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A single ectomycorrhizal fungal species can enable a *Pinus* invasion

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Abstract. Like all obligately ectomycorrhizal plants, pines require ectomycorrhizal fungal symbionts to complete their life cycle. Pines introduced into regions far from their native range are typically incompatible with local ectomycorrhizal fungi, and, when they invade, coinvade with fungi from their native range. While the identities and distributions of coinvasive fungal symbionts of pine invasions are poorly known, communities that have been studied are notably depauperate. However, it is not yet clear whether any number of fungal coinvaders is able to support a Pinaceae invasion, or whether very depauperate communities are unable to invade. Here, we ask whether there is evidence for a minimum species richness of fungal symbionts necessary to support a pine/ectomycorrhizal fungus coinvasion. We sampled a *Pinus contorta* invasion front near Coyhaique, Chile, using molecular barcoding to identify ectomycorrhizal fungi. We report that the site has a total richness of four species, and that many invasive trees appear to be supported by only a single ectomycorrhizal fungus, *Suillus luteus*. We conclude that a single ectomycorrhizal (ECM) fungus can suffice to enable a pine invasion.

Key words: biological invasions; Coyhaique, Chile; ectomycorrhizal fungus; molecular barcoding; Pinus; Suillus luteus.

Introduction

Obligate mutualists, by definition, cannot succeed without their partners. In the context of an invasion, a lack of partners for specific, obligate mutualists is expected to prevent the spread of an exotic obligately mutualistic organism unless mutualists are cointroduced and coinvade with those organisms (Richardson et al. 2000*a*, Nuñez and Dickie 2014). Among the most widespread obligate mutualisms for woody plants is the mycorrhizal symbiosis (Smith and Read 2008). While the degree of specificity of most mycorrhizal symbioses is poorly known, the mutualism between *Pinus* species (Pinaceae) and ectomycorrhizal fungi appears to exhibit specificity that has relevance to the performance of pines as invaders. Here, we take the term "invasive" to refer to organisms that reproduce and spread into previously uncolonized areas in a region to which they have been introduced without further human intervention, following Richardson et al. (2000*b*). Pinaceae species introduced into regions without compatible ectomycorrhizal (ECM) inoculum cannot complete their life cycles, and consequently cannot invade (Kessel 1927, Mikola 1970, Nuñez et al. 2009). A variety of inoculation programs and forestry practices have resulted in the introduction of Pinaceae-compatible inoculum into many regions around the world (Mikola 1970). These programs have been effective in transporting Pinaceae-compatible inoculum into many regions, facilitating the establishment of plantations, but also enabling Pinaceae invasions in some areas (Richardson 1998, Richardson and Rejmanek 2004). However, because inoculation has frequently been with whole soil or duff rather than pure cultures of mycorrhizal fungi, the identities and distributions of ECM fungi cointroduced with Pinaceae species are poorly known (Mikola 1970, Dickie et al. 2010, Hynson et al. 2013).

Chu-Chou and Grace (1988) reported that ECM fungal communities associating with pines in New Zealand are depauperate relative to communities in the native range, an observation echoed by Chapela et al. (2001) in Ecuador and Hynson et al. (2013) in Hawaii. Hynson et al. (2013) also report that communities associating with pines establishing outside plantations are depauperate even relative to the already species-poor communities present inside plantations, with only four species of ECM fungus associating with trees establishing more than 250 m from plantations. In contrast,

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TABLE 1. Richnesses of ectomycorrhizal (ECM) fungi associating with Pinaceae species far from their native ranges as detected in a representative sample of studies.

Location	Total no. ECM fungal species	Co-invading fungal species†	No. Pinaceae species	No. samples	Reference
Brazil	25‡		2		Giachini et al. (2000)
Hawaii	24	18	4	520	Hynson et al. (2013)
New Zealand	14	14	1	25	Dickie et al. (2010)
New Zealand	19		1	84	Walbert et al. (2010)
Ecuador	4		1	47	Chapela et al. (2001)
Seychelles	4		1	11	Tedersoo et al. (2007)
New Zealand	11		1		Chu-Chou and Grace (1988)

Note: Empty cells indicate that no data is available and refer to studies in which the given data point was not collected.
† Fungi detected associating with trees establishing outside plantations.
‡ Sporocarps only; no belowground samples.

ECM-dominated forests in the native range can contain several hundred ECM species, even at local scales (Buee et al. 2009, Taylor et al. 2013). The reasons for the hyperdiversity of native-range ectomycorrhizal communities are still debated (Dickie 2007, Taylor et al. 2013), but the fact that pines can coinvade with a species-poor ECM fungal assemblage indicates that from the plant's perspective, many ECM fungal species are redundant, at least for establishment. In the native range, similarly low ECM fungal species richnesses have been observed on seedlings establishing in the absence of belowground ectomycorrhizal networks (Horton et al. 1998, Baar et al. 1999, Ashkannejhad and Horton 2006).

In cases where Pinaceae species are invading, ECM fungi can be said to enable invasions, in the sense that the invading individuals would not establish and could not invade without those fungi. Previous studies of ECM fungal communities associating with Pinaceae species far from their native range have yielded species richnesses ranging from 4 to 25 species (albeit with different sampling intensities; Table 1). However, because the species richness was not fully assessed in these studies (e.g., the species-effort curves, when included, were not saturated), determining how many ECM fungi coinvaded with each Pinaceae species is difficult. It is therefore also impossible to determine whether some minimum richness of ECM fungi is necessary for Pinaceae invasions to succeed.

Using a site in Chile where only a single Pinaceae species is invasive (*Pinus contorta*, among the most invasive woody plants worldwide; Langdon et al. 2010), and where deliberate inoculation (even in the form of duff or soil movement) has apparently never been used (Mikola 1970), we ask the following question: Are there indications that a minimum number of ECM fungal symbionts are necessary to enable a pine invasion?

METHODS

Study site

In the 1970s, the Chilean Corporacion Nacional Forestal (CONAF) established plantations of Pinaceae species (*Pinus contorta*, *P. ponderosa*, and *Pseudotsuga menziesii*) covering more than 43 000 ha in Chile's Patagonian Aysen region. To the best of our knowledge, compatible ECM inoculum was never deliberately cointroduced by CONAF with forestry trees in Patagonian Chile (Mikola 1970); its presence in the region may be the result of accidental cointroduction with nonsterile seeds or seedlings, or via dispersal from regions where deliberate inoculation was practiced. This study used a site on the Patagonian steppe 45°30′2″ S, 71°42′15″ W) near the city of Coyhaique in the Aysen region (see Plate 1); for details about the site, see Yarrow and Torres (2008) and Langdon et al. (2010; our site is the same as site 5 of that study). The *P. contorta* plantation acting as the seed source for the invasion studied here was established around 1981 (MININCO, personal communication). Although there is a *P. ponderosa* plantation nearby, *P. contorta* is the sole invasive Pinaceae species at this site, forming monocultures with densities as high as 10 000 trees/ha within 100 m of the plantation. Nonreproductive *P. contorta* recruits can be found as far as 3 km from the plantation, but are extremely scarce further than 900 m from the plantation edge.

Field methods

We collected ECM root tips from 104 *Pinus contorta* individuals. We sampled 25 trees inside the plantation and 79 trees along two transects leading away from the plantation perpendicular to the plantation edge to a distance of approximately 800 m (the greatest distance at which we were able to locate *P. contorta*). All trees sampled outside of the plantation were prevailingly downwind of the plantation itself. We sampled trees at 10-m intervals along the transect. We collected ECM root as follows: for each tree, we selected a cardinal direction at random using least significant digits on a stopwatch. We dug downward using a hand trowel on the flank of the tree facing this cardinal direction until we encountered a coarse root belonging to the selected tree. We then traced this coarse root away from the bole of the tree to a fine root cluster. We collected approximately 10 cm of fine root material from the first such cluster encountered and immediately preserved it in

2× CTAB buffer for future molecular analysis. We repeated this procedure on the opposite side of the tree bole, for a total of approximately 20 cm of fine root material collected per tree. To age each tree, we either sawed the bole at ground level and collected a disk, or else collected a core. We sanded disks smooth using a handheld sander. We aged trees by counting rings.

Molecular methods

We examined root tips under a Nikon SMZ645 dissecting microscope (Nikon Instruments, Melville, New York, USA) at 20–40× magnification. We identified ectomycorrhizal root tips by a combination of characteristic swelling, color, and texture changes indicating the presence of a fungal mantle. We sorted ectomycorrhizal root tips by morphological type (morphotype) using mantle color and texture, root tip branching pattern, and presence, color, and texture of extramatrical hyphae or rhizomorphs. We selected one or two exemplars of each morphotype per sample for molecular analysis; this resulted in a total of 445 individual root tips from which we extracted DNA.

We extracted DNA from root tips using a modified glassmilk protocol as in Nuñez et al. (2013). We used primers ITS1f (White et al. 1990) and NLB4 (Martin and Rygiewicz 2005) to amplify the fungal nuclear ribosomal internal transcribed spacer (ITS) region, the official DNA barcode for fungi, using the same conditions used in Nuñez et al. (2013). We re-amplified DNA extracts for which reactions failed with primers NSI1 (Martin and Rygiewicz 2005) and ITS4b (Gardes and Bruns 1993). We digested amplicons with the restriction enzymes *Hinf*I and *Hae*III (New England Biolabs, Ipswich, Massachusetts, USA) following the manufacturer's protocol, and visualized restriction fragment patterns on 3% agarose gels. We selected five to seven exemplars of each unique restriction fragment type for sequencing. We re-amplified the ITS region from the DNA extracts yielding these restriction fragment types, then submitted them for sequencing using standard chemistry on an ABI 3730xl in one direction using ITS1f as the sequencing primer. We uploaded representative sequences of each operational taxonomic unit (OTU) to GenBank under accession numbers KF836006–KF836009.

Data analysis

We grouped sequences into OTUs in Mothur 1.31 (Schloss et al. 2009) using a cutoff of 97% sequence similarity, not counting end gaps and treating internal gaps as a single character. We named OTUs based on BLAST comparisons to GenBank: we considered a sequence conspecific with named GenBank sequences at >97% similarity if at least 60% of the ITS region was alignable. We performed all statistical analyses using the vegan package (Oksanen et al. 2007) in R 3.0.1 (R Development Core Team 2012). We used a permutational ANOVA-like test on redundancy-analysis fitted data (function anova.cca) to test the significance of the effect of distance on community structure. We used this test both on trees outside the plantation, and on the entire data set, treating trees inside the plantation as having zero distance from plantations. We also implemented a permutation test using the function permatfull implemented in vegan (Oksanen et al. 2007) for Spearman's rank correlation coefficient to test the significance of the effect of distance from the plantation on species richness without taking into account community composition. For this test, we computed Spearman's rank correlation coefficient for 10 000 permutations of the community data matrix for which column but not row sums are preserved. Because we expect species richness to decline with distance, the P value for this test is the proportion of correlation coefficients in this distribution that are greater than the observed value.

To examine spatial differences in the distribution of *Suillus luteus* vs. other taxa, we implemented two distance-based statistics. For a given spatial point occupied by a sampled tree, these statistics are defined as the minimum distance, and the mean distance, to a sampled tree where at least one taxon other than *Suillus luteus* was detected. These measures are intended to function as proxies for the local density of non-*Suillus* taxa. We calculated the null distribution for this statistic by calculating the statistic for each tree 5000 times, using identical parameters to the observed distribution, but permuting the community data matrix using permatfull constrained to maintain species abundances. To correct for the multiple tests entailed by calculating the statistic for multiple trees, we used Bonferroni correction.

Results

The majority of root tips (296 out of 445; 67%) yielded ITS amplicons. Root tips not yielding amplicons were concentrated in several samples, probably because the individual root clusters harvested for these samples were senescent or dead. In at least some cases, root clusters which failed to produce amplicons showed signs of senescence such as wrinkling, discoloration, and loss of structural integrity. Consequently, only 71 of the 104 (68.2%) trees from which root tips were harvested yielded molecular data. Trees for which we were unable to gather molecular data were scattered throughout, without any clear patterns. We detected four ectomycorrhizal fungal OTUs associating with *Pinus contorta*: *Suillus luteus*, *Hebeloma mesophaeum*, *Tomentella* cf. *sublilacina*, and species of Hydnaceae. None of these species was a singleton, doubleton, or tripleton; consequently, Chao, ACE, and Jacknife richness estimators for the site all yielded predicted richnesses of 4.0 species. We also saturated the species-accumulation curve for this sampling effort (Fig. 1).

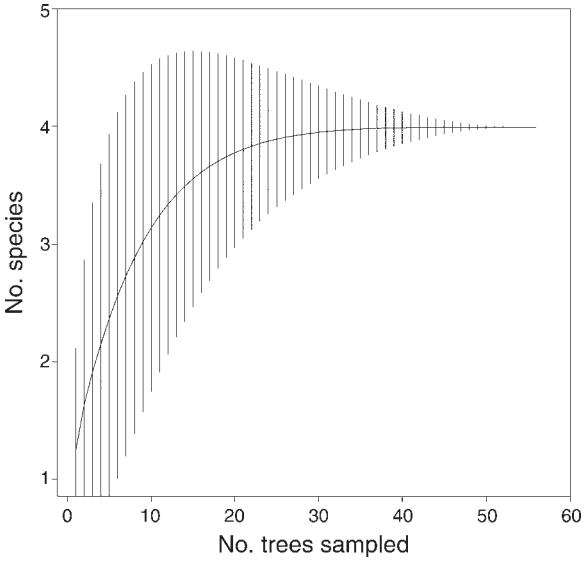

FIG. 1. Species-accumulation curve generated using the exact method implemented in vegan.

A permutational ANOVA-like test (anova.cca in the vegan package) for the effect of distance from the plantation on redundancy-analysis fitted community data for the entire data set revealed that distance had a significant effect on community structure (proportion of variation explained = 0.174; 10 000 permutations; $P < 0.005$). A similar test on a data set restricted to trees outside the plantation showed similar results (proportion of variation explained = 0.07; 10 000 permutations; $P < 0.01$). Species scores along the distance-associated component (positive with increasing distance) for this restricted data set were as follows: *Suillus luteus*, 0.398; Hydnaceae, –0.061; *Tomentella* cf. *sublilacina*., –0.231; *Hebeloma mesophaeum*, –0.311. Similar tests failed to detect a significant effect of tree age on community structure (10 000 permutations; $P > 0.39$). A permutation test for the entire data set revealed that species richness was significantly inversely correlated with distance (Spearman rank correlation coefficient = –0.234; $P = 0.0243$); however, when restricted only to trees outside the plantation this effect was not significant (Spearman rank correlation coefficient = –0.159; $P > 0.47$)

The distance to the nearest tree (i.e., our minimum-distance statistic) associating with a species other than *Suillus luteus* increased with distance from the plantation (Spearman rank correlation coefficient = 0.562, $P < 0.001$). The increasing proportion of the community made up by *S. luteus* with distance from the plantation is visually apparent (Fig. 2a). For 10 sampled trees, the minimum distance to another tree colonized by a non-*Suillus* taxon was significantly greater than expected following Bonferroni correction, using a null model that assumes random distribution of taxa (Fig. 2b). This distance increased with distance from the plantation (Spearman rank correlation coefficient = 0.562, $P < 0.001$). For 25 trees, the mean distance to other trees colonized by non-*Suillus* taxa was significantly greater than expected under the null (Fig. 2c); this mean

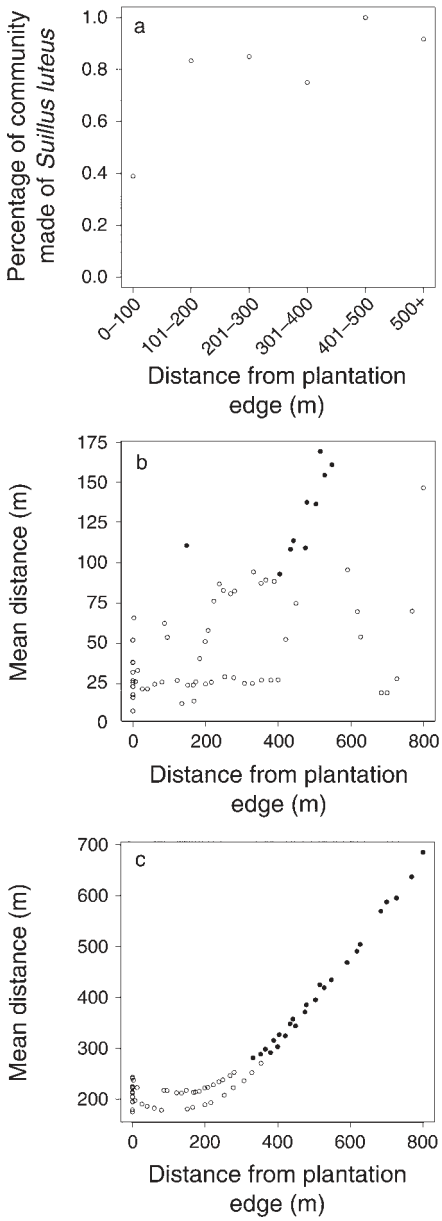

FIG. 2. Distance from the pine plantation plotted against (a) the percentage of the overall mycorrhizal community in that distance class made up by *Suillus luteus*, (b) the minimum distance to any single tree colonized by one of the three ectomycorrhizal (ECM) fungi other than *S. luteus* that we detected, and (c) the arithmetic mean distance to all trees colonized by one of the three ECM fungi other than *S. luteus* that we detected. Trees inside the plantation are considered to be 0 m from the plantation. Distances that are significantly greater than expected are shown as filled circles.

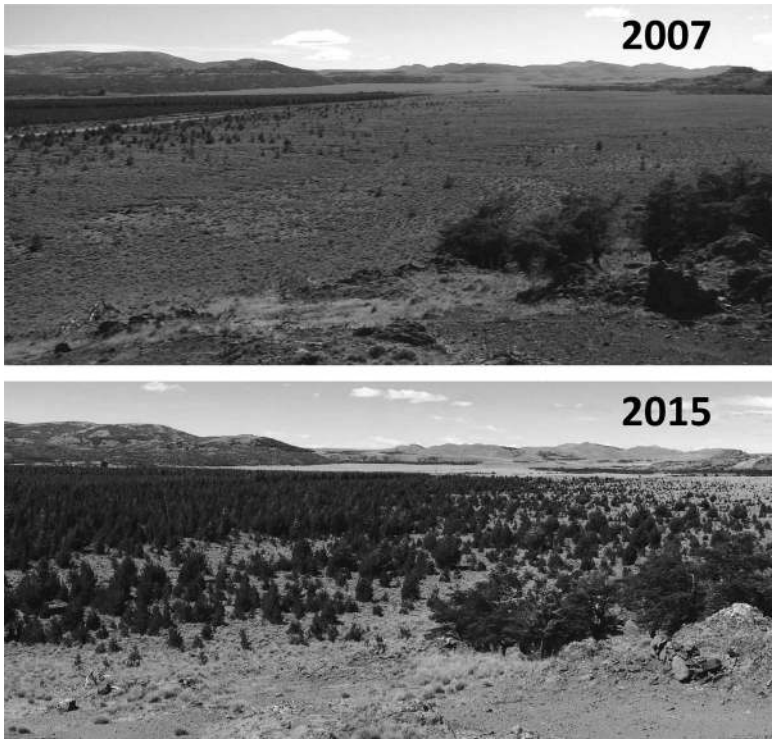

PLATE 1. Invasion of *Pinus contorta* in Coyhaique, Chile. Pictures taken from the same location in January 2007 (photo credit: A. Pauchard) and January 2015 (photo credit: J. Esquivel).

distance also increased with distance from the plantation (Spearman rank correlation coefficient = 0.72, $P < 0.001$).

DISCUSSION

The saturated species-effort curve (Fig. 1) indicates that it is unlikely that there are many undetected ECM fungal species at our site, and that four species represents the true richness. Compared to the hundreds of species associating with Pinaceae species in the native range (Taylor et al. 2013), four species is an exceptionally depauperate community. Even as compared to the species richnesses detected elsewhere associating with invasive or nonnative pines, four species represents a poor community (Table 1). All four species were detected both inside the plantation and associating with invading trees. If invasive species are taken to be those establishing in natural habitats, all four species can be called invasive. The degree to which they invade, however, varies substantially. *Suillus luteus* was detected associating with every tree sampled outside the plantation except five; the five trees with which we did not detect *S. luteus* were the five trees closest to the plantation (all less than 65 m from the plantation edge). In contrast, *Hebeloma mesophaeum* and Hydnaceae species were never detected more than 400 m from the plantation edge, and the occurrence of *Tomentella* cf. *sublilacina* far from the plantation was restricted to two adjacent trees.

The presence of a pine invasion in an area with only four *Pinus*-compatible ectomycorrhizal fungi obviously indicates that pines are capable of invading with only four ECM symbionts. However, the distribution of those four species at the site indicates that a single ECM fungal species may enable invasions. The vast majority of trees further than 250 m from the plantation were colonized exclusively by *Suillus luteus*. For several trees, the minimum distance to a tree colonized by an ECM fungal species other than *Suillus luteus* was significantly greater than expected under the null model. All trees further than approximately 300 m from the plantation had a greater mean distance to trees colonized by non-*Suillus* taxa than expected. The only trees further than 100 m from the plantation edge colonized by non-*Suillus* fungi were also colonized by *S. luteus*. These results suggest that *S. luteus* is unexpectedly common far from the plantation. This in turn suggests that the fact that many trees far from the plantation are colonized only by *Suillus* is unlikely to be due to chance. The declining proportion of inoculum other than *S. luteus* far from the plantation suggests that most trees establishing far from the plantation at our site are probably first colonized by *S. luteus*, and may be capable of surviving to reproductive age without further colonization.

Suillus species possess several traits that make them ideally suited for the role of coinvasive fungi with *Pinus* invasions. First, they produce unusually prolific crops of fruit bodies, increasing the number of spores liberated from a given area of pine-dominated forest (Hedger 1986, Dahlberg and Finlay 1999). Second, these fruit bodies are frequently consumed by local animals, which disperse large numbers of spores through endozoochory (Ashkannejhad and Horton 2006, Nuñez et al. 2013), Third, those spores are unusually resistant, enabling the establishment of a long-lived spore bank, and so avoiding the need for simultaneous codispersal of plant and fungal propagules (Ashkannejhad and Horton 2006, Nara 2008, Nguyen et al. 2012). Fourth, perhaps partly as a combination of those three traits, *Suillus* spores yield mycorrhizal infection after dispersing over exceptionally long distances (Peay et al. 2012). Finally, *Suillus* species are capable of rapidly colonizing roots of pines, permitting quick mycorrhization of establishing trees (Dahlbeg and Finlay 1999, Ashkannejhad and Horton 2006, Nara 2008). In the native range, these characters make *Suillus* species exceptionally important early in succession (Baar et al. 1999, Ashkannejhad and Horton 2006). The importance of *Suillus* spp. in both successional and invasive contexts suggests that similar processes may be operating in both situations, and that our results may pertain to successional processes in the native zone as well as to the invasional context studied here. Consequently, we suggest that the identities of species involved in facilitating succession or comigration in the native zone, rather than the richnesses of these communities per se, may prove important, and that EM plant species may be capable of comigrating or colonizing new territory in the native zone with very depauperate symbiont communities.

Since without anthropogenic nutrient and water inputs, pines rarely survive beyond a year or two in the absence of mycorrhizal inoculum (Mikola 1970, 1973), rapid colonization by *Suillus luteus* is instrumental in permitting survival of pines far from plantations. Presumably, some or much of this colonization is initiated by a resistant spore bank (Baar et al. 1999, Taylor and Bruns 1999, Ashkannejhad and Horton 2006, Nguyen et al. 2012, Hynson et al. 2013), allowing nonsimultaneous arrival of pine seeds and fungal spores. ECM fungal species that lack resistant propagules cannot survive long in the soil and depend for their survival on the pre-existence of established trees. If *S. luteus* permits the establishment of those trees, *S. luteus* not only enables a pine invasion, but also indirectly permits subsequent invasion of other ECM fungi. This may constitute an unusual example of facilitation in invasion (invasional meltdown sensu Simberloff and Von Holle 1999) between members of a single guild. The few studies that have sampled ECM fungi associating with pines invading outside plantations in areas where pines are not native (Nuñez et al. 2009, Dickie et al. 2010, Hynson et al. 2013) also found *Suillus* spp. to be important members of the coinvasive community. This is surprising, given the differences in geographic region and climate (New Zealand, Hawaii, and South America), and the different Pinaceae species sampled (including *Pinus contorta*, *P. radiata*, *P. pinaster*, and *Pseudotstuga menziesii*). The ubiquity of *Suillus* as a coinvasive genus with Pinaceae reinforces its importance as a key facilitator for pine invasions.

Our results highlight the fact that extremely depauperate fungal communities may be sufficient to enable a pine invasion outside the native range or to facilitate range expansion in the native distribution. However, while the ability of a single ECM fungus to enable an invasion demonstrates that high diversity is not needed for coinvasion, it does not suggest that *any* ECM fungus can enable an invasion. *Suillus* species, in the native range, are known to act as pioneer species (Baar et al. 1999, Ashkannejhad and Horton 2006). ECM fungi lacking some of the physiological and ecological adaptations necessary to facilitate invasion may yield slower invasions, or may coinvade only when other, complementary fungi are also present. However, *Suillus* species are found virtually wherever *Pinus* species occur, including almost all sampled regions outside the native range where pines have been introduced (Mikola 1969, Hedger 1986, Chu-Chou and Grace 1988, Dickie et al. 2010, Walbert et al. 2010, Hynson et al. 2013). Wilde (1944) commented that "99 percent of all practicing foresters will not have to lose any sleep over the problem of mycorrhizal infection." Unfortunately, it seems likely that many invasive pines, too, will not have to lose too much sleep over absence of co invasive mycorrhizal fungi, since the presence of a few species with high dispersal ability may secure the inoculum needed for invasion.

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