New Phytologist

Research

The effect of different pine hosts on the sampling of *Rhizopogon* spore banks in five Eastern Sierra Nevada forests

T. A. Rusca, P. G. Kennedy and T. D. Bruns

Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720-3102, USA

Author for correspondence:
Thomas D. Bruns
Tel: +1 510 6427987
Fax: +1 510 6424995
E-mail: pogon@berkeley.edu

Received: *11 November 2005*
Accepted: *11 January 2006*

Summary

• Our primary goal was to determine whether detection of *Rhizopogon* internal transcribed spacer (ITS) groups is affected by the pine species used in seedling bioassays. Our secondary goal was to investigate composition of *Rhizopogon* spore banks in the Eastern Sierra Nevada of California, a previously unsampled region.

• We used seedlings of *Pinus contorta*, *Pinus jeffreyi*, *Pinus lambertiana*, and *Pinus muricata* as bioassay plants and identified the *Rhizopogon* retrieved by internal transcribed spacer (ITS) sequence analysis.

• We found that each of the pine species retrieved all of the abundant *Rhizopogon* ITS groups, but there were significant differences among pines in the richness of *Rhizopogon* ITS groups recovered. *Pinus muricata* recovered all ITS groups found in this study and was significantly better than *P. lambertiana*. *Rhizopogon* communities from the five sampled sites contained six to eight ITS groups per site, with two unique sequence groups and a higher abundance of the *Rhizopogon ellenae* and *Rhizopogon arctostaphyli* groups than at previously sampled sites.

• These results show high cross-receptivity between *Rhizopogon* and pine species, and regional patterns in spore bank composition.

Key words: host specificity, spore bank, *Rhizopogon*, *Pinus*, California.

New Phytologist (2006) **170**: 551–560

doi: 10.1111/j.1469-8137.2006.01689.x

Introduction

Ectomycorrhizal fungi are necessary for the growth and survival of pines and many temperate forest species (Smith & Read, 1997). These fungi are essential for acquisition of mineral nutrients and can protect roots from adverse conditions such as drought and high metal concentrations (Brownlee *et al.*, 1983; Burgess *et al.*, 1993). *Rhizopogon* is a genus of ectomycorrhizal fungi with over 250 described species and varieties, and most of those species are specifically associated with members of the Pinaceae (Molina *et al.*, 1999). Recent work by Kjøller and Bruns (2003) has shown that *Rhizopogon* species form abundant spore banks in pine-dominated forests in California, and these spore banks can be efficiently sampled by bioassaying diluted soil with pine seedlings. Following severe disturbance, such as stand-replacing fire, these spore banks provide most of the mycorrhizal inoculum for newly establishing pine seedlings (Horton *et al.*, 1998; Baar *et al.*, 1999).

Our primary goal was to test the hypothesis that different pine species yield different species of *Rhizopogon* under identical bioassay conditions. This question has practical implications, because some pine hosts are much faster and easier to grow than others, and if one were to design an efficient survey of *Rhizopogon* based on pine bioassays, it would be important to know which pines provide the best assay plants. The question is also important for biological reasons, because if all pines can interact with the same *Rhizopogon* spore banks it could facilitate migrations of both pine and *Rhizopogon* species in diverse floristic settings such as California

where different pine species typically dominate at different elevational and latitudinal zones (Barbour & Major, 1990).

It is well established that some *Rhizopogon* species are specific to either *Pinus* or *Pseudotsuga*, and that host specificity within *Rhizopogon* correlates with the phylogeny of the Pinaceae (Massicotte *et al.*, 1994; Molina *et al.*, 1999; Grubisha *et al.*, 2002). However, *Rhizopogon* specificity within the genus *Pinus* has not been well investigated. *Suillus*, the sister genus to *Rhizopogon*, has been observed to discriminate between hard pines (subgenus *diploxylon*) and soft pine (subgenus *haploxylon*) (Smith & Thiers 1964), and there is some evidence that *Rhizopogon* may also discriminate. For example, two species are known only from single hosts in subgenus *haploxylon*. *Rhizopogon pinyonensis* is so far known only from pinyon pine (Harrison & Smith, 1968) and an apparently undescribed species of *Rhizopogon* was retrieved from bioassays with *Pinus longaeva* (Bidartondo *et al.*, 2001). However, most collections of pine-associated *Rhizopogon* appear to be associated with hard-pines (J. M. Trappe, pers. comm.; T. D. Bruns pers. obs.).

Based on these observations we hypothesized that some *Rhizopogon* species would discriminate between hard and soft pines, but that the majority of species would not discriminate among hosts within a subgenus. To test this hypothesis, we collected soils from five sites and bioassayed it with *Pinus lambertiana*, *Pinus jeffreyi*, *Pinus contorta* and *Pinus muricata* and then determined the taxa of *Rhizopogon* retrieved from each host. The first three hosts are all native to the Sierra Nevada, while the last is restricted the coastal California. *Pinus lambertiana* is a soft pine, while the three others are hard pines. *Pinus muricata* can be germinated within a week but the three montane species require longer vernalization treatments for efficient germinate. For these reasons, *P. muricata* is faster and more convenient to use, and it was the only host used for the previous bioassay survey of coastal and Western Sierra Nevada sites (Kjøller & Bruns, 2003).

Our second goal was to sample composition of *Rhizopogon* spore banks from sites in the Eastern Sierra Nevada as part of a long-term study of *Rhizopogon* distributions throughout the California floristic province. Previous bioassay studies of pine forests were limited to four sites on the coast dominated by *P. muricata*, one site at moderate elevation in the Western Sierra Nevada dominated by *Pinus ponderosa*, and one site at high elevation in the California White Mountains dominated by *P. longaeva* (Bidartondo *et al.*, 2001; Kjøller & Bruns, 2003). From these studies it was determined that the composition of *Rhizopogon* spore banks was similar in the Northern coastal sites and the Western Sierra site, but was very different at the Southern most coastal site and in the White Mountains. The Eastern Sierra sites sampled in the current study contain a different set of pine species, a different climate, and different soils than the other sites sampled, and for these reasons we expected to see differences in the composition of *Rhizopogon* spore banks.

Materials and Methods

Plant material

Pinus l. Douglas, *P. jeffreyi* Grev. & Balf. and *P. contorta* Loudon seeds were provided by the Placerville Nursery (USDA Forest Service). *Pinus muricata* D. Don cones were collected from Point Reyes National Seashore and the seeds were harvested directly from the serotinus cones. All seeds were surface-sterilized in 30% hydrogen peroxide with a drop of Tween-20, for 20 min, and then rinsed with water. *P. lambertiana*, *P. jeffreyi* and *P. contorta* seeds were vernalized, as suggested by the Forest Service Nursery in the following way. Seeds were placed in individual plastic bags with small holes punctured in the bottom and placed under running water for 2 d followed by a 30-d drip-dry period in the cold room (4°C). Seeds were covered by moist paper towels in the plastic bags to retain moisture and were checked every other day to make sure the moist environment was maintained. After 30 d the seeds were rinsed, again, drip-dried, and placed in a new plastic bag and in the cold room for the following lengths of time: *P. jeffreyi*, 33 d; *P. lambertiana*, 61 d; and *P. contorta*, 15 d. Vernalization was timed so that seeds for all species would be ready to plant at approximately the same time. This process was started on 15 July 2003 and ended on 14 October. The *P. muricata* seeds did not require any soaking or drying time and were ready to plant immediately after they were surface sterilized and rinsed.

Soil material

Soil samples were obtained from Eastern Sierra pine sites near Mammoth Lakes area between 18 and 20 June, 2003 from the following five sites: Obsidian Dome (37°45.403′, 119°1.456′, altitude 2588 m); Forest near Obsidian Dome (37°45.433′, 119°1.652′, altitude 2573 m); Inyo Crater Area (37°41.277′, 119°0.370′, altitude 2484.6 m); Fern Lake Trail Head (37°45.754′, 119°6.812′, altitude 2242 m); and Ribbon Falls Area (37°36.194′, 119°4.904′, altitude 2287 m). The first three sites had soil derived from volcanic activity within the last 600–900 yr. Ribbon falls was a young postfire forest derived from a burn less than 10 yr old. *Pinus jeffreyi* was the dominant pine at all sites except the Fern Lake trailhead, where *P. contorta* was more common. *Pinus contorta* was also found at Ribbon falls, and at the forest near Obsidian Dome. The latter site also contains *Pinus monticola*, a soft pine, and *Abies concolor*. Each soil sample consisted of 10 small scoops of soil, approx. 10 cm into the mineral layer. Each scoop was collected 2–3 m from the previous scoop along a haphazardly placed line. To avoid cross-contamination the trowel was wiped clean with an ethanol-soaked paper towel between sites.

Each of these five sample soils was sifted in a no. 10 soil screen to remove large rocks, roots, and other debris from the samples. This process was carried out in a transfer hood to

prevent contamination between soils. All sifting equipment was soaked in a bleach solution between siftings for 10 min, rinsed in water for 5 min, and then dried in an oven. Each sifted soil was transferred to plastic bags and stored in the cold room until needed. Extra soil is archived at the Department of Plant and Microbial Biology, University of California at Berkeley.

Bioassay design

Soil collected from Tomales Point, in a region known to be devoid of *Rhizopogon* (T. D. Bruns, unpublished) was autoclaved for 60 min with a 10-min drying cycle. A total of 300 ml of this sterile soil and 300 ml of sterile coarse sand was mixed with 4.5 ml of the inoculum soil in a sterile quart mason jar (approx. 946 ml), producing an approximate volume of 600 ml per jar. Separate sterile jars were used for each test soil. This dilution step was used because previous studies have shown it to be an efficient way to retrieve *Rhizopogon* from soil (Taylor & Bruns, 1999; Kjøller & Bruns, 2003). Each jar provided enough inoculated soil to fill 12 bioassay tubes (RLC-4 Super 'Stubby' Cell Container; Stuewe & Sons Inc., Corvallis OR, USA) to a volume of 50 ml. Polyester fiber was stuffed to an approximate height of 2–3 cm to prevent any soil added from falling through the drainage slots in the bottoms of the tubes, as well as to allow for sufficient drainage while maintaining a moist environment. For each site 40 tubes were set up for each of the four test pine species (n = 160 tubes per site); this number was selected because in an earlier study 20–40 *Rhizopogon* samples were found to saturate the accumulation curve for a site (Kjøller & Bruns, 2003). In addition 160 uninoculated controls (40 per host) were set up with only sterile Tomales Bay soil. Tubes were watered to moisten the soil and two or three pine seeds were distributed into each tube using sterilized forceps. These were later weeded to achieve one seedling per tube. Approximately 1 cm of sterilized sand was added on top of the seeds. A total of 960 tubes were planted in this way: 5 soil types × 4 hosts × 40 replicates + 160 uninoculated controls.

Isolation of *Rhizopogon*

Seedlings were harvested 5–6 months after planting. Suilloid ectomycorrhizal root tips were identified under a dissecting scope by their clustered morphology, cottony white surfaces, and thick white-branched rhizomorphs (Agerer 1987–2002; Kjøller & Bruns, 2003). Typically one or two mycorrhizal tips were collected from each seedling. This small sampling within seedlings was chosen because we have found that at high levels of sample soil dilution seedlings are typically colonized by one species of *Rhizopogon*. Individual tips were placed in 2 ml Eppendorf screw-top tubes containing three glass beads, snap-frozen in liquid nitrogen, and stored in a −80°C freezer until they were freeze-dried. Individual freeze-dried root tips were crushed in a bead beater and DNA extraction was performed using the Dneasy Tissue Kit (Qiagen Inc., Valencia, CA, USA). Polymerase chain reaction (PCR) amplification was carried out using the internal transcribed spacer (ITS) of the fungal ribosomal DNA with the primers ITS1-F and ITS4, as described by Gardes & Bruns (1993). We initially screened PCR amplicons by restriction fragment length polymorphism (RFLP) analysis to reduce sequencing. Separate aliquots of PCR products were digested with the enzymes *Hinf*-I and *Hha*-I (purchased from New England Biolabs Inc., Beverly, MA, USA), and restrictions digest patterns were visually compared on agarose gels. Multiple samples of all unique RFLP patterns from each host–site combination were sequenced. Sequencing reactions were performed following instructions from the BIGDYE v3.1 chemistry (Applied BioSystems, Foster City, CA, USA) and reactions were run on an ABI 3100 automated sequencer (Applied BioSystems, Foster City, CA, USA). Data obtained were processed using DNA sequence analysis (version 2.01) and SEQUENCE NAVIGATOR software (Applied BioSystems, Foster City, CA, USA).

Sequences analysis and phylogenetic placement

We used phylogenetic analysis to place unknown sequences into terminal groups that contained low levels of nucleotide divergence and no discernible phylogenetic structure. We did this in five steps. (1) We used BLASTN to screen out sequences out were not *Rhizopogon*. (2) We assembled a baseline ITS dataset using previously deposited sequences of *Rhizopogon* derived from identified basidiocarps and from unidentified root samples. These were retrieved from GenBank via Entrez by targeting sequences deposited from several recent papers (Bidartondo *et al.*, 2001; Bidartondo & Bruns, 2002; Grubisha *et al.*, 2002; Kjøller & Bruns, 2003; Kretzer *et al.*, 2003) and by using the key word search: '*Rhizopogon*' and 'internal transcribed spacer'. Retrieved sequences containing only one of the two spacer regions were discarded and identical sequences were dropped. The final baseline data set consisted of 139 *Rhizopogon* sequences derived from GenBank and 113 derived from this study. Accession numbers for the sequences used and produced in this study are given in the supplementary table (Table S1). All of the sequences were then aligned using POA (Lee *et al.*, 2002). We trimmed this alignment at the 5′ and 3′ ends to the point where most of the sequences contained data, but the alignment was otherwise unaltered. (3) We used neighbor-joining (NJ) analysis as implemented in PAUP (Swofford, 2002) to identify groups of highly similar sequences. This 'broad' analysis was used to place the unknown sequences into smaller groups of more similar sequences. (4) We aligned sequences from each group identified by the broad analysis and their closest relatives with POA and analysed these groups separately with parsimony in 'group-specific' analyses. We used heuristic searches using 10 random addition sequences, and set the maxtrees options to 10 000. We then examined the strict consensus tree to look for common

internal structures, and several individual trees to visually exam branch lengths. (5) We used Jacknife analysis as implemented in PAUP with 33.3% deletion, and the fast search option, to examine support for internal branches.

Statistical analyses

Differences in the number of seedlings colonized and ITS group richness among hosts and sites were compared using a series of one-way ANOVAs. The ITS group richness was analysed using both the raw data as well as richness estimates based on rarefaction (Krebs, 1999) to correct for differences in the number of seedlings colonized across hosts. For host comparisons, ITS group richness estimates were rarefied to five seedlings per host per site, which was the minimum number of seedlings colonized on any host (at one site, *P. lambertiana* had only three out of 40 seedlings colonized, but this sample was eliminated from statistical analyses of host effects). When comparing ITS group richness among sites, the data were first rarefied within each host (n = 3–8 depending on host) in order to minimize the differences based on hosts at each site. *Post-hoc* comparisons of hosts and sites were conducted using Tukey HSD tests. Before each ANOVA, variances were determined to be homogeneous using Levene's test ($P > 0.05$). The ITS group richness at each site in this study and that in Kjøller & Bruns (2003) were compared using a two-tailed t-test following rarefactions to standardize sample sizes (n = 8) and limiting comparison to assays with *P. muricata*. The variances were determined to be homogeneous using an F-test. The occurrence of the *Rhizopogon occidentalis* group across hosts was tested using a χ^2 test of independence. All analyses were conducted in JMP 5.0 (SAS Institute Inc, Cary, NC, USA) and considered significant if $P = 0.05$. Bootstrap estimates of *Rhizopogon* ITS group richness were calculated for each site using the pooled data from all four hosts and ESTIMATES software (Colwell, 2005).

Results

A total of 295 of the 800 test seedlings were visually selected for analysis based on suilloid-like morphologies. *Rhizopogon* was confirmed in 285 of these, the other 10 yielded sequences of *Suillus* (6), *Phialophora* (2), *Hebeloma* (1), and an unidentified basidiomycete (1). In addition five seedlings contained two different *Rhizopogon* ITS groups for a total of 290 *Rhizopogon* positive samples. No *Rhizopogon* was found on any of the 160 control seedlings. We recovered 11 *Rhizopogon* ITS groups from the positive bioassays. These groups were based on 113 sequences (Table 1), which represented multiple samples of all RFLP types from each site and usually from multiple hosts. Nine of the groups defined are the same as those recognized in previous study of *Rhizopogon* spore banks in other parts of California, or from roots of the Monotropoideae (Bidartondo & Bruns, 2002; Kjøller & Bruns, 2003), but two are unique to this study. One of them, gr. 10, is placed in section *Amylopogon* as a unique clade with high confidence (several branch > 95% jackknife), and is placed as the sister group to group 8 (*arctostaphyli*) with low confidence (not shown). Group 16, the other unique group, is placed in section *Rhizopogon* in the neighborhood of a sequence identified as *Rhizopogon subsalmonius*, but it is not particularly close to that sequence (not shown). Most of the sequence groups defined in Table 1 contain few sequence differences, no discernible within-group phylogenetic structure and are well separated from other groups in the phylogenetic analyses conducted. Groups 4a and 4b are exceptions to these rules in that they contain more within-group divergence, and differences between the two groups are not well supported. However for the purposes of searching for possible host discrimination among different *Rhizopogon* taxa, we think it is better to divide groups as finely as possible.

Table 1 Summary of internal transcribed spacer (ITS) sequence groups

ITS groups[1]	Total[2]	% Substitutions[3]
gr 4a *roseolus*	36 (28)	0.74, 3.30
gr 4b *vulgaris*	18 (10)	0.93, 3.30
gr 5 *ellenae*	35 (19)	0.47, 1.60
gr 8 *arctostaphyli*	44 (33)	0.69, 2.27
gr 9 *salebrosus*	35 (12)	0.46, 1.70
gr 10 sect. *Amylopogon*	2 (2)	na, 0.0
gr 11 *occidentalis*	27 (8)	0.52, 1.54
gr 13 *fuscorubens*	6 (4)	0.37, 1.09
gr 14 *evadens*	8 (4)	0.43, 1.25
gr 15 *olivaceotintus*	8 (4)	0.89, 2.14
gr 16 sect. *Rhizopogon*	2 (2)	na, 0.25
Other groups	50 (0)	na

[1]Group names are those used by Kjøller & Bruns (2003), or Bidartondo & Bruns (2002), except for the last two that are novel. Underlined names are groups that contain sequence from the holotype of the named species; other names are linked to identified sporocarps primarily from Grubisha *et al*. (2002).
[2]Total number of sequences analysed (number derived from this study).
[3]Mean (and maximum) within group uncorrected substitutions calculated as the number of bases that differ divided by the number of aligned bases.
na, not applicable.

Differences in the distribution of the 11 ITS groups were evident across both hosts and sites (Fig. 1, Table 2). The number of seedlings colonized was not significantly different among hosts (F = 0.577, P = 0.638), however, ITS group richness was significantly different (F = 3.333, P = 0.046). *Pinus muricata* seedlings recovered greater richness of *Rhizopogon* ITS groups than *P. lambertiana*, with *P. jeffreyi* and *P. contorta* being intermediate (Tukey test, $P < 0.05$) (Table 3). The ITS group richness pattern was even stronger when controlling for differences in sample size across hosts using rarefaction (F = 6.137, P = 0.006). Both *P. muricata* and *P. contorta*

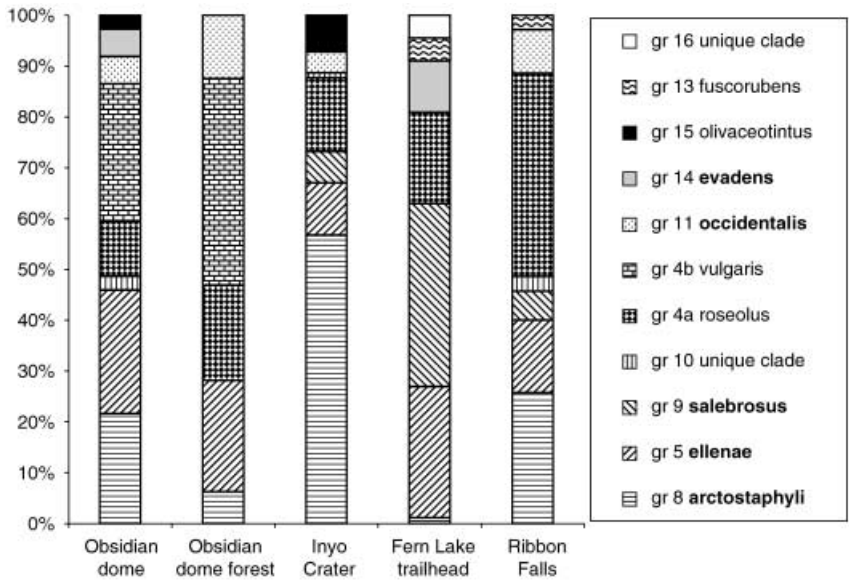

Fig. 1 Composition of *Rhizopogon* spore bank communities at the five sites. Names in bold are based on the inclusion of holotype sequences.

Table 2 *Rhizopogon* internal transcribed spacer (ITS) sequence groups found across sites and host

Site[1]	*Pinus* host	*n*[2]	Proportion of richness[3]	*Rhizopogon* ITS groups[4]										
				4a	4b	5	8	9	10	11	13	14	15	16
IC (7)	*P. muricata*	30	0.71	5		4	15	4					1	
	P. jeffreyi	23	0.57	7		3	7						3	
	P. contorta	25	0.71	1		3	14	2		4				
	P. lambertiana	24	0.57	1	1		19						3	
FL (7)	*P. muricata*	36	0.86	8		10		7			3	6		2
	P. jeffreyi	22	0.43	4				16				1		
	P. contorta	17	1.00	4		7	1	2			1	1		1
	P. lambertiana	16	0.57			6		7				1		1
OF (5)	*P. muricata*	8	0.80	2	3	1				1				
	P. jeffreyi	12	0.80	3	5	2				2				
	P. contorta	10	0.80		5	2	2			1				
	P. lambertiana	3	0.40	1		2								
RF (7)	*P. muricata*	11	0.86	5		2	1	1		1	1			
	P. jeffreyi	12	0.71	4		3	3	1	1					
	P. contorta	8	0.43	4			1			2				
	P. lambertiana	5	0.29	1			4							
OD (8)	*P. muricata*	12	0.63		4	4			1	2			1	
	P. jeffreyi	6	0.38	2	2		1							
	P. contorta	7	0.38	2	3							2		
	P. lambertiana	13	0.38		1	5	7							
Total		290		54	24	54	75	40	2	13	5	11	8	4

[1]IC, Inyo Crater; FL, Fern Lake Trail Head; OF, Forest next to Obsidian Dome; RF, Ribbon Falls; OD, Obsidian Dome; (number) equals total *Rhizopogon* ITS groups recovered at site from all hosts.
[2]Number of seedlings with *Rhizopogon*.
[3]Proportion of *Rhizopogon* richness at a site recovered by each host.
[4]Number of seedlings colonized by the following groups: 4a, *roseolus*; 4b, *vulgaris*; 5, *ellenae*; 8, *arctostaphyli*; 10, unique *amylopogon*; 9, *salebrosus*; 10, *occidentalis*; 13, *fuscorubens*; 14, *evadens*; 15, *olivaceotinctus*; 16, unique sect *Rhizopogon*.

seedlings had significantly higher ITS group richness than *P. lambertiana*, while ITS group richness of *P. jeffreyi* was not significantly different from any other host (Tukey test, $P < 0.05$). The number of seedlings colonized also varied significantly among sites ($F = 10.741$, $P < 0.001$). Over half the seedlings from both Inyo Crater and Fern Lake trailhead were colonized, while less than a quarter of the seedlings were colonized from the forest near Obsidian Dome, Obsidian

Table 3 Recovery of *Rhizopogon* internal transcribed spacer (ITS) sequence groups summarized across sites for each host

Host *Pinus* species	Mean colonized seedlings[1]	Mean richness[2]	Total ITS gr recovered across all sites
P. muricata	19.4 (5.67)	3.28 (0.17) a	11
P. jeffreyi	15.0 (3.26)	2.86 (0.17) ab	9
P. contorta	13.4 (3.39)	3.03 (0.17) ab	9
P. lambertiana	12.2 (3.81)	2.21 (0.58) b	8

[1]Average of number of seedlings with *Rhizopogon* for the five sites (SE). Differences are not significant with ANOVA.
[2]Average number of ITS groups recovered per site when sample sizes are equalized by rarefaction (standard error). Means that differ significantly (Tukey HSD) do not share a common letter.

Table 4 Variation in colonization and *Rhizopogon* internal transcribed spacer (ITS) sequence group richness across five sites in the Eastern Sierra Nevada

Sites	Proportion of seedlings colonized[1]	Observed[2] richness	Estimated richness[3]
Obsidian dome	0.24 (0.4)	8.00	8.77 (0.19)
Obsidian dome forest	0.21 (0.5)	5.00	5.14 (0.26)
Inyo Crater	0.64 (0.4)	7.00	7.39 (0.01)
Fern Lake Trailhead	0.57 (0.12)	7.00	7.40 (0.16)
Ribbon Falls	0.23 (0.4)	7.00	7.88 (0.77)

[1]Proportion of seedling colonized by *Rhizopogon* averaged across the four hosts; SE in parenthesis.
[2]Number of *Rhizopogon* ITS groups recovered by all four hosts.
[3]Bootstrap estimate of richness; SD in parenthesis.

Dome, and Ribbon Falls (Table 4). The observed ITS group richness appeared to vary between sites (Table 4), but it was not significant when sample sizes were controlled within hosts ($F = 0.11$, $P = 0.973$). Accumulation curves for *Rhizopogon* ITS groups appeared to plateau for three of the sites when hosts were pooled (data not shown). Bootstrap estimates of *Rhizopogon* richness were only slightly greater than the observed values for all five sites, and they indicated that at most one ITS group may have been missed at Obsidian Dome and Ribbon Falls sites (Table 4).

When we examined the recovery of individual *Rhizopogon* ITS groups, we found no obvious differences between hosts. Six of the 11 *Rhizopogon* sequence groups were found on all four pines within at least one site (Table 2), and these six included all of the abundant ITS groups. The five groups that were not found on all potential hosts were the rarest: (1) *Rhizopogon olivaceotinctus* group was recovered from eight seedlings across two sites and was not found on *P. contorta*; (2) *Rhizopogon fuscorubens* group was recovered on three seedlings across two sites was not found on *P. jeffreyi* and *P. lambertiana*; (3) *Amylopogon* group 10 was recovered from four seedlings across two sites but was not found on *P. contorta* or *P. lambertiana*; (4) group 16 was recovered from five seedlings at a single site, but was not found from *P. jeffreyi*; and (5) *R. occidentalis* group was recovered on 13 seedlings across four sites, but was not found on *P. lambertiana*. Of these examples only the *R. occidentalis* group appeared to be abundant enough that its absence on *P. lambertiana* may not have been caused by chance. However, the observed pattern was not significantly different from a random pattern ($\chi^2_{9,17} = 11.08$, $P = 0.271$). In addition, we know from inoculation studies that *R. occidentalis* has the ability to colonize *P. monticola* (Molina & Trappe, 1982), which, like *P. lambertiana*, is in the soft pine group. Interestingly, all *Rhizopogon* ITS groups were recovered by *P. muricata*, the only host species tested that is not native to the Sierra.

While the *Rhizopogon* spore banks across the five sites differed in terms of the relative abundance of individual ITS groups (Fig. 1), their higher-level composition and general structure were similar (Fig. 2). Three ITS groups were found in all five locations: *Rhizopogon arctostaphyli*, *Rhizopogon ellenae* and *Rhizopogon roseolus*, and these were often among the most abundant groups. Only group 16 was unique to one site, even though several other groups were consistently low in abundance. At all sites > 80% of the *Rhizopogon*-positive seedlings were colonized by a combination of species in the subgenus *Amylopogon* (groups 5, 8–10) and the *roseolus–vulgaris* clade (gr. 4a and 4b), while species in the remaining five groups were low in abundance (Table 2, Fig. 1). Ignoring differences in taxon identity, the three most abundant ITS groups at a site accounted for 79 ± 2% of the colonized seedlings, and the structure of the communities fit a log normal distribution (Fig. 2).

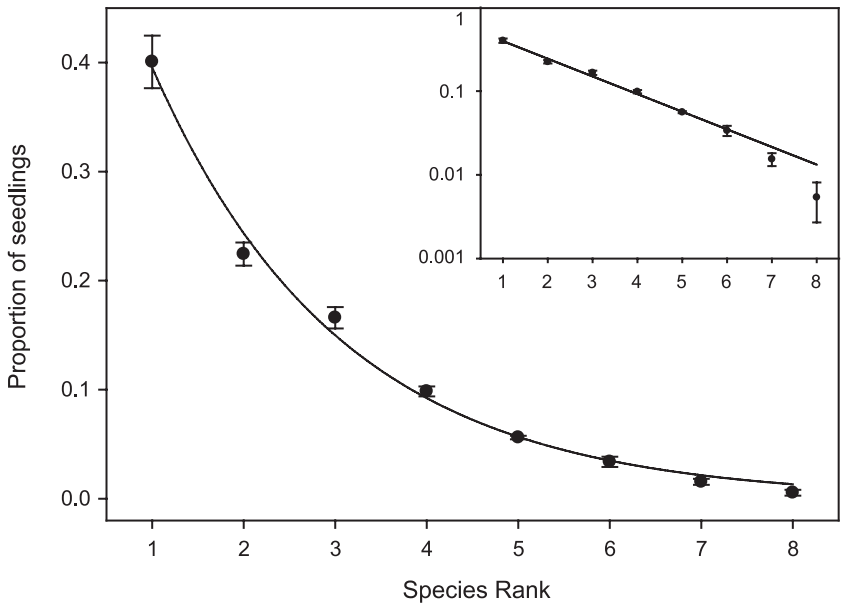

Fig. 2 Composite ranked abundance curve for *Rhizopogon* spore bank communities at five sites. Percentage of *Rhizopogon* positive seedlings colonized by the most (1) to least (8) abundance *Rhizopogon* internal transcribed spacer (ITS) group is averaged across sites. Standard errors are shown. (Inset shows *y*-axis as a log scale.)

Discussion

All four pine species tested appeared to have the ability to associate with all of the *Rhizopogon* ITS groups encountered. We only qualify this statement because particular hosts did not recover several rare types. These missing *Rhizopogon*–pine combinations are probably explained by chance, but because these ITS groups were rare in our sample, we did not have the ability to detect species-specific effects involving them. There could be different biases among hosts for particular taxa of *Rhizopogon*, but if such biases exist they are not obvious (Table 2). The fact that no *Rhizopogon* ITS groups appeared to be specific to the hard or soft pine subgenera is very different from the pattern reported in *Suillus*, where many species that are distinct at the ITS level are associated with either hard or soft pines but not both (Kretzer *et al.*, 1996). Our results with *Rhizopogon* mean that there is broad cross-receptivity among pines; this could be important as pine-dominated vegetation zones move with changing climate or changing fire regimes.

Pinus muricata, the only species not native to the Sierra, actually performed the best, exhibiting the highest level of *Rhizopogon*-positive bioassays, recovering the greatest average richness of *Rhizopogon* across all sites, and missing none of the ITS groups detected in our study. It also recovered significantly higher levels of *Rhizopogon* richness than *P. lambertiana*. This result has practical importance for seedling bioassay studies, because the seeds of *P. muricata* are more easily germinated than many of the montane species (see the Materials and Methods section).

The host specificity of *Rhizopogon* species has been extensively studied among genera of the Pinaceae and arbutoid hosts in the Ericaceae (Massicotte *et al.*, 1994, 1999; Molina & Trappe, 1994). From these studies it is known that *Rhizopogon* specificity varies with context of the assay: syntheses based on mycelial cultures, or dual-host pots exhibit broader host ranges than those based on spore inoculations with single hosts. In the case of mycelial cultures, this may be an artifact of sugar level in the media (Massicotte *et al.*, 1994). However, in the dual-host setting, the lower apparent specificity likely reflects the ecological potential in which a species that has already colonized its primary host by spore may be able to expand via mycelium onto a secondary host (Massicotte *et al.*, 1994). This is relevant to the current study in that single host–spore inoculation assays are the most restrictive setting, yet our single host assays showed no specificity among pine species.

Composition compared among sites in this and other studies

The species richness observed at these Eastern Sierra sites, was slightly higher than that reported on Western Sierra and Coastal sites by Kjøller and Bruns (2003). Excluding their southern coastal site, which was a clear outlier in the earlier study, the three central and northern coastal sites and the Western Sierra site had four to six ITS groups (mean = 5), while the Eastern Sierra sites, varied from five to eight groups (mean = 6.8). The confounding factors of slightly different methods, spatial samples and sample sizes make quantitative comparisons between the two studies difficult. However, ignoring the difference of slightly greater within-site spatial scale of the earlier study (which should bias it toward greater richness), and the culture bias of that study (which may have reduced richness) there was no significant difference in group richness ($T = -1.75$ df = 7, $P = 0.123$). Despite the fact that differences

were not significant, it is clear that the Eastern Sierra sites are at least as species rich as the coast and Western Sierra sites.

The high richness of *Rhizopogon* in the Eastern Sierra sites is interesting because some of the sites are amazingly harsh. Obsidian Dome, for example, is an 600-yr-old volcanic dome of unvegetated rhyolite and obsidian with scattered islands of pine that are separated from the adjacent forest by several hundred meters of bare rock. Our sample from this site included four pine islands, three of which consisted of only one or two trees. Obtaining sufficient soil for these samples was difficult; yet, this site had the highest richness (Fig. 1, Table 4). The adjacent Obsidian Dome forest and the Inyo Crater forest were less harsh, and contain fairly continuous, open forest, but the soil of both was derived from volcanic activity of about the same age as Obsidian Dome. Izzo *et al.* (2005) has shown that even though some of the *Rhizopogon* species are not resident colonizers or sporocarp producers in mature forests, they are present in spore bank samples. In this way they are similar to some post-fire plant species that reside in a seed bank between disturbance events. Thus, the richness of *Rhizopogon* on these harsh sites may simply be a function of their early successional status, or perhaps the drier climate of the Eastern Sierra, which may favor dispersal by mammals over wind dispersal, or greater persistence in the spore bank.

The composition of these Eastern Sierra communities contained some major differences from the coastal sites. The high frequency of the *R. arctostaphyli* and *R. ellenae* groups across the Eastern Sierra sites was one striking difference, as the former group was absent from the coast and the latter was restricted to the Northern most coastal site. Similarly, the *R. roseolus* group, which was found at all five sites in this study, was found at only one of the coastal sites. There were also two novel groups found: group 10, which is related to the *R. arctostaphyli* clade, but distinct from it, and group 16, which is unique relative to all sampled taxa within the genus. Neither of these new groups was particularly abundant, unlike the novel *Amylopogon* clades found at the southern coastal sites (Kjøller & Bruns, 2003).

The effects of soil dilution on perception of the *Rhizopogon* spore banks

The purpose of soil dilution before bioassay is to limit competitive effects among fungal species so that a few good competitors do no dominate the assay and mask the presence of lesser competitors. To achieve this goal, the soil must be diluted enough so that most seedlings are colonized by a single species. The low colonization levels observed (Table 4) are thus in line with this goal, but at the cost of sample size, since the majority of bioassay seedlings yielded no data. There is also the risk of diluting less abundant species to extinction. The differences in percentages of seedlings colonized by *Rhizopogon* observed between sites (Table 4) probably reflect differences in spore concentration. The alternative hypothesis that differences in soil properties affected colonization is less plausible as test soils were diluted 134 : 1 into a common *Rhizopogon*-free soil for all assays. Had we known in advance that some sites had lower spore densities it would have made sense to dilute them less and thereby increase sample sizes.

Table 5 Abundance differences in two *Rhizopogon* internal transcribed spacer (ITS) sequence groups across studies using different soil dilutions

Study	% of sect. *Amylopogon*[1]	% *olivaceotintus* gr.[1]
Current (five sites, 134 × dilution)	52 (8.5)	2.0 (1.5)
Kjøller and Bruns (2003) (five sites, 50 × dilution)	57 (5.8)	4.6 (4.6)
Izzo *et al*. (2006) (six sites, no dilution)	23 (6.4)	20 (5.1)

[1]Average percentage; SE in parenthesis.

Even if different dilutions recover the same species, they are likely to provide rather different views of abundance. The most dilute samples will reveal primarily the most abundant species, but the least dilute samples will reveal some mix of the most abundant and the most competitive of the low abundance species. Evidence of this effect was present in the Taylor & Bruns (1999) study that showed a significant increase in number of seedlings by *Rhizopogon salebrosus* (sect. *Amylopogon*), a species that we now know can be outcompeted in the initial colonization of seedlings by *R. occidentalis* (Kennedy & Bruns 2005), and possibly other taxa. Comparison across studies also suggests that species in section *Amylopogon* increase with dilution (Table 5). In the spore banks at Teakettle forest in the Western Sierra, Izzo *et al.* (2006) used undiluted soils and recovered a much lower frequency of groups from section *Amylopogon* compared with the current study or the Kjøller & Bruns (2003) study. The Izzo *et al.* (2006) study also found a much higher frequency of the *R. olivaceotinctus* group, which is rare in the other studies cited (Table 5). Differences in the *Rhizopogon* communities present among the sites could obviously be a factor, but one of the sites in the Kjøller & Bruns (2003) study was only a few miles away from Teakettle Forest and yet the percentage of seedling colonized by *Amylopogon* taxa and *olivaceotinctus* gr. were 71% and 2%, respectively, vs 23% and 20% at Teakettle. These comparisons point out that abundance estimates are relative measures that are comparable only among studies that use similar dilutions, and that absolute quantification will be difficult to achieve owing to the confounding factor of competition.

Acknowledgements

We thank Tim Szaro for his assistance with teaching the senior author the molecular techniques necessary for this work. We

thank Inyo National Forest and Devil's Pile Post National Monument for cooperation with the fieldwork. Funding was provided by NSF grant DEB-0236096 to T.D. Bruns, and an REU supplement to T.A. Rusca.

References

Agerer R.1987–2002. *Colour atlas of Ectomycorrhizae*. Schwäbisch-Gmünd, Germany: Einhorn-Verlag.

Baar J, Horton TR, Kretzer A, Bruns TD. 1999. Mycorrhizal recolonization of *Pinus muricata* from resistant propagules after a stand-replacing wildfire. *New Phytologist* **143**: 409–418.

Barbour MG, Major J. 1990. *Terrestrial vegetation of California, new expanded edition*. Davis, CA, USA: California Native Plant Society.

Bidartondo MI, Bruns TD. 2002. Fine-level mycorrhizal specificity in the Monotropoideae (Ericaceae): specificity for fungal species groups. *Molecular Ecology* **11**: 557–570.

Bidartondo MI, Baar J, Bruns TD. 2001. Low ectomycorrhizal inoculum potential and diversity from soils in and near ancient forests of bristlecone pine (*Pinus longaeva*). *Canadian Journal of Botany* **79**: 293–299.

Brownlee C, Duddridge JA, Malibari A, Read J. 1983. The structure and function of mycelial systems of ectomycorrhizal roots with special reference to their role in forming inter-plant connections and providing pathways for assimilate and water transport. *Plant Soil* **71**: 433–444.

Burgess TI, Malajczuk N, Grove TS. 1993. The ability of 16 ectomycorrhizal fungi to increase growth and phosphorus uptake of *Eucalyptus globulus* Labill and *E. diversicolor* F-Muell *Plant and Soil* **153**: 155–164.

Colwell RK. 2005. *EstimateS: statistical estimation of species richness and shared species from samples, version 7.5.* http://viceroy.eeb.uconn.edu

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Grubisha LC, Trappe JM, Molina R, Spatafora JW. 2002. Biology of the ectomycorrhizal genus *Rhizopogon*. VI. Re-examination of infrageneric relationships inferred from phylogenetic analyses of ITS sequences. *Mycologia* **94**: 607–619.

Harrison KA, Smith AH. 1968. Some new species and distribution records of *Rhizopogon* in North America. *Canadian Journal of Botany* **46**: 881–899.

Horton TR, Cázares E, Bruns TD. 1998. Ectomycorrhizal, vesicular-arbuscular and dark septate fungal colonization of bishop pine (*Pinus muricata*) seedlings in the first 5 months of growth after wildfire. *Mycorrhiza* **8**: 11–18.

Izzo AD, Meyer M, Trappe JM, North M, Bruns TD. 2005. Hypogeous ectomycorrhizal fungal species on roots and in small mammal diet in a mixed-conifer forest. *Forest Science* **51**: 243–254.

Izzo A, Nguyen DT, Bruns TD. 2006. Spatial structure and richness of the ectomycorrhizal resistant propagule community colonizing hosts with differing seedling establishment patterns. *Mycologia*. (In press.)

Kennedy PG, Bruns TD. 2005. Priority effects determine the outcome of ectomycorrhizal competition between two *Rhizopogon* species colonizing *Pinus muricata* seedlings. *New Phytologist* **166**: 631–638.

Kjøller R, Bruns TD. 2003. *Rhizopogon* spore bank communities: within and among Californian pine forests. *Mycologia* **95**: 603–613.

Krebs CJ. 1999. *Ecological methodology*, 2nd edn. Menlo Park, CA, USA: Addison-Wesley Longman.

Kretzer A, Li Y, Szaro T, Bruns TD. 1996. Internal transcribed spacer sequences from 38 recognized species of *Suillus* sensu *lato*: phylogenetic and taxonomic implications. *Mycologia* **88**: 776–785.

Kretzer AM, Luoma DL, Molina R, Spatafora JW. 2003. Taxonomy of the *Rhizopogon vinicolor* species complex based on analysis of ITS sequences and microsatellite loci. *Mycologia* **95**: 480–487.

Lee C, Grasso C, Sharlow MF. 2002. Multiple sequence alignment using partial order graphs. *Bioinformatics* **18**: 452–464.

Massicotte HB, Molina R, Luoma DL, Smith JE. 1994. Biology of the ectomycorrhizal genus, *Rhizopogon* II. Patterns of host-fungus specificity following spore inoculation of diverse hosts grown in monoculture and dual culture. *New Phytologist* **126**: 677–690.

Massicotte HB, Molina R, Tackaberry LE, Smith JE, Amaranthus MP. 1999. Diversity and host specificity of ectomycorrhizal fungi retrieved from three adjacent forest sites by five host species. *Canadian Journal of Botany* **77**: 1053–1076.

Molina R, Trappe JM. 1982. Patterns of ectomycorrhizal host specificity and potential among Pacific Northwest conifers and fungi. *Forest Science* **28**: 423–458.

Molina R, Trappe JM. 1994. Biology of the ectomycorrhizal genus, *Rhizopogon*. I. Host associations, host-specificity and pure culture syntheses. *New Phytologist* **126**: 653–675.

Molina R, Trappe JM, Grubisha LC, Spatafora JW. 1999. *Rhizopogon.* In: Cairney JWG, Chambers SM, eds. *Ectomycorrhizal fungi key genera in profile*. Berlin, Germany: Springer, 129–161

Smith AH, Thiers HD. 1964. A contribution toward a monograph of the genus *Suillus*. Ann Arbor, MI, USA. (privately printed).

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis*, 2nd edn. New York, NY, USA: Academic Press.

Swofford DL. 2002. PAUP* Phylogenetic analysis using parsimony (*and other methods). Version 4.0d81. Sinauer Associates, Sunderland, MA, USA.

Taylor DL, Bruns TD. 1999. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. *Molecular Ecology* **8**: 1837–1850.

Supplementary Material

The following supplementary material is available for this article online.

Table S1 Accession numbers for internal transcribed spacer (ITS) sequences analyzed and produced

This material is available as part of the online article from http://www.blackwell-synergy.com

About *New Phytologist*

- *New Phytologist* is owned by a non-profit-making **charitable trust** dedicated to the promotion of plant science, facilitating projects from symposia to open access for our Tansley reviews. Complete information is available at **www.newphytologist.org**.
- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication 'as-ready' via *OnlineEarly* – the 2004 average submission to decision time was just 30 days. Online-only colour is **free**, and essential print colour costs will be met if necessary. We also provide 25 offprints as well as a PDF for each article.
- For online summaries and ToC alerts, go to the website and click on 'Journal online'. You can take out a **personal subscription** to the journal for a fraction of the institutional price. Rates start at £109 in Europe/$202 in the USA & Canada for the online edition (click on 'Subscribe' at the website).
- If you have any questions, do get in touch with Central Office (**newphytol@lancaster.ac.uk**; tel +44 1524 594691) or, for a local contact in North America, the US Office (**newphytol@ornl.gov**; tel +1 865 576 5261).

