not surprising (Wingfield and Gibbs 1991). It was interesting that *L. serpens*, a very common associate of several root-infesting *Hylastes* spp. and *Hylurgus ligniperda* (Jacobs and Wingfield 2001; Zhou et al. 2001), was not encountered.

Ophiostoma piceae, Ophiostoma stenoceras, and *Ophiostoma piliferum*-like, which were commonly encountered in this study, are not generally considered to be strict associates of bark beetles. They commonly cause blue stain of coniferous timber and are often encountered in the absence of bark beetle activity (Harrington et al. 2001). Results of this study showed that they also have a close association with *Hylastes attenuatus* and *Hylurgops palliatus* bark beetles. These two beetle species often share a similar niche, which would explain the overlap in their fungal associates. In the field, we observed that these two beetle species constructed galleries in close proximity to each other. This might result in fungal co-colonization of galleries, although the fungi might also be laterally transferred by phoretic mites that move actively in bark beetle galleries (Hofstetter et al. 2006).

Results of this study showed that *I. sexdentatus*, a relatively weakly aggressive bark beetle that appears to be incapable of sustaining successful populations when colonizing vigorous trees, is associated with a relative pathogenic fungus, *Ophiostoma ips*. In contrast, the moderately aggressive *T. piniperda* is mostly associated with the relatively less pathogenic fungus *L. guttulatum*. These results support the view that the more aggressive bark beetle species are often associated with weakly pathogenic fungi (Harrington 1993). However, the fungal associates of *T. piniperda* have been relatively widely studied in the past, and the close association we found of *L. guttulatum* with this insect is unusual. The two most common fungi previously associated with this insect in Europe are *L. wingfieldii* and *Ophiostoma minus* (Gibbs and Inman 1991; Solheim and Längström 1991), which was not the case here.

Many new bark beetle – fungus associations emerged in this study (Table 5). To our knowledge, all isolations made from *Hylastes attenuatus*, *Pityogenes calcaratus*, *Pityophthorus pubescens,* and *B. incanus* are new vector fungal detections. *Ophiostoma piliferum*-like, *Ophiostoma pluriannulatum*, *Ophiostoma ips*, *Ophiostoma olivaceum*, *Ophiostoma quercus*, *Ophiostoma rectangulosporium*-like, *P. fragrans*, and *Diplodia pinea* are newly recorded from *Hylurgops palliatus*. However, *L. procerum*, a root-inhabiting species commonly encountered on this bark beetle (Jacobs and Wingfield 2001; Kirisits 2004), was not isolated. *Leptographium guttulatum*, *L. truncatum*-like, *Ophiostoma olivaceum*, *Ophiostoma pluriannulatum*, *Ophiostoma stenoceras*, and *Ophiostoma rectangulosporium*-like were isolated from *I. sexdentatus* for the first time. However, *Ophiostoma brunneo-ciliatum*, previously reported from *I. sexdentatus* by Lieutier et al. (1989), was not found. *Dryocoetes autographus* had not previously been associated with *L. guttulatum*, *Ophiostoma ips*, *Ophiostoma olivaceum*, *Ophiostoma minus*, *Ophiostoma piliferum*-like, *Ophiostoma stenoceras*, and *Ophiostoma rectangulosporium*-like. In contrast, previous research has shown that this bark beetle species often carries ophiostomatoid fungi belonging to the *Ophiostoma piceae* complex of conifers (Haberkern et al. 2002), records not registered in the present survey. The high frequency of occurrence of *Ophiostoma stenoceras* on *Orthotomicus erosus* is also remarkable.

Also included in Table 5 are new fungal records on *Pinus radiata* and other fungal species previously isolated from *Pinus radiata* in New Zealand that have not been recorded in Spain stands (Kim et al. 2005; Thwaites et al. 2005). Further research is needed to clarify the distribution of different ophiostomatoid species associated with different conifer hosts and different insect vectors in different geographic areas.

The association of *Diplodia pinea* with bark beetles in this study is interesting but is probably incidental. This is because the biology and ecology of this fungus is somewhat different than that of the ophiostomatoid fungi. This species is disseminated primarily by wind and rain, and it usually behaves as an opportunistic pathogen after stress, such as that associated with hail damage or drought (Swart and Wingfield 1991). Apart from *Diplodia pinea*, species such as *Ophiostoma ips*, *Ophiostoma minus*, *Ophiostoma piceae*, and *Ophiostoma pluriannulatum* are important agents of sapstain (Seifert 1993), whereas *Ophiostoma ips*, *Ophiostoma minus*, and *L. wingfieldii* pathogenicity is well recognized (Raffa and Smalley 1988; Lieutier et al. 1989; Yamaoka et al. 1990; Fernández et al. 2004). Therefore, together with the associated bark beetles, these species should be taken into consideration when control measures for sapstain are developed for the Basque Country forestry industry. Fungal species from this group have already been introduced into new environments, both in northern and southern hemispheres. Therefore, studies on bark beetles and their associated fungi will be important for the development of effective quarantine target lists and regulation measures.

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