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Structure and species composition of ectomycorrhizal fungal communities colonizing seedlings and adult trees of *Pinus montezumae* in Mexican neotropical forests

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Abstract

Mexico is a center of diversity for pines but few studies have examined the ectomycorrhizal (ECM) fungal communities associated with pines in this country. We investigated the ECM communities associated with *Pinus montezumae* seedlings and mature trees in a neotropical forest of central Mexico, and compared their structure and species composition. Root tips were sampled on both planted seedlings and naturally-occurring adult trees. A total of 42 ECM Operational Taxonomic Units (OTUs) was found on *P. montezumae*. Diversity and similarity indices showed that community structure was similar for both plant growth stages, but phylogenetic diversity and Chao estimated richness were higher for seedlings. Species composition differed between communities. The dominant OTUs belonged to the families Atheliaceae, Cortinariaceae and Sebacinaceae, although different taxa appeared to colonize seedlings and adults. Only 12 OTUs were shared between seedlings and adults, which suggests that ECM fungi colonizing seedlings are still not fully incorporated into mycelial networks and that ECM taxa colonizing young individuals of *P. montezumae* are likely to come from fungal propagules. Intra-generic diversity could be an insurance mechanism to maintain forest productivity under stressed conditions. This is the first report describing the abundance of Atheliaceae in tree roots in neotropical ecosystems.

Key words: Ectomycorrhizas; Atheliaceae; *Inocybe*; tree growth stages; phylogenetic diversity.

Introduction

The importance of ectomycorrhizal (ECM) fungi for tree growth and development and particularly for seedling establishment has been widely demonstrated (Teste *et al.*, 2004; Nara, 2006; Ding *et al.*, 2011). The ECM fungi favour seedling growth by increasing their uptake of water and nutrients, enhancing fine root longevity, protecting the host against potential root pathogens and by helping to maintain soil structure (Smith and Read, 2008). Thereby, they facilitate natural regeneration of forests, especially where harsh environmental conditions may reduce seedling survival (Horton *et al.*, 1999).

Establishing seedlings are either colonized by ECM fungi present in the form of propagules, such as spores or sclerotia, or by mycelium originating from the roots of mature co-occurring trees (Dahlberg *et al.*, 1997; Simard and Durall, 2004). Mycelial networks are usually present in forest soils where they connect the roots of young and adult trees. They confer a higher potential to those ECM taxa incorporated into the network to colonize emerging root tips because of the carbohydrates they received from the host trees (Jonsson *et al.*, 1999; Teste *et al.*, 2009). This mycorrhizal network-mediated colonization of seedlings has been supported by some evidence demonstrating that seedlings planted near adult trees have higher ECM fungal diversity than those planted in clearcuts (Durall *et al.*, 1999; Cline *et al.*, 2005). The continuity between seedling and mature ectomycorrhizal hosts has been assessed for boreal and temperate forest ecosystems (Jonsson *et al.*, 1999; Matsuda and Hijii, 2004; Nara, 2006; Aučina *et al.*, 2011). However, there is little information concerning ECM fungal communities colonizing seedlings and conspecific trees in neotropical and tropical ecosystems.

Mexico is a center of diversity for the genus *Pinus* with 75 taxa registered in the country (Perry, 1991). Pines are obligate ECM symbionts and depend on their association with ECM fungi to be able to survive and establish successfully (Pérez-Moreno and Read, 2004). Paradoxically, few studies have examined the ECM fungal communities associated with pines in Mexico (Reverchon *et al.*, 2010a). *Pinus montezumae* Lamb. var. *montezumae* is one of the most common pine species in Central and Southern Mexico (Leopold, 1950), especially in forests of the Mexican Transvolcanic Belt growing below 3200 m.a.s.l. It is also an important tree species for reforestation and has a great economic value as it is used for saw timber, cellulose and fire wood (Viveros-Viveros *et al.*, 2007). A previous sporome sampling identified various ECM fungal genera associated with *P. montezumae* such as *Amanita*, *Boletus*, *Cortinarius*, *Hygrophorus*, *Inocybe*, *Laccaria*, *Lactarius*, *Russula*, *Suillus*, and *Tricholoma* (Reverchon *et al.*, 2011). An account of ECM taxa on roots of *P. montezumae* was published recently by Kennedy *et al.* (2011) in a study of Mexican *Alnus* forests where *P. montezumae* was incidentally co-dominating these ecosystems with *Alnus jorullensis*. However, the belowground ECM taxa associated with *P. montezumae* have not been previously described in detail. The objective of the present study was therefore to determine the belowground ECM fungal taxa colonizing *P. montezumae* in Mexican neotropical forests and to compare the ECM community composition of both seedlings and mature trees.

Materials and methods

Study sites

This study was conducted in native, mature pine forests located in Corredor Biológico Sierra del Chichinautzin, a protected area at the southern margin of Mexico City. The study area was extensively

described elsewhere (Reverchon *et al.*, 2010a, 2010b). These forests grow on soils of volcanic origin and are dominated by *P. montezumae*, especially above 2000 m.a.s.l. The mean annual temperature in the region ranges from 10 to 14°C and the mean annual precipitation varies from 1000 to 1200 mm, with 80% occurring during the rainy season (from June to October).

To examine the ECM fungal communities associated with *P. montezumae*, we selected three forest sites within the core zone of the protected area, where no management was allowed. The three sites were located on the slope of volcanoes Chichinautzin (UTM coordinates: X: 482,041, Y: 2,109,907), Guespalapa (X: 482,037, Y: 2,109,903) and Pelado (X: 475,922, Y: 2,114,796) respectively. All sites were established at 3100 m.a.s.l., on slopes of less than 10°, with a southern orientation. Forest turnover at the three sites was approximately 150 years. Soil properties of the three sites are reported in Reverchon *et al.* (2010a).

Sampling design

At each site, five sampling plots of 100 m² were established around five adult *P. montezumae* trees of approximately 80 years old. These trees were chosen as isolated as possible from other ECM hosts. To study the ECM fungal communities associated with seedlings, we planted 80 seeds of *P. montezumae* in each sampling plot, at approximately 3 m from the central adult tree, in October 2006. This approach was chosen because of the low regeneration rates of *P. montezumae* trees in the area, and hence of the low occurrence of naturally-regenerated seedlings. Additionally, since our study sites belonged to a protected area, we were unable to harvest naturally-occurring trees. The seeds were provided by a local nursery and were collected in the Sierra del Chichinautzin area, at 2800 m.a.s.l. Their viability was c. 90%. Prior to planting, the seeds were washed in a solution of 30% hydrogen peroxide for 25 min in order to remove potential germs. In February 2008, all surviving seedlings (74) were harvested carefully to obtain the entire root system. In total, 20, 10 and 44 seedlings were harvested at sites

Chichinautzin, Guespalapa, and Pelado respectively. The low observed survival rates were due to a combination of harsh conditions prevailing during the dry season in the Sierra and to an unusually high predation by moles at the Guespalapa site. The average shoot height of the seedlings after 16 months was 10.1 ± 2.3 cm.

Sampling of the ECM root tips of adult trees was carried out at the end of the rainy season. Four soil cores per plot were collected at 1 m from the trunk of the central pine tree. These soil cores measured 20 cm in depth and 2.5 cm in diameter, except at Chichinautzin site where soil depth was only 6 cm (Reverchon *et al.*, 2010b). At this site, smaller and wider cores were used (5 cm depth, 5 cm diameter) in order to sample the same volume of soil. All soil cores were stored at 4°C for a maximum of two weeks before being processed.

Ectomycorrhizal root tip processing and molecular analysis

The ECM colonization of seedlings was determined by quantifying the extent of conversion of lateral root to mycorrhizas expressed as percentage. While entire root systems were recovered from seedlings, root tips of mature trees were gently teased out of soil cores under running water and examined under a stereo dissecting microscope. Live ECM root tips were separated into putative morphotypes based on standard morphological characters such as color, texture, size, branching patterns and presence of emanating hyphae and rhizomorphs. For each morphotype, up to three healthy ECM root tips were placed in a 1.5 mL microcentrifuge tube and stored at -70°C prior to DNA extraction.

Total DNA was extracted using the REDExtract-N-Amp Plant PCR kit (Sigma-Aldrich). Individual root tips were added to 10 µL of extraction solution and cycled at 65°C for 10 minutes, followed by 95°C for 10 minutes. After incubation, 30 µL of dilution solution were added and the samples were first incubated during one hour at room temperature and then stored at -20°C. Amplification by PCR was carried out in 25 µL reactions containing 5 µL of the extracted DNA, 10 µL of PCR mix (Sigma-

Aldrich), and 0.25 µL of primers ITS1F and ITS 4 (Gardes y Bruns, 1993). PCR cycling parameters were as follows: 94°C for 1 min; 35 cycles at 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min; and a final 8-min 72°C extension. Initial PCR success varied depending on the samples, and each extraction product which did not amplify at the first time went at least through two more PCR rounds.

Successfully-amplified PCR products were then purified through mini-columns (Wizard SV Gel and PCR Clean-Up System, Promega). DNA sequencing was performed with the same primers on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Laboratorio de Biología Molecular, Instituto de Biología, UNAM.

Phylogenetic and statistical analyses

Verified sequences were assigned to a taxonomic category with The Fungal ITS Pipeline 1.0 (Nilsson *et al.*, 2009). Best matches obtained from this pipeline and from the UNITE database (Kõljalg *et al.*, 2005) were used as reference sequences to construct a reference alignment with M-Coffee from the T-Coffee distribution (Notredame *et al.*, 2000). Our sequences were then sequentially aligned to the reference alignment with the “align sequences to profile” options in T-Coffee. All alignments were manually inspected in BioEdit (Hall, 1999). A phylogenetic tree was constructed from the reference alignment with PhyML 3.0 (Guindon *et al.*, 2010) using the GTR model with parameters estimated with ModelGenerator (Keane *et al.*, 2006).

Sequences were clustered into Operational Taxonomic Units (OTUs) with a cutoff value of 93% as recently recommended by Hibbett *et al.* (2011), with the software mothur (Schloss *et al.*, 2009).

Relative abundance of ECM taxa was measured by the number of sequences belonging to each OTU divided by the total number of sequences obtained for seedlings (41) and for adult trees (55).

Rarefaction curves were drawn from values calculated in mothur to compare taxa richness for seedlings and mature trees of *P. montezumae*. Diversity and phylogenetic indices were calculated with mothur

and with the *vegan* (Oksanen *et al.*, 2007) and *picante* (Kembel *et al.*, 2010) packages in R.

Results

On average, 75% of the root tips of seedlings were colonized by ECM fungi (Supplementary Table 1). We recovered 2328 ECM root tips from the 74 harvested seedlings and 1305 root tips from the 46 of the 60 soil cores where the presence of ECM root tips was recorded. From these root tips, we morphologically characterized 32 and 25 morphotypes for seedlings and adult trees respectively. A total of 272 root tips (130 sampled on seedlings and 142 on adult trees) was processed for molecular analysis. In total, 43 OTUs were identified, 26 of which were found on the roots of *P. montezumae* seedlings and 29 associated with adult trees (Supplementary Table 2). One of these OTUs, found in a seedling at Chichinautzin, was assigned to the species *Oidiodendron maius*, an ericoid mycorrhizal fungus widely distributed in subtropical areas (Vallino *et al.*, 2011), and thus was not considered as being part of the ectomycorrhizal community.

The Chao estimated richness was almost twice as high as the actual number of OTUs found on the roots of *P. montezumae* (Table 1). Only 12 OTUs (28%) were common to both ECM fungal communities: Atheliaceae sp.1 and sp.2, *Clavulina* sp., *Inocybe praetervisa* and *Inocybe* sp.4, *Russula abietina* and *Russula* sp.1, Sebacinaceae sp.1 and sp.3, Thelephoraceae sp.2 and sp.3, and *Tomentella* sp.1 (Table 2). In total, 9 different fungal families were identified on the roots of *P. montezumae*, resulting in a large phylogenetic diversity (PD=19.75). Although diversity indices were slightly higher for the ECM fungal community colonizing adult trees, the overlapping of confidence intervals in the rarefaction analysis suggests that there were no significant differences between both community structures (Fig.1). According to the Chao index, a higher richness would be expected in seedlings (69)

than in adult trees (Chao=56), as well as a higher phylogenetic diversity (PDadults=11.996, PDseedlings=12.817). The UniFrac metrics also revealed that both ECM communities were phylogenetically distinct (UniFrac=0.640, $p < 0.0001$). Altogether, these data depict two communities with a similar structure, composed of a few dominant OTUs (Sebacinaceae sp.1, Atheliaceae sp.1 and *Inocybe* sp.4) and of a large number of rare OTUs (Fig.2), but with differences in species composition.

A taxon belonging to the Sebacinaceae (Sebacinaceae sp.1) was predominant on the roots of seedlings. Atheliaceae sp. 1 together with *Inocybe* sp. 4 were the other dominant taxa colonizing seedlings and were also the most abundant taxa on the roots of mature trees (Fig. 2). Members of Cortinariaceae and Thelephoraceae were abundant on colonized roots of both seedlings and adults (Fig. 3). Some fungal genera seemed to be specifically present on seedling roots, such as *Cenococcum*, *Cortinarius* or *Rhizopogon*, whereas taxa associated with older trees generally belonged to the same dominant families: Atheliaceae and Sebacinaceae, and to the genera *Inocybe* and *Russula*.

Discussion

We investigated the ECM species composition on the roots of seedlings and adult trees of *P. montezumae* in mature neotropical forests and showed that while the structure of both ECM fungal communities was similar, their species composition was different. The relatively high richness and phylogenetic diversity found for ECM fungal communities associated with *P. montezumae* confirm the pattern observed by Kennedy *et al.* (2011) who detected 24 ECM taxa from 42 ECM root tips and concluded that *Pinus*-associated fungal communities were more diverse than the ones associated with *Alnus* in montane forests of Central Mexico. This is also in agreement with previous studies of ECM fungal communities associated with conifer species which report phylogenetically diverse belowground

communities (Dahlberg *et al.*, 1997; Peter *et al.*, 2001a; Matsuda and Hijii, 2004; Wang and Guo, 2010).

Diversity and similarity metrics showed that ECM fungal communities colonizing seedlings and adult trees were similar in terms of structure. However, both Chao estimated richness and phylogenetic diversity were higher in seedlings than in adult trees, indicating that the species composition of the two communities was different. While the dominant OTUs belonged to the fungal families Atheliaceae, Cortinariaceae (*Inocybe*) and Sebacinaceae, on both seedlings and adult roots, they were composed of different taxa and were present in different proportions. Only 12 OTUs were common to both seedlings and adults, which suggests that seedlings of *P. montezumae* were still not fully incorporated into mycorrhizal networks. Most of the published works on seedlings and co-occurring trees report a large proportion of ECM taxa common to both growth stages (Jonsson *et al.*, 1999; Cline *et al.*, 2005).

The most abundant OTU was Atheliaceae sp.1, which dominates the ECM community associated with adult trees and was also abundant on the roots of seedlings. Atheliaceae sp.2 was also dominant on adult trees, and overall Atheliaceae was one of the most abundant family in the community (7 OTUs). This suggests that taxa belonging to Atheliaceae may play important ecological roles in nutrient transfer at those sites. This is the first report describing the abundance of Atheliaceae in tree roots in neotropical ecosystems. However, species belonging to Atheliaceae have been reported to be efficient colonizers of seedlings and important components of the belowground ECM community in temperate and boreal forests, in particular in degraded sites (Hedh *et al.*, 2008; Peter *et al.*, 2008; Kalliokoski *et al.*, 2010). A *Piloderma* species was shown to dominate the belowground ECM community in *Pinus sylvestris* forests of Sweden (Jonsson *et al.*, 1999), and high abundances of up to 70% on the root system of adult trees were reported for *Tylospora fibrillosa* from monocultures of Sitka spruce growing in frequently water-logged, anoxic soils in Northern England (Palfner *et al.*, 2005). Some members of the Atheliaceae, such as *Tylospora fibrillosa*, are known to have decomposing and proteolytic abilities

(Cairney and Burke, 1994). Such fungi may therefore provide their host with nutrients which are otherwise unavailable for other ECM fungal species, conferring them a potential advantage to grow on nutrient-poor soils.

The dominant OTU occurring on seedling roots was an uncultured member of Sebacinaceae (sp.1) of which only one sequence was found on adult trees. Altogether, Sebacinaceae was the third most abundant family (16 sequences) but it presented a relatively low OTU diversity (5 OTUs only). The most abundant Sebacinaceae OTUs on adults (sp.4 and sp.5) had no representatives on seedlings, suggesting that the presence and abundance of particular species within the Sebacinaceae family may be affected by the plant growth stage. Cortinariaceae was the most abundant (23 sequences) and one of the most diverse families identified (10 OTUs), and only two OTUs were shared between seedlings and adults. *Inocybe* is a species rich, widely distributed, and commonly encountered genus (Ryberg *et al.*, 2008), especially in harsh environments (Nara, 2006). Whereas this genus has been reported as an abundant component of ECM roots in temperate and boreal ecosystems (Glowa *et al.*, 2004; Tedersoo *et al.*, 2006; Ryberg *et al.*, 2009), our study shows that *Inocybe* is also a dominant ECM component of roots in neotropical forests. A large OTU richness of the genus *Inocybe* has already been reported in previous studies (Gao and Yang, 2010; Vásquez Gassibe *et al.*, 2011) and could have implications for intra-generic functional diversity and therefore for ensuring forest productivity under stressed conditions.

Similarly to what was found in temperate and boreal ecosystems (Taylor and Alexander, 1989; Peter *et al.*, 2001b), there is a poor correlation between the above and belowground ECM communities in the studied neotropical forest, as confirmed by an ECM sporome survey (Reverchon *et al.*, 2011) conducted in parallel to this study. Taxa such as Atheliaceae and Sebacinaceae, which were found abundantly on the root systems of both seedlings and adult trees of *P. montezumae*, were not sampled in the sporome collection because of the inconspicuous nature of the fruiting bodies formed by these

fungi. Our study therefore confirms that the assessment of ECM fungal communities based upon ECM root tips and fruiting body studies provide different partial accounts of the existing ECM diversity (Aučina *et al.*, 2011). Nevertheless, some fungal genera were found to be abundant at both the aboveground and belowground levels. This was the case for *Inocybe* and *Russula*, for which we determined 15 and 17 species respectively in our sporome collection (Reverchon *et al.*, 2011). *Inocybe cookei* Bres. and *I. fastigiata* (Schaeff.) Quél. were particularly dominant in the sporome community at the 3 sites and were proposed to be used as an inoculum source in reforestation programs of the area (Reverchon *et al.*, 2010a).

In this study we sequenced the ITS region of the nuclear rDNA, which is the most commonly sequenced genetic marker for molecular identification of fungi from environmental samples (Nilsson *et al.*, 2010). Bellemain *et al.* (2010) recently suggested that environmental surveys of fungal ITS may be biased towards specific taxonomic groups depending on primer pair selection. They showed that primer ITS1F preferentially amplified basidiomycetes while primer ITS4 preferentially amplified ascomycetes. Moreover, the amplification of the whole ITS region might also be biased towards the amplification of ascomycetes. This is however especially apparent during high-throughput sequencing, which was not employed in this work. Here we used strict PCR conditions which may prevent, in the case of ITS1F, the amplification of certain fungal groups. This, together with the possibility of taxonomic biases created by the use of standardized DNA extraction protocols, could explain our low amplification rate (50%), despite the existence of a prominent mantle on most of the sampled root tips. Nevertheless, and although all PCR-based studies are subject to potential taxonomic biases, our results suggest that the selected primer pair represented a good compromise between the biases towards ascomycetes and basidiomycetes. Only one sequence from ascomycetes was found in our analysis, even when using the whole ITS region was supposed to favour the amplification of this group.

In summary, a total of 42 ECM OTUs were observed in the present study, and fungal species composition varied between growth stages of *P. montezumae*. Although the number of obtained sequences was relatively small, these differences in ECM fungal community composition suggest that seedlings at Sierra del Chichinautzin are still not fully incorporated in the mycelial networks existing in the soil of mature native forests, and that ECM taxa colonizing young individuals of *P. montezumae* are likely to come from fungal propagules. Nevertheless, ECM fungal communities colonizing both seedlings and adults were dominated by members of the Atheliaceae family and by the fungal genus *Inocybe*. The latter was also found to dominate the ECM sporome community of these forests and *Inocybe* species could hence be used as a potential source of inoculum in the reforestation programs carried out in this protected area.

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Figure captions

Fig. 1. Rarefaction curves of ectomycorrhizal taxa richness on seedlings (▲) and adult trees (■) of *Pinus montezumae* with 95% confidence intervals calculated in Mothur (Schloss *et al.*, 2009). OTUs were assigned at 93% similarity for these analyses.

Fig. 2. Rank-abundance curves for the ectomycorrhizal fungal communities colonizing seedlings and adult trees of *Pinus montezumae* at Sierra del Chichinautzin. The values in parentheses represent the total number of sequences obtained for this OTU. Grey bars represent OTUs common to both seedlings and adults; black bars represent OTUs only found on seedlings or adult trees.

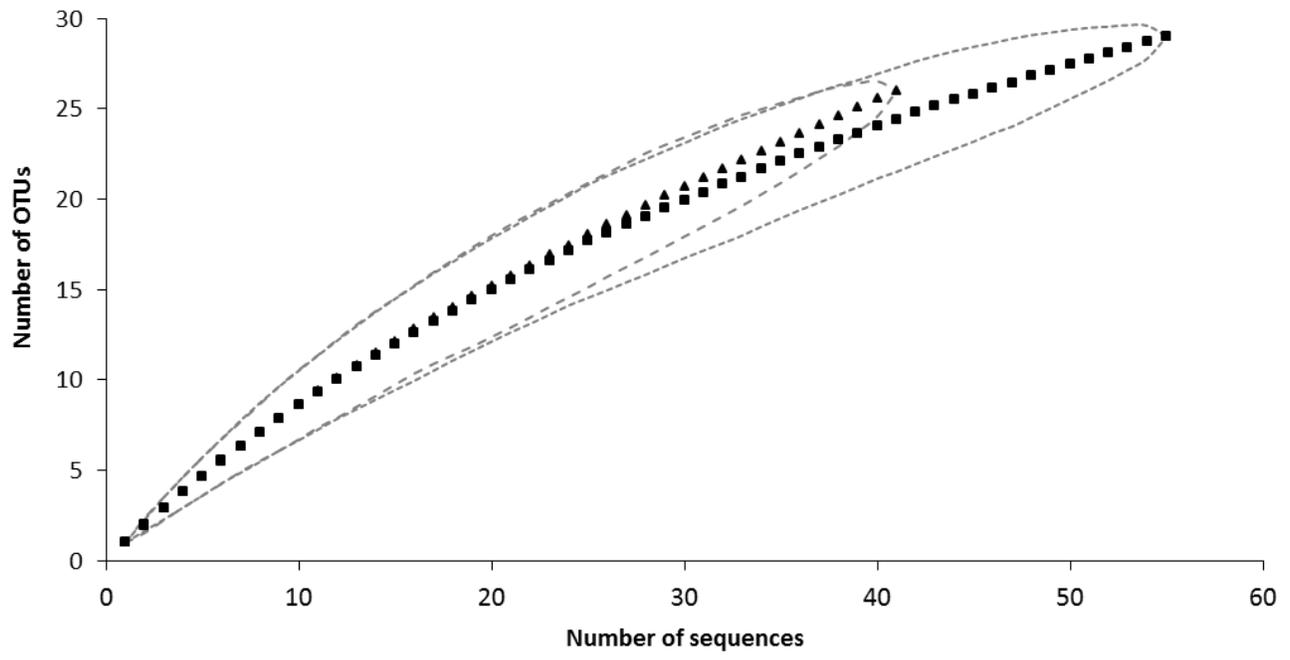
Fig. 3. Maximum likelihood phylogenetic reconstruction of OTUs obtained from rRNA-ITS sequences of ectomycorrhizal fungi associated with *Pinus montezumae*. Bold circles represent nodes supported by aLRT scores above 0.70. Horizontal bars represent the abundance of these OTUs on seedlings and adult trees respectively.

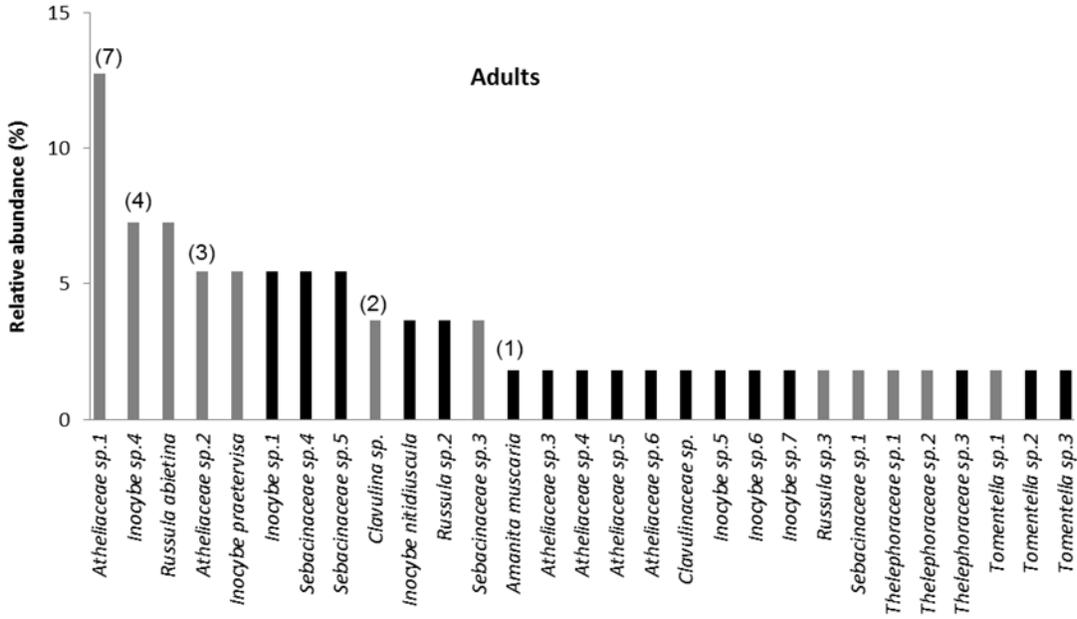
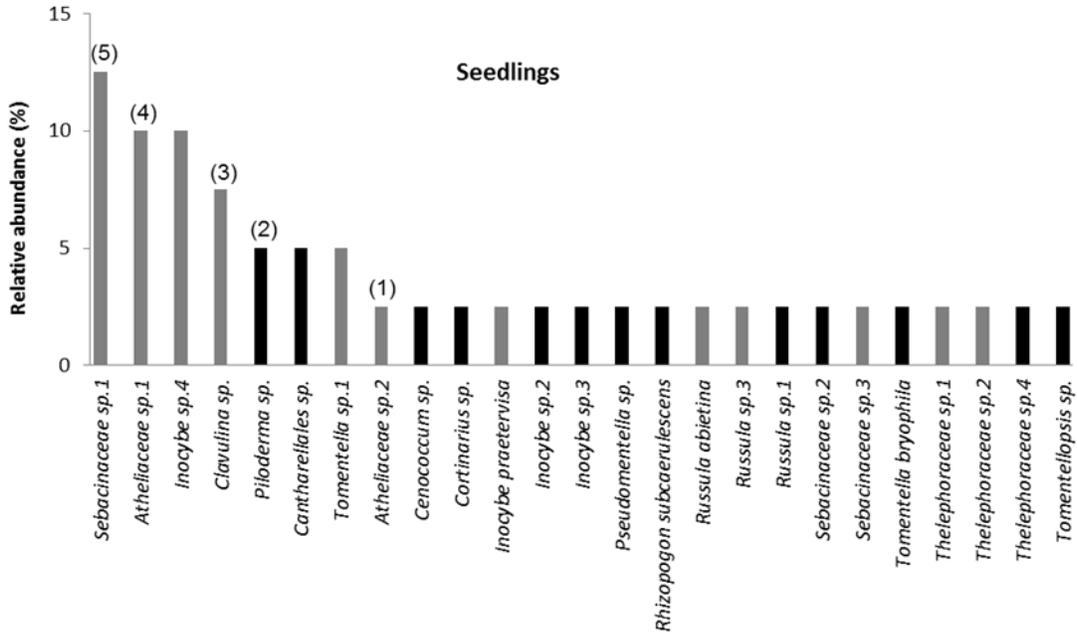
Table 1. Alpha and Beta diversity of ectomycorrhizal fungal communities on seedlings and adult trees of *Pinus montezumae*. Estimated richness and diversity indexes were calculated in the program R (R Development Core Team, 2009)

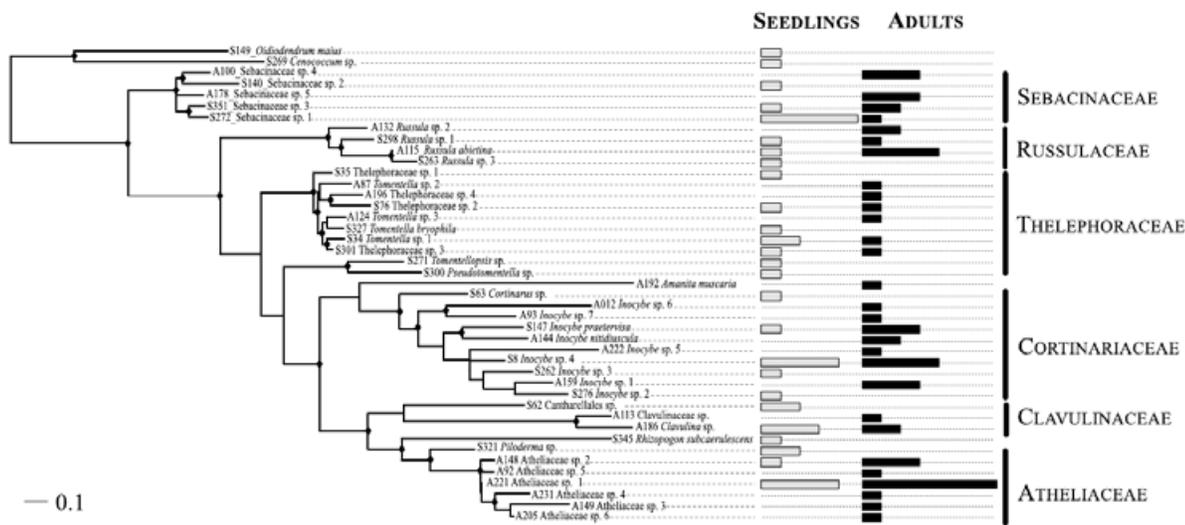
	Alpha diversity			Beta diversity	
	Seedlings	Adults	TOTAL		
Number of observed taxa	25	29	42	Observed shared taxa	12
Chao estimated taxa	69	56	78	Chao estimated shared taxa	27
Shannon diversity index	3.06	3.16	3.45	Sørensen similarity index	0.436
Simpson diversity index	0.034	0.035	0.033	Bray-Curtis similarity	0.396

Table 2. Number of sequences and Operational Taxonomic Units (OTUs) per ectomycorrhizal fungal taxon, in total and shared between seedlings and adult trees of *Pinus montezumae*

	Number of sequences	Total number of OTUs	Number of shared OTUs
<i>Amanita</i>	1	1	
<i>Cenococcum</i>	1	1	
<i>Rhizopogon</i>	1	1	
Clavulinaceae	8	3	1
Russulaceae	10	4	2
Thelephoraceae	14	10	3
Sebacinaceae	16	5	2
Atheliaceae	21	7	2
Cortinariaceae	23	10	2







— 0.1