Ectomycorrhizal host specificity in a changing world: can legacy effects explain anomalous current associations?

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Summary

- Despite the importance of ectomycorrhizal (ECM) fungi in forest ecosystems, knowledge about the ecological and co-evolutionary mechanisms underlying ECM host associations remains limited.
- Using a widely distributed group of ECM fungi known to form tight associations with trees in the family Pinaceae, we characterized host specificity among three unique *Suillus*–host species pairs using a combination of field root tip sampling and experimental bioassays.
- We demonstrate that the ECM fungus *S. subaureus* can successfully colonize *Quercus* hosts in both field and glasshouse settings, making this species unique in an otherwise Pinaceae-specific clade. Importantly, however, we found that the colonization of *Quercus* by *S. subaureus* required co-planting with a Pinaceae host.
- While our experimental results indicate that gymnosperms are required for the establishment of new *S. subaureus* colonies, Pineaceae hosts are locally absent at both our field sites. Given the historical presence of Pineaceae hosts before human alteration, it appears the current *S. subaureus*–*Quercus* associations represent carryover from past host presence. Collectively, our results suggest that patterns of ECM specificity should be viewed not only in light of current forest community composition, but also as a legacy effect of host community change over time.

Introduction

There is widespread recognition that both plant health and ecosystem functioning are strongly influenced by symbiotic interactions with microorganisms (Van der Heijden *et al.*, 2008). In many forest soils, trees form close associations with ectomycorrhizal (ECM) fungi, which facilitate nutrient and water acquisition in exchange for photosynthetically derived sugars (Smith & Read, 2008). Unlike associations between plants and other microbial groups (e.g. arbuscular mycorrhizal fungi or nitrogen-fixing bacteria), a considerable number of ECM fungi exhibit strong patterns of host specificity (Molina *et al.*, 1992). This specificity is most often observed at the level of host family or genus and involves a diverse array of plant lineages (e.g. *Alnus* (Molina, 1979), *Pisonia* (Hayward & Horton, 2012), *Gnetum* (Tedersoo & Põlme, 2012), Pinaceae (Bruns *et al.*, 2002)). Despite some informed speculation (Kropp & Trappe 1982; Bruns *et al.*, 2002; Walker *et al.*, 2014), our current understanding of the ecological and co-evolutionary mechanisms underlying ECM host association patterns remains limited. Plant control of colonization by ECM fungi may take place at multiple stages of mycorrhization, including spore germination, directed mycelial growth, plant–fungal contact, during formation of the Hartig net, or after mycorrhization in response to nutrient transfer

(Fries, 1984; Duddridge, 1986; Ditengou *et al.*, 2015; Hortal *et al.*, 2017). Although both spore and mycelial colonization are thought to occur in response to host-initiated molecular triggers, spores and mycelia probably require distinct molecular signals in order for colonization to occur and it is likely that a plant's ability to trigger spore germination is independent of the ability to ultimately form functional mycorrhizas with a given fungal species (Palm & Stewart, 1984; Kikuchi *et al.*, 2007; Ishida *et al.*, 2008). Because signaling molecule quantity and quality are dependent on host identity (Palm & Stewart, 1984; Massicotte *et al.*, 1994), forest community composition has important ecological consequences for host specificity.

The process of mycorrhization often occurs in the context of many potential host plants. Deviations from expected host specificity patterns may be mediated by either a mycelial- or spore-based mechanism, both of which may be influenced by third-party organisms. For example, the potential for alternative host associations can arise when ECM fungi already established on a primary host simultaneously colonize a second host via mycelial networks, or when proximal plants or microbial organisms trigger spore germination in ECM species that would otherwise exhibit dormancy (Fries, 1984; Hubert & Gehring, 2008; Bogar & Kennedy, 2013; Bogar *et al.*, 2015). The ability of proximal trees to influence ECM community composition has already been

documented as an example of how neighborhood effects can act as an important mediator of host–symbiont interactions (Bogar & Kennedy, 2013). However, extending the context of plant–microbe interactions to encompass all extant community members may still fall short of encompassing the causal agents responsible for patterns of ECM host association.

Legacy effects (defined here as the long-term influence of a species after its local extinction) include anthropogenic disturbance events which can alter community dynamics many years after an event took place (Cuddington, 2011). In forest ecosystems, land use histories are important determinants of both community structure and function, with far-reaching effects on both plants and microbes (Goodale & Aber, 2001; Foster *et al.*, 2003; Fraterrigo *et al.*, 2006). In multi-host stands, disturbance events such as fire, disease and logging can facilitate the asymmetric removal of a given host species (Metz *et al.*, 2012; Hollingsworth *et al.*, 2013; Covey *et al.*, 2015), which may open new niche space for existing hosts as well as put significant pressure on host-specific fungi to associate with nonprimary host trees.

Suillus is one of the most well-known examples of an ECM fungal lineage that exhibits a high degree of host specificity (Dahlberg & Finlay, 1999). Suilloid fungi are noted for their close associations with trees in the family Pinaceae (Molina *et al.*, 1992; Kretzer *et al.*, 1996; Horton & Bruns, 1998; Horton *et al.*, 2005; Nguyen *et al.*, 2016). Unlike many other ECM fungal lineages, *Suillus* species possess both reactive spores (i.e. those that readily germinate in the presence of compatible host roots) (Fries, 1978) and long-distance rhizomorphic mycelium (Agerer, 2001). This combination of traits makes them capable of readily colonizing host roots using either spore germination or mycelial extension from established ectomycorrhizas. Control of host specificity in *Suillus* spp. may occur at both of these stages, although most experimental tests have only been conducted via mycelial colonization (Molina & Trappe, 1982; Duddridge, 1986; Finlay, 1989; but see Liao *et al.*, 2016). For example, in field settings, *S. grevillei* and *S. cavipes* associate exclusively with *Larix*, but will form ectomycorrhizas with *Pinus* hosts in laboratory settings (Finlay, 1989). However, the interaction with novel hosts in laboratory settings has been associated with abnormal cellular development and the accumulation of phenolic compounds as well as anomalies in host nutrient provisioning (Molina, 1979; Malajczuk *et al.*, 1982; Duddridge, 1986; Finlay *et al.*, 1988).

A single species of *Suillus*, *S. subaureus*, has long been cited as a possible exception to the tightly coupled relationship between *Suillus* and the Pinaceae. Sporocarp collection records of *S. subaureus* often include site descriptions that note the absence of known Pinaceae hosts and, instead, the presence of angiosperm trees such as *Quercus* and *Populus* (Smith & Thiers, 1964; Homola & Mistretta, 1977; Kuo & Methven, 2010). Despite much speculation, to our knowledge, the natural host(s) of *S. subaureus* has never been confirmed. If *S. subaureus* associates with hosts outside the Pinaceae, it would represent either host switching or host expansion for a species deeply nested within a clade of host specialists (Kretzer *et al.*, 1996; Nguyen *et al.*, 2016). Such an exception would provide an ideal system for inquiry into the genetic and molecular mechanisms mediating ECM specificity, including the level at which symbiosis is regulated (such as genetic vs epigenetic factors). Finally, understanding the ecological drivers of changing ECM host associations (including host switching or host expansion from specialist to generalist fungi or gymnosperm to angiosperm associates) could have important implications for understanding and predicting plant and fungal range shifts related to anthropogenic disturbance and global change (Dickie *et al.*, 2010; Pickles *et al.*, 2012; Hayward *et al.*, 2015).

In this study, we first investigated the hosts of *S. subaureus* observed at two geographically distant field sites and then, based on those associations, tested four hypotheses in a series of glasshouse bioassays. The first two bioassays, referred to as the *Angiosperm Spore Colonization Bioassay* and the *Gymnosperm Spore Colonization Bioassay*, were conducted to provide experimental evidence of either host expansion (i.e. colonization of multiple phylogenetically distant hosts) or host switching (i.e. colonization of hosts only in specific phylogenetic lineages) for *S. subaureus*. Based on our working knowledge about this study system, we hypothesized (1) that the presence of angiosperm hosts alone would not be sufficient to trigger *S. subaureus* spore germination and thereby prevent mycorrhization, and (2) that the presence of ancestral Pinaceae hosts would be sufficient and/or necessary to trigger spore germination and thereby lead to mycorrhization. In the third bioassay, referred to as the *Mycelial Colonization Bioassay*, we tested the hypothesis that *S. subaureus* mycorrhization on alternative hosts (angiosperms) is possible, but only when the alternative host is co-planted with the primary host (Pinaceae). Finally, in the fourth bioassay, referred to as the *Primary Host Removal Bioassay*, we tested the hypothesis that removal of the primary host would facilitate angiosperm colonization by *S. subaureus*.

Materials and Methods

Site descriptions, field sampling and species identification

Fieldwork was conducted at two locations in the midwestern United States. The first site, Lake Alexander Woods Scientific and Natural Area (SNA), was located in Cushing, MN, USA (46.158609°N, 94.561718°W, elevation *c.* 400 m). Mean annual temperature at the site is 4°C (maximum of 33°C in July and minimum of −32°C in January) and mean annual precipitation is *c.* 700 mm, which comes mostly as rain during the spring and summer months. The predominant soil type is Alstad loam. At the time of sampling, the site was a *c.* 70-yr-old mixed deciduous forest in which conifer trees were locally absent (a single *P. strobus* sapling was present in the area, but was located > 75 m from the nearest *S. subaureus* sporocarp collection). Overstory trees included red oak (*Q. rubra*), paper birch (*Betula papyrifera*), big-tooth aspen (*P. grandidentata*), and sugar maple (*Acer saccharum*). The site is located in the 'Pine Moraines and Outwash Plains Subsection' of the Minnesota floristic designation, where *P. strobus* was a canopy-dominant species before intensive logging in the 19th century. Historical aerial photographs of the site (www.lib.umn.edu/borchert) confirm that logging events were common in the general area

between 1939 (when the earliest photographs were taken for the area) and 1980. At the exact location where samples were collected, the most recent logging event appeared to have taken place before 1955. The second field site was located at Tolleston Dunes National Lakeshore in Hammond, IN, USA (41.604623°N, 87.439874°W, elevation *c.* 180 m). Mean annual temperature is 8°C (maximum of 34°C in July and minimum of −22°C in January) and mean annual precipitation is *c.* 1128 mm. The predominant soil type is sand-silt from the Oakville-Adrian complex. The forest canopy was dominated by mature black oak (*Q. velutina*), paper birch (*B. papyrifera*) and cottonwood (*P. deltoides*). Conifer trees were locally absent at the time of collection, although they are present as part of the mature 'Dune and Swale Complex' characteristic of the Great Lakes shoreline. Historical aerial photographs of the site (https://igs.indiana.edu/IHAPI) confirm that disturbance events (logging or periodic burning) were common in the area before 1973.

In late August 2014, nine *S. subaureus* sporocarps were collected from the MN site. Soil cores (15 × 15 × 15 cm) were taken directly under six of the *S. subaureus* sporocarps. ECM root tips were sieved from the soil and individual ectomycorrhizas exhibiting a suilloid morphology (up to six per core) were extracted for total genomic DNA using the REDExtract-N-Amp plant kit (Sigma-Aldrich). From each sample, the fungal rRNA internal transcribed spacer (ITS) region was PCR-amplified using the primer pair ITS1–F/ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993) as well as a portion of the plant *trn*L chloroplast gene using the primers *trn*C/*trn*D (Taberlet *et al.*, 1991). Amplicons were cleaned using ExoSAP-IT (USB Corp., Cleveland, OH, USA) and sequenced using single-pass Sanger sequencing with either ITS1-F (fungus) or *trn*C (plant) primers at the University of Arizona Genetics Core, Tucson, AZ, USA. In early October 2016, three *S. subaureus* sporocarps were collected from the IN site, along with one soil core taken directly under a sporocarp of *S. subaureus*. Fruiting body and root tips were prepared and sequenced as above.

Angiosperm spore colonization bioassay

Quercus rubra and *Q. macrocarpa* acorns were obtained (Sheffield Seed Co., Locke, NY, USA), cupules were removed and the acorns were surface-sterilized in 10% bleach for 12 h before being rinsed twice, placed into open plastic bags with moistened medium-grade sand (10 ml sand per 30 acorns) and stratified at 4°C for 77 d. In September 2014, *P. tremuloides* and *P. grandidentata* roots were collected from the Cedar Creek Ecosystem Science Reserve in East Bethel, MN, USA. After removing tertiary and secondary roots, the primary root was trimmed to a length of 30 cm and packed in heat-sterilized peat moss. Shoots produced from primary roots (*c.* 12 cm tall) were cut at the stem base, dipped in 1.6% indole butyric acid and rooted in sterilized sand for 30 d before transplanting.

Spores from the nine *S. subaureus* sporocarps were collected following the methods outlined by Kennedy *et al.* (2011) and stored in moistened sterile growth media at 4°C until use. Plant growth medium consisting of a 2 : 2 : 1 mix of peat (no. 0128P,

Premier Horticulture, Quakertown, PA, USA) : forest soil (from the University of Minnesota (UMN) St Paul campus) : sand (Monterrey no. 2/16; Cemex, Marina, CA, USA) was autoclaved for 90 min at 20 psi and 121°C for two consecutive days before adding fungal inoculum. Plant growth medium was inoculated with *S. subaureus* spores at a concentration of 5×10^5 spores ml^{-1} soil. Small cone-tainers (150 ml capacity) were sterilized overnight in 10% NaOCl, rinsed, dried and stuffed with a small amount of synthetic pillow stuffing to keep plant growth media in place. Seedlings were randomly arrayed on benches at the UMN Growth Facilities Greenhouse and grown under a 16 h photoperiod, 24°C : 21°C, day : night, with daily watering, and in the absence of fertilization (Fig. 1a).

Seedlings of *Q. rubra* and *Q. macrocarpa* ($n = 20$ per species per time point) were checked for evidence of colonization at 3 (92 d) and 6 months (185 d) after planting. *P. tremuloides* ($n = 12$) and *P. grandidentata* ($n = 5$) were destructively harvested and checked for evidence of colonization 3 months (92 d) after planting. The 6-month time point for the *Populus* species was not taken due to the small number of *Populus* cuttings that successfully rooted.

Gymnosperm spore colonization bioassay

Fruiting body of *S. americanus* and *S. clintonianus* (previously known as *S. grevillei* in North America (Nguyen *et al.*, 2016)) were collected in autumn 2014 from multiple forests in Minnesota beneath *P. strobus* and *Larix laricina*, respectively. The methods in this second bioassay matched those of the Angiosperm Spore Colonization Bioassay except where specified below. Spores of these two *Suillus* species were prepared from the fresh collections, whereas spores of *S. subaureus* for this second bioassay were from the same stock as above. Seeds of *P. strobus* and *L. laricina* (hereafter referred to as *Pinus* and *Larix*) were sourced from the Badoura State Forest Nursery (Minnesota Department of Natural Resources). A *Q. rubra* treatment was included as a negative control based on the results of the Angiosperm Spore Colonization Bioassay. *Q. rubra* acorns were collected from a parent tree located on the UMN St Paul campus. *Pinus* and *Larix* seeds were surface-sterilized and stratified for 60 d at 4°C following Mujic *et al.* (2015). Stratified seeds were germinated in sterilized plant growth media and grown for 30 d before transplanting into 350 ml cone-tainers. Individual cone-tainers were inoculated with either *S. americanus*, *S. clintonianus* or *S. subaureus* at a concentration of 5×10^5 spores ml^{-1} soil. Two seedlings were planted per cone-tainer, representing two plants of the same host ($n = 6$ pots per treatment = 12 plants per treatment) (Fig. 1b).

Plants were grown in a second UMN glasshouse under the following conditions: 16 h photoperiod, 24°C : 21°C, day : night, daily watering and in the absence of fertilization. Seedling location was randomized and periodically rotated throughout the experiment. At 158–180 d post-inoculation, all replicates with two living plants were harvested. Each replicate was removed from its pot, and the root systems washed of soil and gently teased apart to separate the two plants. Each single root system was divided into nine

Host associations observed in field settings
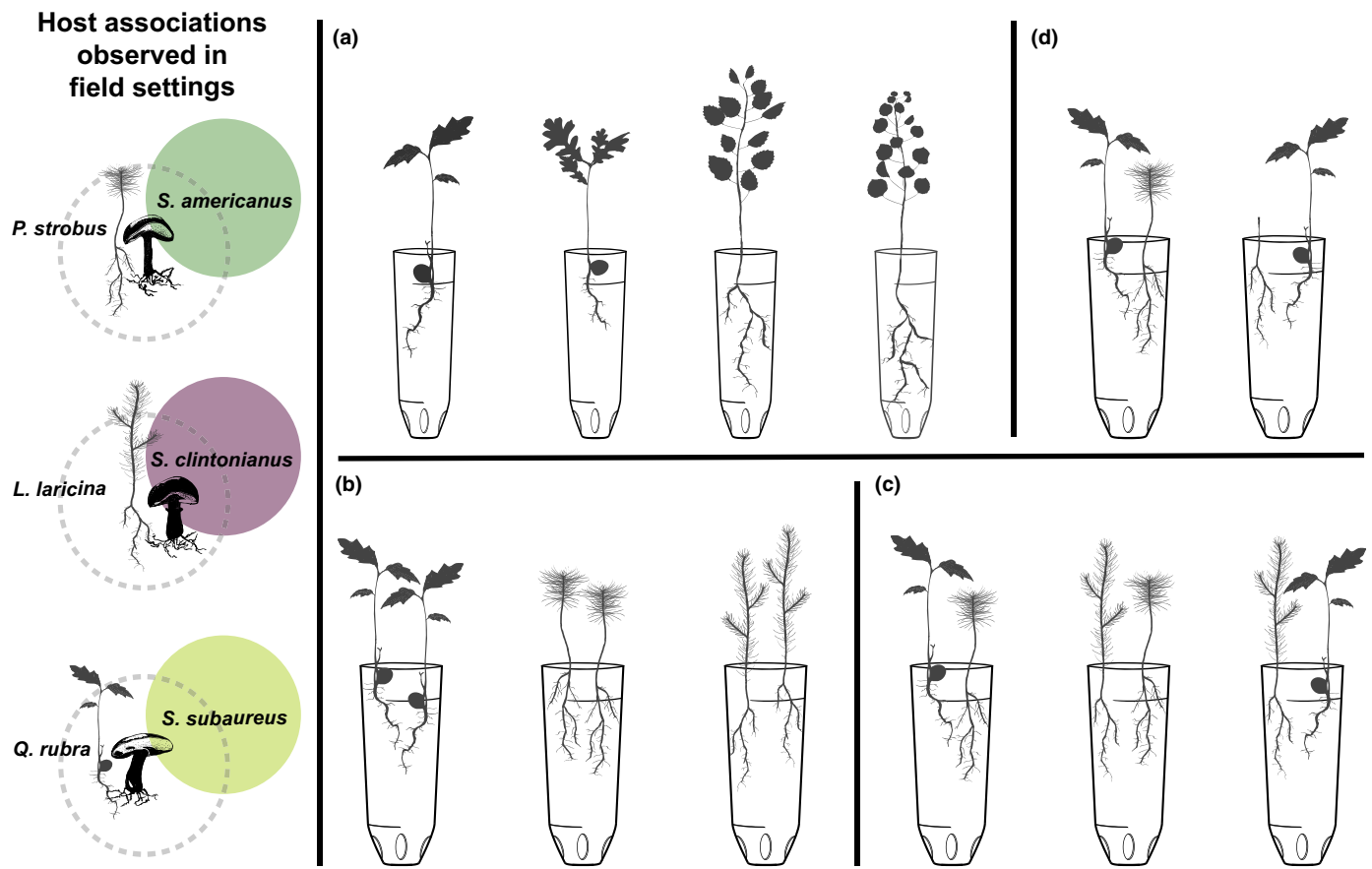
Fig. 1 Summary of experimental design for bioassays. (a) The Angiosperm Spore Colonization Bioassay tested angiosperms as spore germination triggers of *Suillus subaureus*: inoculation of *S. subaureus* spores onto (left to right) *Quercus rubra*, *Q. macrocarpa*, *Populus grandidentata* and *P. tremuloides* by spore. (b) The Gymnosperm Spore Colonization Bioassay tested single host species colonization: inoculation of either *Suillus americanus*, *S. clintonianus* or *S. subaureus* spores onto a single host species, *Pinus strobus*, *Larix laricina* or *Q. rubra*. (c) The Mycelial Colonization Bioassay tested dual host species colonization: inoculation of either *S. americanus*, *S. clintonianus* or *S. subaureus* spores into pots planted with two host species, with all pairwise combinations of *P. strobus*, *L. laricina* and *Q. rubra* represented. (d) The Primary Host Removal Bioassay examined disturbance as a mediator of host expansion: inoculation of *S. subaureus* into pots planted with both *P. strobus* and *Q. rubra*. After sufficient growth (5 months), *P. strobus* seedlings were hewn at the soil line in half the replicates, removing *P. strobus* as a potential carbon source for the fungus.

parts, randomized and scored for percenatge colonization with the aid of a ×10 dissecting microscope. For *P. strobus* and *L. laricina* seedlings, 300 root tips were scored per plant unless < 300 root tips were present, in which case all available root tips were scored. For *Q. rubra* seedlings, 1000 root tips were scored per plant due to the higher abundance of fine roots.

Mycelial colonization bioassay

Plants, growth media and fungal inoculum were prepared, grown and harvested using the same methods and timeline (harvested 158–180 d after inoculation) as described above except that each pot was planted with combinations of two host species, with all host combinations represented ($n = 9$ pots per treatment = 9 plants per treatment) (Fig. 1c).

Primary host removal bioassay

Cone-tainers (350 ml) were co-planted with *P. strobus* and *Q. rubra* and inoculated as above with *S. subaureus* spores using

the methods reported above. After 6 months (180 d) of growth, in half of the pots, *P. strobus* plants were hewn at the soil line, killing the seedling and removing the above-ground portion of the plant ($n = 8$ hewn and $n = 8$ unhewn). Plants were then grown for another 54 d before harvesting and scoring as above (Fig. 1d).

Morphological investigation of mycorrhizas

For all bioassays, representative and anomalous ectomycorrhizas were photographed using an Olympus Stylus TG4 and sequenced to confirm fungal identity. In all three bioassays, ITS sequencing identified that *Suillus* ectomycorrhizas were of the same species inoculated into the pots. Uninoculated controls ($n = 6$ plants) remained uncolonized throughout the experiment. For analyses of Hartig net formation for *S. subaureus* on *Pinus* and *Quercus* hosts, a representative subset of ectomycorrhizas from the bioassays were reserved and stored in formalin-acetic-alcohol fixative (ethanol : acetic acid : formalin : water at 50 : 5 : 10 : 35). To prepare for microcopy, ectomycorrhizas were rinsed in 0.1 M sodium cacodylate buffer (10 min, 3×), post-

fixed overnight at 4°C in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer and dehydrated in an ethanol series. Ectomycorrhizas were then embedded in Embed 812 resin (Electron Microscopy Sciences, Hatfield, PA, USA) and semi-thin sections (0.5 μm thick) were cut on a Leica Ultracut UCT microtome (Leica Microsystems, Buffalo Grove, IL, USA) using a diamond knife. Sections were then stained with 0.5% toluidine blue and observed using a Nikon Eclipse 90i light microscope (Nikon Instruments Inc., Melville, NY, USA) in bright field mode. Images were captured with a Nikon D2-Fi2 color camera using Nikon Elements software.

Statistical analyses

To analyze differences in mycorrhizal colonization by treatment in the bioassays for which colonization was observed, we used a combination of statistical analyses. In the Gymnosperm and Mycelial Colonization Bioassays, we applied separate one-way nonparametric Kruskal–Wallis tests because assumptions of variance homogeneity could not be met due to the lack of colonization in some treatments but not others. Based on the significance of both tests, *post-hoc* Wilcoxon tests were then used to determine specific differences among treatment means for each pair. For the Primary Host Removal Bioassay, we again observed high heterogeneity in colonization across treatments, so applied a one-tailed Mann–Whitney U test. In all cases, significance was determined at $P < 0.05$ using the R programing environment (R Core Team, 2013) and JMP Pro 12 (Cary, NC, USA).

Results

Field analysis

From the six soil cores taken from beneath *S. subaureus* fruiting body at the Minnesota field site, four contained ectomycorrhizas exhibiting characters morphologically associated with *Suillus* species (white to off-white color with thick mantles and notable extramatrical mycelium). A total of 10 root tips were identified as *S. subaureus* in three of the four cores for which suilloid tips were present. Plant DNA was successfully extracted from six of the 10 root tips identified as *S. subaureus*. Of these, three yielded high-quality sequences, with the plant host identified as *Q. rubra* in all cases. The soil core from the Indiana field site also contained tips exhibiting characters morphologically associated with *Suillus* species. Fungal DNA was successfully extracted from six of the eight root tips taken for analysis and identified as *S. subaureus* in all cases. Plant DNA was successfully extracted from all six of those root tips and was identified as the genus *Quercus* in all cases (BLAST confidence was not high enough to identify the host DNA to species, but *Q. velutina* was the only *Quercus* species present at the field site).

Angiosperm spore colonization bioassay

For both the 3- and 6-month time points and for all angiosperm hosts tested, *Q. rubra*, *Q. macrocarpa*, *P. tremuloides* and *P. grandidentata*, spore inoculation failed to result in any colonization by *S. subaureus*.

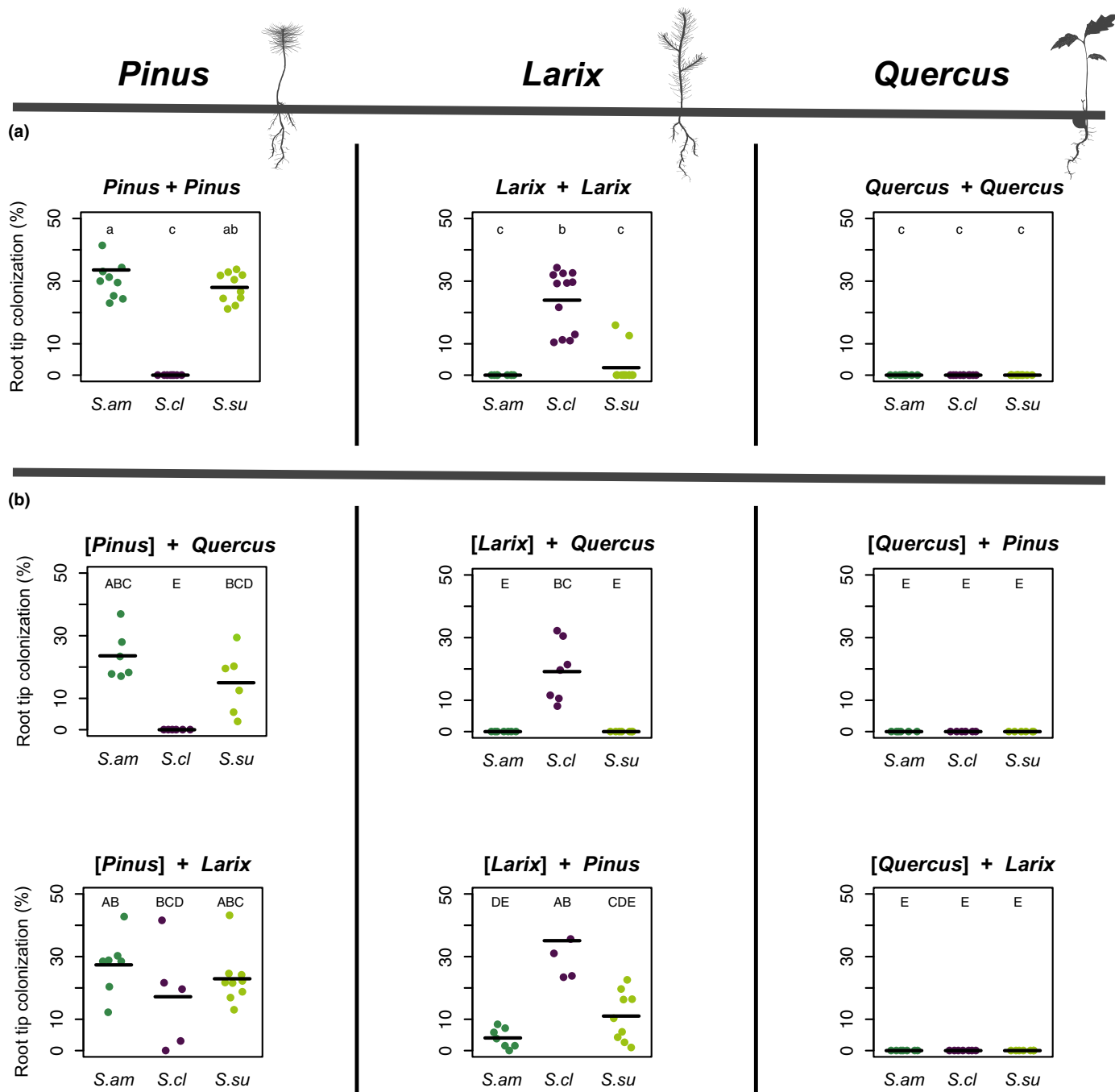### Gymnosperm spore colonization bioassay

On *Pinus*, *S. americanus* and *S. subaureus* colonized at statistically equivalent mean rates of 34% ($n = 10$, with all plants colonized) and 28% ($n = 10$, with all plants colonized), respectively, whereas *S. clintonianus* failed to form ectomycorrhizas on this host (Wilcoxon tests, $P < 0.05$) (Fig. 2). (Colonization rate is defined as the total number of root tips colonized by *Suillus* out of ~300 counted per plant.) On *Larix*, *S. clintonianus* formed ectomycorrhizas at a mean rate of 24% ($n = 12$, with all plants colonized), which was significantly higher than for *S. subaureus* and *S. americanus*, which colonized at 2% ($n = 12$, with two plants colonized at a mean of 14%) and 0%, respectively. Neither *S. americanus* ($n = 10$ plants), *S. clintonianus* ($n = 12$ plants) nor *S. subaureus* ($n = 12$ plants) formed ectomycorrhizas with *Q. rubra* (hereafter referred to as *Quercus*).

Mycelial colonization bioassay

S. americanus formed ectomycorrhizas on *Pinus* at statistically equivalent mean rates of 27% ($n = 7$, with all plants colonized) when co-planted with *Larix* and 24% ($n = 6$, with all plants colonized) when co-planted with *Quercus* (Fig. 2). On *Larix*, *S. americanus* formed ectomycorrhizas at a mean rate of 4% ($n = 7$, with six plants colonized averaging 5% colonization) when co-planted with *Pinus*, but did not form ectomycorrhizas ($n = 7$, with all plants uncolonized) when co-planted with *Quercus* (Wilcoxon test, $P > 0.05$). On *Quercus*, *S. americanus* failed to form ectomycorrhizas regardless of host species pairing. *S. clintonianus* formed ectomycorrhizas on *Larix* at statistically equivalent mean rates of 35% ($n = 5$, with all plants colonized) when co-planted with *Pinus* and 19% ($n = 7$, with all plants colonized) when co-planted with *Quercus*. On *Pinus*, *S. clintonianus* formed ectomycorrhizas at a mean rate of 17% ($n = 5$, with four plants colonized at a mean rate of 21%) when co-planted with *Larix*. This was significantly higher than the 0% colonization of *S. clintonianus* on *Pinus* when co-planted with *Quercus* or on any of the *Quercus* seedlings (Wilcoxon tests, $P < 0.05$). Finally, *S. subaureus* formed ectomycorrhizas on *Pinus* at the statistically equivalent mean rates of 23% when co-planted with *Larix* ($n = 9$, with all plants colonized), 15% ($n = 6$, with all plants colonized) when co-planted with *Quercus*, and 11% on *Larix* when co-planted with *Pinus* (Wilcoxon tests, $P < 0.05$). By contrast, *S. subaureus* did not form ectomycorrhizas on *Larix* when co-planted with *Quercus* ($n = 7$, with all plants uncolonized) and failed to form ectomycorrhizas on *Quercus* regardless of host species pairing.

Morphological description of *S. americanus* and *S. clintonianus* mycorrhizas

On *Larix*, *S. clintonianus* formed typical monopodial–pyramidal ectomycorrhizas with typical root swelling, an off-

Fig. 2 Per cent ectomycorrhizal (ECM) root tip colonization in (a) the Gymnosperm Spore Colonization Bioassay and (b) the Mycelial Colonization Bioassay. In the Gymnosperm Spore Colonization Bioassay, a single species of *Suillus* per pot was inoculated onto a single species of host (two trees per pot) whereas in the Mycelial Colonization Bioassay a single species of *Suillus* per pot was inoculated onto pots containing two host species (two trees per pot). The first [bracketed] host genus indicates the host being quantified, and the second genus indicates the second tree species present in the pot. Different letters above treatments indicate significant differences in colonization among the nine treatments in the Gymnosperm Colonization Assay (lower case) or the 18 treatments in the Mycelial Colonization Assay (upper case) as determined by two separate one-way Kruskal–Wallis tests followed by Wilcoxon *post-hoc* tests for each comparison pair. *S.am*, *Suillus americanus*; *S.cl*, *Suillus clintonianus*; *S.su*, *Suillus subaureus*.

white mantle and prolific extramatrical mycelium (see Supporting Information Fig. S1a). On *Pinus*, *S. clintonianus* formed primarily monopodial ectomycorrhizas with loosely attached mantle hypha and frequent dark patches (Fig. S1b). On *Pinus*, *S. americanus* formed typical bifurcate

ectomycorrhizas with a dense off-white mantle and prolific extramatrical mycelium (Fig. S1c). On *Larix*, however, *S. americanus* formed primarily monopodial ectomycorrhizas, with a loose hyphal mantle and frequent dark patches (Fig. S1b).

Primary host removal bioassay

In cone-tainers where *Pinus* was hewn after 5 months, *S. subaureus* successfully formed ectomycorrhizas on all six *Q. rubra* plants, at a mean colonization rate of 2% (Fig. 3) (Note that colonization rate on *Quercus* is defined as the total number of root tips colonized out of 1000 root tips counted per plant.) In cone-tainers where *Pinus* was unhewn, *S. subaureus* ectomycorrhizas were formed on two of the four *Q. rubra* plants. These two replicates were colonized at individual rates of 4% and 0.3% (mean rate = 2%). While more of the *Quercus* plants were colonized by *S. subaureus* when *Pinus* seedlings were hewn, there was no significant difference in the mean rates of colonization between the two treatments ($n = 8$, $P = 0.225$). To rule out contamination, fungal species identity was confirmed by sequencing the ITS region of individual mycorrhizas as described previously and were identified as *S. subaureus* in all cases.

Morphological description of *S. subaureus* mycorrhizas

Although *S. subaureus* formed ectomycorrhizas on *Pinus*, *Larix* and *Quercus*, the morphology exhibited on each of these hosts differed (Fig. 4). Unlike the nonprimary associations occasionally formed between *S. americanus* and *Larix* or between *S. clintonianus* and *Pinus* in the Gymnosperm Spore Colonization Bioassay, *S. subaureus* ectomycorrhizas were never monopodial and did not exhibit loosely attached mantles or dark discoloration on any of the host species tested. Rather, *S. subaureus* formed ectomycorrhizas that were white to orange (with larger,
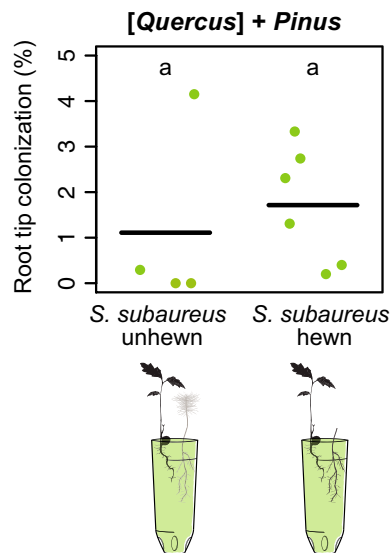
Fig. 3 Per cent ectomycorrhizal (ECM) root tip colonization in the Primary Host Removal Bioassay. In hewn treatments, *Suillus subaureus* colonized all six of *Quercus rubra* seedlings at a mean rate of 2%. In unhewn treatments ($n = 4$), two *Q. rubra* replicates were uncolonized, and two replicates formed ectomycorrhizas at rates of 4% and 0.29%. The first [bracketed] host genus indicates the host being quantified, and the second genus indicates the second species present in the pot. Letters shared above treatments indicate no significant differences in colonization as determined by a Mann–Whitney *U* test.

presumably older, ectomycorrhizas intensifying in color on all hosts), with thick mantles and prolific extramatrical mycelium. On *Pinus*, *S. subaureus* formed bifurcate ectomycorrhizas (like the ectomycorrhizas formed between *S. americanus* and *Pinus*). On *Larix*, *S. subaureus* ectomycorrhizas were monopodial–pyramidal (like the ectomycorrhizas formed between *S. clintonianus* and *Larix*) whereas on *Quercus*, *S. subaureus* ectomycorrhizas were notably coralloid (containing as many as 55 individual lobes per ectomycorrhiza) and, as a unit, several times larger than those formed on either conifer host. Cross-sections of *S. subaureus* mycorrhizas on both *Pinus* and *Quercus* revealed well-developed Hartig net structures on both hosts, with epidermal penetration on *Quercus* and outer cortical cell penetration on *Pinus*.

Discussion

Neighborhood effects as a function of time

Our results clearly demonstrate that the ECM fungus *S. subaureus* can associate with *Quercus* hosts, both in field and in laboratory settings, making this species unique in an otherwise Pinaceae-specific clade. We have also shown that *S. subaureus* can colonize two Pinaceae host species, suggesting that this species is a host generalist rather than a *Quercus* specialist. Because the capacity to colonize alternative hosts can be controlled either at the point of spore germination or during downstream signaling processes, host identity may influence colonization differently depending on whether spores must be germinated in order to establish fungal presence, or whether extant mycorrhizas are already present on neighboring plants (Molina *et al.*, 1992; Kennedy *et al.*, 2011). Consistent with earlier studies (i.e. Molina *et al.*, 1992; Massicotte *et al.*, 1994), our bioassays indicated that the mode of colonization (i.e. spores vs mycelium) strongly affects patterns of ECM host specificity. We observed that the spores of *S. americanus* and *S. clintonianus* germinated only in the presence of their primary hosts, and only formed a few (morphologically anomalous) mycorrhizas on alternative Pinaceae hosts when colonizing via mycelial networks. By contrast, *S. subaureus* germinated in the presence of both Pinaceae hosts and colonized all three hosts by mycelia (Fig. 5). Importantly, the resulting ectomycorrhizas of *S. subauerus* were anatomically typical of functional host associations on all three hosts (Fig. 4). While the bioassay results indicated that only Pinaceae hosts could trigger germination of *S. subaureus* spores, Pineaceae trees were locally absent at both field sites. Because these hosts were historically present at both locations before anthropogenic disturbance events, it appears the current *S. subaureus*–*Quercus* associations represent carryover from past host presence. This pattern echoes other studies highlighting the role of neighborhood effects in structuring ECM fungal host specificity (Bogar & Kennedy, 2013; Bogar *et al.*, 2015), but, because *S. subaureus* fruiting body and mycorrhizas were found in angiosperm-only forests where Pinaceae hosts have long been locally extirpated, the spore germination triggers or mycelial inoculum originating from Pinaceae hosts cannot be considered a neighborhood effect in the traditional definition. Instead, the establishment of new *S. subaureus*
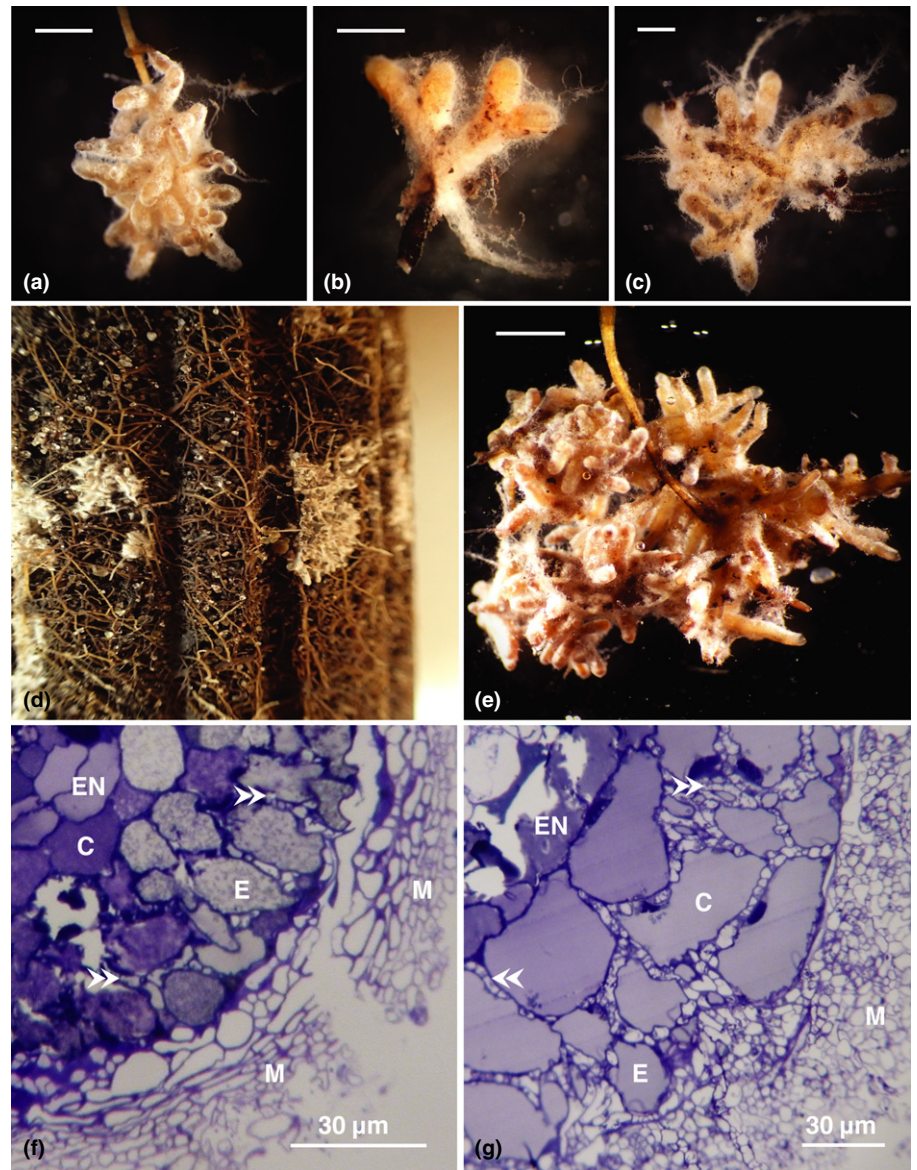
Fig. 4 Ectomycorrhizas formed by *Suillus subaureus* on three different host species, *Quercus rubra*, *Pinus strobus* and *Larix laricina*. Ectomycorrhizas formed a thick white mantle that turned progressively orange with age. On *Q. rubra*, *S. subaureus* developed progressively multi-lobed coralloid structures with surface area per mycorrhiza much larger than that formed on *P. strobus* or *L. laricina*. Bars, 1 mm unless otherwise noted. (a) A young *S. subaureus* mycorrhiza on *Q. rubra*. (b) A mature *S. subaureus* mycorrhiza on *P. strobus*. (c) A mature *S. subaureus* mycorrhiza on *L. laricina*. (d) Large coralloid mycorrhizas and extramatrical hyphae of *S. subaureus* on *Q. rubra*. (e) A mature *S. subaureus* mycorrhiza on *Q. rubra*. (f, g) cross sections of *S. subaureus* on *Q. rubra* (f) and *S. subaureus* on *P. strobus* (g) mycorrhizas, stained with Toluidine blue and visualized with light microscopy. M, mantle; ≫, Hartig net; E, epidermis; C, cortical cells; EN, endodermis.

colonies appears to depend on triggers provided by hosts long absent from the system, suggesting that neighborhood effects should not only be viewed in light of the current host community structure, but as a function of host community change over time.

Evidence for host expansion rather than host switching

The deeply nested phylogenetic location of *S. subaureus* within the genus *Suillus* strongly suggests this species was ancestrally associated with Pinaceae hosts (Nguyen *et al.*, 2016). If *S. subaureus* has lost the ability to colonize hosts in the Pinaceae, it would indicate this fungus has switched its association patterns to now associate exclusively with angiosperm hosts. Alternatively, the ability to colonize both angiosperm and gymnosperm hosts would indicate that this fungus has simply expanded its host range to include angiosperms. In this study, the colonization of *Quercus* seedlings coupled with frequent colonization of *S. subaureus* on *Pinus* seedlings as well as the occasional colonization on *Larix*

seedlings is consistent with a pattern of host expansion rather than host switching. These results add to the growing evidence that host specialization is not necessarily an evolutionary dead-end (Desdevises *et al.*, 2002; Nosil, 2002; Tripp & Manos, 2008; Ouvrard *et al.*, 2015), as famously suggested by Simpson (1953). In contrast to host–pathogen relationships, the evolutionary pressures structuring host range in fungal mutualists has been suggested to ultimately favor the maintenance of host generalism, where the capacity to colonize diverse hosts is assumed to have a positive net impact for both plant and fungal partners (as discussed by Harley & Smith, 1983). However, the high host specificity observed in most *Suillus* species appears to be a derived trait which evolved from an ancestral habit of host generalism (Nguyen *et al.*, 2016), bringing into question the assumption that expanded host range is an evolutionary driver that is beneficial to both partners. Experimental investigation regarding how the functional benefit to each partner might vary by species, and which partner (plant or fungus) controls the mutualism were not

Fig. 5 Summary of differences in host colonization mode exhibited by the three *Suillus* species. Whereas *Suillus americanus* colonized its primary host, *Pinus strobus*, by both spore and mycelium, it only colonized *Larix laricina* via mycelial extension from extant mycorrhizas and did not form mycorrhizas with *Quercus* spp. Similarly, *S. clintonianus* colonized its primary host, *L. laricina*, by both spore and mycelium but colonized *P. strobus* only by mycelium and did not colonize *Quercus* spp. *Suillus subaureus* formed mycorrhizas on both *P. strobus* and *L. laricina* from both spore and mycelium and was additionally able to form mycorrhizas on *Q. rubra* when colonized via mycelium. Brackets indicate the host associations observed in field settings.

investigated in this study. However, examples such as *S. subaureus*, which appear to have the reverted capacity for host generalism, could provide an excellent experimental system for addressing these questions in ECM fungi.

Separating evolutionary pressure vs environmental pressure

In a recent analysis of the ECM genus *Russula*, Looney *et al.* (2016) showed that changes in host association from Pinaceae to angiosperms occurred at a rate 15 times higher than the inverse, suggesting the transition may be a relatively common phenomenon. Long-term disturbance regimes resulting in selective host removal could act as a driver of ECM host expansion by placing pressure on specialist fungi to secure carbon from alternative hosts (given the obligate nature of the ECM symbiosis, it is very unlikely that ECM fungi can meet any significant part of their carbon needs by living saprotrophically; Baldrian, 2009; Kohler *et al.*, 2015). For example, repeated disturbances, such as fire, may favor alternative hosts such as *Quercus* spp. that are able to re-sprout from their existing tree bases (Crow, 1988). In the *S. subaureus* study system, we are unaware of any current populations of this fungus present in either young or mature angiosperm-only forests that have not at one point also contained hosts in the Pinaceae. However, our results suggest that Pinaceae host removal is not immediately necessary to induce angiosperm colonization by *S. subaureus* (and given the recent nature of the human disturbances (< 200 yr), it is not likely that anthropogenic influences are the selective agent directly responsible for inducing this broader host association). Rather, our results offer an example of the fitness advantage of an ECM fungus that is capable of acting as a generalist in the event of local extirpation of its primary host. Given the young age of the hosts used in glasshouse bioassays, future research examining whether the timing of primary host removal (in regard to the age of the respective host trees and the time since mycorrhizal establishment) influences

colonization rates on secondary hosts will also provide greater insight into the relative importance of evolutionary vs environmental pressure as drivers of observed host associations.

Mycorrhizal morphology and colonization patterns are influenced by host identity

Root tip colonization percentages of *S. subaureus* were notably lower on *Q. rubra* compared to *S. subaureus* colonization on *P. strobus* and *L. laricina*. This result is typical of *Quercus* ECM colonization due to the extensive production of fine roots generated by this host genus (He *et al.*, 2010; Chen *et al.*, 2016). Similarly, the difference in Hartig net development on *Quercus* (epidermal penetration) and *Pinus* (outer cortical cell penetration) is typical of angiosperm and gymnosperm ECM development, respectively (Smith & Read, 2008; Watkinson *et al.*, 2015). Less expected were the macro-morphological differences observed among *S. subaureus* on *Quercus* and the two gymnosperm hosts. On *Q. rubra*, *S. subaureus* produced prolific rhizomorphic mycelium and individual ectomycorrhizas exhibited greatly increased biomass and surface area over those produced on Pinaceae hosts (Fig. 4). Microscopic inspection (Fig. 4f,g) coupled with the presence of *S. subaureus* ECM root tips directly under *S. subaureus* sporocarps in the field with no primary host (*Pinus*) in the vicinity suggests that *Quercus*–*S. subaureus* ectomycorrhizas are functional in terms of carbon acquisition by the fungus. Similar results were observed in experimental inoculations by Finlay (1989), who found normal carbon allocation of *P. sylvestris* seedlings to *S. cavipes*, a species strictly associated with *Larix* hosts in field settings. Interestingly, the phosphorus returned from that same experimental association was notably lower than when *P. sylvestris* seedlings were colonized by *Pinus*-specific *Suillus* species. Although we did not measure physiological traits in any of our experiments, and therefore cannot make any inferences about the efficacy of *Quercus*–*S. subaureus*

symbioses, our combined results indicate that *S. subaureus* has the ability to both colonize and persist on both angiosperm and multiple gymnosperm hosts. The reason for the absence of *S. subaureus* on pine in field conditions is not clear, but may reflect edaphic specialization or limited competitive ability by *S. subaureus*, as has been observed with *Suillus* species in other studies (Bidartondo *et al.*, 2001; Kennedy *et al.*, 2011). We are currently testing the competition hypothesis with seedling bioassay experiments, but additional field-based studies are needed to fully understand the ecological factors that make *S. subaureus* rare in both angiosperm-only and mixed host forests.

Resolving the long-standing question of angiosperm hosts for *Suillus*

Although there has been anecdotal evidence of some *Suillus* species being associated with angiosperm hosts under natural conditions (Miller & Miller, 2006), to date, no reliable confirmation of these associations has been established. Seedling inoculation trials claimed that ectomycorrhizas were formed between *S. luteus* and *S. granulatus* and four *Quercus* species (Dixon *et al.*, 1984; Dixon & Johnson, 1992), but in both of those studies, it was not accurately confirmed whether the ectomycorrhizas present belonged to *Suillus* or other ECM fungal species. In laboratory settings, by contrast, Molina & Trappe (1982) were able to successfully synthesize ectomycorrhizas between *S. brevipes*, *S. clintonianus*, *S. cavipes* and *S. lakei* and a number of different host species, including the angiosperm host *Arbutus menziesii*. It was later recognized, however, that the presence of glucose in the growth medium in that and other early ECM synthesis trials effectively reduced the host specificity barriers normally present among many ECM fungi (Duddridge, 1986; Theodorou & Reddell, 1991). Similarly, Murata *et al.* (2015) achieved superficial colonization between *S. luteus* and *Prunus speciosa* when grown in the presence of added glucose and sucrose. In this case, however, ECM colonization consisted of limited mantle development, no Hartig net and frequent dark spotting.

Conclusions and future directions

Moving forward, we believe that assessing the effects of differences in mycorrhizal morphologies on nutrient trading dynamics, determining competitive ability, analyzing the genomic content and expression of *S. subaureus* will all aid in identifying the mechanisms that have facilitated host generalism in this species. Understanding the underlying ecological and evolutionary mechanisms driving host specificity in ECM symbioses is broadly important given the current rate of forest redistribution and changes to community composition caused by anthropogenic processes (Perry *et al.*, 1989; Dickie *et al.*, 2010; Pickles *et al.*, 2012; Bogar *et al.*, 2015; Hayward *et al.*, 2015). Specifically, as forest landscapes undergo host migration and current host species are displaced due to climate change, studying host expansion will help in understanding both how ECM hosts and fungi came to occupy their respective niches and how each will respond to future forest community dynamics.

Author contributions

L.L., N.H.N. and P.G.K. planned and designed the research; N.H.N. and P.G.K. conducted field work; L.L. conducted the bioassays and molecular lab work; L.L. and P.G.K. collected data and performed data analysis and interpretation; L.L. and P.G.K. wrote the manuscript, with editorial contributions from N.H.N.

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Fig. S1 Ectomycorrhizas formed by *Suillus americanus* and *S. clintonianus* on two different host species.

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