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Diversity and density of the EM fungal community present in high elevation Fraser fir forests of Great Smoky Mountains National Park

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Abstract A study of the diversity and density of ectomycorrhizal (EM) fungi in two Fraser fir stands near Clingmans Dome, Great Smoky Mountains National Park was conducted over a period of three years. Plots were established in three naturally occurring age class treatments including mature tree, sapling, and low regeneration (no trees) sites. Lesser vegetation data were determined for nine plant species within all plots including two ferns and two grasses that impeded survival of Fraser fir seedlings. Diversity and densities of vegetation were significantly greater in low regeneration plots as compared to other treatments. For each plot bryophyte mat forest floor percent occurrence/cover was obtained and percent root colonization/sclerotia of the EM fungus taxa were collected from the three management treatments including 11 decomposers and 33 ectomycorrhizal species. *Clavulina cristata* occurred in 22.5% of all plots and had a frequency of 1.7% in low regeneration treatments. Four species of *Laccaria* occurred in

17.3% of all plots, and *Laccaria laccata* and *L. laccata* var. *pallidifolia* were the most common of these species, the former having the highest frequency of occurrence (1.9%) in low regeneration treatments. Four species of *Cortinarius* occurred in 10.3% of the plots, and *Cortinarius anomalus s.l.,* the most common *Cortinarius*, occurred in 4.4% of all plots. Seven of the 44 species had significantly greater percent frequency among the three treatments, and six of those were the most frequent in sapling plot treatments at the two locations. Species found at the two locations were similar, although in 2009 their frequency values were greater than in 2010 and 2011 due to greater total precipitation. Mature and sapling plot frequency values were significantly greater than those for low regeneration sites due to the low establishment of Fraser fir. Significant results for species richness, diversity and evenness between years, locations and treatments are present below. Based on percent frequency values, *Laccaria* could be used in reforestation of Fraser firs in all plots. A project is underway to evaluate seedling establishment and survival following inoculation with *Laccaria* spp. on a low regeneration site at Mount Buckley.

Key words: Fraser fir, fleshy fungi, Basidiomycetes, saprobic, mycorrhizae, vegetation

Introduction: Fraser fir or southern balsam fir (*Abies fraseri* (Pursh) Poir.), is a medium size tree in the red spruce (*Picea rubens* Sarg.)-Fraser fir (spruce-fir) forests of the southern Appalachian Mountains. Fraser fir has a disjunct distribution across several high elevation peaks and ridges from southwestern Virginia to western North Carolina and eastern Tennessee (Smith & Nicholas 2000). It is the only fir species endemic to the southern Appalachian (Oosting & Billings 1951). The Mount Rogers National Recreation Area (NRA) in southwestern Virginia marks the northern extent of Fraser (Pyle & Schafale 1988). The Great Smoky Mountain Nation Park (GRSM) is unique in that it contains the largest contiguous area of spruce-fir forest in the southern Appalachians from Mount Sterling southward to Clingmans Dome (Whittaker 1956). Fraser fir occurs only at high elevations and its primary value is for watershed protection, scenic attraction, habitat for unique biota, and the Christmas tree industry.

The southern Appalachian spruce-fir forest typically ranges in elevation from 1500 to 2000 m (Busing et al. 1988). Fraser fir increases in dominance at higher elevations and forms almost pure stands above 1890 m (Whittaker 1956). It can also occur at elevations as low as 1370 m on north facing slopes in protected coves. Mixed stands of Fraser fir and red spruce are more frequent above 1524 m (Crandall, 1958). This forest type is extremely fragile and is one of most endangered in North America (Noss & Peters 2002).

In the GRSM over 90% of the mature Fraser firs were killed by the balsam woolly adelgid (*Adelges piceae* Ratz.) from 1962 through the late 1970's (Busing 1988; Dull et al. 1988). Since then natural reforestation has occurred in some devastated sites with portions having excellent stand densities. Other sites, however, have very low regeneration and lesser vegetation covering the forest floor. Interestingly, high elevation areas southwest of Clingmans Dome that are capable of supporting spruce-fir forests are occupied by deciduous hardwood forests with grassy balds at the peaks. It is uncertain why the different sites vary in regeneration potential especially since fir seed sources are adequate to establish Fraser fir on all of these sites.

Studies have shown that associations between plant roots and rhizosphere microbes may limit the ability of plants to reestablished themselves and persist in particular habitats (Klironomos 2002, Bardgett et al. 2005, Kardol et al. 2006). In another study, drastic deforestation caused changes in rhizosphere mycobiota (fungi) diversity and frequencies that later affected re-establishment of previous forest communities

(Ingham and Thies 1996). Recovery of the previous mycobiota levels, which include important mycorrhizal fungi, may take years regardless of forest types. Similarly specific mycorrhizal species have been lost on some sites still not reforested with Fraser fir where seedlings cannot outgrow the lesser vegetation. In a 2005 study, ten species of ectomycorrhizal (EM) fungi (*Amanita constricta, Cortinarius sp. (Phlegmacium), Entoloma* spp., *Inocybe napipes, Laccaria laccata, Lactarius chrysorrheus*, *Russula raoultii, Russula* spp. and *Xerocomus badius*) were collected in plots with high Fraser fir regeneration, whereas none were observed in plots with low Fraser fir regeneration (M. Wood, Mississippi State University (MSU), unpublished data). These results suggested that EM fungi are important for reforestation of the Fraser fir. A similar study found that *Elaphomyces muricatus* Fr. produced the most abundant hypogeous sporphores and *Cenococcum geophilum* Fr. was the most commonly found EM fungus in spruce-fir forests near Roan Mountain, North Carolina (Bird & McCleneghan 2005). Basidiomycete populations and diversity also have been evaluated for red spruce and northern hardwoods forests in West Virginia (Bills et al. 1986).

The mature stands of Fraser fir with closed canopies had very little lesser vegetation or shrubs, but bryophyte mats often covered the forest floor and there were abundant Fraser fir seedlings (R. Baird, MSU, personal observations). Although a shrub layer may be absent from some Fraser fir dominated stands, on mesic or low regeneration sites hobblebush (*Viburnum alnifolium* Marsh.), redberry elder (*Sambucus pubens* Michx.), southern mountain cranberry (*Vaccinium erythrocarpum* Michx.), minnie-bush (*Menziesia pilosa* (Michx. ex Lam.) Juss. ex Pers.), and southern bush-honeysuckle (*Diervilla sessilifolia* Buckl.) may occur (Whittaker 1956). Ridge areas may exhibit high heath coverage that includes catawba rhododendron (*Rhododendron catawbiense* Michx.) and Carolina azalea (*Rhododendron carolinianum* Rehd.). Following disturbance, thornless blackberry (*Rubus canadensis* L.) may densely populate canopy gaps (Pauley & Clebsch 1990).

Germination of Fraser fir seed may occur on moss, decaying logs, mineral soil, and moist litter (Pauley & Clebsch 1990). Since moisture plays an important role in determining seedling survival germination on substrates with variable moisture content, such as mineral soil and litter, present a greater potential for seedling death from desiccation (Crandall 1958). As a result, over story shading provides the best conditions for initial germination and establishment of Fraser fir seedlings (Beck 1990). Although seedlings are able to persist in shaded conditions for many years and grow 2.5 to 5.1 cm per year, growth is best in full sunlight (Crandall 1958; Beck 1990) where, under optimal conditions, Fraser fir may grow 1.8 m in 6 to 8 years (Beck 1990).

Ground cover of Fraser fir stands on north facing slopes have very high moss cover (90%), and high wood sorrel (*Oxalis* spp.) and wood fern (*Dryopteris* spp.) cover at 50% and 40%, respectively (Whittaker 1956). Intermediate aged Fraser fir forests have very high moss coverage (80-90%), but vegetation of other strata is usually less than 10%. South facing slopes exhibit decreased moss cover (60% or less) and high heath cover (40-60%). Areas of greater moss cover may be important for seedling establishment due to moisture retention, although thick layers of moss may not be ideal for initial root establishment (Pauley & Clebsch 1990). Wood (MSU, unpublished data) determined that mean percent moss cover averaged across two locations in GRSM was numerically greater for high regeneration Fraser fir plots than for low regeneration plots. Furthermore, mean percent herbaceous cover averaged across two locations was significantly greater for low regeneration Fraser fir plots compared to high regeneration plots. It has been suggested that regeneration of fir may be

dependent on the abundance of bryophyte cover and reduced understory vegetation (M. Clouster (Gray), USDA/FS-GRSM; R. Baird, MSU, personal observations). However, further studies are necessary to determine the role bryophytes plan in improving seedling establishment and survival especially in areas with poor reforestation. This study was done to, 1) determine the density and diversity of EM species on mature, sapling and low regeneration stands of Fraser fir, 2) collect and isolate the fungi observed, 3) document plant and shrub species, and 4) determine correlations between bryophytes and EM fungi in three fir regeneration types.

Materials and Methods

Study Plots: Long-term permanent plots containing pure stands of Fraser fir were established near the Clingmans Dome area at Mount Love (north facing slope) and Mount Buckley (south facing slope) in the GRSM. Four replicate plots (20 × 20 m) of the following three treatments were established at each location. The management treatments were based on naturally occurring age classes: 1) low regeneration plots, defined as containing <10% of plot areas with Fraser fir seedlings and no sapling regeneration; 2) sapling plots, defined as stands with trees >1.37 m tall and <5.0 cm diameter at breast height (dbh=1.37 m), with up to 50 % of plot containing saplings and 50% canopy closure; and 3) mature stands, with trees >15.0 cm dbh and complete enclosed canopy cover. All Fraser fir trees within the plots were numbered and the dbh was measured on the uphill side of each tree using previously defined methods (Schomaker et al. 2007). This data provided tree density, maturity and canopy closure information that are believed to impact occurrence of fungi.

Study Site: Fir sapling/seedling/tree numbers per plot and bryophyte cover were recorded in 15 October 2009. Bryophyte cover was rated as 1 to 10 with 1=< 10 % cover of the forest floor, 2=10 to < 20 % cover, 3=20 to < 30 % cover up to 10=90 to 100 % cover. Lesser vegetation data were obtained on June 10 and 29 October, 2011 and included diversity and density of plant species per plot. Any grasses in a plot were rated as percent cover using the same scale as used for the presence of bryophytes.

Following establishment of 20 x 20 m plots, monthly sampling of all macrofungi was conducted from April through October within each plot at the two locations from 2009-2011. All macrofungi present on the forest floor or on woody debris were collected. All fresh sporophores were photographed for future reference and to aid in identification, macroscopic characteristics of fresh sporophores were recorded for each specimen (Largent 1977; Largent et al. 1977). Colors of sporophores are from *Methuen Handbook of Colour* (Kornerup & Wanscher, 1978). All voucher specimens were numbered as FF 1-420 and are housed at MSU in the fungal herbarium (MISSA) by R. Baird, BCH-EPP Department.

The EM fungus *Cenococcum geophilum* which forms black root tips and sclerotia was evaluated by sampling approximately 1.0 L soil (A0 and A1 horizon) samples per plot on 24 September 2011. All samples were stored in 3.78 L Ziploc storage bags, placed into a cooler, returned to the laboratory and stored at 4.0 °C until processed seven days later. Methods for sampling sclerotia and root morphotypes followed those of Bird & McCleneghan (2005), but 250 ml of soil was sampled per plot rather than the four 15 ml subsamples used in their study. Isolation of *C. geophilum* sclerotia to confirm identity followed the methods of Melin & Mikola (1948) and Trappe (1969). Furthermore, 20 feeder roots were removed from each soil sample by sieving, segmented into 10 cm pieces and observed under a dissection microscope for presence of the black hyphal Hartig Net morphotype as a preliminary identification of *C. geophilum*.

Table 1. Plot locations of Fraser fir sites by treatment near Clingmans Dome in Great Smoky Mountains National Park (UTM NAD27 CONUS).

Coordinates For Fraser Fir Plots near Clingmans Dome			
Mt. Buckley	Low Regeneration	Sapling Stands	Mature Stand
Rep. 1	(P)	(R)	(S)
	35° 33.727	35°	35°
	-83° 30.402	33.738	33.722
		-83°	-83°
		30.498	30.258
Rep. 2	35° 33.719	35°	35°
	-83° 30.435	33.728	33.725
Rep. 3	35° 33.729	-83°	-83°
	-83° 30.282	30.178	30.258
		35°	35°
Rep 4	35° 33.720	33.726	33.734
	-83° 30.388	-83°	-83°
		30.305	30.116
		35°	35°
		33.727	33.748
		-83°	-83°
		30.140	30.042
Mount Love	35° 33.886	35°	35°
Rep. 1	-83° 29.796	33.874	33.869
		-83°	-83°
	35° 33.938	29.788	29.787
Rep. 2	-83° 29.731		
		35°	35°
	35° 33.926	33.944	33.843
Rep. 3	-83° 29.756	-83°	-83°
		29.746	29.796
	35° 33.959		
Rep. 4	-83° 29.677	35°	35°
		34.008	33.964
		-83°	-83°
		29.556	29.672
		35°	35°
		34.007	34.005
		-83°	-83°
		29.541	29.599

Tissue cultures of EM sporophores were made on the three media described below to obtain isolates in pure culture. Macroscopic, microscopic, and DNA sequenced data were used to confirm identification and vouchers specimens were maintained in a repository (discussed below). Tissue cultures were made with a sterile transfer needle directly onto modified Modifed Melin-Norkrans nutrient agar, half strength Hagem and half strength potato dextrose agar media (Lilleskov et al., 2002; Marx et al., 1982; Molina & Palmer, 1982; Sundari & Adholeya, 2003; Turnau et al., 2001). Tissue cultures were attempted from more than one specimen for greater assurance of success. Ten to twenty isolation attempts were done for each EM species collected. Extraction of DNA and PCR protocol followed those previously described (O'Brien et al., 2005; Mata et al., 2007) except using the primers ITS1F and ITS4 (White et al., 1990; Gardes & Bruns, 1993). The ITS sequences of EM fungus cultures was obtained for comparison to the GenBank database using BLAST. When cultures were not available from a field collection, genomic DNA was extracted from sporophore tissues and cloned for 93% of the taxa to obtain clean sequences using the methods of Lickey et al. (2007). The confirmed isolates of EM fungi are maintained using 15.0 % sterile glycerol mixture and stored at -80 °C or in 200 ml glass sterile distilled water (100 ml) test tubes left at room temperature at MSU BCH-EPP Dept.

Statistical Analysis: Four replicate plots for each site, low regeneration, saplings, and mature stands, were selected using a randomized complete block design. All fungus data for species richness, diversity and evenness followed formulas and methods discussed below. Data were analyzed as a series of combined experiments using the GLM procedure of SAS (SAS Institute, Cary, NC) and means were separated using Fisher's protected least significant difference (LSD). Species richness values (SR) and species diversity indices (H') were calculated using Shannon-Weaver index for

evenness (E) (Stephenson 1989, Stephenson et al. 2004) and Coefficient of Community (CC) (Van Dyke 2003). Pearson's correlation coefficient compared plot data between select parameters including the three age class treatments, bryophyte forest floor coverage, and mean numbers of *Cenococcum geophilum* sclerotia.

Results: A total of 44 taxa of fungi were collected over three years from the Fraser fir plots (Table 2). Eleven were decomposers, including three unknowns which were pooled due to their low frequencies. Thirty-three EM taxa were collected with three of those pooled as unknowns. Identifications were based on morphological and/or ITS sequence data. All successfully sequences were deposited in GenBank.

Megacollybia rodmanii R.H. Petersen, K.W. Hughes & Lickey was the most frequent decomposer collected (2.6%) during the study (Table 2). Several *Mycena* spp. were found on fallen logs but their percent occurrence varied yearly even in some of the wetter mature plots during the three year study. Sporophores of the *Mycena* spp. were more abundant during the first year of the study. It was uncertain why the occurrence of saprobic species was low since plots were generally wet throughout the study period. Sapropbic species were more common overall in the mature than in the sapling plots except for *Gymnopus junquilleus* R.H. Petersen & J.L. Mata and the one *Entoloma* sp. which occurred primarily in the sapling plots. Richness, diversity and densities of EM fungi were much greater than that of saprobic fungi present in plots.

Clavulina cristata comprised 22.5% of all sporophores observed during the three year study. A significantly greater number of sporophores (14.2%) occurred in the saplings plots than the low regeneration plots (1.7%) and mature plots were similar to both at 6.6%. It was one of the two most common EM species observed at both locations and had the second highest total occurrences within low regeneration plots. For all EM species, richness and diversity values at 21 to 25 and 2.24 to 2.38, respectively, were compared by months, June, July and August had significantly greater values than April, May, and September at 0 to 8 and 0 to 1.6. Evenness was similar between months and ranged from 0.6 to 0.7.

The four *Cortinarius* spp. (including one *Phlegmacium* sp.) composed 10.3%of the total sporophores collected, with *Cortinarius anomalus s.l.* being the most common species (4.4%) and more abundant in mature Fraser fir plots (3.3%) than in the sapling plots (1.1%) (Table 1). Only the *Phlegmacium* sp. had a significantly greater frequency of sporophores (1.3%) in sapling plots but none were observed in the low regeneration plots. A total of six *Lactarii* were identified during the study (Table 1). Of these, *Lactarius oculatus* (15.5%) and *L. chrysorrheus (*7.5%) were the most common of all six species. For *Lactarius oculatus* the frequency was greatest in mature plots (10.4%), less in saplings plots (4.3%) and lowest in low regeneration plots (0.8%). Whereas the frequency of *Lactarius chrysorrheus* was similar across mature and sapling plots (3.7% and 3.8%, respectively) but significantly lower in low regeneration plots than the other two age classes. Furthermore, the frequency of *Lactarius lignyotellus* and *L. sordidus* was significantly greater in sapling plots than in other plots. All species of *Lactarius* were difficult to culture. The four *Laccaria* species comprised 17.3% of total sporophores observed. *Laccaria laccata* var. *pallidifolia* had the highest percent frequency of sporophores observed (8.3%) with a similar frequency on sapling and mature plots and the highest frequency of all fungi on the low regeneration plots (1.9%). *Laccaria laccata* occurred at a frequency of 5.2% of the total but was not observed on low regeneration plots during the study. *Laccaria nobilis* most commonly occurred in mature and sapling plots

(1.8% and 1.6%, respectively) indicating potential importance in early sapling development and mature stands. Both species were readily isolated and cultured on selective media and in sterile water test tubes.

Isolation of fungus species was difficult even with multiple replications per collection. Fifteen EM taxa were cultured but stored isolates often stopped growing following two or three subcultures (eg. *Russula paludosa* FF 271A, *Russula* sp. FF 278). However, *Laccaria laccata, L. laccata* var. *pallidifolia* and *L. nobilis* isolates FF 162, FF 262, and FF 242/FF 272, respectively, remain stable with minimal sectoring. Unfortunately all attempts to isolate *Clavulina cristata* were unsuccessful due to secondary contamination by anamorphic fungi present within the tissues.

Species richness values for the 44 fungus taxa from the two locations over three years are shown in Table 4. There were no significant differences in numbers between Mount Buckley and Mount Love with 26 and 27 taxa recorded respectively. Species richness was highest in 2009 at 29 compared to 20 in 2010 and 18 in 2011. In 2009, 818 sporophores were observed compared to 328 in 2010 and 285 in 2011. Across years and locations fungi observed in mature and sapling plots had significantly greater species richness values at 25 and 26 respectively as compared to low regeneration plots at 17. Species diversity was significantly greater at Mount Love (2.93) than at Mount Buckley at (2.12). Fungus diversity in 2009 (2.67) and 2010 (2.61), were significantly different than in 2011 (1.97) (Table 4). However, no differences in diversity occurred between low regeneration (2.40), sapling (2.44) and mature plot (2.57) treatments. When evenness was evaluated, all plots at Mount Love had significantly greater species evenness (0.89) than those at Mount Buckley (0.65). Furthermore, evenness of the taxa was greater in 2010 (0.87) than 2011 (0.68) but values for 2009 (0.79) were similar to both.

All major EM species discussed above were found more commonly in 2009 than in 2010 and 2011 (Table 3). This may have been the result of more timely precipitation in 2009 than the other two years (J. Renfroe, Air Quality Specialist, Dept. of Interior/GRSM). For example, precipitation data for May through October in 2009 was 165.6 cm, but decreased to 107.0 cm in 2010 and 99.6 cm in 2011. The 60.0 cm or greater difference in 2009 rainfall data as compared to 2010 and 2011 is likely the main reason for greater species richness and density based on sporophore data. The increased rainfall also was greater just prior to the sampling dates for 2009. Across months no real trends in sporophore frequencies were noted and total values were evenly divided between May, June and July for each year (Table 3).

In the high elevations Fraser fir natural regeneration is very poor or does not occur where lesser vegetation has become established, thus impacting the diversity and density of fungus and bryophyte communities. Nine species of plants, including two grasses, occurred across both locations but primarily in the low regeneration plots (Table 5). Common species included *Dryopteris campyloptera* Clarkson (mountain wood fern), *Vaccinium corymbosum* L. (blueberry), *Rubus canadensis* L. (thornless blackberry) and two grass species *Carex* sp. and *Luzula acuminate* Raf. (hairy wood rush) which were pooled into one data set. All plants including the grasses were particularly abundant in the low regeneration or low regeneration sites and diversity did not change from early to late season.

The vegetation at the two study locations, Mount Buckley and Mount Love, was similar in composition. *Dryopteris campyloptera* and *Angelica triquinata* Michx. (filmy Angelica) had a significantly greater number of populations in the low regeneration plots than the other two treatments (Table 4). When the data were compared by locations only the two pooled grass species had a significantly greater percent

occurrence in the low generation plots as compared to sapling or mature plots (data not shown). As expected, mature and sapling plots had a significantly greater numbers of large trees and saplings than low regeneration plots. In addition, presence of small seedlings in mature plots was significantly greater (mean of 19.3) than in the sapling or low regeneration plots (5.8 and 0.8 respectively).

Since *Cenococcum geophilum* was previously reported to be a common EM fungal symbiont with Fraser firs (Bird and McCleneghan, 2005), sclerotia counts and root morphotypes from plot soil samples were used to measure population levels. Mature plots had a significantly greater mean average of sclerotia (1.2) per 250 ml soil compared to sapling or low regeneration plots (0). Also, the number of feeder roots showing black morphotypes was greatest from mature plots soils (average of 15 of 20 replicate roots samples per treatment) than sapling or low regeneration plots (2.3 and 1.8, respectively). Bryophyte mat coverage, which has been shown to suppress vegetation by impacting seedling survival, were greater in mature stands (6.9%) compared to sapling and low regeneration plots (3.6% and 2.9%, respectively). The correlation coefficient comparing bryophyte mat forest floor cover, and mean numbers of sclerotia across treatments showed numerical or significant correlations between presence of mature trees-sclerotia numbers (~0.053) and bryophyte forest floor percent cover-sclerotia numbers (0.022) while the percent bryophyte cover-mature trees were not correlated (0.14).

Discussion: Although 26 of the 44 taxa of fungi collected were previously reported to occur in GRSM, most sources did not list habitat or associated tree species (Table 1). This study is the first comprehensive study of fleshy fungi, including the root fungus *C. geophilum*, from Fraser firs at high elevations of GRSM near Clingmans Dome. Many of these same fungi are known to occur at mid- or low elevations in the park. Of note, *Megaollybia rodmanii* associated with dead wood, likely represents a complex of four species based on molecular data, and this name may not apply to any species in North America (Hughes et al. 2007). For the species collected in this study *M. rodmanii* is the correct species epithet based on morphological characterization.

The four genera of EM fungi *Clavulina, Cortinarius, Laccaria* and *Lactarius* have been reported from similar ecosystems (Bills et al., 1986; Bird & McCleneghan, 2005). The species of these genera were more common in mature stands and sapling plots than in low regeneration plots. However, *C. cristata* and *L. laccata* var. *pallidifolia* had highest occurrences in low regeneration sites. Both species are widely distributed in conifer and hardwood forest of North America with *C. cristata* more common in conifers than hardwoods (Corner 1950; Coker 1974; Arora 1986) and *L. laccata* var. *pallidifolia* forming symbiotic associations with many tree species at various elevations and in various forest types (Mueller 1991, 1992).

When species frequencies were compared between low regeneration, sapling and mature stand treatments there were differences among 7 of the 44 species observed during the study (Table 1). From these observations six of seven were greater in sapling plots, four of seven in mature and none in low regeneration plots. Even though these fungi were different between treatments, their percent frequencies were low, and only *C. cristata* and *L. laccata* var. *pallidifolia* are important in early growth of seedlings and saplings.

Conducting floristic studies of fungi within specific habitats provides only a portion of species associated within those different ecosystems. It is important to note that many EM fungi rarely sporulate, are not conspicuous and hard to observe, including *Cenococcum geophilum* and the hypogeous fungi

Elaphomyces spp. and *Tuber* spp. (Gardes & Bruns, 1996). While *C. geophilum* was abundant in the spruce-firs forest of the southern Appalachian Mountains hypogeous fungi were infrequently found (Bird & McCleneghan 2005). This study supports these observations, since the sclerotia of *C. geophilum* were only collected from soils of mature tree plots where the canopy was closed and occurrence was correlated with increased bryophyte ground cover. However, none of the soil samples yielded sporophores of *Elaphomyces* or *Tuber* during soil analysis for *Cenococcum* sclerotia levels.

The low regeneration plots at both locations were heavily covered with lesser vegetation, especially with the two grasses and two ferns, making it very different to observe sporophores. Both *Clavulina cristata* and *Laccaria laccata* var. *pallidifolia* had the greatest percent occurrence in low regeneration plots and were often observed within the 0.5 m of Fraser fir seedlings indicating a putative symbiotic association. In addition, *L. nobilis* was not observed in low regeneration plots but often was seen adjacent to the plots and within the vicinity Fraser fir seedlings.

Regeneration of Fraser firs following heavy mortality from balsam woolly adelgid was dramatic (Dull et al., 1988). But many Fraser fir habitats within the park did regenerate with Fraser fir and are now considered artificial balds within the GRSM. Seedlings in balds must have adequate soil moisture (eg. bryophyte mats) and EM associates to survive and compete for space with lesser vegetation such as ferns and grasses. The presence of grasses, together with their associated environmental parameters, seems to be an important factor for seedling survival. Vegetation in low regeneration plots was the greatest compared to the sapling and mature plots (Table 4). Sapling plots had large seedling biomass per plot, reducing available space, and mature trees limited canopy sunlight exposure which resulted in reduced grass/vegetation cover. The sapling and mature tree plots had the greatest numbers and diversity of saprobic and ectomycorrhizal fungi. In turn moisture requirements and occurrence of available hosts are critical for ectomyorrhizal fungi to survive and form basidiomata and ascomata in the often harsh Fraser fir ecosystem.

Bryophytes clearly retain water and serve as reservoirs that aid in the survival of Fraser fir seedlings and enhance ectomycorrhizal formation. This results in greater uptake of mineral nutrients and water for seedling, sapling and mature tree health. The significantly greater occurrences of bryophyte mats in the mature tree plots where seedlings were at their highest densities, suggests that bryophyte water retention is an important parameter for seedling establishment. Outside the mature stands, the lower percentage of bryophyte biomass could be correlated with lower seedling numbers in sapling or low regeneration plots. However, the number of EM fungi in sapling plots was relatively high and seemed to be more directly influenced by presence of saplings than bryophyte coverage or canopy closure as seen in mature stands. The same is true for the significantly greater frequency of *Cenococcum geophilum* in mature plots, reflected in the greater number of feeder roots with the black morphotype of this fungus. From these observations it seems possible that increased frequency of *C. geophilum* is more closely correlated with the forest floor and bryophyte protection due to the closed canopy. Closed canopies support great moisture retention which in turn supports greater bryophyte reproduction and survival.

In conclusion, EM fungi require Fraser fir trees to grow and reproduce on these sites and the tree hosts benefit from fungus uptake mineral nutrient and water. Other research has shown that the loss of EM fungi from forest habitat negatively impact reestablishment of tree host such as Fraser fir (Hagerman et al. 2001,

Reithmeier & Kerrigan 2013). The two ferns and two grasses are believed to be limiting seedling establishment and survival in low regeneration plots and thus indirectly decreasing EM species richness. During this three year study lesser vegetation was observed completely covering one and two year old seedlings, decreasing light availability and root zone competition for water and nutrients. During subsequent sampling dates almost all seedlings previously observed were dead in the low regeneration plots. The massive fern rhizomes hamper successful seed germination and prevent Fraser fir roots from penetrating into the soil. In addition, soils at the two locations are less than 6.0 cm deep and very rocky, preventing easy root establishment and moisture retention which places young seedlings under stress. Bryophytes are important reservoirs for moisture and physiological barriers to grasses but appear to be directly associated with closed canopies of mature Fraser fir stands. *Laccaria* species can be used in artificial reforestation efforts in conifers (Molina & Palmer, 1982). Their occurrences in low regeneration and sapling plots in this study suggest that they are EM associates of Fraser firs. These observations indicate their importance for early establishment of Fraser fir seedlings. Studies to enhance the survival of seedlings at sites where canopy cover is minimal and vegetation levels are high will require artificial introduction of these or other EM fungi. Furthermore, based on soil sclerotia and feeder root morphotype data, *Cenococcum geophilum* is more of a climax species than an early colonizer of seedlings. The high density of lesser vegetation in low regeneration plots on bald habitats at Mount Buckley and Mount Love prevent early establishment of seedlings is critical to the development of Fraser firs in open previously forested areas of GRSM. Lesser vegetation will have to be removed so that Fraser fir can become reestablished in these areas.

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Table 2. Percent occurrence of fungi from three age classes of Fraser fir stands at two locations and across three years by site treatment, near Clingmans Dome in the Great Smoky Mountains National Park.

Taxa/ Main FF voucher Coll. #/GenBank Accession #[a]	Low Regeneration Stands	Saplings	Mature Stand	Percent[a] Occurrences by loc. B-L/total
Saprobic Fungi				
Entoloma sp. FF5	0	1.3	0.1	0.1-1.3/1.4[b]
Gymnopus dryophilus (Bull.) Murrill [CI,II] FF 6 KF007938	0	0	0.7	<1.0
Gymnopus junquilleus R.H. Petersen & J.L. Mata[III] FF 39 KF007938	0	0.7	0	<1.0
Lentaria micheneri (Berk. & M.A. Curtis) Corner [I,II] FF 26	0	0	0.2	<1.0
Leotia lubrica (Scop.) Pers.[I,II] FF 145	0 b[d]	0 b	1.9 a	*0-1.8/1.8
Megacollybia rodmanii R. H. Petersen, K.W. Hughes & Lickey [I,II] FF 9	0.6	0	2.0	0.6-2.0/2.6
Mycena pura (Pers.) P. Kumm. [I,II] FF 35	0	0	0.5	<1.0
Mycena sp. (Pers.) Roussel FF 1,2,3,4	0	0.4	1.6	1.1-0.9/2.0
Saprophytes- Unknown spp. A, B, C FF 2,3,4,5	1.2	0.7	0.8	1.3-1.4/2.7
Ectomycorrhizal Fungi				
Amanita borealisorora Tulloss nom. prov.[IV] FF10	0.2	0.2	0.1	<1.0
Amanita constricta Theirs & Ammirati [I,II] FF 49 KF007934	0.2	0.1	1.0	0.9-0.4/1.3
Amanita flaviconia G.F. Atk. [I,II] FF 20	0	0.4	0.7	0.8-0.3/1.1
Boletus badius (Fr.) Fr. [I,II] FF 45				<1.0
Clavulina cristata (Holmsk.). J. Schrot. [I,II] FF13 KF007936	1.7 b	14.2 a	6.6 ab	*19.0-3.5/22.5

Table 2. Continued

Taxa/ Main FF voucher Coll. #/GenBank Accession #[a]	Low Regeneration Stands	Saplings	Mature Stand	Percent[a] Occurrences by loc. B-L/total
Cortinarius anomalus (Fr.) Fr. [I,II] (wrinkle pileus light colored) FF 133	0	1.1	3.3	0-4.4/4.4
Cortinarius croceus (Schaeff.) Gray [II] (yellow lamellae) *incognitus* FF41	0	0.5	2.3	0.2-2.8/3.0
Cortinarius tortuosus (Fr.) Fr.[II] (Deep brown-no purple stipe) FF 237 KF007937	0	0.3	0	<1.0
Cortinarius, Phlegmacium sp. (deep brown +pur-br stipe) FF 32	0 b	1.3 a	0.9 ab	0.6-2.0/2.6
Inocybe calamistrata (Fr.) Gillet [I,II] FF 135 KF007939	0	0.2	2.0	1.3-1.4/2.2
Inocybe sp. A FF 192 KF007940	0	0.3	2.6	0.1-2.8/2.9
Laccaria laccata (Scop.) Cooke[I,II] FF 48 KF007941	0	1.0	4.2	3.0-2.2/5.2
Laccaria laccata var. *pallidifolia* (Peck) Peck [II] FF 1 FF 55 KF007942	1.9	2.7	3.7	*5.2-3.1/8.3
Laccaria nobilis A.H. Smith[II] FF 126, 162, 242 KF007943	0	1.6	1.8	2.1-1.3/3.4
Lactarius chrysorrheus Fr. FF 187 KF007945	0.6 b	3.8 a	3.6 a	4.6-2.9/7.5
Laccaria proxima (Boud.) Pat.FF 236 KF007944	0	0.1	0	<1.0
Lactarius lignyotus Fr. [I,II] FF 186 KF007946	0.7	0.5	0.1	*0.9-0.4/1.3
Lactarius lignyotellus A.H. Smith & Hesler [I,II] FF 29	0.3 b	2.4 a	0.7 b	0.9-2.5/3.4
Lactarius mucidus Burl. [I,II] FF 147	0	0.7	0	<1.0

Table 2. Continued

Taxa/ Main FF voucher Coll. #/GenBank Accession #[a]	Low Regeneration Stands	Saplings	Mature Stand	Percent[a] Occurrences by loc. B-L/total
Lactarius oculatus (Peck) Burl. [I,II] FF 174 FF 17 KF007947	0.8	4.3	10.4	10.9-4.6/15.5
Lactarius sordidus Peck [I,II] FF 23	0 b	1.1 a	0 b	0.1-1.0/1.1
Ramaria sp. FF 285	0	0	0.1	<1.0
Russula abietina Peck FF 169 KF359677	0 b	1.0 a	0 b	0.6-0.4/1.0
Russula bicolor Burl. FF 57 KF007949	0.1	3.2	2.6	*3.7-2.2/5.9
Russula paludosa Britzelm. FF 185 KF007950	1.3	0.8	0.4	*1.2-1.3/2.5
Russula turci Bres. FF 193 KF007951	0.1	1.5	1.6	*2.1-1.1/3.2
Russula xerampelina (Schaeff.) Fr. FF 157 KF007952	0	0.3	0	<1.0
Russula sp. FF 22	0.7	0.8	0.7	<1.0
Scleroderma citrinum Pers.[I,II] FF 94	0	0.7	0	<1.0
Unknown (Three spp.) FF 7, 146	0	0.4	0	<1.0

[a] Field collections and NCBI accession numbers in bold; NCBI accession numbers ones accessioned into TENN herbarium.

[b]Percent occurrence by location B=Mount Buckley and L=Mount Love of GRSM and total percent occurrences based on 1,507 sporophores observed during the three year study; * indicates significant difference between locations (P≤ 0.05).

[c]When present references showing previous reports for fungus species in GRSM but locations and forest type not always associated with Fraser fir include: [I]Petersen, R. 1978. Checklist of fungi GSMNP, Management Report # 9; [II] http://tenn.bio.utk.edu/fungus/database/fungus-browse-results.asp?GSMNP=GSMNP, TENN collection previously reported; [III]Mata, J.L.; Hughes, K.W.; Petersen, R.H. 2006. An investigation of /Omphalotaceae [*sic*] (*Fungi*, euagarics) with emphasis on the genus *Gymnopus*. *Sydowia* 58: 191-289; and [IV]ehttp://pick4.pick.uga.edu/mp/20q?search= Amanita +borealisorora

[d]Numbers followed by a letter are significantly different using GLM procedure t-test (P≤0.05).

Table 3. Percent occurrence of a taxa per year and month across age classes in Fraser firs stands and two locations near Clingmans Dome in the Great Smoky Mountains National Park.

Taxa/ Main FF voucher Coll. #/GenBank Accession #[b]	Year			Months				
	2009	**2010**	**2011**	**April**	**May**	**June**	**July**	**August**
Entoloma spFF5	1.3	0.1	0	1.5	0	0	0	0
Gymnopus dryophilus FF 6 **KF007938**	0.7	0	0	0.7	0	0	0	0
Gymnopus junquilleus FF 39 **KF007938**	0	0.7	0	0	0	0	0.3	0.4
Lentaria micheneri FF 26	0.2	0	0	0	0.1	0.1	0	0
Leotia lubrica FF 145	1.8	1.0	0	0	0	0	2.8	0
Megacollybia rodmanii FF 9	0.6	2.0	0	0	0.6	0	2.0	0
Mycena pura FF 35	0.5	0	0	0	0.2	0.3	0	0
Mycena sp. FF 1,2,3,4	1.8	0	0.2	2.0	0	0	0	0
Saprophytes- Unknown spp. A, B, C FF 2,3,4,5	1.1	1.6	0	0	0.8	0.4	1.5	0
Ectomycorrhizal Fungi								
Amanita borealisorora FF 10	0.3	0.1	0.1	0	0.2	0.1	0.2	0
Amanita constricta FF 49 **KF007934**	0.9	0	0.4	0	0.5	0.3	0.5	0
Amanita flaviconia FF 20	1.1	0	0	0	0.7	0.4	0	0
Boletus badius FF 45	0.1	0	0.1	0	0	0.2	0	0
Boletus luridiformis [B] (red pored) stains blue FF 180 **KF007935**	0	0	0.2	0	0.1	0	0.1	0
Clavulina cristata FF13 **KF007936**	10.8	1.6	10.2	0	10.5	0.5	11.6	0
Cortinarius anomalus (wrinkle pileus light colored) FF 133	1.1	2.8	0.5	0	0.1	0.4	1.1	2.8
Cortinarius croceus[B] (yellow lamellae) *C. incognitus* FF41	2.7	0.4	0	0	0	0	3.0	0.1
Cortinarius tortuosus (Deep brown-no purple stipe) FF 237 **KF007937**	0	0.3	0	0	0	0.3	0	0

Table 3. Continued

Taxa/ Main FF voucher Coll. #/GenBank Accession #[b]	Year			Months				
	2009	2010	2011	April	May	June	July	August
Cortinarius, Phlegmacium sp. (deep brown +pur-br stipe) FF 32	0.7	1.9	0.1	0	0.3	0.2	0.4	1.7
Inocybe calamistrata FF 135 **KF007939**	1.5	0.3	0.4	0	0	1.2	1.0	0
Inocybe sp. A FF 192 **KF007940**	3.8	1.5	0.1	0	4.5	0.4	0.5	0
Laccaria laccata (Scop.) Cooke FF 48 **KF007941**	3.2	0.8	1.2	0	1.5	1.7	1.1	0.9
Laccaria laccata var. *pallidifolia* FF 55 **KF007942**	5.7	1.2	1.4	0	4.4	2.7	1.0	0.3
Laccaria nobilis FF 126, 162, 242 **KF007943**	1.7	0.3	0.4	0	1.2	0.4	0.7	0.1
Laccaria proxima FF 236 **KF007944**	0	0	0.1	0	0	0	0.1	0
Lactarius chrysorrheus FF 187 **KF007945**	3.5	1.9	2.1	0	1.1	2.1	4.1	0.2
Lactarius lignyotus FF 186 **KF007946**	0.8	0	0.5	0	0.7	0.4	0.2	0
Lactarius lignyotellus FF 29	1.3	0.7	1.5	0	0.3	1.4	1.8	0
Lactarius mucidus FF 147	0.1	0	0.5	0	0	0.5	0.1	0
Lactarius oculatus FF 174 FF 17 **KF007947**	11.2	1.3	3.0	0	7.3	2.4	5.3	0.5
Lactarius sordidus FF 23	0.5	0.1	0.5	0	0.1	0.1	0	0
Ramaria sp. FF 285	0	0	0.1	0	0	0	0.1	0
Russula abietina FF 169 FF 31	0.2	0	0	0	0.1	0.1	0	0
Russula bicolor FF 57 **KF007949**	2.5	1.5	1.9	0	3.2	0.6	2.1	0.1
Russula paludosa FF 185 **KF007950**	0.9	1.6	0	0	0.5	0.4	1.6	0
Russula turci FF 193 **KF007951**	1.8	1.2	0.2	0	1.7	0.5	0.3	0.6

Table 3. Continued

Taxa/ Main FF voucher Coll. #/GenBank Accession #[b]	Year			Months				
	2009	**2010**	**2011**	**April**	**May**	**June**	**July**	**August**
Russula xerampelina FF 157 **KF007952**	0.2	0	0.1	0	0.2	0.1	0	0
Russula sp. Pers. FF 22	0.6	0.1	0.2	0	0.6	0.2	0.1	0
Scleroderma citrinum FF 94	0	0.1	0	0	0	0	0.1	0
Unknown (Three spp.) FF 7, 46	0.4	0	0	0	0.4	0	0	0

Table 4. Species richness (n), diversity (H'), and evenness (E) for Fraser fir fungi identified from two locations in the Great Smoky Mountains National Park over two years.

	(n)	(H')	(E)
Location			
Mt. Love	27.0 A	2.93 A	0.89 A
Mt. Buckley	26.0 A	2.12 B	0.65 B
LSD	6.7	0.52	0.13
Year		**Across Locations**	
2009	29.0 A	2.67 A	0.79 AB
2010	20.0 B	2.61 A	0.87 A
2011	18.0 B	1.97 B	0.68 B
LSD	5.3	0.57	0.10
Stand Type (Age)		**Across Locations**	
Mature Stands[b]	25.0 A	2.57 A	0.80 A
Low Regeneration Stands	17.0 B	2.40 A	0.84 A
Sapling Stands	26.0 A	2.44 A	0.75 A
LSD	6.6	0.67	0.15

[a]=Mean with different letters are significantly different with LSD using the Jackknifing procedures (P≤0.05).

[b]= Each location included three treatments including plots established as low regeneration are those containing <10 % sapling growth of the plot areas. Sapling or regeneration stands are those with stems >1.37 m tall and <5 cm dbh and >50 % of plot area with growing saplings, and mature stands had completely enclosed canopy cover.

Table 5. Plant vegetation from three age classes of Fraser fir from two locations in Great Smoky Mountains National Park, September 2006.

	Tree Age Types- Fraser Fir Plot[A]			
	Low Regeneration	Saplings	Mature Stands	LSD
Angelica triquinata Michx.	12.0a	ob	ob	11.3
Carex sp.-*Luzula acuminata* Raf.	77.5a[B]	17.5b	6.9b	25.9
Viburnum lantanoides Michx.	8.5	6.1	2.3	7.2
Dryopteris campyloptera Clarkson	49.4a	11.9b	0b	30.4
Dryopteris intermedia (Muhl. ex Willd.) A. Gray	7.3	4.5	2.0	10.9
Vaccinium cormybosum L.	20.6	2.5	0.8	35.6
Solidago glomerata Michx.	13.5	6.4	4.5	18.9
Sorbus americana Marsh	2.1	0	0	2.3
Rubus canadensis L.	39.9	27.9	3.3	43.2

[A] Low regeneration sites=those containing <10% saplings per plot areas with saplings containing stems >1.37 m tall and <5.0 cm diameter at breast height (dbh), regeneration or sapling plots= with >50 % of plot area containing saplings, and mature stands= those with completely enclosed canopy cover.

[B] Mean numbers refer to percent of stand/plot for a specific plant species over two locations; mean percent followed by different letter are significantly different ($p \leq 0.05$) using GLM procedure.