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Where are they hiding? Testing the body snatchers hypothesis in pyrophilous fungi

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ABSTRACT

Pyrophilous fungi produce sporocarps after a fire but little is known about their ecology prior to or after a fire event. Recently, the body snatchers hypothesis was proposed that suggests some post-fire fungi form endophytic and/or endolichenic relationships with plants and lichens. To test the body snatchers hypothesis, bryophyte, lichen, club moss, and soil samples were collected from unburned and mixed-intensity burned areas 1–2 y after a 2016 wildfire in the Great Smoky Mountains National Park, and from unburned areas in four states outside the park. Samples were examined for the presence of pyrophilous fungi occurring as endophytes or in lichens using culture-dependent and culture-independent techniques. Culture-dependent methods isolated *Pholiota highlandensis*, a known pyrophilous fungus, from five bryophyte samples. Culture-independent methods identified 22 pyrophilous taxa from bryophyte, club moss, lichen, and soil samples across a range of geographical localities. The 'body snatchers' hypothesis is supported since many bryophyte, lichen, and club moss samples contained pyrophilous taxa suggesting that these fungi occur as endophytes and/or endolichenic fungi until a fire event triggers them to produce sporocarps.

1. Introduction

Wildfires are important abiotic disturbance events that reshape forest and grasslands through the combustion of stored carbon (Hurteau and Brooks, 2011), alter composition and structure of plant communities (Stevens-Rumann and Morgan, 2016), increase spatial heterogeneity (Malone et al., 2018), and alter soil bacterial and fungal communities (Jorgensen and Hodges 1970; Widden and Parkinson 1975). Although wildfires are detrimental to many plant and microbial species, some plant and fungal species have adapted by forming reproductive sporocarps only after a wildfire disturbance event (Seaver, 1909; Moser, 1949; Petersen, 1970). Post-fire recovery processes of forests have been thoroughly reviewed (Lisiewska, 1992). In short, during the first month post-fire, algae and discomycete pioneers appear, the second stage and third stages are characterized by the presence of fire-associated and non-fire associated bryophytes, and the fourth stage is characterized by the colonization of nitrophilous plants (Moser, 1949; Lisiewska, 1992).

Of particular interest, pyrophilous fungi are found throughout the kingdom Fungi and include members within the Ascomycota, Basidiomycota, and Mucoromycota, but most belong to the Ascomycota (Turnau, 1984; Claridge et al., 2009). These fungi are found, for example, in genera such as *Anthracobia*, *Morchella*, *Neurospora* and *Pyronema* in the Ascomycota and in *Coprinopsis*, *Pholiota* and *Psathyrella* in the Basidiomycota. Several categories of pyrophilous fungi have been reported by Moser (1949) and Petersen (1970) and summarized in Lisiewska (1992). The general categories consist of (i) fungal species that only fruit on burned (or heated) soil (e.g., *Anthracobia melaloma*, *Peziza echinospora*, *Pholiota highlandensis*), (ii) fungal species that prefer burned soil conditions but may fruit elsewhere, (iii) randomly encountered fungi (i.e., pre-fire fungi that survived the fire), and (iv) fire-intolerant fungi. Throughout this

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paper, pyrophilous fungi are defined as either category 1-fruiting is observed only after a fire event; or category 2-fruiting does not require a fire event, but is frequently enhanced by fire over that normally observed in unburned areas.

The high temperature during a fire event followed by a post-fire increase in soil alkalinity and reduction in microbial competition favor pyrophilous taxa (Warcup and Baker, 1963; El-Abyad and Webster, 1968; McMullan-Fisher et al., 2011). Exposure to high temperatures is required for some taxa to break spore or sclerotium dormancies (McMullan-Fisher et al., 2011). For example, Warcup and Baker (1963) could only isolate *Anthracobia* from heat treated soil but not from untreated soil. In addition, El-Abyad and Webster (1968) demonstrated that pyrophilous taxa had a higher pH optimum for spore germination and mycelial growth compared to soil fungi. Lastly, an intense fire event kills most of the soil microorganisms presumably reducing post-fire competition levels for pyrophilous fungal taxa (Seaver, 1909; Moore and Korf, 1963; Warcup and Baker, 1963).

The ecological roles of pyrophilous fungi, however, are not well known. It has been suggested that pyrophilous fungi are dormant as spores in the soil spore bank (Jalaluddin, 1967), while others have shown pyrophilous fungi to occur as endophytes before a fire disturbance (Baynes et al., 2012; Davey et al., 2013; Huo et al., 2014; Wang et al., 2016). Several authors have reported that certain pyrophilous species occur as mycorrhizas (Edgar and Paden, 1986; Vrålstad et al., 1998), root pathogens (Weir, 1915), or soil saprobes (Warcup, 1990). However, the ecological niche and ecological reservoir are still unknown for many pyrophilous fungi (Claridge et al., 2009).

Recently, Matheny et al. (2018) proposed that some post-fire fungi form endophytic and/or endolichenic relationships with plants and lichens based on ITS sequence similarity between post-fire fungal sporocarps and endophytic and/or endolichenic fungi, observations previously noted by others (Baynes et al., 2012; Wang et al., 2016). Matheny et al. (2018) described this as the body snatchers hypothesis. If the hypothesis is accurate, pyrophilous taxa should occur as endophytes and/or endolichenic fungi in plants and lichens in pre- and post-fire systems and it should be possible to isolate them into culture from plant and lichen tissues. The goal of this study was to test this hypothesis by sampling bryophytes, club mosses, lichens, and soil in unburned and burned areas after a mixed-intensity wildfire in the Great Smoky Mountains National Park (GSMNP) and in unburned areas in four states outside the park using both culture-dependent and culture-independent methods. This research aims to extend our understanding of the pre- and post-fire ecological roles of pyrophilous fungi beyond their sporocarp formation after a wildfire in forest ecosystems.

2. Material and methods

2.1. Sampling sites

Sampling was conducted in the GSMNP as an extension of a larger investigation documenting the occurrence of pyrophilous fungi within the park after a wildfire swept through the park in November 2016. Soil samples were collected on February 4, 2017, prior to the detection of post-fire aboveground fungal sporocarps, from burned and unburned areas. In March–May 2018, additional samples of bryophytes, lichens and soil were collected within the same GSMNP areas (Table 1). Additional bryophyte and club moss samples were obtained from 2016 through 2018 from various unburned areas in Alaska, Illinois, Indiana, and Pennsylvania (Table 1). These additional samples were collected from sites that showed no visible indication of a recent fire and represented samples outside the perceived GSMNP spore load zone, which was important to consider since environmental sampling techniques do not discriminate between dead, inactive (resting state) and live, actively-growing fungi.

2.2. Culture-dependent methods

Fresh bryophytes were collected from the GSMNP burned areas in April and May 2018 and their endophytes were isolated at the University of Tennessee following protocols outlined in U'Ren et al. (2010). Samples were surface-sterilized with sterile ddH_2O, followed by 30 s in 95% EtOH, 2 min in 0.5% NaOCl solution, 2 min in 70% EtOH, and dried under sterile conditions prior to transferal to 60 mm diam Petri plates containing 2% malt extract agar. Petri plates were wrapped with Parafilm, incubated at room temperature, and monitored for fungal growth. Mycelia emerging from cultured tissues were subcultured on malt extract agar. When mycelium covered the plate, a small piece (ca. 1–2 mm^2) was scraped into a 1.5 mL centrifuge tube, and DNA was extracted in extract-n-amp solution (Sigma-Aldrich). The entire ITS nrDNA region was amplified and sequenced using primers ITS1F, ITS2, ITS3 and ITS4 in various combinations (White et al., 1990; Gardes and Bruns, 1993). Amplification of the nrLSU region was made using primers LR0R (Cubeta et al., 1991) and LR5 (Vilgalys and Hester, 1990). A nBLAST search of GenBank was used to identify the isolates to species where feasible.

2.3. Culture-independent methods

Bryophyte, lichen, and club moss samples collected prior to May 2018 were air dried and stored between newspaper at room temperature. All fresh samples were processed at the University of Illinois within 1 week. Bryophyte samples were examined at 10 x, mixed species clumps were separated by species and thoroughly cleaned with water to remove foreign debris. Whole specimens were surface sterilized according to Petrini (1986). Bryophytes and lichens were surface sterilized for 30 s in 96% EtOH, 1 min in NaOCl solution (3% available Cl), and 30 s in 96% EtOH solution with a final rinse for 30 s in distilled water. Club mosses were surface sterilized for 1 min in 96% EtOH, 3 min in NaOCl solution (3% available Cl), and 30 s in 96% EtOH solution with a final rinse for 30 s in distilled water. All samples were agitated throughout the entire surface sterilization process, and solutions and containers were replaced for each sampling location and condition to prevent cross contamination. Surface sterilized samples were stored in a flow hood in uncapped sterile 30 ml tubes to dry overnight, cut by hand with sterile scissors, and 0.3–0.5 g dry sample was placed into separate MP FastDNA™ Spin Kit (MP Biomedicals) homogenization tubes. Soil samples were stored at −20 °C until time of DNA extraction, at which time 0.5 g of frozen sample was added to separate homogenization tubes. For DNA extraction, a MP FastDNA™ Spin Kit for Soil isolation kit (MP Biomedicals) was used following the instruction protocols but with the following modifications: samples were stored at −20 °C overnight, homogenization was performed for 12 min using a Vortex Genie™, samples were placed on ice for 5 min, homogenized for 8 additional min, and protein precipitation was completed twice. Samples were eluted in 100 μl of DNA/Pyrogen-free water, and quality and quantity were initially assessed using both gel electrophoresis and Nanodrop. One additional sample using 0.5 g DNA/Pyrogen-free water (negative control) was processed at the same time as the environmental samples. Amplification of the ITS1 nrDNA region was performed using the forward primer ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and reverse primer ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′). Amplification of the ITS2 nrDNA region was performed using the forward primer ITS7 (5′-GTGARTCATCGAATCTTTG-3′) and reverse

Table 1
Culture-dependent and culture-independent samples.

Method	Determination	Category	Condition	Location/approximate GPS	Collection date	Herbarium accession #
Culture-dependent						
	Ditrichum pallidum	Bryophyte	burned	GSMNP Table Mt Pine zone 35.6796N; −83.4781W	4-20-2018	TENN-B-0102889
	Ceratodon purpureus	Bryophyte				TENN-B-0102890
	Ditrichum pallidum	Bryophyte				TENN-B-0102891
	Funaria hygrometrica	Bryophyte				TENN-B-0102893
	Trematodon longicollis	Bryophyte				TENN-B-0102894
	Marchantia polymorpha	Bryophyte		GSMNP Cove Mt trail 35.6956N; −83.5331W	5-15-2018	TENN-B-0102895
	Polytrichum commune	Bryophyte	burned	GSMNP Baskins Creek lower trail 35.6781N; −83.4784W	4-24-2018	TENN-B-0102892
Culture-independent						
	Dicranum sp.	Bryophyte	unburned	GSMNP Baskins Creek 35.6772N; −83.4785N	5-06-2018	ILLS2962
	Hypnum sp.	Bryophyte				ILLS2938
	Atrichum angustatum	Bryophyte				ILLS2965
	Hypnum sp.	Bryophyte				ILLS2961
	Thuidium delicatulum	Bryophyte				ILLS2947
	Leucobryum sp.	Bryophyte				ILLS2949
	Brachythecium sp.	Bryophyte	burned	GSMNP Baskins Creek lower trail 35.6781N; −83.4784W	5-08-2018	ILLS2967
	Usnea cornuta	Lichen				ILLS82150
	Dicranum sp.	Bryophyte				ILLS2959
	Leucodon julaceus	Bryophyte				ILLS2945
	Lobaria quercizans	Lichen				ILLS82149
	Rhizomnium sp.	Bryophyte				ILLS2940
	Dicranum sp.	Bryophyte				ILLS2942
	Thuidium delicatulum	Bryophyte				ILLS2955
	Myelochroa aurulenta	Lichen				ILLS82151
	Atrichum angustatum	Bryophyte				ILLS2950
	Polytrichum commune	Bryophyte	burned	GSMNP Table Mt Pine zone 35.6796N; −83.4781W	5-08-2018	ILLS2958
	Atrichum angustatum	Bryophyte				ILLS2957
	Atrichum angustatum	Bryophyte				ILLS2960
	Atrichum crispulum	Bryophyte				ILLS2963
	Bryaceae	Bryophyte				ILLS2937
	Leucobryum sp.	Bryophyte				ILLS2953
	Soil	Soil	unburned	GSMNP Baskins Creek 35.6772N; −83.4785N	3-04-2018	98-TGU
	Soil	Soil			2-04-2017	15-TGU
	Soil	Soil	burned	GSMNP Lower trail Baskins Creek 35.6781N; −83.4786W	3-17-2018	97-BCT
	Soil	Soil		35.6777N; −83.4785W	2-04-2017	13-BCT
	Soil	Soil	burned	GSMNP Table Mt Pine zone 35.6796N; −83.4781W	3-17-2018	95-BCH
	Soil	Soil		35.6790N; −83.4774W	2-04-2017	14-BCH
	Conocephalum conicum	Bryophyte	unburned	Indiana	5-09-2018	ILLS2939
	Climacium americanum	Bryophyte		39.6085N; −86.9676W		ILLS2956
	Atrichum angustatum	Bryophyte				ILLS2964
	Huperzia serrata	Club moss				ILLS281300
	Polytrichum commune	Bryophyte				ILLS2951
	Diphasiastrum sp.	Club moss				ILLS281301
	Anomodon attenuatus	Bryophyte	unburned	Illinois 39.4687N; −88.1558W	10-31-2017	ILLS2952
	Bryoandersonia sp.	Bryophyte				ILLS2941
	Polytrichum commune	Bryophyte				ILLS2948
	Thuidium delicatulum	Bryophyte				ILLS2946
	Brachythecium sp.	Bryophyte				ILLS2954
	Leucobryum glaucum	Bryophyte	unburned	Pennsylvania 41.0535N; −77.3305W	12-25-2016	ILLS2944
	Bryoandersonia sp.	Bryophyte				ILLS2966
	Anomodon attenuatus	Bryophyte				ILLS2943
	Pohlia sp.	Bryophyte	unburned	Alaska 61.6275N; −149.0776W	7-14-2017	ILLS2972
	Cinclidium sp.	Bryophyte		63.8100N; −148.9646W		ILLS2971
	Hylocomium splendens	Bryophyte		64.1328N; −145.6814W	7-12-2017	ILLS2973
	Polytrichum commune	Bryophyte				ILLS2974

Table 2
Number of samples containing pyrophilous fungi from culture-dependent and culture-independent sampling.

Fungal species	Category[b]	Other unburned areas[a]		Great Smoky Mountains National Park						
		Samples		Unburned			Burned			
		Bryophytes 2016–2018	Club mosses 2018	Bryophytes 2018	Soil 2018	Soil 2017	Bryophytes 2018	Lichens 2018	Soil 2018	Soil 2017
Culture-dependent total number of samples[b]							7			
Pholiota highlandensis	1						5			
Culture-independent total number of samples[c]		15	2	6	1	1	12	3	2	2
Anthracobia melaloma	1			3			8	1	1	1
Ascocoryne cylichnium	unknown	2		2			4		1	
Bulgaria inquinans	unknown	1		1			4			
Coniochaeta ligniaria	unknown						2			
Cotylidia undulata	2	2	1	3		1	5	1	1	1
Geopyxis carbonaria	1	1		1						1
Gymnopilus decipiens	1								1	1
Laccaria laccata	2	1		1		1	3		2	1
Morchella exuberans	1									1
Neurospora crassa	2	3		3			11	1		1
Peziza echinospora	1			1						
Peziza saccardoana	unknown				1					
Pholiota castanea	1						1			
Pholiota highlandensis	1	3		2	1	1	10	1	2	2
Plicaria acanthodictya	unknown						2			
Plicaria anthracina	1						4			
Psathyrella pennata	1			1			1		1	1
Pyronema domesticum	1						1			1
Rhizina undulata	1			2			2			
Lyophyllum anthracophilum	1	1		2			3			
Thelephora terrestris	unknown	2	1			1				
Tricharina praecox	1						1			

[a] Locations include Alaska, Illinois, Indiana, and Pennsylvania.

[b] Pyrophilous fungi classification was categorized as follows: category 1. fruiting is observed only after a fire event; category 2. fruiting does not require a fire event, but is frequently enhanced by fire over that normally observed in unburned areas; Unknown. defined by previous authors as pyrophilous but do not fit category 1 or category 2.

[c] Indicates total number of samples in each category.

primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). Amplification was completed using the Fluidigm Access Array (Brown et al., 2016). The final Fluidigm amplicon construct was as follows: Illumina Adapter - Fuidigm-specific primer pad (C1) - Forward Primer - Amplicon - Reverse Primer - Fuidigm-specific primer pad (C2) - Illumina Adapter. The final amplicons were size selected into <500 nt and >500 nt subpools, remixed together by nM concentration in a 1X:3X proportion, and sequenced using the Illumina MiSeq v2 platform rapid 2 × 250 nt paired-end reads. All amplification and sequencing steps were performed at the Roy J. Carver Biotechnology Center at the University of Illinois.

2.4. Illumina read processing and taxonomic assignment

Illumina reads were processed in QIIME 1.9.1 (Caporaso et al., 2010). Forward reads were analyzed to maximize sequence usage, sequence quality was set to 25, and singletons were removed during pick_open_reference_otus.py by setting the –min_otu_size to 5. Sequences were clustered into OTUs using a 97% similarity threshold using the UNITE Qiime_ver7_97_s_01.12.2017 database using the uclust algorithm (Caporaso et al., 2010; Edgar, 2010) (S1, S2, S3, S4, S5). Taxonomic assignment for each OTU was completed with CONSTAX (Gdanetz et al., 2017) using the UNITE database (Abarenkov et al., 2010) for both ITS1 and ITS2 reads (S6, S7). To provide additional support for each taxonomic assignment, taxonomic names were verified in QIIME 1.9.1 using the BLAST algorithm (Altschul et al., 1990) against a QIIME compatible NCBI database containing a total of 870,044 sequences (88,129 Animal, 270,414 Plant, 456,750 Fungi, 54,639 Protists, and 11 Archaea) constructed on June 27, 2018 using entrez_qiime v2.0 (Baker, 2016). This database was parsed from the NCBI database using the following criteria: internal transcribed spacer 1, length of 300–2500 bp, and two filters (bacteria and uncultured). The CONSTAX OTU taxonomic assignment was retained for non-pyrophilous fungal OTUs and for congruent pyrophilous OTU taxonomic assignments from both aforementioned taxonomic assignment methods. When conflicts between taxonomic assignments occurred for presumed pyrophilous OTUs, each OTU was compared against the full NCBI nucleotide database and against the UNITE database to determine if the conflict arose due to: (1) low identity match against the best NCBI match, (2) absent species level reference sequence in UNITE, or (3) no consensus at the species level using CONSTAX. If the taxonomic conflict could be identified, the most appropriate OTU taxonomic assignment was retained. All supplemental data files (S1–S8) are publicly available from the University of Illinois Databank at https://doi.org/10.13012/B2IDB-1530363_V1.

3. Results

3.1. Culture-dependent results

A total of 27 fungal endophytes was isolated from seven surface-sterilized bryophytes (Table 1), of which eight (30%) were *P. highlandensis*. *Pholiota highlandensis* was isolated from five of the seven (83%) bryophyte samples: *Ceratodon purpureus*, *Ditrichum pallidum* (two samples), *Funaria hygrometrica* (a fire-response bryophyte), and *Polytrichum commune* (Table 2). No pyrophilous fungal isolates were obtained from *Marchantia polymorpha* and *Trematodon longicollis*.

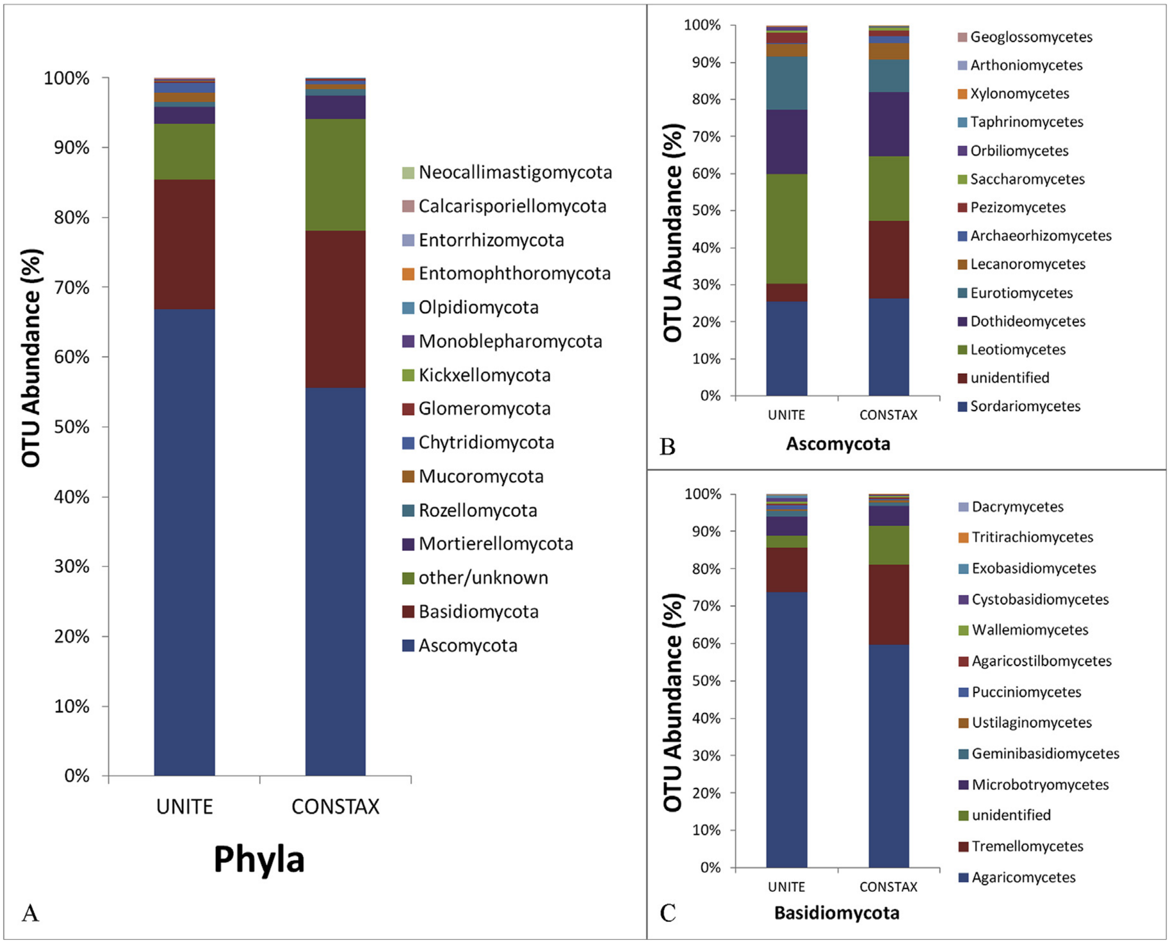

Fig. 1. Distribution of ITS1 OTUs per fungal phylum (A) and at the class level for the Ascomycota (B) and Basidiomycota (C).

3.2. *Culture-independent results*

Illumina sequencing of 35 bryophyte, six soil, three lichen, and two club moss samples produced a combined 1,548,713 forward reads for both ITS1 and ITS2 sequences, of which 78% passed quality filtering (S1, S2). No sequences from the negative control passed quality filtering. The resulting 1,203,727 sequences clustered into 5,132 ITS1 and 3,545 ITS2 OTUs (S6, S7). Rarefraction analyses indicated that sampling depth was adequate for most samples (data not shown). The ITS1 OTUs consisted of 56–67% Ascomycota, 19–23% Basidiomycota, 8–16% unidentified, and 6–7% other fungi (Fig. 1A), while the ITS2 OTUs consisted of 54–67% Ascomycota, 18–19% Basidiomycota, 8–22% unidentified, and 5–7% other fungi depending on the taxonomic assignment method used (data not shown). Ascomycota classes with the most numerous OTUs were Dothideomycetes, Leotiomycetes, and Sordariomycetes (Fig. 1B), whereas Basidiomycota classes with the most numerous OTUs included Agaricomycetes, Microbotryomycetes, and Tremellomycetes (Fig. 1C).

Overall, 792 OTUs were assigned to genus or species level (S8), of which 22 taxonomic assignments were known pyrophilous fungi (Table 3, Fig. 2). The remaining OTUs consisted of endophytes and soil fungi. Of the 22 pyrophilous OTUs, three were found only in soils while the other 19 were found to form endophytic and/or endolichenic associations. The most frequently encountered pyrophilous species in Ascomycota were *Anthrocobia melaloma* and *Neurospora crassa*. The most frequently encountered pyrophilous species in Basidiomycota were *Cotylidia undulata* and *Pholiota highlandensis* (Fig. 2F; Table 2).

Pyrophilous fungi were found in numerous moss species: *Atrichum angustatum*, *A. crispulum*, *Bryoandersonia* sp., *Cinclidium* sp., *Climacium americanum*, *Dicranum* sp., *Hylocomium splendens*, *Hypnum* sp., *Leucobryum* sp., *Polytrichum commune*, *Rhizomnium* sp. and *Thuidium* sp., in the club moss *Huperzia serrata*, and in the lichen *Lobaria quercizans*. Nine pyrophilous fungal OTUs were found outside of the GSMNP in bryophyte and club moss species (Table 2). Pyrophilous fungal OTUs found in bryophyte samples from both burned and unburned GSMNP areas and outside of the GSMNP were *Ascocoryne cylichnium*, *Cotylidia undulata*, *Laccaria laccata*, *Neurospora crassa*, *Pholiota highlandensis*, and *Thelephora terrestris*. Soil samples from burned and unburned GSMNP areas collected in 2017 and 2018 contained 13 pyrophilous fungal OTUs, of which only three were found exclusively in soil samples: *Gymnopilus decipiens* (2017 and 2018 GSMNP burned area), *Morchella exuberans* (2017 GSMNP burned area), and *Peziza saccardonana* (2018 GSMNP unburned area) (Table 2). ITS relative abundance/sample and total read count for bryophyte samples (seven from GSMNP, two from Alaska) were equal to or greater than those from 2018 GSMNP soil samples.

4. Discussion

This is the first study to use culture-dependent and culture-independent techniques to investigate whether pyrophilous fungi

Table 3
Summary of pyrophilous fungi from culture-dependent and culture-independent sampling. Newly discovered ecologies in this study are shown in bold text.

Fungal species	Category[a]	Nutritional mode	Nutritional mode reference	Fire-association reference
Culture-dependent				
Pholiota highlandensis	1	saprobe, **endophytic**	**this study**	Moser (1949); Petersen (1970)
Culture-independent				
Anthracobia melaloma	1	saprobe, **endophytic**	**this study**	Seaver (1928); Moser (1949)
Ascocoryne cylichnium	unknown	**endophytic**	**this study**	Adamczyk et al. (2012)
Bulgaria inquinans	unknown	saprobe, biotrophic, **endophytic**	Itzerott (1967); Döring and Triebel (1998); **this study**	Adamczyk et al. (2012)
Coniochaeta ligniaria	unknown	saprobe, endophytic	Hirose et al., (2013); Rosa et al., (2013)	Wicklow (1975) (as *C. discospora*) per Mahoney and LaFavre (1981)
Cotylidia undulata	2	saprobe, **endophytic**	Korotkin et al., (2018); **this study**	Monti et al. (1992)
Geopyxis carbonaria	1	biotrophic, endophytic	Vrålstad et al., (1998); U'Ren et al., (2012)	Seaver (1928); Beug et al., (2014)
Gymnopilus decipiens	1	saprobe	Holec (2005)	Moser (1949)
Laccaria laccata	2	ectomycorrhizal	Machón et al. (2009)	Monti et al., (1992); Adamczyk et al., (2012)
Morchella exuberans	1	saprobe		Beug et al., (2014); Miller et al., (2017)
Neurospora crassa	2	saprobe, endophytic	Huo et al. (2014)	Petersen (1970);
Peziza echinospora	1	mycorrhizal		Moser (1949); Petersen (1970)
Peziza saccardoana	unknown	saprobe, **endophytic**	**this study**	Monti et al. (1992)
Pholiota castanea	1	saprobe, **endophytic**	**this study**	Matheny et al. (2018)
Pholiota highlandensis	1	saprobe, **endophytic**	**this study**	Moser (1949); Petersen (1970)
Plicaria acanthodictya	unknown	saprobe, **endophytic**	**this study**	Dougoud, 2007
Plicaria anthracina	1	saprobe, **endophytic**	**this study**	Moser (1949); Dougoud, 2007
Psathyrella pennata	1	saprobe, **endophytic**	**this study**	Moser (1949)
Pyronema domesticum	1	mycorrhizal		El-Abyad and Webster (1968); Monti et al., (1992)
Rhizina undulata	1	biotrophic	Jalaluddin M (1967); Egger (1986)	Seaver (1928); Moser (1949)
Lyophyllum anthracophilum	1	saprobe, **endophytic**	**this study**	Singer, 1969
Thelephora terrestris	unknown	saprobe, **endophytic**	**this study**	Adamczyk et al. (2012)
Tricharina praecox	1	saprobe, **endophytic**	**this study**	Yang and Korf (1985)

[a] Pyrophilous fungi classification was categorized as: category 1. fruiting is observed only after a fire event; category 2. fruiting does not require a fire event, but is frequently enhanced by fire over that normally observed in unburned areas; Unknown. defined by previous authors as pyrophilous but do not fit category 1 or category 2.

possess endophytic or endolichenic life history stages during the course of their life cycles. Here we identified 22 previously recognized pyrophilous fungal taxa (Table 3) from many bryophyte, club moss, and lichen samples from the GSMNP burned and unburned sites and from unburned locations outside of the GSMNP, providing evidence for the body snatchers hypothesis (Fig. 3). Numerous non-pyrophilous endophytic and soil fungi were also identified (S8), but the evaluation of these groups of fungi was beyond the scope of this paper.

The current state of the body snatchers hypothesis, proposed by Matheny et al. (2018) with *P. highlandensis* as the model (Fig. 2F; Fig. 3), still needs further investigation to determine the extent and duration of the endophytic relationship. It is possible that this association is a transient relationship due to the close temporal and spatial approximation of pyrophilous bryophyte species and pyrophilous fungal germinating spores (Claridge et al., 2009, Fig. 2A–C). However, the frequency of occurrence of pyrophilous fungi as endophytes suggests otherwise. In our study, seven 2018 bryophytes within the GSMNP contained equal to or greater ITS1 relative abundance per sample and total read count of the 22 pyrophilous fungi than the 2018 soil samples suggesting that the duration of this association was maintained at least until the second spring following the GSMNP fire event. In addition, two Alaskan bryophyte samples collected in a location with no indication of a recent fire event also contained equal to or greater relative abundance of pyrophilous taxa (see Table 2) than compared to the 2018 GSMNP soil samples (Table 4). Similarly, U'Ren et al. (2012) noted that more than 50% of endophytes (particularly within bryophytes) and endolichenic fungi within Arizona's fire-dominated montane forests belonged to Pyronemataceae and related Pezizomycetes taxa including *Anthracobia*, *Geopyxis*, and *Pyronema*. Tedersoo et al. (2013) and Hansen et al. (2013) suggested that these fungi could most likely obtain some nutrients from their host. It is also important to note that some bryophytes and lichens are not completely destroyed during a fire event. Hylander and Johnson (2010) demonstrated that bryophytes can survive fire events through small-scale refugia, typically associated with boulders and vertical rocks. In addition, post-fire observations of unburned lichen fragments were noted in burned areas within the GSMNP.

Other studies have identified pyrophilous fungi as endophytes in other vascular and non-vascular plants, providing additional support for the body snatchers hypothesis (Table 3). Baynes et al. (2012) investigated a fire-adapted grass, *Bromus tectorum*, and reported that 39% of the endophytes they isolated were known fire-adapted or heat tolerant fungi including the genus *Morchella*. Although we did not sample vascular plants in our study, we did find a known post-fire morel (*Morchella exuberans*) occurring in a February 2017 soil sample (Miller et al., 2017). Interestingly, this morel was not found in the March 2018 soil samples after fruiting prolifically in April 2017 (Table 2).

Species of *Anthrocobia* are presumed saprotrophs that may act as important post-fire soil stabilizers (Claridge et al., 2009, Fig. 2D). One recent study suggested that *Anthrocobia* species can form associations with lichens (Tedersoo et al., 2013). *Anthrocobia* was isolated from a surface-sterilized *Physcia caesia* thallus from Arizona and from a surface-sterilized *Lecanora oreinoides* thallus from North Carolina (U'Ren et al., 2010; U'Ren et al., 2012). In our study, *Anthrocobia* was identified as an endolichenic fungus from *Lobaria quercizans* and as an endophyte of the mosses *Atrichum angustatum*, *A. crispulum*, a Bryaceae species, *Dicranum* sp., *Hypnum* sp., *Leucobryum* sp., *Leucodon julaceus*, and *Thuidium delicatulum*.

Wang et al. (2016) investigated the genus *Geopyxis* (Pyronemataceae) and commented on the vast number of endophytic and endolichenic *Geopyxis* environmental sequences. In particular, the pyrophilous *G. carbonaria* (Fig. 2E) has been previously reported as an endophyte of *Pleurozium schreberi* and as endolichenic with *Cladonia mitis* from Alaska. *Geopyxis delectans*, also pyrophilous, has been reported as endolichenic with *Pseudevernia intensa* from

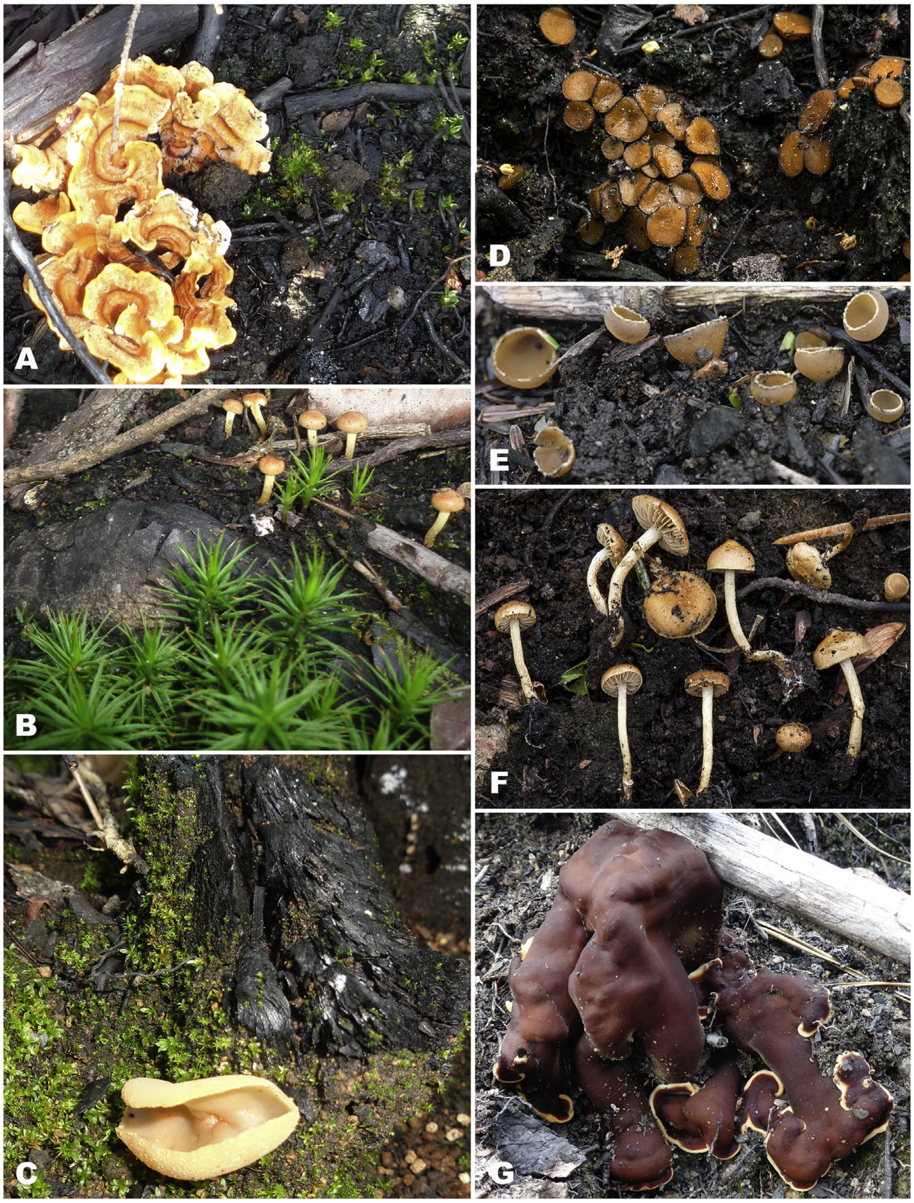

Fig. 2. Mosses and fungi growing on burned soil or wood after a fire in the Great Smoky Mountains National Park. A-C. Various species of mosses and pyrophilous fungi growing together after a severe burn. D. *Anthracobia melaloma*. E. *Geopyxis carbonaria*. F. *Pholiota highlandensis*. G. *Rhizina undulata*.

Arizona and *Umbilicaria proboscidea* from Alaska (U'Ren et al., 2012). In our study, *G. carbonaria* was found from two bryophyte samples, *Clinclidium* sp. from Alaska and *Leucobryum* sp. from the GSMNP unburned area, which reinforces the conclusion that *G. carbonaria* is a widespread endophyte and endolichenic fungus that then produces apothecia exclusively in post-fire environments.

In another study, Davey et al. (2013) obtained two pyrophilous OTU matches to *Ascocoryne cylichnium* and *Thelephora terrestris* while investigating bryophyte-associated communities of four moss species along an elevational gradient using culture-independent techniques. In our study, *A. cylichnium* was found in several bryophyte samples such as *Climacium americanum* from Indiana, and *Atrichum angustatum*, *Dicranum* sp., *Hypnum* sp., *Leucobryum* sp. and *Polytrichum commune* from the GSMNP. In addition, *Thelephora terrestrisi* was found in a club moss (*Huperzia serrata*) and in a bryophyte (*C. americanum*) from Indiana, and in a bryophyte (*Hylocomium splendens*) from Alaska. *Thelephora terrestris* is widely recognized as a root symbiont (ectomycorrhizal) of a wide array of vascular plants, including greenhouse seedlings (Marx et al., 1970; Agerer and Weiss, 1989). We recorded sporocarps of *T. terrestris* on <2 year-old burned soils in the GSMNP (during the second summer after the fire) among numerous recently germinated seedlings of *Pinus pungens* (e.g., TENN-F-074475). In addition, *T. terrestris* has been recorded on recently burned sites in Europe (Adamczyk et al., 2012). Our assumption was that *T. terrestris* was among the earliest successional ectomycorrhizal formers on *Pinus* seedlings. Our culture-independent results suggest the life history traits of *T. terrestris* may be more complex than previously thought, and that *T. terrestris* is an important constituent of early post-fire successional habitats.

Huo et al. (2014) isolated *Neurospora crassa* from *Pinus sylvestris* and showed that this fungus had lifestyle plasticity and could occur as an endophyte, pathogen, or saprobe. In this study, we found OTU matches to *N. crassa* from many bryophyte samples and one lichen

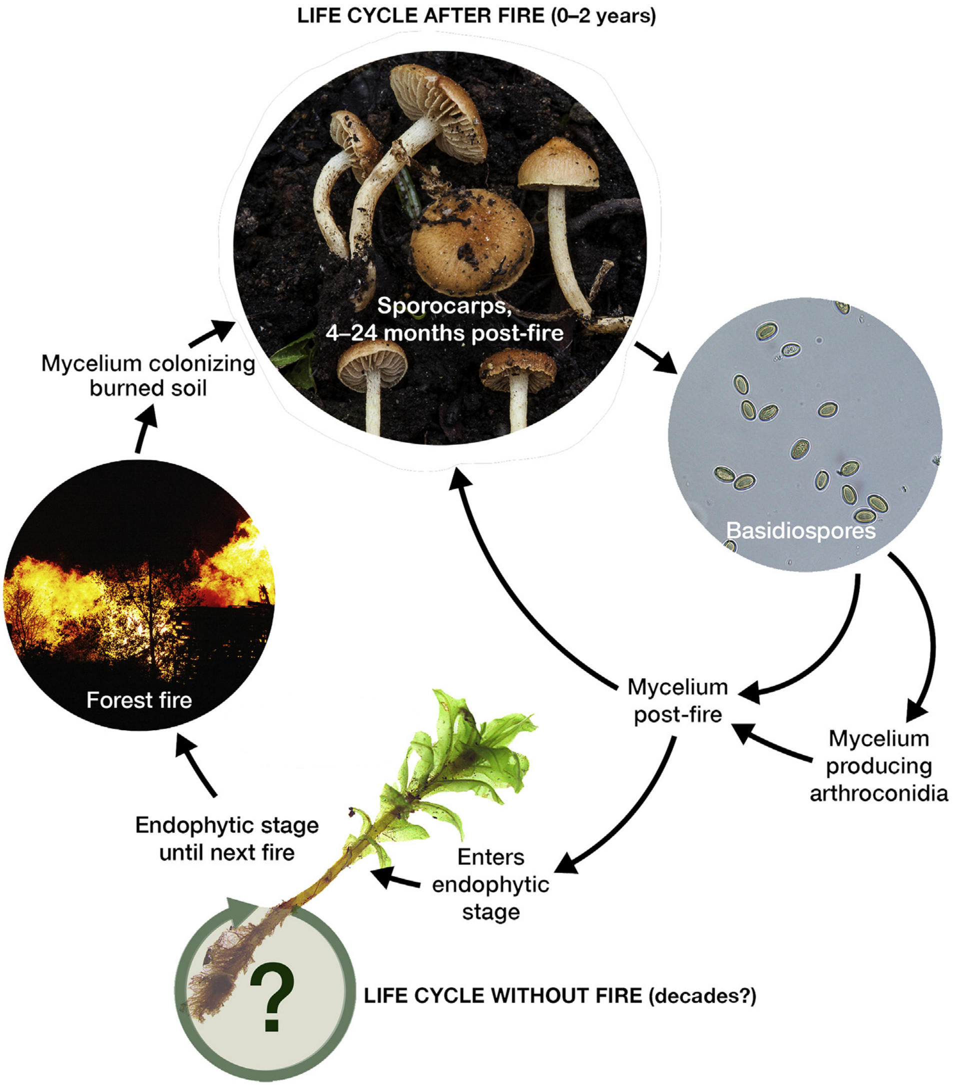

Fig. 3. Diagram of the body snatchers hypothesis with *Pholiota highlandensis* as the model. After a fire event, *P. highlandensis* produces sporocarps, basidiospores enter soil and produce mycelium and arthroconidia. The mycelium in the soil is capable of producing sporocarps repeating the cycle. Alternatively, mycelium is capable of associating with some plant and lichen species. This endophytic association persists until a fire event occurs and the cycle repeats. Factors that lead to this association and duration are not fully resolved and need further study.

(*Lobaria quercizans*) from the GSMNP and from bryophyte samples outside the GSMNP. Rosa et al. (2013) isolated *Coniochaeta ligniaria* as an endophyte from a perennial daisy, *Smallanthus sonchifolius*. We found fungal OTU matches to *C. ligniaria* from two bryophyte samples (*Dicranum* sp. and *P. commune*) within GSMNP burned areas.

In this study, we identified 22 pyrophilous fungi from samples of bryophytes, lichens, and club mosses using culture-independent techniques. One weakness of culture-independent techniques is that any DNA on or within the sample can be sequenced without regard to the state of the organism (inactive, actively growing, or dead) thereby increasing the importance of proper surface sterilization. We cannot discount the possibility that some epiphytic fungi, spores or external DNA survived the sterilization procedure. However, by using new sterilization equipment and solutions for each site and condition, and assaying samples outside the GSMNP spore load zone, we believe we have adequately addressed the potential for false positives from incomplete surface sterilization. In fact, some samples may have been over-sterilized as eight bryophyte samples contained $\leq$10 OTUs. In addition, we note that during this investigation OTU taxonomic determination was only as reliable as the reference databases since we discovered taxonomic discrepancies between UNITE and NCBI databases. This was resolved by using additional reference sequences from GSMNP pyrophilous fungal sporocarps collected in 2017 or by examining the reference database to see if a particular species was absent. Therefore, it is important to understand the limitations of each reference database and verify OTU taxonomic assignments using multiple methods (Raja et al., 2017).

It appears there is substantial evidence for the body snatchers hypothesis proposed by Matheny et al. (2018) that expands the life history traits and ecological roles of pyrophilous fungi. Our results support the idea that many pyrophilous fungal species from North America (at least 19) form endophytic and/or endolichenic associations. From our 22 OTUs, only three were found exclusively from soil samples, which suggests these fungi are probably not residing solely in dormancy as spore banks or subterranean organs such as sclerotia. Therefore, pyrophilous fungi have thus likely evolved multiple mechanisms that maintain their diversity in systems prone to disturbance by heat or fire.

Table 4
Relative abundance of taxa in Table 3. Only species within the ITS1 dataset are shown. Samples with low abundance (count data <10) and samples with low reads per sample (<3000) removed.

Sample Id	Condition	Location	Table 3 Pyrophilous fungi ITS1 OTU relative abundance (%)	Non-pyrophilous endophytes/Soil fungi ITS1 OTU relative abundance (%)	Reads per sample (#)	ITS1 read count for Table 3 taxa (#)
Atrichum angustatum	unburned	GSMNP[a]	4.5%	95.5%	10595	480
Hypnum sp.			2.2%	97.8%	3968	86
Leucobryum sp.			0.05%	99.95%	18444	10
Dicranum sp.	burned		0.5%	99.5%	3803	19
Dicranum sp.			0.1%	99.9%	14043	12
Polytrichum commune			1.4%	98.6%	17977	257
Atrichum angustatum			34.3%	65.7%	15968	5478
Atrichum angustatum			9.7%	90.3%	9675	943
Atrichum crispulum			11.8%	88.2%	11450	1350
Bryaceae			8.5%	91.5%	8641	735
Leucobryum sp.			1.4%	98.6%	22222	314
Climacium americanum	unburned	Indiana	0.2%	99.8%	17614	40
Cinclidium sp.	unburned	Alaska	1.8%	98.2%	21507	386
Hylocomium splendens			0.8%	99.2%	12176	101
Soil 2017			10.6%	89.4%	6589	696
Soil 2018	burned	GSMNP	0.8%	99.2%	12473	101

[a] GSMNP = Great Smoky Mountains National Park.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2019.100870.

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