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Co-invasive exotic pines and their ectomycorrhizal symbionts show capabilities for wide distance and altitudinal range expansion

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ABSTRACT

We asked if exotic *Pinus elliotti* seedlings can survive and form ectomycorrhizas at higher elevations and long distances from their current range, and which ECM partners disperse to these soils. We selected three plots at four grassland sites along an altitudinal gradient (900, 1600, 2200, and 2700 m asl) established at *c.* 110, 3000, 6000, and 9000 m from the closest pine plantation, respectively. We combined field experiments with glasshouse assays to assess survival and ECM fungi in roots and soils. A pine plantation close to the lowest site was also selected for DNA metabarcoding of soils. Pine seedlings survived at all altitudes but not all formed mycorrhizas. They formed mycorrhizas with *Suillus granulatus* at 900, 1600, and 2200 m asl (i.e. up to 6000 m from the closest pine plantation), and with *Rhizopogon pseudoroseolus* and *Thelephora terrestris* at lower altitudes and distances. Twelve ECM fungal OTUs were found in grasslands and 34 were detected in the pine plantation. Although richness and abundance of ECM fungi decreased with increasing distance from the pine plantation, there was at least one non-native ECM fungal species present in each sampling site, even at 2700 masl and 9000 m distance from the closest plantation. This study provides evidence that the availability of suitable fungal symbionts might constrain but not hinder the expansion of a pine species over wide distances and altitudinal zones even in areas with no native ECM fungi.

1. Introduction

Biological invasions are recognized as a major threat to biodiversity (Sala et al., 2000). The mechanisms behind non-native plant expansion on an exotic range have been shown to be complex and variable across ecosystems (Levine et al., 2003).

The altitudinal expansion of non-native species over high elevation environments is gaining interest in the last decade and is increasingly documented in the literature (e.g. McDougall et al., 2011; Alexander et al., 2011; Pollnac and Rew, 2014; Tecco et al., 2016). It was recently shown that non-native species with broad climatic tolerances, rather than specialized stress tolerants, are capable of expanding from low to high elevations along the corridors of introduction (Alexander et al., 2011; Tecco et al., 2016). This broad climatic tolerance combined with enough residence time in the region (e.g. Haider et al., 2010; Pyšek et al., 2011) may underlie the success of non-native species at high elevational ranges.

Many plant species form obligatory symbiotic interactions, such as mycorrhizas, to establish, grow, and reproduce (Smith and Read, 2008). Therefore, most exotic plant species also need to rely on compatible belowground mutualists in their new environments (Nuñez and Dickie, 2014). For these plants, three major strategies have been proposed (Dickie et al., 2010): (a) those that can establish symbiosis with the native organisms in the invasive range (novel mutualisms), (b) those that establish symbioses with native organisms that are also native to the home range of the invasive plant (cosmopolitan mutualisms), and (c) those that establish symbioses with invasive organisms (co-invaders).

Pines are globally distributed woody invaders (Richardson, 2006; Rejmánek and Richardson, 2013). They establish obligatory symbioses with ectomycorrhizal (ECM) fungi and cannot establish and survive for long periods without them (Allen, 1991). Therefore, both symbionts, the pine and the fungi, need to co-invade in habitats in which compatible ECM fungal symbionts are absent in native plant communities. It is now well documented that both pines and ECM fungi are able to disperse or escape from plantations and to get co-established in pine-free habitats (e.g. Dickie et al., 2010; Hayward et al., 2015a, 2015b; Hynson et al., 2013; Nuñez

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et al., 2009, 2013; Salgado Salomón et al., 2011). However, the evidence is restricted to horizontal expansion (i.e. similar altitudes) and short distances from the plantations (i.e. ≤ 1000 m). It is not known whether pines and ECM fungi can co-occur and survive outside plantations at longer distances and elevations in which they might be constrained by climate conditions and/or dispersal limitations. Moreover, previous studies on pine expansion have identified those fungal species that colonize pine roots, but did not provide evidence on the complete pool of ECM fungi that can disperse over considerable distances and provide suitable conditions for the invasion front of pines. In other words, we do not know their capabilities to expand to altitudinal zones in which they currently are not present.

Despite the traditional belief that mountains are less prone to invasions than other ecosystems, there is increasing attention to the expansion of alien species over high elevation environments (e.g. Pauchard et al., 2009). In central Argentina, the Sierras de Córdoba mountain range includes a wide elevational gradient (500–2790 m asl) that is threatened by an incipient spread of woody alien species from lower altitudes (Giorgis et al., 2011). Extensive plantations of *Pinus elliottii* were established in the lower belts of these mountain ranges. In certain areas, some individuals have already escaped from plantations and have expanded to higher altitudes, but not above 1850 m asl (Giorgis et al., 2011). In these ecosystems there are no native ECM host plants.

Expansion of woody aliens over altitudinal ranges has been poorly studied in comparison to other growth forms such as herbs (McDougall et al., 2011). Moreover, most of the evidence on the occurrence of non-native flora along elevational gradients comes from species that are already established in their non-native ranges. However, current altitudinal limits of plant distribution may not necessarily reflect their actual climatic tolerance (Araújo and Pearson, 2005; Tecco et al., 2016). This could be the case of *P. elliottii*, i.e. we cannot currently predict if climatic factors and/or lack of compatible fungal symbionts (e.g. Peay et al., 2012) will constrain their expansion to more distant areas and higher altitudes where they currently are absent. For this reason, we combined field experiments with glasshouse essays and DNA metabarcoding analysis of soil samples taken along an altitudinal gradient that is threatened by an incipient spread of exotic *Pinus elliottii* to assess: (a) if pines can survive and form ectomycorrhizas at elevations outside of their current range and (b) which fungal partners are present in soils at different altitudes (900–2700 m asl) involving long distances from plantations (i.e. 100–9000 m).

2. Materials and methods

2.1. Study site

The study was conducted in the Sierras Grandes mountain range in central Argentina. The experimental plots were placed along an altitudinal gradient ranging from 900 m asl to 2700 m asl (Linderos road, 32° 50'S, 64° 90'W), near the highest peak of the mountain range. The gradient comprises the following vegetation belts described by Cabrera (1976) for the Mountain Chaco District: (1) the upper portion of Chaco mountain woodlands, which is distributed from 400 to 1300 m asl; (2) an intermediate belt devoid of forest currently occupied by mountain grasslands and shrublands (1300–1700 m asl); and (3) a mosaic of high mountain grasslands and *Polylepis australis* (Rosaceae) woodlands (above 1700 m asl). There are no ectomycorrhizal species in these ecosystems. Along the gradient, temperatures vary from temperate-warm to temperate-cold. Specifically, mean annual temperature at the lower end of the gradient (900 m asl) is 15.7 °C dropping to 7.4 °C near the summit at 2700 m asl (Marcora et al., 2008). There is no frost-free period over the 1800 m asl. Mean annual precipitation varies between 750 and 970 mm, with most rainfall concentrated in the warmer months, from October to April (Cabido, 1985; Colladon et al., 2010). The main economic activity is livestock rearing that had begun in the early 17th century and had completely replaced the native herbivore (*Lama guanicoe*) by the beginning of the 20th century (Díaz et al., 1994).

2.2. Study species

Pinus elliottii (slash pine) is native to the southeastern United States. The rainfall in its native range averages about 1270 mm and is concentrated mainly in the warmer period. The mean annual temperature in the slash pine region is 17 °C (Burns and Honkala, 1990).

Together with other *Pinus* species, it was introduced to central Argentina in the 1960s (Ferchmin, 1969) where it now reproduces and expands naturally (Giorgis and Tecco, 2014). In the sampling region, several ECM fungi have been observed to fruit in *P. eliottii* plantations, such as *Endogone* sp., *Rhizopogon* spp., *Inocybe* sp., *Paxillus* sp., *Scleroderma* spp., *Suillus* spp., *Amanita muscaria*, and *Thelephora terrestris* (Nouhra, 1999; Nouhra et al., 2008, 2012; pers. obs.).

2.3. Experimental design

We transplanted seedlings and tested their survival along the contrasting environmental conditions that characterize the altitudinal gradient. The field experiment was complemented with glasshouse assay evaluating whether soils from different altitudes contained mycorrhizal inocula to colonize roots.

Four sites were selected along the altitudinal gradient, placed at intervals of *c*. 400–600 m asl (965, 1600, 2248, and 2685 m asl). All sites were grasslands established on ridges with similar gentle slopes and high solar insolation (see Tecco et al., 2016 for more details). At each altitudinal site, three plots (4 × 4 m) were selected within a livestock exclosure. These sites were established at a distance of *c*. 110, 3000, 6000, and 9000 m, respectively, from the closest pine plantation. We cannot discard the occurrence of some scattered pine seedlings in between the plots and the plantations but they would not be a significant source of fungal propagules in comparison with the thousands of hectares of mature pine plantations. Moreover, pines are absent above 1850 m asl (Giorgis et al., 2011). For simplicity, altitudinal sites will be hereafter referred as: 900, 1600, 2200, and 2700 m asl.

2.4. Seedling production

Seeds were provided by the Instituto Nacional de Tecnología Agropecuaria Montecarlo (Misiones, Argentina). The seeds were surface sterilized with 10% sodium hypochlorite for 10 min, submerged in water for 24 h and then stored at 4 °C for 45 d. Then, they were germinated in a glasshouse in an autoclaved mix of sand and native soil (2:1v v^{-1}). After 20–25 d, these seedlings were used for glasshouse and field experiments.

2.5. Glasshouse experiment

Seedlings were transplanted at the same time to 48 pots (500 cm^3). All pots contained 450 ml of autoclaved mix of sand and native soil (2:1 v v^{-1}). Soil suspensions from each altitude were used as soil inocula. We diluted a soil fraction in sterile water (1:5 v v^{-1}) and added 40 ml of this solution to six pots. In the same way, a sterilized soil fraction was diluted in sterile water and 40 ml of this solution was added to another six pots as control for possible

contamination (i.e., 12 pots *per* altitude, six in sterilized and six in non-sterilized soils, 48 pots in total). Plants were grown in the glasshouse under temperatures ranging from 15 °C to 25 °C and without water stress (daily watering with tap water). The pots were rotated weekly to avoid any potential artifacts related to their position in the glasshouse.

After 160 days, plants were harvested, washed, and were separated into shoots and roots. Shoots were dried at 60 °C for 72 h, and were weighed. Roots were carefully separated from soil and were subjected to morphotyping and molecular analyses as described below.

2.6. Field experiments

Two field experiments were carried out. In both experiments the seedlings were transplanted to three plots at each altitude. They were protected with cylindrical cages staked to the ground. The 45 cm diameter × 25 cm height cages were built on a stainless steel cylindrical structure covered with a 1 mm mesh stainless net (to allow water run). The upper extreme of the cylindrical cages was covered (after seedling addition) with a 1 cm mesh stainless net. Seedlings were placed within the cages, avoiding the 10 cm belt adjacent to the cage edge. Although cages might have modified the natural environmental conditions, it constituted a precaution to prevent any accidental movement in an area were the invasive species are still absent. Nevertheless, any potential effects of cages (e.g. shading) were presumably uniform for all altitudinal sites and were not expected to mask the overall altitudinal differences in environmental conditions.

2.6.1. Field experiment 1

From November 16, 2011 to November 2, 2012.

Four seedlings were transplanted to each of three plots at each altitude at the beginning of the growing season (12 *per* altitude, 48 seedlings in total). In this experiment only seedlings at lower altitudes survived after nearly 1 yr (Table 1) and, therefore, we were not able to assess mycorrhizal colonization at higher altitudes. In addition, we were not able to distinguish if mortality was due to harsh climate in winter at higher altitudes or to the lack of fungal inocula, although many seedlings survived without ectomycorrhizas at lower altitudes, as discussed below. Because of this, we carried out a second field experiment.

2.6.2. Field experiment 2

From January 3, 2013 to October 11, 2013. Plants from this experiment were harvested in two stages (before and after winter).

Eight seedlings (four *per* cage) were transplanted to each of three plots at each altitude (24 *per* altitude, 96 seedlings in total). Of these, a total of 56 survived the first 3 months (April 14, 2013), before the onset of winter. At this time, a total of 36 were harvested (see Table S1). Of the remaining 20 seedlings, 19 survived the winter (October 11, 2013). Because we suspected that seedlings could die in winter, we decided to collect more seedlings before winter in order to have a higher number of root systems to examine for ECM colonization.

Plants from both experiments were washed, separated into shoots and roots. Shoots were dried at 60 °C for 72 h, and were weighed. Roots were carefully separated from soil and were subjected to morphotyping and molecular analyses described below.

Special efforts were made to preclude any propagule or ramet left in the study site after harvesting the seedlings. Although accidental plant remnant has successfully been avoided, plots are still monitored due to other ongoing experiments (Tecco et al., 2016).

2.7. Ectomycorrhiza morphotyping

Roots from each harvested seedling were removed from the soil and were gently rinsed with water. For each sample, all roots were placed under a stereomicroscope for examination at 10–40 × magnification. The ECM root tips were separated carefully from the roots of non-ECM plants and were sorted according to morphotypes based on their morphological and anatomical features. Criteria for sorting included diameter, branching pattern, mantle color, morphology of emanating hyphae and rhizomorphs, as described in Agerer (1991).

ECM colonization was calculated as the number of ECM root tips divided by the total number of root tips (Gehring and Whitham, 1994). The percentage of colonization by each ECM morphotype was calculated for each sample by dividing the number of root tips colonized by each ECM morphotype by the total number of root tips and multiplying by 100 (Helm et al., 1999).

2.8. Molecular identification of ECM root samples

Clusters of ECM root tips belonging to one individual morphotype from each seedling were inserted into 1.5 ml microtubes

Table 1
Number of pine seedlings that were alive at the end of experiments to be harvested and analyzed for mycorrhizal colonization. Glasshouse data refers to seedlings grown in soils from different altitudes, not including individuals grown with sterile inoculum. Field experiment I corresponds to the first cohort harvested after winter; and Field experiment II refers to the second cohort harvested both before and after winter. Three ECM fungal species were found in roots of harvested seedlings. Details on the altitudinal provenance and number of pine seedlings in which those ECM associations were recorded are provided (see supplementary information in Table S1).

Altitude (masl)	Experiment	Harvested individuals	Colonized individuals	*Suillus granulatus*	*Rhizopogon pseudoroseolus*	*Telephora terrestris*
900	Glasshouse	6	0	–	–	–
	Field exp. I (af. w.)	9	9	7	2	–
	Field exp. II (bef. w.)	4	3	3	–	–
	Field exp. II (af. w.)	1	0	–	–	–
		6	0	–	–	–
1600	Glasshouse	6	1	1	–	–
	Field exp. I (af. w.)	10	10[a]	8	–	–
	Field exp. II (bef. w.)	10	5[a]	3	–	1
	Field exp. II (af. w.)	6	4	3	–	–
2200	Glasshouse	6	1	1	–	–
	Field exp. I (af. w.)	–	–	–	–	–
	Field exp. II (bef. w.)	11	1	1	–	–
	Field exp. II (af. w.)	10	0	–	–	–
2700	Glasshouse	6	0	–	–	–
	Field exp. I (af.w.)	–	–	–	–	–
	Field exp. II (bef.w.)	11	0	–	–	–
	Field exp. II (af.w.)	2	0	–	–	–

[a] Some root fungi did not amplify.

containing 500 μl 2% CTAB DNA extraction buffer (2% cetyltrimethylammonium bromide, 100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, and 20 mM EDTA) and were stored at −20 °C. One to five root tips from each morphotype *per* seedling were subjected to DNA extraction by CTAB chloroform method (Rogers and Bendich, 1994). 50 μL of extracted DNA was resuspended in TE buffer. DNA presence in the extracts was checked on 1% agarose gels. 36 out of 39 extracts were positive. Extracts were diluted for PCR mixes (1: 10 and 1: 20). The full ITS rDNA repeat, including the 5.8S region, was amplified via PCR with ITS1F and ITS4 as well as ITS1F and ITS4B primer pairs (Gardes and Bruns, 1993; White et al., 1990). PCR reactions were performed in 50 μL reaction tubes with 1.1 × Reddy Mix™ PCR Master Mix (2.5 mM $MgCl_2$) (Thermo Fisher Scientific Inc., ABgene® UK) according to the manufacturer's instructions. Cycling conditions consisted of 2 min of activation at 94 °C, followed by 35 cycles of 45 s at 94 °C, 30 s at 50 °C and 60 s (+1 s/cycle) at 72 °C, and a 10 min final extension at 72 °C. PCR products were checked for positive amplification on 1% agarose gels and the amplified products were sent to Macrogen Inc. (Seoul, South Korea) for purification and sequencing using the BigDye™ terminator kit and run on ABI 3730XL. ECM voucher material has been deposited at CORD herbarium (Córdoba, Argentina).

2.9. Diversity of ECM taxa in the soil at the sampled altitudinal sites

To characterize the richness and abundance of ECM taxa present in the soil at the experimental sites, soil samples were taken in November of 2014 at the same locations for fungal DNA metabarcoding using deep sequencing as follows. Ten soil cores, each ca. 4 cm in diameter and 10–15 cm deep, were randomly taken more than 1 m from each other and were pooled to form a composite sample for each of the three plots at each of the four altitudinal sites. In addition, ten soil samples were taken at each of three plots in the pine plantation next to the lowest site (i.e., 15 composite samples in total). Genomic DNA was extracted from 0.5 g of dry soil using NucleoSpin® Soil kit (Macherey-Nagel Gmbh & Co., Düren, Germany), according to the manufacturers protocol. The ITS2 region (ca. 250 bp) of the nuclear ribosomal rDNA repeat was PCR amplified as described in Geml et al. (2014), including negative control. 250 μL of the sample was used for emulsion PCR according to the Ion PGM™ 200Xpress™ Template Kit manual and sequenced by an Ion Torrent Personal Genome Machine (PGM; Life Technologies, Guilford, CT, U.S.A.) at the Naturalis Biodiversity Center.

The initial clean-up of the raw sequence data (1,306,069 sequence reads) was carried out using the online platform Galaxy (https://main.g2.bx.psu.edu/root), in which the sequences were sorted according to samples and adapters (identification tags) were removed. The primers were removed and poor-quality ends were trimmed off based on 0.02 error probability limits in Geneious Pro 5.6.1 (BioMatters, New Zealand). Subsequently, sequences were filtered using USEARCH v.8.0 (Edgar, 2010) based on the following settings: all sequences were truncated to 200 bp and sequences with expected error > 0.5 were discarded. The resulting 551,691 high-quality sequences were grouped into 6990 operational taxonomic units (OTUs) with USEARCH at 97% sequence similarity, following other fungal metabarcoding studies (e.g., Bjorbækmo et al., 2010; Geml et al., 2010; Bellemain et al., 2013), while simultaneously excluding 6396 putative chimeric sequences. We assigned sequences to taxonomic groups based on pairwise similarity searches against the curated UNITE fungal ITS sequence database containing identified fungal sequences with assignments to Species Hypothesis groups (Kõljalg et al., 2013). After discarding global singletons and OTUs that did not have at least 80% similarity to any fungal sequence in UNITE, the final dataset contained 3024 OTUs. For this paper, OTUs representing ECM fungi were selected for further analyses according to Tedersoo et al. (2010) and Tedersoo and Smith (2013).

2.10. Statistical analyses

Ectomycorrhizal colonization, survival rates and OTU richness were compared among the altitudinal sites using analysis of variance (ANOVA), with means compared with Tukey's HSD test. In the case of ectomycorrhizal colonization, plots were nested within altitude. When data did not meet the assumptions and could not be corrected by log-transformation they were rank-transformed and the analyses were run on the rank data (Zar, 1999). The non-parametric analyses yielded the same conclusions as parametric ANOVAs run on the untransformed data, suggesting that it had sufficient power (Zar, 1999). These analyses were carried out with the Infostat Statistical Package (Di Rienzo et al., 2013) and R (Faraway, 2002).

We also constructed a rarefaction curve of soil ECM fungal OTUs vs. the number of sampled sites using PC-ORD v. 6.0 (McCune and Grace, 2002).

3. Results

3.1. Glasshouse experiment

ECM colonization occurred in seedlings inoculated with soil suspensions from intermediate altitudes (1600 and 2200 m asl). The proportion of individuals colonized by fungal symbionts was quite low (Table 1) but in both cases, seedlings showed more than 50% root colonization (white bars in Fig. 1). The morphotypes were identified as *Suillus granulatus* based on molecular analyses (Table 1). Pine roots in the control treatments were not colonized by any ECM fungi. No mortality was recorded among individuals from any treatments during the 4 months of the experiment (data not shown).

3.2. Field experiment 1

Seedlings grown in the field were colonized by ECM fungal symbionts at the two lower elevations (900 and 1600 m asl). ECM colonization at higher altitudes could not be assessed in this first cohort as no seedlings overwintered. Survival at the lower elevations was high (Fig. 2A). All surviving seedlings at both altitudes were colonized by ECM fungi (Table 1) with an average colonization of 40% (black bars in Fig. 1). Most of the individuals were colonized by *S. granulatus* at both altitudes (seven and eight individuals at 900 and 1600 m asl, respectively), while two individuals from the lower altitude (900 m asl) were colonized by *Rhizopogon pseudoroseolus* (Table 1). One strain assigned to Chaetosphaeriales sp. was also identified from the molecular analysis of the ECM root tips, but it is considered not mycorrhizal. There were no significant differences in ECM colonization rate (black bars, Fig. 1).

3.3. Field experiment 2

ECM colonization was recorded in seedlings of the second cohort in all but the highest elevation (Table 1, Fig. 1). More than half of the seedlings harvested before and after winter were colonized by ECM at 900 and 1600 m asl, while just one individual was colonized at 2200 m asl and no colonization was recorded in seedlings of the 2700 m asl (Table 1). In all cases *S. granulatus* was the most frequent symbiont while one individual at 1600 m asl was colonized by *Thelephora terrestris* (Table 1). Mycorrhizal colonization rates among seedlings harvested before winter averaged 10% at lower altitudes and slightly less at 2200 m asl (dark grey bars in

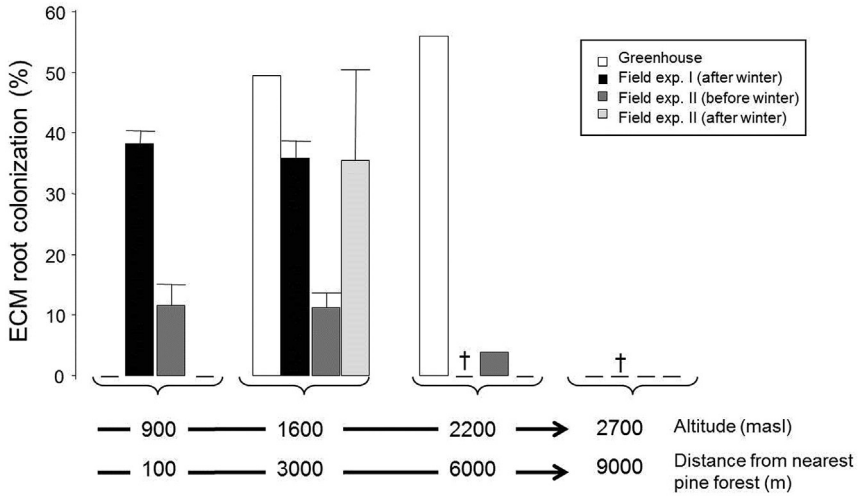

Fig. 1. Percentage of ectomycorrhizal colonization in pine seedlings along the altitudinal gradient and with increasing distances from pine forestations. The figure summarizes the three experiments, featuring the greenhouse assays with soils from different altitudes/distances and the field assessments with two cohorts of seedlings before and after winter. Absence of data (__) implies absence of ECM colonization on harvested seedlings, except for two cases (†) in which no seedling survived (see Table 1 for further details on the number of colonized seedlings). Distance from nearest pine forest increases with altitudinal sites (i.e. at distances of 110, 3000, 6000, and 9000 m, respectively). Colonized seedlings did not show significant differences in mycorrhizal colonization between altitudes in any experiment.

Fig. 1). Nearly 40% colonization rates were recorded at 1600 in overwintering seedlings (light grey bar in Fig. 1).

At the end of the growing season (i.e., before winter), individuals survived at all altitudes but the survival rates were highest at intermediate elevations (Fig. 2B). Despite the low *n* (Table S1), after 9 months in the field (i.e., after winter) survival rates were high at all four elevations (Fig. 2B).

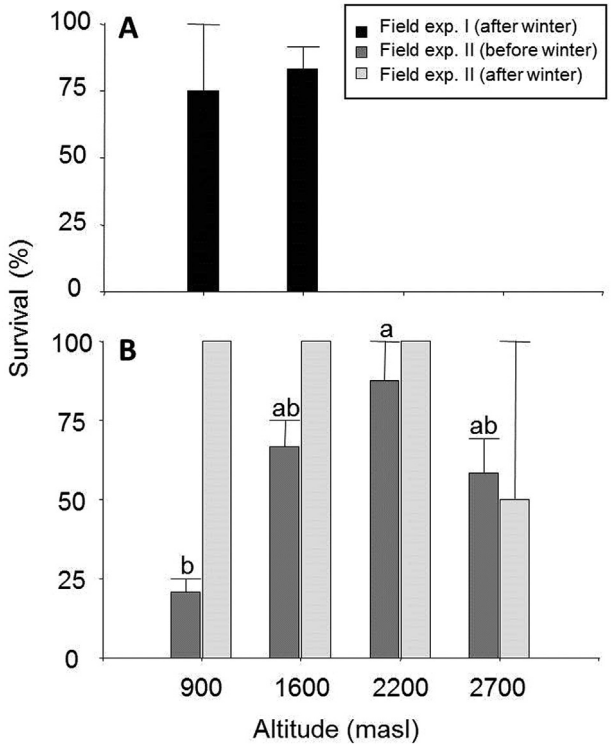

Fig. 2. Survival rates of pine seedlings in the field along the altitudinal gradient in (A) the first cohort harvested after winter ($F = 0.04$; $p = 0.7676$) and (B) in the second cohort, harvested before ($F = 5.75$; $p = 0.0214$) and after winter ($F = 1.00$; $p = 0.4789$). Means of survival rates include both ECM and non-ECM seedlings (see Table 1 for more details). Bars with different letters are significantly different (Tukey test, $P < 0.05$).

3.4. Presence of ECM fungi in the soil at different altitudes

A total of 36 OTUs in the soil samples were putatively identified as ECM using DNA metabarcoding. They belonged to the following phylogenetic lineages:/endogone,/meliniomyces,/pyronemataceae,/wilcoxinia,/amanita,/inocybe,/laccaria,/amphinema-tylospora,/pisolithus-scleroderma,/suillus-rhizopogon,/paxillus-gyrodon,/cantharellus,/clavulina,/russula-lactarius,/sebacina,/tuber-helvella, and/tomentella-thelephora (Table 2). The rarefaction curve (Fig. 3) generated based on the accumulating number of OTUs with increased number of sites indicated that almost all ECM fungi inferred to occur in the region of study likely have been sampled, with 38 observed ECM fungal OTUs and first and second order Jackknife estimates of 39.37 and 40.67, respectively.

Out of the 36 ECM fungal OTUs, 34 were detected in the pine plantation and 12 were found at sites without pines at various elevations. Twenty-four OTUs were restricted to the plantation, while two lineages (*Meliniomyces bicolor* and *Scleroderma areolatum*) were detected in the altitudinal plots but not in the plantation, albeit in low frequency. The richness of ECM fungal OTUs and the number of their representative sequences was notably lower outside plantations and decreased monotonically with increasing distance and altitude ($F = 291.58$, $p < 0.0001$) (Fig. 4, Table 2). All three ECM fungal species that colonized the pine seedlings at different elevations (Table 1) were present in the respective soil samples as well (Table 2).

4. Discussion

4.1. Co-invasion by pine and fungi

It has been previously shown that *Pinus* species and their associated fungal symbionts are able to co-invade *Pinus*-free areas at distances shorter than 1000 m from pine plantations (e.g. Nuñez et al., 2009; Dickie et al., 2010; Salgado Salomón et al., 2011; Hynson et al., 2013; Hayward et al., 2015b). In this study, we provide evidence showing that pines can establish symbioses with their fungal partners at substantially greater distances (at least 6000 m) from the nearest pine plantation and over elevations where ECM host plants are absent. Specifically, the results show

Table 2
Ectomycorrhizal fungal OTUs found in soils along the altitudinal gradient with number of sequence reads. Only OTUs with ≥95% similarity to known ECM fungi are listed.

ECM taxa			Altitudinal gradient (masl)				
Linage	Species hypothesis	Blast ≥ 95% similitud	Pine plantation 900	900	1600	2200	2700
/endogone	SH033627.06FU	*Endogone lactiflua*	2				
/meliniomyces	SH207167.06FU	*Cadophora finlandica*	319				
/meliniomyces	SH207167.06FU	*Cadophora finlandica*	3				
/meliniomyces	SH207170.06FU	*Meliniomyces bicolor*				2	
/wilcoxina	SH227977.06FU	*Wilcoxina* sp	54				
/wilcoxina	SH227976.06FU	*Wilcoxina mikolae*	355				
/amanita	SH200305.06FU	*Amanita muscaria*	2	4	2		
/inocybe	SH200562.06FU	*Inocybe curvipes*	36				
/inocybe	SH226291.06FU	*Inocybe jacobi*	4				
/inocybe	SH201882.06FU	*Inocybe sindonia*	1354		1		
/laccaria	SH205137.06FU	*Laccaria* sp	277				
/amphinema-tylospora	SH229868.06FU	*Tylospora* sp.	407				
/amphinema-tylospora	SH229868.06FU	*Tylospora* sp.	13				
/pisolithus-scleroderma	SH205932.06FU	*Scleroderma* sp.	568	1			
/pisolithus-scleroderma	SH238662.06FU	*Scleroderma areolaum*		2			
/suillus-rhizopogon	SH191162.06FU	*Rhizopogon pseudoroseolus*	328	28	1		
/suillus-rhizopogon	SH190954.06FU	*Suillus granulatus*	40	10	7	4	1
/paxillus-gyrodon	SH239907.06FU	*Paxillus involutus*	1	1			
/cantharellus	SH211497.06FU	*Sistotrema* sp	2	1			
/clavulina	SH191669.06FU	*Clavulina* sp	670				
/russula-lactarius	SH201190.06FU	*Russula* sp	2150				
/sebacina	SH226216.06FU	*Sebacina* sp	5221				
/sebacina	SH226216.06FU	*Sebacina* sp	34				
/sebacina	SH226216.06FU	*Sebacina* sp	2				
/sebacina	SH017339.06FU	Sebacinaceae sp	51				
/sebacina	SH017339.06FU	Sebacinaceae sp	11				
/tuber-helvella	SH200481.06FU	*Tuber* sp	148				
/tomentella-thelephora	SH195956.06FU	*Thelephora terrestris*	77	11	1	2	
/tomentella-thelephora	SH202449.06FU	*Tomentella coerulea*	2	2			
/tomentella-thelephora	SH021838.06FU	*Tomentella* sp	12				
/tomentella-thelephora	SH195962.06FU	*Tomentella* sp	60				
/tomentella-thelephora	SH206268.06FU	Thelephoraceae sp	10				
/tomentella-thelephora	SH202676.06FU	Thelephoraceae sp	44				
/tomentella-thelephora	SH023960.06FU	Thelephoraceae sp	105				
/tomentella-thelephora	SH222939.06FU	Thelephoraceae sp	3				
/tomentella-thelephora	SH006309.06FU	Uncultured ECM fungi	1				10

OTUs sequences <150 pb and <95% similitude to a reference taxa are not included.

that pine seedlings have the potential to overwinter, survive and form ectomycorrhizas at notably long distances from plantations and elevations with different climatic conditions than those in which they currently occur. It is worth mentioning that we cannot discount the occurrence of some scattered pine seedlings in between plots at low altitudes and the plantations. These seedlings, however, would not be a significant source of fungal propagules in comparison with the thousands of hectares of mature pine plantations.

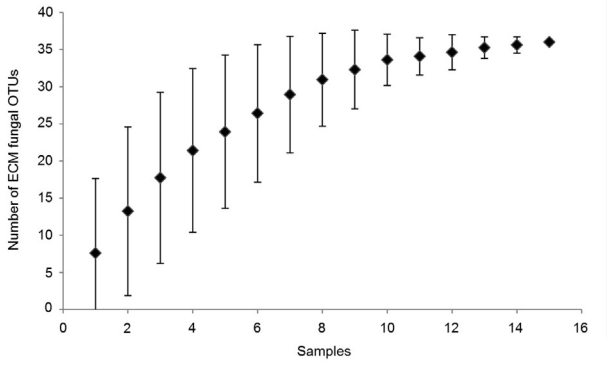

Fig. 3. Rarefaction curve of the total number of ECM fungal OTUs detected in the soil samples.

4.2. Invasive fungi

Previous studies on pine expansion have identified those fungal species that colonize pine roots, but not the whole community of ECM fungi that can disperse over considerable distances and

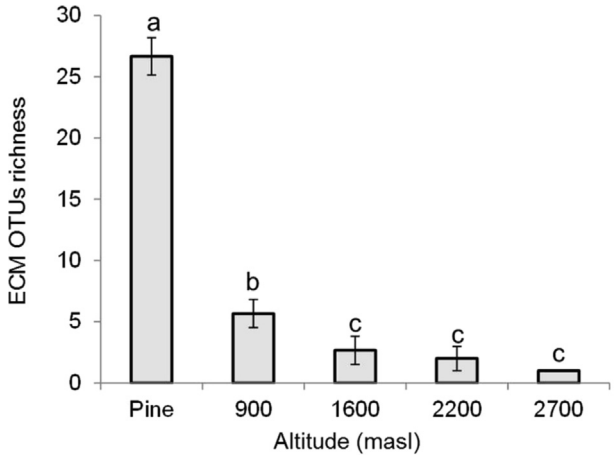

Fig. 4. Comparison of ECM fungal OTU richness among the elevational sampling sites and the pine plantation (900 masl). Bars with different letters are significantly different (Tukey test, $p < 0.05$).

provide suitable conditions for the invasion front of *Pinus*. In the present study, the molecular analysis of soil samples revealed the presence of several ECM fungal propagules at various elevations and distances from plantations. However, the ECM fungal richness and sequence counts outside plantations were notably lower than those recovered from within the plantation and they decreased with elevation (Table 2, Fig. 4). This is consistent with other studies showing decreasing ECM fungal propagules with increasing isolation from fruit body sources (e.g. Collier and Bidartondo, 2009; Peay et al., 2012). Despite the occurrence of propagules belonging to several species of ECM fungi in pine-free sites at different distances and altitudes (i.e. 12 OTUs, Table 2), only *S. granulatus*, *R. pseudoroseolus* and *T. terrestris* were able to establish symbioses with pine seedlings outside plantations (Table 1). These species belong to genera previously shown to be early successional colonizers (Chu-Chow, 1979) and frequently reported as co-invaders with pine species (e.g. Hayward et al., 2015b and references therein). In particular, *S. granulatus* is the only species that was present at the four elevation sites, albeit only one sequence was registered at the highest altitude (i.e. 2700 m asl). This suggests that this species has the highest dispersal efficiency. Because the soil DNA metabarcoding analysis and the assessment of fungal colonization of pine roots were not carried out simultaneously, the direct comparisons between them should be interpreted with caution.

4.3. Pine survival and formation of ectomycorrhizas

At the highest elevation (i.e. 2700 m asl), two of three pine seedlings overwintered in the second experiment, but none of them formed ectomycorrhizas. Considering that soil ECM fungal sequence counts were very low at this site, the results suggest that fungal propagule pressure (i.e., the scarcity of symbionts), rather than climate, may constrain pine expansion over these altitudes. Because pines have been expanding in other areas of the study region, presumably together with their ECM fungal symbionts, it is reasonable to expect that this constraint will decrease with time.

At the second-highest altitude (i.e. 2200 m asl), all pine seedlings were able to overwinter and individuals forming ectomycorrhizas were observed in two of the three experiments (glasshouse and the second field experiment, Table 1, Fig. 1). Despite the presence of two other ECM fungal species at this elevation, only *S. granulatus* colonized the roots. These findings confirm that *S. granulatus* is able to disperse and form ectomycorrhizas over high altitudes and notably longer distances than previously reported. This is particularly relevant because this species in itself might be sufficient to promote pine invasion, as has recently been shown for *S. brevipes* in southern Chile (Hayward et al., 2015a). Unlike in our first field experiment, pines survived at all elevations in the second experiment. This could be attributed to lower temperatures during the winter of 2012 (field experiment 1) than those during the winter of 2013 (field experiment 2) (average minimum temperatures at 2100 m asl for July 2012 and 2013 were −3.1 and 0.72, respectively).

Plots at the two lowest elevations were located in pine-free areas but at the same altitudes at which pine already occur in other areas. At these elevations, survival rates were lower than or similar to those at higher elevations, suggesting that the findings reported here reveal a real potential of pine expansion over higher elevations. Similar findings were recently reported for arbuscular mycorrhizal invasive trees in the study area (Tecco et al., 2016).

4.4. Fungal strategies

The ability of fungi to disperse and to form ectomycorrhizas depends on traits such as fruit body type, dispersal vectors, and spore biology. ECM fungi associated with pines in the sampled region exhibit various life style strategies. For example, spores of the subhypogeus *R. pseudoroseolus* are mainly dispersed by mammals, *T. terrestris* by wind, while *Suillus* spp. is dispersed by both of them (Ashkannejhad and Horton, 2006). In temperate forests of southern South America, three species of invasive *Rhizopogon* and *Suillus luteus* were dispersed by exotic deer and boars (Nuñez et al., 2013). Only exotic boar (*Sus scrofa*) has been reported for the mountains from central Argentina (Morando and Polop, 1997) and is the only candidate for mycophagous dispersal vector in the region. Despite the fact that the plots in our study were situated in an exclosure established in April 2008 (Tecco et al., 2016), precluding recent events of animal-mediated spore dispersal in those soils, it has been shown that *Rhizopogon* species persist in soils for several years, and probably for decades (Bruns et al., 2009). Moreover, they germinate and increase infectivity with time when stimulated by host presence. Therefore, spores of *R. pseudoroseolus* could have been dispersed before the establishment of the exclosures and may have persisted in the spore bank retaining viability. Resistant spore bank in soil has also been reported for *Suillus* but to a lesser degree than for *Rhizopogon* (Ashkannejhad and Horton, 2006).

Besides this, a dikaryotic fungal thallus is also needed for a functional ECM symbiosis (Smith and Read, 2008). Typically, this thallus is the result of the fusion of hyphae from two haploid spores of opposite mating types (Alexopoulos et al., 1996). Because feces of animals that disperse fungi contain large quantities of spores, *Rhizopogon* would not be constrained by the possibility of forming the dikaryotic thallus once deposited in soils.

On the other hand, wind-dispersed spores from epigeous fruit bodies disperse randomly and depend on the probability of the encounter between hyphae originated in two haploid spores of opposite mating types. This probability sharply decreases with increasing distances from fruit bodies (Galante et al., 2011; Peay et al., 2012). In addition, spore infectivity in certain species usually decreases after 1 yr as shown for a *Suillus* species (Ashkannejhad and Horton, 2006). Despite these constraints, *S. granulatus* can produce an important percentage of binucleate spores with the ability to establish a dikaryon (Jacobson and Miller, 1994). This capability may enable this species to form a functional symbiosis from a single spore explaining the success in forming ecotmycorrhizas at long distances from fruit body sources observed here.

In the case of the wind-dispersed *T. terrestris*, it has been also shown that spore persistence in soils is low (Nguyen et al., 2012). However, the congeneric *T. americana* also produces binucleate, albeit probably homokaryotic, spores (Horton, 2006). This could be also expected for *T. terrestris* since it frequently colonizes seedling roots in disturbed habitats and is a common contaminant in glasshouse assays. Whether the mycelia from these spores can form a functional ectomycorrhiza remains to be elucidated. Moreover, spores of this species germinate faster in the presence of host roots (Birraux and Fries, 1981), consistent with the ruderal life strategy typical of pioneering species.

In contrast to these successful pioneers, other fungal species dispersed outside plantations but did not form ectomycorrhizas with the sampled seedlings. This suggests that, rather than dispersal capabilities, spore traits might determine the ability of these fungi to colonize new roots and expand outside their current range. For instance, among them, *Amanita* and *Scleroderma* have low spore germination rates while *Inocybe* decreases infectivity after 1 yr (Nara, 2009).

Nonetheless, it is likely that more ECM species will be able to colonize pine roots at the elevational sites in the near future. One such candidate species is *Amanita muscaria*, found up to 1600 m in the sampled sites, that has been introduced to various regions of

the Southern Hemisphere with pine plantations and is a well-known invasive fungus in Australia (Johnston and Buchanan, 1998) and New Zealand (Dickie et al., 2010).

Altogether, the evidence suggests that different spore biological strategies, including dispersal vector, nuclei number, infectivity and persistence in spore bank, might be at play in the dispersal and establishment dynamics of exotic ECM fungi. Further studies on dispersal and reproductive traits may allow disentangling the relative importance of these mechanisms. This knowledge would be essential for designing management strategies for these invasive species.

5. Conclusions

Seedlings of exotic *P. elliottii* can survive at higher altitudes than those in which they currently occur in the mountain ranges of central Argentina. Several putatively exotic ECM propagules disperse over those altitudes and long distances from plantations but only *Suillus granulatus,* and to a lesser degree *Rhizopogon pseudoroseoulus* and *Telephora terrestris*, are able to disperse and form ectomycorrhizas. Overall, we provide novel evidence anticipating that the availability of suitable fungal symbionts might constrain but not hinder the expansion of a pine species over wide distances and altitudinal zones even in areas with no native ECM fungi.

Because pine plantations alter hydrologic regimes in invaded ecosystems (e.g. Le Maitre et al., 2002; Jobbágy et al., 2013), these findings suggest urgent intervention over expanding pines and their associated fungal symbionts since water provision for cities and towns established at the bottom of the mountain ranges would ultimately be affected.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2016.11.002.

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