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ORIGINAL PAPER

Ophiostomatoid fungi including two new fungal species associated with pine root-feeding beetles in northern Spain

Pedro Romón · Z. Wilhelm De Beer · Mercedes Fernández · Julio Diez · Brenda D. Wingfield · Michael J. Wingfield

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Abstract Many bark beetles live in a symbiosis with ophiostomatoid fungi but very little is known regarding these fungi in Spain. In this study, we considered the fungi associated with nine bark beetle species and one weevil infesting two native tree species (*Pinus sylvestris* and *Pinus nigra*) and one non-native (*Pinus radiata*) in Cantabria (Northern Spain). This included examination of 239 bark beetles or their galleries. Isolations yielded a total of 110 cultures that included 11 fungal species (five species of *Leptographium* sensu lato including *Leptographium absconditum* sp. nov., five species of *Ophiostoma* sensu lato including *Ophiostoma cantabriense* sp. nov, and one species of *Graphilbum*). The most commonly encountered fungal associates of the bark beetles were *Grosmannia olivacea, Leptographium procerum*, and *Ophiostoma canum*. The aggressiveness of the collected fungal species was evaluated using inoculations on two-year-old *P. radiata* seedlings. *Leptographium wingfieldii*, *Leptographium guttulatum*, and *Ophiostoma ips* were the only species capable of causing significant lesions.

Keywords *Ophiostoma* · *Leptographium* · Root-feeding beetles · Two new fungal species

P. Romón (✉) · B. D. Wingfield
Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
e-mail: Pedro.Romon@fabi.up.ac.za

Z. W. De Beer · M. J. Wingfield
Department of Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa

M. Fernández
Department of Agroforestry Sciences, University of Valladolid, ETSIIAA Palencia, Avda. Madrid 57, 34071 Palencia, Spain

J. Diez
Department of Plant Production and Forest Resources, University of Valladolid, ETSIIAA Palencia, Avda. Madrid 57, 34071 Palencia, Spain

Introduction

Bark beetles (Coleoptera: Scolytinae) that infest coniferous trees carry many different ophiostomatoid fungi including species related to *Ophiostoma*, in the Ophiostomatales; and those related to *Ceratocystis* in the Microascales. Some of these fungi (i.e.: *Grosmannia clavigera* (Owen et al. 1987), *Leptographium terebrantis* (Parmeter et al. 1989), *L. wingfieldii* (Lieutier et al. 1989; Jankowiak 2006), *Ophiostoma ips* (Raffa and Smalley 1988; Fernández et al. 2004), *O. minus* (Yamaoka et al. 1990; Jankowiak 2006), *Ceratocystis laricicola* (Redfern et al. 1987) and *C. polonica* (Christiansen and Solheim 1990) can result in lesions when inoculated onto trees. However, with the exception of *L. wageneri* that causes black stain root disease (Harrington and Cobb 1988; Jacobs and

Wingfield 2001), they are not considered primary pathogens and their role in killing trees has been questioned (Six and Wingfield 2011). In this regard, fungi such as *Ophiostoma ips, O. minus, O. piceae, O. piliferum* and *O. pluriannulatum* are best considered as agents of sapstain (Seifert 1993), leading to significant financial losses to the forestry industry that can amount to as much as 50 % reduction in the price of wood used for furniture or construction (Maderas Elorriaga Company pers. comm.).

Knowledge of the ophiostomatoid fungi in the Iberian Peninsula is very limited (De Ana Magán 1982; 1983; Fernández et al. 2004; Villarreal et al. 2005; Romón et al. 2007; Bueno et al. 2010; Pestaña and Santolamazza-Carbone 2010; Romón et al. 2014), and only three studies have dealt with their taxonomy. For example, a new species, *Leptographium gallaeiciae*, was described invalidly from stressed *Pinus pinaster* trees (De Ana Magán 1983). Duong et al. (2012) suggested that this species belongs to the *Grosmannia serpens* complex, but validation of the name will be possible only if the original material can be located (De Beer et al. 2013). Other fungi in this group, *Ophiostoma sejunctum* (Villarreal et al. 2005), *Ophiostoma nebulare*, *Ophiostoma euskadiense* and *Graphilbum crescericum* (Romón et al. 2014) were described only very recently, suggesting that these fungi have been incompletely studied in the area.

The primary aim of this study was thus to conduct surveys of fungi associated with several beetles colonizing native and introduced conifers in Spain and to consider their identity. A second objective was to consider the aggressiveness of these fungi on *P. radiata* seedlings as an indication of their relative ability to colonise infected trees.

Materials and methods

Collection of samples and isolation of fungi

During 2012, beetles and galleries of *Hylurgops palliatus, Hylastes attenuatus, Hylastes angustatus, Hylastes ater, Hylurgus ligniperda, Orthotomicus laricis, Orthotomicus erosus, Tomicus piniperda, Gnatothrichus materiarius* (Coleoptera: Scolytinae) and *Hylobius abietis* (Coleoptera: Entiminae) were collected from trap logs in three mature stands of *P. sylvestris*, *P. nigra* and *P. radiata* in Cantabria province (northern Spain). In April 2012, five almost totally buried trap logs, 0.5 m long and 0.2 m in diameter (bark thickness about 1 cm), were set out in each stand. After seven weeks, the logs were inspected. All beetles from a single gallery were removed using sterilized tweezers, placed individually in sterile Eppendorf tubes, and identified using a MOTIC dissecting microscope and several taxonomic keys (Balachowsky 1949; Grüne 1979; Gil and Pajares 1986; López et al. 2007). Complete galleries, including the cambium up to 2 cm away from the main tunnel, were removed and placed in separate, clean paper bags. The galleries, together with the beetles present in them, were treated as single samples.

Individual beetles were carefully crushed on the surface of 2 % MEA (20 g malt extract, 20 g agar and 1,000 ml distilled water), amended with 0.05 % cycloheximide and 0.04 % streptomycin, using sterilised tweezers. Beetles from different galleries were incubated in separate Petri dishes at 25 °C in the dark for two weeks. Cultures were purified by transferring hyphal tips from the edges of individual colonies, or spore masses from the apices of emerging ascomata or conidiophores to fresh 2 % MEA. Beetle galleries were maintained in humid chambers at 25 °C and 70 % RH in the dark for three weeks. Spore masses accumulating at the apices of ascomata or conidiophores produced in the galleries, were carefully transferred onto 2 % MEA using a sterile needle. From each sample, only one isolate per fungal taxon was recorded and subsequently used for frequency calculations. Frequencies of occurrence of fungi were computed using the formula of Yamaoka et al. (1997) where F = (NF/NT) 100 (%) and F represents the frequency of occurrence (%) of the fungus, NF represents the number of samples from which fungi were isolated, and NT represents the total number of samples from which isolations were made. All cultures were accessioned in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Cultures of new taxa were deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

PCR amplification, sequencing and phylogenetic analyses

Eppendorf tubes (2-ml) containing 1 ml of malt extract broth at 2 % were inoculated by transferring

hyphal tips from the edges of individual colonies. After 4 days of incubation at 25 °C, DNA was extracted using Prepman Ultra Sample Preparation Reagent (Applied Biosystems). PCR amplification was performed with primers ITS1-F (5′-CTTGGTCA TTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCG CTTATTGATATGC-3′) (White et al. 1990). DNA was amplified in 50 μL PCR reaction volume with 5 μL of 10X Reaction Buffer, 5 μL of $MgCl_2$ (25 mM), 5 μL of dNTPs (10 mM), 1 μL of each primer (10 μM), 1.5 μL of DNA solution and 0.5 μL of Super-Therm Taq polymerase. Reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems) with an initial denaturation step of 2 min at 95 °C. This step was followed by 40 cycles of denaturation at 95 °C (30 s), annealing at 52–55 °C (30 s) and elongation at 72 °C (1 min). A final extension was conducted for 8 min at 72 °C.

In the case of the purported new *Ophiostoma* sp., amplicons were also obtained for the β-tubulin gene region using primers T10 (5′-ACGATAGGTTCACC TCCAGAGAC-3′) or Bt2a (5′-GGTAACCAAATCG GTGCGCTTTC-3′) with Bt2b (5′-GGTAACCAAAT CGGTGCTGCTTTC-3′) (Glass and Donaldson 1995). A portion of the calmodulin gene region was also amplified with primers CL1 (5′-GARTWCAAG GAGGCCTTCTC-3′) and CL2A (5′-TTTTGCATCA TGAGTTGGAC-3′) (O'Donnell 2000). PCR conditions to amplify the calmodulin gene region were the same as those for ITS1–5.8S–ITS2, whereas for the β-tubulin gene region an initial denaturation step of 4 min at 95 °C, followed by 35 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 47–52 °C and elongation for 1 min at 72 °C, with a final elongation step of 7 min at 72 °C were included.

In the case of the purported new *Leptographium* sp., the internal transcribed spacer region two and partial large subunit (ITS2-LSU) of the ribosomal DNA were amplified using the primers ITS3 (5′-GCATCGATGA AGAACGCAGC-3′) and LR5 (5′-TCCTGAGGGAA ACTTCG-3′) (White et al. 1990). The beta-tubulin gene region was also amplified as described above. Furthermore, part of the elongation factor 1-alpha (EF1-α) gene was amplified using the primers EF1F (5′-TGCGGTGGTATCGACAAGCGT-3′) and EF2R (5′-AGCATGTTGTCGCCGTTGAAG-3′) (Jacobs et al. 2004). PCR conditions for ITS2-LSU and EF1-α amplification were the same than those for ITS1–5.8S–ITS2 except respective annealing temperatures of 55 and 60 °C.

PCR products were visualized under UV illumination on a 1 % agarose gel stained with Gelred (Biotium), run in a Wide Mini-Sub Cell GT Electrophoresis System (BioRad) and then digitalized in a White-Ultraviolet Transilluminator Gel Documentation System (UVP). Amplification products were purified using the High Pure PCR Product Purification Kit (Roche), sequencing prepared with ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, precipitated by sodium acetate-cold ethanol protocol in an Eppendorf 5415R centrifuge, and dried in an Eppendorf 5301 concentrator.

Sequencing was performed on an ABI PRISM 377 Autosequencer. Forward and reverse sequences were aligned and consensus sequences determined using ContigExpress, Vector NTI Advance 11.5.0 (Invitrogen). BLAST searches were conducted for preliminary identifications, after which datasets that included all most up-to-date GenBank sequences were compiled in MEGA5 (Tamura et al. 2011). Sequences were aligned online with MAFFT6 (Katoh et al. 2002), using the FFT-NS-i option. Datasets were analysed using maximum likehood (ML), maximum parsimony (MP) and Bayesian inference (BI). DNA alignments were converted to appropriate file formats for analyses by using ALTER (Glez-Peña et al. 2010). ML analyses were performed using PhyML3.0 (Guindon et al. 2006) after determining the substitution model in jModelTest (Posada 2008). Support for the nodes was estimated from 1,000 bootstrap replicates. MP analyses were conducted using PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford 2003). Random stepwise addition heuristic searches were performed with tree-bisection-reconnection (TBR) branch swapping active. Alignment gaps were treated as a fifth character state. Ten trees were saved per replicate and branches of zero length were collapsed. Confidence levels were estimated by performing 1,000 bootstrap replicates (Felsenstein 1985) with fast-stepwise addition. BI analyses were carried out with MrBayes3.1.2 (Ronquist and Huelsenbeck 2003). Markov Chain Monte Carlo chains were run for five million generations using the best fitting model selected by the Akaike Information Criterion in MrModeltest2.3. Trees were sampled every 100 generations. Burn-in values were determined using Tracer1.4. All sampled trees lower than the burn-in

values were discarded and a 50 % majority rule consensus tree was then constructed.

Biodiversity

The Shannon–Weaver diversity index was used to compare the diversity of fungal taxa on different insects. This index $H = -\Sigma(P_i \times \ln P_i)$ 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 $P_i > 1/S$.

Culture characteristics

Isolates representing the same species were grown and crossed in all possible combinations on 2 % agar to which autoclaved pine twigs had been added and on oat meal agar to induce production of ascomata. Slide cultures were made to observe asexual state structures and these were mounted in lactophenol on glass slides and examined with a Zeiss Axioskop microscope. Fifty measurements were made for each taxonomically characteristic structure. All qualitatively and quantitatively informative characters including those of mycelium, conidiophores, and conidia were characterized and compared with the closely related species using relevant taxonomic keys and protologues. Measurements are presented in the format (minimum−) mean minus standard deviation—mean plus standard deviation (−maximum).

For each purportedly new taxon as well as phylogenetically closely related species, the optimal growth temperature for two isolates was determined by growing them at temperatures ranging from 5–35 °C at 5 °C intervals in Sanyo MIR-253 incubators. A 5 mm diameter agar disk was taken from the actively growing margin of a fresh colony of each isolate and inoculated onto the agar surface of six 2 % MEA replicate plates for each temperature. Colony diameters were measured after 8 days and mean minimum, optimum and maximum growth temperatures were calculated. Mean growth was compared among isolates using ANOVA and Tukey's test.

Fig. 1 Phylogram based on ML analyses of ITS1-5.8S-ITS2 rDNA sequences, showing where fungal associates of pine bark beetles in Spain groups within the Ophiostomatales. Spanish isolates of known species are *shaded*, while those of novel taxa are printed in *bold* type. ML and MP bootstrap support values (1,000 replicates) are indicated at the nodes. BI probabilities (above 90 %) are indicated by *bold lines* at the relevant branching points. *Bootstrap values lower than 75 %, *T* ex-type isolates, *Scale bar* total nucleotide difference between taxa, *ML* maximum likelihood, *MP* maximum parsimony, *BI* Bayesian inference

Pathogenicity tests

Other than one isolate for each of *L. wingfieldii* and *O. saponiodorum*, two randomly selected isolates of each of the remaining nine collected fungal species were used in a pathogenicity test on two-year-old *P. radiata* seedlings. The plants were placed in a greenhouse under natural lighting conditions and they were watered three times a week during the experiment.

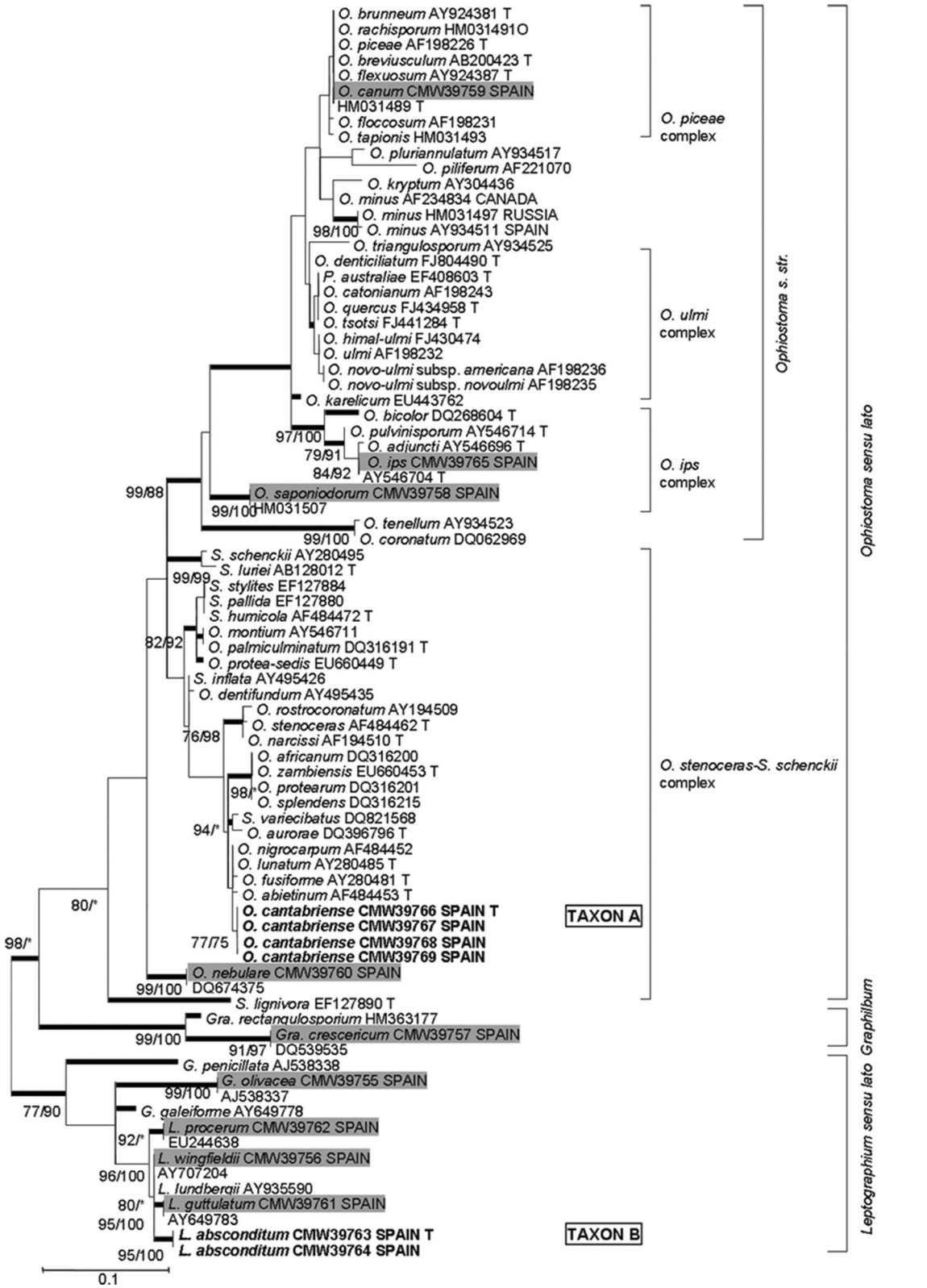
On 29 July 2013, 24 seedlings were inoculated with each of the 20 selected isolates plus control (sterile agar). The inoculations of the 504 seedlings were made by cutting a bark flap (4 × 8 mm) with a sterile scalpel, placing inoculum on the exposed surface and covering it with the bark flap and a Parafilm strip. Inoculum was taken for the margins of 14-day-old cultures growing at 25 °C and consisted of a 5 mm disc of fungus growing on 2 % MEA or sterile agar in the case of the controls.

After 6 weeks, inoculated plants were harvested and the bark was removed from the inoculum site. The lengths of the necrotic lesions were measured and the percentages of yellowing seedlings were determined. The data were analysed using analysis of variance (ANOVA). Treatment differences were further evaluated by Tukey's test.

Results

Sequencing and phylogenetic analyses

ITS1-5.8S-ITS2 sequences of the isolates obtained from bark beetles in Spain confirmed the presence of nine known species and two undescribed species (Fig. 1). The amplified ITS regions of isolates


O. brunneum AY924381 T
O. rachisporum HM031491O
O. piceae AF198226 T
O. breviusculum AB200423 T
O. flexuosum AY924387 T
O. canum CMW39759 SPAIN
HM031489 T
O. floccosum AF198231
O. tapionis HM031493
O. piceae complex
O. pluriannulatum AY934517
O. piliferum AF221070
O. kryptum AY304436
O. minus AF234834 CANADA
O. minus HM031497 RUSSIA
98/100 O. minus AY934511 SPAIN
O. triangulosporum AY934525
O. denticiliatum FJ804490 T
P. australiae EF408603 T
O. catonianum AF198243
O. quercus FJ434958 T
O. tsotsi FJ441284 T
O. himal-ulmi FJ430474
O. ulmi AF198232
O. novo-ulmi subsp. americana AF198236
O. novo-ulmi subsp. novoulmi AF198235
O. ulmi complex
Ophiostoma s. str.
O. karelicum EU443762
O. bicolor DQ268604 T
97/100 O. pulvinisporum AY546714 T
79/91 O. adjuncti AY546696 T
84/92 O. ips CMW39765 SPAIN
AY546704 T
O. ips complex
99/88 O. saponiodorum CMW39758 SPAIN
99/100 HM031507
O. tenellum AY934523
99/100 O. coronatum DQ062969
Ophiostoma sensu lato
S. schenckii AY280495
99/99 S. luriei AB128012 T
S. stylites EF127884
S. pallida EF127880
S. humicola AF484472 T
82/92 O. montium AY546711
O. palmiculminatum DQ316191 T
O. protea-sedis EU660449 T
S. inflata AY495426
O. dentifundum AY495435
O. rostrocoronatum AY194509
76/98 O. stenoceras AF484462 T
O. narcissi AF194510 T
O. africanum DQ316200
O. zambiensis EU660453 T
98/* O. protearum DQ316201
O. splendens DQ316215
94/* S. variecibatus DQ821568
O. aurorae DQ396796 T
O. nigrocarpum AF484452
O. lunatum AY280485 T
O. fusiforme AY280481 T
O. abietinum AF484453 T
O. stenoceras-S. schenckii complex
80/* O. cantabriense CMW39766 SPAIN T
O. cantabriense CMW39767 SPAIN
98/* 77/75 O. cantabriense CMW39768 SPAIN
O. cantabriense CMW39769 SPAIN
TAXON A
99/100 O. nebulare CMW39760 SPAIN
DQ674375
S. lignivora EF127890 T
Gra. rectangulosporium HM363177
99/100 Gra. crescericum CMW39757 SPAIN
91/97 DQ539535
Graphilbum
G. penicillata AJ538338
G. olivacea CMW39755 SPAIN
99/100 AJ538337
77/90 G. galeiforme AY649778
L. procerum CMW39762 SPAIN
92/* EU244638
L. wingfieldii CMW39756 SPAIN
96/100 AY707204
L. lundbergii AY935590
80/* L. guttulatum CMW39761 SPAIN
95/100 AY649783
L. absconditum CMW39763 SPAIN T
TAXON B
95/100 L. absconditum CMW39764 SPAIN
Leptographium sensu lato
0.1

representing the two new taxa (A, B) were respectively 543 and 598 bp in size. ITS sequences of taxa A and B grouped in *Ophiostoma* sensu lato and *Leptographium* sensu lato respectively.

The ITS sequences of taxon A showed that it grouped close to *O. abietinum* and related species in the *Sporothrix schenckii—O. stenoceras* complex, but it did not sufficiently distinguish between these species. Amplicons for the β-tubulin and calmodulin regions for these isolates (Table 1) were 231 and 574 bp respectively. ITS sequence accounted for a total of 5, 3 and 6 substitutions between taxon A and *O. abietinum*, *O. lunatum* and *O. fusiforme*. Similarly, beta-tubulin sequences (Fig. 2a) accounted for a total of 6, 4 and 4 substitutions between taxon A and *O. abietinum, O. lunatum* and *O. fusiforme*. Calmodulin sequences (Fig. 2b) respectively accounted for a total of 9, 9 and 9 substitutions between taxon A and *O. abietinum*, *O. lunatum* and *O. fusiforme*. Taxon A accounted for a total of 4, 6 and 27 differences from the recently described *O. euskadiense* (data not shown) respectively for ITS, beta-tubulin and calmodulin sequences.

The ITS sequences of taxon B showed that it was closely related to *L. lundbergii*. The ITS2-LSU and elongation factor 1-alpha sequences were 570 and 587 bp respectively. A beta-tubulin sequence could not be obtained for this taxon. For each of the sequence datasets, ML, MP and BI resulted in trees with similar topologies. Phylograms obtained with ML are presented for all the datasets (Figs. 1, 2, 3) with nodal support obtained from ML, MP and BI indicated on the trees. GenBank accession numbers of published sequences are shown in the phylogenetic trees, while accession numbers of sequences obtained in the present study are presented in Table 1. Statistical values resulting from the respective phylogenetic analyses are presented in Table 2. DNA sequence matrices are available from TreeBase ID15019.

Culture characteristics

Taxon A did not grow at 10 °C, whereas the closely related species *O. abietinum* and *O. fusiforme* colonised similar areas, and it was the only species able to grow at 35 °C together with *O. fusiforme* to a lesser extent (Fig. 4). The colony diameters of Taxon B were generally smaller than those of closely related *Leptographium* species, except at 20–25 °C where they all

Table 1 Origin, hosts and Genbank accession numbers for fungal isolates sequenced in this study

Species	CMW no.[a]	CBS no.[b]	PREM[c]	Insect vector/Host	Collector	Country	ITS	β-tubulin	Calmodulin	ITS2-LSU	EF1-α
O. cantabriense sp. nov. (A)	39766[d]	136529	60890	*Hylastes attenuatus*/*Pinus sylvestris*	P. Romón	Spain	KF951554	KF951544	KF951540	–	–
	39767	136530	60891	*Orthotomicus laricis*/*P. sylvestris*	P. Romón	Spain	KF951555	KF951545	KF951541	–	–
	39768	136531	60892	*O. laricis*/*P. sylvestris*	P. Romón	Spain	KF951556	KF951546	KF951542	–	–
	39769	136532	60893	*O. laricis*/*P. sylvestris*	P. Romón	Spain	KF951557	KF951547	KF951543	–	–
L. abscondidum sp. nov. (B)	39763[d]	136527	60888	*O. laricis*/*Pinus nigra*	P. Romón	Spain	KF951558	–	–	KF951550	KF951552
	39764	136528	60889	*O. laricis*/*P. nigra*	P. Romón	Spain	KF951559	–	–	KF951551	KF951553

[a] CMW, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

[b] CBS, Centraalbureau voor Schimmelcultures, The Netherlands

[c] PREM, South African National Collection of Fungi, South Africa

[d] Ex-type culture

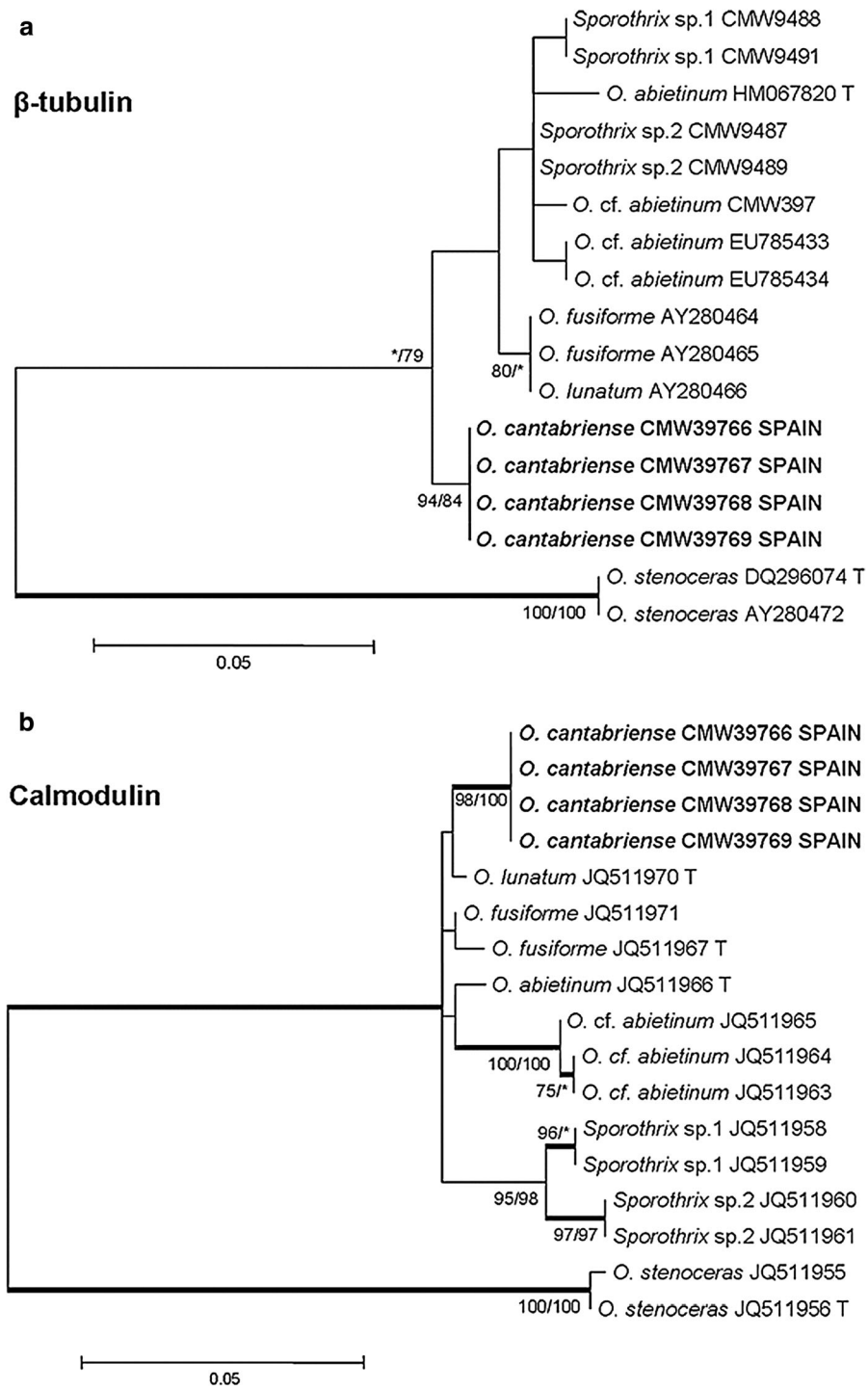
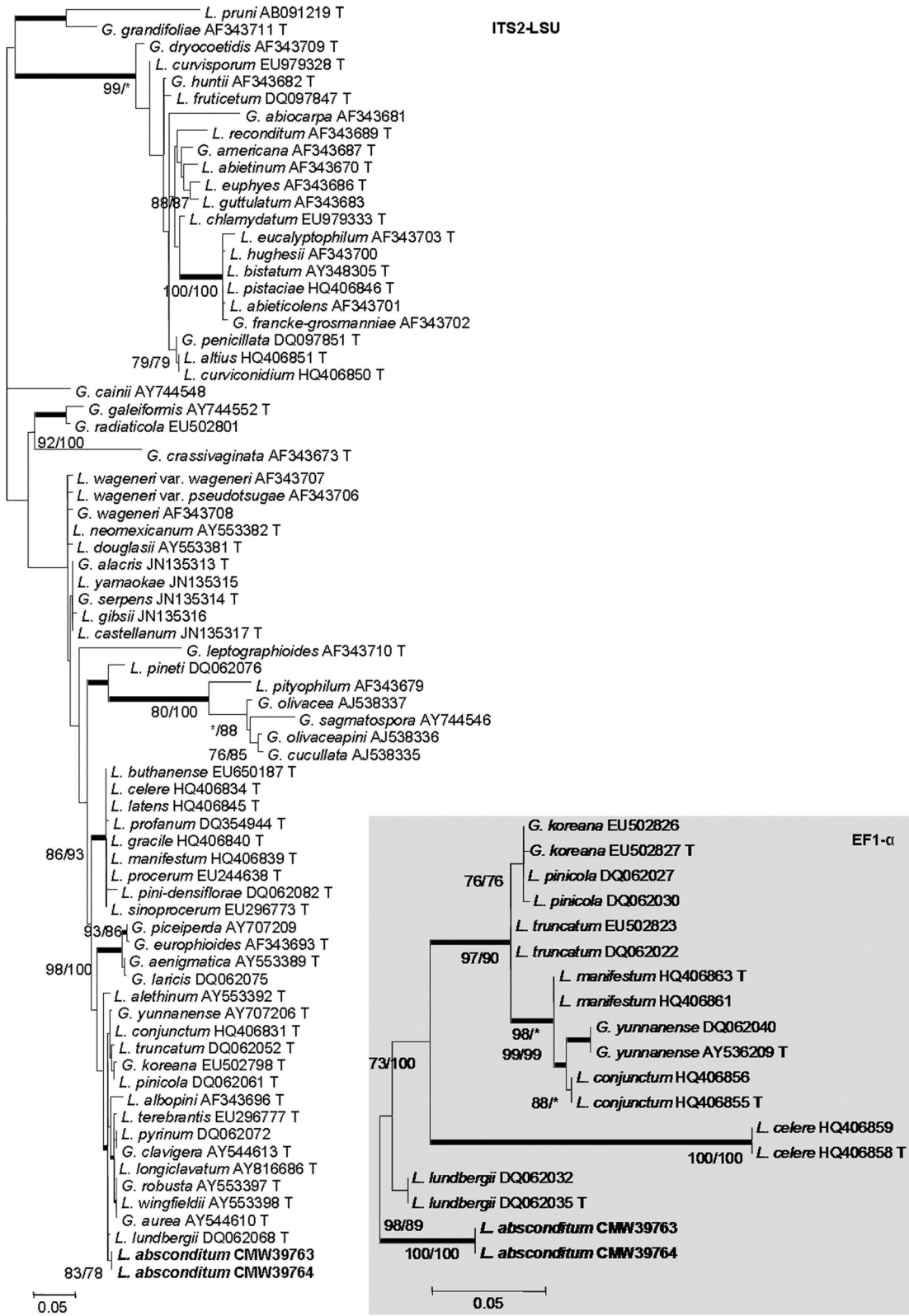


Fig. 2 Phylogram based on ML analyses of β-tubulin **(a)**, and calmodulin **(b)** sequences. ML and MP bootstrap support values (1,000 replicates) are indicated at the nodes. BI probabilities (above 90 %) are indicated by *bold lines* at the relevant branching points. *Bootstrap values lower than 75 %, *T* ex-type isolates, *Scale bar* total nucleotide difference between taxa, *Bold* new species, *ML* maximum likelihood, *MP* maximum parsimony, *BI* Bayesian inference

covered the entire surface of the Petri dishes. Taxon B was the only *Leptographium* sp. able to grow at 30 °C. The optimum temperatures for growth of Taxon A and Taxon B were 30 and 20 °C respectively, with the cultures having an average diameter of 34 and 63 mm respectively in 8 days.


ITS2-LSU
L. pruni AB091219 T
G. grandifoliae AF343711 T
G. dryocoetidis AF343709 T
L. curvisporum EU979328 T
99/*
G. huntii AF343682 T
L. fruticetum DQ097847 T
G. abiocarpa AF343681
L. reconditum AF343689 T
G. americana AF343687 T
L. abietinum AF343670 T
L. euphyes AF343686 T
88/87
L. guttulatum AF343683
L. chlamydatum EU979333 T
L. eucalyptophilum AF343703 T
L. hughesii AF343700
L. bistatum AY348305 T
100/100
L. pistaciae HQ406846 T
L. abieticolens AF343701
G. francke-grosmanniae AF343702
G. penicillata DQ097851 T
79/79
L. altius HQ406851 T
L. curviconidium HQ406850 T
G. cainii AY744548
G. galeiformis AY744552 T
G. radiaticola EU502801
92/100
G. crassivaginata AF343673 T
L. wageneri var. wageneri AF343707
L. wageneri var. pseudotsugae AF343706
G. wageneri AF343708
L. neomexicanum AY553382 T
L. douglasii AY553381 T
G. alacris JN135313 T
L. yamaokae JN135315
G. serpens JN135314 T
L. gibsii JN135316
L. castellanum JN135317 T
G. leptographioides AF343710 T
L. pineti DQ062076
L. pityophilum AF343679
80/100
G. olivacea AJ538337
G. sagmatospora AY744546
*/88
G. olivaceapini AJ538336
76/85
G. cucullata AJ538335
L. buthanense EU650187 T
L. celere HQ406834 T
L. latens HQ406845 T
L. profanum DQ354944 T
86/93
L. gracile HQ406840 T
L. manifestum HQ406839 T
L. procerum EU244638 T
L. pini-densiflorae DQ062082 T
L. sinoprocerum EU296773 T
93/86
G. piceiperda AY707209
G. europhioides AF343693 T
98/100
G. aenigmatica AY553389 T
G. laricis DQ062075
L. alethinum AY553392 T
G. yunnanense AY707206 T
L. conjunctum HQ406831 T
L. truncatum DQ062052 T
G. koreana EU502798 T
L. pinicola DQ062061 T
L. albopini AF343696 T
L. terebrantis EU296777 T
L. pyrinum DQ062072
G. clavigera AY544613 T
L. longiclavatum AY816686 T
G. robusta AY553397 T
L. wingfieldii AY553398 T
G. aurea AY544610 T
L. lundbergii DQ062068 T
L. absconditum CMW39763
83/78
L. absconditum CMW39764
0.05
EF1-α
G. koreana EU502826
G. koreana EU502827 T
76/76
L. pinicola DQ062027
L. pinicola DQ062030
L. truncatum EU502823
97/90
L. truncatum DQ062022
L. manifestum HQ406863 T
L. manifestum HQ406861
98/*
G. yunnanense DQ062040
99/99
G. yunnanense AY536209 T
73/100
L. conjunctum HQ406856
88/*
L. conjunctum HQ406855 T
L. celere HQ406859
100/100
L. celere HQ406858 T
L. lundbergii DQ062032
L. lundbergii DQ062035 T
98/89
L. absconditum CMW39763
100/100
L. absconditum CMW39764
0.05

◀ **Fig. 3** Phylogram of species in *Leptographium* sensu lato based on ML analyses of ITS2-LSU and EF1-α sequences. ML and MP bootstrap support values (1,000 replicates) are indicated at the nodes. BI probabilities (above 90 %) are indicated by *bold lines* at the relevant branching points. *Bootstrap values lower than 75 %, *T* ex-type isolates, *Scale bar* total nucleotide difference between taxa, *Bold* new species *ML* maximum likelihood, *MP* maximum parsimony, *BI* Bayesian inference

Biodiversity and aggressiveness

The collected fungi included, in order of total relative abundance, *Grosmannia olivacea*, *Leptographium procerum*, *Ophiostoma canum*, Taxon A, *O. nebulare*, *O. ips*, *Graphilbum crescericum*, *L. guttulatum*, Taxon B, *L. wingfieldii* and *O. saponiodorum* (Table 3).

Grosmannia olivacea was the most frequently encountered fungus on *O. laricis*, *H. attenuatus*, *H. palliatus, H. angustatus* and *H. ligniperda* colonizing *P. sylvestris*; and on *O. laricis* and *H. ater* colonizing *P. nigra*. No isolates of *G. olivacea* were obtained from *P. radiata*. *Leptographium procerum* was most commonly found on *H. angustatus* and *H. palliatus* colonizing *P. radiata*. The frequency of occurrence of *O. canum* from *H. palliatus* colonizing *P. radiata* was 27.3 % and from *T. piniperda* colonizing *P. sylvestris* was 25.0 %. Taxon A occurred on 3.6 and 10.3 % of *H. attenuatus* and *O. laricis* individuals infesting *P. sylvestris*, respectively. *Ophiostoma nebulare*, a recently described species from *H. attenuatus* infesting *P. radiata* in Basque Country (northern Spain) (Romón et al. 2014), occurred on 9.1 and 6.2 % of *H. palliatus* and *H. attenuatus* individuals respectively colonizing *P. radiata*. *Ophiostoma ips* was the most frequently encountered fungus on *O. erosus* colonizing the native pine species included in the present study. *Graphilbum crescericum*, recently described from *H. ater*, *O. erosus* and *H. palliatus* infesting *P. radiata* in Basque Country (northern Spain) (Romón et al. 2014), occurred on 3.6 and 3.2 % of *H. attenuatus* and *O. laricis* individuals colonizing *P. sylvestris* and *P. nigra* respectively. *Leptographium guttulatum* was the most frequently encountered fungus on *T. piniperda* colonizing *P. radiata*, whereas *L. wingfieldii* was present on 25 % of the *T. piniperda* specimens colonizing *P. sylvestris*. Taxon B occurred on 3.2 % of *O. laricis* individuals infesting *P. nigra*, whereas *O. saponiodorum* occurred on 7.1 % of *H. ater* colonizing the same host tree species. No fungal species was isolated from the weevil *H. abietis* (Table 3).

The fungal community associated with *T. piniperda* colonizing *P. sylvestris* had the greatest diversity. In contrast, the highest level of species-richness was obtained for the community associated with *O. laricis* colonizing *P. nigra*, which included six species dominated by *G. olivacea*. The same fungal species dominated the communities associated with *O. laricis* colonizing *P. sylvestris*, *H. ater* colonizing *P. nigra* and *H. angustatus* colonizing *P. sylvestris* (Table 3). The fungal communities associated with buried *P. sylvestris*, *P. nigra* and *P. radiata* baiting logs had mean diversities of 0.47, 0.21 and 0.13 respectively.

In the pathogenicity tests, *L. wingfieldii*, *L. guttulatum* and *O. ips* caused plants to have yellow needles after 2 weeks (Fig. 5a). None of the other inoculated fungi nor the control treatment gave rise to external symptoms on the plants. They also induced lesions of similar size to those on the plants inoculated with sterile agar as controls. Average lesion lengths for the three most aggressive species were 18.1 (±9.5), 10.8 (±9.7) and 5.4 (±7.3) cm respectively (Fig. 5b).

Taxonomy

Based on sequence comparisons, morphology and growth comparisons in culture, two groups of isolates from bark beetles colonizing pines in Spain were found to represent undescribed species of *Ophiostoma* and *Leptographium* in the Ophiostomatales. They are described as follows:

TAXON A

Ophiostoma cantabriense P. Romón, Z. W. De Beer & M. J. Wingf., sp. nov. (Figure 6).

MycoBank MB 807054.

Distribution: Cantabria province, Spain.

Host: *Pinus sylvestris* (Pinaceae).

Etymology: *cantabriense*, referring to Cantabria where this species was first collected.

Description: Sporothrix-like asexual state: conidiophores (17.5–) 12.3–63.0 (−77.4) µm long; conidia fusiform (3.9–) 3.9–5.2 (−5.6) × (1.1–) 1.2–1.7 (−1.7) µm. Sexual state not observed.

Table 2 Statistics from the different phylogenetic analyses

Dataset	Coding genes		Maximum Likelihood			Maximum Parsimony						Bayesian inference	
	Exons	Introns	Subst. model	Pinvar	Gamma	PIC	No. of trees	Tree length	CI	RI	HI	Subst. model	Burn-in
ITS	–	–	GTR+G	0	0.4410	404	100	1,589	0.475	0.844	0.525	GTR+G	500
β-tubulin	4–6	4–5	HKY+G	0	0.0990	27	1	30	0.933	0.957	0.067	HKY+G	500
Calmodulin	3–6	3–4	TPM1uf+G	0	0.2540	94	8	109	0.963	0.975	0.037	TPM1uf+G	500
ITS2-LSU	–	–	TrN+G	0	0.2470	126	100	407	0.548	0.881	0.452	TrN+G	500
EF1-α	3–4	23	TrN+G	0	0.3730	117	48	145	0.897	0.940	0.103	TrN+G	500

Subst. Model best fit substitution model; *Pinvar* proportion of invariable sites; *Gamma* Gamma distribution shape parameter; *PIC* parsimony informative characters; *CI* consistency index; *RI* retention index; *HI* homoplasy index

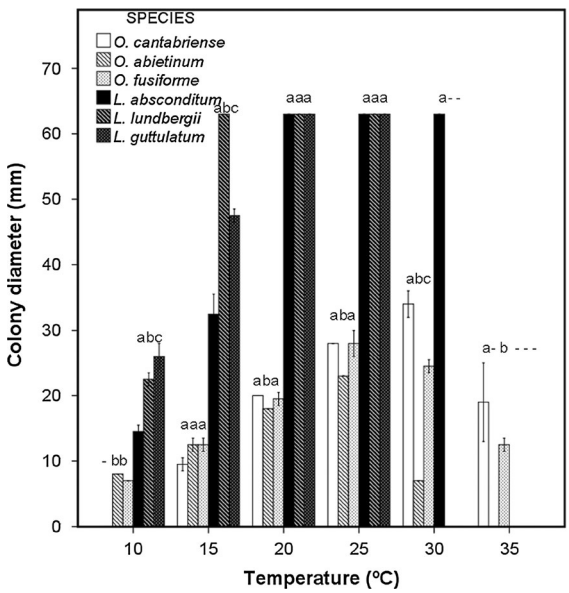


Fig. 4 Mean growth on MEA (two isolates per tested species, ±standard deviation) of *O. cantabriense*, *L. absconditum* and closely related species (groups respectively with *white* and *dark bars*) at a range of temperatures after 8 days in the dark. Means with different letter are significantly different within each species group and temperature ($P > 0.05$), by ANOVA followed by Tukey's test

Culture characters: Colonies with optimal growth at 30 °C on 2 % MEA, reaching 34 mm diameter in 8 days. Colonies white. Very little aerial mycelium.

Specimens examined. SPAIN, Cantabria. *Hylastes attenuatus* beetle infesting a buried log of *Pinus sylvestris*, Jun. 2012, P. Romón, PREM 60890 (holotype); ex-type culture CMW39766; *ibid* CBS136529). SPAIN, Cantabria. *Orthotomicus laricis* beetle infesting a buried log of *Pinus sylvestris*, Jun. 2012, P. Romón, PREM 60891 (paratype); ex-culture CMW39767; *ibid* CBS136530). SPAIN, Cantabria. *Orthotomicus laricis* beetle infesting a buried log of *Pinus sylvestris*, Jun. 2012, P. Romón, PREM 60892 (paratype); ex-culture CMW39768; *ibid* CBS136531). SPAIN, Cantabria. *Orthotomicus laricis* beetle infesting a buried log of *Pinus sylvestris*, Jun. 2012, P. Romón, PREM 60893 (paratype); ex-culture CMW39769; *ibid* CBS136532).

TAXON B

Leptographium absconditum P. Romón, Z.W. De Beer & M.J. Wingf., sp. nov. (Figure 7).

MycoBank MB 807055.

Distribution: Cantabria province, Spain.

Host: Pinus nigra (Pinaceae).

Etymology: *absconditum*, referring to the hidden status of this species.

Description: Leptographium-like asexual state: conidiophores (20.2–) 30.2–88.1 (−92.8) µm long; conidia obovoid with truncate bases (7.9–) 8.1–9.6 (−9.7) × (3.2–) 3.4–4.3 (−4.3) µm. Sexual state not observed.

Culture characters: Colonies with optimal growth at 20 °C on 2 % MEA, reaching 63 mm diameter in 8 days. Colonies olivaceous. Abundant aerial mycelium.

Specimens examined. SPAIN, Cantabria. *Orthotomicus laricis* beetle infesting a buried log of *Pinus nigra*, Jun. 2012, P. Romón, PREM 60888 (holotype); ex-type culture CMW39763; *ibid* CBS136527). SPAIN, Cantabria. *Orthotomicus laricis* beetle infesting a buried log of *Pinus nigra*, Jun. 2012, P. Romón, PREM 60889 (paratype); ex-culture CMW39764; *ibid* CBS136528).

Table 3 Isolation number and frequencies of fungi associated with bark beetles specimens and their galleries in *Pinus sylvestris* (S), *Pinus nigra* (N) and *Pinus radiata* (R) (in order of total relative abundance)

	H. palliatus			*H. attenuatus*			*H. angustatus*			*H. ater*			*H. ligniperda*		
	S	N	R	S	N	R	S	N	R	S	N	R	S	N	R
G. olivacea	1	–	–	2	–	–	5[a]	–	–	1	4	–	3	–	–
	16.6 %			7.1 %			71.4 %			11.1 %	28.6 %		30.0 %		
L. procerum	–	–	3	1	–	–	1	–	2	1	1	–	–	–	–
			27.3 %	3.6 %			14.3 %		66.6 %	11.1 %	7.1 %				
L. guttulatum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L. absconditum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L. wingfieldii	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
O. canum	–	–	3	–	–	–	–	–	–	–	–	–	2	–	–
			27.3 %										20.0 %		
O. cantabriense	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–
				3.6 %											
O. nebulare	–	–	1	–	–	1	–	–	–	–	1	–	–	–	–
			9.1 %			6.2 %					7.1 %				
O. ips	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
O. saponiodorum	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–
											7.1 %				
Gra. crescericum	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–
				3.6 %											
Total no. samples	6	2	11	28	14	16	7	–	3	9	14	–	10	–	–
Total no. isolates	1	–	7	5	–	1	6	–	2	2	7	–	5	–	–
H	0.29	–	0.91	0.49	–	0.16	0.51	–	0.27	0.48	0.90	–	0.68	–	–
S	1	–	3	4	–	1	2	–	1	2	4	–	2	–	–
E	–	–	0.83	0.35	–	–	0.73	–	–	0.69	0.65		0.98	–	–

Table 3 continued

	O. laricis			*O. erosus*			*T. piniperda*			*G. materiarius*			*H. abietis*[b]			Collection no.
	S	N	R	S	N	R	S	N	R	S	N	R	S	N	R	
G. olivacea	22 76.0 %	24 38.1 %	–	–	–	–	1 25.0 %	–	–	1 11.1 %	–	–	–	–	–	CMW39755
L. procerum	1 3.4 %	5 8.0 %	–	–	–	–	–	–	–	2 22.2 %	–	–	–	–	–	CMW39762
L. guttulatum	–	–	–	–	–	–	–	–	2 100 %	–	–	–	–	–	–	CMW39761
L. absconditum	–	2 3.2 %	–	–	–	–	–	–	–	–	–	–	–	–	–	CMW39763
L. wingfieldii	–	–	–	–	–	–	1 25.0 %	–	–	–	–	–	–	–	–	CMW39756
O. canum	2 7.1 %	2 3.2 %	–	–	–	–	1 25.0 %	–	–	–	–	–	–	–	–	CMW39759
O. cantabriense	3 10.3 %	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CMW39766
O. nebulare	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CMW39760
O. ips	–	1 1.6 %	–	1 100 %	1 50.0 %	–	–	–	–	–	–	–	–	–	–	CMW39765
O. saponiodorum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CMW39758
Gra. crescericum	–	2 3.2 %	–	–	–	–	–	–	–	–	–	–	–	–	–	CMW39757
Total no. samples	29	63	–	1	2	–	4	–	2	9	3	–	–	6	–	
Total no. isolates	28	36	–	1	1		3	–	2	3	–	–	–	–	–	
H	0.73	0.89	–	0	0.34	–	1.03	–	0	0.57	–	–	–	–	–	
S	4	6	–	1	1	–	3	–	1	2	–	–	–	–	–	
E	0.52	0.49	–	–	–	–	0.94	–	–	0.82	–	–	–	–	–	

[a] In shaded are represented dominant species for each bark beetle associated fungal community. A species is considered dominant if $P_i > 1/S$ where P_i is proportion of total sample represented by species i and S (species richness) is number of competing species present 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)

[b] *Hylobius abietis* weevil specimens were collected from logs surface

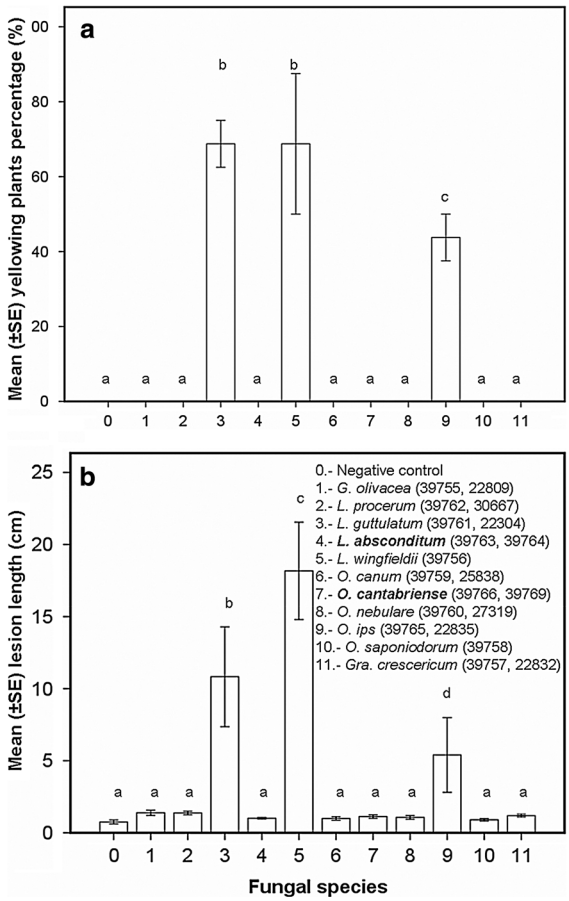


Fig. 5 Mean yellowing plants percentage **(a)** and lesion length **(b)** of collected fungal species after 6 weeks. Means with different letter are significantly different ($P > 0.05$), by ANOVA followed by Tukey's test. All isolates accession numbers belong to the CMW culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria

Discussion

This study includes the first detailed consideration of the ophiostomatoid fungi associated with root-infesting bark beetles in Spain. Isolations yielded a total of 110 fungal cultures that included nine known ophiostomatoid fungal species and two new taxa. Pathogenicity tests showed that several of these fungi are able to produce lesions in inoculated seedlings and this gives some indication of their ability to colonise infected trees.

Ophiostoma cantabriense, described as new in the present study, resides in the *S. schenckii—O. stenoceras* complex, which is characterized by species with orange-section-shaped allantoid ascospores without sheaths, and sporothrix-like asexual states (De Beer et al. 2003; De Beer and Wingfield 2013). The complex includes several species associated with human sporotricosis (Marimon et al. 2007), soil (De Meyer et al. 2008), hardwoods (Aghayeva et al. 2004) or *Protea* infructescences (Roets et al. 2008; 2010). *Ophiostoma cantabriense*, mainly isolated from *O. laricis* colonizing *P. sylvestris*, showed high ITS1-5.8S-ITS2 homology with the type strain of *Ophiostoma lunatum* (CMW10563, AY280485, Aghayeva et al. 2004). The main morphological differences between these species are fusiform, longer conidia and also larger conidiophores in the new species (Table 4).

Leptographium absconditum formed a discrete well-supported ITS clade close to *L. lundbergii* complex. ITS2-LSU and elongation factor 1-alpha sequences confirmed this position. *Leptographium lundbergii* represents the type of the genus *Leptographium*, has broadly truncate conidia, and it is distinguished from *L. truncatum* by slow colony growth, sparse sporulation and larger conidia (Jacobs et al. 2005). *Leptographium absconditum*, described in the present study, was exclusively isolated from the bark beetle *Orthotomicus laricis* colonizing *Pinus nigra*. The main morphological differences between *L. absconditum* and *L. lundbergii* are growth at 30 °C and smaller colony diameters between 10 and 15 °C, smaller conidiophores and larger conidia in the new species (Table 4).

Grosmannia olivacea was the dominant species amongst the fungal communities associated with *O. laricis*, *H. angustatus* and *H. ater*. No isolates of this fungus were obtained from *P. radiata*. The same occurred with *O. cantabriense*, *O. ips*, *G. crescericum*, *L. absconditum*, *O. saponiodorum* and *L. wingfieldii*, ratifying the known European distribution of this fungus in a low but uniform frequency with the bark beetle *T. piniperda* colonizing *P. sylvestris* (Jacobs and Wingfield 2001). In contrast, *L. procerum* was most commonly found on *H. angustatus* and *H. palliatus* colonizing *P. radiata*. Similarly, *O. nebulare* occurred on *H. palliatus* and *H. attenuatus* colonizing *P. radiata* and *L. guttulatum* was the most frequent fungus on *T. piniperda* colonizing *P. radiata*.

Various ophiostomatoid fungi known to be associated with root-feeding bark beetles in Europe where not encountered in this study. These include *Grosmannia radiaticola*, *L. lundbergii*, *L. truncatum*

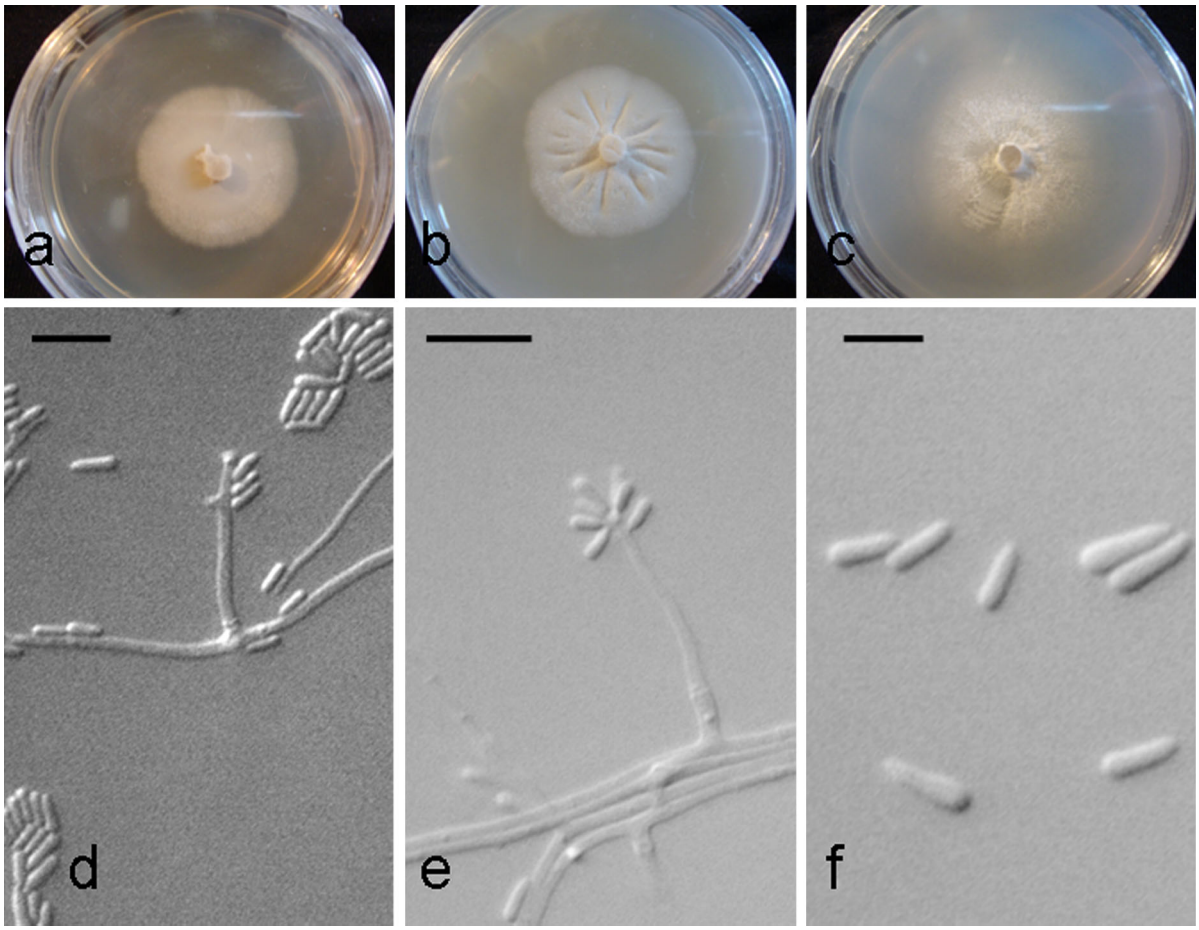


Fig. 6 *Ophiostoma cantabriense* (CMW39766). **a–c** Growing respectively on 2 % MEA, PDA and OA after 8 days at 25 °C; **d–e** Sporothrix-like conidiophores in different growing stages. *Scale bars* = 10 μm; **f** Fusiform conidia. *Scale bar* = 5 μm

(Jankowiak and Bilański 2013b), *L. guttulatum*, *O. quercus*, *O. ips*, *O. pluriannulatum*, and *L. wingfieldii* (Romón et al. 2007), *L. guttulatum*, *L. lundbergii*, *G. serpens*, *Pesotum* spp. (Wingfield and Gibbs 1991), *Graphium aureum*, *G. penicillata*, *L. lundbergii*, *G. serpens*, *O. floccosum*, *O. ips*, *O. minus*, *O. piceae*, *O. piliferum* (Mathiesen 1950; Mathiesen-Käärik 1953), all of which are known associates of *H. ater* and *H. ligniperda*. Their absence from the present study could be due to various factors including time and intensity of sampling, different host trees or the area sampled. In this regard, it was also interesting that *G. serpens*, a very common associate of several root-infesting *Hylastes* spp. and *H. ligniperda* in some parts of England (Wingfield and Gibbs 1991), Sweden (Mathiesen 1950; Mathiesen-Käärik 1953) and South Africa (Jacobs and Wingfield 2001), was not encountered. The absence of fungi such as *L. procerum* and *O. quercus* on *H. abietis* (Jankowiak and Bilański 2013a) was also noteworthy.

Many new bark beetle-fungus associations emerged from this study. To the best of our knowledge, all isolations made from *H. angustatus* and *G. materiarius* are new fungus-vector records. All records, except those for *L. procerum* and *G. olivacea*, are new for *H. palliatus*, *H. ater* and *H. ligniperda*. All fungal species other than *O. ips*, were newly recorded from *O. laricis*. *Tomicus piniperda* has also not previously been associated with *G. olivacea* (Mathiesen 1950; Mathiesen-Käärik 1953; Wingfield and Gibbs 1991; Jacobs and Wingfield 2001; Kirisits 2004; Romón et al. 2007; Jankowiak and Bilański 2013b). *Orthotomicus erosus*, *T. piniperda*, *G. materiarius* and *H. abietis* are not root-feeding insects and the frequencies

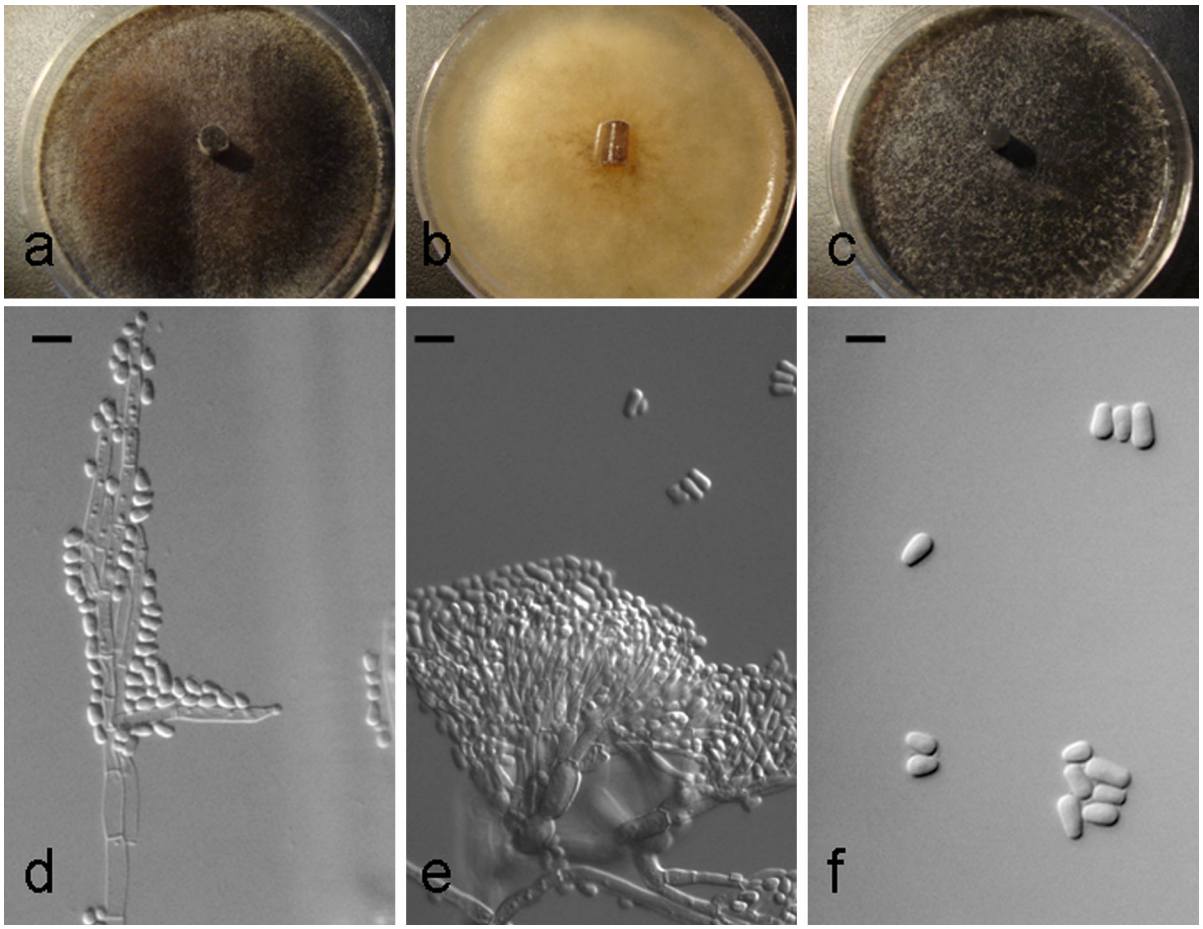


Fig. 7 *Leptograhium absconditum* (CMW39763). **a–c** Growing respectively on 2 % MEA, PDA and OA after 8 days at 25 °C; **d–e** Leptographium-like conidiophores in different growing stages. *Scale bars* = 10 μm; **f** Obovoid truncate conidia. *Scale bar* = 5 μm

Table 4 Characters comparison of new species with closely related species (measurements in μm)

	O. cantabriense	*O. abietinum* Marmolejo & Butin (1990)	*O. fusiforme* Aghayeva et al. (2004)	*L. absconditum*	*L. lundbergii* Jacobs et al. (2005)	*L. guttulatum* Jacobs et al. (2001)
Conidiophore length	(17.5–)	5.0–50.0	14.3–53.9	(20.2–)	2.0–4.0	(200.0–)
	12.3–63.0		(−72)	30.2–88.1		365.0–465.0
	(−77.4)			(−92.8)		(−810.0)
Conidia shape	Fusiform	Clavate-cilindrical	Fusiform-guttuliform	Obovoid with truncate bases	Broadly ellipsoid with truncate bases	Oblong to slightly obovoid, conspicuously guttulate
length	(3.9–)	4.0–7.5	3.2–5.9	(7.9–)	(6.0–)	(4–)
	3.9–5.2		(−8)	8.1–9.6	7.0–11.0	5.0–9.0
	(−5.6)			(−9.7)	(−15.0)	(−10)
width	(1.1–)	1.0–2.0	1.1–1.9	(3.2–)	2.0–4.0	2.0–3.0
	1.2–1.7		(−2.1)	3.4–4.3		
	(−1.7)			(−4.3)		
Culture color	White	White	White	Olivaceous	Olivaceous	Olivaceous

Measurements are presented in the format (minimum−) mean minus standard deviation—mean plus standard deviation (−maximum) where possible

of isolation of their associated fungi must be considered with caution.

Leptographium wingfieldii, *L. guttulatum* and *O. ips* were much more aggressive than the other fungi evaluated in the pathogenicity tests. This study confirms the results of earlier inoculation studies (Owen et al. 1987; Raffa and Smalley 1988; Lieutier et al. 1989; Parmeter et al. 1989; Langström et al. 2001; Solheim et al. 2001; Fernández et al. 2004; Lieutier et al. 2004; Jankowiak 2006) that demonstrated that *L. wingfieldii* and *O. ips* have high levels of aggressiveness. The aggressiveness in inoculation tests has not previously been tested for *L. guttulatum*. Studies on pathogenicity of the isolates used in this study should also be conducted on large pine trees with equivalent inoculation loads. Among the other inoculated fungi, *L. procerum* appeared to be non-pathogenic to *P. radiata* seedlings; although it has been associated with the decline of *Pinus strobus* in various parts of the United States (Lackner and Alexander 1982; Alexander et al. 1988), and the death of *Pinus tabuliformis* trees in China in association with the introduced red turpentine beetle *Dendroctonus valens* (Lu et al. 2009). Besides its aggressiveness in inoculations, *O. ips* is an important agent of sapstain (Seifert 1993) and this is most likely also true for *L. wingfieldii* and *L. guttulatum* as they are frequently isolated from stained wood.

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