Survival of *Suillus pungens* and *Amanita francheti* ectomycorrhizal genets was rare or absent after a stand-replacing wildfire

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Summary

• We looked for evidence of mycelial survival by *Suillus pungens* and *Amanita francheti* following a stand-replacing wildfire. These species were selected because we had previously mapped and genotyped their fruiting bodies in the pre-fire forest.
• Mycelial survival was investigated in two ways. First, we sampled seedlings in areas where these species had fruited abundantly before the fire, and second, we collected and genotyped mushrooms of *S. pungens*.
• Neither species was detected on seedlings within the areas sampled, and *A. francheti* was not detected in any above- or below-ground samples after the fire. Genetic evidence from *S. pungens* revealed that post-fire genets were small and numerous, and none were found to be identical to the genets sampled prior to the fire.
• From these results we conclude that *A. francheti* was not a common survivor or an early colonist of the post-fire forest, and that spores are the primary means by which *S. pungens* recolonized. If mycelial survival occurred in either species, it must have been relatively rare.

Key words: Fire survival, post-fire recolonization, ectomycorrhizal fungi, AFLP genotypes.

Introduction

Bishop pine, *Pinus muricata*, is a closed-cone pine endemic to coastal areas of California, USA. It is a short-lived pine with a maximum life span of 120 yr (Keeley & Zedler, 1998), but it thrives in settings where severe crown fires reoccur at approximately 40-yr intervals (Sugnet, 1985). Mature pines are typically killed in such fires, but the abundant seed cache released from the closed cones results in the re-establishment of a dense even-aged pine forest (Vogl *et al.*, 1977). Most other plants in these communities also have life histories that enable them to re-establish after severe fire (Heady *et al.*, 1977; Vogl *et al.*, 1977). Examples include *Baccharis pilularis*, a dominant shrub which resprouts vigorously after fire, and *Ceanothus* spp. which re-establish from their soil seed-bank.

We were interested in how ectomycorrhizal fungi that are associated with Bishop pine re-establish after such fires. Three basic possibilities exist: (1) they could re-establish from resistant propagules, which are cached in the soil; (2) they could re-establish by dispersal of new propagules from adjacent unburned areas; and (3) they could survive as mycelia. To examine these three possibilities, we studied a pre- and post-fire bishop pine forest at Point Reyes National Seashore, CA, USA. The pre-fire forest, which was approximately 35–40 yr old, had a mycorrhizal community that was dominated by members of the Russulaceae, and *Tomentella sublilacina*, with several *Amanita* species as common subdominants (Gardes & Bruns, 1996; Horton & Bruns, 1998; Taylor & Bruns, 1999). In the first two years following the fire, some of these same species were found on the newly established seedlings, but overall the community was dominated by *Rhizopogon*, *Wilcoxina*, and *Tuber* species (Horton *et al.*, 1998; Baar *et al.*, 1999; Grogan *et al.*, 2000a). Baar *et al.* (1999) compared the ectomycorrhizal composition in bioassays conducted before and after the fire to that seen on naturally established post-fire seedlings and showed that resistant propagules that survived

the fire were the most likely means of re-establishment for *Rhizopogon* and *Tuber* spp. A *Hebeloma* species, which was abundant at some sites, appeared to establish through newly dispersed propagules; it was not detected in the pre-fire forest either as a common resident or as resistant propagules (Baar *et al.*, 1999; Taylor & Bruns, 1999). *Wilcoxina* species, which were present as resistant propagules prior to the fire, may have increased their abundance through post-fire dispersal, or perhaps heat activation of soil-borne propagules (Baar *et al.*, 1999).

Clear evidence for survival of mycelium was absent, but some observations and prior reports suggested that it would be at least possible and perhaps likely. Pathogenic fungi such as *Armillaria gallica* and *Phellinus weirii* appear to persist as saprobes and recolonize living trees after fire (Dickman & Cook, 1989; Smith *et al.*, 1992). Several studies have shown that ectomycorrhizas can survive for months or even years after trees have been cut or roots have been severed (Harvey *et al.*, 1980; Ferrier & Alexander, 1985; Parsons *et al.*, 1994; Hagerman *et al.*, 1999a), and pine seedlings that re-established within 2 months after the Pt. Reyes fire, were well within the time-frame where one might expect survival of ectomycorrhizal roots. *Russula* species that were the pre-fire dominants were found immediately after the fire on seedlings (Baar *et al.*, 1999; Horton *et al.*, 1998; Grogan *et al.*, 2000a) and, in at least one case, *Russula* mycorrhizae were observed on a seedling that was adjacent to *Russula*-like mycorrhizae on dead tree roots (T. W. Horton, unpublished). In addition, *Russula* species found on new seedlings tended to occur at greater depths, where one might expect root survival to be greatest (Baar *et al.*, 1999). The lack of evidence for a *Russula* spore-bank (Taylor & Bruns, 1999) and the speed with which they recolonized also supported the idea that mycelial survival may have occurred. However, the only definitive evidence would have been to show that the same fungal genotypes occurred at the same location before and after the fire. Unfortunately, this type of evidence was not possible for the *Russula* species at Pt. Reyes because we had very limited pre-fire data on individual genotypes. The scarcity of genetic data was due primarily to the fact that these were not common fruiters and could not be isolated into pure culture from root tips.

We had genotypic data for *Suillus pungens* and *Amanita francheti* for one pre-fire site, and for that reason we targeted these two species to look for evidence of mycelial survival. Mushrooms of these species, which were abundant in the pre-fire forest, were mapped, collected, and genotyped. The latter was done by either single-stranded conformational polymorphisms (SSCPs) of isolated randomly amplified polymorphic DNA (RAPD) fragments (Bonello *et al.*, 1998), or by amplified fragment length polymorphisms (AFLPs) (Redecker *et al.*, 2001). The results showed that most of the *S. pungens* fruiting was from a single large genet (Bonello *et al.*, 1998), while *A. francheti* had multiple small genets (Redecker *et al.*, 2001). The mycorrhizae of these species could be found directly under their sporocarps; those of *S. pungens* were relatively low in abundance, while those of *A. francheti* were locally high in abundance (Gardes & Bruns, 1996; Taylor & Bruns, 1999).

We used two methods to test for mycelial survival of these two species. First, we sampled post-fire seedlings 9 months after the fire in areas where we had collected and archived freeze-dried sporocarp samples from the two years before the fire. Second, when *S. pungens* began to fruit abundantly in the post-fire forest, we mapped, collected, and genotyped its mushrooms in the same site where we had pre-fire data for this species. We then compared pre- and post-fire genotypes.

Materials and Methods

Materials

The study site was located on a low ridge within Pt. Reyes National Seashore adjacent to Limantour road in a small *P. muricata* D. Don stand. The area surrounding the stand was occupied by a coastal shrub community dominated by *Baccharis pilularis*. The site is within 4 km of the Pacific Ocean (38°03′16″ N, 122°51′12″ W), and the elevation is 170 m. Precipitation is about 80 cm yr^{-1}, most of which comes as rain during the winter months (Evens, 1993). Fog drip during the dry summer helps to lessen the effect of the long rainless period, which usually occurs between April and November. The soil is part of the Pablo–Bayview complex, a shallow, well-drained, gravelly loam (Anonymous, 1985).

On 3–7 October 1995, a high-intensity crown wildfire burned 5000 ha of the forest and shrub community at this site. All trees at the site were killed. The fire completely burned off the litter and humus layers. The first pine seedlings appeared at the site after 4 wk. In 1999, when post-fire mushrooms for this study were collected, the site was already covered densely by 1–2 m high *P. muricata* and by non-ectomycorrhizal shrubs.

On July 1996, we sampled naturally established *P. muricata* seedlings within site C (Gardes & Bruns, 1996; Bonello *et al.*, 1998). Ten seedlings were sampled within an area where the large *S. pungens* genet had fruited abundantly before the October 1995 fire (Bonello *et al.*, 1998), and 10 seedlings were sampled within an area were *A. francheti* genets had fruited abundantly and had been genotyped prior to the fire. Each seedling was the center of a soil core (10 cm diameter, 40 cm depth); this sampling method recovered the entire root system for most of the seedlings, which were generally less than 15 cm tall. All soil cores were kept at 4°C and processed within 1 wk of field sampling. Each soil core was sprayed with tap water over a sieve to rinse the soil off the root system of each seedling. The washed roots of each seedling were examined using stereo microscopes, and all mycorrhizal roots were collected and morphotyped. The minimum depth at which mycorrhizal roots occurred was estimated from the length of

the tap root. All mycorrhizal roots were then flash-frozen and lyophilized. We did not attempt to identify identical morphotypes among seedlings based on morphology, but instead relied on molecular analysis. The mycorrhizal fungi were identified using methods described by Gardes & Bruns (1996) but using both ITS1F/ITS4 (fungal-specific) and ITS1F/ITS4B (Basidiomycete-specific) primer combinations for polymerase chain reactions (PCRs). Restriction fragment length polymorphisms (RFLPs) of the nuclear ribosomal internal transcribed spacer region were carried out using the endonucleases *Alu*I and *Hin*fI (New England Biolabs Inc., Beverly, MA, USA).

A total of 49 *S. pungens* mushrooms were used for AFLP analysis; 14 were collected prior to the fire and 35 after it. Mushrooms were collected from the same bishop pine stand at Point Reyes National Seashore that had been sampled by Bonello *et al.* (1998). The pre-fire age of the trees was estimated to be about 40 yr (Gardes & Bruns, 1996), which coincides with the typical fire return interval in this community (Sugnet, 1985). The locations of mushrooms were mapped to the nearest 0.5 m within an area of 1200 m^2. The first batches of mushrooms were collected in 1994 and 1995 before the wildfire in October 1995; the genet sizes derived from these have already been published (Bonello *et al.*, 1998). Seven single-mushrooms genets and one large multi-mushroom genet were found. We used AFLPs to re-examine the genotypes of the freeze-dried mushroom samples from the seven unique genets and from seven of the mushrooms from the large multi-mushroom genet. The first *Suillus* basidiocarps after the fire were found in December 1998, but were not of sufficient quality to be used for AFLPs. Sound fruitbodies used in this study were collected from December 1999 to January 2000; all sound mushrooms collected during that period were genotyped.

Methods

Amplified fragment length polymorphism data were produced using a Perkin-Elmer Applied Biosystems (Foster City, CA, USA) plant mapping kit for small genomes. Reactions were prepared according to the manufacturer's manual with the following exceptions: for restriction ligation, a master mix that contained all components except DNA was prepared. For 10 samples we used: 10 µl ligase buffer (New England Biolabs), 10 µl 0.5 M NaCl, 5 µl bovine serum albumin (1 mg ml^{-1}), 10 µl *Mse*I adaptor, 10 µl *Eco*RI adaptor, 2.5 µl *Mse*I (4000 U ml^{-1}; New England Biolabs), 0.5 µl *Eco*RI (100 000 U ml^{-1}; New England Biolabs), 0.3 µl ligase (2×10^6 U ml^{-1}; New England Biolabs) and 6.7 µl double-distilled water. For each reaction, 5.5 µl of this mix was combined with 4.5 µl of DNA solution. Preselective and selective PCRs were performed with half the volumes indicated in the manual. The primer combinations used were: *Eco*RI-AT/*Mse*I-CT and *Eco*RI-AA/*Mse*I-CT. Aliquots of 0.75 µl of the selective PCRs were analysed in each lane of the gel with 0.3 µl of GeneScan (ROX) 500 size standard (PE Applied Biosystems, Foster City, CA, USA). Data were collected on an ABI 377 sequencer with GENESCAN software (PE Applied Biosystems).

Electropherograms were scored manually for the presence or absence of bands of the same apparent size. Markers that did not show a clear presence–absence pattern were excluded. The data matrices obtained by this procedure were checked in PAUP (Swofford, 2002) for their similarity by parsimony analyses to identify identical patterns.

Results

Nineteen of the 20 seedlings sampled were mycorrhizal. The mean minimum depth at which mycorrhizas were present was 12.9 cm (SE 1.5 cm). Neither *S. pungens* nor *A. francheti* were found on any of the roots. The Ascomycetes identified in mycorrhizal roots of 14 seedlings included *Cenococcum* sp. and six unidentified ITS-RFLP types. The latter were tentatively identified as ascomycetes partly by morphology (thin mantles, limited swelling and uncolonized tip) and by their lack of amplification with ITS1F/ITS4B. Their identification was not pursued further, as it was clear that they were not our targeted species. The Basidiomycetes identified in ectomycorrhizal roots of 10 seedlings included *Russula xerampelina* s.l. (eight seedlings), *Russula amoenolens* (one seedling), and *Rhizopogon salebrosus* (referred to as *R. subcaerulescens* in previous studies at Point Reyes) (one seedling). This sample of 20 seedlings was initially meant to be the first of several samples, from which we would isolate and genotype the target species. However, the fact that neither species was recovered in this sample caused us to curtail our seedling sampling effort and to wait for fruiting of these species. *Suillus pungens* obliged by producing abundant fruitbodies 4 yr and 5 yr after the fire, while fruitbodies of *A. francheti* were not seen until January 2002.

An AFLP analysis was completed on all mushroom samples, although four of the 49 samples were analyzed for only one of the two primer sets. A total of 31 variable fragments were scored when both primers were employed and 17 when only a single primer was employed. We tested for sufficiency, reproducibility, and scoring errors, as described in Redecker *et al.* (2001). Briefly, we tested whether sufficient markers had been scored by calculating the probability of encountering the same genotype by chance given a randomly mating population and assuming that the observed allele frequencies reflected the population frequencies. This approach showed that the highest probability of encountering the same genotype when both primers were scored was 4×10^{-5}, and was 7.5×10^{-3} when only one primer was scored. We tested for reproducibility by repeating twice the complete extraction, ligation, amplification and analysis of three samples; all replicate samples yielded identical fragment patterns. In

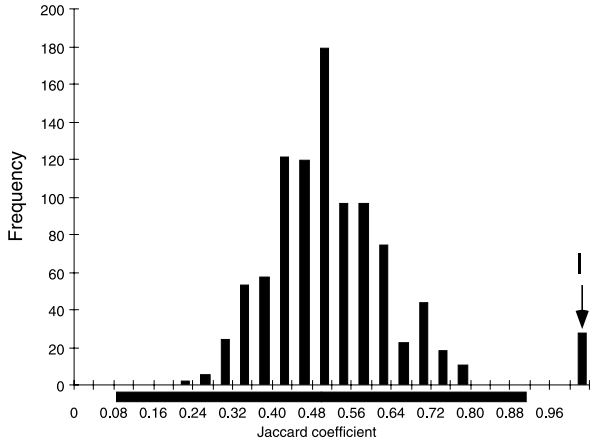

Fig. 1 Distribution of pair-wise genetic distances among all S. *pungens* genotypes. Jaccard distances are essentially the proportion of shared amplified fragment length polymorphisms fragments. I, comparisons among identical genotypes. Three standard deviations from the mean are indicated (bar). Both pre- and post-fire genotypes are included.

addition, all seven independent samples that were derived from the previously identified genet were found to be identical with AFLPs. For all nearly identical samples, we tested for scoring errors by examining their electropherograms side by side. We also calculated pairwise Jaccard similarity indices (S_J) using the program package LE PROGICIEL R (Casgrain & Legendre, 1999) and produced histograms of these pairwise Jaccard indices using Microsoft Excel 98 for Macintosh (Microsoft Corporation, Redmond, WA, USA). We then used these distributions to test: (1) whether the samples we diagnosed to be identical were significantly outside the randomly mating population and (2) whether any samples scored as different also fell outside this range (Redecker *et al.*, 2001). All of the putatively identical samples were more than three standard deviations beyond the mean of pairwise distances (Fig. 1), demonstrating that they were unlikely to be identical by chance. No samples scored as different fell outside this three standard-distribution range, and the distribution was symmetric (Fig. 1). This suggests that scoring errors or somatic mutations did not cause us to erroneously classify identical genets as different. Although the small number of pre-fire genotypes limits the usefulness of comparisons between the pre- and post-fire populations, the symmetry of the combined distribution is consistent with the expected distribution from a single population.

All post-fire genets of *S. pungens* were small in size compared with the large one found before the fire (Fig. 2). The large genet identified in a previous study of the pre-fire site was confirmed with the AFLP technique. This genet had a minimum size of 40×15 m and comprised six of the pre-fire samples analysed here (Fig. 2). By contrast, the largest distance between two mushrooms of one genet in the post-fire forest was 1.6 m. The Bonello *et al.* (1998) study that originally identified the large pre-fire genet, was based on single-stranded conformational polymorphisms of isolated RAPD bands. This technique was very time-consuming and technically demanding. The current work, which is based on AFLP analysis, was much faster, and with it we confirmed the existence of the same large genet from our archived freeze-dried samples. This was reassuring, because we have applied AFLP analyses to several other species where we have only found small genets (Redecker *et al.*, 2001).

None of the post-fire genets were found to be identical to pre-fire genets (Figs 1 and 2). This is despite the fact that several post-fire fruitbodies were located within the apparent territory of the large pre-fire genet, and several other post-fire mushrooms were collected in the immediate area of genotyped pre-fire mushrooms. Thus, there was no evidence that any of the post-fire genotypes were present before the fire.

Discussion

Amanita francheti was clearly not a common colonizer after fire. It has never been found below ground in any of our post-fire study sites (Horton *et al.*, 1998; Baar *et al.*, 1999; Grogan *et al.*, 2000a). This includes two sites where it had been abundant in the pre-fire forest (e.g. the present one, and the Taylor & Bruns, 1999 site). It was also not seen fruiting in the post-fire forest for the first five years after the fire. These results suggest that it was not a common survivor or colonizer in the immediate years after the fire. In retrospect, this made it a poor choice in which to look for evidence of mycelial survival, but this was unknown at the time this study was initiated. Its lack of colonization is interesting: if we assume that it will again become common it provides a good example of a late-successional species. This is consistent with the pre-fire genet results that showed that it frequently established by spore within the undisturbed 40-yr-old forest (Redecker *et al.*, 2001). This species is not specific to the bishop pine forest. In the immediate area it can also be found with Douglas fir (*Pseudotsuga menziesii*) and elsewhere in California it is found throughout the coast and Sierra foothills in conifer, hardwood and mixed forests (Thiers, 1982; Arora, 1986). These other communities, with different fire regimes, may provide a spore source for later colonization of regenerating forests within Point Reyes. The slow colonization after fire was not characteristic of all other species of *Amanita*. For example, *Amanita gemmata* was present before the fire and may have actually increased its importance after the fire (Baar *et al.*, 1999; Horton *et al.*, 1998; Grogan *et al.*, 2000b).

Unlike *A. francheti*, the fate of *S. pungens* is tied directly to the bishop pine forest, as this tree is its only known host in the immediate area. This means that *S. pungens*, like its host, has experienced stand-replacing fires approximately every 40 yr (Sugnet, 1985; Vogl *et al.*, 1977). Thus, it is not surprising that *S. pungens* was able to recolonize rapidly after such a fire and fruit abundantly. Similarly, post-fire studies elsewhere

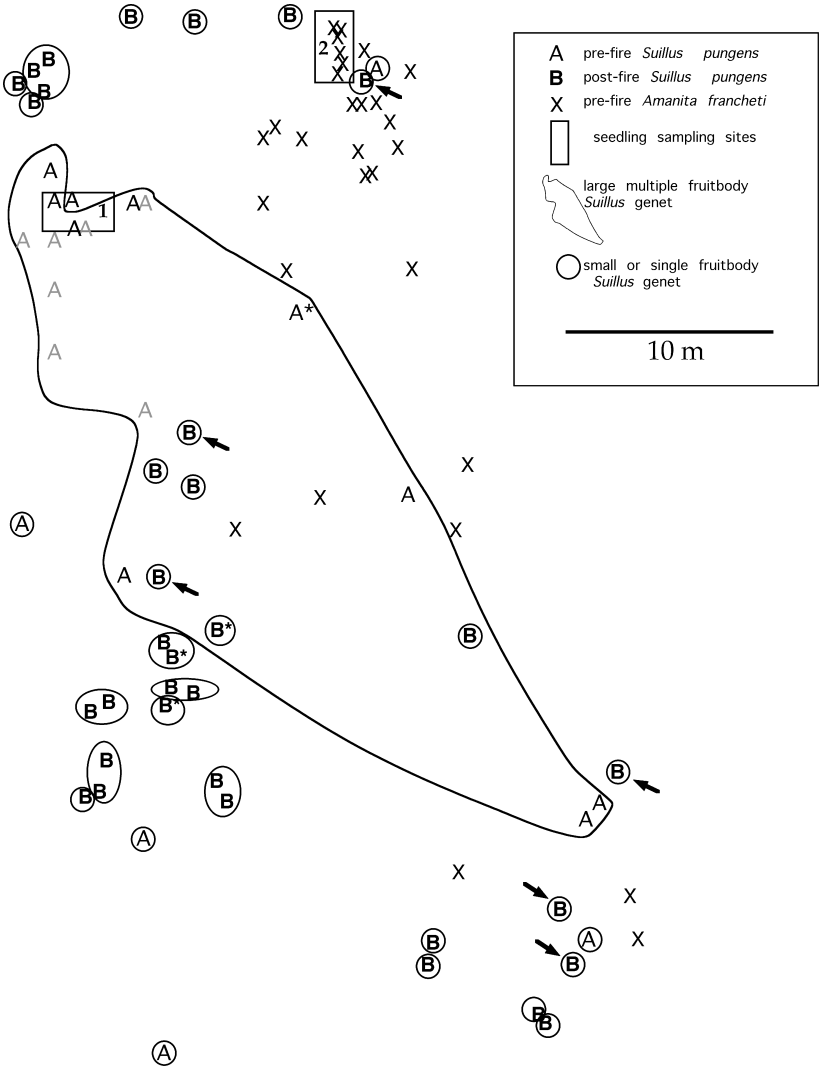

Fig. 2 Pre- and post-fire genet structure of *Suillus pungens*. Locations of pre-fire (A) and post-fire (B) mushrooms are indicated. Those enclosed by a common ellipse or polygon share a common amplified fragment length polymorphism (AFLP) genotype. Arrows indicate post-fire mushrooms that were collected near pre-fire genets. An asterisk indicates a mushroom for which only one of the two AFLP primer sets was successfully run. Pre-fire members of the large genet that were not re-analyzed with AFLP are shown in gray. Note that no genotypes seen prior to the fire were recovered after the fire. Rectangles 1 and 2 show locations of seedling samples that targeted *S. pungens* and *Amanita francheti*, respectively. Pre-fire mapped mushrooms of *A. francheti* in the area of the sample frame are shown (X); no post-fire fruiting was seen until January 2002.

often list *Suillus* species as abundant (Visser, 1995; Torres & Honrubia, 1997). Perhaps it is more surprising that it was not an abundant mycorrhizal type immediately after the fire. Its absence on the seedlings sampled in this study is reinforced by its absence or rarity at three other nearby sites that were studied in the year following the fire (Baar *et al.*, 1999; Horton *et al.*, 1998; Grogan *et al.*, 2000a). We recently resampled mycorrhizae within the study area and confirmed that *S. pungens* is still not a common mycorrhizal type even though it is now a common fruiter (M. Bidartondo & T. Bruns, unpublished). This pattern is similar to that seen in the pre-fire forest (Gardes & Bruns, 1996).

We cannot exclude mycelial survival in *S. pungens*, because we could have missed the fruiting of surviving genets, or perhaps they had not yet fruited. However, it is clear that spores were quantitatively the primary means by which *S. pungens* recolonized abundantly in the years after the fire. It is uncertain whether colonization was from newly dispersed spores derived from unburned pine forest, or from a spore bank, as seen with *Rhizopogon* species (Baar *et al.*, 1999; Taylor & Bruns, 1999), but two observations favor the idea that newly dispersed spores were the primary agent. First, we have rarely found *Suillus* in our bioassays of soils from these forests (Baar *et al.*, 1999; Taylor & Bruns, 1999; R. Kjøller & T. Bruns, unpublished). Therefore, if *S. pungens* does 'stockpile' spores in the soil, they are either unresponsive to our greenhouse bioassay conditions, or they are in much lower abundance than *Rhizopogon* spores. Second, if *S. pungens* formed a spore bank, it should be most dense in the immediate area of recent fruitings, yet neither our seedling sample nor its post-fire fruiting pattern seems to support this (Fig. 2).

Most of the post-fire genotypes found were represented by single mushrooms, but four were represented by two to three adjacent mushrooms. Assuming that these genets established in 1996, the first growing season after the fire, the observed size of the largest genet is consistent with a minimum mycelial expansion rate of 40 cm yr^{-1}. This is close to the minimum expansion rate of the large pre-fire genet, which was estimated

to be 50 cm yr^{-1} (Bonello *et al.*, 1998). Both rates are likely to be underestimations because the genets probably extend beyond the borders defined by fruitbodies, and because both rates assume that establishment started in the first post-fire season. The latter assumption now seems reasonably accurate for the large older genet because the current study shows that spore establishment dominates in the post-fire forest. The pattern of multiple small genets in a young post-disturbance forest and fewer, larger genets in older forests has been previously reported for *Suillus bovinus* and *Suillus variegatus* by Dahlberg (1997) and Dahlberg & Stenlid (1990, 1994). They suggested that most genets initially establish by spore following disturbance and then die or are out-competed over time, while only a few continue to expand vegetatively. Our current study is consistent with that view for *S. pungens*. One difference is that we can say with some confidence that the genets are new, because we had data on the predisturbance population at this site.

The question that remains is whether mycelial survival is a primary means through which any species of ectomycorrhizal fungi recolonize following forest death and regeneration. Our lack of evidence for survival in *S. pungens* and *A. francheti* following fire, and the pattern of apparent spore establishment in other *Suillus* species after disturbance (Dahlberg & Stenlid, 1990, 1994; Dahlberg, 1997), suggests that different taxa should be the target of future studies. Hagerman *et al.* (1999a) found that in a clear-cut subalpine *Picea engelmanni*/*Abies lasiocarpa* forest some ectomycorrhizal morphotypes persisted longer than others on the dying roots. In particular, a *Lactarius* morphotype increased its frequency relative to other types over the three years of the study. However, seedlings that established in these sites were colonized primarily by taxa such as *Wilcoxina*, *Mycelium radicis atrovirens* and *Hebeloma*-like, all of which are known to colonize well by spore or sclerotia; thus, the significance of mycelial persistence was unclear (Hagerman *et al.*, 1999b). In our system, the prime candidates for survival would also be members of the Russulaceae, such as *R. amoenolens*, and *R. xerampelina*-like, as these were the dominant colonizers of bishop pine roots prior to the fire and common recolonizers of seedlings immediately after the fire. However, recent data from other *Russula* species in our area show that spore establishment may be much more important than mycelial spread even in undisturbed forests (Redecker *et al.*, 2001). These results cast doubt on the role of mycelial survival as a quantitatively important process for ectomycorrhizal fungi in forest regeneration; however, the critical data for some of the most relevant taxa are lacking.

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