Molecular identification of ectomycorrhizas associated with ponderosa pine seedlings in Patagonian nurseries (Argentina)

C. Barroetaveña, M.B. Pildain, M.E. Salgado Salomón, and J.L. Eberhart

Abstract: Ponderosa pine (*Pinus ponderosa* Dougl. ex P. & C. Laws.), an ectomycorrhiza (EM) dependent species, has been widely introduced in Patagonia, Argentina. This study used morphotyping, restriction analysis, and sequencing of EM root tips from ponderosa pine seedlings in two nurseries to assess the complete EM fungus (EMF) richness, to confirm doubtful identities of commonly reported morphotypes, and to evaluate the efficiency of morphotyping compared with molecular analysis. This interdisciplinary approach together with the fact that is the first study in which Patagonian nurseries EMF are genetically evaluated contributes to the general knowledge of this important group of fungi. Sequencing revealed the presence of 11 taxa. Basidiomycetes included Thelephoraceae (*Tomentella* sp.), Atheliaceae (*Amphinema byssoides* (Pers.) J. Erikss.), Hydnangiaceae (*Laccaria* sp.), Rhizopogonaceae (*Rhizopogon roseolus* (Corda) Th. Fr.), and Cortinariaceae (*Hebeloma mesophaeum* (Pers.) Quel.). Ascomycetes included Pezizaceae (*Wilcoxina mikolae* (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf and *Wilcoxina* sp.) and Tuberaceae (*Tuber* sp.). Morphotyping proved to be useful for certain EMF species (*R. roseolus*, *H. mesophaeum*, *A. byssoides*, and to a lesser extent *Tuber* sp.) in which some morphological features are conspicuous and unique. Our detection of *W. mikolae* and *Wilcoxina* sp. are new records for ponderosa pine in Patagonia. All of the EM taxa identified are common to pine plantations and nurseries around the world, and no indigenous EM associated with native *Nothofagus* spp. were found.

Résumé : Le pin ponderosa (*Pinus ponderosa* Dougl. ex P. & C.Lawson), une espèce qui dépend des champignons ectomycorhiziens (CEM), a été introduit à grande échelle en Patagonie (Argentine). Dans cette étude, nous avons utilisé la caractérisation morphologique, l'analyse des fragments de restriction et le séquençage des apex racinaires ectomycorhizés provenant de semis de pin ponderosa dans deux pépinières pour évaluer la richesse complète des CEM, confirmer l'identité incertaine des morphotypes communément rapportés et évaluer l'efficacité de la caractérisation morphologique comparativement à l'analyse moléculaire. Cette approche interdisciplinaire, couplée au fait qu'il s'agit de la première étude qui porte sur une évaluation génétique des CEM dans les pépinières de la Patagonie, contribue à la connaissance générale de cet important groupe de champignons. Le séquençage a révélé la présence de 11 taxons. Les basidiomycètes incluaient des théléphoracées (*Tomentella* sp.), des athéliacées (*Amphinema byssoides* (Pers.) J. Erikss.), des hydnangiacées (*Laccaria* sp.), des rhizopogonacées (*Rhizopogon roseolus* (Corda) Th. Fr.) et des cortinariacées (*Hebeloma mesophaeum* (Pers.) Quel.). Les ascomycètes incluaient des pézizacées (*Wilcoxina mikolae* (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf et *Wilkoxina* sp.) et des tuberacées (*Tuber* sp.). La caractérisation morphologique s'est avérée utile pour certaines espèces de CEM (*R. roseolus*, *H. mesophaeum*, *A. byssoides* et à un moindre degré *Tuber* sp.) chez lesquelles certaines caractéristiques morphologiques sont évidentes et uniques. Notre détection de *W. mikolae* et de *Wilkoxina* sp. constitue une première sur le pin ponderosa en Patagonie. Tous les taxons de CEM qui ont été identifiés sont présents dans les pépinières et les plantations de pin partout dans le monde et aucun CEM indigène associé aux *Nothofagus* spp. indigènes n'a été observé.

[Traduit par la Rédaction]

Introduction

Ponderosa pine (*Pinus ponderosa* Dougl. ex P. & C. Laws.) is the most widely planted species in the vast grasslands on the piedmont of the Patagonian Andes in Argentina. Plantations were initiated around 50 years ago and there are approximately 50 000 ha currently forested (Andenmatten et al. 2002). The species grows naturally on the eastern side of the Cascade Mountains of northwestern North America and forms symbiotic associations with at least 157 ectomycorrhizal fungi (EMF) in its natural distribution area (Barroetaveña et al. 2007). However, papers

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dealing with morphotypes descriptions are scarce (Goss 1960; Riffle 1973; Massicotte et al. 1999*a*; Stendell et al. 1999). A previous survey of the EMF associated with ponderosa pine introduced into Patagonia has detected 18 taxa fruiting in both nurseries and plantations (Barroetaveña 2004). In nurseries, 11 taxa of EMF were found fruiting and 15 ectomycorrhizal morphotypes were described (Barroetaveña and Rajchenberg 2003). Because sporocarp abundance and diversity do not necessarily correlate with the EMF present on the roots (Gardes and Bruns 1996; Dahlberg 2001; Yamada and Katsuya 2001) and because morphotyping can be imprecise, it is highly probable that there are still undetected EMF species present in this region. Several E-strain-like morphotypes (Yu et al. 2001) have been described in previous morphological studies in Patagonia (Barroetaveña and Rajchenberg 2003; Barroetaveña 2004; Salgado Salomón et al. 2009). It still remains unclear whether these morphotypes are all E-strain and which EMF species are involved, as the species forming this type of mycorrhiza do not usually form fruiting bodies (Smith and Read 2008) and the ectomycorrhizas (EM) are difficult to separate morphologically.

Nurseries are the only source of EMF inoculum for ponderosa pine planted in Patagonian grasslands, as the native flora has arbuscular symbionts (Fontenla et al. 1998). Since plantations have been established with seedlings from regional nurseries, it seems appropriate to conduct the study of EMF richness in Patagonia by using molecular tools on colonized root tips from those nurseries. Moreover, fresh and turgid root tips are very difficult to find in Patagonian plantations because soils are sandy and periodically exposed to strong water stress (Barroetaveña 2004).

The use of morphotype characters is considered problematic by some authors because it is not precise enough to accurately describe EMF communities, is time consuming to learn, may not be consistent among laboratories, and provides only a selective biased window on diversity (Mehmann et al. 1995; Dahlberg 2001). Moreover, it has been repeatedly shown that there is a poor correlation between morphotyping and the results of molecular analysis (Mah et al. 2001; Menkis et al. 2005). The application of DNA-based molecular techniques in EM research has replaced the use of morphological methods of EM identification to a great extent (Horton and Bruns 2001), either complementing other methods (Sakakibara et al. 2002) or on its own. Molecular techniques present their own bias, randomly sampled root tips do not allow quantitative descriptions of EMF communities, and it is very expensive and time consuming to run large numbers of root tips without previous morphological characterization (Sakakibara et al. 2002). Techniques utilized for the study of root-associated fungal community diversity include comparisons of restriction fragment length polymorphisms (RFLPs) (Gardes and Bruns 1996; Kårén and Nylund 1997) and the use of sequences from the internal transcribed spacer (ITS) regions (Egger et al. 1991; Gardes and Bruns 1996). Direct sequencing of fungal DNA from root tips has proved to be a sensitive method for detection of potentially all EM fungi, and both inter- and intraspecific genetic variability can be evaluated (Dahlberg 2001).

The objectives of this research were to assess the EMF diversity on ponderosa pine root tips from two nurseries in Patagonia (Argentina) using polymerase chain reaction (PCR) – RFLP analysis and direct sequencing to confirm doubtful identities of previously described EM roots and to evaluate the efficiency of morphotyping compared with molecular analysis.

Materials and methods

Nurseries, seedlings, and soil attributes

The two oldest bareroot nurseries in the region were selected for the study. Both of them belong to the National Institute of Agriculture and Cattle Technology (INTA). Nursery 1 is located at Trevelin (Chubut Province, Argentina) and has been producing exotic tree seedlings for 45 years. Nursery 2 has been located at Las Golondrinas (Chubut Province, Argentina) for 25 years. These two nurseries have previously been sampled and were found to have a high number of EMF species producing sporocarps (Barroetaveña and Rajchenberg 2003; Barroetaveña 2004). Climatic characteristics and nursery management are shown in Table 1. A systematic sampling of thirty 2-year-old seedlings was carried out in each nursery in February 2008. To sample each seedling, a group of four or five was excavated with a shovel in a predetermined pattern and the one with the most intact root system was used. Seedlings were put in soil in plastic bags and stored not more than 10 days in the shade, with appropriate watering, until processed.

Soil and seedling parameters were analyzed to establish basic attributes of each nursery that could influence EM richness. Composite soil samples of five sites were taken in each nursery to evaluate soil texture, content of nutrients, clay, silt, and sand percentage composition, pH in 1:1 dilution of soil in water, electrical conductivity, soil organic matter percentage using loss on ignition, and total N percentage using the Kjeldahl method. Soil texture was loamy in both soils but they differed in N content (Table 1). From each seedling, shoot length and diameter at the root collar was recorded. All secondary roots were dissected from the main root, measured to obtain total root length, and submerged in a tray with water to quantify the total number of root tips (Table1). Differences in shoot length, diameter at the root collar, total root length, and total number of root tips between nurseries were analyzed using a t test for independent samples or Mann–Whitney test, after checking for homogeneous variances with the Levenne test and normality with the Shapiro–Wilk test, using SPSS 11.5 for windows. Seedling size parameters differed between the two nurseries, with bigger seedlings as measured by shoot length ($p = 0.001$, t test), collar diameter ($p < 0.0001$, t test), and total root length ($p < 0.0001$, Mann–Whitney test) and with more total root tips ($p < 0.0001$, Mann–Whitney test) in nursery 1 where chemical fertilization was applied (Table 1).

Morphotype classification

Morphotypes were characterized, determined, and classified according to Goodman et al. (1996) and Agerer (1991) as well as the reference work of Barroetaveña and Rajchenberg (2003), Barroetaveña (2004), and Barroetaveña et al. (2005) in which most of the morphotypes found in ponderosa pine plantations and forest nurseries in Patagonia are described. Concise morphological descriptions were made

Table 1. Nursery characterization including soil description and climatic description, nursery management, and seedling morphometry.

	INTA Trevelin nursery (1)	INTA Golondrinas nursery (2)
Soil and climate		
pH H_2O	4.90	5.57
Electrical conductivity (dS/m)	0.24	0.25
% organic matter	10.61	10.56
% organic C	5.31	5.28
% total N	0.407	0.254
Relationship C/N	13	21
% clay	9	14
% silt	41	49
% sand	50	37
Textural class	Loamy	Loamy
Annual precipitation (mm)	1030	921
Minimum absolute temperature (°C)	–15	–11.2
Maximum absolute temperature (°C)	38	37.3
Mean annual temp. °C	10	9.8
Nursery management		
Sowing	October 2006	September 2006
Pruning	March 2007 (machine)	Ringing in 2007
Watering	Aspersion	Aspersion
Fertilization	Chemical, one application presowing	Green manure
Fungicides	Captan to seeds	Captan to seeds, 420 g/100m^2 potassium chloride (two applications)
Herbicides	None	Koltar (preemergence, one application) Fordor (postemergence, one application)
Seedlings morphometry		
Stem height (cm)	15.17 (SE 0.31) a	13.27 (SE 0.43) b
Stem diameter (mm)	49.57 (SE 1.26) a	34.62 (SE 1.38) b
Root length (cm)	281.93 (SE 15.62) a	119.60 (SE 8.45) b
Total number of root tips	296.57 (SE 15.62) a	130.07 (SE 15.62) b

Note: Different letters indicate significant differences at $p = 0.001$ (t test). Ec: electrical conductivity (in dS/m); %OM: organic matter percentage; %OC: organic carbon percentage; %N: percentage ot total nitrogen.

using a Wild M3Z dissecting microscope with 10–40× magnification and a Zeiss-Axioscop compound microscope focusing on distinctive features easily recognizable. The total number of root tips assigned to each morphotype was recorded for each seedling and the percentage of each morphotype was calculated as a proportion of the total number of EM tips for each seedling. This procedure provided a measure of the relative frequency of each EM type on each seedling. These data were then averaged to produce a mean relative frequency for each EM type in each nursery (Table 2).

Molecular methods

Selected tips of each EM type were preserved in 1.5 mL Eppendorf tubes containing 300 µL of 2× CTAB lysis buffer (100 mmol/L Tris–HCl (pH 8.0), 1.4 mol/L NaCl, 20 mmol/L EDTA, and 2% CTAB) at the end of each day to avoid overgrowth and contamination. Five individual root tips from different plants and nurseries were selected from each morphotype for DNA extraction. DNA was also extracted from sporocarps of *Rhizopogon roseolus* (Corda) Th. Fr.) (OSC Herbarium 129167), *Rhizopogon ellenae* (A.H. Sm.) (OSC Herbarium 129168), *Hebeloma mesophaeum* (Pers.) Quel. (OSC Herbarium 129169), *Amphinema byssoides* (Pers.) J. Erikss. (OSC Herbarium 129166), and *Suillus luteus* (L.) Roussel (OSC Herbarium 129165). DNA extraction was carried out individually to each tip following the method described by Avis et al. (2003) using a plant DNA extraction kit (REDExtract-N-Amp Plant PCR Kit; Sigma, St. Louis, Missouri).

DNA extractions of the sporocarps and root tips were subjected to PCR and RFLP analysis of the internal transcribed spacer (ITS). The ITS region (ITS1, 5.8S, and ITS2) of the rRNA operon was amplified using primer set ITS1-F/ITS4 (White et al. 1990; Gardes and Bruns 1993). PCR was performed using the REDExtract-N-Amp Plant PCR Kit with PCR cycling conditions modified from Gardes and Bruns (1993). Cycling conditions included an initial denaturation at 94 °C for 4 min followed by 35 PCR cycles (93 °C, 35 s; 55 °C, 53 s; 72 °C, 30 + 5 s per cycle). Amplified ITS products were visualized on an agarose gel stained with ethidium bromide under UV illumination. ITS amplicons obtained for the root tips were digested with the restriction endonucleases *Hin*f1 and *Dpn*2 (Promega, Madison, Wisconsin). The resulting PCR–RFLP fragments were separated on an agarose gel stained with ethidium bromide and visualized under UV illumination. RFLP profiles obtained with each enzyme were scored and the fragment size was

Table 2. Morphotypes descriptions, mean relative abundance in each nursery, and PCR identity.

Morphotype	Branching	Texture	Colour	Abundance in nursery 1 (%)	Abundance in nursery 2 (%)	PCR identity
M1 (patchy, white mycelium)	Dichotomous	Irregular, sometimes shiny patches; emanating hyphae abundant	White	15.05	6.91	Two not identified, two *A. byssoides*
M2 (woolly, yellow)	Irregular	Smooth, thin mantle with woolly, yellowish emanating hyphae	Light brown	0.31	0.03	Two *H. mesophaeum*, two *A. byssoides*, one *W. mikolae*
M3 (white mycelium with emanating hyphae)	Dichotomous	Similar to M1 but mantle more homogeneous; sometimes shiny with abundant emanating hyphae	White mantle; emanating hyphae translucent	1.55	0.23	*H. mesophaeum*
M4 (woolly, yellow, nacreous)	Dichotomous, irregular	Similar to M2 but yellowish with woolly emanating hyphae	Yellow surface; emanating hyphae white–yellowish	2.48	0	One *Laccaria* sp., one *Tomentella* sp., three *H. mesophaeum*
M5 (E-strain type)	Nonramified, dichotomous simple or multiple	Surface smooth, no emanating hyphae; not inflated.	Brown	21.30	10.3	Three *Laccaria* sp., one *Wilcoxina* sp., one uncultured Ascomycete
M6 (*Rhizopogon* type)	Dichotomous, sympodial and clusters	Felty with abundant rhizomorphs	Mantle and rhizomorphs white, lightly shiny	15.18	1.94	*R.. roseolus*
M7 (short, woolly, yellow, nacreous)	Abundant, short, dichotomous branching	Smooth, thin mantle with woolly, yellowish emanating hyphae	Yellowish	0.12	0	*W. mikolae*
M8 (yellow E-strain)	Dichotomous	Smooth with no emanating hyphae	Yellow–tan	2.55	0.02	Four *Laccaria* sp., one *W. mikolae*
M9 (robust E-strain)	Short, dichotomous	Surface smooth, no emanating hyphae; not inflated	Brown	1.02	0.14	Two *W. mikolae*, two *Wilcoxina* sp., one *Tuber* sp.
M10 (E-strain with mycelium)	Dichotomous	Surface smooth with white, wooly emanating hyphae	Brown	2.47	17.26	One *P. ostracoderma*, one *Laccaria* sp., one *Cylindrocarpon* sp., one *W. mikolae*, One *Tomentella* sp.
M11 (E-strain ramified with mycelium)	Dichotomous	Surface smooth with scarce to abundant emanating hyphae	Brown	0.61	0	Four *W.mikolae*, one *Laccaria* sp.
M12 (yellow E-strain with mycelium)	Not branched or dichotomous	Smooth with scarce emanating hyphae	Yellow	1.52	0.45	Three *Tuber* sp., two *W. mikolae*
M13 (white–yellowish mycelium with fringes)	Not branched or dichotomous	Mantle in patches; abundant emanating hyphae grouped in plumose rhizomorphs	Yellowish, shiny patches; white emanating hyphae	1.41	0	*H. mesophaeum*
M14 (white mycelium with emanating hyphae, immature)	Short, dichotomous branching	Mantle with irregular, inconspicuous, nacreous patches; emanating hyphae scarce but always present	White mantle and emanating hyphae	0.19	0	Two *W. mikolae*, one *Laccaria* sp., one *Tomentella* sp.
M15 (white E-strain)	Short, nonramified	Smooth with scarce to null emanating hyphae	White on top and grey–brown at the base	0	1.03	*W. mikole*

Table 2 (*concluded*).

Morphotype	Branching	Texture	Colour	Abundance in nursery 1 (%)	Abundance in nursery 2 (%)	PCR identity
M16 (*Suillus* type)	Dichotomous, multiple branching	Smooth with rhizomorphs	Brown mantle and rhizomorphs	0	0.09	One *W. mikole*, four contaminated
M17 (grey E-strain)	Dichotomous branching	Smooth with no rhizomorphs	White–greyish; opaque	0	0.29	*Laccaria* sp.

Note: Mean relative abundance for each EM type was calculated as its proportion of the total number of EM tips for each seedling averaged across the nursery.

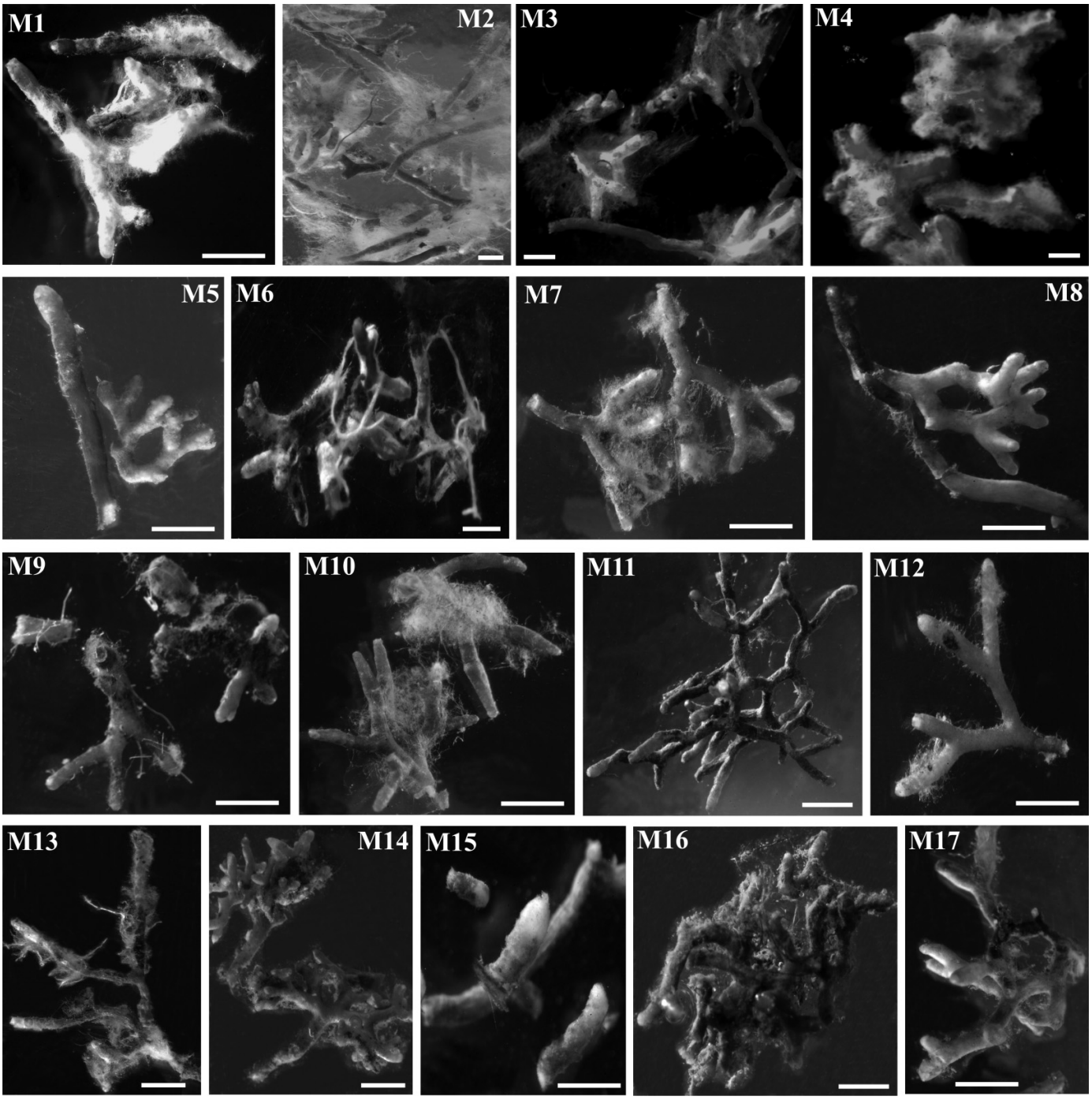
estimated. One representative sample among those sharing an identical RFLP profile was chosen for sequencing. PCR products were purified prior to sequencing using ExoSAP-IT (USB, Cleveland, Ohio). Sequencing was performed by the Center for Genome Research and Biocomputing Core Laboratory at Oregon State University using an ABI Prism 3730 genetic analyzer (Applied Biosystems, Foster City, California). Sequences were identified by querying the GenBank database using the nucleotide–nucleotide (blastn) blast search option on the National Center for Biotechnology Information Web site (Altschul et al. 1997). Identities above 98% were treated as species-level matches, while identification at the genus level was based on BLAST consensus above 90%.

Results

A total of 17 morphotypes were detected (Fig. 1) with 14 types on seedlings in nursery 1 and 12 in nursery 2. Table 2 shows the main features of each EM morphotype and the mean relative abundance of each type for each nursery. While nine morphotypes were common to both nurseries (M1, M2, M3, M5, M6, M8, M9, M10, and M12), five were exclusively found in nursery 1 (M4, M7, M11, M13, and M14) and three exclusively found in nursery 2 (M15, M16, and M17). The most abundant morphotypes were M5, M6, and M1 in nursery 1 and M10, M5, and M1 in nursery 2 (Table 2). Of the 17 described morphotypes, eight were E-strain like (M5, M8, M9, M10, M11, M12, M15, and M17).

ITS amplicons of a sample from each RFLP pattern were directly sequenced revealing the presence of 11 taxa (Tables 2 and 3). BLAST searches allowed identification of the EMF associated with the roots to different taxonomic levels, with three identified to species, seven to genus, and one only to the Ascomycetes class. Sequencing revealed the presence of the following Basidiomycetes: *Tomentella* sp. (Theleporaceae), *Amphinema* sp. (Atheliaceae), *Laccaria* sp. (Hydnangiaceae), *R. roseolus* (Rhizopogonaceae), and *Hebeloma* sp. (Cortinariaceae). The Ascomycetes identified by sequencing were *Wilcoxina mikolae* (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf, *Wilcoxina* sp. (Pezizaceae), *Tuber* sp. (Tuberaceae), and the non-EM taxa *Cylindrocarpon* sp. (Nectriaceae) and *Peziza ostracoderma* Korf 1961 (Pezizaceae). Restriction maps for both restriction endonucleases *Hin*f1 and *Dpn*2 were determined from sequence data. *Wilcoxina mikolae* and *Wilcoxina* sp. presented the same number of RFLP fragments but these differed in size (data not shown). The RFLP pattern of M6 was the same as that for the *R. roseolus* sporocarp; the identity of that morphotype was later confirmed by sequencing and BLAST search with 98% affinity. BLAST searches of M2(morphotype replicates b and d), M3, M4(a, b, and d), and M13 matched with *H. psammophilum* with an affinity of 98%, but the RFLP pattern was the same as those obtained for our *H. mesophaeum* sporocarp, a species that was not present in the BLAST database (Table 3). When sequenced, morphotypes M1(a and c) and M2(a and e) matched an *Amphinema* sp. and the RFLP profile matched that of our *A. byssoides* sporocarp. Considering previous morphological studies (Barroetaveña and Rajchenberg 2003; Barroetaveña 2004), morphotyping, and the similarity in RFLP patterns, we could

Fig. 1. Habitus of the 17 ectomycorrhizal morphotypes found in both surveyed nurseries. M1: patchy, white mycelium; M2: woolly, yellow; M3: white mycelium with EH; M4: woolly, yellow, nacreous; M5: E-strain type; M6: *Rhizopogon* type; M7: woolly, yellow, nacreous, short; M8: yellow E-strain; M9: robust E-strain; M10: E-strain with mycelium; M11: E-strain ramified with mycelium; M12: yellow E-strain with mycelium; M13: white–yellowish mycelium with fringes; M14: white mycelium with EH, inmature; M15: white E-strain; M16: *Suillus* type; M17: grey E-strain. Bars = 1 mm.



confirm the presence of *H. mesophaeum* and *A. byssoides* in the nurseries analyzed.

A total of 85 root tips were subjected to RFLP analysis. Although the RFLP patterns did not always coincide with the groups based on morphology, from the 17 described morphotypes, 65% had at least three replicates with the same band pattern (Table 3). Of these, six produced one RFLP pattern for the five replicates (M3, M6, M7, M13, M15, and M17), two produced the same RFLP pattern for four replicates (M8 and M11), and three presented the same RFLP pattern for three replicates (M4, M5, and M12). The most frequent RFLP pattern was that represented by morphotype M7 (*W. mikolae*). Other RFLP patterns observed frequently were those represented by M17 and M3 with 15 and 14 tips, respectively (Table 3).

Table 3. RFLP patterns and ITS sequence identity of morphotypes from ponderosa pine nurseries in Patagonia.

Morphotype	Identified by:	Classification and BLAST similarity	% sequence similarity	GenBank No.
M1 a and c	RFLP and ITS sequence	EU649087 *Amphinema* sp.: *A. byssoides*	95	GU969246
M1 d and e	RFLP	*H. mesophaeum*		
M2 a and e	RFLP	*A. byssoides*		
M2 b and d	RFLP and ITS sequence	AB211272 *H. mesophaeum*	98	GU969248
M2 c	RFLP	*W. mikolae*		
M3 a, b, c, d, and e	RFLP	*H. mesophaeum*		
M4 a, b, and d	RFLP	*H. mesophaeum*		GU969248
M4 c	RFLP and ITS sequence	AJ534899 *Laccaria* sp.	100	GU969249
M4 e	RFLP and ITS sequence	AB253523 *Tomentella* sp.	96	GU969250
M5 a	RFLP and ITS sequence	EU5622601 uncultured Ascomycete	98	GU969251
M5 b, c, and e	RFLP and ITS sequence	AJ534899 *Laccaria* sp.	100	GU969252
M5 d	RFLP and ITS sequence	DQ069052 uncultured *Wilcoxina*	99	GU969252
M6 a, b, c, d, and e	RFLP and ITS sequence	AJ419210 *R. roseolus*	99	GU969254
M7 a, b, c, d, and e	RFLP and ITS sequence	DQ069000 *W. mikolae*	100	GU969255
M8 a, c, d, and e	RFLP and ITS sequence	AJ534899 *Laccaria* sp.	100	GU969256
M8 b	RFLP and ITS sequence	DQ069000 *W. mikolae*		
M9 a and b	RFLP and ITS sequence	DQ069000 *W. mikolae*	100	GU969257
M9 c	RFLP and ITS sequence	DQ069052 uncultured *Wilcoxina*	99	GU969258
M9 d	RFLP and ITS sequence	GQ267493 *Tuber* sp.	100	GU969259
M10 a	RFLP and ITS sequence	DQ974687 *Cylindrocarpon* sp.	95	GU969260
M10 b	RFLP	*Tomentella* sp.		
M10 c	RFLP and ITS sequence	EU819461 *P. ostracoderma*	98	GU969261
M10 d	RFLP and ITS sequence	DQ069000 *W. mikolae*	100	GU969262
M10 e	RFLP and ITS sequence	AJ534899 *Laccaria* sp.	100	GU969263
M11 a, b, c, and d	RFLP and ITS sequence	DQ069000 *W. mikolae*	100	GU969264
M11 e	RFLP and ITS sequence	AJ534899 *Laccaria* sp.	100	GU969265
M12 a and c	RFLP and ITS sequence	AY748861 *Tuber* sp.	100	GU969266
M12 b and d	RFLP and ITS sequence	DQ069000 *W. mikolae*	100	GU969267
M12 e	RFLP and ITS sequence	AY748861 *Tuber* sp.	100	GU969268
M13 a, b, c, and e	RFLP	*H. mesophaeum*		
M14 a	RFLP	*Laccaria* sp.		
M14 b and d	RFLP	DQ069000 *W. mikolae*	100	GU969270
M14 c	RFLP	AB253523 *Tomentella* sp.	96	GU969269
M15 b, c, and d	RFLP and ITS sequence	DQ069000 *W. mikolae*	100	GU969271
M16 a, b, c, and d	RFLP			
M16 e	RFLP	*W. mikolae*		
M17 a, b, c, and d	RFLP and ITS sequence	AJ534899 *Laccaria* sp.	100	GU969247

Note: Letters a, b, c, d, and e correspond to morphotype replicates. The absence of a certain letter indicates that the DNA isolation from these EM tips failed.

Comparing morphotypes with RFLP pattern and sequencing results, we found that almost all morphotypes described as "E-strain" in this study presented at least one replicate corresponding to the genus *Wilcoxina* and another to the genus *Laccaria*. Only E-strain morphotypes M15 and M17 (*W. mikolae* and *Laccaria* sp., respectively) matched with one taxon. *Wilcoxina mikolae* was also identified in all replicates of morphotype M7 (not classified as E-strain) and by four RFLP replicates of morphotype M11 as well as in M2, M8, M9, M10, M12, M14, and M16 (Table 2). *Laccaria* sp. presented the same RFLP profile in all replicates of M17 and in four replicates of M8, indicating that they correspond to the same species of *Laccaria*, which was also present in M4, M5, M10, M11, and M14 in at least one replicate. The *Laccaria* morphotype was not distinguished by conspicuous morphological features and was found distributed in several morphotype groups (Table 2).

Rhizopogon roseolus was identified in only one morphotype group (M6) with all five replicates (Table 2). Its dichotomous to clustered branching, felty, plectenchymatous, white mantle covered by abundant translucent crystals, simple septa emanating hyphae, and the abundant rhizomorphs, coinciding with the description of Massicotte et al. (1999*b*), make this morphotype easy to recognize. Emanating hyphae ramified in right angles, had occasional contact anastomosis, and were 5–6 μm in diameter. Rhizomorphs were restricted to one point, slightly plumose with smooth margins, slightly ramified in acute angles, anatomically were slightly differentiated, compact, with central hyphae wider than others with dissolved septa, coinciding with DEEMY (Agerer and Rambold 2004–2010). *Hebeloma mesophaeum* was consistently represented by all of the RFLP replicates of two morphotypes, M3 and M13, and by some EM tips also in M4 and M2 (Table 2), indicating that the species has a broad

morphological variation that could include both descriptions. The distinctive features are a white to slightly yellow, cottony, shiny mantle, continuous or in patches, and abundant emanating hyphae sometimes forming plumose rhizomorphs. Microscopically, the outer mantle was plectenchymatous, loosely organized, with interhyphal spaces and the inner mantle was also plectenchymatous but compact. Emanating hyphae were clamped, 3–4 μm in diameter, with smooth or warty walls, ramified in right angles with H-shaped, simple septa anatomosis. Rhizomorphs were restricted to one point, not ramified, anatomically were slightly differentiated, compact, with central hyphae wider than others with dissolved septa and with vesicles with thick walls, not amyloid, coinciding in general with DEEMY (Agerer and Rambold 2004–2010) except for the presence of rhizomorphs, which were also described for the *Hebeloma* sp. – *Pinus strobus* L. morphotype (Ursic and Peterson 1997). *Amphinema byssoides* was present in two RFLP replicates in the M1 and M2 groups and was not found within any other morphotype group. Our description coincides with Massicotte et al. (1999*a*) and Harniman and Durall (1996) in that this morphotype is branched with yellow–white to yellow–brown tips with loosely woolly, patchy mantle and abundant, clamped emanating hyphae. Yellow mycelial strands and granulated hyphae described as typical for this species were not observed. *Tuber* sp. was identified in three root tips from M12 and one root tip of M9 (Table 2). Distinguishing features of this EM include simple or dichotomous branched tips with a smooth, yellow mantle. Microscopically, the outer mantle was plectenchymatous, very loosely organized with abundant interhyphal spaces; the inner mantle was also plectenchymatous but more compact with still interhyphal spaces. The emanating hyphae were simple septa and no cystidia were observed. This description partially coincides with *Tuber* sp. morphotype as described by Pacioni and Comandini (1999).

The molecular data showed a different distribution of fungal species in comparison with the described morphotype data. No taxon appeared exclusively in one nursery, as all 11 taxa were detected in both, indicating that the same EMF are present in both soil textures and nutritional situations.

Discussion

Direct amplification of fungal DNA in combination with morphotyping allowed us to make a detailed assessment of the EMF community associated with the studied conifer nurseries. This interdisciplinary approach together with the fact that is the first study where Patagonian nurseries EMF are genetically evaluated contributes to the general knowledge of this important group of fungi. Our results show that certain EM species (*R. roseolus*, *H. mesophaeum*, *A. byssoides*, and to a lesser extent *Tuber* sp.) form EM with distinctive morphological features that allow reliable identification. It was also evident that different fungi may form indistinguishable morphotypes and that mycorrhizal roots with similar morphologies may be formed by different taxa. This was also observed by, e.g., Menkis et al. (2005) and Pestaña Nieto and Santolamazza Carbone (2009) who found that only 4 out of 33 morphotypes and 7 out of 15 morphotypes, respectively, consisted of only one fungus when molecular tools were used, while Trocha et al. (2006) showed that a number of EM classified as the same morphotypes have important inter- and intraspecific variation. In our study, *W. mikolae* and *Laccaria* sp., although characterized by some distinguishable features at certain stages, appeared widely dispersed within different morphotypes. *Tomentella* sp. and *Wilcoxina* sp. always appeared dispersed within different morphotypes, indicating that they have no clear diagnostic features that allow them to be distinguished under a dissecting microscope. We cannot distinguish from our data whether roots that were morphologically different from each other, yet had the same RFLP patterns, were examples of tips with two EMF occupying the same root, extraradical mycelium amplifying more robustly than the underlying EMF, duo-mycorrhizas (Agerer 2006), or morphologies that we could not separate. These difficulties in identifying EM types are present to some degree whether using morphotyping or RFLP and sequencing to assess the root systems.

Wilcoxina sp. and *W. mikolae* detected during our study are new records for ponderosa pine in Patagonia, and they are the only taxa not previously mentioned for the region from sporocarp surveys and were the only E-strain-associated taxa found in these Patagonian nurseries. They are characterized by a thin mantle (sometimes absent) (Fig. 1, M7 and M15) and, as reported by Yu et al. (2001), the presence of a Hartig net and various degrees of intracellular hyphal penetration into epidermal and cortical cells. Previous studies have reported *A. byssoides*, *H. mesophaeum*, *Hebeloma hiemale* Bres., *Laccaria tortilis* (Bolton) Cooke, *R. roseolus*, and *Thelephora terrestris* Ehrh. fruiting in both nurseries. An unidentified *Tuber* sp. was also reported fruiting in Nursery 1, while *Inocybe kauffmanii* A.H. Smith, *Rhizopogon subolivascens* A.H. Smith, and *Scleroderma areolatum* Ehrenb. were reported in nursery 2 (Barroetaveña and Rajchenberg 2003; Barroetaveña 2004; Barroetaveña et al. 2005). Some of these species were not detected in this study by PCR–RFLP analysis of root tips. They could have been missed somehow because of either seasonal shifts, low morphotype abundance, or misidentication. *Suillus luteus*, widely dispersed and one of the most abundant fruiters in ponderosa pine plantations in Patagonia (Barroetaveña et al. 2005), was not detected either. Very low morphotype abundance already reported for *Suillus pungens* Thiers & A.H. Sm. (Bruns et al. 2002) is a possible explanation. Although performing seasonal samplings and increasing field collection data could improve species richness assessment, these results demonstrates clearly that a combination of morphotyping, molecular typing, and monitoring of fruiting bodies is essential to obtain a complete picture of EM communities.

Conclusions about relative abundance can only be made for *R. roseolus*, which formed only one morphotype, the second most abundant in nursery 1. No conclusions can be drawn about the abundance of the other identified taxa because they were dispersed within different morphotypes, as previously mentioned. The species of *Wilcoxina* have been found growing in EM associations with many tree species (Massicotte et al. 1999*a*; Yu et al. 2001). The genus *Laccaria* is also associated with a wide variety of tree species and is frequent and abundant in nurseries and during the first

years after planting (Kropp and Mueller 1999). Species of *Hebeloma* often appear at early successional stages and different strains have been used as commercial inoculum for tree seedlings (Marmeisse et al. 1999). In Patagonia, *H. mesophaeum* sporocarps have been widely found in nurseries and in up to 20-year-old plantations (Barroetaveña et al. 2005). *Amphinema byssoides* is a very frequent and widely distributed species in nurseries of the western United States as well as in mature boreal forests (Castellano and Molina 1989). In Patagonia, it has been shown to occur frequently in *P. ponderosa* and *Pseudotsuga menziesii* (Mirb.) Franco plantations of different ages and under different precipitation conditions. Similarly, *T. terrestris* in *Pinus* spp. plantations in western Australia fruit in a wide range of conditions (Dunstan et al. 1998). The genus *Rhizopogon* has wide ecological amplitude (Molina et al. 1999); several species of this genus have been reported as dominant EMF in Pinaceae plantations introduced in Australia, New Zealand, and South America. In ponderosa pine plantations in Patagonia, *R. roseolus* has been widely detected and has been found to form abundant sporocarps (Barroetaveña et al. 2005). *Tuber* spp. have been reported both from seedlings in natural forests (Walker et al. 2005) and from artificially inoculated seedlings (Pacioni and Comandini 1999). *Tomentella* is a genus with several ECM species with very wide geographical distribution and variety of tree partners (Erland and Taylor 1999). *Thelephora atra* Weinm. (syn. *Tomentella atramentaria* Rostr.) has been previously reported in ponderosa pine nurseries and plantations in Patagonia (Barroetaveña et al. 2005). It remains to be clarified whether the *Tomentella* sp. we report is the same species. Thus, all taxa found during this study have wide ecological amplitudes, which may explain why EMF species presence was not affected by differences in nursery attributes such as N availability and seedling size (Table 1).

Cylindrocarpon sp. and *P. ostracoderma* are not EMF but are commonly found on plant roots as weak pathogens (Chakravarty and Unestam 1987) or saprophytes (Dennis 1981). These two taxa were identified associated with morphotype M10 where each analyzed tip represented a different fungal species. This morphotype, described as "E-strain with mycelia'' (Table 2), probably represented senescent EM with an overgrowth of *Cylindrocarpon* sp. or *P. ostracoderma* hyphae.

The fungal taxa identified in this study belong to the most common EMF on pine plantations and nurseries around the world. Our results show that no indigenous fungi associated with native *Nothofagus* spp. were found related to *P. ponderosa* seedlings. Only the cosmopolitan species *T. terrestris* and *H. mesophaeum* have been registered from both native forest and exotic conifer plantations in Patagonia (Barroetaveña et al. 2007). These results support previous reports where the EMF species are repeated in different plantations and nurseries within the Southern and Northern Hemispheres (Barroetaveña et al. 2007). The strong evidence that the majority of the EMF species found in ponderosa pine plantations of Patagonia are introduced is correlated with the fact that the original seedlings used for the first ponderosa pine plantations in Patagonia came from Estación Forestal Puerto Anchorena, in Isla Victoria, Río Negro, Argentina. This nursery started its production in 1925 with imported seeds and seedlings (Koutché 1942). After that, two public forest experimental stations with nurseries were established in Patagonia that imported seeds from the United States or from pine stands at Isla Victoria and, lately, from local stands (Barroetaveña et al. 2005). EMF inoculum has probably been introduced and spread with seedlings, either as mycelium or as spores (Dunstan et al. 1998), helping to explain the low EMF species diversity in these nurseries and the presence of the same species in both nurseries. Spread of these fungi in the region has been promoted by the common practice of covering seedbeds with pine leaf litter taken from established pine plantations. This practice is performed in almost all local bare-root nurseries to avoid frost damage (Barroetaveña and Rajchenberg 2003).

In this study, we demonstrated that RFLP analysis and direct sequencing of fungal DNA isolated from EM root tips are powerful tools for identification of fungi in the Patagonian region. Morphotyping proved to be useful to our objectives only for certain EMF species in which some morphological features are conspicuous and unique. This is the first attempt to combine molecular techniques with preliminary mycorrhizal morphotype assessment in cultivated pine nurseries in Patagonia. The knowledge of the species present will allow the assessment of their distribution in the Patagonian environment, which becomes rapidly drier going east from the Andes. It also expands the opportunities for EMF species selection to be used in inoculation of seedlings in nurseries. The results presented here provide a basis for future investigations of fungal community richness in native *Nothofagus* forests, biological and ecological interpretation of succession, and development of forest plantations with controlled EM inoculations.

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