Fungal endophytic communities on twigs of fast and slow growing Scots pine (*Pinus sylvestris* L.) in northern Spain

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ARTICLE INFO

ABSTRACT

Most plant species harbour a diverse community of endophytic, but their role is still unknown in most cases, including ecologically and economically important tree species. This study describes the culturable fungal endophytic community of *Pinus sylvestris* L. twigs in northern Spain and its relationship with diametric growth of the host. In all, 360 twig samples were collected from 30 Scots pines in fifteen stands. Isolates were obtained from all twig samples and 43 fungal taxa were identified by morphogrouping and subsequent ITS rDNA sequencing. All isolates were Ascomycetes, being Dothideomycetes and Sordariomycetes the most abundant classes. Half of the species were host generalists while the others were conifer or pine specialists. We found three new endophytic species for the Pinaceae: *Biscogniauxia mediterranea*, *Phaeomoniella effusa* and *Plectania milleri*, and additional six new species for *P. sylvestris*: *Daldinia fissa*, *Hypocrea viridescens*, *Nigrospora oryzae*, *Ophiostoma nigrocarpum*, *Penicillium melinii* and *Penicillium polonicum*. The endophytic community of fast and slow growing trees showed differences in species composition, abundance and evenness, but not in diversity. *Phoma herbarum* was associated to fast growing trees and *Hypocrea lixii* to those growing slow. Our results support the hypothesis that some endophytic species may affect growth of *P. sylvestris*.

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Introduction

Research on endophytes of woody plants has so far concentrated mainly on describing species and infection rates of fungal inhabitants of various plant tissues, while in most cases their ecological role has remained poorly understood.

Present knowledge on the microbial community structure of internal fungi of even the most common tree species is still patchy. Many species described as endophytes may simply be parasites tolerated by the host tree while other species can be regarded as opportunistic or latent pathogens, and some fungi, endophytic in one plant species, can be pathogenic to

other plant species (Carroll 1988; Ganley et al. 2004; Slippers & Wingfield 2007). Some of them can also be primary decomposers waiting inactively for senescence of the tissue they have colonized (Müller et al. 2001). A number of fungal species described as endophytes on various plants may actually be insignificant to their host when occurring only as ungerminated spores which have survived surface sterilization procedures, for instance in a stomatal cavity (Schulz & Boyle 2005).

A typical trait of endophytic fungi is their common ability to produce biologically active compounds in tests for fungicidal, antibacterial, and herbicidal activities (Schulz et al. 2002). On the other hand, plants control their internal fungi and the relation has been described as a delicately balanced association where the host not only tolerates all the secondary metabolites excreted by the endophyte but retards growth of the invader without eradicating it (Schulz et al. 2002). When this balance is disturbed by either a decrease in plant defense or a rise in fungal virulence, disease develops.

The significance of the vast majority of fungal endophytes to their host trees is so far unknown. However, in some cases an endophytic fungus has been shown to promote host fitness, for instance by decreasing the palatability of the host tissue to herbivores through the production of toxic metabolites (Carroll 1988; Miller et al. 2008), acting as antagonists against pathogens (Webber 1981; Arnold et al. 2003) or inducing endophyte-mediated resistance against pathogens (Ganley et al. 2008). In non-woody plants several endophytic fungi have shown the ability to increase plant growth as well as stress tolerance (Ernst et al. 2003; Gasoni & De Gurfinkel 1997; Mucciarelli et al. 2003; Rademacher 1994). In some studies the inoculation of woody plants with endophytic fungi has increased growth of the host. Binucleate *Rhizoctonia* strains enhanced considerably root growth of Scots pine and Norway spruce (*Picea abies*) seedlings (Gronbeg et al. 2006). Also *Trichoderma harzianum* and *Laccaria laccata* when inoculated to blue pine seedling roots improved growth and biomass of the host (Ahangar et al. 2012). Moreover, dark septated root endophytes in Scots pine seedlings growing under elevated CO_2 concentrations produced an increase of nutrient use efficiency and a slight carbon gain (Alberton et al. 2009). Interestingly, also the diversity of microbes living in tree organs may be related to the growth rate of their host tree as shown by Korkama et al. (2006) who found higher species richness and diversity of ectomycorrhizal infections in roots of fast than in slow growing clones of Norway spruce.

Some fungal and bacterial species associated to plant tissues are able to produce plant growth regulators (Rim et al. 2005; Hamayun et al. 2009a; Hamayun et al. 2009b; Ahmad et al. 2010; You 2012; Kang et al. 2014). Production of plant growth regulators can proceed jointly by both the host and the endophyte. Cottonwood (*Populus trichocarpa*) harbours yeasts which are able to enhance seedling growth by producing indol acetic acid, IAA, a plant growth regulator. For producing IAA, the yeast needs L-tryptophan, provided by the host (Furukawa et al. 1996).

The first step to investigate the interaction of endophytes with a certain host species is to find out what endophytic species thrive in various tissues and how abundant they are. Variation of the endophytic community between host individuals and different environments may give clues on the interaction between the host species and its various inhabitants. The aim of this study was 1) to characterize the endophytic community of *Pinus sylvestris* twigs, and 2) to find out if the structure of this community is related to the growth rate of Scots pine. *Pinus sylvestris* was chosen because of its high ecologic and economic significance in European forests. We tested the hypotheses that 1) Scots pine twigs inhabit a number of culturable fungal species not previously described in this host species and that 2) the endophytic community of fast and slow growing trees differs.

Materials and methods

Study area and sampling method

The study was carried out in 2005 at Palencia province (northern Spain). The study area is located between UTM coordinates 4.685.000 to 4.741.000 (latitude) and 342.000 to 398.000 (longitude), where the altitude ranges from 800 to 1000 m.a.s.l., the climate is Mediterranean with a slight Atlantic influence, mean temperature is 11.5 °C and annual rainfall 519 mm. This area represents a transition from agricultural lands (southwards) to Cantabric Mountains (northwards), where forest lands cover 32 % of the surface, consisting of conifer, broadleaved, and mixed forests including riparian sites, and shrublands (Sanz-Ros et al. 2008).

Fifteen circular Scots pine plots (Ø 15 m) were selected from the Spanish National Forest Inventory grid (NFI) (Fig 1). The selected Scots pine stands were uniform in age and height (20–32 y and 7.4–13.0 m, respectively) but varied in basal area (9.13–30.3 m2ha^{-1}) and density (187–1368 trees ha^{-1}). Stand characteristics are presented in Table 1.

For twig sampling two healthy trees were randomly chosen from each plot, thus the intensity of the sampling was 1 tree90 m^{-2}. Tree growth rate and age were measured from increment cores taken with a Pressler borer at 1.3 m height, mounted, sanded, and analysed with the WinDendro® scanning software (Regent Instruments Canada Inc). Scanning results were used for calculation of tree diameter (without bark) (D), age of the tree, diametric growth rate (DGR), and relative diametric growth rate (RDGR), as shown in equations 1 to 3. Since the endophyte community is not likely to be influenced solely by the current year growth, both growth variables were calculated also for the last 5, 10, and 15 y before this study (Table 2). Equations for applied variables are as follows:

$$D_i = \left(\sum_1^n Tree\ ring\ width \right) \Big/ N \tag{1}$$

$$DGR_i = (D_n - D_i)/n - i \tag{2}$$

$$RDGR_i = (D_n - D_i)/D_i \tag{3}$$

where n is the tree age in years, N is the number of trees, and i represents a reference age of the tree n−i years ago.

Four healthy twigs with a diameter of 0.2–1.5 cm were cut from each sampling tree from the external part of the crown at 3–4 m above ground level during summer 2005. Each twig was divided with a sterilized knife into 1.5 cm segments, and three of them were randomly chosen for fungal isolation, resulting
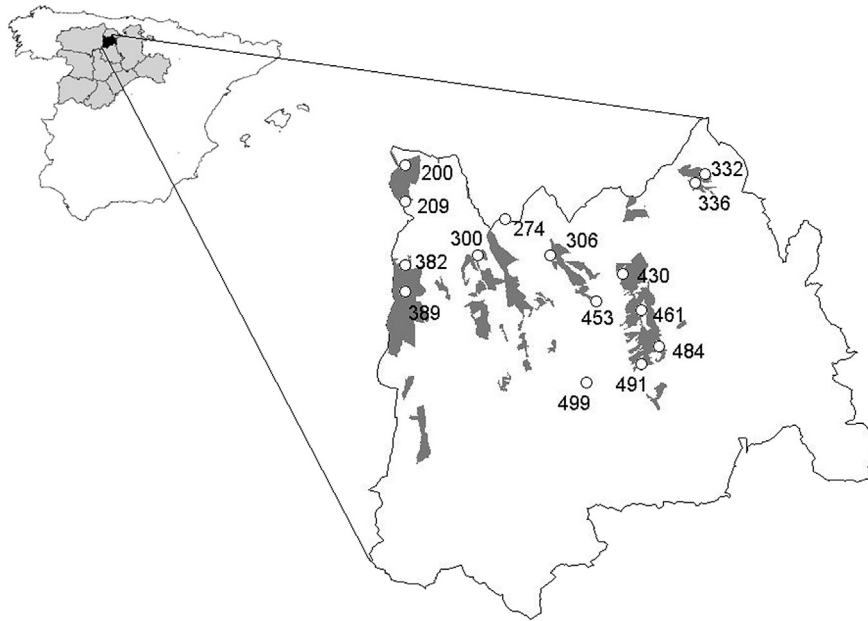
Fig 1 — **Location of the study area and sampling plots (O). In the left Spain and Balear Islands are represented, Castilla y León Region is showed in grey and the study area in black, located at Palencia Province. In the right grey areas represent *Pinus sylvestris* distribution, and numbers are Spanish NFI plot codes.**

in 12 segments per tree. Twigs were stored at 4 °C and processed within the next 48 h. Summarizing, from each plot eight different twigs were selected from two different trees, of which 24 segments were randomly chosen for fungal isolation, making a total of 360 segments obtained from 30 different trees.

Isolation and morphogrouping of the isolates

Twig segments were surface sterilized by shaking them for 1 min in 96 % ethanol, 5 min in 6 % sodium hypochlorite,

1 min in 70 % ethanol, and finally two times for 2 min in sterile distilled water. Thereafter the segments were placed on Petri dishes of 9 cm diameter (four segments per plate) containing a generalist medium for fungal culture, potato dextrose agar (PDA; Scharlau®), previously autoclaved for 20 min at 121 °C. Cultures were incubated in darkness at 23 °C.

After 4 d all outgrowing fungi were transferred by taking a ca. 9 mm² piece of agar from the edge of each colony to fresh medium, and during 1 m a weekly check was carried out in order to find new colonies. Fungal isolates were counted and stored at 4 °C. Finally, assemblages were grouped according

Table 1 — **Mean stand characteristics of the Scots pine sampling plots and properties of the endophytic fungal community isolated from Scots pine twigs.**

	Scots pine stand characteristics					Endophytic fungi of Scots pine twigs				
Plot	Species[a] composition	Age (y)	Height (m)	Basal area (m² ha⁻¹)	Density (trees ha⁻¹)	Species richness	Simpson diversity	Simpson evenness	Shannon diversity	Shannon evenness
P200	Pure Ps	27	12.2	34.5	987	12	9.00	0.75	4.67	1.88
P209	Ps, Pp, Pn	32	11	12.9	249	16	10.24	0.64	5.16	1.86
P274	Ps, Pn	20	8.9	12.3	516	7	5.83	0.83	3.69	1.90
P300	Ps, Pn	26	11.6	30.3	1022	11	6.19	0.56	4.21	1.75
P306	Ps, Qp	30	9.3	10.4	1085	13	7.52	0.58	4.56	1.78
P332	Ps, Pn, Qp	25	10	21.5	704	4	1.94	0.49	1.87	1.35
P336	Ps, Pn	31	13	24.6	690	13	6.03	0.46	4.29	1.67
P382	Ps, Qp, Pn	32	10.6	21.5	1093	7	6.00	0.86	3.73	1.92
P389	Pure Ps	32.5	7.4	20.1	1368	8	5.48	0.69	3.56	1.71
P430	Ps, Qp	29	11.2	18.6	810	9	7.72	0.86	4.21	1.91
P453	Ps, Pn	33.5	12.3	26.6	679	7	3.59	0.51	3.07	1.58
P461	Pure Ps	22	9.6	9.1	187	13	9.00	0.69	4.74	1.85
P484	Ps, Pn	28	12	27.6	704	8	5.19	0.65	3.21	1.54
P491	Ps, Pn	26.5	10.6	21	584	10	5.42	0.54	3.93	1.71
P499	Ps, Pn	25	8.4	23.4	1206	8	6.37	0.80	3.92	1.88

a Tree species are showed by its dominance in each plot. Ps = *Pinus sylvestris*; Pn = *Pinus nigra*; Pp = *Pinus pinaster*; Qp = *Quercus pyrenaica*.

Table 2 – Fungal endophytic community characteristics and Scots pine growth parameters.

Variable	Units	Description
Richness	Number of species	Number of endophytic species
DSim	–	Simpson's diversity index
ESim	–	Simpson's evenness index
DSha	–	Shannon's diversity index
ESha	–	Shannon's equitability index
Age	y	Averaged plot age
D5	mm	Tree diameter 5 y ago (without bark)
D10	mm	Tree diameter 10 y ago (without bark)
D15	mm	Tree diameter 15 y ago (without bark)
DGR5	mm y^{-1}	Diametric growth rate of the last 5 y period
DGR10	mm y^{-1}	Diametric growth rate of the last 10 y period
DGR15	mm y^{-1}	Diametric growth rate of the last 15 y period
RDGR5	mm y^{-1}	Relative diametric growth rate of the last 5 y
RDGR10	mm y^{-1}	Relative diametric growth rate of the last 10 y
RDGR15	mm y^{-1}	Relative diametric growth rate of the last 15 y
DGR5cat	–	Categorized DGR5
DGR10cat	–	Categorized DGR10
DGR15cat	–	Categorized DGR15
RDGR5cat	–	Categorized RDGR5
RDGR10cat	–	Categorized RDGR10
RDGR15cat	–	Categorized RDGR15
Density	Trees ha^{-1}	Plot density of trees per hectare
BA	m^2 ha^{-1}	Basal area of plot
MH	meters	Mean height of plot
DH	meters	Dominant height of plot

to colony morphology on PDA, and they are called here as 'colonial morphotypes' (CMs). Seventy eight CMs were distinguished based on colony colour, size, texture, and presence of aerial hyphae (Wang et al. 2005).

The relative abundance (RA) of each fungal taxon (i.e. species identified by molecular means) is calculated as the number of fungal isolates expressed as percents of the total number of twig segments investigated from a plot.

Identification of isolates

The CMs were identified by molecular methods as follows. One isolate of each CM was transferred to a cellophane membrane (Surface Specialities, Wigton Cumbria, UK) on MOS-agar (Müller et al. 1994), and cultivated at 19 °C in darkness for a week. Total DNA was isolated according to Vainio et al. (1998) including minor modifications. The protocol included I) cell disruption with quartz sand using a FastPrep® cell disrupter (Qbiogene, Inc., Cedex, France) for 2 × 20 s, II) two or three phenol:chloroform:isoamyl alcohol (25:24:1) and one chloroform:isoamyl alcohol (24:1) extraction (a third extraction with phenol:chloroform:isoamyl alcohol was done when dark pigments still existed in the extract after two extractions), III) precipitation with polyethylene glycol (PEG) and drying, and IV) resuspension of the DNA in 50 μl of TE-buffer (pH 8.0), containing 10 mM Tris–HCl and 1 mM ethylenediaminetetraacetic acid (EDTA).

The internal transcribed spacer (ITS) region of the rDNA was amplified with primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) according to White et al. (1990) and Vainio & Hantula (2000). Shortly, 1 μM of each primer, 0.4 mM of each deoxynucleotide (dNTP), 1 U of Dynazyme II

Taq polymerase II (Finnzymes Ltd, Espoo, Finland), 1× Dyna-buffer and 1 μl of template were mixed to 50 μl and subjected to a thermal program consisting of 10 min at 95 °C, followed by 13 cycles at 95 °C for 35 s (s), at 55 °C for 55 s and at 72 °C for 45 s, 13 cycles at 95 °C for 35 s, at 55 °C for 55 s, and at 72 °C for 2 min, and finally 13 cycles at 95 °C for 35 s, at 55 °C for 55 s, and at 72 °C for 3 min. PCR products were purified with the Jet Quick PCR Purification Spin Kit (Genomed, Löhne, Germany), according to the manufacturer's instructions.

DNA samples were sequenced with the automated sequencing apparatus (Li-Cor Global Edition IR2 System; Li-Cor Inc., Lincoln, NE, USA), using the Therm EXCEL™ II DNA Sequencing Kit-LC (Epicentre®; Madison, WI, USA) and following the manufacturer's instructions. Finally, the sequences were aligned with LI-COR software (ALIGN IR version 2.0). Consensus sequences were identified by blasting them against sequences deposited in GenBank (NCBI) database (Benson et al. 2002). The fungal assemblages were named according to the best GenBank match if at least 97.2 % matched with a sequence consisting of at least 393 bp. Sequences generated in this study have been submitted to GenBank (Table 3).

A maximum parsimony tree was constructed from the sequences in order to reveal the phylogenetic relationships of the isolated endophytes. The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei & Kumar 2000) with search level 3, in which the initial trees were obtained with the random addition of sequences (ten replicates). The tree is drawn to scale, with branch lengths

calculated using the average pathway method (Nei & Kumar 2000) and are in the units of the number of changes over the whole sequence. All alignment gaps were treated as missing data. There were a total of 642 positions in the final dataset, out of which 418 were parsimony informative.

For *Biscogniauxia mediterranea* sequences comparison, a Neighbor-Joining tree was built. The bootstrap consensus tree was inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Nei & Kumar 2000) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 641 positions in the final dataset. All Phylogenetic analyses were conducted in MEGA 4 (Tamura *et al.* 2007).

Table 3 − **Fungal endophytic taxa isolated from *Pinus sylvestris* twigs and identified according to BLAST of their ITS-sequences. The isolates are listed according to decreasing relative abundance (RA).**

Fungal taxon[a]	Short name	Class	Order	AN[b]	QC[c]	MI[d]	RA[e]
Phoma herbarum Westend.	PhoHer	Dothideomycetes	Pleosporales	JX421706	100	99.1	36
Hormonema dematioides Lagerb. & Melin	HorDem	Dothideomycetes	Dothideales	JX421710	99.3	99.5	34
Alternaria infectoria E.G. Simmons	AltInf	Dothideomycetes	Pleosporales	JX421701	100	98.2	31
Biscogniauxia mediterranea (De Not.) Kuntze	BisMed	Sordariomycetes	Xylariales	JX421711	100	99.3	26
Hypocrea lixii Pat.	HypLix	Sordariomycetes	Hypocreales	JX421742	100	99.5	20
Leotiomycetes sp.	Leosp	Leotiomycetes	Unknown	JX421713	83.1	98	20
Alternaria alternata (Fr.) Keissl.	AltAlt	Dothideomycetes	Pleosporales	JX421734	100	100	20
Penicillium glabrum (Wehmer) Westling	PenGla	Eurotiomycetes	Eurotiales	JX421729	99.3	99.1	18
Trichoderma atroviride P. Karst	TriAtr	Sordariomycetes	Hypocreales	JX421707	99.6	99.7	17
Pezicula eucrita (P. Karst.)	PezEuc	Leotiomycetes	Helotiales	JX421708	99.8	100	16
Fusarium verticillioides (Sacc.) Nirenberg	FusVer	Sordariomycetes	Hypocreales	JX421719	99	99	12
Sordaria fimicola (Roberge ex Desm.) Ces. & De Not	SorFim	Sordariomycetes	Sordariales	JX421743	100	99.7	11
Phaeomoniella effusa Damm & Crous	PhaEff	Chaetothyriomycetes	Chaetothyriales	JX421744	87.3	99.4	9
Aureobasidium pullulans (De Bary) G. Arnaud ex Cif., Ribaldi & Corte	AurPul	Dothideomycetes	Dothideales	JX421738	100	100	8
Beauveria bassiana (Bals.-Criv.) Vuill.	BeaBas	Sordariomycetes	Hypocreales	JX421709	99.8	99.5	6
Phoma macrostoma Mont.	PjoMac	Dothideomycetes	Pleosporales	JX421747	100	99.1	6
Geomyces sp.	GeoPan	Leotiomycetes	Not assigned	JX421704	90.6	100	6
Ophiostoma nigrocarpum (R.W. Davidson) de Hoog	OphNig	Sordariomycetes	Ophiostomatales	JX421721	100	100	5
Unidentified CM 64	UnkH64	—	—	—	—	—	5
Unidentified CM 66	UnkH66	—	—	—	—	—	5
Hypocrea viridescens Jaklitsch & Samuels	HypVir	Sordariomycetes	Hypocreales	JX421750	100	99.3	4
Fimetariella rabenhorstii (Niessl) N. Lundq.	FimRab	Sordariomycetes	Sordariales	JX421715	99.6	99.8	4
Sarocladium strictum (W. Gams) Summerbell	SarStr	Sordariomycetes	Hypocreales	JX421705	98.6	99.5	4
Lophodermium conigenum (Brunaud) Hilitzer	LopCon	Leotiomycetes	Rhytismatales	JX421746	100	97.2	4
Sarea resinae (Fr.) Kuntze	SarRes	Lecanoromycetes	Agyriales	JX421720	95.1	99.2	4
Unidentified CM 80	UnkH80	—	—	—	—	—	4
Trichoderma viride Pers.	TriVir	Sordariomycetes	Hypocreales	JX421740	100	99.1	3
Penicillium melinii Thom	PenMel	Eurotiomycetes	Eurotiales	JX421739	100	100	3
Daldinia fissa Lloyd	DalFis	Sordariomycetes	Xylariales	JX421716	98.9	98.9	2
Alternaria tenuissima (Nees) Wiltshire	AltTen	Dothideomycetes	Pleosporales	JX421745	100	99.8	2
Unidentified CM 77	UnkH77	—	—	—	—	—	2
Xylaria sp.	Xylsp1	Sordariomycetes	Xylariales	JX421748	100	98.4	1
Nigrospora oryzae (Berk. & Broome) Petch	NigOry	Sordariomycetes	Trichosphaeriales	JX421751	100	99.8	1
Plectania milleri Paden & Tylutki	PleMil	Pezizomycetes	Pezizales	JX421749	99.7	98	1
Penicillium polonicum K.M. Zalessky	PenPol	Eurotiomycetes	Eurotiales	JX421702	100	100	1
Penicillium minioluteum Dierckx	PenMin	Eurotiomycetes	Eurotiales	JX421741	100	99.8	1
Aspergillus fumigatus Fresen.	AspFum	Eurotiomycetes	Eurotiales	JX421732	100	99.5	1
Unidentified CM 33	UnkH33	—	—	—	—	—	1
Unidentified CM 82	UnkH82	—	—	—	—	—	1
Unidentified CM 84	UnkH84	—	—	—	—	—	1
Unidentified CM 87	UnkH87	—	—	—	—	—	1
Unidentified CM 88	UnkH88	—	—	—	—	—	1
Unidentified CM 89	UnkH89	—	—	—	—	—	1

a Closest related sequences in GenBank. Fungal species nomenclature follows National Center of Biotechnology information.
b GenBank accession number for ITS rDNA sequences.
c Query coverage.
d Maximum identity.
e Relative abundance.

Statistical analysis

Diversity of endophytes was obtained for each plot by calculation of the Shannon diversity index H (DSha, Eq. 4) and Simpson diversity index D (DSim, Eq. 5). Also species evenness was estimated calculating Shannon equitability index J (ESha, Eq.6) and Simpson equitability index E (ESim, Eq. 7) (Begon *et al.* 2006).

$$H = -\sum_{i=1}^{S} p_i * \ln p_i \qquad (4)$$

$$D = 1 \Big/ \sum_{1}^{S} p_i^2 \qquad (5)$$

$$J = H/\ln S \qquad (6)$$

$$E = \left(1 \Big/ \sum_{1}^{S} p_i^2\right) \Big/ S \qquad (7)$$

where p_i is the proportion of the ith species and S is plot species richness. The species accumulation curves (rarefaction curves) and bootstrap estimates of total richness were inferred using the software EstimateS, version 9 (Colwell 2011).

Sample-based rarefaction curves were computed according to Gotelli & Colwell (2001), and the 95 % confidence intervals according to Colwell *et al.* (2004). For inferred richness several non-parametric estimators were computed: Mao Tau Estimator (Colwell *et al.* 2004), Incidence-based Coverage Estimator of species richness, ICE (Chazdon *et al.* 1998; Chao *et al.* 2000), First and Second order Jackknife richness estimator, Jack 1 and Jack 2 (Burnham & Overton 1978; Smith & van Belle 1984), and Chao 1 and Chao 2 estimators (Chao 1984; 1987).

For multivariate analysis we excluded those fungal taxa which were isolated less than four times. Principal Component analysis (rotated with varimax normalized) was used with the aim of reducing data dimensionality and to test which factors explained best the system variance. Variables included were summarized and described in Table 2.

Canonical correspondence analysis (CCA) was carried out to investigate if endophyte assemblages differed according to diametric growth (D, DGR, and RDGR, see Table 2) and age. The statistical significance was tested by means of a Monte Carlo permutation test with 499 permutations of total inertia. Statistical analyses were carried out using the software Canoco 4.5 (Ter Braak & Šmilauer 2002) and Statistica (release 6.0, Tulsa, KA, USA), both software were also used for building the figures included in the results section.

One-way analysis of variance (ANOVA) was used to determine the relationship between the relative abundance of a specific fungal species and the diametric growth rate (DGR) and relative DGR. The variables were categorized into 3 levels (low, medium and high) so that medium level corresponded to mean ± standard deviation, and the rest of values were assigned to low or high. Differences in growth among DGR categories were assessed by using ANOVA and Tukey HSD post-hoc multiple range comparison test.

Alignment of multiple DNA sequences and cluster analyses were done with Mega 4.0 software (Center for Evolutionary Functional Genomics, Biodesign Institute, Arizona State University). DNA alignments were submitted to TreeBase, submission number 16763.

Results

Fungal isolates were obtained from all investigated twigs. A total of 360 isolates were assigned into 77 different CMs and one representative of each CM was characterized by ITS sequencing. Ten CMs could not be identified due to their stunted growth and death or in a few cases due to bacterial contamination. The number of these unidentified isolates represented 6 % of all isolates. In average 2.3 colonial morphotypes represented one fungal taxon as 33 species were identified by blasting the ITS sequences in GenBank (Table 3). All identified species were Ascomycetes. Summarizing, 43 taxa were counted (including 33 ITS-sequences and 10 unknown CM groups). Sequences were included in a maximum parsimony tree to reveal their grouping into taxonomic units (Fig 2).

The most frequently isolated class was Dothideomycetes (43.1 % of total number of isolates) followed by Sordariomycetes (32.1 %), Eurotiomycetes (7.7 %), Leotiomycetes (7.1 %), Chaetothyriomycetes (2.5 %), Lecanoromycetes (1.1 %), and Pezizomycetes (0.3 %) (Fig 3).

The most frequently isolated species were *Phoma herbarum*, followed by *Hormonema dematioides*, *Alternaria infectoria*, *Biscogniauxia mediterranea*, *Hypocrea lixii*, *Leotiomycetes* sp, *Alternaria alternata*, and *Penicillium glabrum*. These species were found in 27—60 % of plots (i.e. in 4—9 of the 15 plots), and they represented 63 % of the total number of isolates obtained. The number of fungal taxa obtained from individual trees ranged from 3 to 13, and plots from 4 to 16. From the 43 fungal taxa, 18 appeared solely in one plot and were named as 'rare fungi', representing 41.9 % of all fungal taxa, but just 7.7 % of the 360 fungal isolates obtained. The 25 remaining taxa were referred to as 'frequent fungi' and they represented 58.1 % of all found taxa and 92.3 % of total fungal isolates, and were used for multivariate analysis. Rare taxa in each plot ranged from 0 to 5, and after removing them, the number of frequent taxa per plot still ranged from 3 to 13.

The Class showing the highest percentage of fungal species was I) Sordariomycetes: including orders Hypocreales, Ophiostomatales, Sordariales, Trichosphaeriales, and Xylariales. The second most numerous class was II) Dothideomycetes: including the orders Pleosporales and Dothideales, followed by Class III) Eurotiomycetes: all belonging to the Order Eurotiales, and class IV) Leotiomycetes: including orders Helotiales, Rhytismatales, and Leotiomycetes Incertae Sedis. Finally, the three less frequent classes were V) Chaetothyriomycetes, VI) Lecanoromycetes, and VII) Pezizomycetes, including orders Chaetothyriales, Agyriales, and Pezizales respectively.

The taxon accumulation curve (Fig 4) showed that our sampling intensity provided a reasonable estimate of fungal endophytes richness of the twigs of *Pinus sylvestris* in the study area. The actual species number was estimated to be 63, 67, 73, 76, and 90 using Jack 1, ICE, Chao 1, Jack 2, and Chao 2 estimates respectively. This means that the 43 taxa found here represent ca. half of the species richness actually present.
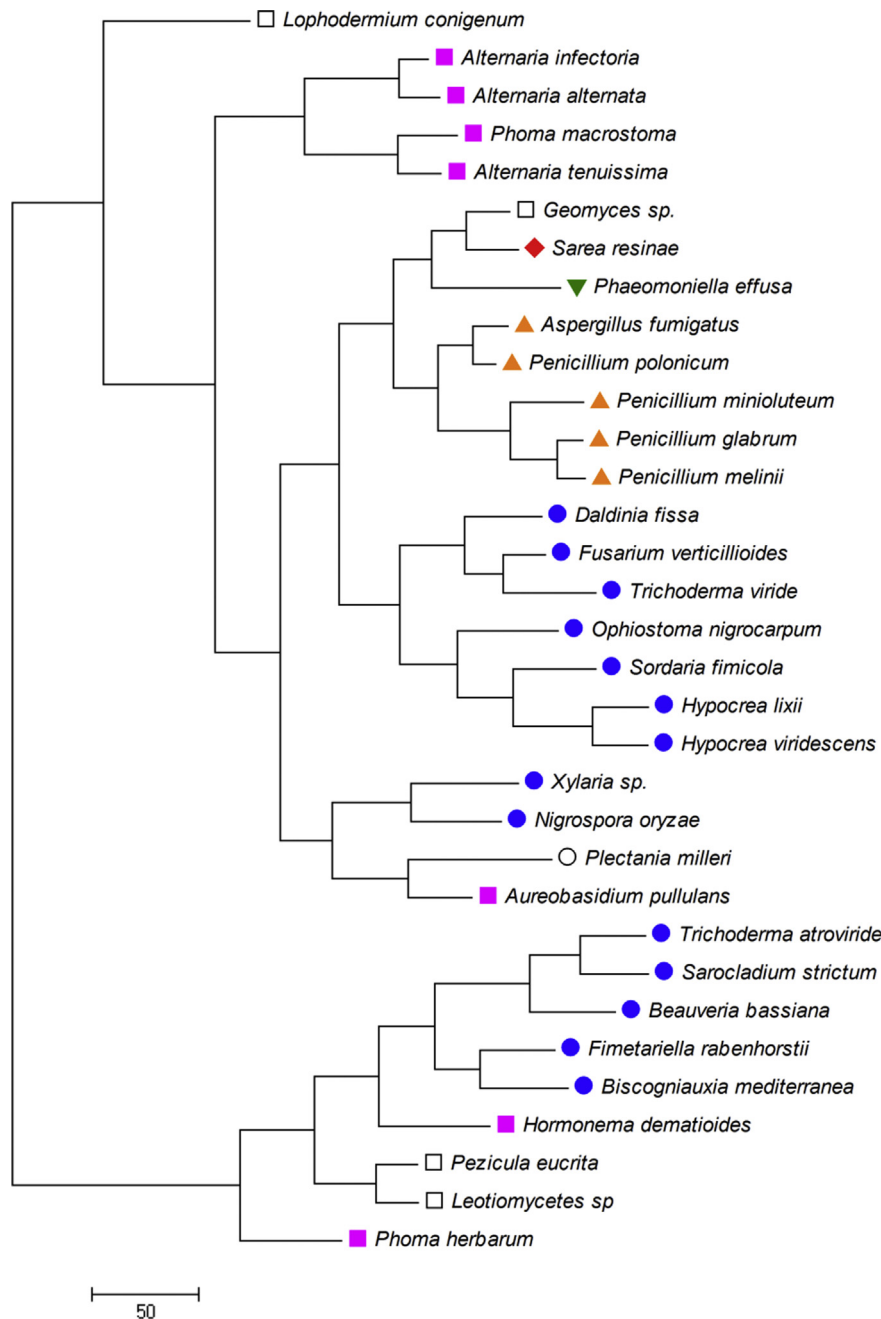
Fig 2 – Maximum parsimony tree of the 33 different ITS-sequences found among all 360 endophytic isolates. Their best GenBank match is given and taxonomic class denoted by symbols (● = Sordariomycetes; ■ = Dothideomycetes; ▲ = Eurotiomycetes: ▼ = Chaetothyriomycetes; □ = Leotiomycetes: ◆ = Lecanoromycetes; ○ = Pezizomycetes). Branch lengths are given as numbers of changes over the whole sequence (scale given below the tree).

Fungal diversity and evenness varied largely between the sampling plots (Table 1). The Shannon diversity index (DSha) ranged from 1.87 to 5.16 and Simpson diversity index (DSim) from 1.94 to 10.24. Evenness varied in the range 1.35–1.92 (Shannon's Equitability index, ESha) and 0.46–0.86 (Simpson's Equitability index, ESim).

A principal component analysis (PCA) including endophyte isolation frequencies and tree characteristics in each plot revealed a relation between tree growth and the endophytic

community structure. PCA yielded three factors (Fig 5) which accounted 69 % of total variance. The first principal component (explaining 30.4 % of variance) separates accumulated growth (D5, D10, and D15) from tree relative growth rate variables (RDGR5, RDGR10, and RDGR15). The second principal component (explaining 22.9 % of variance) separates plot DGR variables (DGR15, DGR10, and DGR5) from variables of fungal evenness (ESim). Factor 1 in Fig 5 suggests a correlation between diversity (Richness, Dsha, and Dsim) and relative
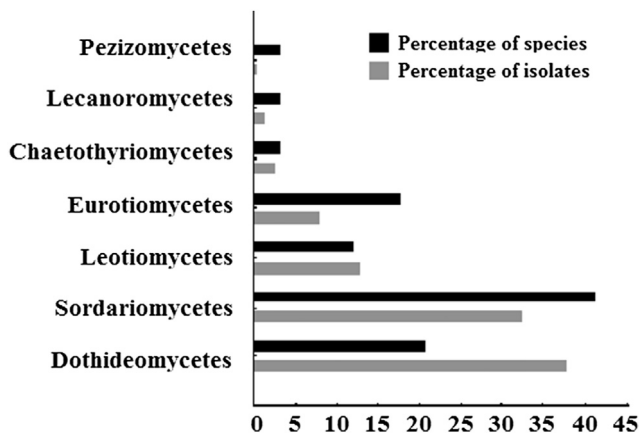
Fig 3 – Characterization of Classes, showing relative abundance of total isolates collected orders and species of each class within Phylum Ascomycota, Subphylum Pezizomycotina.

diametric growth (RDGR) or negative correlation with tree size (D). In regression analyses, however, no significant relationships were found between fungal diversity and tree growth, size, basal area or tree density. Factor 2 suggests that trees with high DGR harbour a higher proportion of dominating fungal species (low evenness, Esha, and Esim) compared to trees with low DGR, in which the abundance of fungal species is more even. These results were supported by regression analysis between evenness and DGR ($p < 0.05$). On the other hand, regression analyses did not showed significant relationships between fungal evenness and diameter, basal area or tree density.

The interrelationship between host diametric growth and the endophytic community structure of twigs is also revealed by a CCA. Monte Carlo permutation test revealed significant
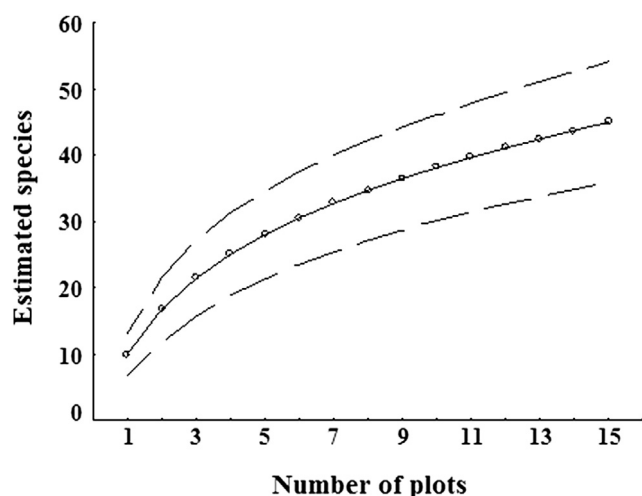

Fig 4 – Taxon accumulation curve of fungal endophyte species as a function of the number of P. sylvestris plots sampled. Solid line: number of fungal taxa calculated by the Mao Tau estimator. Dashed lines: upper and lower 95 % confidence limits of the estimate of taxa number. The curve is based on 50 randomizations of sample order.
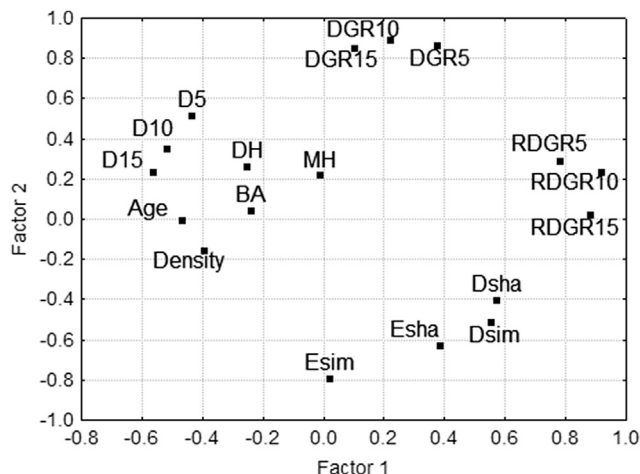

Fig 5 – Relation between twig endophyte community characteristics and host tree growth features as revealed by principal component analysis. Host tree variables: BA: Basal area; MH = Mean height; DH = Dominat height; D5, D10, and D15 = Plot average stem diameter 5.10, and 15 y ago, respectively; DGR5, DGR10, and DGR15 = Plot average annual diametric growth during last 5, 10, and 15 y, respectively; RDGR5, RDGR10, and RDGR15 = Plot relative DGR during last 5, 10, and 15 y, respectively. Endophyte community variables: Richness = number of fungal taxa/plot; DSha = Shannon's diversity index; DSim = Simpson's diversity index; ESha = Shannon's equitability index; ESim = Simpson's equitability index.

relationships between DGR of the host tree and the presence of certain endophyte species, some of which show a trend to be associated to high growth (Fig 6, lower circle cluster) and other to low growth (Fig 6, upper circle cluster).

The presence of three endophytic species in twigs correlated significantly with DGR15 of the host tree. Both *Fimetariella rabenhorstii* and *Phoma herbarum* occurred more frequently in fast growing trees ($p = 0.008$ and $p = 0.051$, respectively, ANOVA). However, only four isolates of *F. rabenhorstii* (Table 2) were encountered and therefore this result should be verified with a larger dataset. On the other hand, *H. lixii* was significantly associated ($p = 0.014$) to trees with low DGR.

We examined the sequences identified as *B. mediterranea* more closely because this species was one of the most frequent ones in this study (Table 3) and because it is a well known pathogen of oaks and has also been found in some other hosts, such as poplar, ash and apple trees (Fig 7). The twenty six isolates identified as *B. mediterranea* were grouped initially in four CMs, and four ITS-sequences were obtained, differing maximally by 14 base pairs. To visualize the relationship of our sequences with those of isolates occurring on other host species we ran a neighbour joining tree of our sequences together with other ITS-sequences deposited in GenBank (Fig 4). Isolates obtained from Scots pine in this study are closely related to isolates from insects in Portugal, three of the CMs (corresponding to accession numbers JX421711, JX421730, and KP113664) representing 24 isolates, are closely related to those obtained from isolates of insects retrieved
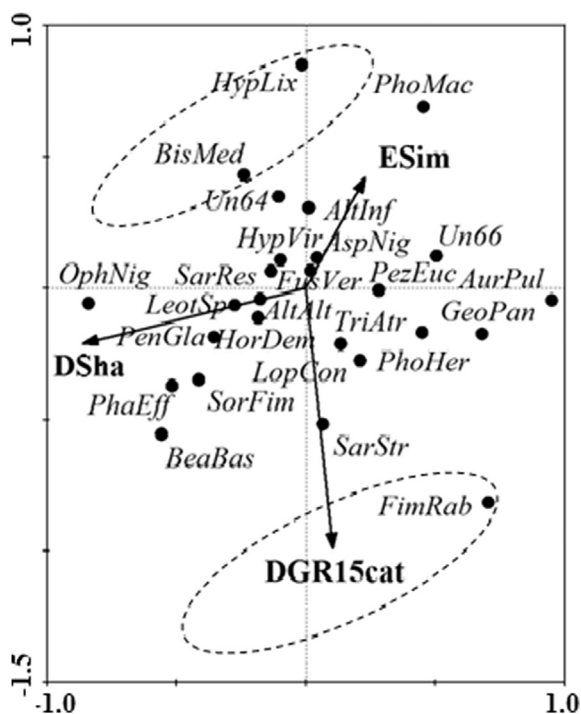
Fig 6 − Biplot between fungal species and plot variables calculated by a CCA using all variables described in Table 2. Black circles and abbreviated names in italics represented fungal species (Table 3), and vectors with bold names represented the following plot variables: **DSha** = Shannon's diversity index; **DGR15** = Diametric growth rate of the trees (categorized); **ESim** = Simpson's equitability. Dash line ellipses indicate fungal endophytes associated to high diametric growth (lower ellipse) or low diametric growth (upper ellipse).

from a documents archive in Portugal and from *Quercus castaneifolia* forest in Iran, while the fourth CM (KP113665) is more related to isolates obtained from *Populus x euramericana* in Spain, but different to the other three CMs.

Discussion

A diverse fungal community including forty three different fungal endophytic taxa was found inhabiting twigs of healthy Scots pines in northern Spain. We found nine new fungal species not earlier described as endophytes of *Pinus sylvestris* supporting our first hypothesis. Species composition showed differences between fast and slow growing pines and some of the species were found to be associated to tree diametric growth. Hence, both of our initial hypotheses were supported. Low fungal evenness and presence of *Phoma herbarum* are associated with high diametric growth, while high fungal evenness and presence of *Hypocrea lixii* are related to low diametric growth of their host trees.

The fungi identified in this study that have already been described as Scots pine endophytes in earlier reports were *Alternaria alternata*, *A. infectoria*, *A. tenuissima*, *Aspergillus fumigatus*, *Aureobasidium pullulans*, *Beauveria bassiana*, *Fimatariella*

rabenhorstii, *Fusarium verticillioides*, *Geomyces sp.*, *H. lixii*, *H. viridescens*, *Hormonema dematioides*, *Lophodermium conigenum*, *P. herbarum*, *P. macrostoma*, *Sarocladium strictum*, *Sordaria fimicola*, *Penicillium glabrum*, *P. minioluteum*, *Sarea resinae*, *Trichoderma atroviride*, *T. viride*, and *Xylaria* sp (Cooke 1973; Carroll *et al.* 1977; Russo & Bradley 1979; Diwani & Millar 1987; Land *et al.* 1993; Kubátová 2000; Anderson *et al.* 2003; Pirttilä *et al.* 2003; Lygis *et al.* 2004; Kwasna 2008; Zamora *et al.* 2008; Giordano *et al.* 2009; Peršoh *et al.* 2010; Menkis & Vasaitis 2011; Terhonen *et al.* 2011), while the following species have been found in other pine species: *Daldinia fissa*, *Nigrospora oryzae*, *Ophiostoma nigrocarpum*, *Penicillium melinii*, *P. polonicum*, *Pezicula eucrita* (Smith 1965; Carroll & Carroll 1978; Kowalski & Zych 2002; Guo *et al.* 2003; Kim *et al.* 2005; Wang *et al.* 2005; Zamora *et al.* 2008; Botella & Diez 2010; Kowalski & Andruch 2011).

Hence, 29 out of all 33 fungal taxa (excluding the ten unidentified colonial morphotypes) obtained in this study have been earlier reported from pines, while 22 out of 33 were previously reported in Scots pine, so that six species (27.3 % of identified taxa) were reported, to our knowledge, for the first time to inhabit Scots pine: *D. fissa*, *H. viridescens*, *Nigrospora oryzae*, *O. nigrocarpum*, *Phaeomoniella effusa*, *P. melinii*, *P. polonicum*, and *Plectania milleri*. In addition, three species, *B. mediterranea*, *P. effusa*, and *P. milleri* are new endophytic species for the *Pinaceae*.

Fifteen species found here can be regarded as host-generalists because they have been described also from broad-leaved trees: *A. alternata*, *A. tenuissima*, *A. pullulans*, *B. bassiana*, *B. mediterranea*, *F. oxysporum*, *H. lixii*, *H. dematioides*, *N. oryzae*, *P. glabrum*, *S. strictum*, *S. fimicola*, *T. harzianum*, *T. viride*, and *Xylaria* sp. (Chapela 1989; Fisher & Petrini 1992; Bettucci & Alonso 1997; Collado *et al.* 1999; Santamaría & Diez 2005; Gonthier *et al.* 2006; Jaklitsch *et al.* 2006; Unterseher & Schnittler 2010; Martín-García *et al.* 2011). Therefore, half of the taxa inhabiting Scots pine twigs were host-generalists, and the other half were coniferous or pine specialists. In this sense, dominant endophytic species are usually presumed to be host-specialists because each host species usually harbours a characteristic assemblage of endophytic fungi (Saikkonen 2007).

Regarding fungal classes distribution, our result is in accordance with earlier studies (Zamora *et al.* 2008; Botella & Diez 2010) showing that the fungal community of the canopy of several pine species in northern Spain is dominated by *Ascomycetes* and more specifically, by the classes Dothideomycetes and Sordariomycetes, which represented 46 % and 34 % of total isolated fungi. Studies on pine species from other countries also showed them as the more frequently isolated classes (Ganley & Newcombe 2006; Arnold *et al.* 2007).

The species richness and diversity found in this study for twig endophytes of *Pinus sylvestris* is similar to those obtained in previous studies on pine twigs (Table 4) (Zamora *et al.* 2008; Botella & Diez 2010; Martínez-Álvarez *et al.* 2012), and higher than in pine needles or cones (Ganley & Newcombe 2006; Arnold *et al.* 2007; Peršoh *et al.* 2010), but lower than those obtained from roots or sapwood (Giordano *et al.* 2009; Menkis & Vasaitis 2011).

The cumulative curves for species richness (Fig 4) show that more species would have been found if more samples
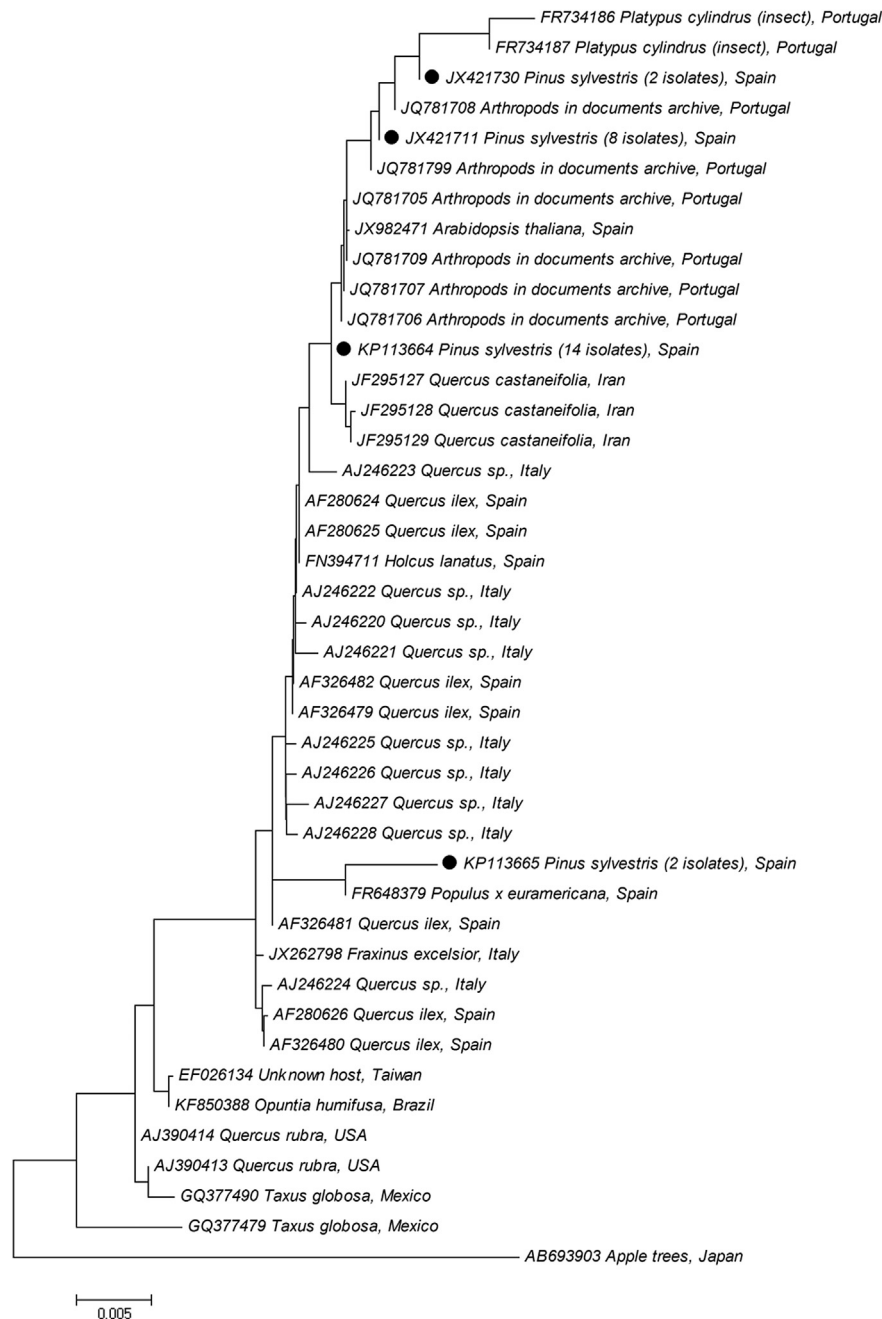
Fig 7 — **Neighbour joining tree (Bootstrap 1000 replicates) of *Biscogniauxia mediterranea* ITS sequences deposited in GenBank. Four of them were obtained in this study from pine and are indicated with a black dot (●). Each of them represents one colony morphotype and the number of total isolates is given in parenthesis. The branch lengths refer to the number of base substitutions per site (scale given below the tree).**

had been taken, but in this context the dominant and abundantly occurring species are of greatest interest because they are more likely to be host-specialists (Saikkonen 2007). Even if half of the actual species remained undetected we can assume to have encountered most dominant species culturable on PDA because the majority of fungal species in healthy trees of natural environments generally occur at very low numbers and just a few are frequent (Müller & Hallaksela 1998; Ragazzi *et al.* 2003; Saikkonen 2007;

Linaldeddu *et al.* 2010). The isolation method used in this study favours medium and fast growing fungi, and thus, some of the slow growing species may have remained undetected, as well as all species not culturable on PDA. The high proportion of successful isolations (i.e. isolates were obtained from every twig segment plated) and high number of species found (33 different sequences from 360 isolates) suggest that at least some of the twig segments may have inhabited more than one species. Therefore, the species richness and diversity

Table 4 – **Summary of the number of fungal endophytes identified in previous studies in different forest tree species, obtained from different organs/tissues.**

Tree species	Number of taxa	Tissue	Location	References
Quercus cerris	15	Bud, twigs, and leaves	Northern Italy	Ragazzi *et al.* 2001
Fagus sylvatica	17	Branches	SW Britain and Southern France	Chapela & Boddy 1988
Pinus monticola	21	Needles and cones	USA	Ganley & Newcombe 2006
Pinus taeda	23	Needles	Eastern USA	Arnold *et al.* 2007
Pinus sylvestris and *Viscum album*	25	Needles and stem	Germany	Peršoh *et al.* 2010
Quercus robur	25	Shoots, twigs, and sapwood	Northern Italy	Gonthier *et al.* 2006
Quercus cerris, Q. pubescens, and *Q. robur*	28	Leaves and twigs	Northern Italy	Ragazzi *et al.* 2003
Pinus nigra, P. pinaster, P. sylvestris, and *P. uncinata*	45	Needles and twigs	Northern Spain	Zamora *et al.* 2008
Populus tremula	48	Leaves, twigs, and bark	Northern Spain	Santamaría & Diez 2005
Pinus nigra	49	Shoots	Southern Poland	Kowalski & Zych 2002
Eucalyptus grandis	52	Twigs	North Uruguay	Bettucci & Alonso 1997
Fagus sylvatica and *Pinus sylvestris*	53	Stem and xylem	United Kingdom	Petrini & Fisher 1988
Quercus ilex and *Q. faginea*	54	Leaves, twigs, and bark	Central Spain	Collado *et al.* 2000
Pinus sylvestris	56	Roots (nursery)	Western Lithuania	Menkis & Vasaitis 2011
Pinus nigra, P. pinea, P. sylvestris, Quercus ilex, and *Q. pyrenaica*	59	Leaves and stem (nursery)	Northern Spain	Martín-Pinto *et al.* 2004
Eucalyptus nitens	64	Leaves, branches, and stem	SE Australia and SW Britain	Fisher *et al.* 1993
24 tree species of dry tropical forest	81	Leaves	Southern india	Suryanarayanan *et al.* 2003
Pinus sylvestris, Betula pendula, and *Juniperus communis*	85	Roots	Eastern Lithuania	Lygis *et al.* 2004
Abies alba	116	Needles	SE Poland	Kowalski & Andruch 2011
Pinus sylvestris	143	Sapwood	Western Italy	Giordano *et al.* 2009
Quercus ilex	149	Leaves, twigs, and bark	Central Spain	Collado *et al.* 2000
Heisteria concinna and *Ouratea lucens*	347	Leaves	Panama	Arnold *et al.* 2000

indices found in this investigation must be regarded as minimum estimates. DNA methodology would have revealed a higher number of microbes in our samples but pure cultures of the organisms may be important for further studies on their significance as for instance inoculation studies.

Factor analysis showed that endophytic fungal diversity (Richness, DSim, and DSha) is not significantly correlated to tree growth or tree size (Fig 5), while for mycorrhizal fungi a relationship between mycorrhizal diversity and spruce growth has been found (Korkama *et al.* 2006). Instead, fungal evenness (ESim) correlates negatively with DGR, ($p < 0.05$), suggesting that there are some dominant species occurring more frequently in fast growing trees, while in slow growing trees the fungal species abundance is more even.

Our results suggest that particular species or clusters of them are related to the DGR of *P. sylvestris*. It is not possible based on the present results to speculate if there is any causality between pine growth and the twig endophyte community structure. One possibility is that the availability and/or quality of nutrients usable by fungi differ in slow and fast growing pines, or that tissue structure of fast/slow growing trees represents different niches for fungal growth. However, enhanced growth due to production of plant growth regulators by endophytes has been observed in roots and stems of woody plants (Hietala *et al.* 1994; Gronberg *et al.* 2006; Xin *et al.* 2009). *Phoma herbarum* and other *Phoma* species have been found to be able to produce gibberellins (Rim *et al.* 2007; Hamayun *et al.* 2009), and the presence of *Phoma* spp. has been previously detected

in different pine tissues, such as roots (Menkis & Vasaitis 2011), sapwood (Giordano *et al.* 2009), needles and twigs (Zamora *et al.* 2008). As *P. herbarum* was the most common endophyte in our samples of fast growing pines, further research on the mechanism of its influence on the host would be justified.

Hypocrea lixii (anamorph *Trichoderma harzianum*) is a cosmopolitan species and a well known mycoparasite (Chet & Inbar 1994) that has been found to promote growth of pine seedlings when inoculated together with the mycorrhizal *Laccaria laccata* (Ahangar *et al.* 2012). However, our results show an association of this species with low growth of pines, which is in accordance with previous studies describing decrease in growth of Scots pine trees infected with *H. lixii* (Giordano *et al.* 2009). Different genotypes of this species may have a different effect on host growth, or perhaps biotic interactions with other organisms are explaining its association to low growing trees.

The presence of some well known pathogens, such as *F. oxysporum*, *F. verticillioides*, and *Biscogniauxia mediterranea* in healthy tissues of Scots pine is noteworthy. Fungi pathogenic to one host but endophytic in other host tree species are not uncommon (Chapela 1989; Rodríguez *et al.* 2012). *Biscogniauxia mediterranea* is known as the causal agent of charcoal disease on *Quercus* spp. (Jiménez *et al.* 2005), mainly on *Quercus suber* (Muñoz *et al.* 2007), and it has been reported as a pathogen also on *Pistacia vera*, *Juglans* sp. (FAO 2007), and *Persea bombycina* (Dutta *et al.* 2010). Charcoal disease outbreak is often

preceded by a long latent period, i.e. the fungus is able to exist as an endophyte in oak (Vannini *et al.* 2009). Still, its rather common presence (53 % of investigated plots) also in Scots pine twigs as an endophyte appears surprising. *Biscogniauxia mediterranea* may be harmful to Scots pine, as it shows a trend (*p* = 0.06, ANOVA) of occurring more frequently in trees with low DGRs (Fig 6).

Recently, *B. mediterranea* has been reported as an endophyte in twigs of hybrid poplars in Spain (Martín-García *et al.* 2011) and also from grasses (Sánchez *et al.* 2010), so this fungus seems to be able to dwell in a broad range of different host species. According to their ITS sequences most of the isolates obtained from pine (24 out of 26) are more closely related to those obtained from insects (Portugal) and *Quercus castaneifolia* (Iran) than to those obtained from oak forests in Spain (Fig 4). But further investigations are needed to elucidate if they represent the same biological species, and if isolates from different tree species show any host specificity, i.e. can strains dwelling endophytic in pine or poplar be pathogenic to oak? Several *Quercus pyrenaica* stands are spread within the study area, but presence of *B. mediterranea* on oak was not investigated in our study. The significance of *B. mediterranea* to Scots pine deserves further investigations because our study shows that the presence of the fungus is associated with low growth of the pine host (Fig 6), and reduced ring growth is a well known symptom of charcoal canker caused by this fungus on *Quercus* spp. (Vannini *et al.* 1996).

Our results revealed a number of new endophytic species living in *P. sylvestris* and suggest that some of the species are involved in mechanisms affecting host growth. Inoculation studies are needed to disclose possible causal effects by these fungi on host plant growth. Abundant presence of fungi in twigs of *P. sylvestris* as found with the applied culturing method does not exclude the possibility that additional species could be found using other methods.

Acknowledgements

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