Fungal Diversity
DOI 10.1007/s13225-010-0061-1

Phylogenic diversity of fungal endophytes in Spanish stands of *Pinus halepensis*

Leticia Botella · Julio Javier Diez

Received: 7 July 2010 / Accepted: 10 September 2010

Abstract Fungal endophytes were collected from 168 *Pinus halepensis* trees sampled in 55 Spanish stands. In total, 229 endophytic isolates were grouped into 92 morphotypes according to their mycelium features. Thus, twigs enclosed 63.76% of the total endophyte isolates and needles 36.24%. Likewise, twig samples also yielded a higher endophyte species richness. Analysis of ITS rDNA region generated 38 different Ascomycota taxa and confirmed the endophytic stage of several pathogens previously associated with the *Pinus halepensis* decline in Spain. *Naemacyclus minor*, *Brunchorstia pinea*, *Lophodermium pinastri*, *Phomopsis* sp., *Diplodia pinea*, *Pestalotiopsis besseyi* and *Truncatella angustata* were isolated. Sequencing of LSU rDNA region verified the 38 taxa and contributed to infer their phylogenic relatedness using the Neighbour-Joining method. In the bootstrap consensus tree five classes were observed. Dothideomycetes resulted to be the dominant class because of its high isolation frequency (52.4%) and richest species composition (39.5%). On the contrary, class Eurotiomycetes was the least abundant (3.5%) and along with class Pezizomycetes harboured the lowest species richness (7.9%). Class Sordariomycetes and Leotiomycetes had intermediate abundance and species composition percentages. This study represents the first work concerning the taxonomy of the fungal endophytic community of the Mediterranean host species *Pinus halepensis* using molecular tools. The data provided here may help to establish the cause of *Pinus halepensis* decline.

L. Botella (✉) · J. J. Diez
Department of Plant Production and Forest Resources,
Sustainable Forest Management Research Institute,
University of Valladolid-INIA,
Avda. Madrid 44,
34004 Palencia, Spain
e-mail: lbotella@pvs.uva.es

Keywords Endophytes · rDNA · Pine decline · *Pinus halepensis* decline · Phylogeny · Aleppo pine

Introduction

The interest in endophytic mycobiota of forest trees has increased over the years, and many recent works have dealt with these organisms (Guo et al. 2003, 2008; Hoff et al. 2004; Saikkonen et al. 2004; Arnold 2007; Saikkonen 2007; Ganley and Newcombe 2006; Hoffman and Arnold 2008; Hu et al. 2007; Slippers and Wingfield 2007; Sieber 2007; Thomas et al. 2008). Their role in forest structure, still unclear (Saikkonen 2007, Sieber 2007; Slippers and Wingfield 2007; Hyde and Soytong 2008), seems to be intricate and changing in an evolutionary and ecological context (Saikkonen 2007). Fungal endophyte species coinhabit with saprotrophs and pathogenic species in forests; and a single fungal species can also evolve different lifestyles as a result of environmental variations influence on fungal genome (Carroll 1988; Freeman and Rodríguez 1993; Promputtha et al. 2007; Sieber 2007). This fact makes fungi genetically highly diverse (Arnold et al. 2000).

Understanding endophyte diversity is considered essential for establishing endophyte implications on forests (Arnold 2007). Endophyte diversity clearly depends on host identity (Higgins et al. 2007) and on geographic situation (Carroll et al. 1977; Fisher et al. 1993; Saikkonen 2007; Thomas et al. 2008). Composition of endophytes is strongly affected by local climatic conditions (Suryanarayanan et al. 2000; Arnold and Herre 2003), even in monospecific host stands (Fisher et al. 1993). However, highly diverse forests, such as tropical ecosystems, contain the most endophytic species richness (Arnold et al. 2000; Fröhlich et al. 2000, Thomas et al. 2008). As although there is less host-tree specificity there

Published online: 30 September 2010

is considerably higher host diversity (Saikkonen 2007). Consequently, temperate areas such as Mediterranean region (Saikkonen et al. 1998) appear to have lower endophyte richness (Arnold 2007).

Endophyte research can benefit from molecular techniques that may infer genetic structure and taxonomy composition of endophyte communities. In this sense, multi-locus analyses are a much-needed tool to obtain consistent information from evolutionary and ecologically determinant loci (Hoffman and Arnold 2008; Hyde and Soytong 2008; Rungjindamai et al. 2008; Cai et al. 2009). Consequently, sequencing of the internal transcribed spacer (ITS) and the partial large subunit (LSU) of nuclear ribosomal DNA (rDNA) was considered the functional framework for our study.

Pinus halepensis Mill. (Aleppo pine) is a pine species native to western part of Mediterranean region. In Spain, it is one of the most abundant conifers and both virtual natural and reforested stands are widespread (Gil et al. 1996). *Pinus halepensis* has suffered from unfavourable environmental conditions in recent years, especially in the north-western Iberian Peninsula where it grows outside its optimum natural habitat (Abelló 1998). In 1998 the General Direction for the Nature Conservation (DGCN) confirmed that *Pinus halepensis* stands were suffering a generalized decline, however, few reasons have been put forward to explain this situation (SPCAN 1998). Santamaría et al. 2007 described some fungal species in declined north-western stands, and Botella et al. (2010) suggested a compendium of abiotic and biotic factors contributing to the development of the decline over the whole Iberian Peninsula. Saprotrophs, pathogens and endophytes fungi were recorded. Likewise, no studies so far have assessed phylogenic relationships among endophytic fungi of *Pinus halepensis*; thereby significant insights into evolutionary associations of fungal lineages with this particular Mediterranean host are still undetermined (Hoffman and Arnold 2008).

The overall objectives of our study were: 1) to characterize genetically fungal endophytic diversity in needles and twigs of *Pinus halepensis* forests in Spain and 2) to explain the phylogenetic relationship among endophytes.

Materials and methods

Sampling

During the spring of 2006, 55 *Pinus halepensis* stands were chosen randomly (Table 1). They all belonged to the European Network of Forest Damages (Level 1), Spain, 2005, whose coordinates were supplied by the General Direction for the Nature Conservation (DGCN). They were distributed throughout Mediterranean coast. Within each stand, three symptomatic trees were selected and from each tree three branches were cut down, in total 168 trees were processed. The samples were stored immediately at 4°C until further processing.

Fungal isolation

Following pilot tests we established the optimum conditions for surface sterilization (i.e.: Zamora et al. 2008). From every branch four twigs and four needles were cut into pieces of 15 mm. Under sterile conditions, tissue segments were surface sterilized through 1 min dipping in 70% ethanol, 5 min for needles and 10 min for twigs in a 2% sodium hypochlorite solution and, finally, three times in sterile distilled water. The pieces were then plated in PDA medium (potato dextrose agar) and stored at 20°C in the dark for 1 month. The outgrowing colonies were transferred to agar-water medium in order to take a single hyphal by a Pasteur pipette and under a microscope. Finally, a piece of the isolated mycelium was subcultured in PDA medium.

Fungal isolates were selected and grouped together according to the morphological characters such as the spore production, spore length and morphology, aerial mycelium colour, texture and form, exudates and the growth rate (Bills 1996; Hawksworth et al. 1995; Lacap et al. 2003).

Genomic DNA extraction and PCR

Genomic DNA was isolated following the protocol described by Vainio et al. 1998. PCR was carried out according to the recommended conditions described by the manufacturer of Dynazyme II DNA-polymerase (Finnzymes Ltd, Espoo, Finland). A concentration of 2 μM was used to amplify ITS rDNA region (*ca* 600 bp) and LSU rDNA (*ca* 600 bp). ITS primers used were 1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and 4 (5′-TCCTCCGCTTATTGATATGC-3′) and LSU primers were LR0R (5′-ACCCGCTGAACTTAAGC-3) and LR16 (5′-TTCCACCCAAACACTCG-3) (Vilgalys and Hester 1990; White et al. 1990; Gardes and Bruns 1993). For ITS amplification samples were denaturized by 10 min incubation at 95°C after which 34 cycles of amplification were carried out in the following way: 13 times: 35 s at 95°C, 55 s at 55°C and 45 s at 72°C; 13 times; 35 s at 95°C, 55 s at 55°C and 2 min at 72°C and finally, 9 times, 35 s at 95°C, 55 s at 55°C and 3 min at 72°C. After these cycles the reaction was followed by 7 min of extension at 72°C. For LSU the protocol was the following one: samples were denaturized by 3 min at 94°C; 36 cycles of 30 s at 94°C, 30 s at 54°C, 1 min at 72°C; and finally, 10 min at 72°C.

DNA amplification products were checked with UV light after running 1 h and 30 min in 1% TAE-buffer at 90 V/30 cm

Table 1 Stand code, location, province and coordinates of stands

Stand code	Location	Province	UTM coordinates	
			X	Y
1-TOR	Tordehumos	Valladolid	321200,4915	4627200,704
2-ARG	Arganda	Madrid	463900,3793	4457700,772
3-COL	Colmenar	Málaga	376500,303	4077500,161
4-ALB	Albuñuelas	Granada	440300,7262	4087200,816
5-ZUJ	Zújar	Granada	510400,6468	4161700,504
6-BAZ	Baza	Granada	512800,7935	4146100,895
7-BEN	Benatae	Granada	530300,1867	4244500,413
8-PUE	P. Don Fabrique	Granada	553600,5125	4200100,218
9-YES	Yeste	Granada	548700,5954	4231300,647
10-MOG	Mogón	Granada	503100,3661	4208600,263
11-AMA	Amarguillas	Granada	531200,9997	4132800,852
12-VVA	Vva. del Arzobispo	Granada	516700,6061	4226600,976
13-MOR	Moratalla	Murcia	599200,7342	4222700,51
14-ALM	Almudema	Murcia	668100,7494	4200900,895
15-SAB	El Sabinar	Murcia	583200,6651	4220400,947
16-FOR	Fortuna	Murcia	665700,0495	4216500,384
17-CIE	Cieza	Murcia	631300,6902	4227500,031
18-VILL	Villena	Alicante	674600,1633	4265700,816
19-ELC	Elche	Alicante	711500,7908	4239200,091
20-COF	Cofrentes	Valencia	662700,0886	4343700,846
21-POR	La Portera	Valencia	660300,8232	4359200,944
22-BUÑ	Buñol	Valencia	676400,7438	4361600,174
23-CHE	Cheste	Valencia	690100,7933	4379500,63
24-AND	Andilla	Valencia	685300,1158	4410600,333
25-UTI	Utiel	Valencia	655500,9372	4390400,423
26-TUE	Tuéjar	Valencia	669200,4909	4408300,92
27-REQ	Requena	Valencia	674000,577	4377100,713
28-SIN	Sinarcas	Valencia	653100,0025	4405900,484
29-NER	Nerpio	Albacete	564700,5223	4233700,915
30-PAR	Paracuellos	Cuenca	604900,2654	4398900,887
31-OLA	Villar de Olaya	Cuenca	567800,396	4425300,551
32-ALA	Alarcón	Cuenca	575100,1572	4378700,632
33-MIN	Minglanilla	Cuenca	625700,2567	4370100,505
34-FUE	Fuendetodos	Zaragoza	675100,0773	4583800,163
35-ARI	Ariza	Zaragoza	578200,3207	4569700,503
36-MEQ	Mequinenza	Zaragoza	774400,1369	4582300,652
37-CAS	Caspe	Zaragoza	758200,5817	4579900,331
38-NON	Nonaspe	Zaragoza	776800,7142	4566800,575
39-ANR	Andorra	Teruel	714600,7448	4541900,1
40-BEL	Belmonte de S. José	Teruel	749200,6988	4531100,954
41-MAE	Maella	Zaragoza	765400,8934	4533400,863
42-MON	Monroyo	Teruel	751600,8874	4515500,384
43-MUS	La Mussara	Tarragona	841400,8231	4576100,758
44-BAT	Batea	Tarragona	779100,1543	4551200,377
45-HOR	Horta de Sant Joan	Tarragona	781500,3178	4535700,619
46-NAV	Navás	Barcelona	896800,5554	4647400,023
47-SUR	Súria	Barcelona	882900,5153	4629600,597

Table 1 (continued)

Stand code	Location	Province	UTM coordinates	
			X	Y
48-SAR	Sarral	Barcelona	855200,5209	4593900,45
49-GRA	La Granadella	Lleida	806700,3067	4586900,248
50-CER	Cerviá de Garrigués	Lleida	822900,7352	4589200,825
51-MAI	Maiá de Montcal	Girona	973100,7366	4690100,784
52-MOC	Monistrol de Calders	Barcelona	915300,6057	4634300,733
53-LLI	LliÇa D´Amunt	Barcelona	933800,6463	4621100,355
54-BAN	Banyoles	Girona	975500,8188	4674600,709
55-CEN	S. Martí de Centelles	Barcelona	931500,9993	4636600,046

in 1% agarose gels (FMC BioProducts, Rockland, ME, USA) which contained 1x TAE (40 mM Tris-acetate pH 8.0, 1 mM EDTA) and 10 μl of GelRed™ Nucleic Gel Acid Gel Stain (Biotium). The marker used to estimate the lengths of the amplification products was λ-DNA Hind III–ΦX174Hae III (DyNAzyme™ DNA Polymerase Kit).

Sequencing

PCR products were purified with NucleoSpin® Extract II 10/2007 Rev. 06 (Macherey-Nagel Gmbh and Co.KG). Then, 1 μl of purified PCR product was pipetted into 9 μl of 1x tracking dye. Next, samples were loaded in a 1% agarose gel, which contained 10 μl of GelRed™ and 1x TAE buffer. The running was performed in a 1x TAE buffer during 10–15 min at 90 V/30 cm, and afterwards observed under UV light and photographed. Concentration was determined by visual comparison between each sample and a series of known standard concentrations of λ-DNA (5, 10, 20, 40, 80 and 160 ng/μl). Sequencing of the ITS and LSU rDNA was developed through Secugen company, Madrid, Spain.

Phylogenic analysis

ITS rDNA sequences of morphological grouped isolates were obtained to determine preliminary identification at higher taxonomy level using BLAST search. LSU rDNA sequences were performed for representative isolates based on ITS rDNA genotypes and for clarifying the generic and species level of isolates. The LSU rDNA sequences were organized and align for phylogenic analysis. All sequences were submitted in the EMBL/GenBank database (Table 2).

All sequences were adjusted using the Chromas Pro version 1.49 beta (Technelysium Pty Ltd). They were compiled and aligned using Geneious Pro 4.8 software package (Biomatters Ltd). Manual corrections of aligned database, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.1 (Tamura et al. 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 552 positions in the final dataset.

Results

Molecular identification

From 168 trees sampled in this study, 2,016 twigs and 2,016 needles were processed. In total, 229 endophyte isolates grew out (Table 2) and 92 endophytic fungi were classified as morphotypes according to their morphological characters. Consequently, the ITS rDNA gene region was sequenced for all morphotypes and identified by comparing with the EMBL/GenBank database. As a result, 38 unique ITS rDNA genotypes were obtained. Based on ITS rDNA data we delimited taxa with ≥97% ITS rDNA similarity considering differences in sequence length. As well, we used ≥97% LSU rDNA to verify the identity of the 38 endophytic isolates (Table 2).

Phylogenic species richness and isolation frequency

All endophytic taxa were included in Phylum Ascomycota and subphylum Pezizomycotina within five classes:

Table 2 Endophytic taxa identified and their isolation frequency

Code	Taxa[a]	T/N[b]	S[c]	ITS[d]	LSU[d]
BL-E26	*Alternaria* sp.	37/12	43	FN868462	FN868857
BL-E6	*Aureobasidium pullulans*	16/5	20	FN868454	FN868849
BL-E16	*Camarosporium brabeji*	2/0	2	FN868461	FN868856
BL-E21	*Chaetomium globosum*	0/1	1	FN868476	FN868871
BL-E14	*Chalastospora gossypii*	9/0	9	FN868458	FN868853
BL-E25	*Davidiella tassiana*	2/0	2	FN868485	FN868880
BL-E5	*Diplodia pinea*	7/1	8	FN868481	FN868876
BL-D6	*Dothideomycetes* sp.	2/1	3	FN868453	FN868848
BL-E13	*Epicoccum nigrum*	3/0	3	FN868456	FN868851
BL-D11	Fungal sp. BL11	2/0	2	FN868450	FN868845
BL-D5	*Fusarium larvarum*	2/0	2	FN868469	FN868864
BL-E1	*Fusarium proliferatum*	4/11	14	FN868470	FN868865
BL-E34	*Gremmeniella abietina*	1/0	1	FN868466	FN868861
BL-E18	*Naemacyclus minor*	0/24	24	FN868468	FN868863
BL-D3	*Leotiomycetes* sp.	4/2	6	FN868467	FN868862
BL-D2	*Leptosphaeria* sp.	1/0	1	FN868449	FN868844
BL-E32	*Lophodermium* sp.	0/2	2	FN868465	FN868860
BL-E31	*Lophodermium pinastri*	0/11	11	FN868464	FN868859
BL-E20	*Paraconiothyrium* sp.	1/0	1	FN868460	FN868855
BL-E22	*Penicillium* sp.	1/1	2	FN868482	FN868877
BL-E24	*Penicillium aurantiogriseum*	0/3	3	FN868484	FN868879
BL-E23	*Penicillium purpurogenum*	2/1	3	FN868483	FN868878
BL-E10	*Pestalotiopsis besseyi*	3/2	5	FN868480	FN868875
BL-D7	*Peziza varia*	3/0	3	FN868472	FN868867
BL-D9	*Pezizomycetes* sp.	1/1	1	FN868473	FN868868
BL-E27	*Phaeomoniella* sp.	3/0	3	FN868455	FN868850
BL-D1	*Phaeosphaeria* sp.	1/1	2	FN868448	FN868843
BL-E15	*Phoma herbarum*	1/0	1	FN868459	FN868854
BL-E12	*Phomopsis* sp.	5/0	5	FN868477	FN868872
BL-E29	*Pleospora herbarum*	8/0	8	FN868457	FN868852
BL-E35	*Preussia* sp.	1/1	2	FN868452	FN868847
BL-E17	*Pyronema* sp.	7/1	7	FN868474	FN868869
BL-E30	*Sordaria fimicola*	3/0	3	FN868475	FN868870
BL-E9	*Trichoderma viridae*	1/1	2	FN868471	FN868866
BL-E11	*Tryblidiopsis pinastri*	6/1	6	FN868463	FN868858
BL-E4	*Truncatella angustata*	3/0	3	FN868479	FN868874
BL-E7	*Ulocladium* sp.	3/0	3	FN868451	FN868846
BL-D10	*Xylaria sp.*	1/0	1	FN868478	FN868873

[a] Closest related sequences in Genbank. These species may have not been correctly named in GenBank. [b] Number of occurrences of every fungus in twigs (T) and needles (N). [c] Number of stands where the fungus was isolated (S). [d] Genbank accession number for ITS and LSU rDNA region

Dothideomycetes, Sordariomycetes, Leotiomycetes, Eurotiomycetes and Pezizomycetes (Fig. 1). According to the percentages of species composition (Table 3), the richest and most abundant class was Dothideomycetes represented by orders Pleosporales (15 species), Botryosphaeriales (1 species) and Capnodiales (1 species). Class Sordariomycetes was represented by orders Hypocreales (3 species), Xylariales (3 species), Sordariales (2 species) and Diaporthales (1 species). This class was the second most diverse in respect of the species composition but the third regarding the fungal occurrence frequency. Orders Helotiales (3 species) and Rhytismatales (3 species) corresponded to Class Leotiomycetes. In this case, the identified species had a high occurrence frequency. Least abundant classes were class Pezizomycetes comprising order Pezizales (3 species) and Eurotiomycetes represented by order Eurotiales (3 species). Although these two last classes presented the same number of species identified, the occurrence frequency of class Pezizomycetes was slightly higher.

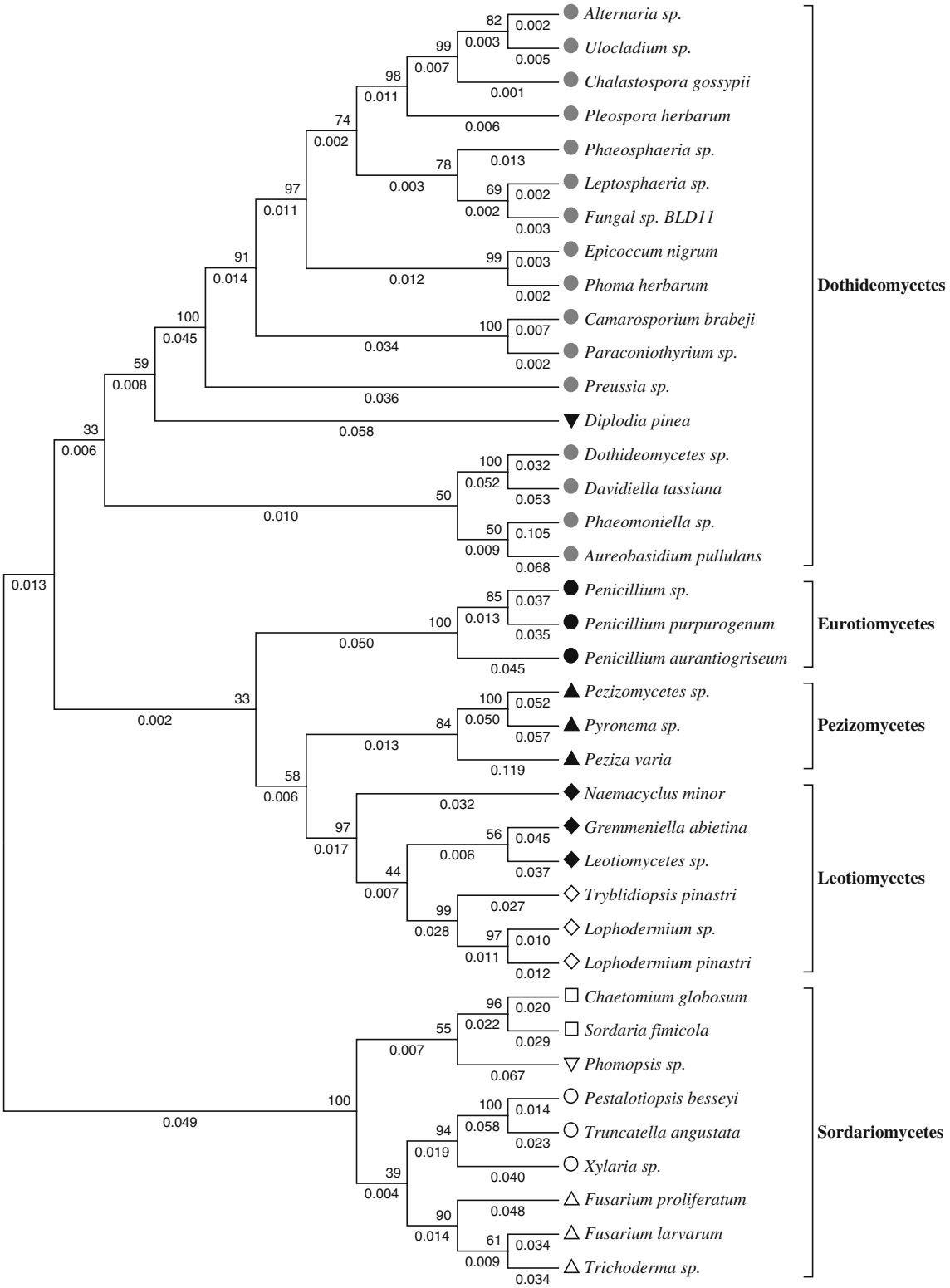

Fig. 1 Evolutionary relationships of 38 taxa. Phylogenic tree based on NJ analysis of LSU rDNA data for classes Dothideomycetes, Eurotiomycetes, Pezizomycetes, Sordariomycetes and Leotiomycetes. Numbers on branches indicate distances among different taxa. Symbols represent the different orders appeared in the analysis: ▼ Botriosphaeriales, ◊ Rhytismatales, ♦ Helotiales, ■ Capnodiales, ● Pleosporales, ○ Xylariales, ● Eurotiales, △ Hypocreales, □ Sordariales, ▲ Pezizales, ▽ Diaporthales

Table 3 Species richness and isolation frequency of the different classes appeared

Classes	Species richness (%)	Isolation frequency (%)
Dothideomycetes	39.47	52.40
Sordariomycetes	23.70	16.16
Leotiomycetes	15.80	22.27
Eurotiomycetes	7.89	3.49
Pezizomycetes	7.89	5.68

Isolation frequency in every stand and tissue type where each endophyte was collected from can be observed in Table 2. Overall, twigs appeared to have richer fungal isolation frequency. From the 229 total endophyte isolates, 146 (63.76%) occurred in twigs and 83 (36.24%) in needles. In the same way, twig samples also presented higher endophyte species richness. Fourteen endophyte species were isolated from both tissue types, twigs and needles, 19 endophytes occurred only in twigs and five species only in needles.

Alternaria alternata was the most abundant species (25.76%) followed by *Aureobasidium pullulans* (9.17%), "*Cyclaneusma niveum*" (10.48%), *Fusarium proliferatum* (6.55%) and "*Lophodermium pinastri*" (4.88%). On the contrary, "*Leptosphaeria* sp.", *Phoma herbarum, Paraconiothyrium* sp. and "*Brunchorstia pinea*", appeared only in one location and just in twigs (Table 2). The other taxa had an intermediate occurrence frequency.

Discussion

The number of endophyte species detected in plant species worldwide is increasing every year. Between 500,000 and 600,000 fungal endophyte species were thought to exist (Schmit and Mueller 2007), and approximately 465,000 of them are not described yet (Sieber 2007). These data can be explained assuming the high genetic variability of endophytic fungi (Arnold et al. 2000) in addition to the large number of host species but also because of difficulties in the identification process. Frequently, endophytic isolates do not sporulate in culture media so morphological description turns into an insufficient method for fungi classification (Baayen et al. 2002; Lacap et al. 2003). However, since "morphotype" concept was defined (Hawksworth et al. 1995), cultural characteristics of fungi are helpful as complementary data for the molecular identification (Guo et al. 2003; Lacap et al. 2003). Phylogenetic analysis of morphospecies has been increasingly used in the identification for endophytic fungi (Huang et al. 2006; Albrectsen et al. 2010; Pinruan et al. 2010; Sánchez Márquez et al. 2010), however there are also problems with this methodology. Many taxa can only be identified to family level as no similar sequences can be blasted in GenBank (Cai et al. 2009; Pinruan et al. 2010), while identification is based on blasting with a similar sequence. There is not guarantee however, that the similarly blasted species have been corrected identified; for example, it has been shown that 96% of names under *Colletotrichum gloeosporioides* have been wrongly applied (Cai et al. 2009).

Trying to minimize this problem, we used sequencing of the ITS rDNA region as a useful and quick method of endophyte identification and LSU rDNA sequences as phylogenetically informative locus (Hoffman and Arnold 2008). Both rDNA sequence GenBank comparison based on ≥97% rDNA similarity. Previous studies have used 90–97% ITS rDNA sequence similarity as a proxy for species boundaries in Fungi (e.g. 97%: O'Brien et al. 2005). One study has explicitly shown that groups based on 90% ITS rDNA similarity are highly congruent with phylotypes based on a second locus (Arnold et al. 2007).

Members of Family Pinaceae have been investigated in-depth for the presence of fungal endophytes (Carroll et al. 1977; Hata et al. 1998; Müller and Hallaksela 1998, 2000; Guo et al. 2003, 2008; Ganley and Newcombe 2006; Arnold et al. 2007; Sieber 2007; Zamora et al. 2008; Giordano et al. 2009). Thus, Leotiomycetes has been designated as the predominant class within gymnosperms and in particular, order Helotiales constitutes the closest taxon from an evolutive point of view (Berbee and Taylor 2001; Scheneider et al. 2004). In our study, class Leotiomycetes is represented by recognised pathogenic fungi of *Pinus* sp. (Table 2) and specifically of *Pinus halepensis* in Spain (Santamaría et al. 2008; Botella et al. 2010). Members of order Rhytismatales produce mostly foliar infections, however, we isolated *Tryblidiopsis pinastri*, which mainly affects twigs. In addition, *Lophodermiun pinastri* was found with an important distribution causing needle cast and an undetermined *Lophodermiun* sp. occurred in two stands. Besides, within helotialean fungi, we isolated *Brunchorstia pinea*, anamorph state of *Gremmeniella abietina*. This is a well-known pathogen with a worldwide distribution, which was found first in *Pinus halepensis* in Spain eleven years ago (Santamaría et al. 2003). *Naemacyclus minor* was the second most taxon isolated in this study.

Class Dothideomycetes is reported to be most common in family Cupressaceae (Arnold 2007). In the present study, class Dothideomycetes not only had the richest species composition (39.47%) but also the highest isolation frequency (52.4%) (Table 3). Thus, this second percentage can be explained because solely *Alternaria alternata* appeared in 43 of the total 55 stands. Class Dothideomycetes usually harbour generalists and opportunistic taxa

(Table 2) as *Davidiella tassiana*, *Ulocladium* sp., *Epicoccum nigrum*, *Phoma herbarum, Pleospora herbarum, Aureobasidium pullulans* and *Phaeomoniell*a sp. and others as *Camarosporium brabeji*, *Paraconiothyrium* sp. Conversely, *Diplodia pinea*, belonging to order Botryosphaeriales, occurred mainly in twigs in a total of eight stands. This fungus has been also considered pathogenic agent involved *Pinus halepensis* decline in Spain (Santamaría et al. 2008; Botella et al. 2010).

We also identified *Leptosphaeria* sp. and *Phaeosphaeria* sp. in one and two stands, respectively. They both have a very close phylogenic relationship (Morales et al. 1995; Câmara et al. 2002). However, fungal endophyte BL11 appears even more related to *Leptosphaeria* sp. This endophytic fungus has a singular importance for our research group because it was already isolated in a previous study (Santamaría et al. 2007). An in vitro experiment determined a successful antibiotic activity against Spanish isolates of *Gremmeniella abietina*. According to the Genbank search completed for ITS and LSU rDNA sequences, an unclassified fungal endophyte isolated from asymptomatic photosynthetic tissue in *Cuppressus arizona* (Hoffman and Arnold 2008) would be the most probable record. However, we collected this endophyte from twigs (Table 2) and no morphological comparison has been developed. Therefore, further experiments are needed to carry out for its characterisation and final identification.

Sordariomycetes commonly found in family Fagaceae (Arnold 2007) was the second more abundant class in our study (Table 3). It is mostly composed of foliar endophytes (Arnold et al. 2007a). In our case, only the ubiquitous fungus *Chaetomium globossum*, the generalist *Fusarium proliferatum* and *Pestalotiopsis besseyi,* described as endophyte of pines (Guo 2002), appeared in needles (Table 2).

Class Eurotiomycetes, represented by Eurotiales (*Penicilium* sp., *P. purpurogenum* and *P. aurantiogriseum*), constitutes the least abundant class (Table 3). Endophytic fungi are relatively few in this clade (Arnold 2007). Finally, class Pezizomycetes is scarcely represented by three species (Table 3), one of them *Peziza varia* is usually collected in forests as a wood decomposer, whereas our molecular study suggests that it has an endophytic stage. Thus, endophytes are capable of colonizing inner plant tissues. Once inside, their ecological role can change into parasitism if their hosts are under environmental stress or into saprophytism if hosts die (Wang et al. 2006; Promputtha et al. 2007).

In conclusion, our objective of providing a framework for phylogenetic classification of endophytic fungi in *Pinus halepensis* in Spain resulted in 38 fungal endophyte taxa. Twenty-two taxa were identified to species level based on blast search similarity with public database accessions. Since we analyzed two loci we tried to avoid erroneous identification but it cannot be ruled out that a mayor multiloci analysis comparing type sequences would further corroborate this identification. In this sense, further sequencing should be applied for future similar studies.

One endophyte, Fungal sp. BL11 may be a novel species. Because of it has been shown to harbour antibiotic properties, further morphological characterization should be performed. Within all taxa isolated, Dothideomycetes was the predominant class and Leotiomycetes mainly harboured pathogenic fungi associated with the *Pinus halepensis* decline in Spain in advance. Likewise, they may play an important role in the current Aleppo pine development and in the future of Spanish stands.

Acknowledgements This research was supported by the Ministry of Culture and Science of Spain (Project: AGL2005-02141/FOR and AGL2008-03622) and by grants provided by the University of Valladolid. We would like to thank the Data Centre of the Protective Service of Nocive Agents (CENDANA) for supplying us environmental data on the locations sampled in this study.

References

Abelló MA (1998) Historia y Evolución de las Repoblaciones Forestales en España. Universidad Complutense de Madrid, Madrid, p 749

Albrectsen B, Björkén L, Varad A, Hagner Å, Wedin M, Karlsson J, Jansson S (2010) Endophytic fungi in European aspen (*Populus tremula*) leaves—diversity, detection, and a suggested correlation with herbivory resistance. Fungal Divers 41:17–28

Arnold AE (2007) Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biol Rev 21:51–66

Arnold AE, Herre EA (2003) Canopy cover and leaf age affect colonization by tropical fungal endophytes: ecological patterns and process in *Theobroma cacao* (Malvaceae). Mycologia 95:388–398

Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA (2000) Are tropical fungal endophytes hyperdiverse? Ecol Lett 3:267–274

Arnold AE, Henk DA, Eells RL, Lutzoni F, Vilgalys R (2007) Diversity and phylogenetic affinities of foliar endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia 99:185–206

Baayen RP, Bonants PJM, Verkley G, Carroll GC, van der Ha A, Weerdt M, van Brouwershaven IR, Schute GC, Maccheroni W, de Blanco GC, Azevedo JL (2002) Non pathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*, identified as cosmopolitan endophyte of woody plants, *G. mangiferae* (*Phyllosticta capitalensis*). Phytopathology 92:464–477

Berbee ML, Taylor JW (2001) Fungal molecular and geologic time. In: McLaughlin DL, McLaughlin EG, Lemke PA (eds) Systematics and evolution. Springer-Verlag, Berlin

Bills GF (1996) Isolation and analysis of endophytic fungal communities from woody plants. In: Carris LM, St Paul MN, Redlin SC (eds) Endophytic fungi in grasses and woody plants. APS, USA, pp 31–65

Botella L, Santamaría O, Díez JJ (2010) Fungi associated with the decline of *Pinus halepensis* in Spain. Fungal Divers 40:1–11

Cai L, Hyde KD, Taylor PWJ, Weir BS, Waller J, Abang MM, Zhang JZ, Yang YL, Phoulivong S, Liu ZY, Prihastuti H, Shivas RG,

McKenzie EHC, Johnston PR (2009) A polyphasic approach for studying *Colletotrichum*. Fungal Divers 39:183–204

Câmara MPS, Palm ME, van Berkum P, O'Neill NR (2002) Molecular phylogeny of *Leptosphaeria* and *Phaeosphaeria*. Mycologia 94:630–640

Carroll G (1988) Fungal endophyte in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology 69:2–9

Carroll G, Müller E, Sutton BC (1977) Preliminary studies on the incidence of needle endophytes in some European conifers. Sydowia 29:87–103

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:779–783

Fisher PJ, Petrini O, Sutton BC (1993) A comparative study of fungal endophytes in leaves, xylem and bark of Eucalyptus in Australia and England. Sydowia 45:338–345

Freeman S, Rodríguez RJ (1993) Genetic conversation of a fungal pathogen to nonpathogenic, endophtic mutualist. Science 260:75–78

Fröhlich J, Hyde KD, Petrini O (2000) Endophytic fungi associated with palms. Mycol Res 104:1202–1212

Ganley RJ, Newcombe G (2006) Fungal endophytes in seeds and needles of *Pinus monticola*. Mycol Res 110:318–327

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorhizae and rust. Mol Ecol 2:113–118

Gil L, Díaz-Fernández PM, Jiménez MP, Roldán M, Alía R, Agúndez D, De Miguel J, Martín S, De Tuerto M (1996) Las regiones de Procedencia del *Pinus halepensis* Mill. en España. Organismo Autónomo de Parques Nacionales, Madrid

Giordano L, Gonthier P, Varese GC, Miserere L, Nicolotti G (2009) Mycobiota inhabiting sapwood of healthy and declining Scots pine (*Pinus sylvestris* L.) trees in the Alps. Fungal Divers 38:69–83

Guo LD (2002) *Pestalotiopsis besseyi*, a new record of endophytic fungi from Pine in China. Mycosystema 21:455–456

Guo LD, Guo RH, Wang Y, He WH, Zheng WH, Hyde HD (2003) Molecular identification of white morphotype strains of endophytic fungi from *Pinus tabulaeformis*. Mycol Res 107:680–688

Guo LD, Huang GR, Wang Y (2008) Seasonal and tissue age influences on endophytic fungi of *Pinus tabulaeformis* (Pinaceae) in the Dongling Mountains, Beijing. J Integr Plant Biol 50:997–1003

Hata K, Futai K, Tsuda M (1998) Seasonal and needle age-dependent changes of the endophytic mycobiota in *Pinus thunbergii* and *Pinus densiflora* needles. Can J Bot 76:245–250

Hawksworth DL, Kirk PM, Sutton BC, Pegler DN (1995) Ainsworth & Biby's dictionary of the fungi, 8th edn. CAB International, Wallingford

Higgins KL, Arnold AE, Miadlikowska J, Sarvate SD, Lutzoni F (2007) Host affinity and geographic structure among boreal and arctic endophytes from three major plant lineages. Mol Phylogenet Evol 42:543–555

Hoff JA, Klopfenstein NB, McDonald GI, Tonn JR, Kim MS, Zambino PJ, Hessburg PF, Rogers JD, Peever TL, Carris LM (2004) Fungal endophytes in woody roots of Douglas-fir (*Pseudotsuga menziesii*) and ponderosa pine (*Pinus ponderosa*). Forest Pathol 34:255–271

Hoffman A, Arnold AE (2008) Geographic locality and host identity shape fungal endophyte communities in cuppressaceous trees. Mycol Res 112:331–334

Hu HL, Jeewon R, Zhou DQ, Zhou TX, Hyde HD (2007) Phylogenetic diversity of endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp.: evidence from rDNA and tubulin gene phylogenies. Fungal Divers 24:1–22

Huang WY, Cai YZ, Hyde KD, Corke H, Sun M (2008) Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Divers 33:61–75

Hyde HD, Soytong K (2008) The fungal endophyte dilemma. Fungal Divers 33:163–173

Lacap DC, Hyde KD, Liew ECY (2003) An evaluation of the fungal "morphotype" concept based on ribosomal DNA sequences. Fungal Divers 12:53–66

Morales VM, Jasalavich CA, Pelcher LE, Petrie GA, Taylor JL (1995) Phylogenetic relationships among several *Leptosphaeria* species based on their ribosomal DNA sequences. Mycol Res 99:593–603

Müller MM, Hallaksela AM (1998) Diversity of Norway spruce needle endophytes in various mixed and pure Norway spruce stands. Mycol Res 102:1183–1189

Müller MM, Hallaksela AM (2000) Fungal Divers in Norway spruce: a case study. Mycol Res 104:1139–1145

O'Brien HE, Miadlikowska J, Lutzoni F (2005) Assessing host specialization in symbiotic cyanobacteria associated with four closely related species of the lichen fungus Peltigera. Eur J Phycol 40:363–378

Pinruan U, Rungjindamai N, Choeyklin R, Lumyong S, Hyde K, Jones E (2010) Occurrence and diversity of basidiomycetous endophytes from the oil palm, *Elaeis guineensis* in Thailand. Fungal Divers 41:71–88

Promputtha I, Lumyong S, Dhanasekaran V, McKenzie EHC, Hyde HD, Jeewon RA (2007) Phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. Microb Ecol 53:579–590

Rungjindamai N, Pinruan U, Choeyklin R, Hattori T, Jones EBG (2008) Molecular characterisation of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeis guineensis*, in Thailand. Fungal Divers 33:139–161

Saikkonen K (2007) Forest structure and fungal endophytes. Fungal Biol Rev 21:67–74

Saikkonen K, Faeth SH, Helander ML, Sullivan TJ (1998) Fungal endophytes: a continuum of interactions with host plants. Annu Rev Ecol Syst 29:319–343

Saikkonen K, Wäli P, Helander M, Faeth SH (2004) Evolution of endophyte-plant symbiosis. Trends Plant Sci 9:275–280

Saitou N, Nei M (1987) The neighbor-joining method—a new method for reconstructing phylogenetic trees. Mol Bio Evol 4:406–425

Sánchez Márquez S, Bills G, Domínguez Acuña L, Zabalgogeazcoa I (2010) Endophytic mycobiota of leaves and roots of the grass *Holcus lanatus*. Fungal Divers 41:115–123

Santamaría O, Pajares JA, Diez JJ (2003) First report of *Gremmeniella abietina* on *Pinus halepensis* in Spain. Plant Pathol 52:425–425

Santamaría O, González MA, Pajares JA, Díez JJ (2007) Effect of fungicides, endophytes and fungal filtrates on in vitro growth of Spanish isolates of *Gremmeniella abietina*. Forest Pathol 37:251–262

Santamaría O, Botella L, Diez JJ (2008) *Gremmeniella abietina* in North-western Spain: distribution and associated mycoflora. Acta Silv Lign Hung, Spec. Edition:137–145

Scheneider H, Shuettpelz E, Pryer KM, Cranfill R, Magallon S, Lupia R (2004) Ferns diversified in the shadow of angiosperms. Nature 428:553–557

Schmit JP, Mueller GM (2007) An estimate of the lower limit of global Fungal Diversity. Biodivers Conserv 16:99–111

Sieber B (2007) Endophytic fungi of forest trees: are they mutualists? Fungal Biol Rev 21:75–89

Slippers B, Wingfield JM (2007) Botryosphaeriaceae as endophytes and latents pathogens of woody plants: diversity, ecology and impact. Fungal Biol Rev 21:90–106

SPCAN (1998) Dirección General para la Conservación de la Naturaleza-Servicio de Protección de los Montes contra Agentes Nocivos. Distribución de Daños Soflamado Observados en *Pinus halepensis*

Suryanarayanan TS, Senthilarasu G, Muruganandam V (2000) Endophytic fungi from *Cuscuta reflexa* and its host plants. Fungal Divers 4:117–123

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. PNAS 101:11030–11035

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24(8):1596–1599

Thomas SE, Crozier J, Aime MC, Evans HC, Holmes KA (2008) Molecular characterisation of endophytic morphospecies associated with the indigenous forest tree, *Theobroma gileri*, in Ecuador. Mycol Res 112:852–860

Vainio EJ, Korhonen K, Hantula J (1998) Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. Mycol Res 102:187–192

Vilgalys R, Hester M (1990) Rapid genetic identification and maping of enzimatically amplified ribosomal DNA from several *Cryptococcus species*. J Bacteriol 172:4238–4246

Wang Z, Binder M, Schoch CL, Johnston PR, Spatafora JW, Hibbet DS (2006) Evolution of helotialean fungi (Leotiomycetes, Pezizomycotina): a nuclear rDNA phylogeny. Mol Phylogenet Evol 41:295–312

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylonetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego

Zamora P, Martínez-Ruiz C, Diez JJ (2008) Fungi in needles and twigs of pine plantations from northern Spain. Fungal Divers 30:171–184