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Multigene phylogenies of Ophiostomataceae associated with Monterey pine bark beetles in Spain reveal three new fungal species

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Abstract: *Ophiostoma* species, some of which cause sapstain in timber and/or are mild pathogens, are common fungal associates of bark beetles (Coleoptera: Scolytinae). Three new Ophiostomataceae from Spain are recognized in the present study based on comparisons of sequence data for three gene regions as well as morphological characteristics. The new taxa are described as *Ophiostoma nebulare* sp. nov., *Ophiostoma euskadiense* sp. nov. and *Graphilbum crescericum* sp. nov.

Key words: β-tubulin gene, calmodulin gene, morphology, rRNA internal transcribed spacers, sequencing

Introduction

Adaptation facilitating insect dispersal, such as erect ascomata and conidiomata bearing sticky spores, has arisen frequently in the evolution of fungi in the Ascomycota. This morphological convergence has resulted in a confused taxonomy for species collectively treated in the so-called ophiostomatoid fungi (Wingfield et al. 1993, Seifert et al. 2013). These fungi all have morphologically similar sexual states residing in two phylogenetically unrelated orders, the Microascales and Ophiostomatales. The majority of known ophiostomatoid species belong to the Ophiostomatales, and traditionally the sexual (teleomorph) and asexual (anamorph) states of these fungi were classified in different genera. Thus one species could have two or sometimes three names, each representing a different state. However, in 2011 the International Code of Nomenclature for Algae, Fungi and Plants (ICN) was emended and currently only allows one species name for each fungus, with the oldest genus name having priority (Hawksworth 2011, Hawksworth et al. 2011). The application of the new rules inevitably led to emended concepts for several of the ophiostomatoid genera, as well as name changes in the Ophiostomatales (de Beer and Wingfield 2013, de Beer et al. 2013). These changes have to be considered in all studies dealing with the biodiversity of these fungi.

Bark beetles that infest conifers carry many different ophiostomatoid fungi including those related to *Ophiostoma* (Jacobs and Kirisits 2003; Kim et al. 2003; Zhou et al. 2004, 2006; Kirisits 2007; Romon et al. 2007; Linnakoski et al. 2008, 2009; Masuya et al. 2009; Jankowiak and Kolarik 2010; Linnakoski et al. 2010; Paciura et al. 2010) and *Ceratocystis* (Harrington and Wingfield 1998, Harrington et al. 2002, van Wyk et al. 2004, Viiri and Lieutier 2004, Yamaoka et al. 2009, Reid et al. 2010). Although many of these fungi have the ability to cause lesions when inoculated into conifers (e.g. *Grosmannia clavigera* [Owen et al. 1987], *Leptographium terebrantis* [Parmeter et al. 1989], *L. wingfieldii* [Jankowiak 2006], *Ophiostoma ips* [Raffa and Smalley 1988], *O. minus* [Jankowiak 2006], *Ceratocystis laricicola* [Redfern et al. 1987] and *C. polonica* [Christiansen and Solheim 1990]), most are not considered pathogens in their own right (Six and Wingfield 2011). The only species able to cause disease independently of its beetle vectors is *L. wageneri*, the causal agent of black stain root disease (Morrison and Hunt 1988). Other species, such as *O. ips*, *O. minus*, *O. piceae*, *O. piliferum* and *O. pluriannulatum*, are best considered as agents of sapstain (Seifert 1993).

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Knowledge of bark beetle-associated fungi in the Iberian Peninsula is limited (de Ana Magán 1982, 1983; Fernández et al. 2004; Villarreal et al. 2005; Romón et al. 2007). Only two studies deal with the taxonomy of these fungi. One (de Ana Magán 1983) erroneously described a new species, *Leptographium gallaeiciae*, that later was identified as *Ophiostoma serpens* (Jacobs and Wingfield 2001). Another fungus in this group, *Ophiostoma sejunctum* (Villarreal et al. 2005), has been described, suggesting that fungi in the region deserve more study. *Pinus radiata* (Monterey pine) is the most economically important conifer species in Spain with exotic plantations covering an area of 270 000 ha. Romón et al. (2007) studied the biodiversity and spatiotemporal ecological segregation of several ophiostomatalean fungi differentially associated with 14 insect species colonizing *P. radiata* in northern Spain. The present study considers the identity, nomenclature and phylogenetic relationships of three new species, collected by Romón et al. (2007), revealed by multigene sequencing and phylogenetics.

MATERIALS AND METHODS

Isolates.—All isolates used in this study were deposited both in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and in the Spanish Type Culture Collection (CECT), University of Valencia, Valencia, Spain. Isolates of the new taxa also were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, and their corresponding dried holo- and paratypes were deposited in the National Collection of Fungi of South Africa (PREM). The origin, number, collection and GenBank numbers of the isolates and sequences used in the phylogenetic analyses are presented (TABLE I).

DNA extraction, PCR amplification, DNA sequencing and phylogenetic analysis.—Two milliliter Eppendorf tubes containing 1 mL malt extract broth at 2% (wt/vol) were inoculated by transferring hyphal tips from the edges of individual colonies. After 15 d static incubation at 25 C, DNA was extracted using Prepman Ultra Sample Preparation Reagent (Applied Biosystems). PCR amplification was performed with primers ITS1-F (5′–CTTGGTCATTTAGAGGAAGTAA–3′) and ITS4 (5′–TCCTCCGCTTATTGATATGC–3′) (White et al. 1990) to amplify the ITS1–5.8S–ITS2 region of rDNA. The template DNA was amplified in a 50 µL PCR reaction volume, consisting of 5 µL 10× reaction buffer, 5 µL MgCl2 (25 mM), 5 µL dNTPs (10 mM), 1 µL each primer (10 µM), 1.5 µL DNA solution and 0.5 µL Super-Therm Taq polymerase. PCR reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems) with an initial denaturation step of 2 min at 95 C, 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 8 min at 72 C.

In cases where ITS sequences were not sufficient to distinguish species, amplicons also were obtained for the β-tubulin gene with primers T10 (5′–ACGATAGGTTCACCTCCAGAGAC–3′) or Bt2a (5′–GGTAACCAAATCGGTGCGCTTTC–3′) with Bt2b (5′–GGTAACCAAATCGGTGCTGCTTTC–3′) (Glass and Donaldson 1995) and part of the calmodulin gene with primers CL1 (5′–GARTWCAAGGAGGCCTTCTC–3′) and CL2A (5′–TTTTGCATCATGAGTTGGAC–3′) (O'Donnell 2000, Romeo et al. 2011). PCR conditions for calmodulin gene amplification were the same as those for ITS, whereas for β-tubulin the cycle included an initial denaturation step of 4 min at 95 C, followed by 35 cycles of denaturation for 1 min at 95 C, annealing 1 min at 47–52 C and elongation 1 min at 72 C, with a final elongation step of 7 min at 72 C. PCR products were viewed under UV illumination on a 1% agarose gel stained with Gelred (Biotium), run in a Wide Mini-Sub Cell GT Electrophoresis System (BioRad) and digitalized in a white-ultraviolet transilluminator Gel Documentation System (UVP). Amplification products were purified with the High Pure PCR Product Purification Kit (Roche).

Sequencing was performed with ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 377 autosequencer. Forward and reverse sequences were aligned and consensus sequences determined with ContigExpress, Vector NTI Advance 11.5.0 (Invitrogen). BLAST queries were conducted for preliminary identifications, after which datasets that included all the most up-to-date GenBank sequences were compiled in MEGA 5 (Tamura et al. 2011). Sequences were aligned online with MAFFT 6 (Katoh et al. 2002). Datasets were analysed with maximum likehood (ML), maximum parsimony (MP) and Bayesian inference (BI). ML analyses were performed with PhyML 3.0 (Guindon et al. 2006) after determining the substitution model in jModelTest 0.1.1 (Posada 2008). Support for nodes was estimated from 1000 bootstrap replicates. MP analyses were conducted with PAUP*: phylogenetic analysis using parsimony (*and other methods) 4.0b10 (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 was estimated by performing 1000 bootstrap replicates (Felsenstein 1985) with fast-stepwise addition. BI analyses were carried out with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Markov chain Monte Carlo was run 5 000 000 generations with the best-fitting model selected by the Akaike information criterion in MrModeltest 2.3 (http://www.abc.se/~nylander). Trees were sampled every 100 generations. Burn-in values were determined with Tracer 1.4 (http://tree.bio.ed.ac.uk/software/tracer). All sampled trees lower than the burn-in values were discarded and a 50% majority rule consensus tree was constructed.

GenBank accession numbers of published sequences are revealed in the phylogenetic trees, while accession numbers of sequences obtained in the present study are presented TABLE I. Statistical values resulting from the respective

TABLE I. Origin, hosts and GenBank accession numbers for fungal isolates sequenced in this study

Species	CMW no.[a]	CECT no.[b]	CBS no.[c]	PREM[d]	Insect vector/host	Collector	Country	ITS	β-tubulin	Calmodulin
Reference species										
O. abietinum	22310[T]	—	125.89	—	*Abies vejari*	J. Marmolejo	Mexico	—	HM067820	**JQ511966**[e]
O. cf. abietinum	397	—	—	—	*Orthotomicus erosus*	G. Tribe	South Africa	DQ396788	—	**JQ511965**
	26262	—	—	—	*Pinus tabuliformis*	M. Lu	China	EU785446	EU785434	**JQ511964**
	26269	—	—	—	*P. tabuliformis*	M. Lu	China	EU785445	EU785433	**JQ511963**
	28030	—	—	—	*P. sylvestris*	R. Linnakoski	Russia	HM031511	HM031568	**JQ511969**
O. fusiforme	7131	—	112925	57487	*Quercus petraea*	T. Kirisits	Austria	AY280497	AY280464	**JQ511971**
	9968[T]	—	112912	57486	*Populus nigra*	D. Aghayeva	Azerbaijan	AY280481	AY280461	**JQ511967**
O. gossypinum	1118[T]	—	—	—	*P. ponderosa*	R.W. Davidson	Mexico	—	—	**JQ511972**
O. lunatum	10563[T]	—	112927	57489	*Carpinus betulus*	T. Kirisits	Austria	AY280485	AY280466	**JQ511970**
O. stenoceras	2344	—	—	—	*Eucalyptus smithii*	G.H.J. Kemp	South Africa	AY280491	AY280472	**JQ511955**
	3202[T]	—	237.32	—	Pine pulp	H. Robak	Norway	AF484462	DQ296074	**JQ511956**
S. curviconia	17163	—	541.84	—	*P. radiata*	H.L. Peredo	Chile	—	—	**JQ511968**
Sporothrix sp.1	9488	—	—	—	*Dendroctonus mexicanus*	M.J. Wingfield	Mexico	AY546720	—	**JQ511959**
	9491	—	—	—	*D. mexicanus*	M.J. Wingfield	Mexico	AY546721	—	**JQ511958**
Sporothrix sp.2	9487	—	—	—	*D. mexicanus*	M.J. Wingfield	Mexico	AY546694	—	**JQ511961**
	9489	—	—	—	*D. mexicanus*	M.J. Wingfield	Mexico	AY546695	—	**JQ511960**
New species										
O. nebulare sp. nov. (A)	27319	20637	122135	59832	*Hylastes attenuatus*	P. Romón	Spain	**DQ674375**	—	**JQ438828**
	27900	20638	122134	59833	*H. attenuatus*	P. Romón	Spain	**DQ674376**	—	**JQ438829**
O. euskadiense sp. nov. (B)	27318	20631	122138	59829	*Hylurgops palliatus*	X.D. Zhou	Spain	**DQ674369**	**EF396344**	**JQ438830**
	27898	20632	122137	59830	*H. attenuatus*	P. Romón	Spain	**DQ674370**	**GU566608**	**JQ438831**
	27899	20633	122136	59831	*H. attenuatus*	X.D. Zhou	Spain	**DQ674371**	**GU566609**	**JQ438832**
Gra. crescericum sp. nov. (C)	22828	20669	130864	60713	*H. palliatus*	P. Romón	Spain	—	—	**JQ438835**
	22829	20670	130865	60714	*Hylastes ater*	P. Romón	Spain	**DQ539535**	—	**JQ438836**
	22830	20671	—	60715	*H. ater*	P. Romón	Spain	**DQ539536**	—	**JQ438837**
	22831	20672	130866	60716	*O. erosus*	P. Romón	Spain	**DQ539537**	—	**JQ438838**
	22832	20673	—	60717	*O. erosus*	P. Romón	Spain	**DQ539538**	—	**JQ438839**

[a] CMW, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[b] CECT, Spanish Type Culture Collection, University of Valencia.
[c] CBS, Centraalbureau voor Schimmelcultures, the Netherlands.
[d] PREM, South African National Collection of Fungi, South Africa.
[e] Accession numbers of the sequences produced in this study appear in boldface.
[T] Ex-type culture.

TABLE II. Statistics from the different phylogenetic analyses

Dataset	Coding genes		Maximum likelihood			Maximum parsimony					Bayesian inference	
	exons	introns	Subst.[a] model	Pinvar[b]	Gamma[c]	PIC[d]	No. of trees	Tree length	CI[e]	RI[f]	HI[g]	Subst. model	Burn-in
ITS	—	—	GTR+I+G	0.0680	0.4630	442	100	1634	0.497	0.874	0.503	GTR+I+G	500
β-tubulin	4–6	4–5	TPM2+G	0	0.1780	39	1	45	0.933	0.952	0.067	TPM2+G	500
Calmodulin	3–6	3–4	HKY+G	0	0.2090	398	4	114	0.965	0.978	0.035	HKY+G	500

[a] Subst. Model = best fit substitution model.
[b] Pinvar = proportion of invariable sites.
[c] Gamma = Gamma distribution shape parameter.
[d] PIC = parsimony informative characters.
[e] CI = consistency index.
[f] RI = retention index.
[g] HI = homoplasy index.

phylogenetic analyses are presented (TABLE II). DNA sequence matrices are available from TreeBase at http://purl.org/phylo/treebase/phylows/study/TB2:S12569.

Culture characteristics and morphology.—Isolates representing the same species were grown and crossed in all possible combinations on 2% water agar and oatmeal agar with autoclaved pine twigs to induce production of perithecia (Grobbelaar et al. 2010). Perithecia and ascospores and/or slide cultures to observe anamorph structures 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, conidia, perithecia and ascospores, were characterized and compared with the most phylogenetically related species using relevant taxonomic keys and protologs. The measurements are presented as (minimum–) mean minus standard deviation – mean plus standard deviation (–maximum).

For each putative new taxon as well as closely related species, the optimal growth temperature for two isolates was determined by growing them at 5–35 C at 5 C intervals in Sanyo MIR-253 incubators. A 5 mm diam 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 d, and mean minimum, optimum and maximum growth temperatures were calculated. Mean growth was compared among isolates with ANOVA and Tukey test.

RESULTS

PCR, sequencing and phylogenetic analysis.—ITS1-5.8S-ITS2 sequences of the isolates obtained from bark beetles in Spain (Romon et al. 2007) confirmed the presence of 12 well defined and commonly occurring species (FIG. 1) and revealed three new taxa. The amplified ITS regions of isolates representing the taxa (A, B, C) were respectively 489, 532 and 537 bp long. ITS sequences of taxa A and C indicated that these two groups of isolates were different than all known species (FIG. 1) and respectively grouped in *Ophiostoma* sensu lato and *Graphilbum*. However, the ITS sequences of taxon B showed that it grouped near *O. abietinum* and related species in the *Sporothrix schenckii-O. stenoceras* complex but did not sufficiently distinguish among these species. For this reason β-tubulin and calmodulin sequences also were produced for these isolates, as well as for reference species for which sequences of these gene regions were not available (TABLE I). The β-tubulin amplicons of isolates of taxon B was 279 bp. Calmodulin gene sequences from isolates of the three species were respectively 612, 566 and 542 bp. For each of the sequence datasets, MP, ML and Bayesian analyses resulted in

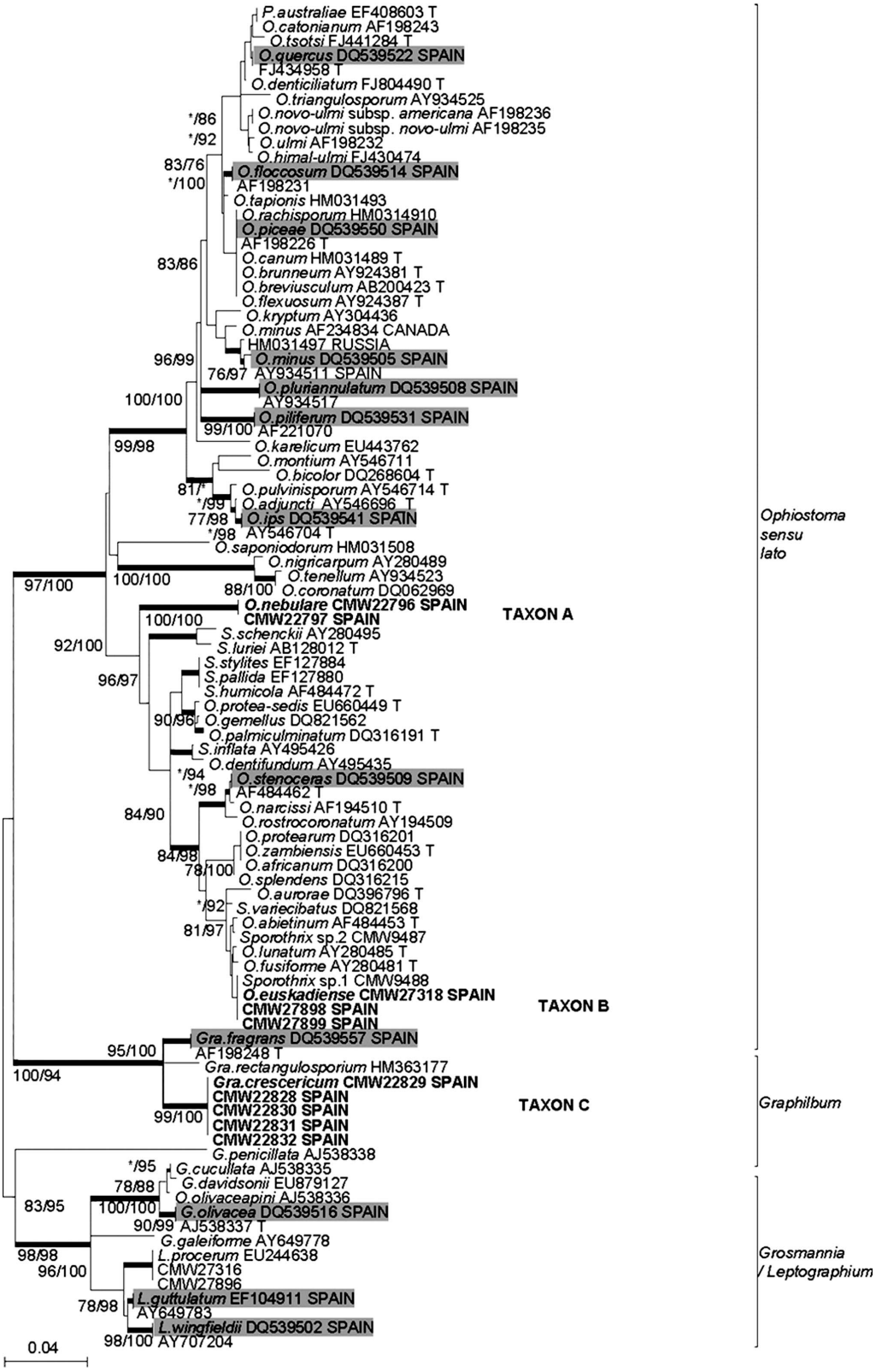

FIG. 1. Phylogram based on ML analyses of ITS1-5.8S-ITS2 rDNA sequences, showing where fungal associates of pine bark beetles in Spain from the study of Romón et al. (2007) groups within the Ophiostomatales. Spanish isolates of known species are shaded, while those of novel taxa are printed in boldface. ML and MP bootstrap support values (1000 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. Bar = total nucleotide difference between taxa. ML = maximum likelihood. MP = maximum parsimony. BI = Bayesian inference.

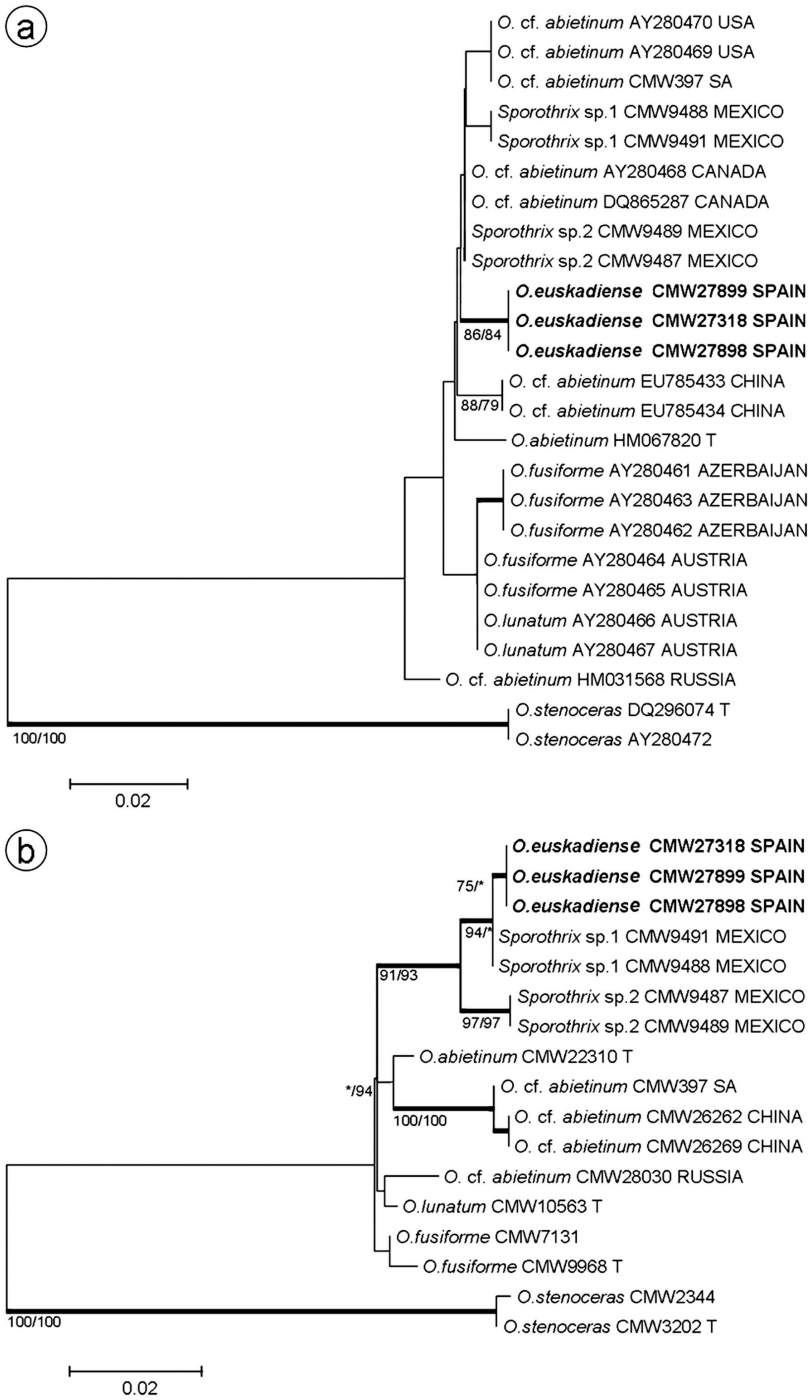

FIG. 2. a. Phylogram based on ML analyses of beta-tubulin (a) and calmodulin (b) gene sequences of the *O. abietinum* subcomplex. ML and MP bootstrap support values (1000 replicates) are indicated at the nodes. BI probabilities (above 90%) are indicated by bold lines at the relevant branching points. * = bootstrap values lower than 75%. T = ex-type isolates. Bar = total nucleotide difference between taxa. Boldface = new species. ML = maximum likelihood. MP = maximum parsimony. BI = Bayesian inference.

trees with similar topologies. Phylograms obtained with ML are presented for all the datasets (FIGS. 1, 2), with nodal support obtained from ML, MP and Bayesian inference indicated on the trees.

Culture characteristics and morphology.—Cultures representing the three new species were white, with little aerial mycelium, and morphologically similar in culture, except for taxon A that had a creamy color

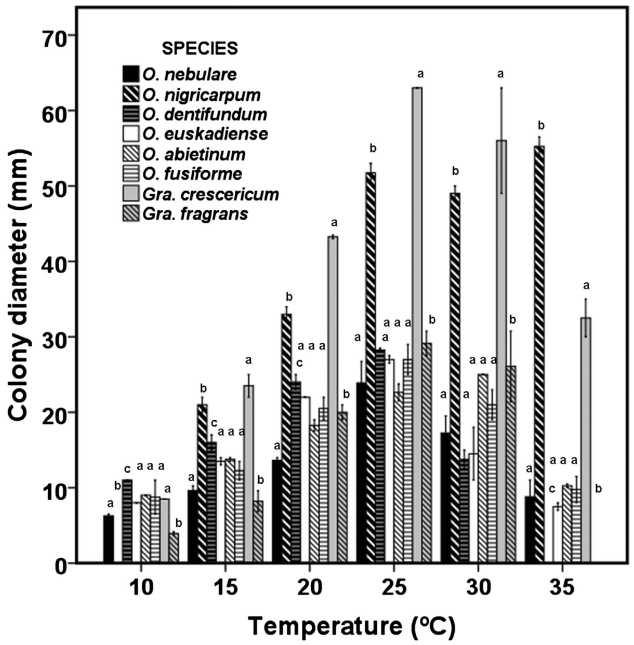

FIG. 3. Mean growth on MEA (two isolates per tested species, ± standard deviation) of *O. nebulare*, *O. euskadiense*, *Gra. crescericum* and closely related species (groups respectively with black, dark gray and light gray bars) at a range of temperatures after 8 d in the dark. Means with different letter are significantly different within each species group and temperature ($P > 0.05$), by ANOVA followed by Tukey test.

on malt extract agar. Isolates representing taxon B produced abundant ascomata in culture. Growth comparisons showed that isolates representing taxon C grew faster than isolates in taxa A and B at all tested temperatures (FIG. 3), whereas taxon B isolates grew faster than taxon A at 10, 15 and 20 C. The optimum temperature for growth of isolates in taxa A, B and C was 25 C, with an average culture diameter of 24.2, 14.4 and 60.4 mm respectively in 8 d (FIG. 3).

TAXONOMY

Based on sequence comparisons and morphology, three groups of isolates from bark beetles colonizing *P. radiata* in Spain were found to represent undescribed species of *Ophiostoma* and *Graphilbum* in the order Ophiostomatales (TABLE III). These are described as follows:

Taxon A:

Ophiostoma nebulare P. Romón, Z.W. de Beer, M.J. Wingf., sp. nov. FIG. 4
MycoBank MB564952

Perithecial bases dark, (83.44–)86.56–101.18(–105.94) µm diam. Perithecial necks dark black, (169.50–)140.54–293.21(–365.86) µm long, (24.61–)25.66–30.81(–30.93) µm wide at base, (8.56–)8.93–12.88(–13.94) µm wide at the apex. Ostiolar hyphae present (8.20–)9.58–15.20(–16.21) µm long and (2.07–)2.11–2.39(–2.47) µm wide. Ascospores allantoid, (3.00–)3.12–4.23(–6.52) × (1.28–)1.42–1.79(–1.88) µm. Sporothrix-like anamorph: conidiophores (20.00–)20.23–20.74(–20.87) µm long; conidia obovoid with truncate bases, (2.53–)2.91–3.70(–3.73) × (1.13–)1.14–1.35(–1.44) µm. Colonies with optimal growth at 25 C on 2% MEA, reaching 24.20 mm diam in 8 d. Colonies whitish to cream with age, changing the media to dark creamy. Little aerial mycelia. Isolation frequency 1.2% from *Hylastes attenuatus*.

Etymology: Referring to the fact that this species causes malt extract agar to change from a dark honey to a dark-creamy color.

Holotype: SPAIN, Basque Country, Morga, Bizkaia, *Hylastes attenuatus* infesting *Pinus radiata*, Jul 2004, P. Romón (PREM 59832, ex-type culture CMW27319 = CECT20637 = CBS122135).

Additional specimens examined: SPAIN, Basque Country, Morga, Bizkaia, *Hylastes attenuatus* infesting *Pinus radiata*, Jul 2004, P. Romón (PREM 59833, ex-paratype culture CMW27900 = CECT20638 = CBS122134).

Taxon B:

Ophiostoma euskadiense P. Romón, Z.W. de Beer, M.J. Wingf., sp. nov. FIG. 5
MycoBank MB564953

Perithecial bases dark, (54.19–)57.64–66.31(–69.69) µm diam. Perithecial necks (201.32–)204.15–213.28(–219.12) µm long, (9.58–)10.18–12.91(–13.98) µm wide at base, (5.26–)5.62–8.83(–9.66) µm wide at the apex. Ostiolar hyphae present (36.67–)41.22–49.13(–49.83) µm long and (3.07–)3.10–3.31(–3.37) µm wide. Ascospores allantoid, (3.15–)3.18–3.56(–3.56) × (1.90–)1.91–2.01(–2.00) µm. Sporothrix-like anamorph: conidiophores (10.02–)10.22–10.76(–10.82) µm long; conidia clavate, (2.10–)2.21–2.95(–3.70) × (1.00–)1.21–1.81(–1.80) µm. Colonies with optimal growth at 25 C on 2% MEA, reaching 14.36 mm diam in 8 d. Colonies shiny white to yellowish in the center with age. Little aerial mycelia. Isolation frequency 0.2 and 0.4% respectively from *Hylurgops palliatus* and *Hylastes attenuatus*.

Etymology: Referring to the Basque Country (Euskadi) where this species first was collected.

Holotype: SPAIN, Basque Country, Morga, Bizkaia, *Hylurgops palliatus* infesting *Pinus radiata*, Jul 2004, X.D. Zhou (PREM 59829, ex-type culture CMW27318 = CECT20631 = CBS122138).

Additional specimens examined: SPAIN, Basque Country, Morga, Bizkaia, *Hylastes attenuatus* infesting *Pinus radiata*, Jul 2004, P. Romón (PREM 59830, ex-paratype culture CMW27898 = CECT20632 = CBS122137); SPAIN, Basque Country, Morga, Bizkaia, *Hylastes attenuatus* infesting *Pinus*

TABLE III. Characters comparison of new *Ophiostoma* species, within the *Ophiostoma stenoceras-Sporothrix schenckii* complex, with closely related species (measurements in μm)

	Ophiostoma nebulare sp. nov.	*Ophiostoma nigrocarpum* Davidson (1966)	*Ophiostoma dentifundum* Aghayeva et al. (2005)	*Ophiostoma euskadiense* sp. nov.	*Ophiostoma abietinum* Marmolejo and Butin (1990)	*Ophiostoma lunatum* Aghayeva et al. (2004)	*Ophiostoma fusiforme* Aghayeva et al. (2004)	*Graphilbum crescericum* sp. nov.	*Graphilbum curvicollis* Olchowecki and Reid (1974)
Perithecia base diam	(83.44–) 86.56–101.18 (–105.94)	50–80	(122–) 153–216 (–261)	(54.19–) 57.64–66.31 (–69.69)	105–170	59.5–178.3 (–204.5)	121.5–273.8	—	60–125
Neck length	(169.50–) 140.54–293.21 (–365.86)	120–160	(439–) 567–1345 (–1571)	(201.32–) 204.15–213.28 (–219.12)	450–650	162.4–554.2 (–700)	301.8–985 (–1168)	—	(60–) 100–200
Width at base	(24.61–) 25.66–30.81 (–30.93)	15–25	(20–) 24–36 (–41)	(9.58–) 10.18–12.91 (–13.98)	19.00–24.50	15.3–33.4 (–40.5)	21.8–33.7 (–44.9)	—	(12–) 20–30
Width at apex	(8.56–) 8.93–12.88 (–13.94)	10–12	(7–) 10–17 (–20)	(5.26–) 5.62–8.83 (–9.66)	9.50–11.50	7.5–10.4 (–13.8)	9.1–13.5 (–18)	—	6.0–10
Ostiolar hyphae length	(8.20–) 9.58–15.20 (–16.21)	—	(18–) 20–52 (–55)	(36.67–) 41.22–49.13 (–49.83)	13–19	13.6–56.9 (–61.7)	16.6–94.5 (–142.5)	—	2.0–10
Width	(2.07–) 2.11–2.39 (–2.47)	—	1–2.5	(3.07–) 3.10–3.31 (–3.37)	2–3	1.01–1.7 (–2.7)	1.71–2.2 (–2.6)	—	0.7–1.0
Ascospores	Allantoid	Allantoid	Allantoid	Allantoid	Allantoid	Allantoid	Allantoid	—	Globose
Length	(3.00–) 3.12–4.23 (–6.52)	3–4	(2–) 2.5–3.5 (–4)	(3.15–) 3.18–3.56 (–3.56)	3–4.5	3.1–3.9 (–4.3)	3.4–4.3 (–5.4)	—	(2.5–) 3.0–4.0 (–5.5)
Width	(1.28–) 1.42–1.79 (–1.88)	1–1.3	1–1.5	(1.90–) 1.91–2.01 (–2.00)	2–2.5	0.7–1.2	0.8–1.3 (–1.6)	—	0.7–1.0
Conidia shape	Obovoid truncate	Broadly ellipsoidal	Fusiform	Clavate	Clavate-cilindrical	Curved, crescent	Fusiform-guttuliform	Globose-subglobose	Clavate to broadly clavate
Length	(2.53–) 2.91–3.70 (–3.73)	3–5	(4–) 4.5–7.5 (–10)	(2.10–) 2.21–2.95 (–3.70)	4.0–7.5	2.3–4.8 (–6.2)	3.2–5.9 (–8)	(4.39–) 4.52–5.73 (–6.18)	(2.0–) 3.5–5.0 (–7.0)
Width	(1.13–) 1.14–1.35 (–1.44)	1.5–3	1–1.5	(1.02–) 1.22–1.76 (–1.82)	1–2	1.5 (–1.6)	1.1–1.9 (–2.1)	(1.74–) 2.00–3.16 (–3.34)	0.7–1.5 (–2.5)
Culture color	White-cream	Light to dark gray	Hyaline to white	White	White	White	White	White	White

Note: Measurements are presented in the format (minimum–) mean minus standard deviation – mean plus standard deviation (–maximum) where possible.

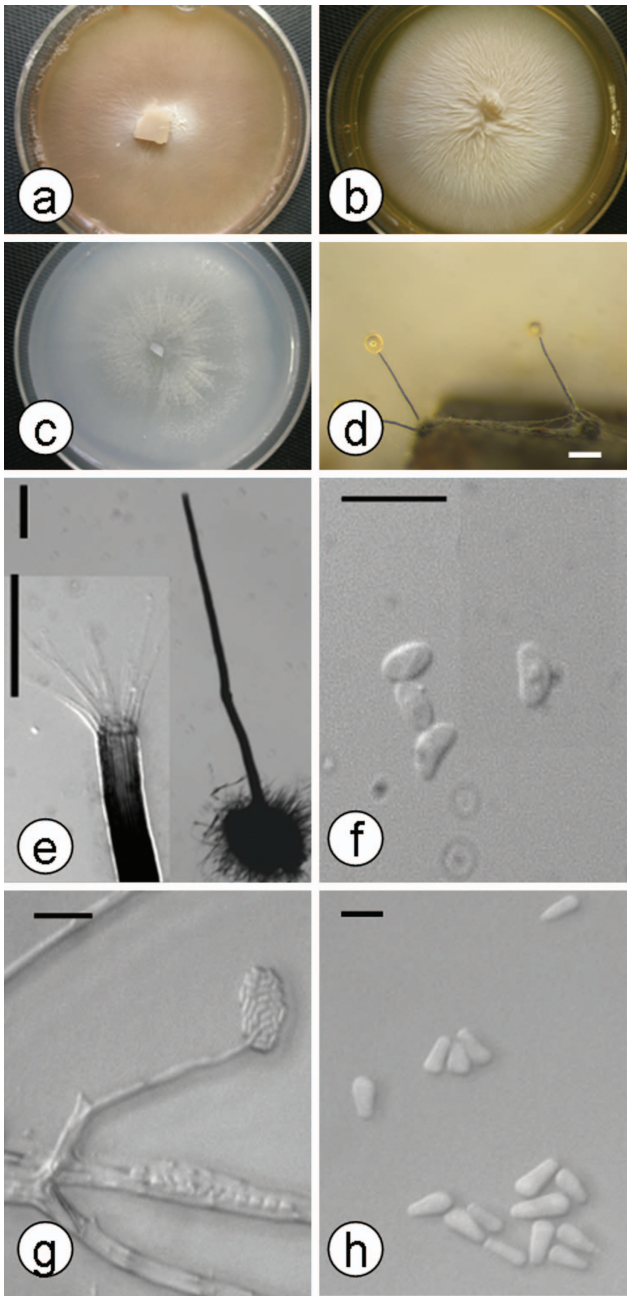

FIG. 4. *Ophiostoma nebulare* (CMW27319). a–d. Growing respectively on 2% MEA, PDA, OA and WA-twigs (bar = 2000 µm). e. Perithecium (bar = 100 µm) with ostiolar hyphae (bar = 25 µm). f. Allantoid ascospores (bar = 5 µm). g. Sporothrix-like conidiophore (bar = 10 µm). h. Obovoid conidia with truncate bases (bar = 5 µm).

radiata, Jul 2004, X.D. Zhou (PREM 59831, ex-paratype culture CMW27899 = CECT20633 = CBS122136).

Taxon C:

Graphilbum crescericum P. Romón, Z.W. de Beer, M.J. Wingf., sp. nov. FIG. 6
MycoBank MB564954

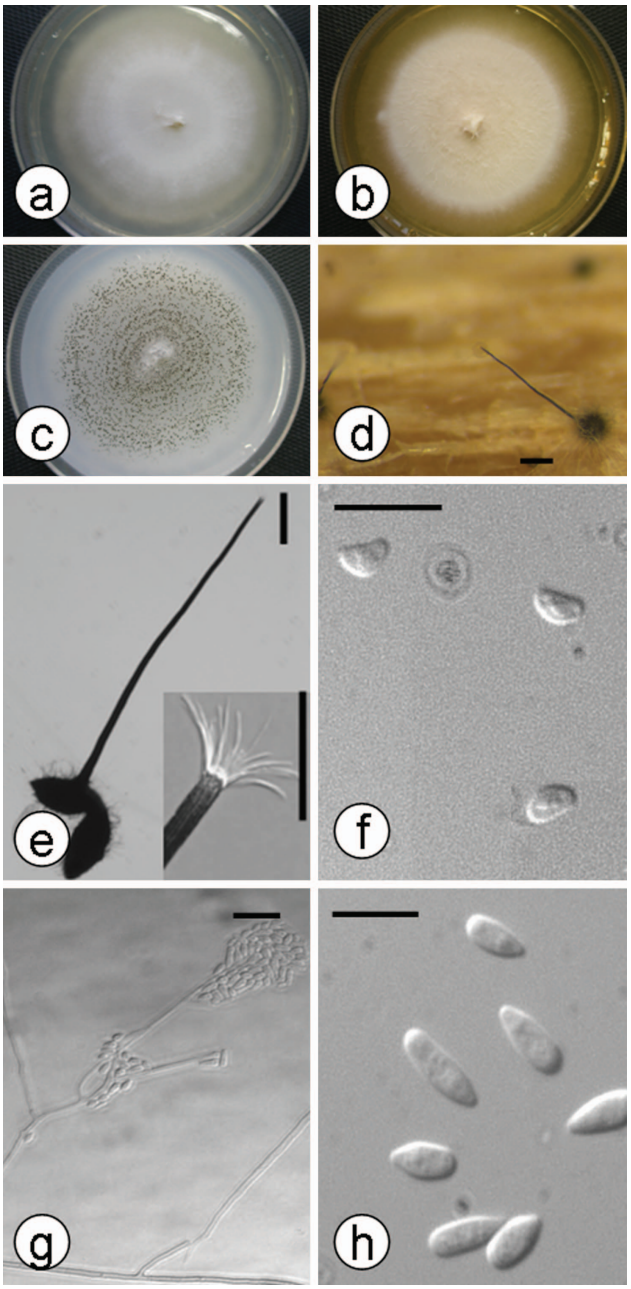

FIG. 5. *Ophiostoma euskadiense* (CMW27318). a–d. Growing respectively on 2% MEA, PDA, OA and WA-twigs (bar = 2000 µm). e. Perithecium (bar= 100 µm) with ostiolar hyphae (bar = 25 µm). f. Allantoid ascospores (bar = 5 µm). g. Sporothrix-like conidiophore (bar = 10 µm). h. Clavate conidia (bar = 5 µm).

Hyalorhinocladiella-like anamorph: conidiophores (16.32–)17.22–58.28(–69.92) µm long; conidia globose-subglobose, (4.39–)4.52–5.73(–6.18) × (1.74–)2.00–3.16(–3.34) µm. Colonies with optimal growth at 25 C on 2% MEA, reaching 60.44 mm diam in 8 d. Colonies white. Isolation frequency 0.2, 2 and 1% respectively from *Hylurgops palliatus*, *Hylastes ater* and *Orthotomicus erosus*.

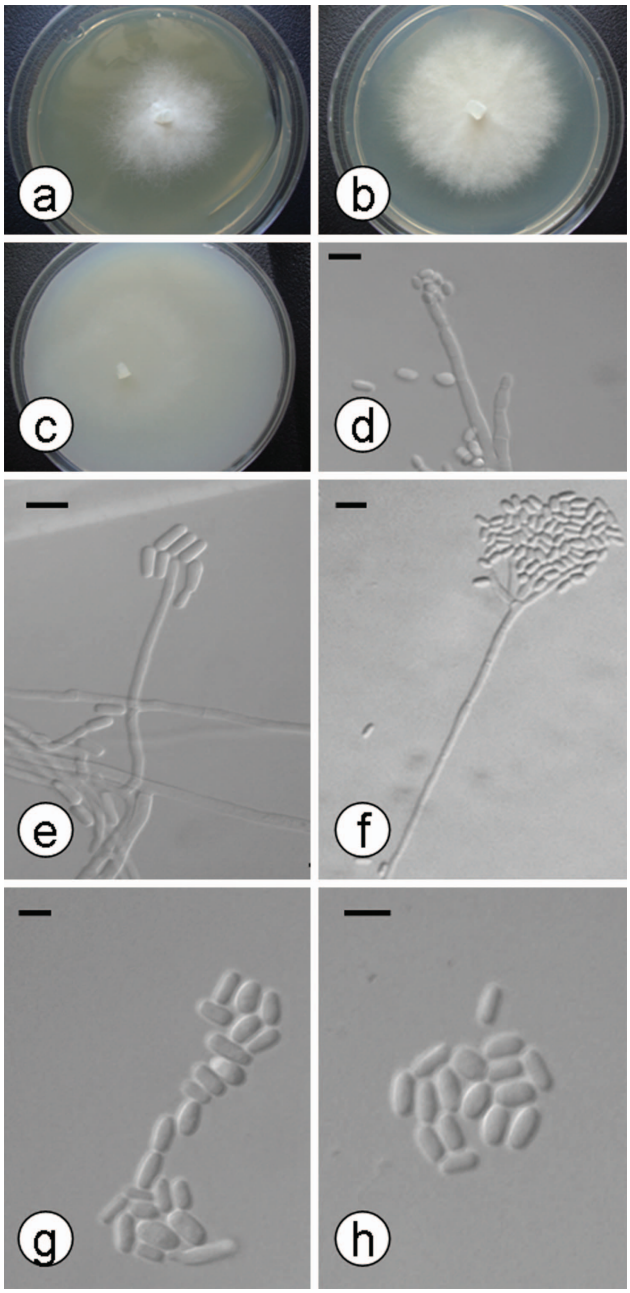

FIG. 6. *Graphilbum crescericum* (CMW22828). a–c. Growing respectively on 2% MEA, PDA and OA. d–f. Hyalorhinocladiella-like conidiophores in different growing statuses (bars = 10 µm). g–h. Globose-subglobose conidia (bars = 5 µm).

Etymology: Referring to the rapid mycelial growth of this fungal species.

Holotype: SPAIN, Basque Country, Morga, Bizkaia, *Hylurgops palliatus* infesting *Pinus radiata*, Jul 2004, P. Romón (PREM 60713, ex-type culture CMW22828 = CECT20669 = CBS130864).

Additional specimens examined: SPAIN, Basque Country, Morga, Bizkaia, *Hylastes ater* infesting *Pinus radiata*, Jul 2004, P. Romón (PREM 60714, ex-paratype culture CMW22829 = CECT20670 = CBS130865); SPAIN, Basque Country, Morga, Bizkaia, *Hylastes ater* infesting *Pinus radiata*, Jul 2004, P. Romón (PREM 60715, ex-paratype culture CMW22830 = CECT20671); SPAIN, Basque Country, Morga, Bizkaia, *Orthotomicus erosus* infesting *Pinus radiata*, Jul 2004, P. Romón (PREM 60716, ex-paratype culture CMW22831 = CECT20672 = CBS130866); SPAIN, Basque Country, Morga, Bizkaia, *Orthotomicus erosus* infesting *Pinus radiata*, Jul 2004, P. Romón (PREM 60717, ex-paratype culture CMW22832 = CECT20673).

DISCUSSION

Romón et al. (2007) collected 1323 insects belonging to 14 species. Isolations yielded a total of 920 fungal cultures that included several mildly pathogenic species, such as *L. wingfieldii* (Jankowiak 2006), *O. minus* (Jankowiak 2006) and *O. ips* (Raffa and Smalley 1988), and well known species that cause sapstain, such as *O. ips*, *O. minus*, *O. piceae* and *O. pluriannulatum* (Seifert 1993). The molecular and morphological methodology used in the present study lets us describe two new fungal species residing in the *S. schenckii-O. stenoceras* complex in *Ophiostoma* sensu lato (*O. nebulare*, *O. euskadiense*) and a new species of *Graphilbum* (*G. crescericum*).

The *S. schenckii-O. stenoceras* complex is characterized by orange section-shaped allantoid ascospores without a sheath, a sporothrix-like anamorph and an absence of intron 4 and presence of intron 5 in the β-tubulin gene (de Beer et al. 2003, de Beer and Wingfield 2013, Zipfel et al. 2006). The complex includes several species associated with human sporotricosis (Marimon et al. 2006, 2007), soil (de Meyer et al. 2008), hardwoods (Aghayeva et al. 2004) or *Protea infructescences* (Roets et al. 2010). It is interesting that most species in the complex do not have specific bark beetle associates, while some species have been shown to be vectored by mites (Roets et al. 2008). The possibility that the newly described species also are vectored by mites phoretic on bark beetles should be studied further.

Among the isolates analyzed in the present study, *O. nebulare* formed a discrete, well supported clade that is peripheral to the major lineage of the *S. schenckii-O. stenoceras* complex (FIG. 1) and not distant from the *O. nigricarpum* complex. The ITS1-5.8S-ITS2 sequence of *Ophiostoma nebulare*, exclusively isolated from the root-feeding bark beetle *Hylastes attenuatus*, was homologous with that of *O. nigricarpum* (CMW650, AY280489; Aghayeva et al. 2004). The main morphological differences between *O. nebulare* and *O. nigricarpum* are growth at 10 C and smaller colony diameters at 15–35 C, cream-colored mycelia in MEA medium with age, smaller *Sporothrix* conidia having a different shape, broader perithecium bases,

longer perithecium necks and ostiolar hyphae and slightly longer ascospores. A β-tubulin sequence could not be obtained for this species.

Based on ITS sequences alone members of the *O. euskadiense* clade could not be distinguished from species in the *O. abietinum* subcomplex. ITS1-5.8S-ITS2 sequence differences were only two-point mutations of cytosine instead of thymine in positions 17 and 174 and two changes of thymine rather than cytosine in positions 173 and 530 bp. ITS sequence accounted for a total of zero and seven substitutions among *O. euskadiense* and *Sporothrix* sp.1 and *Sporothrix* sp.2 indicated by Zhou et al. (2004). Similarly β-tubulin sequences accounted for a total of five, four and three substitutions between *O. euskadiense* and *O. abietinum*, *Sporothrix* sp.1 and *Sporothrix* sp.2 respectively. Comparative growth did not reflect significant differences among all tested temperatures (FIG. 3). However calmodulin sequences (FIG. 2b) and morphology data (TABLE III) clearly separated these species within the *O. abietinum* subcomplex. Calmodulin sequence accounted for a total of 15, two and seven substitutions between *O. euskadiense* and *O. abietinum*, *Sporothrix* sp.1 and *Sporothrix* sp.2 respectively. The ITS1-5.8S-ITS2 sequence of *Ophiostoma euskadiense*, also mainly isolated from *H. attenuatus*, shared a high degree of homology with the type strain of *Ophiostoma abietinum* (CBS 125.89, AF484453; de Beer et al. 2003). The main morphological differences between these two species are shorter and clavate *Sporothrix*-type conidia, a narrower perithecium base, perithecia with shorter necks and slightly shorter ascospores. The beta-tubulin sequence included intron 5 but not intron 3 or intron 4 as characteristic of the complex.

Graphilbum crescericum did not have a sexual state and had the highest homology with *Gra. rectangulosporium* in what was formerly known as the *Pesotum fragrans* complex. De Beer et al. (2013) revealed that this complex represented a phylogenetically distinct lineage in the Ophiostomatales, for which they reinstated the older genus name, *Graphilbum*. They redefined the genus, previously considered an anamorph genus, based on the one fungus one name principles adopted in the ICN (Hawksworth 2011), to accommodate species known from either their sexual or asexual states or both. At present *Graphilbum* contains six known species, *Gra. fragrans* (Mathiesen-Käärik 1954, pesotum-type conidiophores), *Gra. nigrum* (Davidson 1958, slightly narrower conidia and sparse surface growth), *Gra. sparsum* (Davidson 1971, slightly smaller conidia and slow growth), *Gra. curvicollis* (Olchowecki and Reid 1974, slightly smaller clavate conidia and mycelium mostly immersed), *Gra. microcarpum* (Yamaoka et al. 2004, dark brown to black conidiophores), *Gra. rectangulosporium* (Ohtaka et al. 2006) and seven undescribed taxa, one of which is described here as *Gra. crescericum*. All these species have hyalorhinocladiella- to pesotum-like anamorphs, except *Gra. rectangulosporium*, for which no anamorph has been observed (Ohtaka et al. 2006).

Some ophiostomatoid species are mild pathogens and/or agents of bluestain. Nothing is known regarding the pathogenicity of the new *Ophiostoma* spp. described in the present study, whose pathogenic and saprophytic capabilities should be studied further. The discovery of a relatively large number of new taxa strongly reflects the fact that these fungi have been poorly studied in the introduced conifer stands of Spain. It is likely that similar studies on other conifers in Spain and/or southern Europe will yield additional new taxa in the Ophiostomatales. These not only will enhance our knowledge of this intriguing group of fungi but also the understanding of the fungal diversity associated with conifers in the region.

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