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Ophiostomatoid fungi isolated from three different pine species in Argentinian Patagonia

A. de Errasti[1] | M. B. Pildain[1,2] | M. Rajchenberg[1,2]

[1]Centro de Investigación Forestal CIEFAP, Esquel, Chubut, Argentina

[2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

Correspondence
Andres de Errasti, Centro de Investigación y Extensión Forestal CIEFAP, Esquel, Chubut, Argentina.
Email: aderrasti@ciefap.org.ar

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Summary

Pine plantations in Argentinian Patagonia cover *ca.* 95,000 ha in Chubut, Río Negro and Neuquén provinces. Exotic bark beetles (*Orthotomicus laricis*, *Hylastes ater* and *Hylurgus ligniperda*) commonly occur in freshly cut logs, stumps and slash. These beetles are vectors of "ophiostomatoid" fungi which include primary tree pathogens as well as important agents of blue stain. The aim of this study was to identify these beetle-associated fungi. Sawing mills and pine plantations were surveyed three consecutive years. Fungal isolates from stained logs, processed wood and insect galleries were identified based on morphological and DNA sequence comparisons of ITS and β-*tubulin* gene regions. Two *Grosmannia*, one *Graphilbum* and three *Ophiostoma* species were identified. *Ophiostoma piliferum* and *O. peregrinum* sp. nov. were the most frequently isolated taxa. *O. peregrinum* occurred in all provinces, colonizing different conifer species and, interestingly, also the native broadleaved species *Nothofagus dombeyi*. Pine plantation forestry in southern South America includes Argentina, Brazil, Chile, Paraguay and Uruguay. Emerging data from Argentina, Chile and Uruguay revealed some coincidences between these countries, but also several differences, probably, as a result of multiple introduction events.

1 | INTRODUCTION

Pine species are native to the Northern Hemisphere; however, they have been introduced into Southern Hemisphere countries such as Argentina, Australia, Brazil, Chile, New Zealand, South Africa and Uruguay. Afforestation in southern Argentina extends over an area of 95,000 ha, mostly with ponderosa pine (*Pinus ponderosa* Doug.) and, to a lesser extent, with Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco), lodgepole pine (*Pinus contorta* Dougl.) and Monterey pine (*Pinus radiata* D Don.) (Loguercio & Deccechis, 2006). Recently harvested pine logs, fresh stumps and slash are usually attacked by bark beetles (Coleoptera, Scolytinae).

Bark beetles build their galleries in the phloem layer of woody plants, where they lay eggs and their brood feed and develop (Raffa, Phillips, & Salom, 1993; Six & Wingfield, 2011). Many species are economically important forest pests, although a large number of species infest only stressed or dying trees (Paine, Raffa, & Harrington, 1997; Wood, 1982). Interaction between conifer species, bark beetles (Coleoptera, Scolytinae) and blue-stain fungi (Ascomycota, Sordariomycetidae) is well documented (Jacobs, Krokene, Solheim, & Wingfield, 2010; Kirisits, Konrad, Wingfield, & Chhetri, 2013; Kirisits & Offanthaler, 2002; Krokene, Roux, Solheim, & Wingfield, 2010; Krokene & Solheim, 1998; Malloch & Blackwell, 1993; Six & Wingfield, 2011; Solheim, Krokene, & Langstrom, 2001).

Blue stain is a dark discoloration of wood caused by the presence of pigmented fungal hyphae (Seifert, 1993a). Blue-stain species are known as the "ophiostomatoid" fungi (Wingfield, Seifert, & Webber, 1993), a convenient term proposed for a polyphyletic assemblage of species with similar, convergent morphologies, presumably reflecting coevolution with insects (De Beer, Seifert, & Wingfield, 2013). More than 300 ophiostomatoid species are included in two different orders: Microascales Lutr. ex Benny & Kimbr. and Ophiostomatales Benny & Kimbr. (De Beer et al., 2013). The latter is a monotypic order, with Ophiostomataceae comprehending *Aureovirgo* J.A. van der Linde, Z.W. de Beer & Jol. Roux, *Ceratocystiopsis* Upadhyay & Kendr., *Fragosphaeria* Shear, *Graphilbum* Upadhyay & Kendr., *Hawksworthiomyces* Z.W. de

Beer, Marinc., M.J. Wingf., *Leptographium* s.l. (including *Grosmannia* Goid.), *Ophiostoma* Syd. & P. Syd., *Raffaelea* Arx & Henneb. and *Sporothrix* Hektoen & C.F. Perkins (De Beer, Duong, & Wingfield, 2016).

There is a growing mistrust towards international trade of stained timber, based on the occurrence of primary pathogens among sap-stain species (Brasier, 1991; Cobb, 1988; Henry, Moses, Richards, & Riker, 1944), but also on the possible presence of other pests and pathogens on stained lumber (arthropods, bacteria and other fungi). Bark beetles represent the majority of intercepted insects at border customs in countries that analyse such data (Brockerhoff, Bain, Kimberley, & Knizek, 2006; Wingfield, Roux, Wingfield, & Slippers, 2013). The introduction of these insects and their fungal partners into new areas is likely to undergo complex new interactions (Wingfield et al., 2013). In Patagonia, three bark beetle species were introduced along with pine, *Hylastes ater* (Paykull), *Hylurgus ligniperda* (Fabricius) and *Orthotomicus laricis* (Fabricius) (Lanfranco, Ide, Ruiz, Peredo, & Vives, 2002; Mausel et al., 2007; Tiranti, 2010), although no information on their fungal partners is available.

Identification of causal agents constitutes a first step to improve current management strategies and contributes to understand the movement of these fungi globally. The aim of this study was to provide information on the economically important staining fungi and their beetle associates.

2 | MATERIALS & METHODS

2.1 | Sampling and isolation

Six pine plantations and 15 sawing mills in Chubut, Río Negro and Neuquén provinces (Patagonia, Argentina) were surveyed for ophiostomatoid fungi and bark beetles, every 3 months, three consecutive years (2009–2011). Samples were taken from three different hosts: ponderosa pine (86.6% of all samples), Monterey pine (10.8%) and Scots pine (2.6%). Dead and recently cut trees showing symptoms of infection by "ophiostomatoid" fungi, including wood staining and the production of typical fruiting bodies, were selected for sampling. Samples were taken to the laboratory in plastic bags to maintain a moist environment. When sporulation structures were present, isolates were obtained by lifting spore masses from the apices of ascomata or synnemata and transferring these to 2% (w/v) malt extract agar (MEA; 20 g agar, 20 g malt extract). When no fruiting bodies were observed, wood tissue was incubated in sealed moistened plastic bags for 5–25 days, until sporulation was evident, after which spore masses were transferred to isolation media. Axenic cultures were obtained by transferring single hyphal tips to non-inoculated plates. Isolates used in this study are maintained in the culture collection at Centro Forestal CIEFAP, Argentina. Duplicates of type cultures and holotype specimens were deposited at BAFC Herbarium (BAFC) and culture collection, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina.

2.2 | Morphology

Morphological features were assessed for seven, 14- and 21-day-old cultures on MEA and using structures on host tissue. Cultures were incubated at 25°C in the dark. Colony colours were described using the Munsell color charts (Munsell, 1912). Conidiophores and ascomata were mounted on microscope slides in distilled water or distilled water and phloxine for microscopic examination. For species descriptions, fifty measurements of each taxonomic structure were made for type specimens. Averages (mean), standard deviation (*SD*), minimum (min) and maximum (max) measurements are presented for each structure as (min-) mean minus *SD* – mean plus *SD* (-max).

2.3 | DNA extraction, PCR and DNA sequencing

DNA was extracted from fungal mycelium (ca.100 mg) grown in malt extract agar (2% Difco malt extract agar) incubated for 2 weeks in the dark at 25°C, using a Ultraclean Microbial DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA) and following the manufacturer's protocols. Two gene regions were amplified for sequencing and phylogenetic analyses. The small subunit partial sequence, internal transcribed spacer 1, 5.8S, internal transcribed spacer 2 and partial large subunit (ITS) of the ribosomal DNA were amplified with primers ITS1-F (Gardes & Bruns, 1993) and ITS4 (White, Bruns, Lee, & Taylor, 1990). A portion of the β-*tubulin* gene (BT) was amplified with primers Bt2a and Bt2b (O'Donnell & Cigelnik, 1997). Conditions for PCR amplification and sequencing were performed as described by Zipfel, De Beer, Jacobs, Wingfield, and Wingfield (2006). PCR products were purified using a QIAGEN Gel Extraction kit (QIAGEN Inc.) and were sequenced at a DNA synthesis and sequencing facility (Macrogen, Seoul, Korea). All sequences were checked manually, and consensus sequences were constructed with MEGA 5.05 (Tamura, Peterson, Stecher, Nei, & Kumar, 2011).

2.4 | Phylogenetic analyses

BLAST searches using the BLASTn algorithm were performed to retrieve similar sequences from GenBank. Accession numbers of these sequences are presented in the corresponding phylogenetic trees. Data sets were compiled in MEGA 5.0.5. Alignments were made online in MAFFT 7 (Katoh, 2013) using the E-INS-i strategy and default settings. All sequences generated in this study were deposited in GenBank. Data sets were analysed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). MP analyses were conducted in PAUP* 4.0b10 (Swofford, 2003). Gaps were treated as missing data. One thousand bootstrap replicates were performed to determine the branch node confidence. Tree bisection and reconnection (TBR) was selected as the branch-swapping algorithm. The tree length (TL), Consistency Index (CI) and Retention Index.

were recorded. ML analyses were conducted with PhyML 3.0 (Guidon & Gascuel, 2003). Substitution models were selected using the Akaike information criterion (AIC) in jModelTest 3.7 (Posada & Crandall, 1998). Confidence supports were estimated with 1,000 replication bootstrap analyses. For Bayesian inference (BI), four MCMC chains were run simultaneously from a random starting tree for 1,000,000 generations. Trees were sampled every 100th generation. Log files for each run were viewed in Tracer 1.6.0 (evolve.zoo.ox.ac.

uk/software.html/tracer) to determine convergence. Trees sampled at burn-in (15%) were discarded, and posterior probabilities were calculated from a majority rule consensus tree regenerated from the remaining trees.

3 | RESULTS

3.1 | Sampling, isolation and morphological identification of fungi

A total of 160 isolates of blue-stain fungi were obtained from *Pinus* species. Morphological identification grouped 125 isolates in *Ophiostoma*, 12 in *Leptographium* s.l. (including *Grosmannia*) and 23 in *Graphilbum*. *Ophiostoma* isolates were further assigned to a specific taxon or species complex: 49 were identified as *O. piliferum* (Fr.:Fr.) Syd. & P. Syd., 57 were similar to *O. piceae*, and the remainder 19 were included in the *O. ips* complex. Insect and host associations were registered for each isolate (Table 1).

3.2 | DNA sequence comparisons

A subset of 25 representative isolates (17 *Ophiostoma*, four *Leptographium* s.l. and four *Graphilbum*) was included in DNA sequence comparisons (Table 2), together with available sequences from online databases (GenBank). Different ITS data sets were compiled for each genus. BT data sets were constructed for each species complex separately, to align sequences with similar exon/intron arrangement.

Alignment of *Ophiostoma* ITS matrix consisted of 17 sequences from Argentina and 66 sequences representing most of the known species within the genus (De Beer & Wingfield, 2013; De Beer et al., 2016). After final alignment, 780 characters including gaps were considered. In total, 519 uninformative characters were excluded before the phylogenetic analysis based on parsimony, which resulted in 101 trees of equal length (TL = 889, CI = 0.75, RI = 0.82). A transitional model (TIM1 + I + G) was selected for ML and BI analyses. Sequences from Patagonia resided in three different groups.

Group A (Figure 1) included isolates BAFC4503, CIEFAP422, CIEFAP443, CIEFAP450, CIEFAP469, CIEFAP470, CIEFAP471, CIEFAP472, CIEFAP473, CIEFAP474 and species from the *O. piceae* complex sensu Yin, Wingfield, Zhou, and De Beer (2016). *O. piceae* complex BT data set included 40 sequences, 10 from Argentina. Alignment of BT sequences resulted in a matrix of 390 characters, of which 340 were considered uninformative for parsimony analysis. The most parsimonious trees had a TL = 67, CI = 0.84 and RI = 0.94. ML and BI searches were performed assuming a general time-reversible model (GTR + I + G). Isolates BAFC4503, CIEFAP422, CIEFAP443, CIEFAP450, CIEFAP469, CIEFAP470, CIEFAP471, CIEFAP472, CIEFAP473 and CIEFAP474 grouped together with CMW29495, an isolate from Norway. This clade clearly represents a new species and is described on the taxonomy section as *Ophiostoma peregrinum* sp. nov.

Group B ITS results clustered isolates CIEFAP360 and CIEFAP361 with the species included in the *Ophiostoma. ips* complex (Figure 1). *O. ips* complex BT data set included 39 sequences, two from this study. After final alignment, data matrix consisted of 279 characters, of which 70 were parsimony informative. Best tree had a TL = 105, CI = 0.84 and RI = 0.95. The "Tamura & Nei" substitution model (TrN + G) was used to run ML and BI searches. Topology of different trees obtained from ML and BI inference agreed on the identification of CIEFAP360 and CIEFAP361 as *O. ips* (Rumbold) Nannf. (Figure 1, group B).

ITS results positioned isolates CIEFAP475, CIEFAP476, CIEFAP477, CIEFAP602 and CIEFAP603 near *O. piliferum* (Figure 1, group C). *O. piliferum* BT data set included five sequences from Argentina and 23 sequences retrieved from GenBank. Final matrix consisted of 279 characters (221 of them uninformative for MP). Most parsimonious trees had 95 steps (CI = 0.87, RI = 0.96). ML and BI analyses were performed assuming a general time-reversible model (GTR + G). Isolates CIEFAP475, CIEFAP476, CIEFAP477, CIEFAP602 and CIEFAP603 were identified as *O. piliferum* (Figure 1, group C).

TABLE 1 Blue-stain fungi, hosts, number of isolates collected and isolates obtained from beetle galleries

				Isolates from insect galleries		
Taxa	Hosts	Number of isolates	Isolates by percentage	*Orthotomicus laricis*	*Hylurgus ligniperda*	*Hylastes ater*
G. huntii	*P. sylvestris*	1	1.92	0	0	0
	P. ponderosa	2		0	0	2
G. radiaticola	*P. radiata*	9	5.77	0	1	1
Graphilbum sp. 1	*P. sylvestris*	3	14.75	0	1	0
	P. ponderosa	20		4	6	1
O. ips	*P. ponderosa*	13	12.18	1	1	0
	P. radiata	6		0	1	0
O. peregrinum	*P. ponderosa*	48	33.97	11	6	0
	P. radiata	5		1	0	0
O. piliferum	*P. ponderosa*	49	31.41	3	9	0

G, *Grosmannia*; *O*, *Ophiostoma*; *P*, *Pinus*.
Total isolates = 156.

TABLE 2 Representative isolates included in the phylogenetic analyses

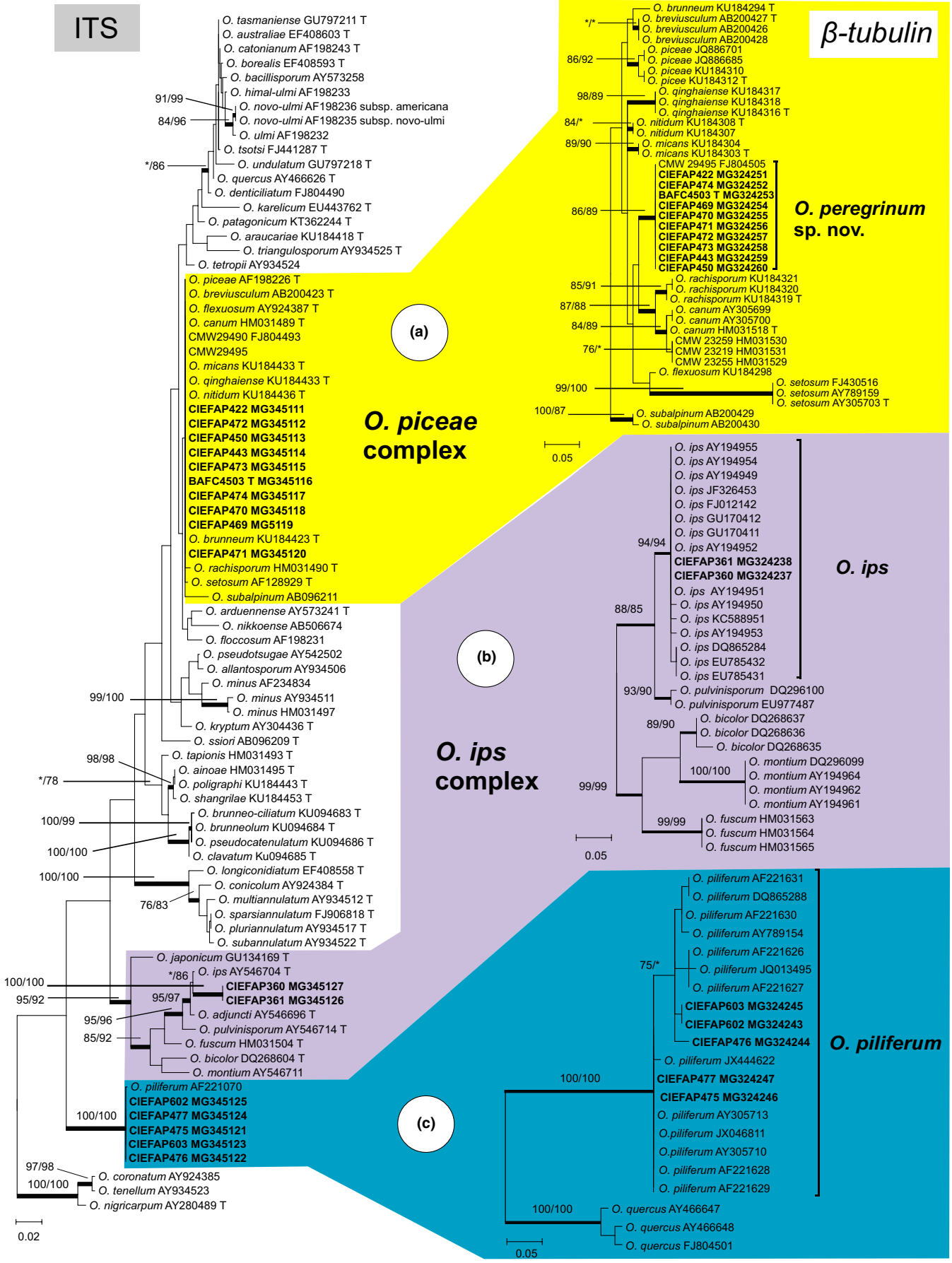
Species	Isolate	Host	Province	ITS	BT
Grosmannia huntii	CIEFAP306	*P. sylvestris*	NQN	MG345129	MG324241
Grosmannia huntii	CIEFAP307	*P. ponderosa*	NQN		MG324242
Grosmannia radiaticola	CIEFAP362	*P. radiata*	CHB	MG345128	MG324240
Grosmannia radiaticola	CIEFAP363	*P. radiata*	CHB		MG324239
Graphilbum sp1	CIEFAP478	*P. ponderosa*	CHB	MG345132	MG324249
Graphilbum sp1	CIEFAP433	*P. sylvestris*	RN	MG345133	MG324248
Graphilbum sp1	CIEFAP467	*P. ponderosa*	CHB	MG345130	MG324250
Graphilbum sp1	CIEFAP468	*P. sylvestris*	RN	MG345131	
Ophiostoma ips	CIEFAP360	*P. ponderosa*	NQN	MG345127	MG324237
Ophiostoma ips	CIEFAP361	*P. radiata*	CHB	MG345126	MG324238
O. peregrinum	CIEFAP422	*P. ponderosa*	NQN	MG345111	MG324251
O. peregrinum (holotype)	CIEFAP426/ BAFC4503cc	*P. radiata*	CHB	MG345116	MG324253
O. peregrinum	CIEFAP474	*P. ponderosa*	NQN	MG345117	MG324252
O. peregrinum	CIEFAP469	*P. ponderosa*	CHB	MG345119	MG324254
O. peregrinum	CIEFAP470	*P. ponderosa*	NQN	MG345118	MG324255
O. peregrinum	CIEFAP471	*P. ponderosa*	NQN	MG345120	MG324256
O. peregrinum[a]	CIEFAP472	*Ps. menziesii*	RN	MG345112	MG324257
O. peregrinum	CIEFAP473	*P. ponderosa*	NQN	MG345115	MG324258
O. peregrinum[a]	CIEFAP443	*Ps. menziesii*	RN	MG345114	MG324259
O. peregrinum[a]	CIEFAP450	*N. dombeyi*	CHB	MG345113	MG324260
O. piliferum	CIEFAP475	*P. ponderosa*	NQN	MG345121	MG324246
O. piliferum	CIEFAP476	*P. ponderosa*	NQN	MG345122	MG324244
O. piliferum	CIEFAP477	*P. ponderosa*	CHB	MG345124	MG324247
O. piliferum	CIEFAP602	*P. ponderosa*	NQN	MG345125	MG324243
O. piliferum	CIEFAP603	*P. ponderosa*	NQN	MG345123	MG324245

P, *Pinus*; Ps, *Pseudotsuga*; N, *Nothofagus*; CHB, Chubut; NQN, Neuquén; RN, Río Negro.
[a]Isolates from different study (de Errasti 2016).

Leptographium s.l. ITS data set included two sequences from this study and 41 sequences retrieved from GenBank. Final matrix consisted of 802 characters including gaps (249 were parsimony informative). Nine best trees were obtained after MP analyses (TL = 725, CI = 0.62, RI = 0.83). ML and BI analyses were carried out assuming a transitional substitution model (TIM3 + G). One sequence from Patagonia (CIEFAP 306) was identical to *Grosmannia huntii* (Rob.-Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf. (Figure 2, group A) and the other (CIEFAP362) clustered with *G. radiaticola* (J.J. Kim, Seifert & G.H. Kim) Zipfel, Z.W. de Beer & M.J. Wingf., *G. galeiformis* (B.K. Bakshi) Zipfel, Z.W. de Beer & M.J. Wingf. and related species (Figure 2, group B). *Grosmannia huntii* BT data set consisted of 27 sequences obtained from GenBank and two sequences from this study. Character matrix consisted of 279 characters. Most parsimonious trees were constructed based on 75 informative characters (TL = 111, CI = 0.80 and RI = 0.96). A transitional model (TIM2 + G) was used to run ML and BI searches. Isolates CIEFAP306 and CIEFAP307 were identified as *G. huntii* (Figure 2, group A). *Grosmannia radiaticola* BT data set included 17 sequences, two from this study. After alignment, final matrix included 294 characters (256 of them, uninformative for MP). Best trees (26) had a TL = 26, CI = 0.93 and RI = 0.96. A transitional (TIM3 + G) substitution model was selected for ML and BI analyses. Isolates CIEFAP362 and CIEFAP363 were identified as *G. radiaticola* (Figure 2, group B).

Graphilbum ITS data set consisted of 23 sequences retrieved from GenBank and four sequences from Argentina. Final matrix consisted of 813 characters, 608 of them, parsimony uninformative. Best trees had 360 steps, CI = 0.81 and RI = 0.91. For ML and BI searches, a transversional model (TVM + G) was selected. Sequences representing isolates CIEFAP433, CIEFAP467, CIEFAP468 and CIEFAP478 clustered with *Graphilbum fragrans* (Math.-Käärik) Z.W. de Beer, Seifert & M.J.

FIGURE 1 Phylogram obtained from ML analyses of the ITS and β-*tubulin* regions of *Ophiostoma* species. Novel sequences obtained in this study are printed in bold type. MP and ML bootstrap support values (1,000 replicates) above 75% are indicated at the nodes as MP/ML. Posterior probabilities (above 95%) obtained from BI are indicated by bold lines at the relevant branching points. *bootstrap values lower than 75%. T = ex-type isolates. Colour boxes indicate groups including Argentinian isolates. Scale bar = total nucleotide difference between taxa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O. borealis EF408593 T
O. bacillisporum AY573258
O. himal-ulmi AF198233
O. novo-ulmi AF198236 subsp. americana
O. novo-ulmi AF198235 subsp. novo-ulmi
O. ulmi AF198232
O. tsotsi FJ441287 T
O. undulatum GU797218 T
O. quercus AY466626 T
O. denticiliatum FJ804490
O. karelicum EU443762 T
O. patagonicum KT362244 T
O. araucariae KU184418 T
O. triangulosporum AY934525 T
O. tetropii AY934524
O. piceae AF198226 T
O. breviusculum AB200423 T
O. flexuosum AY924387 T
O. canum HM031489 T
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CMW29495
O. micans KU184433 T
O. qinghaiense KU184433 T
O. nitidum KU184436 T
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CIEFAP473 MG345115
BAFC4503 T MG345116
CIEFAP474 MG345117
CIEFAP470 MG345118
CIEFAP469 MG5119
O. brunneum KU184423 T
CIEFAP471 MG345120
O. rachisporum HM031490 T
O. setosum AF128929 T
O. subalpinum AB096211
O. arduennense AY573241 T
O. nikkoense AB506674
O. floccosum AF198231
O. pseudotsugae AY542502
O. allantosporum AY934506
O. minus AF234834
O. minus AY934511
O. minus HM031497
O. kryptum AY304436 T
O. ssiori AB096209 T
O. tapionis HM031493 T
O. ainoae HM031495 T
O. poligraphi KU184443 T
O. shangrilae KU184453 T
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O. longiconidiatum EF408558 T
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O. bicolor DQ268604 T
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O. piliferum AF221070
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O. tenellum AY934523
O. nigricarpum AY280489 T
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(a)
O. piceae complex
(b)
O. ips complex
(c)
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O. breviusculum AB200428
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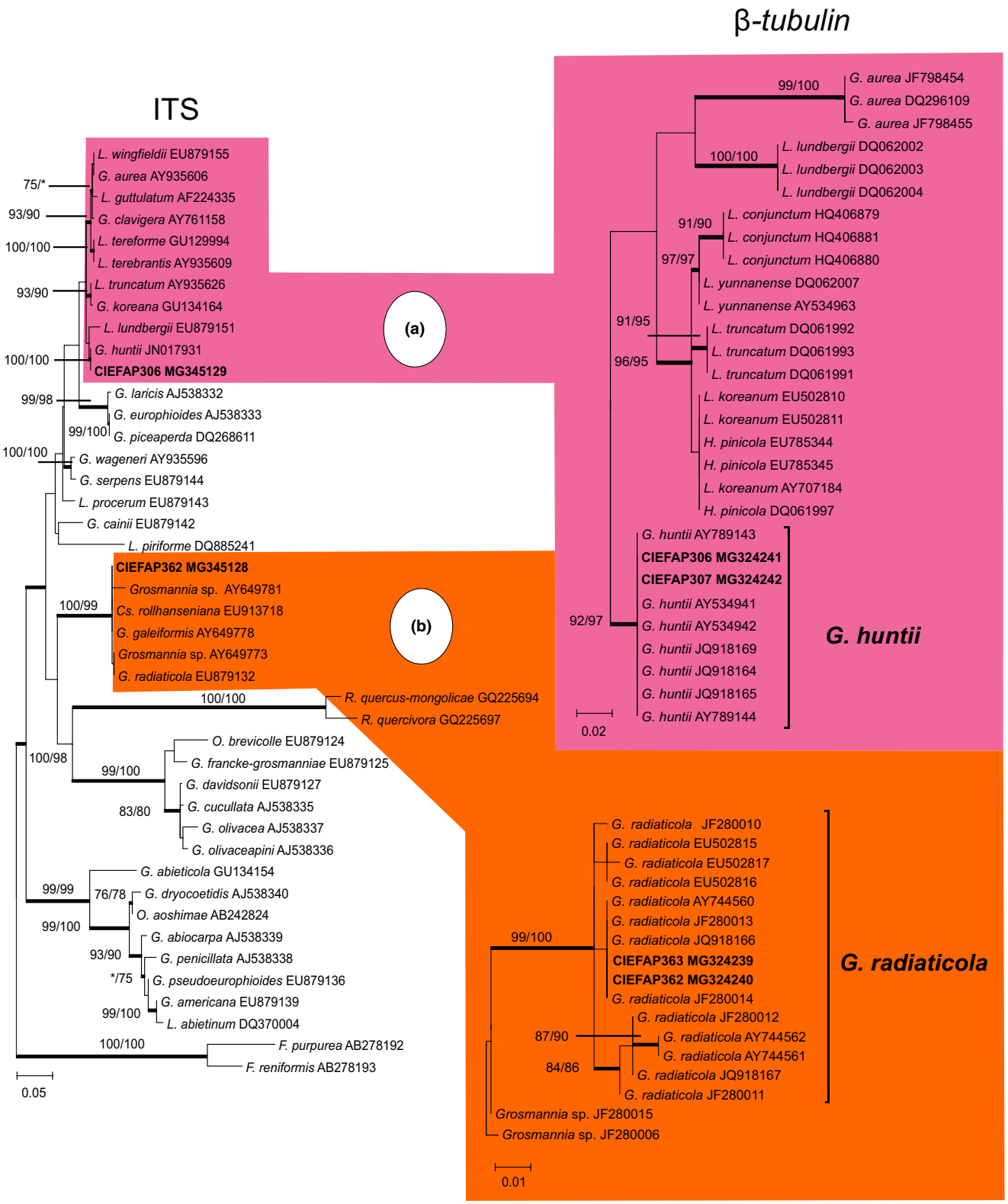

FIGURE 2 Phylogram obtained from ML analyses of the ITS and β-*tubulin* regions of *Grosmannia/Leptographium* species. Novel sequences obtained in this study are printed in bold type. MP and ML bootstrap support values (1,000 replicates) above 75% are indicated at the nodes as MP/ML. Posterior probabilities (above 95%) obtained from BI are indicated by bold lines at the relevant branching points. *bootstrap values lower than 75%. T = ex-type isolates. Colour boxes indicate groups including Argentinian isolates. Scale bar = total nucleotide difference between taxa

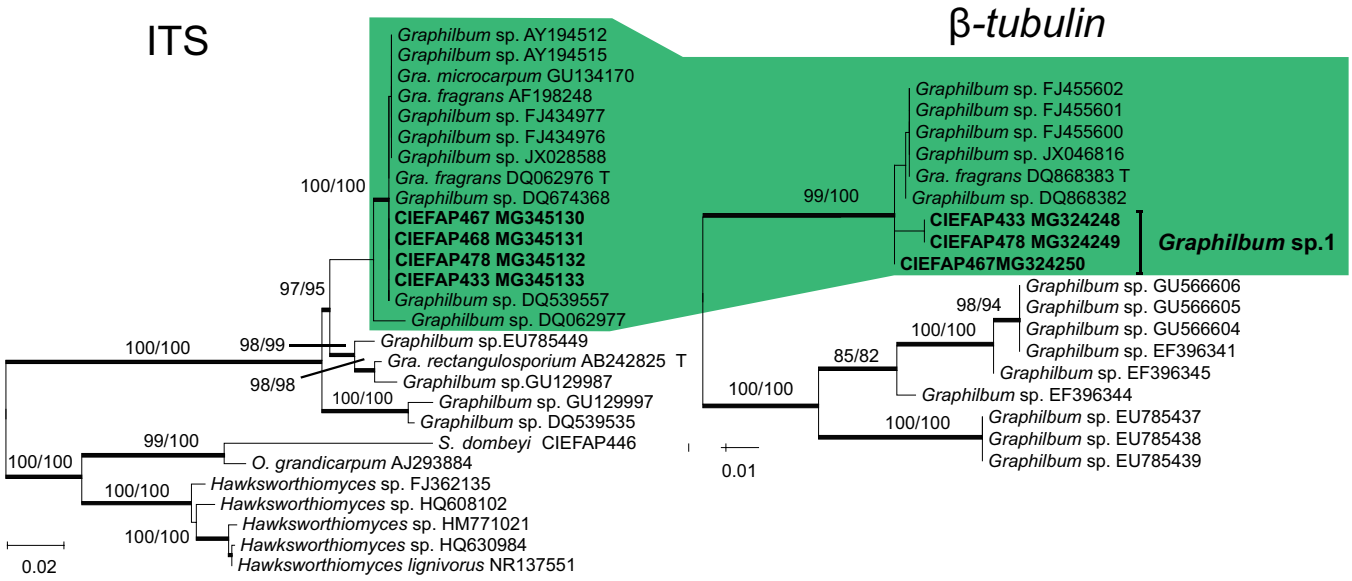

FIGURE 3 Phylogram obtained from ML analyses of the ITS and β-*tubulin* of *Graphilbum* species. Novel sequences obtained in this study are printed in bold type. MP and ML bootstrap support values (1,000 replicates) above 75% are indicated at the nodes as MP/ML. Posterior probabilities (above 95%) obtained from BI are indicated by bold lines at the relevant branching points. *bootstrap values lower than 75%. T = ex-type isolates. Colour boxes indicate groups including Argentinian isolates. Scale bar = total nucleotide difference between taxa

Wingf., *G. microsporum* (Yamaoka & Masuya) Z.W. de Beer, Masuya & Yamaoka and other isolates not formally described (Figure 3). *Graphilbum* BT data set included 20 sequences, and of these, four were obtained from Argentinian isolates. Four trees of equal length (233 steps), CI = 0.88 and RI = 0.95, were obtained from MP analyses. A transitional (TIM3 + G) substitution model was selected for ML and BI searches. *Graphilbum* species from Argentina were closely related to the type species, *G. fragrans* (Math.-Käärik) Z.W. de Beer, Seifert & M.J. Wingf., and to other isolates of Asia and Europe, most of them not formally described.

3.3 | Taxonomy

Based on phylogenetic analyses, the isolates employed in this study could be assigned to six different taxa. Of these, three were *Ophiostoma* species, two were included in *Leptographium* and one resided in *Graphilbum*. Four taxa were identified as *Grosmannia huntii*, *G. radiaticola*, *O. ips* and *O. piliferum*. One taxon could not be identified with certainty and is referred to as *Graphilbum* sp1. The other taxon characterized in this work clearly represents a novel species and is described below.

Ophiostoma peregrinum de Errasti & Rajchenb., *sp.nov* – Mycobank MB 818859; Figure 4.

Etymology: the Latin word "*peregrinum*" is a derivation from the adverb "*peregre*" literally meaning "from abroad," referring to the previous record of this species in Norway.

Ascomata not seen. Pesotum-like macronematal anamorph present. Synnemata simple, dark brown (11YR 1/2) at the base, becoming paler towards the apex, (89) 215,5–427,2 (734) µm long, (19) 31,2–43,2 (48,5) wide at the base, (11) 15,2–27,2 (24,4) wide at the apex, below conidiogenous apparatus. Conidiogenous apparatus consisting of 2–3 (6) verticilate rows of conidiophores, producing sticky spore masses at the apex. Conidiogenous cells hyaline, elongated, tapering towards the apex (9,5) 10,2–17,2 (18,5) × 1–2 µm; conidia hyaline, one-celled, smooth, oblong, clavate or obovoid (2.5) 3–4.5 (5.5) × 1–1.5 µm. Sporothrix-like synanamorph present. Conidiogenous cells micronematous, mononematous, hyaline, (2,5) 6,2–18,7 (26,5) × (1) 1,2–1.4 (1.8) µm, apical part consisting of swollen clusters bearing pointed denticles; conidia hyaline, one-celled, smooth, oblong, clavate or obovoid (2) 3,6–8,8 (12,5) × (1) 1,2–1,5 (3,5) µm. Secondary conidia very frequent. Culture characteristics – Mycelium superficial and embedded on the agar, no aerial mycelium present. Pesotum-like anamorph dominant in cultures. Colonies hyaline the first week, later becoming light to dark brown (19YR 1/2) during the second week. Colony margin smooth, growth rate at 25°C, 2.7 (±0.5) mm/d.

Host range: *Pinus ponderosa*, *Pinus radiata*, *Pseudotsuga menziesii* (de Errasti, 2016), *Nothofagus dombeyi* (de Errasti, 2016), *Betula pendula* (Linnakoski, de Beer, Rousi, Solheim, & Wingfield, 2009).

Distribution: ARGENTINA, Andes region, from Tierra del Fuego in the south to Neuquén in the north. Norway, Østfold, Hobøl (Linnakoski et al., 2009).

Insect associations: *Hylurgus ligniperda*, *Orthotomicus laricis*, *Scolytus ratzeburgi* Janson, *Xylechinus nahueliae* (Schedl).

Specimens examined: *Holotype* ARGENTINA, Chubut, Dto. Futaleufú, EEA INTA Esquel, Campo Experimental Aldea Escolar, on *Pinus radiata*, May 2009, A. de Errasti, BAFC4503 (=CIEFAP426). Neuquén, Dto. Los Lagos, Lago Lácar, Ea. Quechuquina, on *P. ponderosa*, May 2009, A. de Errasti (CIEFAP422), Lago Lolog, CORFONE S.A., on *P. ponderosa*, December 2011, A. de Errasti (CIEFAP473). Dto. Las Lajas, Aluminé, CORFONE S.A., on *P. ponderosa*, May 2010, A. de Errasti (CIEFAP474). Río Negro, Parque Nacional Nahuel Huapi, Isla Victoria, on *Pseudotsuga menziesii* (de Errasti, 2016), May 2010, A. de

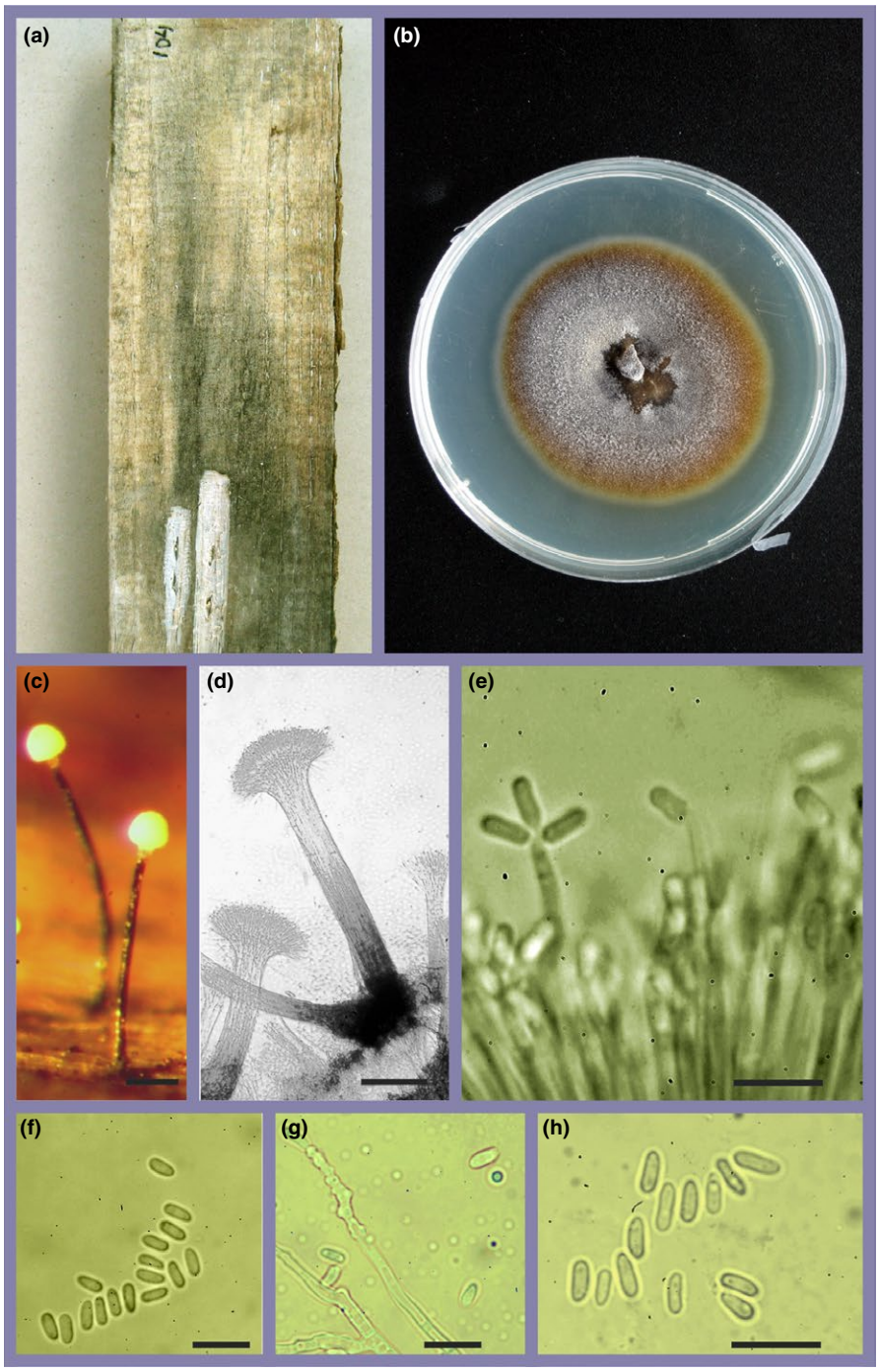

FIGURE 4 *Ophiostoma peregrinum sp. nov.* (a) Stained wood. (b) 14-day-old culture on MEA. (c) Pesotum-like synnemata on natural substrate. (d) Pesotum-like synnemata on MEA. (e) Pesotum-like conidiogenous cells. (f) Pesotum-like conidia. (g) Sporothrix-like conidiogenous cells. (h) Sporothrix-like conidia. Reference barr: (c, d) = 100 μm, (e, f, g, h) = 10 μm

Errasti (CIEFAP443), Parque Nacional Los Alerces, Lago Futalaufquen, "Arroyo Cascada" trail, associated *with Xylechinus nahueliae* galleries on *Nothofagus dombeyi* (de Errasti, 2016), November 2011, A. de Errasti/B. Hurley/J. Roux. (CIEFAP450).

Notes: *Ophiostoma peregrinum* was first isolated by Linnakoski et al. (2009). Based on molecular data, the authors linked these isolates (CMW29490 and CMW29495) to *O. canum*, although recognized them as different taxa. In 2010, Linnakoski and co-workers compared these isolates with new data obtained from Fennoscandia and they found *O. canum* more related to *O. rachisporum* and other isolates (CMW23253/5) than to CMW29490 and CMW29495. Our results agree with these evidences, with *O. peregrinum* always peripheral to the *O. canum* clade. The consistency of the phylogenetic data and the high isolation frequency of this fungus in Patagonia demand a formal description for this fungus, even if the statistical support of the phylogenetic trees is not optimal.

4 | DISCUSSION

The present study represents the first detailed study of the ophiostomatoid fungi affecting pine plantations in Patagonia, Argentina. A three-year survey, in three different provinces, and the collection of more than 250 samples allowed the identification of the most relevant

staining organisms. *Ophiostoma piliferum and O. peregrinum* sp. nov. were the most frequently isolated organisms, representing 66% of all isolates. *Ophiostoma ips*, *Grosmannia huntii*, *Grosmannia radiaticola* and a *Graphilbum* species represent the remaining 34% of total isolations.

One of the most relevant results of this work is, certainly, the finding of the new species *Ophiostoma peregrinum*: this taxon was first isolated by Linnakoski et al. (2009) in Norway, associated with *Scolytus ratzeburgi* on *Betula* trees, but it was not described formally. Several works in Fennoscandia had addressed the diversity of bark beetle-associated fungi on conifer and broadleaved forests (Jacobs et al., 2010; Kamgan Nkuekam et al., 2010; Krokene & Solheim, 1998; Linnakoski et al., 2008, 2010, 2012), but none of them reported *O. peregrinum*. In Patagonia, a three-year survey of blue-stain fungi on native broadleaved forests (de Errasti, 2016) found only a single isolate of *O. peregrinum*, associated with *Xylechinus nahueliae* on *Nothofagus dombeyi* (Fagales), indicating this species is not frequent in these forests either. On the contrary, *O. peregrinum* was one of the most frequent and important sap stainers of pine plantations in Patagonia, found in all provinces surveyed, isolated from different conifer hosts (de Errasti, 2016), and associated with different bark beetle species (*Hylurgus* and *Orthotomicus*).

Ophiostoma peregrinum and closely related taxa (*O. piceae* complex) are usually heterothallic, with the exception of *O. raquisporum* being homothallic. Homothallic species of *Ophiostoma*, or heterothallic species with both mating types present, produce the sexual state in vitro rather easily (Seifert, 1993b). None of the 57 isolates of *O. peregrinum* produced the sexual state in vitro, indicating this species is probably heterothallic with only one mating type present in the region. If this assumption is correct, *O. peregrinum* was probably introduced along with pine seedlings from the Northern Hemisphere. The different isolation frequency of this species from exotic conifer plantations vs native broadleaved forests in Patagonia could reflect differences in niche competing species diversity and/or differences in vector dynamics.

Ophiostoma piliferum and *O. ips* are widespread in Europe, North America and several Southern Hemisphere countries (Peredo & Alonso, 1988; Thwaites, Read, Schirp, Grinter, & Farrell, 2013; Thwaites et al., 2005; Zhou, De Beer, Ahumada, Wingfield, & Wingfield, 2004; Zhou et al., 2007). In Argentina, these typical blue-stain species were isolated with high (30%) and moderate (11%) frequency, respectively, associated with *Orthotomicus laricis* and *Hylurgus ligniperda*. Their occurrence in Chubut and Neuquén provinces indicates that they are widely distributed.

Kirschner (1998, 2001) reported 14 ophiostomatoid species associated with *Orthotomicus laricis* in Europe, with *Ophiostoma ips* being the only coincidence compared with the fungal associates of this beetle in Patagonia. Mathiesen (1950) studied ophiostomatoid species associated with *Hylastes ater* in Sweden. Five fungal species are mentioned, but none of them were found in this study. Nevertheless, these reports based on morphological identification criteria should not be considered until molecular confirmation is available.

Previous works in Chile (Zhou et al., 2004) reported *Grosmannia huntii* associated with *Hylastes ater* on *Pinus radiata*. This interaction has also been reported from New Zealand (Reay, Thwaites, & Farrell, 2005) and Argentina (Gómez, Greslebin, & Rajchenberg, 2011) affecting young pine seedlings. In the present study, *G. huntii* has been isolated as a saprobe from *Hylastes ater* galleries and stained timber, occurring only in Neuquén Province (Northern Patagonia). *Grosmannia radiaticola* has been isolated from *Hylastes ater* and *Hylurgus ligniperda* galleries on *Pinus radiata* in Chile (as *G. galeiformis* [Linnakoski et al., 2012; Zhou et al., 2004]). The same fungus was reported from *Cyrtogenius luteus* and *Hylurgus ligniperda* on *P. taeda* and *P. elliotti* in Uruguay (Alonso et al., 2014). The low isolation frequency of *Grosmannia* species (7%) suggests that they are of minor importance concerning blue-stain management strategies.

Currently, the genus *Graphilbum* includes six known species and seven undescribed taxa (De Beer & Wingfield, 2013). Most species are known only by their asexual states, and proper identification is only possible through DNA sequence comparisons. However, data sets of variable regions, necessary to achieve a reliable identification (β-*tubulin*, *tef* 1-α), are fragmentary. Thus, isolates from Argentina could not be assigned to a specific taxon with confidence. From 23 isolates, half of these were present inside *Orthotomicus laricis*, *Hylastes ater* and *Hylurgus ligniperda* galleries. Their isolation frequency was moderate (14.75%).

Pine plantation forestry in southern South America includes Argentina, Brazil, Chile, Paraguay and Uruguay. Bark beetles species and their fungal associates have been extensively studied in Chile. Emerging data from Argentina and Uruguay indicate some coincidences between these countries and Chile (e.g., the presence of *O. ips* and *G. radiaticola*, associated with *Hylurgus* and *Hylastes*), but also several differences. In this regard, *Ceratocystiopsis minuta* and *Ophiostoma abietinum* are only registered in Chile and Uruguay, respectively (Alonso et al., 2014; Zhou et al., 2004), and *Ophiostoma peregrinum* and *Graphilbum* sp. 1 are only known from Argentina. This scenario could indicate multiple introduction events, alerting once again on the perils of uncontrolled international trade.

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ORCID

A. de Errasti http://orcid.org/0000-0001-6869-6014
M. Rajchenberg http://orcid.org/0000-0001-5031-5148

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