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Characterization of juvenile maritime pine (*Pinus pinaster* Ait.) ectomycorrhizal fungal community using morphotyping, direct sequencing and fruitbodies sampling

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Abstract Using ectomycorrhizal root tip morphotyping (anatomical and morphological identification), molecular analysis (internal transcribed spacer region amplification and sequencing), and fruitbody sampling, we assessed diversity and composition of the ectomycorrhizal fungal community colonizing juvenile *Pinus pinaster* Ait. under natural conditions in NW Spain. Overall, we found 15 Basidiomycetes and two Ascomycetes. Members of the family Thelephoraceae represented up to 59.4% of the samples. The most frequent species was *Tomentella sublilacina* followed by *Thelephora terrestris*, *Russula drimeia*, *Suillus bovinus*, and *Paxillus involutus*, while the less frequent were *Pseudotomentella tristis*, *Lactarius subdulcis*, *Russula ochroleuca*, and *Entoloma conferendum*. From October 2007 to June 2008, we sampled 208 sporocarps belonging to seven genera and nine species: *Thelephora terrestris*, *Paxillus involutus*, *Suillus bovinus*, *Xerocomus badius*, *Scleroderma verrucosum*, *Amanita gemmata*, *A. rubescens*, *Amanita* sp., and *Russula* sp. The species belonging to the genus *Amanita*, *X. badius* and *S. verrucosum* were not found on root samples. By comparing our results with a bibliographic review of papers published from 1922 to 2006, we found five genera and six species which have not been previously reported in symbiosis with *P. pinaster*. This is the first time that the diversity of the ectomycorrhizal fungal community associated with *P. pinaster* was investigated using molecular techniques. Considering that only 38% of the genera found by sequencing were found as fruitbodies, we conclude that integrating morphotyping and sporocarps surveys with molecular analysis of ectomycorrhizas is important to documenting the ectomycorrhizal fungus community.

Keywords Ascomycete · Basidiomycete · Ectomycorrhiza · Fungal diversity · ITS

Introduction

Mycorrhizal symbiosis has evolved as a mechanism of survival for plants and fungi, allowing both to survive in areas with poor natural conditions such as depleted soil, extreme temperatures, and periodic droughts (Harley and Smith 1983; Molina et al. 1992). Root colonization by mycorrhizal fungi affects carbohydrate flow from leaves to roots, promotes the mobilization of soil phosphorous and nitrogen, increases water uptake, contributes to the aggregation and stabilization of soil particles, and enhances plant tolerance to salinity, herbivory, root pathogens and toxic heavy metals (Ingham and Molina 1991; Smith and Read 1997; Bogeat-Triboulot et al. 2004). Ectomycorrhizal (ECM) symbiosis involves approximately 8,000 species of higher plants and 7,000–10,000 fungal species worldwide (Taylor and Alexander 2005). Ectomycorrhizal fungi are functionally important in temperate forest ecosystems and

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play an influential role in forest community dynamics (Smith and Read 1997).

Different methods have been used to determine diversity and structure of ECM fungus communities (Egger 1995; Dahlberg 2001). Currently, studies from North America and Europe confirm that many dominant ECM fungal symbionts, especially members of *Ascomycota*, *Corticiaceae*, and *Thelephoraceae*, do not produce obvious sporocarps aboveground (Gardes and Bruns 1996; Gehring et al. 1998; Jonsson et al. 1999; Dahlberg 2001). Consequently, to count frequency and abundance of sporocarps do not accurately reflect ECM fungal community structure and species diversity.

On the other hand, morphotyping is a well-developed but time-consuming approach to identify ECM fungi colonizing roots. This method involves the description of morphological and structural characteristics of ectomycorrhizae from root tips and, in comparison with sporocarps sampling, allows determination of the host species (Agerer 1987–2002; Massicotte et al. 1999). However, morphotyping has its limitations because: (1) the number of unidentified species is still high (Nylund et al. 1995), (2) the morphology of a particular fungal taxon can change with different hosts and environments (Egger 1995), and (3) an individual taxon may produce different morphologies and similar morphologies may be produced by different taxa (Egger 1995; Wurzburger et al. 2001; Menkis et al. 2005).

The methodological advance with the most impact has been the application of high-resolution molecular tools that allow identification of individual mycorrhizae using polymerase chain reaction (PCR) and the internal transcribed spacer (ITS) of the fungal nuclear ribosomes (Gardes and Bruns 1993; Egger 1995; Gehring et al. 1998; Horton and Bruns 2001; Sakakibara et al. 2002).

Most research on diversity and ecology of the ECM fungus communities in maritime pine (*Pinus pinaster* Ait.) has studied the sexual structures of fungal symbionts (Fernández de Ana Magán and Rodríguez Fernández 2000; Ágreda and Fernández 2003; Martín-Pinto et al. 2006; Fernández-Toirán et al. 2006). Other studies have investigated which species could be associated with *P. pinaster*, using seed infection under controlled conditions, even if these associations are not necessarily found in the field (Pera and Álvarez 1995).

Currently, *P. pinaster* is the most extensively distributed forest species in Galicia (NW Spain). According to the last forest survey, monospecific stands represent 389,489 ha and mixed stands with *Eucalyptus globulus* Labill. or other broad leaved species represent 243,735 ha, which means that 44% of the Galician forested area is covered with *P. pinaster* (Ministerio Medio Ambiente 2006). The success of *P. pinaster* on disturbed soils may be attributed in part to the wide range of its fungal symbionts (Pera and Álvarez 1995).

The aim of the present research was to investigate the above and belowground ECM fungal community associated with juvenile *P. pinaster* in a natural regeneration stand by combining mycorrhizal root tip description, molecular identification by PCR and sequencing, and sporocarps sampling. We are unaware of previous work on the ECM community associated with this conifer using molecular methods. Moreover, we performed a bibliographic search to collect all the available data on ectomycorrhizal fungi that are symbionts of maritime pine. The results obtained in our work increase the knowledge of the diversity of the ECM fungi hosted by young pines and provide background information to study the potential ecological role of these fungal symbionts especially important for those environments stressed by anthropogenic disturbance, soil pollution, and climatic change.

Materials and methods

Study area

The study site was located at Catoira (42°38′ N, 8°41′ W; Pontevedra, Galicia, NW Spain) in a patch of natural regeneration (5 years old) of a logged mature forest, approximately 60-year-old, where maritime pine was the dominant tree species. Soil is umbric regosol type from unconsolidated materials and with an umbric A-horizon (Macías and Calvo de Anta 2008). Associated vegetation was composed of patches of trees [*Pyrus cordata* Desv., *Frangula alnus* Miller, *Quercus robur* L., *Pinus radiata* Don.], shrubs [*Ulex* sp., *Erica* sp., *Rubus* sp., *Calluna vulgaris* (L.) Hull and *Daboecia cantabrica* (Hudson) K. Koch.] and herbs [*Agrostis curtisii* Kerguelen, *Mentha rotundifolia* L., *Pteridium aquilinum* (L.) Kunth, *Digitalis purpurea* L., *Asphodelus albus* Willd. *Narcissus bulbocodium* L., *Potentilla erecta* (L.) Rauschel., *Lithodora prostrata* (Loisel) Griseb].

Galicia is characterized by an Atlantic humid climate without large frost periods and with a uniformly distributed annual precipitation over 700 mm. Nonetheless, the autumn of 2007 was considered the driest of the last 50 years in Pontevedra (Consellería de Medio Ambiente 2008).

Root sampling

Root samples were collected during June–July 2007 from a 5-year-old plot of maritime pine that measured 70×100 m. We collected fine roots (<2-mm diameter) from 45 randomly selected pine saplings. The conifers were marked with plastic tags for weekly sporocarp production checks (described later). All samples (approximately 200 cm of roots per tree) were collected using a trowel and digging

around the tree to a maximum depth of 10–20 cm following the root system connected to each plant (Fig. 1). Pine roots were carefully collected with soil to minimize damages to ectomycorrhizal tips, placed in plastic bags, and transported to the laboratory. Samples were washed gently under running tap water and stored at 4°C until examined.

We calculated the relative abundance of the ECM fungal genera as the ratio of the mycorrhizal root tips of each taxon to the total root tips sampled.

Morphotyping

Morphological characteristics used to categorize the root tips into types were described by Agerer (1987–2002). Mycorrhizal tips were sorted using a dissection microscope into morphotypes based on color, shape, texture, ramification type, and occurrence and abundance of the emanating hyphae or rhizomorphs. The morphotypes received a brief description and an identity code. For every sampled tree, a subsample of each morphotype (depending on its abundance, it ranged from 10 to 100 mg) was placed individually in a 1.5-mL Eppendorf tube and stored at −20°C for molecular analysis.

Molecular analysis

Fungal DNA was extracted from frozen individual ECM root tips using an EZNA Fungal DNA kit (Omega Bio-Tek, USA) according to the manufacturer's instructions. DNA samples were run by gel electrophoresis, with 1.5% agarose gel and 1 μL of ethidium bromide, at 60 V for 15 min. Results were visualized under a UV transilluminator. We obtained a gross estimation of DNA concentration and molecular size using Lambda Phage (Sigma, USA) and BioMarker Low (Bioventures, USA), respectively. PCR was replicated twice for each sample using first the universal primer pair ITS1/ITS4 and then the basidiomycete-specific primer pair ITS1F/ITS4B. The PCR reaction mix consisted of 1 μL of undiluted DNA (concentration ranged between 10 and 50 ng/μL), 23.8 μL sterile ultrapure water, 3 μL of 10× PCR buffer–$MgCl_2$, 0.6 μL of 0.2 mM dNTPs, 0.5 μL of each primer, and 0.6 μL of 1 U/μL of Taq enzyme (BioTaq, Bioline, USA). The thermocycling pattern used was 94°C for 3 min (one cycle); 94°C for 30 s, 55°C for 30 s and 72°C for 1 min (40 cycles); and 72°C for 10 min (one cycle). Successful amplifications were verified by gel electrophoresis at 60 V for 30 min. When PCR products were not successfully amplified, the eluted DNA was purified with PowerClean DNA Clean-up kit following manufacture's instructions (MoBio Laboratories, USA), and PCR reaction was repeated. PCR products were purified and sequenced by Macrogen Laboratories (www.macrogen.com).

Using the software BioEdit (version 7.0.8, http://www.mbio.ncsu.edu/BioEdit/bioedit.html), sequences were edited and manually corrected before alignment to obtain a consensus sequence. All sequences were identified to genus or species level by querying the GenBank database, using the nucleotide–nucleotide (blastn) BLAST search option, available through the National Center for Biotechnology Information and the UNITE (Kõljalg et al. 2005) online database through blastn algorithm, searching sequences from INSD, the International Nucleotide Sequence Database (GenBank, EMBL, DDBJ). We considered only sequences with 90–100% similarity. Assignment to taxonomic categories was performed using the following criterion: sequence similarity ≥98% gives a match for species identification, while sequences with similarity between 90% and 97% were identified to genus groups.

Sporocarps sampling

From October 2007 to June 2008, we collected weekly samples of all the fruitbodies growing up to 2 m from marked trees. Sporocarps were placed individually in plastic bags, transported to the laboratory, and identified. The abundance of sporocarps and mycorrhizal root tips were compared by genus. Relative abundance of the fruitbodies was calculated as the ratio of the fruitbodies of each genus over the total amount of fruitbodies. The collected specimens were

Fig. 1 Root sampling method

dehydrated and conserved at the Mycoteca of the Sección de Fitopatología of the Centro de Investigación e Información Ambiental de Lourizán (Pontevedra, NW Spain).

Results

The study of 18,463 root tips yielded 15 different morphotypes (Table 1). Direct sequencing of 107 root samples revealed the presence of 15 Basidiomycetes and two Ascomycetes (Table 2). Members of the family Thelephoraceae represented up to 59.4% of the examined root tips. The most common species was *Tomentella sublilacina* followed by *Thelephora terrestris*, *Russula drimeia*, *Suillus bovinus*, and *Paxillus involutus* (Fig. 2). The less abundant species included *Pseudotomentella tristis*, *Lactarius subdulcis*, *Russula ochroleuca*, and *Entoloma conferendum*.

We found 208 sporocarps belonging to seven genera and nine species: *Thelephora terrestris*, *Paxillus involutus*, *Suillus bovinus*, *Xerocomus badius*, *Scleroderma verrucosum*, *Amanita gemmata*, *A. rubescens*, *Amanita* sp., and *Russula* sp. The species belonging to the genus *Amanita* and the species *X. badius* and *S. verrucosum* were not found on root tips. The most common species were *S. bovinus* and *P. involutus* (Fig. 3). Whereas these two species produced 86% of the fruitbodies, on the root systems, they only represented 18%. Only 38% of the genera described from root tips were seen as fruitbodies.

Table 1 Description of the ectomycorrhizal morphotypes based on the external morphology

Morphotype	Morphology, color and surface habit	Emanating hyphae	Rhizomorphs
A	Dichotomous, brown and whitish. Cylindric, not inflated, straight unramified ends, smooth surface.	Infrequent and wavy	Infrequent whitish roundish
B	Dichotomous or coralloid, brown and whitish. Hydrophobicity. Tapering, sinuous and tortuous unramified ends, smooth surface. In some cases, globular sclerotia	Infrequent and wavy	Abundant whitish; margins rather smooth. Globular sclerotia
C	Dichotomous, dark brown or black.	Abundant and brownish	Infrequent brown and roundish
D	Dichotomous, reddish and white straight cilindric unramified ends. Brown older parts. Not smooth surface.	Infrequent and wavy	Abundant whitish roundish
E	Senescent, brownish wrinkled unramified ends and very thin mantled	Lacking	Lacking
F	Dichotomous whitish and brown straight or bent, cylindric unramified ends. Covered with soil particles. Brown older parts.	Abundant and brownish	Abundant whitish roundish with fan-like connection to mantle
G	Dichotomous or coralloid ochre and whitish unramified ends. Covered with soil particles. Not smooth surface, Woolly mantle surface	Abundant	Infrequent whitish roundish
H	Dichotomous ochre or orange straight or bent, cylindric unramified ends. Brown older parts. Constricted between older and younger parts. Brilliant surface	Infrequent and straight	Lacking
I	Dichotomous yellowish straight, cylindric unramified ends. Brown older parts. Constricted between older and younger parts. Loosely or densely grainy surface	Infrequent and wavy	Infrequent yellowish roundish
J	Dichotomous brown and whitish straight cylindric unramified ends. Brown older parts. Smooth surface	Infrequent and wavy or tortuous	Infrequent brownish roundish
K	Dichotomous or coralloid brown tapering unramified ends	Abundant brownish and straight	Lacking
L	Dichotomous grayish or whitish straight cylindric unramified ends. Hydrophobicity. Presence soil particles and smooth surface	Lacking	Infrequent whitish roundish
M	Dichotomous or coralloid, yellowish straight cylindric unramified ends. Brown older parts. Grainy surface	Lacking	Lacking
N	Dichotomous orange or brown bent and constricted between older and younger parts. Cylindric unramified ends. Brown older parts. Lost orange coloration in contact with air	Infrequent and not striking	Infrequent brownish roundish
O	Dichotomous ochre or whitish bent cilindric or tapering unramified ends. Brown older parts. Presence soil particles and smooth surface	Infrequent not striking	Infrequent brownish roundish; connected mantle at restricted points

Table 2 Ectomycorrhizal fungal taxa identified by direct sequencing compared with the results of morphotyping

Accession number[a]	Length	Closest BLAST match[b]	Phylum	Sequence similarity (%)	Total score	Query coverage (%)	Morphotypes
FJ188349	732	*Entoloma conferendum* (AF538624)	B	98	1,160	86	B
FJ188350	850	*Gomphidius* sp. (DQ534570)	B	94	1,022	73	E
FJ188351	846	*Lactarius subdulcis* (AJ889963)	B	98	1,452	98	A, B, N
FJ188352	511	*Meliniomyces* sp. (EF093171)	A	96	867	99	D
FJ188353	833	*Paxillus involutus* (EU486436)	B	98	1,459	99	A, B
FJ188354	515	*Phialophora* sp. (DQ069046)	A	97	846	96	A, E, H
FJ188355	719	*Pseudotomentella tristis* (AJ889979)	B	99	1,203	90	C, O
FJ188356	660	*Pseudotomentella* sp. (AF274769)	B	92	824	87	B, K
FJ188357	721	*Russula drimeia* (EU557320)	B	99	1,230	92	C, I, L
FJ188358	675	*Russula ochroleuca* (AM087261)	B	99	1,212	99	B, H, M
FJ188359	749	*Scleroderma* sp. (EU718119)	B	93	944	81	B, E
FJ188360	822	*Suillus bovinus* (AB036902)	B	98	1,426	99	E, G
FJ188361	567	*Thelephora terrestris* (EU427330)	B	99	1,040	100	A, F, H, J
FJ188362	801	*Thelephora* sp. (DQ822828)	B	97	1,338	97	B, I, J
FJ188363	617	*Tomentella sublilacina* (AF272929)	B	99	1,055	94	H
FJ188364	746	*Tomentella* sp. (DQ482015)	B	96	1,242	99	C
FJ188365	841	*Tomentellopsis* sp. (AY641459)	B	97	1,306	92	D, H

For all sequences the *E* value is equal to zero
A Ascomycota, *B* Basidiomycota
[a] Accession number submitted into GenBank database
[b] Closest named BLAST species and genera were used. The accession number is indicated in parentheses

The result of the bibliographic review of publications from 1922 to 2006 is provided as Electronic supplementary material (S1). There are 40 genera and 199 species of ectomycorrhizal fungi, of which 193 species were Basidiomycetes and only five species Ascomycetes. By comparing our results with this list, we found five genera (*Tomentella*, *Pseudotomentella*, *Tomentellopsis*, *Phialophora*, and *Meliniomyces*) and six species (*Tomentella sublilacina*, *Russula drimeia*, *Pseudotomentella tristis*, *Russula ochroleuca*, *Scleroderma verrucosum*, and *Entoloma conferendum*) that have not been previously documented in symbiosis with *P. pinaster*.

Discussion

Through use of morphotyping and molecular analysis, we found that the ECM fungal community in *Pinus pinaster* is more diverse than fruitbody studies have shown. We added five genera and six species to the list of fungal symbionts hosted by *P. pinaster*.

Morphological classification prior to molecular analysis can be useful to expedite the process of identification (Wurzburger et al. 2001; Sakakibara et al. 2002). However, morphotyping is subject to bias due to natural degradation of the morphological characteristics which may be the result of bad conservation, phenotypic expression, tip age, environmental conditions, time spent for identification, and the skill level of the observer (Wurzburger et al. 2001; Horton and Bruns 2001; Sakakibara et al. 2002; Burke et al. 2005). In our study, we found that old ectomycorrhizal root tips were often brownish, which enhanced mismatches during the process of morphotype creation. However, in other cases, the presence of diagnostic morphological characters such as the globular sclerotia and rhizomorphs of *P. involutus*, the coralloid ramifications of *S. bovinus*, or the reddish color of *Tomentellopsis submollis* contributed to successful identifications (Fig. 2).

The use of morphotyping and molecular methods can lead to contradictory results in determining ECM fungal diversity of pines largely due to similarity in morphology by different fungal taxa (Menkis et al. 2005; Wurzburger et

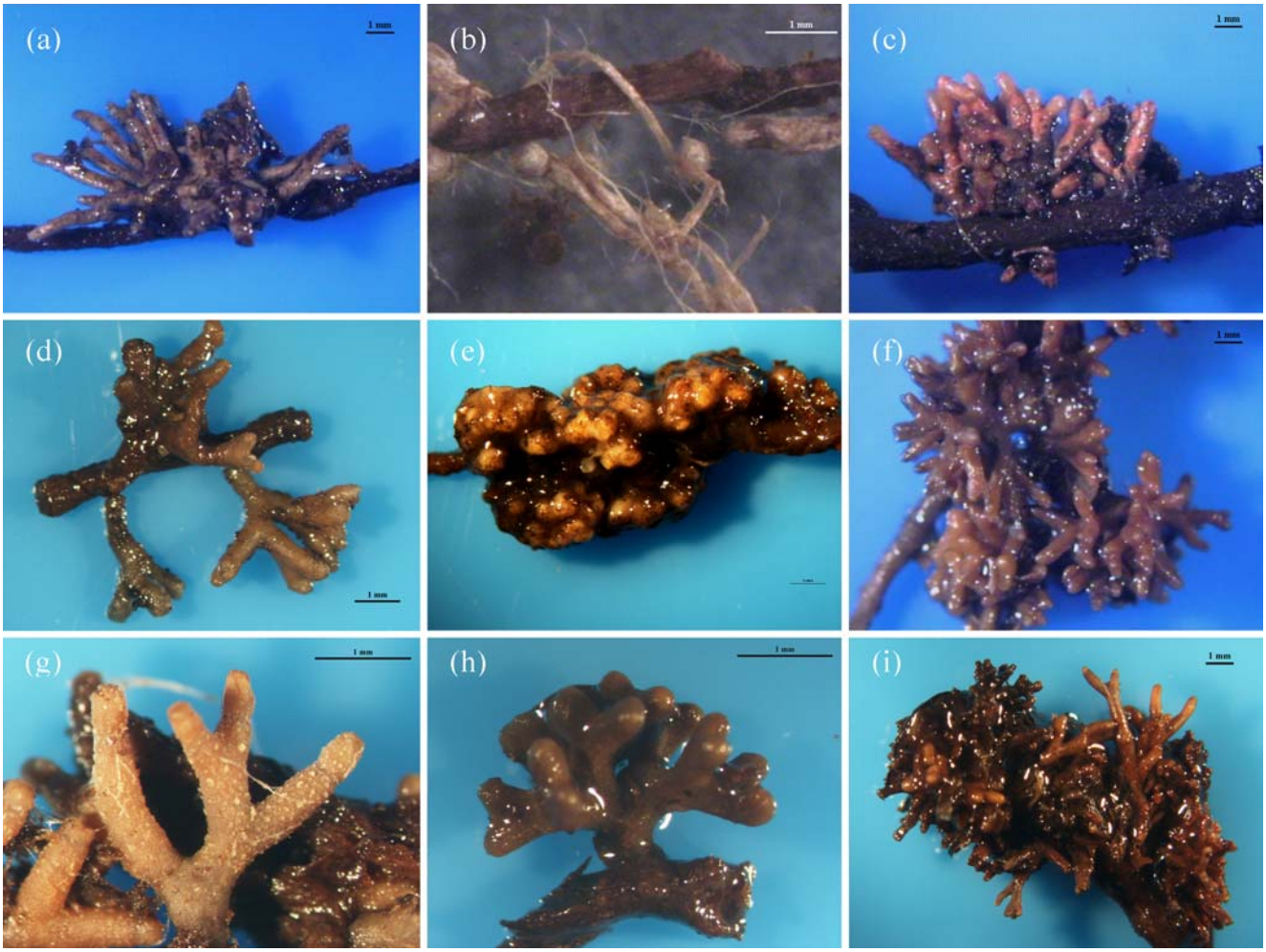

Fig. 2 Ectomycorrhizas of *Pinus pinaster*: *Paxillus involutus* (**a**); rhizomorphs and sclerotia of *Paxillus involutus* (**b**); *Tomentellopsis submollis* (**c**); *Pseudotomentella tristis* (**d**); *Suillus bovinus* (**e**); *Tomentella sublilacina* (**f**); *Russula drimeia* (**g**); *Russula ochroleuca* (**h**); *Lactarius subdulcis* (**i**)

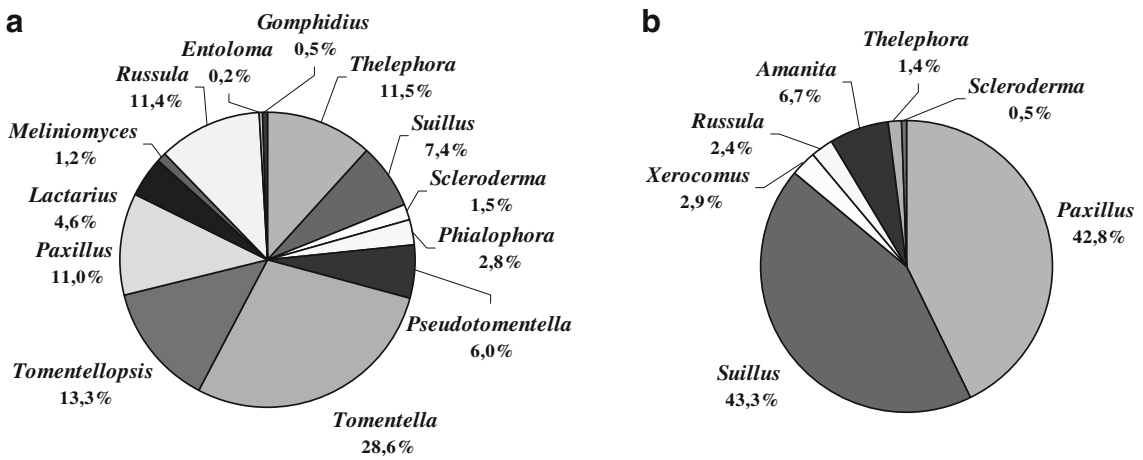

Fig. 3 Relative abundance of the genera described by examining ectomycorrhizal root tips (**a**) and sporocarps (**b**)

al. 2001). Our study showed similar results. By comparing fungal taxa identified by molecular analysis with the groups obtained by morphotyping (Table 2), we found that for seven morphotypes only one taxon of fungus was detected by sequencing, while the remaining eight represented two to seven different taxa each. However, we do not disregard the possibility that this pattern could be due to the ability of the observer and the characters used for morphotyping.

Compared to sporocarp sampling, morphotyping and DNA sequencing provide the most powerful tools in ECM fungal community studies (Horton and Bruns 2001). In our research, only 38% of the genera described from root tips have been found as fruitbodies, which means that species that do not form obvious fruiting structures may be abundantly represented as mycorrhizal root tips (Gardes and Bruns 1996; Kåren et al. 1997; Gehring et al. 1998; Jonsson et al. 1999). In fact, while *S. bovinus* and *P. involutus* sporocarps were the most abundant, their presence belowground constituted only 7.4% and 11% of root tips, respectively (Fig. 3).

Moreover, a high percentage of the fungal species do not form conspicuous sporocarps or form thin resupinates which are not detected during sampling (Gardes and Bruns 1996; Horton 2002; Tedersoo et al. 2003). In particular, Thelephoroid species such as *Thelephora* and *Tomentella* could easily be missed due to their resupinate fruitbodies (Gardes and Bruns 1996). In fact, during the field surveys, we found only three fruitbodies of *Thelephora terrestris* over a total 208 sporocarps. Our results are consistent with other studies that show that members of the family Thelephoraceae and non-Thelephoroid resupinates are among the most abundant and frequent taxa on ECM roots in conifer communities in Europe (Taylor and Bruns 1999; Kõljalg et al. 2000; Wurzburger et al. 2001; Horton and Bruns 2001).

One of the most significant advances from molecular approaches has been the realization that many Ascomycetes are mycorrhizal fungal symbionts (Horton and Bruns 2001) forming ecto- or ericoid mycorrhizae on trees of the family Ericaceae (Bougoure 2006). In our study, the phylum Ascomycota was represented by *Phialophora* sp. and *Meliniomyces* sp. The dark-septate root endophytes (DSE) fungus *P. finlandia* was previously reported to form symbiosis with conifers of temperate forests (Trevor et al. 2001; Tedersoo et al. 2003; Menkis et al. 2005). Although the mycorrhizal status of DSE is still uncertain, these fungi are often associated with herbs, shrubs, and trees of alpine, boreal and northern temperate ecosystems (Caldwell et al. 2000). The fungus *Meliniomyces variabilis* was described for the first time by Hambleton (2005), who placed it within the species complex referred as the "*Hymenoscyphus ericae* aggregate" which includes *H. ericae* (Leotiomycetes), many unnamed taxa, and *Cadophora finlandica*. Nonetheless, the mycorrhizal status of *M. variabilis* is not yet clear. It demonstrated little or no colonization with ericoid and ectomycorrhizal hosts but did not form true ectomycorrhizae (Hambleton 2005).

To conclude, as noted for other forest trees (Gardes and Bruns 1996), above and belowground diversity need to be considered simultaneously to understand the ecological importance of ECM fungal species diversity within the *P. pinaster* ecosystem.

Our study represents a first glance to the ectomycorrhizal fungal community hosted by *P. pinaster* using molecular methods. Further genetic characterization of species diversity and succession is needed, at a more large temporal and spatial scale, to elucidate the major processes involved in the distribution of the fungal species.

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